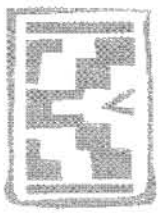
Onderstepoort Journal of Veterinary Research, 70:255–263 (2003)



Leishmania donovani-derived lipophosphoglycan plus BCG induces a Th1 type immune response but does not protect Syrian golden hamsters (*Mesocricetus auratus*) and BALB/c mice against *Leishmania donovani*

W.K. TONUI[1], S.S. MPOKE[1], A.S. ORAGO[2], S.J. TURCO[3], P.A. MBATI[4] and G.M. MKOJI[1]

ABSTRACT

TONUI, W.K., MPOKE S.S., ORAGO, A.S., TURCO, S.J., MBATI, P.A. & MKOJI, G.M. 2003. *Leishmania donovani*-derived lipophosphoglycan plus BCG induces a Th1 type immune response but does not protect Syrian golden hamsters (*Mesocricetus auratus*) and BALB/c mice against *Leishmania donovani*. *Onderstepoort Journal of Veterinary Research*, 70:255–263

The efficacy of *Leishmania donovani*-derived lipophosphoglycan (LPG) plus *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) as a vaccine candidate against visceral leishmaniosis in susceptible BALB/c mouse and Syrian golden hamster (*Mesocricetus auratus*) models was investigated. Following a triple vaccination with a total dose of 150 µℓ BCG plus 60 µg or 30 µg of LPG for hamsters and BALB/c mice respectively, there were no noticeable side effects both locally and systemically; implying that the molecule was safe at this dosage level. Vaccinated animals demonstrated an activation of both the humoral as well as cell-mediated responses to LPG, which correlated with resistance against the disease. Protection by LPG plus BCG, was however, poor as the remaining immunized animals showed disease progression leading to severity of the disease as illustrated by emaciation, mass loss and heavy splenic parasitaemia in hamsters. These data nevertheless suggest that it may be rewarding to further evaluate the potential of LPG as a vaccine candidate in leishmaniosis using other adjuvants, which may enhance its immunogenicity.

Keywords: Bacille Calmette-Guérin, BALB/c mice, *Leishmania donovani*, lipophosphoglycan, Syrian golden hamsters

INTRODUCTION

The *Leishmania donovani* parasite is a protozoan that causes a severe, debilitating and often fatal disease called visceral leishmaniosis (VL) which is a public health problem in areas of Latin America, Africa, India and southern Europe.

Although VL has received considerable attention, efforts toward vaccination against leishmaniosis have focussed almost exclusively on localized cutaneous disease (reviewed by Handman 2001). Lipophosphoglycan (LPG) a glycoconjugate present on the surface of the *Leishmanias* expressed in promastigotes and amastigotes (Turco 1988, McNeely & Doyle 1996) and reported to promote intracellular survival of these parasites, has been shown to protect mice and to elicit T-cell responses in infected mice (Handman & Mitchell 1985; McConvile, Bacic, Mitchell & Handman 1987; Russell & Alexander 1988; Elhay, Kelleher, Bacic, McConville, Tolson Pearson & Handman 1990) and humans (Kemp, Theander, Handman, Hey, Kurtzhals, Hviid, Srrensen, Were, Koech & Kharazmi 1991; Mendonca,

[1] Centre for Biotechnology Research & Development, Kenya Medical Research Institute, P.O. Box 54840, Nairobi, Kenya. E-mail: tonuiwk@hotmail.com; wtonui@nairobi.mimcom.net

[2] Zoology Department, Kenyatta University, Nairobi, Kenya

[3] Department of Biochemistry, Kentucky University, KY, USA

[4] Department of Zoology, University of the North, South Africa

Accepted for publication 9 May 2003—Editor

Russell & Coutinho 1991). Studies have shown that the lymphocyte proliferation is associated with the protein component and not the glycan of LPG (Jardim, Tolson, Turco, Pearson & Olafson 1991; Russo, Turco, Burns & Reed 1992).

Studies in our laboratory have shown that LPG is an excellent candidate as a transmission blocking vaccine against *Leishmania major* infections (reviewed Tonui 1999; Tonui, Mbati, Anjili, Orago, Turco, Githure & Koech 2001a, b). In these studies, sand flies which fed on BALB/c mice immunized with *L. major*-derived LPG (Tonui *et al.* 2001a) or monoclonal antibodies raised against LPG (Tonui, Ngumbi, Mpoke, Orago, Mbati, Turco & Mkoji 2003) showed that parasite development was inhibited at the log phase (procyclic) of the parasite. 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 (Tonui *et al.* 2001b). These results prompted us to further evaluate LPG as a vaccine candidate in leishmaniosis. Our goal has been to develop a vaccine that can be used both to reduce transmissible infections within the sand fly and disease severity within the mammalian host.

MATERIALS AND METHODS

Isolation of lipophosphoglycan

The LPG antigen was extracted from *L. donovani* promastigotes, purified and quantitated by phosphate analysis as has previously been described (McConvile *et al.* 1987; Orlandi & Turco 1987). Lyophilised LPG was dissolved to desired concentrations in sterile phosphate buffered saline before vaccinations.

Animals and vaccination protocol

BALB/c mice and Syrian golden hamsters (*Mesocricetus auratus*) used in this study were obtained from the Kenya Medical Research Institute's (KEMRI) animal house and maintained under conventional conditions. A group of 15 hamsters, matched by sex, were each immunized by three subcutaneous (s.c.) injections of 20 µg/mℓ LPG mixed with 50 µℓ BCG (Pasteur Mérieux, Lyon, France). Injections were given at 2-week intervals (Ingonga, Mbati, Anjili, Mutani, Wishitemi, Odongo, Robert & Githure 1996). Another group of 15 hamsters, also matched by sex were used as controls. Five of these animals received 50 µℓ BCG alone and five others received phosphate-buffered saline (PBS) alone. The last five of this group remained naive.

Vaccination of BALB/c mice was done as has previously been described (Handman, Symons, Balwia, Curtis & Scheerlinck 1995). Briefly, 15 mice aged 6–8 weeks, matched by sex, were immunized intraperitoneally (i.p.) three times every 2 weeks with 10 µg/mℓ of purified LPG mixed with 50 µℓ of BCG as an adjuvant. Another group of 15 mice were used as controls. Five of these animals received 50 µℓ BCG alone and five others received phosphate-buffered saline (PBS) alone. The remaining five of this group remained naive. All animals were weighed weekly.

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 was used to examine T-cell responses to LPG antigen while the other group was challenged by inoculation of live *L. donovani*.

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 footpads with 5 µg of LPG antigen. This amount of antigen had been determined in preliminary studies to be the minimum for DTH elicitation. The degree of skin induration was measured after 72 h, and those above 5 mm in diameter were recorded as positive (Yang, Rogers & Liew 1991).

Humoral responses

Enzyme-linked immunosorbent assay (ELISA) was used to determine for antibody levels present in the serum of immunized animals (Voller, Bartlett & Bidwell 1976). Briefly, 100 µℓ of 10 µg of LPG antigen was used for coating U-well polyvinyl chloride plates overnight. After washing with PBS–(0.05%), Tween 20 and blocking with boiled casein, serial dilutions of sera were added to the plates and incubated at 37°C for 1 h. A 1/2000 dilution of goat anti-mouse IgG-HRP conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), followed by 0.4 mg/mℓ substrate [o-phenylenediamine o-diaminobenzene dihydrochloride (Sigma®) and 30 % hydrogen peroxide (Sigma®) was used. Colour reaction was stopped by using 25 µℓ of IN HCl (BDH, UK) and read on a Titertek® ELISA plate reader at a wavelength of 405 nm. To ensure consistency, negative

and positive control reference antibody preparations were used.

T-cell proliferation assay

Animals were killed and cells were prepared from their spleens 7 days post-immunization as has previously been described (Gicheru, Olobo, Anjili, Orago, Modabber & Scott 2001). Briefly, lymphocytes isolated from spleens from two of the animals in each group adjusted to a final concentration of 3 x 10^6/mℓ in 100 µℓ complete RPMI 1640 medium were stimulated with 20 µg LPG. Control wells received the same concentration of cells plus complete RPMI 1640 only. Cultures set up in duplicates, were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 for 5 days and pulsed with 0.5 Ci per well (20 µℓ per well) of ^{3}H-thymidine (Amersham, Life Science, USA) 1.85 mBg/mℓ over the last 18 h and harvested on glass fibre filters (Titertek, Micro titration Equipment, UK). Incorporation of ^{3}H-thymidine into DNA was determined by liquid scintillation spectrophotometry. Proliferation was expressed as counts per min in stimulated cultures minus counts per min in unstimulated cultures.

Interferon-gamma (IFN-γ) and Interleukin-4 (IL-4) production

Lymphocytes isolated from spleens from two of the BALB/c mice in each group adjusted to a final concentration of 2 x 10^6/mℓ in 100 µℓ complete RPMI 1640 medium were stimulated with 20 µg LPG. Control wells received the same number of cells plus complete RPMI 1640 only. Cultures set up in triplicates, were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2. Culture supernatants were collected from the wells after 48 h of stimulation, and the concentrations of IFN-γ and IL-4 in culture supernatants were determined by ELISA. Protocols outlined in the BD Pharmingen's manual were followed. Briefly, polystyrene micro-ELISA plates (Dynatech Laboratories, Sussex, UK) were coated overnight with 50 µℓ of a 2-µg/mℓ concentration of capture monoclonal antibody to rat anti-mouse IFN-γ or IL-4 using (BD Pharmingen) diluted in bicarbonate buffer, pH 9.6. Plates were washed twice with Elisa solution consisting of 0.05% Tween 20 diluted in PBS and blocked with a blocking buffer consisting of 0.1 % Tween 20 and 3 % bovine serum albumin diluted in borate buffered saline for 2 h at room temperature. The plates were washed again twice, 50 µℓ per well standards and samples diluted in 0.05 % foetal bovine serum in PBS were added followed by incubation overnight at 4 °C. Plates were washed again four times and diluted biotinylated rabbit anti-mouse IFN-γ or IL-4 detection polyclonal antibodies added. Plates were incubated for 45 min at room temperature and washed again six times with Elisa wash. Diluted avidin-peroxidase (Sigma®) was added and incubated for 30 min at room temperature. Finally the plates were washed eight times with Elisa wash and 100 µℓ of diluted TMB (TMB Peroxidase Development System, Kirkegaard & Perry, MD, USA) added. The colour reaction was stopped by adding 50 µℓ per well of sulphuric acid (1:7 dilution) and read at 450–650 λ dual wave lengths.

Flow cytometry

For T and B cell analyses, respectively, phycoerythrin-conjugated hamster anti-mouse CD3 monoclonal antibody (Becton Dickinson or BD, USA) and fluorescein-conjugated hamster anti-mouse Ly-5 (B220) (BD, USA) was used. Briefly, 50 µℓ of whole blood from immunized groups and the controls were loaded into a tube containing 2 µℓ FITC anti CD3 or CDR 45/B200 labelled monoclonal antibodies. The mixture was incubated at room temperature for 30 min and 2 mℓ of lysis solution added. Following incubation for 10 min at room temperature, the cells were centrifuged at 1500 rpm (800 *g*) for 5 min and washed twice with PBS. Finally, fluorescence was monitored by flow cytometry on a fluorescence-activated cell sorter (BD, Heidelberg, Germany) counting 10 000 cells per sample.

Challenge of vaccinated animals

The *L. donovani* (strain NLB-065) used was isolated from a kala-azar patient prior to the start of these experiments. Parasites were cultured as has been previously been described (Mbati, Anjili, Lugalia, Mwanyumba, Tonui, Robert & Githure 1995). Both immunized and control animals were each challenged with 10^8 virulent *L. donovani* metacyclic promastigotes in 0.1 ml PBS i.p. (Mbati, Abok, Orago, Anjili, Kagai, Githure & Koech 1994). Animals were left in order to allow the infection to develop for a period of at least 6 months. At the end of the experiments, their spleens, livers, and bone marrow were removed and cultured for parasites. Parasite loads in these tissues were determined using the methods of Chulay & Brycesson (1983).

Statistical analysis

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

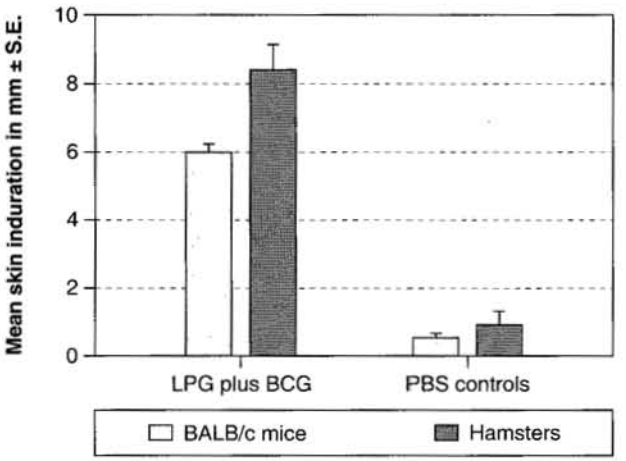


FIG. 1 Delayed-type-hypersensitivity responses in BALB/c mice and hamsters following a triple vaccination with LPG plus BCG

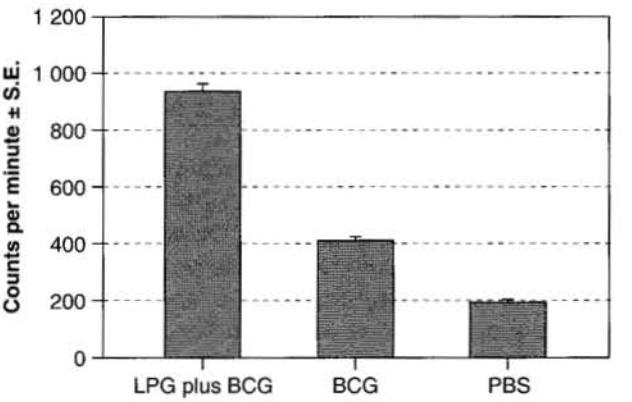


FIG. 2 Lymphocyte proliferation responses in BALB/c mice immunized with LPG plus BCG, BCG and PBS controls

TABLE 1 Antibody responses (IgG) of BALB/c or hamsters to LPG following a triple vaccination with LPG plus BCG

Immunizing antigen	Antigen concentration (µg or µℓ)	Optical density (OD)
Hamsters		
LPG plus BCG	60 µg LPG + 150 µℓ BCG	0.640
BCG	150 µℓ	0.149
PBS controls	150 µℓ	0.061
BALB/c mice		
LPG plus BCG	30 µg LPG + 150 µℓ BCG	0.708
BCG	150 µℓ	0.124
PBS controls	150 µℓ	0.065

TABLE 2 Comparison of the percentage of CD3 and CD45 cells drawn from BALB/c mice following immunization with the respective antigen and after challenge infection with virulent *L. donovani* parasites

Group	Cell phenotype (mean counts of positive cells in 10 000 gated events)					
	After one immunization		After three immunizations		After challenge with *L. donovani*	
NAIVE	CD3 20.53	CD45 48.86	CD3 524.9	CD45 548.02	CD3 543.89	CD45 568.98
PBS	21.5	49.23	535.7	550.65	467.37	441.19
BCG	25.66	36.52	577.09	507.48	528.52	546.2
LPG + BCG	40.93	51.58	518.81	519.91	412.25	389.75

RESULTS

Safety of LPG molecule and delayed type hypersensitivity responses

Previous attempts to vaccinate BALB/c mice using *L. major*-derived LPG have been successful against *L. major* (Handman & Mitchell 1985; McConvile *et al.* 1987). Therefore, in order to determine whether such results could be extrapolated to visceral leishmaniosis caused by *L. donovani*, susce tible BALB/c mice and Syrian golden hamsters (*Mesocricetus auratus*) were vaccinated with *L. donovani*-derived LPG plus BCG.

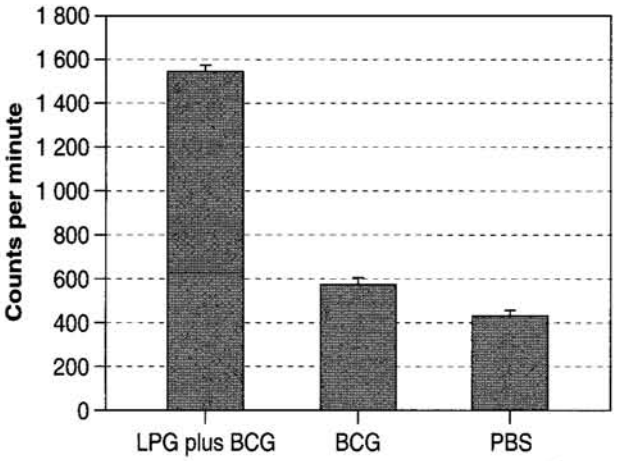


FIG. 3 Lymphocyte proliferation responses in hamsters immunized with LPG plus BCG, BCG or PBS controls

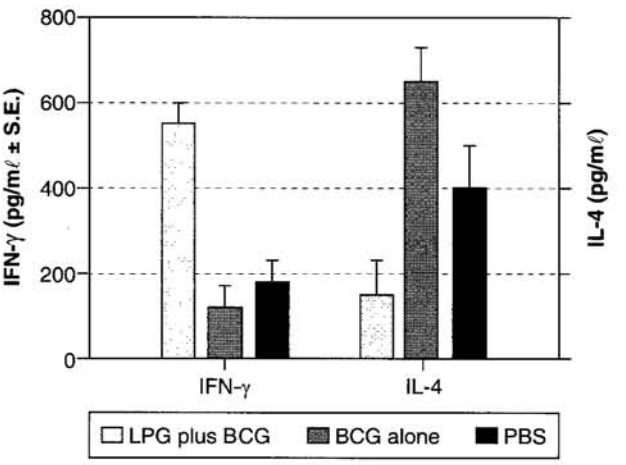


FIG. 4 IFN-γ and IL-4 responses in BALB/c mice following vaccination with LPG plus BCG, BCG alone or PBS

Following triple vaccination with a total dose of 60 µg or 30 µg of LPG plus 150 µℓ BCG for hamsters and BALB/c mice, respectively, 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, with the mean skin induration of 8.38 and 5.996 mm in diameter for hamsters and BALB/c mice, respectively (Fig. 1). Animals in control groups BCG or PBS were not tested for DTH responses using LPG, to avoid any exposure to the antigen before challenge.

Antibody levels

Sera from hamsters or mice immunized with LPG plus BCG tested by ELISA revealed significant IgG responses to LPG antigen compared to animals inoculated with BCG alone or PBS (controls), indicating a successful immunization ($P < 0.05$). LPG plus BCG-immunized hamsters showed average optical density (OD) values of 0.640 whereas BCG and PBS-immunized controls showed an average of 0.149 and 0.061, respectively (Table 1). LPG plus BCG-immunized mice showed OD values of 0.708 whereas BCG or PBS controls showed 0.124 and 0.065, respectively.

In vitro lymphocyte proliferation to LPG

In Fig. 2 and Fig. 3 the counts per min following stimulation of lymphocytes from vaccinated animals or their controls are represented. Data showed that cells count from animals immunized with LPG plus BCG was higher compared to those of their control animals previously immunized with BCG or PBS. These data suggests that cells from animals vaccinated with LPG plus BCG showed higher proliferation on re-stimulation with the LPG antigen.

Secretion of Interferon gamma (IFN-γ) and Interleukin 4 (IL-4)

In order to assess antigen-specific IFN-γ or IL-4 production, lymphocytes from BALB/c mice immunized with LPG plus BCG, BCG or PBS were stimulated with the LPG antigen for 48 h. Results showed that supernatants from animals previously immunized with LPG plus BCG expressed significantly higher IFN-γ levels 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 (Fig. 4). Results of hamster immunizations are not featured due to the fact that the reagents for the hamster models are not available.

B and T-cell phenotypes

Fluorescence activated cell sorter analyses indicated no significant differences in terms of T and B-cell populations between animals immunized with LPG plus BCG and those given BCG alone or the PBS controls (Table 2).

Protection conferred by vaccination with LPG

Mean body mass

In these experiments, mean body mass as a criterion for monitoring wasting and emaciation following challenge infection was used. The mean body mass of immunized animals and their controls were

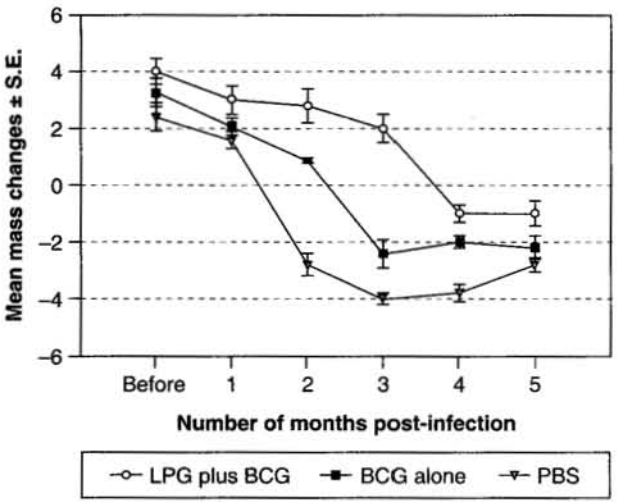


FIG. 5 Mean mass loss in immunized hamsters following challenge with virulent *L. donovani*

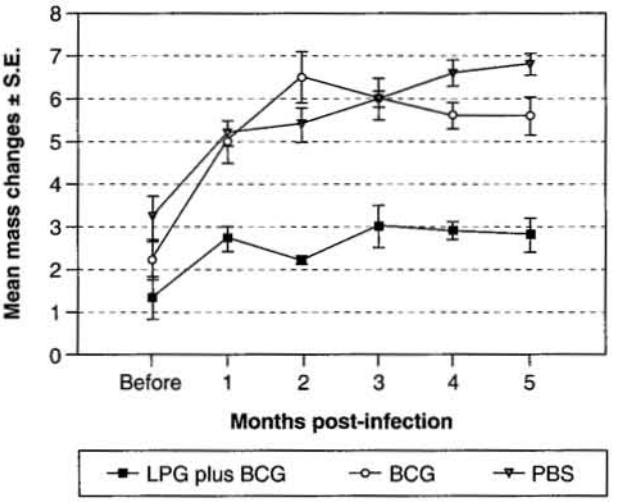


FIG. 6 Mean mass gain in immunized BALB/c mice following challenge with virulent *L. donovani*

monitored following challenge with virulent *L. donovani promastigotes* for a period of 5 months. Results showed that hamsters previously immunized with LPG plus BCG and later challenged with *L. donovani* had average but higher mass loss compared to BCG-immunized controls (Fig. 5). The PBS controls showed gradual loss of mass, which was the highest in the three groups. In all the experimental groups, wasting, emaciation and eventually death of animals accompanied mass loss. The initial mass increase in all the experimental animals was a result of splenomegaly due to infection rather that chronological factors (data not shown). Overall, the mean mass loss in LPG plus BCG, BCG and PBS controls were 5.5, 4 and 6 g, respectively.

Unlike hamsters, BALB/c mice showed an increase in body mass throughout the experiment. The group, which had received PBS alone, showed the highest mean mass gain compared to the BCG and the LPG plus BCG immunized groups. The LPG plus BCG group showed an average increase in mass throughout the experiment. The BCG group showed the highest increase in body mass, which were statistically significant, compared to the control and the LPG plus BCG groups ($P < 0.05$). Overall, there was an increase of 1, 2 and 4 g respectively for the PBS, LPG plus BCG and the BCG groups (Fig. 6).

Spleen parasite loads

After 6 months the experiments were stopped and the remaining animals killed. At this stage all the hamsters from the BCG-immunized groups had died and only four animals from the LPG plus BCG and three from the PBS control groups had survived. Surviving animals were killed and their spleens were cultured for parasites and touch smears were made on glass slides. Results showed that spleen biopsies from the dead and the surviving animals were enlarged and yielded parasites in culture. Furthermore, there were no significant differences in the parasite loads in the spleen of all the surviving animals. All animals showed severe parasitaemia levels (4+) in their spleens.

At the end of experiments 9, 1 and 5 BALB/c mice survived in the LPG plus BCG, BCG and PBS groups, respectively. On sacrificing, five out of nine (55.5%) spleen biopsies from *L. donovani* infected LPG-immunized BALB/c mice were negative for parasites, while all cultures from the PBS and BCG-treated mice yielded parasites (0% negative). The parasite loads in LPG plus BCG-immunized mice were low (1+) whereas those of BCG and PBS showed an average of 3+.

DISCUSSION

Many studies have demonstrated the importance of T-cell responses in recovery and resistance to leishmaniosis. It is therefore of considerable importance to use T-cell responses to evaluate the immunostimulatory potential of *Leishmania* antigens (Russo *et al.* 1992). Glycolipid molecules which include LPG have also been shown to be presented to T-cells by a special subset of MHC class 1 proteins known as CD1 (Sugita, Moody, Jackman, Grant, Rosat, Behar, Peter, Porcelli & Brenner 1998;

Moody, Ulrich, Muhlecker, Young, Gurcha, Grant, Rosat, Brenner, Costello, Besra & Porcelli 2000; Sieling, Ochoa, Jullien, Leslei, Sabet, Rosat, Burdick, Rea, Brenner, Porcelli & Modlin 2000). Previous experiments in our laboratory have suggested the potential of using sera raised against LPG to inhibit parasite survival within the sand fly and to reduce *L. major* transmission to a new host (reviewed by Tonui 1999). Other attempts to vaccinate BALB/c mice using *L. major*-derived LPG have also been successful against *L. major* (Handman & Mitchell 1985; McConvile *et al.* 1987). Therefore, our study sought to determine whether such results could be extrapolated to visceral leishmaniosis caused by *L. donovani* using susceptible BALB/c mice and hamsters models.

In this study, the induction of cell-mediated immunity (CMI) following vaccination was determined by measuring delayed-type hypersensitivity, *in vitro* lymphocyte proliferation and gamma interferon (IFN-γ) production. LPG plus BCG-immunized animals developed positive DTH responses, which are commonly used as an indication of resistance against the disease (Carvalho, Badaro, Reed, Jones & Johnson 1985; Gajewski, Schell, Nau & Fitch 1989; White & MacMahon-Pratt 1990; Kemp, Kurtzhals, Bendtzen, Poulsen, Hansen, Koech, Kharazmi & Theander 1993; Kemp 1997). Lymphocytes from LPG plus BCG-immunized animals also proliferated on re-stimulation with the LPG antigen. This ability to induce proliferation was shown to be related to proteins associated with LPG, which enhance the antigenicity of purified LPG for human T-lymphocytes (Jardim *et al.* 1991; Mendonca *et al.* 1992). In this study, supernatants from re-stimulated lymphocytes expressed high levels of IFN-γ compared to IL-4 levels on re-stimulation with the LPG antigen. The production of high levels of IFN-γ is also a characteristic of resistance and subsequently protection to *Leishmania* infections (Kemp 1997).

In our experiments, mean body mass as a criteria for monitoring wasting and emaciation, and parasite loads for disease severity following infection with *L. donovani* were used. Results showed that hamsters previously immunized with LPG plus BCG showed an average mass loss compared to the PBS controls. BCG-immunized hamsters, on the other hand, showed smaller mass loss compared to the LPG plus BCG or the PBS controls. In all the experimental groups, wasting, emaciation and eventually death of animals accompanied mass loss by the 5th month. Surviving animals were killed and examination of their spleens showed high levels of parasitaemia in their smears and parasites on culture. In BALB/c mice, the highest number of surviving animals had been immunized with LPG plus BCG (60 %) compared to those, which had been immunized, with BCG alone (7 %) or PBS (30 %). On sacrificing them, five out of 9 (55.5 %) of the spleens from *L. donovani* infected LPG-immunized BALB/c mice were negative for parasites, while all cultures from the PBS and BCG treated mice yielded parasites (0 % negative). The parasite loads in LPG plus BCG-immunized mice were also lower compared to those previously immunized with BCG alone or PBS. From these experiments, it can be concluded that though BCG alone showed some partial protection to both BALB/c and hamsters this protection was short lived and the animals succumbed to the disease. In the group of the hamsters immunized with LPG plus BCG, 27 % did survive but this is not statistically significant as 20 % of those previously immunized with PBS survived. It is not clear why BCG conferred some protection in these experiments although antigenic cross-protectivity between *Mycobacteria* and *Leishmania* is known (Sharifi, Fekri, Aflatonian, Khamesipour, Nadim, Mousavi, Momeni, Dowlati, Godal, Zicker, Smith & Modabber 1998). Other studies have also shown that BCG alone accelerated resolution of both L. *major* and *L. donovani infections* in CBA/J mice or BALB/c (Weintraub & Weinbaum 1977; Connell, Medina-Acosta, McMaster, Bloom & Russell 1993; Streit, Recker, Donelson & Wilson 2000). This has also been obtained in human vaccine trials where a proportion of the volunteers who received BCG alone showed positive leishmanin skin conversion in the Sudan (Khalil, Hassan, Zijlstra, Mukhtar, Ghalib, Breyma, Ibrahim, Kamil, Elsheikh, Babiker & Modabber 2000).

Other experiments have shown that LPG provided excellent protection to *L. major* infections in BALB/c mice (Handman & Mitchell 1985; McConvile *et al.* 1987; Russell & Alexander 1988). Protection depended on the use of adjuvants such as liposomes or *Corynebacterium parvum* and on the integrity of the molecule. Not only was the water-soluble form of LPG lacking the glycosylphosphadidylinositol anchor not protective, but also it exacerbated the disease (Mitchell & Handman 1986). A purified water-soluble form of LPG was used in our study and this may explain the low immunogenicity and the partial protection observed in immunized rodents. These results suggest that other adjuvants apart from BCG should be considered in the pursuit of increasing the immunogenicity of this molecule in vaccination studies.

Currently, it is widely believed that there is no optimal animal model for studying *L. donovani* unlike in *L. major* where the BALB/c mouse has proved to be the best model. Though both the BALB/c and hamster models can be manipulated to define mechanisms of visceral disease they have a disadvantage of lacking many of the clinicopathological features observed in active human VL. However, the use of either model has it's own advantages. The disadvantage of using the BALB/c mouse or other susceptible mouse strains is that they develop acute infection with *L. donovani* but progressive clinical disease does not ensue. On the other hand, visceral parasitism in hamsters is uncontrolled which results in progressive disease and ultimately death (Pearson, Cox, Jeronio, Castrache, Drew, Evans & Eduardo de Alencar 1992). Therefore, it can be concluded that the hamster model is suitable for evaluating disease progression and severity whereas the BALB/c mouse is suitable for evaluating the correlates for protection.

In conclusion, although vaccination with LPG plus BCG induced a Th1 response, which correlated with protection, the disease course was not altered in hamsters and therefore protection was low and not significantly different compared to that of the BCG or PBS controls. Since the LPG molecule shows the presence of correlates of protection, the results obtained in these experiments agree with other published reports (Handman 2001) that it may be rewarding to re-evaluate the potential of LPG as a vaccine candidate in leishmaniosis.

ACKNOWLEDGEMENTS

This manuscript is published with the approval of the Director, Kenya Medical Research Institute (KEMRI). We thank Mr Charles Were, Mr Charles Magiri, Mr Jonathan Koskei, Mr Lucas Ogutu, the late Mr Kagoiya Mugo, Mr Kamwana Wambugu and Mr Haron Sang from KEMRI for their technical assistance. This study received financial support in part from the International Society for Infectious diseases (ISID), International Atomic Agency (IAEA) and the Kenya Medical Research Institute.

REFERENCES

CARVALHO, E.M., BADARO, R., REED, S.G., JONES, T.C. & JOHNSON, W.D. 1985. Absence of gamma-interferon and interleukin-2 production during active visceral leishmaniosis. *Journal of Clinical Investigations*, 76:2066–2069.

CHULAY, J.D. & BRYCESSON, A.D.M. 1983. Quantitation of amastigotes of *Leishmania donovani* in smears of splenic aspirates from patients with visceral leishmaniosis. *American Journal for Tropical Medicine and Hygiene*, 32:475–479.

CONNELL, N.D., MEDINA-ACOSTA, E., MCMASTER, W.R., BLOOM, B.R. & RUSSELL, D.G. 1993. Effective immunization against cutaneous leishmaniosis with recombinant Bacille Calmette-Guérin expressing the *Leishmania* surface proteinase gp63. *Proceedings of the National Academy of Sciences USA*, 90:11473–11477.

ELHAY, M., KELLEHER, M., BACIC, A., MCCONVILLE, M.J., TOLSON, D.L., PEARSON, T.W. & HANDMAN, E. 1990. Lipophosphoglycan expression and virulence in ricin-resistant variants of *Leishmania major*. *Molecular Biochemical Parasitology*, 40:255–268.

GAJEWSKI, F.T., SCHELL, R.S., NAU, G. & FITCH, W.F. 1989. Regulation of T-cell activation: difference among T-cell subsets. *Immunological Reviews*, 111:79–110.

GICHERU M., OLOBO, J., ANJILI, C., ORAGO, A.S., MODABBER, F. & SCOTT, P. 2001. Vervet monkeys vaccinated with killed *Leishmania major* parasites and interleukin-12 develop a Type1 immune response but are not protected against infection challenge. *Infection and Immunity*, 69:245–251.

HANDMAN, E. 2001. Leishmaniosis. Current status of vaccine development. *Clinical Microbiology Reviews*, 14:229–243.

HANDMAN, E., SYMONS, F.M., BALWIA, T.M., CURTIS, J. & SCHEERLINCK, J.P.Y. 1995. Protective vaccination with promastigote surface antigen 2 from *Leishmania major* is mediated by a TH1 type of immune response. *Infection and Immunity*, 63:4261–4267.

HANDMAN, E. & MITCHEL, G.F. 1985. Immunization with *Leishmania* receptor for macrophages protective against cutaneous leishmaniosis. *Proceedings of the National Academy of Sciences USA*, 82: 5910–5914.

INGONGA, P., MBATI, P.A., ANJILI, C.O., MUTANI, A., WISHITEMI, B., ODONGO, S., ROBERT, L.L. & GITHURE, J.I. 1996. The effect of immune sera from hamsters immunized with sand fly gut and whole body extract antigens on the fecundity and mortality of *Phlebotomus duboscqi* (Diptera: Psychodidae). *Acta Tropica*, 60:269–279.

JARDIM, A., TOLSON D.L., TURCO, S.J., PEARSON T.W. & OLAFSON, R.W. 1991. The *Leishmania donovani* lipophosphoglycan T-lymphocyte-reactive component is a tightly associated protein complex. *Journal of Immunoology*, 147: 3538–3544.

KEMP, M. 1997. Regulator and effector functions of T-cell subsets in human *Leishmania* infections. *Acta Pathologica, Microbiologica et Immunologica Scandinavica Suppl.*, 68: 1–33.

KEMP, M., KURTZHALS, J.A., BENDTZEN, K., POULSEN, L.K., HANSEN, M.B., KOECH, D.K., KHARAZMI, A. & THEANDER, T.G. 1993. *Leishmania donovani*-reactive Th1- and Th2-like T-cell clones from individuals who have recovered form visceral leishmaniosis. *Infection and Immunity*, 61: 1069–1073.

KEMP, M., THEANDER, T.G., HANDMAN, E., HEY, A.S., KURTZHALS, J.A.L., HVIID, L., SØRENSEN, A.L., WERE, O.B., KOECH, D.K. & KHARAZMI, A. 1991. Activation of human T-lymphocytes by *Leishmania* lipophosphoglycan. *Scandinavian Journal of Immunology*, 33:219–224.

KHALIL, E.A., HASSAN, A.M., ZIJLSTRA, E.E., MUKHTAR, M.M., GHALIB, H.W., BREYMA, M., IBRAHIM, M., KAMIL, A., ELSHEIKH, M., BABIKER, A. & MODABBER, F. 2000. Autoclaved *Leishmania major* vaccine for prevention of visceral leishmaniosis: a randomised, double-blind, BCG-controlled trial in Sudan. *The Lancet*, 356:1565–1569.

MBATI, P.A., ABOK, K., ORAGO, A.S., ANJILI, C.O., KAGAI, J.M., GITHURE, J.I. & KOECH, D.K. 1994. Establishment of appropriate inoculum dose of *Leishmania donovani* promastigotes required to establish a visceral infection in laboratory rodent models. *Africa Journal of Health Sciences,* 1: 165–168.

MBATI, P.A., ANJILI, C.O., LUGALIA, R., MWANYUMBA, P., TONUI, W.K., ROBERT, L.L. & GITHURE, J.I. 1995. Experimental immunization against cutaneous leishmaniosis using *Leishmania major* sub-cellular fractions alone or in combination with *Phlebotomus duboscqi* gut antigens. *East African Medical Journal*, 72:519–522.

MCCONVILLE, M.J., BACIC, A., MITCHELL, G.F. & HANDMAN, E. 1987. Lipophosphoglycan of *Leishmania major* that vaccinates against cutaneous leishmaniosis contain an alkylglycerophospholinositol lipid anchor. *Proceedings of the National Academy of Sciences USA*, 84:8941–8945.

MCNEELY, T.B. & DOYLE, P.S. 1996. Isolation of lipophosphoglycans from *Leishmania donovani* amastigotes. *Archives of Biochemistry and Biophysics*, 334:1–8.

MENDONCA, S.C., RUSSELL, D.G. & COUTINHO, S.G. 1991. Analysis of the human T-cell-responsiveness to purified antigens of *Leishmania*; lipophosphoglycan (LPG) and glycoprotein (GP63). *Clinical Experimental Immunology,* 83:472–478.

MITCHELL, G.F. & HANDMAN, E. 1986. The glycoconjugate derived from a *Leishmania major* receptor for macrophages is a suppressonic, disease–promoting antigen in murine cutaneous leishmaniosis. *Parasite Immunology*, 8:255–263.

MOODY, D.B., ULRICH, T., MUHLECKER, W., YOUNG, D.C., GURCHA, S.S., GRANT, E., ROSAT, J.P., BRENNER, M.B., COSTELLO, C.E., BESRA, G.S. & PORCELLI, S.A. 2000. CD1c-mediated T-cell recognition of isoprenoid glycolipids in *Mycobacterium tuberculosis* infection. *Nature*, 404:884–888.

ORLANDI, P.A. & TURCO, S.J. 1987. Structure of the lipid moiety of *Leishmania donovani* lipophosphoglycan. *Journal of Biological Chemistry*, 262:10384–10391.

PEARSON, R.D., COX, G., JERONIO, S.M.B., CASTRACHE, J., DREW, J.S., EVANS, T. & EDUARDO DE ALENCAR, J. 1992. Visceral leishmaniosis: a model for infection-induced cachexia. *American Journal for Tropical Medicine and Hygiene*, 47:8–15.

RUSSELL, D.G. & ALEXANDER, J. 1988. Effective immunization against cutaneous leishmaniosis with defined membrane antigens reconstituted in liposomes. *Journal of Immunology,* 140:1274–1279.

RUSSO, D.M., TURCO, S.J., BURNS, J.M. JR & REED, S.G. 1992. Stimulation of human T lymphocytes by *Leishmania* lipophosphoglycan-associated proteins. *Journal of Immunology,* 148:202–207.

SHARIFI, I., FEKRI, A.R., AFLATONIAN, M.R., KHAMESIPOUR, A., NADIM, A., MOUSAVI, M.R., MOMENI, A.Z., DOWLATI, Y., GODAL, T., ZICKER, F., SMITH, P.G. & MODABBER, F. 1998. Randomised vaccine trial of single dose of killed *Leishmania major* plus BCG against anthroponotic cutaneous leishmaniosis in Bam, Iran. *The Lancet,* 351: 1540–1543.

SIELING, P.A., OCHOA, M.T., JULLIEN, D., LESLEI, D.S., SABET, S., ROSAT, J.P., BURDICK, A.E., REA, T.H., BRENNER, M.B., PORCELLI, S.A. & MODLIN, R.L. 2000. Evidence of human CD4+ T-cells in the CD1-restricted repertoire: derivation of mycobacteria-reactive T-cells from leprosy lesions. *Journal of Immunology,* 164:4790–4796.

STREIT, J.A., RECKER, T.J., DONELSON, J.E. & WILSON, M.E. 2000. BCG expressing LCR1 of *Leishmania chagasi* induces protective immunity in susceptible mice. *Experimental Parasitology*, 94:33–41.

SUGITA, M., MOODY, D.B., JACKMAN, R.M., GRANT, E.P., ROSAT, J.P., BEHAR, S.M., PETER, S.J. PORCELLI, S.A. & BRENNER, M.B. 1998. CD1—a new paradigm for antigen presentation and T-cell activation. *Clinical Immunology and Immunopathology,* 87:8–14.

TONUI, W.K. 1999. *Leishmania* transmission-blocking vaccines: a review. *East African Medical Journal*, 76:93–96.

TONUI, W.K., MBATI, P.A., ANJILI, C.O., ORAGO, A.S., TURCO, S.J., GITHURE, J.I. & KOECH, D.K. 2001a. Transmission blocking vaccine studies in leishmaniosis. I: Lipophosphoglycan (LPG) is a promising transmission blocking vaccine candidate against cutaneous leishmaniosis *East African Medical Journal*, 78:84–89.

TONUI, W.K., MBATI, P.A., ANJILI, C.O., ORAGO, A.S., TURCO, S.J., GITHURE, J.I. & KOECH, D.K. 2001b. Transmission blocking vaccine studies in leishmaniosis: II. Effect of immunization using *L. major*-derived 63 kilo Dalton glycoprotein, lipophosphoglycan and whole parasite antigens on the course of *L. major* infection in BALB/c mice. *East African Medical Journal*, 78:90–92.

TONUI, W.K., NGUMBI, P., MPOKE S., ORAGO, A.S., MBATI, P.A., TURCO, S.J. & MKOJI, G.M. 2003. *Leishmania major-Phlebotomus duboscqi* interactions: inhibition of anti-LPG antibodies and characterization of two proteins with shared epitopes. *East African Medical Journal*, in press.

TURCO, S.J. 1988. The lipophosphoglycan of *Leishmania. Parasitology Today,* 4:255–257.

VOLLER, A., BARTLETT, A. & BIDWELL, D.E. 1976. Enzyme-linked immunoassays for parasitic disease. *Transactions of the Royal Society for Tropical Medicine and Hygiene,* 70:98–106.

WEINTRAUB, J. & WEINBAUM, F.I. 1977. The effect of BCG on experimental cutaneous leishmaniosis in mice. *Journal of Immunology,* 118:2288–2290.

WHITE, A.C., JR & MCMAHON-PRATT, D. 1990. Prophylactic immunization against experimental *Leishmania donovani* infection by use of a purified protein vaccine. *Journal of Infectious Diseases* 161:1313.

YANG, D.M., ROGERS, M.V. & LIEW, F.Y. 1991. Identification and characterization of host-protective T-cell epitopes of a major surface glycoprotein (gp63) from *Leishmania major. Immunology*, 72:3–9.