CHAPTER 6

EFFICACY OF LIPOPHOSPHOGLYCAN AGAINST EXPERIMENTAL VISCERAL LEISHMANIASIS
INTRODUCTION:

Protozoan parasite of the genus *Leishmania* affects mammalian hosts, and causes a wide spectrum of diseases, depending on the host and on the *Leishmania* species involved. In humans, the infection ranges from self healing lesions to disseminated cutaneous disease or highly destructive mucosal lesions and from asymptomatic infection to fatal visceral dissemination, causing one of the World's major health problems.

Lipophosphoglycan (LPG) is one of the major *Leishmania* cell surface glycoconjugate and has been reported to exist on the surface of all *Leishmania* species of promastigote stage (Turco and Descoteaux, 1992) and also on the surface of *L. major* amastigotes (Glaser et al., 1991, Moody et al., 1993) and the flagellar pocket of *L. mexicana* (Bahr et al., 1993). The abundance and presence of LPG throughout the various stages of the life cycle of parasite suggest that it plays an important role in the biology and infectivity of *Leishmania* organisms. (Tolson et al., 1990). Several studies have examined LPGs potential as a chemically defined vaccine against cutaneous leishmaniasis. Administration of immunoaffinity purified *L. major* LPG along with adjuvant *C. parvum*, to genetically resistant mice was found to induce full protection against challenge with promastigotes whereas, partial protection was observed in susceptible mice (Handman and Goding 1985; Handman and Mitchell, 1985; McConville et al., 1987). Similar protection against *L. mexicana* in CBA/Ca mice was also reported using homologous LPG reconstituted into liposomes (Russell and Alexander, 1988: Kahl et al., 1990). However, it has been recently found that LPG purified by immunoaffinity is heavily contaminated with peptide. Mendonca et al. (1991) reported that T-lymphocytes from cutaneous leishmaniasis patients respond to purified *L. braziliensis* LPG, whereas proteinase K-treated LPG did not stimulate any response. Subsequent T-cell proliferation studies showed that the LPG-associated proteins were potent stimulators of T-cells in leishmaniasis patients (Moll et al., 1989; Jardim et al., 1991; Russo et al., 1992) as well as in mice immunized with protein contaminated LPG (Jardim et al., 1991). In view of these observations the mechanism of protection and use of LPG as a vaccine against leishmaniasis is open to question. Furthermore, use of LPG in protecting against visceral leishmaniasis has not yet been evaluated.

Studies on the potential of promastigote LPG in providing protection against *L. donovani* infection in susceptible golden hamsters was carried out. The results demonstrate that pretreatment of hamsters with LPG provides significant protection against *L. donovani* infection. Furthermore, the protective efficacy of LPG molecules was found to be significantly enhanced on its incorporation in cholesterol containing phosphatidylcholine liposomes.
MATERIALS AND METHODS:

Isolation and purification of egg phosphatidylcholine (PC):
The egg yolk was collected in grinder from one dozen egg and to this acetone (150-200 ml) was added. This was mixed properly and filtered. Filtrate was discarded and solute was again treated with acetone. This step was repeated 9-10 times until filtrate became colourless or materials looked like white powder. It was dried in vacuo for 1.0 hr. The dried material was taken in round bottom flask and added 1.0-1.5 litre of absolute alcohol and kept on magnetic stirrer for 1-1.5 hr, filtered it and filtrate was evaporised at 40 - 45°C. The residue was dissolved in minimum amount of petroleum ether (b.p 60-80°C) and precipitated with chilled acetone. The solvent from precipitated material was removed by decantation. The precipitate was dissolved again in petroleum ether immediately to avoid oxidation of egg phosphocholine. Finally, it was precipitated with chilled acetone and all precipitate was dissolved in minimum amount of chloroform and rotavaporised, dried under vacuum and stored at -20°C. This dried material was passed through column chromatography over neutral alumina (Grade III) using increasing amounts of methanol in chloroform as the eluent. Elution with 7-10% methanol in chloroform gave pure egg PC. Fractions were analyzed by thin layer chromatography using silica gel G-60 TLC plates. The plates were developed in chloroform/methanol/water (65:25:4) and the spot was identified by staining them with iodine vapour followed by molybdenum-blue spary (Goswami and Frey, 1971).

Preparation of lipid liposomes:
Liposomes were prepared by the method of Gupta and Bali, 1981. using egg phosphocholine in presence or absence of cholesterol. Egg PC and cholesterol were taken in the round bottom flask and dissolved in minimum amount of chloroform. The solvent was evaporated in a rotavapor resulting in formation of a lipidfilm. For complete dryness, it was kept under vacuum for 1.0 hr. A ratio of 1 μg LPG to 1.0 mg phospholipid and 15% cholesterol of lipid was maintained in all experiments.

Neutral liposomes: Neutral liposomes were prepared using neutral phospholipid and cholesterol.

Multilamellar vesicles: Multilamellar vesicles (MLV) were prepared as described earlier (Senior and Gregoriadis, 1982; Woeff and Gregoriadis, 1984). Lipid film was prepared as mentioned above. The film was dissolved in 50 mM PBS (pH 7.4) vortexed and sonicated at 20°C for 30 min using water bath sonicator. The MLV was used immediately for immunization experiment.

Small Unilamellar vesicles: Small unilamellar vesicles (SUV) were prepared through probe
sonication of MLV in the presence of ice for 30 min or more, with 50 pulse cycle in 3 Hz frequency. Then preparations were centrifuged at 30,000×g for 15-20 min and collected supernatant. Again small unilamellar vesicles were washed with PB5 (50 mM, pH 7.4) by centrifugation at 30,000×g. These preparations were used immediately for immunization, although storage at -20°C did not appear to alter their stability.

Negatively charged liposomes: Multilamellar and unilamellar vesicles were prepared as described above. 7% dicetylphosphate to phospholipid was added during the preparation of film. A ratio of 1 μg LPG to 1.0 mg phospholipid (egg PC) and 15% cholesterol of lipid was maintained in all preparations.

Positively charged liposomes: All preparations were prepared as mentioned above. Stearylamine (14%) of lipid (w/w) was added during the preparations of film. All preparations were used immediately for immunization.

Incorporation of LPG into liposomes:

LPG was incorporated into the liposomes by dissolving the film in 50 mM PBS (pH 7.4) followed by adding LPG stock as required amount before sonication. LPG incorporation was different in various liposomised preparations. Small unilamellar vesicles were incorporated more LPG as compared to multilamellar vesicles. The percent of incorporation varied between 85-95%.

**IN VIVO EFFICACY OF LIPOSOMISED LPG:**

For evaluating immunization: immunoprophylactic efficacy of liposomised LPG, various protocols were tried, which are summarized below.

**Protocol-1:**

Male syrian golden hamsters (35-45 gm) were given intraperitoneal injection of LPG alone (10 μg of LPG/animal) and in various liposome incorporated forms, like (SUV) and (MLV) liposomes. Each group was subdivided into two groups, which consisted of 5-7 animals group. On day 21 post LPG administration, the hamsters were challenged with 1x10^7 promastigotes of infective *L. donovani* strain Dd8. One set of subgroup was immunized again after 14 days (booster dose). The animals were sacrificed on day 45 and/or 60 post infection and the percent infected splenic macrophages were counted and percent protection was calculated.
Percent protection (PP) = \( \frac{N_1 - N_2}{N_1} \times 100 \)

Where \( N_1 \) is the number of amastigotes 100 cells in control, \( N_2 \) is the number of amastigotes 100 cells in the treated group of animals.

Protocol-II:

Initially three groups were taken and subdivided into two groups. Each sub group contained 7 animals. Different concentration of LPG (10, 20, 30 \( \mu \)g/animal) incorporated in small unilamellar vesicles in presence or absence of cholesterol were inoculated into hamsters by intraperitoneal route and challenged with \( 1 \times 10^7 \) promastigotes 21 days after LPG challenge. Percent protection was calculated as mentioned above.

RESULTS:

LPG provides protection against \( L. \) donovani infection in susceptible golden hamsters:

The potential of LPG in protecting susceptible golden hamsters against \( L. \) donovani infection was studied by administration of a single dose of 10 \( \mu \)g/animal of purified LPG alone or incorporation in various liposomised preparations, 21 days prior to infection. The percent protection against \( L. \) donovani infection in susceptible golden hamsters on pretreatment with LPG and its liposomised preparations is summarized in figure 1. Pretreatment of animals with LPG alone or in various liposomised form provided significant protection against \( L. \) donovani infection. The group of animals receiving LPG alone showed a relatively low protection of about 22 percent. This protective efficacy of LPG was found to be significantly enhanced on its incorporation into liposomes. For LPG incorporated in cholesterol containing multilamellar liposomes about 35 percent protection was observed. Incorporation of LPG in unilamellar vesicles enhanced the protecting efficacy to 41 percent. A maximum protection of about 48 percent was observed for LPG incorporated in cholesterol containing unilamellar liposomes, which is more than double of that observed for LPG alone. These observations suggest that small unilamellar vesicles (SUV) in presence of cholesterol responded better as compared to other preparations.

Optimization of conditions for pretreatment with LPG:

Effect of variation of pretreatment schedule with liposomized LPG on the protection against \( L. \) donovani infection in hamsters was studied. Figure 2 shows the protection compared to control observed in animals treated with a constant dose (10 \( \mu \)g/animals) on day 2, 10, 14, 21 and 28 prior to infection. A low protection of about 32 percent compared to control
Figure 1: Protection against *L. donovani* infection in Syrian golden hamsters on pretreatment with LPG alone and its various liposomised forms. The LPG concentration in all the preparations was kept constant at 10 μg/animal. Values are represented as mean ±SE (n=20). The various abbreviations used are as follows: LPG MLV = LPG incorporated in cholesterol containing multilamellar vesicle; LPG SUV = LPG incorporated in unilamellar vesicle and LPG: SUV CH = LPG incorporated in cholesterol containing unilamellar vesicle.
Figure 2: The effect of various pretreatment schedules using liposomised LPG (10 μg/animal) on the protection against *L. donovani* infection in susceptible golden hamsters. The protection was compared to control with treated animals on day 2-28 prior to infection. Values are represented as mean ± SE (n=15)
was observed for day 2 pretreatment with liposomised LPG. This was found to be enhanced significantly to about 41 and 48 percent on increasing the LPG pretreatment time to day 10 and 21, respectively, prior to infection. The results indicate that pretreatment of animals with liposomised LPG anywhere between 20 to 30 days is optimum for getting maximum protection against L. donovani infection. The effect of multiple doses of LPG alone or its various liposomised preparations was also studied. Two doses 10 μg animal each of LPG was administered in a gap of 14 days. The results of the studies are summarized in Fig. 3. Although the booster dose of LPG incorporated in various liposomised preparations showed a slight enhancement in percent protection but almost no change was observed in case of LPG alone. The percent protection observed with liposomised preparations by the administration of booster dose was increased about 1.15 fold. The study suggests that single dose of LPG administration prior to challenge with promastigotes was sufficient in providing optimum protection against L. donovani infection. Effect of intraperitoneal and intracardial administration of LPG preparations on efficacy was also studied. A higher percent protection by intracardial administration as compared to intraperitoneal route was observed. Although administration by intracardial route was found to provide better protection than intraperitoneal route but intraperitoneal route is preferred because the intracardial administration of any vaccine/chemoprophylactic agent is risky, difficult as well as it requires expertise hand to perform it. Furthermore, the intraperitoneal administration is easy to perform, well accepted by others and risk factors are low as compared to intracardial route.

Dose dependent LPG mediated protection against L. donovani infection in hamsters: Studies on concentration dependence of liposomised LPG in protecting hamsters against L. donovani infection was carried out for optimization of dose required to give maximum protection. 10, 20 and 30 μg LPG was incorporated in liposomes and administered intraperitoneally in golden hamsters 21 days prior to infection. A dose dependent protection by liposomised UR6 LPG was observed (Table 1 and Figure 4). For increase in concentration of LPG incorporated in SUV from 10 to 20 μg/animal, an increase in percent protection from 50 to 68 percent was observed. Further increase of LPG concentration to 30 μg/animal showed no significant enhancement in percent protection. These results indicate that single administration of 20 μg of LPG incorporated in cholesterol containing SUV is the most effective dose for achieving maximum protection by UR6 LPG against L. donovani infection in susceptible golden hamsters. Liposomes injected intravenously into the blood circulation are readily taken up by the mononuclear phagocyte system (Gregoriadis, 1988; Raz et al., 1981; PerezSolar et al.,
Figure 3: The effect of multiple doses of LPG alone or its various liposomised preparations. Susceptible golden hamsters were pretreated with LPG and its various liposomised preparations on day 14 prior to infection with *Leishmania donovani* promastigotes of strain Dd8. The percent protection compared to control in these treated group of animals was calculated on day 45 post infection. Values are represented as mean ±SE (n=10). The various abbreviations used are as follows: LPG MLV = LPG incorporated in cholesterol containing multilamellar vesicle; LPG SUV = LPG incorporated in unilamellar vesicle and LPG SUV CH = LPG incorporated in cholesterol containing
Figure 4: The protection against *L. donovani* infection in susceptible golden hamsters on pretreatment with various dose of liposomised LPG incorporated in SUV in presence and absence of cholesterol. Syrian golden hamsters were administered a single dose of desired concentration of LPG in SUV, 21 days prior to infection with *L. donovani* promastigotes of strain Dd8. The results presented were obtained on day 45 post infection by sacrificing animals. Values are represented mean ±SE (n=15). The various abbreviations used are as follows: LPG SUV = LPG incorporated in unilamellar vesicle and LPG SUVCH = LPG incorporated in cholesterol containing unilamellar vesicle.
TABLE 1:

Protection against *L. donovani* infection in susceptible syrian golden hamsters on pretreatment with varying dose of UR6 LPG incorporated in unilamellar liposome.

<table>
<thead>
<tr>
<th>LPG concentration in liposomes</th>
<th>Mean number of amastigotes /100 splenic macrophages</th>
<th>Protection (%)</th>
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<tbody>
<tr>
<td>1. Control</td>
<td>25.38±3.2</td>
<td>-</td>
</tr>
<tr>
<td>2. 10 µg/animal</td>
<td>12.56±1.22</td>
<td>50</td>
</tr>
<tr>
<td>3. 20 µg/animal</td>
<td>8±0.92</td>
<td>68</td>
</tr>
<tr>
<td>4. 30 µg/animal</td>
<td>7.56±1.13</td>
<td>69</td>
</tr>
</tbody>
</table>

Values represent mean±SE (n=15).

Syrian golden hamsters were given single i.p. injection of desired concentration of LPG incorporated in cholesterol containing unilamellar liposomes 21 days prior to infection with *L. donovani* promastigotes of strain Dd8. The results were obtained on day 45 post infection by sacrificing animals.
1985). It has been suggested that negatively charged MLVs are more readily taken up by murine peritoneal macrophages (Mehta et al., 1982) and human peripheral blood monocytes (Mehta et al., 1984). Hence, studies using negatively and positively charged liposomes were also carried out. 20 and 30 µg of LPG were incorporated both in negatively charged (Diocetylphosphate) and positively charged (stearylamine) egg PC SUV and MLV liposomes. The results of these studies are summarized in Table 2. With negatively charged liposomes showed no significant change in percent protection as compared to neutral liposomes was observed.

DISCUSSION:

The importance of T-cell responses in recovery and resistance to leishmaniasis is well demonstrated. It is therefore, of considerable importance to use these responses to evaluate the immunostimulatory potential of Leishmania antigens. LPG is the major surface glycoconjugate of all Leishmania promastigotes and has been reported to be both immunogenic and antigenic. Hence the induction of immune responses using LPG might be an effective approach to inhibit parasite survival and replication in the mammalian host. LPG has been used successfully to immunize mice against cutaneous infection with L. major (Handman and Mitchell, 1985; McConville et al., 1987), as well as to elicit T-cell response in leishmaniasis patients (Kemp et al., 1991; Mendonca et al., 1991). Mice immunized with LPG produce LPG specific IgG and increases frequency of L. major reactive T-cells, although these cells do not respond to LPG in vitro (Moll et al., 1989). Mendonca et al. (1991) have demonstrated that LPG stimulated PBMC in cutaneous leishmaniasis patients, but that treatment of LPG molecule with proteinase K abolished its stimulatory activity. It has been demonstrated that protein copurified with LPG was responsible for the observed T-cell stimulation (Russo et al., 1992). LPG which is associated with protein contaminants elicited in vitro proliferative responses and IFN-γ production in cutaneous, mucocutaneous and cured visceral leishmaniasis patients. However, protein free LPG and repeating disaccharide units of LPG did not stimulate LPG specific T-cell (Russo et al., 1992). Similarly, purified glycolipids are weak stimulator whereas, the protein associated glycolipid was found to be high stimulator of T-Cell response. A LPG associate protein (Mr 11000) was isolated and it was found that lymphocyte stimulation was associated with the protein component of LPG and not the glycan (Jardim et al., 1991; 1995; Pimenta et al., 1994).

After establishing that LPG from L. donovani promastigotes (strain UR6) inhibits attachment of promastigotes of strain Dd8 to macrophages and also provide protection to macrophages against L. donovani infection (Ali et al., 1995), we set out to explore whether pretreatment of hamsters with UR6 LPG can provide protection against L. donovani infection. Pretreatment of hamsters with UR6 LPG or its liposomized preparation anywhere between 10 to
TABLE 2:

Protection against *L. donovani* infection in susceptible syrian golden hamsters on pretreatment with 20 μg/animal single administration of UR6 LPG alone or on incorporation in liposomes.

**A: Neutral liposomes**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean number of amastigotes /100 splenic macrophages</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 45</td>
<td>Day 60</td>
</tr>
<tr>
<td>1. Control</td>
<td>26-2.2</td>
<td>41.22+2.8</td>
</tr>
<tr>
<td>2. LPG alone</td>
<td>19-1.7</td>
<td>27</td>
</tr>
<tr>
<td>3. MLV</td>
<td>25-1.9</td>
<td>-</td>
</tr>
<tr>
<td>4. MLV LPG</td>
<td>15.5-2.0</td>
<td>26.3+1.2</td>
</tr>
<tr>
<td>5. SUV</td>
<td>25-1.2</td>
<td>-</td>
</tr>
<tr>
<td>6. SUV LPG</td>
<td>9.5-1.6</td>
<td>17.66+1.3</td>
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**B: Negatively charged liposomes:**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean number of amastigotes /100 splenic macrophages</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 45</td>
<td>Day 60</td>
</tr>
<tr>
<td>1. Control</td>
<td>28-3.2</td>
<td>42.33+2.8</td>
</tr>
<tr>
<td>2. MLV</td>
<td>28-1.9</td>
<td>-</td>
</tr>
<tr>
<td>4. MLV LPG</td>
<td>19.2-1.2</td>
<td>30.33+1.24</td>
</tr>
<tr>
<td>5. SUV</td>
<td>27.3+1.7</td>
<td>-</td>
</tr>
<tr>
<td>6. SUV LPG</td>
<td>9.5-1.6</td>
<td>15.66+1.6</td>
</tr>
</tbody>
</table>

a: Egg phosphatidylcholine liposomes containing cholesterol.
b: Egg phosphatidylcholine liposomes containing cholesterol and dicetyl phosphate.
Values are represented as mean ± SE (n=20).
The various abbreviations used are as follows: MLV = multilamellar liposomes; SUV = unilamellar liposomes; MLV LPG = LPG incorporated in cholesterol containing multilamellar liposomes and SUV LPG = LPG incorporated in unilamellar liposomes.

Susceptible Syrian golden hamsters were pretreated with LPG and its various liposomised forms on day 21 prior to infection with L. donovani promastigotes of strain Dd8. The percent protection compared to control was calculated on day 45 (by spleen biopsy) and on day 60 post infection by killing the respective animals of the groups.