THE IMMUNOCHEMISTRY AND IMMUNOBIOLOGY OF
LEISHMANIA DONOVANI LIPOPHOSPHOGLYCAN

by

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mimeograph or other means, without the permission of the author.
Using intact *Leishmania donovani* promastigotes or purified *L. donovani* lipophosphoglycan (LPG) as immunogens, four LPG-specific monoclonal antibodies (mAbs) were derived. Two of these mAbs (CA7AE and BF9CC) specifically bound to an epitope consisting of the repeating phosphorylated Gal-(1,4)-Man disaccharide portion of the LPG molecule. MAb CA7AE also bound antigens in *L. donovani* promastigote-conditioned culture medium; specifically, the secreted forms of LPG (phosphoglycan) and acid phosphatase, demonstrating that major secreted glycoconjugates of *L. donovani* share phosphorylated carbohydrate epitope(s). The two other mAbs (L157 and L98) bound to a parasite-derived protein component that was discovered to be tightly associated with the phosphocarbohydrate core region of the LPG molecule (LPG-associated protein; LPGAP). These are the first defined epitopes of LPG.

Immunochemical assays were used to analyze the distribution of the LPG repeat and LPGAP epitopes over a wide sampling of *Leishmania* species and strains. MAb CA7AE recognized, to varying extents, epitopes from most of the *Leishmania* species examined; both as parasite surface-exposed, membrane-bound molecules and as antigens released into parasite-conditioned culture medium. The anti-LPGAP mAbs bound to all twenty *Leishmania* and *Trypanosoma* strains assayed but not to the surface of living parasites. None of the anti-LPG mAbs bound the amastigote form of the parasites. Experiments involving amastigote-to-promastigote in vitro transformation showed that the CA7AE epitope was expressed on the surface of transforming cells within 5 hours of culture at 26°C. The epitope was excreted into the culture supernatant within 15 hours. In addition, the mAb CA7AE epitope was detected in 50% of sera tested from *L. donovani*-infected (Kala-azar-positive) patients.

Murine macrophages, infected with *L. donovani* promastigotes, were examined by immunofluorescence for the expression of LPG epitopes. The CA7AE epitope, detected as early as 5-10 minutes post infection (p.i.), was initially localized to the immediate area of internalization of the promastigote into the macrophage with even distribution over the entire macrophage surface by 25 minutes p.i. These epitopes remained on the macrophage surface until approximately 88 hours p.i. Acetone permeabilization of the macrophages, prior to mAb probing, exposed LPG epitopes present within the macrophages to at least 160 hours p.i. Treatments which inhibited
macrophage phagolysosomal degradation processes had no effect on epitope expression whereas reagents that affected macrophage membrane flow, and thus phagocytosis, drastically reduced or abolished expression. Purified LPG or de-lipidated LPG were also shown to bind to a variety of different cell types but in a temperature-independent manner. The early and continued expression of LPG epitopes on the macrophage surface suggests the possibility that LPG epitopes may be involved in the immune response which is directed to Leishmania-infected macrophages.

Lymph node cells from mice primed with L. donovani LPG or LPGAP or with living, virulent L. donovani promastigotes were specifically stimulated to proliferate in vitro by the LPGAP moiety of the LPG molecule. The T cell response was antigen-specific, dose-dependent, and non-mitogenic. In addition, purified T lymphocytes primed with purified LPG or LPGAP were not stimulated by LPG or LPGAP in vitro unless promastigote-infected or LPG-pulsed or LPGAP-pulsed macrophages were added. Recognition of LFGAP epitopes was an MHC-restricted event. LPGAP epitopes specifically stimulated CD4+CD8-, IL-2 secreting T lymphocytes and that active antigen processing by macrophages was required for T cell stimulation. Both L. donovani LPG and L. tropica LPG which have antigenically different repeat epitopes but which share LPGAP epitopes stimulated lymphocyte proliferation independent of the LPG source used for priming. The T cell stimulation caused by LPGAP was not species-specific and since the responding T cells were of the Th1 phenotype and recognized epitopes in amastigotes, the LPGAP epitopes are very likely of considerable importance in Leishmania-specific immunity. The data suggests that Leishmania LPGAP is a potential vaccine candidate for leishmaniasis.
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DLT
Thought, I love thought.

But not the jiggling and twisting of already existent ideas

I despise that self-important game.

Thought is the welling up of unknown life into consciousness,

Thought is the testing of statements on the touchstone of the conscience,

Thought is gazing on to the face of life, and reading what can be read,

Thought is pondering over experience, and coming to a conclusion.

Thought is not a trick, or an exercise, or a set of dodges,

Thought is a man in his wholeness wholly attending.*

*D.H. Lawrence, "Thought"
GENERAL INTRODUCTION

*Leishmania* parasites have a digenetic life cycle that includes both intracellular amastigotes and extracellular promastigotes. In the mammalian host the initial interaction is between promastigotes and macrophages. Recent evidence has demonstrated the importance of the leishmanial major surface glycoconjugate, lipophosphoglycan (LPG), in this interaction. In addition, LPG has been implicated to play a variety of roles in other parasite-host immunobiological interchanges. Therefore, it is important that parasite molecules such as LPG be identified, characterized and their importance with respect to parasite biology be examined. The resulting information will be invaluable for our understanding of basic host-parasite relationships and for their application to medicine via improved vaccination and epidemiological control of this serious parasitic disease.

1. Biology of *Leishmania* parasites and pathology of Leishmaniasis.

1.1 Overview.

The *Leishmania* parasites are protozoans that exist as several distinct species and cause a wide spectrum of disease phenotypes. The life cycle of the leishmanial parasite is relatively simple. The organisms multiply in mononuclear phagocytes of the mammalian host and in the gut lumen of the sandfly vector. In the sandfly they exist as free or attached flagellated promastigotes. The promastigotes are introduced into the mammalian host by the bite of an infected sandfly and are soon taken up by macrophages, where they differentiate into intracellular amastigotes which lack flagella.

The mechanisms and molecules involved in the recognition and uptake by macrophages are areas of intense study. The survival and replication of the intracellular parasites are probably dependent on differentiation into amastigotes but clearly there are other factors involved as well, including the parasite's ability to evade killing by the phagolysosomal system of the host macrophage. Leishmanial surface molecules are important in host-parasite and vector-parasite interactions and possibly play a role in the spectrum of disease manifestations and virulence properties characteristic of the different *Leishmania* species. Much research on the biochemistry and immunology of *Leishmania* has been performed and, as with many other parasites, the work has been done with a variety of species. Perhaps *L. donovani* is the most important of these since it causes a debilitating and often fatal visceral disease.
1.2 Old and New World species distribution and disease manifestations.

Leishmaniasis is the general name given to a spectrum of debilitating, chronic diseases caused by infection with parasites of the genus *Leishmania*. Collectively, the *Leishmania* spp. are responsible for economically and socially devastating diseases in the developing world and for this reason, leishmaniasis has been included among the six major parasitic diseases targeted for intensive research and control effects by the UNDP/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases. No reliable estimate of the global magnitude of the problem is available but as many as 20 million people are thought to be infected and conservative estimates suggest that more than 400,000 new cases occur each year (World Health Organization 1984). Leishmaniasis is endemic on five continents, with the exclusion of Australia and Antarctica, and higher prevalence occurs towards the equator (see Figure 1).

The classification of *Leishmania* infecting man was originally based on the disease types occurring in the patients from whom the parasites had been isolated: 1) *L. tropica* (cutaneous leishmaniasis), 2) *L. braziliensis* (mucocutaneous leishmaniasis), and 3) *L. donovani* (visceral leishmaniasis) (Handman 1986). However, this system was soon found to be inadequate since it was realized that the clinical manifestations of disease are the expression of both genetic variability of the host and of the parasite (Handman 1986). Recently, criteria based on intrinsic properties of the organism have allowed a more useful classification of *Leishmania*. These criteria include isoenzyme variant type, nuclear and kinetoplast DNA analysis by restriction fragment length polymorphism, pulsed field gradient gel electrophoresis, cDNA and genomic sequencing, and monoclonal antibodies to specific parasite antigens (reviewed in Alexander and Russell 1985; Wirth et al. 1996).

The complexity of the clinical manifestations of leishmaniasis and the inadequate taxonomic classification of the organisms led in the 1960s to the concept of a disease spectrum analogous to the situation in leprosy (Bloom and Godal 1983, Handman 1986). This concept correlates various clinical forms of the disease with a spectrum of histopathological changes in the host that correspond to different levels of immunoreactivity as measured by delayed-type hypersensitivity. The diseases are generally classified, on clinical grounds, into four main complexes (see Table 1):

Parasites of the *L. tropica* complex (*L. tropica*, *L. major*, *L. aethiopica*) are known to cause cutaneous leishmaniasis throughout the Mediterranean region, the Middle East, the southern Soviet Union and parts of Africa. Infection is self-limiting and begins with a small lesion at the site of the sand fly bite. The lesion ulcerates, either quickly in the case of *L. major* or slowly in *L. tropica* infections. Lesions usually heal over a period of
Figure 1. Distribution of leishmaniasis worldwide. A. Visceral leishmaniasis. B. Cutaneous and mucocutaneous leishmaniasis. (World Health Organization 1984).
<table>
<thead>
<tr>
<th>Species</th>
<th>Disease</th>
<th>Tendency to self-cure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Old World species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. donovani</em></td>
<td>V</td>
<td>None</td>
</tr>
<tr>
<td><em>L. d. infantum</em></td>
<td>V</td>
<td>None</td>
</tr>
<tr>
<td><em>L. major</em></td>
<td>C</td>
<td>Rapid</td>
</tr>
<tr>
<td><em>L. tropica</em></td>
<td>C</td>
<td>Slow</td>
</tr>
<tr>
<td><em>L. aethiopica</em></td>
<td>DC</td>
<td>Slow</td>
</tr>
<tr>
<td><strong>New World species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. d. infantum</em></td>
<td>V</td>
<td>Rare</td>
</tr>
<tr>
<td><em>L. mexicana pifanoi</em></td>
<td>C</td>
<td>Variable</td>
</tr>
<tr>
<td><em>L. m. amazonensis</em></td>
<td>C, DC</td>
<td>Variable</td>
</tr>
<tr>
<td><em>L. m. mexicana</em></td>
<td>C</td>
<td>Variable</td>
</tr>
<tr>
<td><em>L. m. venezuelensis</em></td>
<td>C</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. m. garnhami</em></td>
<td>C, DC</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. aethiopica</em></td>
<td>DC</td>
<td>Slow</td>
</tr>
<tr>
<td><em>L. braziliensis braziliensis</em></td>
<td>MC</td>
<td>None</td>
</tr>
<tr>
<td><em>L. b. panamensis</em></td>
<td>C</td>
<td>Variable</td>
</tr>
<tr>
<td><em>L. b. peruviana</em></td>
<td>C</td>
<td>Mostly</td>
</tr>
<tr>
<td><em>L. b. guayanensis</em></td>
<td>C</td>
<td>Variable</td>
</tr>
<tr>
<td><strong>Other species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. enriettii</em></td>
<td></td>
<td>- guinea pigs</td>
</tr>
<tr>
<td>(non-human pathogens)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. naiffi</em></td>
<td></td>
<td>- 9-banded armadillo</td>
</tr>
<tr>
<td><em>L. tartenolae</em></td>
<td></td>
<td>- lizards</td>
</tr>
</tbody>
</table>

* Based on information compiled from the World Health Organization (1984), Alexander and Russell (1985) and Wirth et al. (1986).

1 V, visceral leishmaniasis; C, cutaneous leishmaniasis; DC, diffuse cutaneous leishmaniasis; MC, mucocutaneous leishmaniasis,
Infection by parasites of the *L. donovani* complex (*L. d. donovani, L. d. infantum, L. d. chagasi*) results in a systemic disease found in Africa, Asia and South America. There is an involvement of the liver, spleen and bone marrow with hyperplasia of reticuloendothelial cells in these organs resulting in the characteristic hepatosplenomegaly of Kala-azar. As the disease progresses an increasing hyperplastic response results in anaemia, leukopaenia and other symptoms. Kala-azar is usually fatal if not treated. Occasionally there is skin involvement, the Post-Kala-azar cutaneous leishmaniasis syndrome, which may occur after apparently successful drug treatment of visceral disease. (World Health Organization 1984).

New World cutaneous leishmaniasis can be divided into two complexes: those diseases caused by parasites of the *L. mexicana* complex and those caused by the *L. braziliensis* group. *L. mexicana* causes a cutaneous lesion, very similar to that caused by *L. major*, that is usually localized but which may spread along lymphatics to involve greater areas of the skin (diffuse cutaneous leishmaniasis). The most devastating of the *L. braziliensis* group is the subspecies *L. b. braziliensis* which also metastasizes, but many years after healing of the primary cutaneous lesion, and causes a condition known as mucocutaneous leishmaniasis. Spread of the infection throughout the oronasopharyngeal regions results in disfiguring erosion of the nasal septum and palate with destruction of cartilage and mucous membranes. (World Health Organization 1984).

1.3 **Treatment of leishmaniasis.**

a) **Chemotherapy.**

Biochemically logical drug development involves recognition of parasite-specific biochemical pathways and synthesis of drugs that act on those pathways. The standard first-line anti-leishmanial chemotherapeutic agent, due to its high cure rate and the low level of toxicity, is pentavalent antimony complexed to carbohydrate in the form of sodium stibogluconate (Pentostam) or meglumine antimonate (Glucantime) (World Health Organization 1984; Berman 1988). Pentavalent antimony is believed to work through the inhibition of amastigote bioenergetics. One mechanism of action is thought to be by inhibition of glycolytic and fat oxidation pathways, since glucose metabolism of *Leishmania* parasites is unusual in that many glycolytic enzymes are contained in a subcellular organelle, the glycosome (reviewed in Berman 1988).

The other commonly used drugs for leishmaniasis are amphotericin B and pentamidine (World Health Organization 1984). Both pentamidine and amphotericin B
have demonstrated efficacy in Kala-azar, and amphotericin B is very effective in mucosal leishmaniasis (Berman 1988). However, the use of these agents is limited by the need for parenteral administration and by their severe toxicity and relatively high relapse rates (Berman 1988). Such side effects are presumably due to the interaction of the drugs with the different cell types of the host, including those not harboring the parasites. These drugs are generally used when antimony is not available or in antimony-nonresponsive disease (World Health Organization 1984).

Cutaneous leishmaniasis, due to the *L. tropica* complex in the Old World and to the *L. mexicana* complex in the New World, do not frequently undergo lymphatic metastasis and generally self-cure in a few months to a year (see above). Therefore, these diseases constitute relatively uncomplicated problems. In addition, as pentavalent antimony is given parenterally, patients may not view 20 injections (Berman 1988) as being clearly preferable to the possibility of a few months of a skin ulcer. Furthermore, there is little evidence that antimony treatment cures cutaneous leishmaniasis at a rate significantly faster than that seen in untreated controls (Berman 1988). Since therapy for any disease should entail less morbidity than that due to the disease and should be effective, it has often been difficult to decide on the appropriate therapy for cutaneous leishmaniasis.

b) Vaccination

A discussion of vaccination against leishmaniasis should be divided between the cutaneous and visceral forms of the disease. In the former, there is a long history of successful human vaccination studies in the Soviet Union, the Middle East, and South America, while in contrast, only rare human studies, with no repeatable success, exist in the literature pertaining to the visceral disease (Handman 1986). Recent reviews (Greenblatt 1985; Handman 1986; Muller et al. 1989) have summarized the various reports of vaccination attempts in both man and experimental animals.

The simple cutaneous form of leishmaniasis is generally a mild disease in man, giving rise to a self-curing localized lesion, and has been extensively studied in both humans and mouse model systems (reviewed in Muller et al. 1989). Development of a vaccine for cutaneous leishmaniasis, especially that caused by *L. major*, *L. braziliensis*, or *L. m. amazonensis*, has recently been the focus of much attention (reviewed in Greenblatt 1988). It is now possible to cite many approaches which give complete or partial protection in model systems. These include injection of: 1) killed, irradiated and avirulent organisms; 2) parasite products; and 3) parasite fractions. In some cases, these antigens protect susceptible animals. Vaccine trials in human volunteers have been carried out with killed promastigote preparations (Antunes et al. 1986). Highly sensitive BALB/c mice have been vaccinated with irradiated (Howard et al. 1982; Barral-Netto et al. 1987) and
killed (Howard et al. 1984; Scott et al. 1987a) parasites, as well as crude and semi-pure parasite fractions (Scott et al. 1987b; Frommel et al. 1988). T cell mechanisms involved in mediating protection in such models, which appears to be T cell-mediated (see Chapter 3), have been extensively studied (reviewed in Muller 1989). Recently, the ability of several purified parasite antigens to prevent the development of cutaneous lesions in both sensitive and resistant mouse strains has been examined. The \textit{L. m. amazonensis} glycoprotein 63 (GP63; promastigote major surface protease) (Russell and Alexander 1988) or glycoprotein M-2 (Champsi and McMahon-Pratt 1988) or \textit{L. major} LPG (Handman and Mitchell 1985; McConville et al. 1987) or GP63 peptides (Jardim et al. 1990; Yang et al. 1991) have been shown to reduce or prevent lesion development in mice challenged with \textit{Leishmania} parasites.

Few parallel studies on vaccination against \textit{L. donovani} parasites which cause the visceralizing, fatal form of leishmaniasis, have been undertaken. Studies of humans suggest that at least 15% of the population exposed to the parasite develop asymptomatic infections which eventually self-cure without recourse to chemotherapy (Jan et al. 1986; Badaro et al. 1986). Experiments with animal models have shown that mice vaccinated with killed parasites plus adjuvant exhibit marked reductions in the level of parasitemia compared with controls (Holbrook et al. 1981; Jarecki-Black et al. 1985; Jarecki-Black et al. 1986; O'Daly and Cabrera 1986); however, few attempts have been made to define the antigens or immunologic mechanisms involved in this protection. The ability of \textit{L. donovani} GP63 or LPG to induce protection against a challenge by this parasite have not been significantly examined although recent studies (Jaffe et al. 1990c; White and McMahon-Pratt 1990) have demonstrated the ability of other \textit{L. donovani} antigens to provide significant protection by reducing parasitemia levels after parasite challenge.

Attention has also focused on the antigenic cross-reactivities between leishmanial species. If these could be exploited, either avirulent organisms or organisms causing less serious forms of leishmaniasis might be used to protect against those organisms causing serious disease. One example would be to vaccinate with \textit{L. major} to protect against mucocutaneous leishmaniasis which is caused by \textit{L. b. braziliensis}. In mouse models, \textit{L. major} generally protects against \textit{L. mexicana} sp. and \textit{L. braziliensis panamensis}, and yet irradiated \textit{L. mexicana mexicana} is not protective against itself (Greenblatt 1985). In addition, \textit{L. major} is able to cross-protect against \textit{L. tropica} but, strangely, the reverse is not true (World Health Organization 1984). While complicated, the important point from studies on cross-protection such as these is that by selection of the appropriate antigen or by stimulation of the correct immune pathway, it appears possible to develop protection against a fairly wide range of organisms. However, the potential of this particular route is
complicated by the problem of how to measure vaccine effectiveness without an actual challenge.

The solid immunity observed following convalescence from simple cutaneous leishmaniasis and from cured Kala-azar has suggested that vaccination to prevent leishmaniasis is within reach of conventional immunization methods (Greenblatt 1985). However, at this time, no vaccine for any human parasite, including Leishmania, is available. It is possible that only a few leishmanial antigens are relevant for the induction of host-protective immunity. The challenge facing those interested in developing a defined-antigen vaccine for immunoprophylaxis is to identify host-protective effector mechanisms and the parasite target antigens of these host responses. Furthermore, in leishmaniasis, the genetic diversity of the human population may be a factor since individuals may develop variable immune responses to different parasite antigens (Handman 1986). Certainly more knowledge is required on the nature and structure of the leishmanial host-protective antigens and the leishmanial molecules involved in virulence. Similarly, more research is required on the appropriate presentation and delivery of candidate vaccine molecules.

2. Leishmania Surface Membrane Molecules.

2.1 Lipophosphoglycan

The major cell surface glycoconjugate of leishmanial parasites is LPG (King et al. 1987). LPG is essentially a tripartite molecule, consisting of a polymer of repeating phosphorylated saccharide units linked via a phosphosaccharide core to a glycosylphosphatidylinositol (GPI) lipid anchor (Turco 1988). Its relative abundance, unique structure, and cellular location suggest several important roles in interactions between Leishmania parasites and host systems. A more comprehensive consideration of LPG is found in the Introductions to Chapters 1-3.

2.2 Promastigote surface protease

A ~63,000 Da glycoprotein (GP63) protease was first described at the surface of promastigotes (Colomer-Gould et al. 1985; Etges et al. 1985). GP63 is by far the most abundant integral membrane glycoprotein of leishmanial promastigotes, as the ~500,000 copies expressed on the surface of a promastigote represent 0.5-1.0% of the total cellular protein depending on the species and strain of the parasite (Bordier 1987). GP63 is a membrane-bound zinc endo-peptidase (Etges et al. 1986; Bouvier et al. 1989; Chaudhuri et al. 1989) which is active at the surface of fixed and live promastigotes (Etges et al. 1986). Recently a related protein which also exhibits acid proteinase activity was reported in the
amastigote stage of the parasite (Chaudhuri et al. 1989; Medina-Acosta et al. 1989; Frommel et al. 1990). Like LPG, GP63 is attached to the promastigote plasma membrane by a glycosylphosphatidylinositol (GPI) anchor (Bordier 1987; Schneider et al. 1990).

GP63 is known to have a role in attachment of promastigotes to host macrophages (Russell and Wilhelm 1986; Chang and Chang 1986). Attachment to macrophages in the absence of serum opsonins is mediated by GP63 (and by LPG; see Chapter 2) interacting predominantly with macrophage complement receptor 3 (CR3). L. major promastigote GP63 contains an Arg-Gly-Asp (RGD) sequon which has been proposed to bind to CR3 (Russell and Wright 1988; Russell et al. 1989). However, GP63 from L. donovani chagasi has recently been shown to lack an RGD sequence (Miller et al. 1990), and as GP63-coated beads bound by macrophages are not phagocytosed (Russell and Wright 1988), this suggests that other endogenous ligands (e.g. LPG) are necessary for successful promastigote infectivity. Both L. d. donovani (Wilson and Pearson 1986) and L. d. chagasi (Wilson and Hardin 1990) promastigotes have also been demonstrated to bind to macrophages through the mannosyl/fucosyl receptor by GP63 carbohydrate components. In the presence of non-immune serum, metacyclic promastigotes activate complement and GP63 is thought to bind to C3bi-binding sites within CR3 (Russell and Wright 1988). In addition, strains of L. mexicana amazonensis (Kink and Chang 1987), L. b. braziliensis (Kweider et al. 1987) and L. d. chagasi (Wilson et al. 1989) displaying higher levels of GP63 on their surface, have been shown to be more infective than wild-type parasites. These studies, together with the fact that GP63 is up-regulated in metacyclic, infective, stationary-phase promastigotes (Ouaissi 1988), further demonstrates the importance of GP63 interaction with macrophage receptors.

Other functions have been demonstrated or at least suggested for GP63. Bordier (1987) and Chaudhuri and Chang (1988) have suggested that GP63 may cleave host complement factor C3 facilitating binding of the parasite to the macrophage surface. GP63 has also been proposed to enhance parasite survival after parasite entry into host cells. In contrast to the neutral to basic pH optimum reported for L. major and L. m. mexicana promastigote GP63 (Bouvier et al. 1990), the GP63 from L. m. amazonensis is a protease capable of degrading lysosomal enzymes and it exhibits optimal activity under the acidic conditions prevailing in the phagolysosome (Chaudhuri and Chang 1988). An intracellular protective role is further supported by the observation that proteins within GP63-bearing liposomes were inhibited from intracellular degradation when they were taken up by macrophages and this protection was lost with heat denaturation of GP63, which destroys its enzymatic activity (Chaudhuri et al. 1989). Furthermore, pretreatment of infective L.
*b. b*raziliensisi promastigotes with anti-GP63 monoclonal antibodies (mAbs) inhibited intracellular parasite survival (Kweider et al. 1987).

2.3 Acid Phosphatase

Gottlieb and Dwyer (1981; 1982) were the first to identify and characterize a surface membrane acid phosphatase in *L. donovani*. This is a mannose-containing glycoprotein (Glew et al. 1982) present on both promastigotes and amastigotes although qualitative differences exist between the promastigote and amastigote molecules (Glew et al. 1988). Remaley et al. (1985b) subsequently identified and characterized three acid phosphatases (AcPase-P1, P2, P3) with different pH optima and molecular characteristics. The tartrate-resistant AcPase-P1 accounts for more than 70% of the total cellular acid phosphatase activity (Remaley et al. 1985b) and has been shown to be quantitatively and qualitatively different between virulent and avirulent *L. donovani* promastigotes (Katakura and Kobayashi 1988).

The physiological role of these AcPases is not well understood. Gottlieb and Dwyer (1981; 1982) have proposed that the AcPases may serve a nutritional role by hydrolysing phosphomonoesters and thus supplying a source of inorganic phosphate for parasite growth. It has also been suggested that the AcPases could facilitate infection by modifying the metabolism of the host cell through the dephosphorylation of host cell phosphoproteins (Remaley et al. 1985b; Glew et al. 1988). Indeed, while not shown for macrophages, *L. donovani* AcPase has been shown to inhibit toxic oxidative metabolite production of neutrophils (Remaley et al. 1984; Remaley et al. 1985a; reviewed in Glew et al. 1988).

A tartrate-sensitive secreted AcPase has also been purified from culture supernatants of promastigotes of *L. donovani* (Lovelace et al. 1986; Bates and Dwyer 1987), *L. tropica* (Jaffe et al. 1990a), and *L. mexicana* (Menz et al. 1991; Ilg et al. 1991), and although its function is unknown, it does suggest the potential of the extracellular AcPase to contribute to parasite pathogenicity by acting at sites distal to the parasite. More than 90% of the AcPase activity produced by *L. donovani* promastigotes accumulates in the extracellular growth medium (Gottlieb and Dwyer 1982). The AcPase is secreted regardless of whether the organism is grown in serum-supplemented or in chemically defined medium (Glew et al. 1988) and secretion is not regulated by environmental inorganic phosphate concentrations (Bates and Dwyer 1987).
2.4 5’- and 3’-Nucleotidase

It is hypothesized that a nutritive role is associated with two specific phosphomonoesterase activities. Both 5’- and 3’-nucleotidases (NTases) have been located in the *L. donovani* membrane (Dwyer and Gottlieb 1984). *Leishmania* spp. are unable to synthesize purines *de novo* and are therefore dependent upon an exogenous supply of preformed purines (Glew et al. 1988). Therefore the leishmanial 5’-NTase which can hydrolyze both ribonucleotides and deoxyribonucleotides and the 3’-NTase which is specific for ribonucleotides appear to provide the parasite with the purine nucleosides required for growth (Glew et al. 1988). Hassan and Coombs (1987) have shown that the specific activities of 3’-NTase and 5’-NTase are nearly the same in promastigotes and amastigotes. It is presumed that the nucleotide substrates for the NTases arise in the gut of the sandfly vector and lysosomes of host macrophages from the nuclease-mediated hydrolysis of DNA and RNA (Dwyer and Gottlieb 1984). In addition, when *L. donovani* promastigotes are transferred to media lacking specific nutrients (e.g. purines) the parasites respond with an increased expression of the 3’-NTase, consistent with its proposed role in purine acquisition (Sacci et al. 1990).

3. The Leishmanial Parasite in the Laboratory.

3.1 General life cycle.

Leishmanial parasites are transmitted into the mammalian host through the bite of a feeding sandfly. In the midgut of the flies the parasites exist as motile promastigotes and when they have entered a mammalian host the promastigotes are ingested by cells of the mononuclear phagocyte system. Inside these cells they transform into smaller, nonmotile amastigotes whose intracellular parasitism culminates in the symptoms and pathologies associated with the disease leishmaniasis. A more detailed discussion of the *Leishmania* parasite's life cycle is found in Chapter 2.

3.2 Promastigotes

As promastigotes can generally be readily cultured in artificial media, they are frequently used for research investigations and are a reasonably well understood form of the *Leishmania* parasite. Promastigote cultures are maintained *in vitro* between 21-26°C to simulate the temperature of the vector (Handman 1986). Various media enriched with rabbit blood or serum were traditionally used for long-term promastigote growth but in recent years a variety of tissue culture media (M199, MEM, RPMI 1640, SM) supplemented with fetal bovine serum have become the reagents of choice (Handman
Serum-free, defined media have been variable in their ability to support growth of various leishmanias (Steiger and Steiger 1976; Chang and Hendricks 1985; Handman 1986). It should also be noted that recent experiments have shown that culture medium components are able to regulate carbohydrate surface configurations of L. major promastigotes and, thereby, antigenic expression. When promastigotes of the same clone were grown in different media their lectin-mediated agglutination profiles were dissimilar and both quantitative and qualitative variation was seen in the antigenic glycoconjugates released into the media (Jacobson and Schnur 1990).

One aspect of the leishmanial parasite's life-cycle is the development (termed "metacyclogenesis") of promastigotes from a noninfective stage to an infective stage (see Chapter 2). This has been demonstrated for promastigotes growing both within the sandfly midgut and in axenic culture (reviewed in Sacks 1989). However, when Leishmania are maintained as promastigotes by long-term serial passage in vitro, their infectivity for laboratory hosts may decrease progressively (Giannini 1974; Nolan and Herman 1985). As was demonstrated by Katakura and Kobayashi (1985) who showed that a more virulent line of L. donovani promastigotes could be obtained from a less infective original strain after serial passages in mice, cyclic passage through a vertebrate host is required to prevent loss of parasite virulence. It is possible that in nature parasite populations consist of a mixture of organisms of varying degrees of virulence and long-term culture may select for a certain population (Handman et al. 1983). Another possibility is that avirulent mutants arise constantly and in the absence of selective pressure for infectivity in the in vivo situation, they outgrow the virulent organisms.

3.3 Amastigotes

Studies involving the amastigote have been hampered by difficulties in isolating this intracellular pathogen from the host macrophage and in culturing amastigotes for extended periods in vitro. A number of procedures have been described for isolating highly pure amastigotes (Dwyer 1976; Channon et al. 1984; Glaser et al. 1990). Amastigotes may also be cultured in vitro for short periods in primary explants of peritoneal macrophages or blood-derived human monocytes (Handman 1986). Long-term, continuous cultures of amastigotes in a macrophage line have been described for L. donovani and L. mexicana (Beres and Marr 1979; Chang 1980). While L. major amastigotes do not seem amenable to this process, some success has been obtained in growing this species in a slow growing mouse macrophage line (Handman 1986). Axenic transformation of promastigotes to amastigotes at elevated temperatures has been described.
for *L. mexicana* and *L. b. braziliensis* (Handman 1986). However, it still remains to be established whether these are nonflagellated promastigotes or true amastigotes.

### 3.4 Animal models

Numerous animal species including dogs, foxes and rodents may be naturally infected with *Leishmania* spp. and many of them serve as the natural reservoir for the human disease (World Health Organization 1984). For example, the dog is a reservoir of *L. donovani* and *L. braziliensis peruviana* and the rat has been shown to harbour at least three *Leishmania* species and is believed to play a role in the maintenance of *L. donovani* in Italy (World Health Organization 1984). The disease pattern observed in the reservoir animal may or may not be similar to that observed in man and even greater caution must be exercised when extrapolating from experimental models to man. Laboratory experiments may bypass the natural, superficial, intradermal route of infection and may use much larger numbers of parasites for infection than would occur with the bite of a sandfly. However, a considerable amount of information has been gained from experimental studies of immune responses in animal models of leishmanial infections. The wide spectrum of immune responses in laboratory animals, the availability of animals of particular genetic constitutions and the possibility of modulating the immune responses of the host in the laboratory have provided interesting insights in three major areas of study: 1) the genetic aspects of susceptibility and resistance; 2) the relative importance of various cell populations in immune responses and effector mechanisms and 3) the potential means of immunizing against the disease. Animals are also routinely used for the maintenance of parasite strain infectivity (see above). Because of their ready availability and their susceptibility to most *Leishmania* parasites, the Syrian hamster is usually the animal of choice for maintaining parasite virulence by serial passaging (Chang and Hendricks 1985).

The disease patterns produced by injection of *Leishmania* spp. into various strains of inbred mice vary widely, depending upon both host factors and parasite factors. For example, while some mouse strains are highly resistant to infection with *L. major* and their macrophages *in vitro* do not support parasite growth, others show intermediate susceptibility and their lesions heal rapidly leaving the animals resistant to reinfection (Handman 1986). In addition, a "nonhealer" phenotype is exemplified by BALB/c mice which are extremely susceptible and usually fail to heal their lesions. Infections using *L. donovani* parasites have shown that the resistant or susceptible phenotype is controlled by alleles at a single locus, designated *Lsh* (Handman 1986).

*L. enrietti* infection of guinea pigs has also been used as a model for human cutaneous leishmaniasis. The immune responses to this parasite are similar to those
described for man. However, the main criticism is that \textit{L. enrietti} does not infect man (World Health Organization 1984). In addition, recent studies have suggested that various East African non-human primates, such as vervet monkeys (\textit{Ceropithecus aethiops}), may serve as suitable models for \textit{Leishmania} spp. infections (Githure et al. 1986b; Githure et al. 1987).

In summary, \textit{Leishmania} parasites are spread by sandflies and cause a spectrum of debilitating and often fatal diseases collectively known as the leishmaniases. Proteins and other molecules on the cell surface have been shown to be involved in many of the cell-cell interactions that occur between the parasites and their host and between the parasites and their vectors. Many studies are underway to gain further understanding of these host-parasite interactions and the specific molecules involved. In addition, \textit{Leishmania} promastigotes can be grown in culture, amastigotes can be purified from infected hamsters and infective promastigotes can be used to infect macrophages \textit{in vitro}. Therefore, both life cycle stages (and their relevant antigens) can be used to study macrophage-parasite interactions as never before.
4. Thesis Rationale and Research Objectives.

4.1 Rationale.

_Leishmania donovani_ parasites have a life cycle which includes both intracellular and extracellular stages each being specialized for interaction with the host and with the sandfly vector. In the host animal one of the major interactions is between promastigotes and macrophages. This interaction is only now being studied at a molecular level and recent evidence suggests the importance of the leishmanial major surface glycoconjugate, LPG, in this interaction. It is important that relevant molecules, such as LPG, on the surface of leishmanial parasites be identified and their importance in terms of _Leishmania_ biology (host-parasite interactions, differentiation, immunity) be assessed. The information gained will be useful for our understanding of basic host-parasite relationships and for their application to human medicine through improved prophylaxis and epidemiological control of the most serious form of leishmaniasis caused by _L. donovani_.

4.2 Research Objectives.

a) To perform an immunochemical analysis of the membrane-bound and secreted forms of the LPG molecule of _L. donovani_ parasites.

b) To study the distribution of LPG in various _Leishmania_ species and during the life cycle of the _L. donovani_ parasite.

c) To examine the possible role(s) of _L. donovani_ LPG in parasite immunobiology.
5. Collaborations.

Collaborations with the following individuals and institutions were established to facilitate the experiments described throughout this thesis:

a) **Dr. S.J. Turco, Dr. T.B. McNeely and K. Greis** (University of Kentucky College of Medicine, Lexington, Kentucky, U.S.A.)
   - have provided various wild-type and LPG-deficient mutant *L. donovani* strains and milligram quantities of purified *L. donovani* and *L. tropica* LPG, purified chemically derived and characterized *L. donovani* LPG fragments, and purified *L. donovani* excreted factor (LPG moiety). Also were an intellectual resource regarding LPG biochemistry and LPG-associated cell biology.

b) **Dr. L.F. Schnur** (Hebrew University-Hadassah Medical School, Jerusalem, Israel)
   - has provided various *Leishmania* species and strains, serotyped these strains for the results shown in Chapter 2 and provided human sera samples from *Leishmania*-infected individuals. Dr. Schnur also provided information on serotyping-associated immunochemistry.

c) **A. Jardim** (University of Victoria)
   - performed the chemical and chromatographic procedures required for the isolation and characterization of the LPG associated protein from *L. donovani* LPG. In addition, aspects of some of the T cell stimulation experiments were performed in collaboration with Mr. Jardim (see Chapter 3).

d) **F. Li and A.G. Sigurdson** (University of Victoria)
   - have provided purified secreted acid phosphatase from *L. donovani* promastigotes.

e) **Dr. H-s. Teh** (University of British Columbia, Vancouver, Canada)
   - provided the technical background and allowed the author access to his laboratory for the determination of the phenotypes of the T cells responding to *L. donovani* LPG epitopes (see Chapter 3).
CHAPTER 1. IMMUNOCHEMICAL ANALYSIS OF LPG.

Introduction

1.1 Structural Features of LPG.

The major cell surface glycoconjugate of leishmanial parasites is lipophosphoglycan (LPG). Structurally, LPG is essentially a tripartite molecule consisting of a polymer of repeating saccharide units linked through monophosphate bridges and attached via a carbohydrate core to the parasite membrane by a lipid anchor. The general structure of leishmanial LPG is shown in Figure 2A. LPG from promastigotes of \textit{L. donovani} (Orlandi and Turco 1987; Turco et al. 1987; Turco et al. 1989), \textit{L. major} (McConville et al. 1987; McConville et al. 1990a), and \textit{L. mexicana} (McConville et al. 1990b) have been characterized. A comparison of the LPGs from \textit{L. donovani} and \textit{L. major} promastigotes is shown in Figure 2B. Though as yet incompletely characterized, the LPG of \textit{L. tropica} promastigotes has been suggested to have a similar chemical structure (Jaffe et al. 1990b). LPG-type molecules have been reported on all \textit{Leishmania} sp. examined to date (Turco 1988) and there is speculation that the structural polymorphisms of LPG amongst the various parasite species may contribute to the different tissue tropisms of various \textit{Leishmania} sp. and the resulting differences in disease patterns (Turco 1990).

a) The lipid anchor.

The hydrophobic anchor of \textit{L. donovani} and \textit{L. major} LPG (and probably of all leishmanial species) is a novel lyso-1-O-alkylphosphatidylinositol lipid (Orlandi and Turco 1987; McConville et al. 1987; Turco 1988). The aliphatic chain in both species consists of either C$_{24}$ or C$_{26}$ saturated, unbranched hydrocarbon. Although the C-2 hydroxyl group of the glycerolipid backbone of mature LPG is unsubstituted, it has been suggested that it is acylated and deacylated during LPG biosynthesis (McConville and Bacic 1989; Rosen et al. 1989). The LPG lipid anchor shares limited structural homology with those of other GPI-anchored molecules including the \textit{Trypanosoma brucei} variant surface glycoprotein (VSG) and the \textit{L. major} promastigote surface protease (GP63) (reviewed in Thomas et al. 1990). Like a number of other GPI-anchored molecules (e.g. VSG), LPG is susceptible to hydrolysis by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), resulting in the release of the 1-0-alkylglycerol moiety (Orlandi and Turco 1987; Turco 1988). The main differences between the anchor of LPG and the other GPI-anchored proteins are: 1) LPG has a lyso-alkyl-PI whereas most GPI-anchored proteins have a conventional diacylated PI; and 2) the lack of diversity of the glycerol-linked aliphatic substituents on LPG (Ferguson et
Figure 2. 
A. General structural features of LPG from *Leishmania.*
B. Comparison of LPG from *L. donovani* and *L. major.*

**A.** General structural features of LPG from *Leishmania.*

B. Comparison of LPG from *L. donovani* and *L. major.*
al. 1988; Thomas et al. 1990). In fact, whereas GPI anchoring has been found to occur in only a few mammalian cell surface proteins, it seems to be the predominant form of membrane attachment in the protozoans (Ferguson and Williams 1988). Indeed, *Leishmania* parasites are one of the few-reported organisms to use different glycolipids to anchor two distinct surface macromolecules (LPG and GP63) (Turco 1988).

b) The phosphosaccharide core.

Structural characterization of the carbohydrate core region has been reported for LPGs of both *L. donovani* (Turco et al. 1989) and *L. major* (McConville et al. 1990a) promastigotes. For both parasites, the LPG phosphosaccharide core has the sequence:

\[ P_0 - 6 \text{Gal}_p(\alpha 1 - 6) \text{Gal}_p(\alpha 1 - 3) \text{Gal}(\beta 1 - 3) \text{Man}_p(\alpha 1 - 3) \text{Man}_p(\alpha 1 - 4) \text{GlcN}_p(\alpha 1 - 6) \text{myo-inositol} \]

In the above structure approximately 60% (*L. major*) or 100% (*L. donovani*) of the mannose residues distal to the glucosamine are phosphorylated on the 6 position. Subscripts indicate whether hexoses are in the pyranose (\(p\)) or furanose (\(f\)) forms. The site of attachment of the phosphoglycan repeat polymer appears to be predominantly through the terminal galactose 6-phosphate residue, suggesting that LPG is organized as a linear molecule (McConville et al. 1990a). However, other evidence has implied that a small portion (< 20%) of LPG molecules may have two saccharide chains branching from the core mannose (distal to the glucosamine) to form a Y-shaped LPG structure (Turco 1988; McConville et al. 1990a).

One noteworthy feature characteristic of the LPG core region is the [\(\text{Man}_p(\alpha 1 - 4) \text{GlcN}_p(\alpha 1 - 6) \text{myo-inositol}-1-\text{P}_0\)] sequence. This sequence is homologous to the corresponding arrangement in the glycosyl-phosphatidylinositol (GPI)-anchored proteins characterized to date; for example, the *T. brucei* VSG (Ferguson et al. 1988), the Thy-1 glycoprotein antigen from rat brain (Homans et al. 1988), and the GP63 glycoprotein of *L. major* promastigotes (Schneider et al. 1990). The presence of this sequence in these (widely) diverse macromolecules may indicate that it is conserved in all GPI-anchors. However, beyond the [\(\text{Man}_p(\alpha 1 - 4) \text{GlcN}_p(\alpha 1 - 6) \text{myo-inositol}-1-\text{P}_0\)] sequence, the structure of the LPG phosphosaccharide core appears to diverge completely from the carbohydrate cores of these other GPI-anchored proteins which exist as branched structures (reviewed in Thomas et al. 1990) as contrasted to the predominantly linear moiety for LPG. Other unusual features of the *Leishmania* LPG core structure are: 1) the presence of an uncommon internal galactofuranose core residue (uncommon as a glycoconjugate residue - where known, this sugar is usually present as a non-reducing terminal sugar in complex glycopeptides and glycolipids of protozoans or as an internal polysaccharide moiety in
bacterial macromolecules, Homans 1990); and 2) the glucosamine is unacylated (Turco 1988).

c) The repeating phosphosaccharide polymer.

A prominent feature of all three characterized LPG molecules is the presence of a common repeating backbone sequence in all phosphoglycan moieties of \([\text{PO}_{4}-6\text{Galp}(\beta 1-4)\text{Man}_p\alpha 1-]\). *L. donovani* LPG has an average of 16 such repeat units (Turco et al. 1987) whereas *L. major* has an average of 27 units (McConville et al. 1990a). This repeat unit may be variably substituted with other sugars on the 3 position of the galactose residues (Turco 1990). In *L. donovani* LPG, there is negligible substitution on the backbone sequence (Turco et al. 1987) whereas in the *L. mexicana* LPG approximately 25% of the galactose residues are substituted with \(\beta\text{Glc}\) residues (McConville et al. 1990a). The *L. major* LPG is more complex as approximately 87% of the galactose residues in the backbone sequence are substituted with galactose or linear saccharide chains containing from 2-4 saccharide residues (including galactose, glucose and arabinose; see Figure 2B) (McConville et al. 1990a). In addition, evidence suggests that the non-reducing terminus of the *L. major* phosphoglycan repeat is capped exclusively with the neutral disaccharide \([\text{Man}_p(\alpha 1-2)\text{Man}_p\alpha 1-]\) (McConville et al. 1990a) whereas *L. donovani* LPG is capped by a heterogeneous mixture of di-, tri-, and tetr saccharides of mixed composition (S. Turco, personal communication). A unique and characteristic feature of LPG is the extreme sensitivity of the mannosyl(\(\alpha 1\))phosphate bonds of the polysaccharide repeat to mild acid hydrolysis (Turco 1990). Another unusual aspect of the repeat moiety is the presence of 4-substituted mannose, which is extremely unusual in eukaryotic glycoproteins; the only other known occurrence of this molecule is in mannan of ivory nuts (Turco 1990).

d) The tertiary structure of LPG.

Preliminary molecular modelling studies (Homans 1990) of the phosphoglycan moiety of *L. donovani* LPG indicated that the \([\text{PO}_{4}-6\text{Galp}(\beta 1-4)\text{Man}_p]\) backbone sequence exists in an extended, linear configuration with the mannose and phosphate residues buried within the helix, and the galactose residues projecting in a "star-like" configuration on the outside of the helix. Since it is the galactose residues that are substituted by other saccharides on different *Leishmania sp.* (see earlier), the additional glucose residues of *L. mexicana*, for example, further extend the helix in a lateral direction, giving LPG the appearance of a "bottlebrush" (Homans 1990). This morphology could explain the fact that LPGs are immunochemically distinct, despite sharing common structural features (see later).

It has been estimated that there are approximately \(1.25 \times 10^6\) or \(5 \times 10^6\) copies of LPG on the surface of a *L. donovani* (Orlandi and Turco 1987) or *L. major* (McConville and Bacic 1990) promastigote respectively. Therefore, LPG, by its preponderance and
large cross-sectional surface area, as a result of lateral extensions by repeat backbone sequence substitutions, accounts for at least 25% of the total cell surface area of promastigotes.

1.2 Secreted LPG Epitopes.

One potentially important observation regarding LPG, that accounted for its original name of "excreted factor" (Schnur et al. 1972), is its release from promastigotes and subsequent appearance in culture medium. This is not unprecedented as, for example, the VSG molecule has also been shown to be shed from African trypanosomes by the action of an endogenous, membrane-bound phospholipase (reviewed in Thomas et al. 1990). While LPG is susceptible to PI-PLC cleavage (Orlandi and Turco 1987), no evidence of this enzymatic mechanism in leishmanial parasites has been demonstrated. The mechanism by which leishmanial glycoconjugates are released from the promastigote surface or, alternatively, are secreted, is unknown. Extracellular LPG occurs in two structurally distinct forms. One form binds very tightly to albumin in the tissue culture medium, presumably interacting with a hydrophobic binding pocket of albumin, facilitating LPG release from the promastigote surface (Turco et al. 1987). Partial characterization of this form of LPG revealed no obvious structural differences in comparison to cellular LPG (Turco et al. 1987). The other released form is a hydrophilic moiety called phosphoglycan (PG). While as yet incompletely characterized, *L. donovani* PG is thought to consist of a carbohydrate polymer of approximately nine units of the phosphorylated P04-6Gal(1-4)Man disaccharide repeat of the LPG molecule (S. Turco, personal communication). While the significance of extracellular LPG and/or conversion of LPG to the excreted PG has yet to be determined, it has been suggested that secreted LPG/PG could act as a "conditioning agent" for parasite infection of macrophages (Slutzky et al. 1979; Eilam et al. 1985). Further discussion regarding LPG/PG functions is found in Chapters 2 and 3.

In addition to PG, at least 40 different proteins are found in promastigote-conditioned culture medium, approximately half of which are glycosylated (Bates et al. 1988). Using rabbit antisera, evidence was obtained that suggested that PG shared antigenic cross-reactivity with both leishmanial surface membrane antigens and shed extracellular glycoproteins (Kaneshiro et al. 1982). Jaffe et al. (1990b) recently demonstrated for *L. tropica* promastigotes that the tartrate-sensitive secreted acid phosphatase (AcPase), the major glycoprotein secreted by leishmanial promastigotes *in vitro* (Bates and Dwyer 1987; Bates et al. 1988), shares carbohydrate epitopes with LPG/PG. Another group (Bates et al. 1990) has shown that *L. donovani* LPG/PG and secretory AcPAse share carbohydrate
epitopes and that it is the mild acid-labile phosphorylated disaccharide repeat moiety that is shared between these different glycoconjugates.

1.3 Other glycolipids.

In addition to LPG and glycoproteins the glycoinositolphospholipids (GIPLs) represent a third class of glycoconjugates on the membranes of *Leishmania* (McConville and Bacic 1989). Although some GIPLs are expressed on the surface of the parasite while others are internal they all have a structure which is similar to each other and to the membrane anchors of LPG, GP63 and other GPI-anchored proteins (Thomas et al. 1990). Monoclonal and polyclonal antibodies to GIPLs show that at least the surface oriented GIPLs most likely are present in all species of *Leishmania*, although there may be some differences in antigenic structure between species (Handman and Hocking 1983; Elhay et al. 1988). Six GIPLs were originally isolated and chemically characterized from an avirulent LPG-deficient *L. major* strain (Handman et al. 1986; McConville and Bacic 1989). All the GIPLs, as their salient structural feature, contained a mannose- and galactose-containing moiety (that demonstrates a sequence with an increasing length of glycan chain from GIPLs 1-6) glycosidically linked via a non-N-acetylated glucosamine to either l-alkyl or alkyl-acyl-phosphatidylinositol (McConville and Bacic 1989; McConville et al. 1990b). Although the function of the GIPLs is unknown, it has been suggested that these glycolipids may have a role as biosynthetic precursors to *Leishmania* LPG (McConville and Bacic 1989). This hypothesis is based on the finding that the GIPLs have the same monosaccharide composition as the glycosylinositol core of *L. donovani* and *L. major* LPGs (Turco et al. 1989; McConville et al. 1990a) and that while the lipid moieties of the GIPLs and LPG are structurally similar, some GIPLs exist as lso-alkyl-PI while others as l-alkyl-2-acyl-PI (Orlandi and Turco 1987; McConville et al. 1987).

The existence of an unusual lipid-containing glycoconjugate is not unique to the LPG of *Leishmania*. Several examples are found in other protozoan parasites, including a ceramide-anchored glycosylphosphoinositide that has been described in *Trypanosoma cruzi* (Previato et al. 1990), and a xylose-rich uronic acid glycoconjugate in *Leptomonas samueli* (Turco 1990). The structure of these glycoconjuagtes and their functions are unknown. A lipophosphoglycan from bloodstream forms of *T. b. brucei* has also been reported (Hublart et al. 1988; Krakow et al. 1989), and has been suggested to represent the glycolipid precursor to the GPI anchor of VSGs. In addition, phenolic glycolipids of *Mycobacterium leprae* have also been characterized (reviewed in Gaylord and Brennan 1987).
1.4 LPG mutants.

A powerful approach used for studying structure, biosynthesis, and function of complex carbohydrates in microorganisms is the production of glycosylation mutants. Therefore, it should be noted that several LPG-deficient promastigote mutants have been reported. *L. donovani* LPG-deficient mutants were selected for resistance to the cytotoxic lectin ricin agglutinin and subsequently shown to be defective in the synthesis of LPG (King and Turco 1988). In addition, an LPG-deficient strain was obtained after many years of serial passage of an infective *L. major* clone in blood-agar medium (Handman et al. 1986). Several of the *L. donovani* mutants were used in this thesis.

In this dissertation, the first objective was to define the immunochemical structure of membrane-bound and secreted forms of *L. donovani* LPG. Here is described the derivation of anti-LPG monoclonal antibodies and their use to define epitopes of LPG and LPG-associated molecules. In addition, the discovery of a protein component tightly associated with the LPG carbohydrate moiety is discussed.
Materials and Methods

Parasites. *Leishmania donovani* 1S2D promastigotes (Dwyer 1977) were a cloned population obtained via Dr. R.W. Olafson (University of Victoria) from Dr. D. Dwyer (NIH, Bethesda, MD). *L. donovani* LD3 promastigotes are a recent hamster-passaged, highly infective clone of *L. donovani* 1S2D and were obtained from Dr. S. Turco (University of Kentucky, Lexington, KY). *L. donovani* R2D2 and C3PO promastigotes were derived from *L. donovani* 1S2D parasites after mutagenesis and selection for resistance to killing by ricin agglutinin and their inability to synthesize LPG (King and Turco 1988). The LPG-deficient mutants (R2D2 and C3PO) were also obtained from Dr. S. Turco. *Trypanosoma brucei rhodesiense* ViTat 1.1 cloned procyclic culture forms (PCF) have been described elsewhere (Richardson et al. 1986; Richardson et al. 1988).

All parasites, unless otherwise stated, were grown at 26°C in SM medium (Cunningham 1973) containing 10% heat-inactivated fetal bovine serum (FBS) and 0.1% gentamycin sulphate. Alternatively, for the serum-free experiments, *L. donovani* LD3 and C3PO promastigotes were grown in MEDIUM 199 (Gibco laboratories, Grand Island, NY) containing 0.1% gentamycin sulphate and 5.0 ug/ml hemin or SM medium containing 10% heat-inactivated FBS.

Preparation of lipophosphoglycan. LPG from *L. donovani* 1S2D promastigotes was extracted, purified and quantitated by phosphate analysis as described previously (Orlandi and Turco 1987). Dr. S. Turco provided the LPG, its subfractions and fragments (see below) for all of the experiments included in this thesis.

Preparation of phosphoglycan, 1-0-alkylglycerol, phosphosaccharide-inositol core, and the phosphorylated disaccharide from LPG. Cellular phosphoglycan (cPG), the delipidated form of LPG (i.e. repeat polymer + phosphosaccharide core), was derived by treating *L. donovani* 1S2D LPG with phosphatidylinositol-specific phospholipase C (PI-PLC) for 16 hr at 37°C as described previously (Orlandi and Turco 1987), except that no detergent was added.

PI-PLC treatment was used to liberate the 1-0-alkylglycerol from the entire carbohydrate portion (PG) as described previously (Orlandi and Turco 1987). The products were separated by partitioning with chloroform and water.

The phosphosaccharide-inositol core was produced as described previously (Orlandi and Turco 1987). Briefly, mild acid hydrolysis (0.02 N HCl, 100°C, 5 min) was used to cleave the phosphorylated disaccharides from LPG. Phenyl-coupled Sepharose was
then used to separate the repeat fragments from the phosphosaccharide-PI fragment. The latter fragment was then subjected to PI-PLC treatment as described above to remove the lipid anchor.

The phosphorylated disaccharide ($P_4$-Gal-$B_1,4$-Man) moiety of LPG was isolated and purified as described elsewhere (Turco et al. 1987).

**Preparation of LPG fragments.** Dephosphorylated LPG, trifluoroacetic acid (TFA)-treated LPG, periodate-treated LPG, and nitrous acid-treated LPG were all produced as described previously (Orlandi and Turco 1987). LPG was hydrolysed with 10% NH$_4$OH to generate dephosphorylated fragments or with 0.2% TFA (pH 1.75, 2 hr, 100$^\circ$C) to cleave the galactofuranosylmannose bond as well as the phosphorylated disaccharide units leaving a fragmented phosphosaccharide core-PI. Periodate treatment cleaves vicinyl hydroxyl groups, while nitrous acid treatment cleaves the glucosaminlyinositol bond.

**Enzymatic and chemical treatments of LPG.** Pronase digestion of LPG was performed by incubating 200 ug of LPG, dissolved in a minimum volume of PBS pH 7.4, with 200 ul of 4 ug/ml *Streptomyces griseus* pronase (Sigma Chemical Co., St. Louis, MO) in 40 mM N-ethylmorpholine pH 8.0 for 4 hr at 37$^\circ$C. Alternatively, LPG was treated by incubating 165 ug LPG with a 25:1 molar ratio of either bovine pancreatic trypsin (Sigma) or proteinase K (EM Chemicals, Hawthorne, NY) in 100 mM N-ethylmorpholine, pH 8.0 for 16 hr at 37$^\circ$C. After digestion, the enzyme-treated LPG samples were dried down onto enzyme-linked immunosorbent assay (ELISA) microwell plates (see below). Proteinase K digestion of LPGAP (see below) was carried out in SDS-PAGE sample buffer at 55$^\circ$C for 3 hr prior to electrophoresis and immunoblotting with the anti-LPGAP mAb L98 (see below). The activity of the different enzymes was confirmed by showing the reduced ability of the anti-procyclin mAb TBRP1.477 to bind to living, enzyme-treated tylonosome PC in immunofluorescence as described previously (Richardson et al. 1988).

LPG was exposed to anhydrous trifluoroacetic anhydrous methanesulphonic acid (TFMSA) to cleave glycosidic linkages (Edges et al. 1981) leaving peptide bonds intact (Tam et al. 1986). To 60 ug of lyophilized LPG dissolved in 100 ul of anhydrous trifluoroacetic acid was added 10 ul of anhydrous TFMSA and the reaction allowed to proceed for 30 min. The TFMSA and solvent were removed by extensive lyophilization and the products were further purified by C-8 reverse phase HPLC using 0.1% trifluoroacetic acid and isopropanol in a linear gradient to 60% organic modifier in 60 min. The TFMSA cleavages were performed by A. Jardim (University of Victoria).
Isolation of LPG core-associated protein. Separation of the LPG core-associated protein (LPGAP) was performed by octyl Sepharose reverse phase chromatography using a 1.0 X 50 cm column (Pharmacia Canad1, Baie D'Urtre, Quebec). The column was developed with a reciprocal linear gradient beginning with 0.1 M ammonium acetate and progressing to 100% n-propanol over 2 hr at a flow rate of 0.3 ml/min. This was a modification of the procedure described by McConville et al. (1987). The column effluent was monitored for carbohydrate using both the phenol-sulfuric acid method (Beeley 1985) and ELISA employing the LPG phosphorylated disaccharide repeat-specific mAb CA7AE while the LPGAP was detected using the anti-LPGAP mAb L98. Since the mAb L98 reactive material eluted in a complex 280 nm absorbing region of the octyl Sepharose chromatogram which showed residual LPG contamination, it was pooled and re-chromatographed on the same column to improve separation from LPG. A. Jardim performed the chromatographic separation to isolate LPGAP from LPG and D.L.T. conducted the ELISAs during the isolation experiments.

Other antigens. The disaccharides Gal-β1,4-Man and Gal-β1,4-Glc (lactose) were purchased from Sigma.

The purification and characterization of the phosphorylated glycan obtained from L. donovani LD3 promastigote-conditioned culture medium (media PG; mPG) is described elsewhere (Greis et al. manuscript in preparation). Briefly, mPG consists of a carbohydrate polymer similar in nature to approximately nine units of the phosphorylated Gal-β1,4-Man disaccharide repeat of the LPG molecule (S. Turco, personal communication).

The Acid Phosphatase (AcPase) from L. donovani 1S2D and R2D2 parasites was purified from stationary phase promastigote-conditioned culture medium and supplied by F. Li (University of Victoria; manuscript in preparation).

The control antigens, human transferrin and BSA, were purchased from Sigma.

Derivation and screening of anti-L. donovani monoclonal antibodies. Inbred BALB/c female mice were purchased from Charles River Laboratories (St. Constant, Quebec) and were maintained in the Animal Care Unit at the University of Victoria. All mice were used at 4-10 weeks of age. A BALB/c mouse was injected intraperitoneally (i.p.) with 2 X 10^7 heat-killed (56°C, 30 min) L. donovani 1S2D late-log to stationary phase promastigotes in 0.2 ml phosphate buffered saline (PBS), pH 7.4. The mouse was injected i.p. with the same antigen preparation at three and six weeks after the initial priming. Approximately 7 days after each of the three i.p. injections, a tail bleed was performed and the serum titrated in indirect ELISA against L. donovani 1S2D lysates. Finally, at 9 weeks, the mouse was
injected intravenously (i.v.) with the same antigen preparation. Three days later the spleen was removed, a cell suspension was made and hybridomas were produced using standard fusion protocols (Kohler and Milstein 1975) with SP2/0 parental myeloma cells (Schulman et al. 1978) as the fusion partner. Hybridomas secreting anti-*L. donovani* antibodies were selected after testing supernatants by: 1) indirect ELISA on lysate of *L. donovani* promastigotes, 2) indirect ELISA on glutaraldehyde-fixed (Richardson et al. 1986) 1S2D and R2D2 promastigotes, 3) immunofluorescence on living *L. donovani* 1S2D and R2D2 promastigotes, 4) binding to promastigote antigens in immunoblotting, and 5) indirect ELISA on purified *L. donovani* LPG (see below for protocol). Stable hybridoma lines were obtained after double cloning by limiting dilution (Oi and Herzenberg 1980).

**Other mAbs and antisera.** In another fusion, a female BALB/c mouse was immunized as for the anti-*L. donovani* promastigote mAbs (described above) except that the immunizing antigen consisted of 12.5 µg (first injection) or 25 µg (second and third injection) of purified *L. donovani* 1S2D LPG in 0.2 ml sterile PBS, pH 7.4. After fusion, hybridomas secreting anti-LPG antibodies were selected by indirect ELISA using pure LPG as solid-phase antigen (see below for protocol). The two mAbs obtained from this fusion were designated L157 and L98. The immunizations, fusion, and screening of these mAbs on pure LPG was performed by R.P. Beecroft, University of Victoria. An antiserum used in antigen-capture ELISA was prepared by immunizing rabbits with crude membrane preparations from *L. donovani* 1S2D promastigotes. The antiserum was produced by T.W. Pearson (University of Victoria). The anti-urokinase mAb 3.3.12 and anti-human transferrin mAb HT1-3 were supplied by T.W. Pearson and R.P. Beecroft.

MAbs TBRP1.247 and TBRP1.477 (used as controls) are specific for the procyclin molecule of *T. brucei* spp. and have been described elsewhere (Richardson et al. 1986; Richardson et al. 1988).

**Enzyme-linked immunosorbent assay.** Indirect ELISA was performed using a variety of solid-phase adsorbed antigens. Intact promastigotes were adsorbed to round-bottom polyvinyl chloride microplates (Becton Dickinson Labware, Oxnard, CA) and fixed using glutaraldehyde as previously described (Richardson et al. 1986). For the production of parasite lysates, *Leishmania* promastigotes or trypanosome PCF were diluted in distilled water to an equivalent original concentration of 5 X 10⁶ parasites/ml and 100 µl/well of this suspension were dried onto the ELISA microplate wells (Costar, Cambridge, MA) by overnight incubation at 37°C in a dry incubator. Purified LPG, LPG subfractions and fragments and control antigen (human transferrin) were coated by drying 100 µl volumes of
10 ug/ml onto the wells. All indirect ELISA were performed using standard methods (Engvall and Perlmann 1971). Undiluted tissue culture supernatants or dilutions of mAb-containing ascites fluids were used as first antibody and a 1/2000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG/IgM (Tago, Burlingame, CA) as second antibody. The substrate was 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) purchased from Sigma. Colour development was measured at 405 nm using an EIA auto-reader (Bio-Tek Instruments Inc., Burlington, VT).

Inhibition ELISA was performed by incubation overnight at 4°C of predetermined dilutions of ascites fluids (containing mAbs) with various concentrations of inhibitors and using these incubation mixtures as first antibody in indirect ELISA.

The isotype of the anti-L. donovani promastigote and anti-LPG mAbs was determined by an antigen capture ELISA technique using a kit purchased from American Qualex (La Mirada, CA) according to the instructions supplied by the manufacturer.

**Antigen-capture ELISA.** A double-antibody antigen-capture ELISA was used to detect molecules containing epitopes bound by anti-L. donovani promastigote or anti-LPG mAbs in supernatants from cultures of Leishmania promastigotes or trypanosome PCF. ELISA microplate wells (Costar, Cambridge, MA) were coated with the mAbs by addition to each well of 100 ul of a 1:1000 dilution of ascites fluid in PBS and incubation for 3.5 hr at 37°C. After the plate was washed three times with PBS containing 0.05% Tween 20 detergent (PBS-Tween), unbound protein binding sites were blocked by adding 200 ul of PBS containing 0.5% bovine serum albumin (BSA; Sigma) to each well and incubating at 37°C for 1 hr with agitation. The wells were washed three times with PBS-Tween, and 100 ul of parasite culture supernatant (diluted in PBS-Tween containing 0.5% BSA) were added to each well. Promastigote or PCF culture supernatants were prepared by centrifuging (four times minimum) culture supernatants from stationary-phase cultures at 2500 g for 20 min on a TJ-6 centrifuge (Beckman Instruments Inc., Palo Alto, CA). After incubation for 1 hr at 37°C with agitation, the plate was washed three times quickly with PBS-Tween and then three times for 10 min with PBS-Tween. One-hundred ul of rabbit anti-L. donovani 1S2D crude membrane serum (1:1000 in PBS-Tween/0.5% BSA) were then added to the wells. After incubation for 1 hr at 37°C with agitation, the plates were washed as described above and 100 ul of a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Tago, Burlingame, CA) were added. After incubation for 1 hr at 37°C, the plates were washed as described above, and 100 ul of the substrate ABTS was added to each well. Colour development was measured as for indirect ELISA.
**Immunofluorescence.** Indirect immunofluorescence was performed on suspensions of live parasites essentially as previously described (Parish et al. 1985). Undiluted tissue culture supernatants or dilutions of mAb-containing ascites fluids were used as first antibody. A 1/40 dilution of affinity purified goat anti-mouse IgG/IgM-FITC (Caltag, South San Francisco, CA) was utilized as the second antibody in all immunofluorescence procedures. Immunofluorescence was observed using a Zeiss standard binocular microscope fitted with an epifluorescence attachment and a Zeiss NeoFluor 63/1.25 oil immersion objective.

**Polyacrylamide gel electrophoresis.** SDS-PAGE was performed according to Laemmli (1970) using a minigel apparatus (BioRad, Richmond, CA). Proteins in parasite lysates (3 X 10^5 parasites/well) or purified antigens (30 ug LPG/well; 10 ug LPGAP/well) were stacked using a 3% gel and were separated on a 10% gel at 10 mA. Molecules were visualized by staining with Coomassie blue R250, by silver staining (Menil et al. 1984) or by immunoblotting (see below). Low molecular weight standards (BioRad Labs., Mississauga, Ontario) were run on each gel.

**Immunoblotting.** Electrophoretic transfer of proteins from SDS-PAGE gels onto nitrocellulose sheets and subsequent detection of antigens were performed by the procedure of Towbin et al. (1979) extensively modified to reduce background binding of antibodies (Birk and Koepsell 1987) and to allow blotted antigens to renature (Bestagno et al. 1987). Undiluted tissue culture supernatants or mAb-containing ascites fluids (diluted 1:1000) were used as first antibody and binding was detected using ^125^I-labelled goat anti-mouse IgG/IgM antibodies (Amersham, Arlington Heights, Il) or peroxidase-conjugated goat anti-mouse IgG/IgM antibodies (Tago) and ABTS substrate. For detection involving ^125^I-labelled second antibodies, immunoblots were exposed at -70°C to Fuji RX X-ray film in a cassette containing intensifying screens. The immunoblot of LPGAP and the anti-LPGAP mAb L98 was performed by A. Jardim.

**Radiolabelling of antigens excreted into promastigote-conditioned culture medium.** For biosynthetic labelling of antigens excreted by *Leishmania* parasites into culture medium, *L. donovani* LD3 and C3PO promastigotes were incubated with ^35^S-methionine, ^3H^-2,3-mannose, or D-[6-^3^H(N)]-glucosamine hydrochloride. Biosynthetic labelling was performed as follows: PBS-washed promastigotes were resuspended at 1 X 10^7 parasites/ml in 5.0 ml of serum-free SM medium (with 5.0 ug/ml hemin) containing 500 uCi ^35^S-methionine. Alternatively, the same number of cells were resuspended in 5.0 ml of SM medium containing 10% heat-inactivated FBS and either 200 uCi of ^3H^-2,3-mannose or
125 uCi of D-[6-\textsuperscript{3}H(N)]-glucosamine hydrochloride. After 16 hr incubation at 26\textdegree{}C, the cells were washed 2 times in ice-cold PBS containing 1\% D-glucose, and resuspended to the same concentration in SM medium containing 10\% FBS. At 4, 12, and 24 hr intervals, samples were harvested, the promastigotes removed by extensive centrifugation, and the parasite-free culture supernatants assayed for the presence of radiolabelled antigens by an antigen-capture ELISA using the anti-LPG repeat mAb CA7AE. The protocol for the antigen-capture ELISA was as follows: flexible, round-bottom polyvinyl chloride microplates (Becton Dickinson) were coated with mAb CA7AE, washed and blocked as described above. After washing with PBS-Tween to remove the blocking solution, 100 ul of radiolabelled promastigote culture supernatants (diluted as above) were added to each well. After one hr incubation at 37\textdegree{}C, the plates were washed and then the microplates were allowed to air dry at room temperature. The individual wells in the microplates were then cut out of the plate (using scissors), cut in half again, and placed in FORMULA-963 aqueous counting cocktail (NEN Research Products, Boston, MA). Counts per minute were determined on a Beckman LS8000 Liquid Scintillation counter (Beckman Instruments Inc., Irvine, CA).
Results

Derivation of anti-\textit{L. donovani} monoclonal antibodies.

In one fusion, heat-killed \textit{L. donovani} 1S2D promastigotes were used as the immunogen and lysates of the homologous promastigotes as antigen in ELISA for initial selection of relevant hybridomas. Of 500 original wells (supernatants), 117 contained antibodies which bound \textit{L. donovani} lysates (data not shown). Forty-eight of these were tested in ELISA for binding to glutaraldehyde-fixed wild-type (1S2D) and the LPG-deficient mutant (R2D2) promastigotes. The premise was that the R2D2 parasites, by lacking LPG, might have additional or differently exposed surface epitopes accessible to antibody binding. Of the forty-eight supernatants twenty-three bound to the wild-type cells and of these fourteen also bound to the R2D2 mutant (data not shown). All forty-eight hybridomas were also tested by immunofluorescence against living \textit{L. donovani} 1S2D and R2D2 promastigotes to complement the glutaraldehyde-fixed ELISA results for antibody specificity to surface exposed epitopes. Fifteen of the hybridomas bound surface epitopes of \textit{L. donovani} 1S2D and/or R2D2 promastigotes. In addition, the twenty-three hybridomas were also assayed for binding to lysates of the 1S2D and R2D2 parasites. The binding specificities for the selected hybridomas are presented in Table 2. All twenty-three hybridomas were doubly cloned and then tested for their ability to bind antigens excrated into promastigote-conditioned culture medium or antigens from promastigote lysates by immunoblotting. These results are also shown in Table 2. Three of the mAbs bound only to the wild-type cells and not to the R2D2 mutants and were therefore selected as being potentially LPG-specific. Only two of these mAbs bound strongly to purified LPG in ELISA. These mAbs were designated CA7AE and BF9CC.

Using high performance thin layer chromatography and immunoblotting of separated leishmanial membrane preparations (Petry and Eisen 1989) it was shown that several of the mAbs, in addition to those recognizing LPG (i.e. AA1BD, AC2AC, AH5FH, BE1AH, CE5AA DD2FC, DD4ED, and BF11FH), were specific for other leishmanial non-LPG glycolipids. This work was performed in collaboration with K. Petry (Fred Hutchinson Cancer Research Center, Seattle, WA). Further characterization of these mAbs was not performed.

In the other fusion (performed by R.P. Beecroft and F. Li), purified LPG from \textit{L. donovani} was used as immunogen and as solid-phase antigen in ELISA for selection of relevant hybridomas. Only two positive wells were found in the first screening and doubly cloned hybridomas were derived from each of them. The two clones were designated LPG157 (L157) and LPG98 (L98).
It was thought that the anti-LPG mAbs could be very useful for purifying LPG, LPG precursors, and/or excreted factor from parasite preparations. Therefore, several attempts were made to purify the anti-LPG mAbs from ascites fluids. Initial procedures using protein A affinity chromatography were unsuccessful, most likely due to the mAbs in question being of IgG1 and IgM isotypes. Gel permeation chromatography did prove relatively successful in purifying these mAbs to relative homogeneity (determined by SDS-PAGE and silver staining; data not shown), but when subsequently coupled onto resins for affinity chromatography, the reactivity of the mAbs for their substrates were lost. Three other fusions, using purified LPG or heat-killed or living *L. donovani* LD3 promastigotes, were performed in order to derive anti-LPG mAbs of isotypes and stabilities more conducive to affinity chromatography isolation procedures. While a number of mAbs were obtained, all were of the IgM isotype and recognized the repeat moiety of the LPG molecule (data not shown). No further attempts to derive anti-LPG mAbs suitable for immunoaffinity chromatography were made.
Table 2. Binding of anti-*L. donovani* 1S2D promastigote mAbs to various antigens.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Isotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Immunofluorescence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ELISA&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Sand.</th>
<th>Immuno-blots&lt;sup&gt;e&lt;/sup&gt;</th>
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<td>R2D2&lt;sup&gt;g&lt;/sup&gt;</td>
<td>ViTat&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1S2D&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>CA3JE</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>CA7AE</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
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<td>-</td>
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<tr>
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<td>+</td>
<td>-</td>
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</tr>
<tr>
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<td>G2B</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
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<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
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<td>+</td>
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</tr>
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<td>+</td>
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<td>G2B</td>
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<td>+</td>
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</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>477&lt;sup&gt;j&lt;/sup&gt;</td>
<td>G1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

see next page for footnotes
a Determined using an antigen capture (sandwich) ELISA.

b Immunofluorescence was performed on living parasites. Binding was considered positive if bright fluorescence was observed on healthy, motile cells. (+) indicates weaker, patchy fluorescence.

c Indirect ELISA was performed on parasite whole cell lysates (1S2D, R2D2, and ViTat) using 5 X 10^5 parasites/well or purified L. donovani LPG at 1.0 ug/well. Binding was considered positive if the absorbance in the ELISA reaction was at least 8 times background. (+) indicates absorbance value of 2-4 times background.

d An antigen capture (sandwich) ELISA was used to determine the ability of the mAbs to trap antigens excreted into L. donovani 1S2D promastigote-conditioned culture medium. Absorbance values of greater than 6 times background were considered positive.

e Immunoblotting used L. donovani lysates as antigen.

f L. donovani 1S2D promastigotes (wild-type).

g L. donovani R2D2 promastigotes (LPG-deficient mutant).

h T. b. rhodesiense ViTat 1.1 PCF.

i LPG was purified from L. donovani 1S2D promastigotes.

j MAb TBRPI.477 is specific for procyclin on T. brucei spp. (Richardson et al. 1986; Richardson et al. 1988).

* Immunoblot profiles were essentially non-specific. MAbs bound multiple, similar bands for both Leishmania and Trypanosoma spp.
Characterization of anti-lipophosphoglycan monoclonal antibodies.

The specificity of the four anti-LPG mAbs was determined by indirect ELISA using purified LPG and purified subfractions of LPG (Table 3). To facilitate the reading of Table 3, a schematic representation of the LPG from *L. donovani* is shown below it. All four mAbs reacted with the intact LPG molecule but could be subdivided into two groups based on their binding to fractions of LPG. The two mAbs from the *L. donovani* promastigote fusion (IgM isotype) bound to the complete carbohydrate portion of LPG (phosphoglycan; phosphorylated disaccharide repeat + phosphosaccharide core) but did not bind to the phosphosaccharide core alone nor to the alkylglycerol portion. The two mAbs from the LPG fusion (mAbs L157 and L98, both IgG1 isotype) bound to the complete carbohydrate and to the phosphosaccharide core of the LPG molecule but not to the alkylglycerol moiety. None of the antibodies bound to the control antigen human transferrin.

The anti-LPG mAbs were tested for their ability to bind LPG in immunoblots. Both anti-core mAbs L157 and L98 bound purified LPG as a broad band between approximately 15 and 30 kDa with a more intensely staining region at the central portion of the smear at about 20 kDa (Figure 3). None of the four anti-LPG mAbs bound *Leishmania* sp. promastigote lysates in immunoblotting (data not shown). This could be due to reduced amounts of antigen being present in the parasite lysates in comparison to when substantial amounts of purified LPG were used. The anti-LPG repeat mAbs, CA7AE (lane C) and BF9CC, did not bind in immunoblots when either antigen preparation was used.

The ability of various disaccharides to inhibit the binding of the anti-LPG mAbs to LPG was tested in an inhibition ELISA. The results obtained using mAb CA7AE are shown in Figure 4. Strong inhibition was seen with the P04-6Gal(81-4)Man disaccharide. Purified LPG also strongly inhibited the binding to solid-phase bound LPG. No inhibition of binding was seen with lactose (Gal(81-4)Glс) or the non-phosphorylated Gal(81-4)Man. The results obtained for mAb BF9CC were similar (data not shown). None of the antigens tested (except purified LPG) were able to inhibit binding of the anti-LPG core mAbs to solid-phase bound LPG (data not shown). Based on their shared isotype and similar binding specificities to a variety of antigens (see Tables 2 and 3 and Figure 4), it seemed probable that mAbs CA7AE and BF9CC were the same antibody or, at least, recognized the same epitope. Therefore, only mAb CA7AE was used as an anti-LPG repeat antibody for the remainder of the experiments in this thesis.

The epitopes recognized by the anti-LPG core mAbs were further characterized by assaying the mAbs against chemically-derived LPG fragments. A comparison of the recognition of LPG and LPG fragments by the anti-LPG mAbs is shown in Figure 5. While
all three mAbs reacted with the intact LPG molecule (column A) and only the anti-LPG core mAbs L157 and L98 bound to purified phosphosaccharide core (column B), none of the mAbs bound to dephosphorylated LPG (column C). Cleavage of the galactofuranose bond within the LPG core region eliminated the binding of the anti-LPG core mAb L157 and decreased the binding of the other anti-LPG core mAb, L98, by ~50% (column D). All the mAbs bound equally well to periodate-treated LPG (column E). Except for the anti-core mAb L157, in which binding was reduced by ~40%, the mAbs bound as well to nitrous acid-treated LPG (column F) as to untreated LPG. None of the antibodies bound to the control antigen human transferrin (column G).
Table 3. Binding of anti-LPG mAbs to purified LPG and LPG subfractions in ELISA.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>mAb&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CA7AE</td>
</tr>
<tr>
<td>Entire LPG</td>
<td>+&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phosphoglycan</td>
<td>+</td>
</tr>
<tr>
<td>Phosphosaccharide core</td>
<td>-</td>
</tr>
<tr>
<td>1-O-Alkylglycerol</td>
<td>-</td>
</tr>
<tr>
<td>Human transferrin</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ascites fluids containing mAbs were used at a dilution of 1:1000.

<sup>b</sup> The control mAb was specific for human serum transferrin.

<sup>c</sup> Binding was considered positive if the absorbance in the ELISA reaction was at least 8 times background.

Components of LPG

\[
\text{REPEAT} \quad \text{CORE} \quad \text{ANCHOR}
\]

\[
\text{Gal-} \quad \text{P}^\text{O}_4\text{-Gal-Man} \quad \text{P}^\text{O}_4\text{-Gal-Gal-Gal_1-Man(P}^\text{O}_4\text{-Man-GlcN} \quad \text{Lyso-Pl}
\]
Figure 3. Detection of epitopes in purified *L. donovani* 1S2D LPG by immunoblotting. Lane A, anti-urokinase mAb 3.3.12; lane B, anti-LPG mAb L98; lane C, anti-LPG mAb CA7AE; lane D, anti-LPG mAb L157. Values for the molecular weight markers are times 1000.
Figure 4. Binding of anti-LPG repeat mAb CA7AE to purified *L. donovani* LPG in an inhibition ELISA. The data points represent single well values from a single experiment. Results from only one of three experiments are shown since the standard deviations from all points for the three assays were less than 0.04 OD units from the mean. Inhibitors: ■, purified *L. donovani* 1S2D LPG; ●, PO₄-6Gal-β1,4-Man; ○, Gal-β1,4-Man; ▲, Gal-β1,4-Glc.
Figure 5. Epitope mapping of *L. donovani* 1s2D LPG by indirect ELISA. Antigens tested were as follows: A, intact LPG; B, Phosphosaccharide core; C, Dephosphorylated LPG; D, TFA-treated phosphoaccharide core-PI; E, Periodate-treated LPG; F, nitrous acid-treated LPG; G, human transferrin. Antibodies were: ■, anti-LPG repeat mAb CA7AE; ○, anti-LPG core mAb L157; □, anti-LPG core mAb L98. The data points represent single well values from a single experiment. Results from only one of two experiments are shown since the standard deviations from all points for the two assays were less than 0.05 OD units from the mean.
binding of promastigote-excreted antigens by anti-LPG mAbs.

An antigen-capture ELISA was performed on purified antigens and *L. donovani* promastigote-conditioned culture medium using the anti-LPG mAbs. As shown in Figure 6A, the anti-LPG repeat mAb CA7AE was able to bind antigens released by wild-type *L. donovani* LD3 promastigotes into the culture medium as well as purified *L. donovani* 1S2D LPG, media Phosphoglycan (mPG) and cellular Phosphoglycan (cPG). The LPG-deficient mutant *L. donovani* C3PO did not release epitopes recognized by mAb CA7AE (data not shown) nor did this mAb bind the control antigen human transferrin. The same antigens were tested in an inhibition ELISA to determine their ability to prevent mAb CA7AE from binding to LPG as solid-phase antigen. The results shown in Figure 6B corroborate those observed for the antigen-capture ELISA (Figure 6A) with the exception that in this assay mPG was not recognized by mAb CA7AE. Neither of the anti-LPG core mAbs L157 and L98 were able to bind any of the antigens as presented in either of these two ELISA assays (data not shown), except for LPG and cPG.

The major glycoprotein secreted by leishmanial parasites *in vitro* is the tartrate-sensitive acid phosphatase (AcPase) (Bates and Dwyer 1987; Bates et al. 1988). The LPG phosphacylated disaccharide repeat epitope has been reported to be on the secreted AcPase of *L. donovani* promastigotes (Bates et al. 1990). To substantiate this, an indirect ELISA against AcPase purified from promastigote-conditioned culture medium was performed using the anti-LPG repeat mAb CA7AE. The results are shown in Figure 7. MAb CA7AE was able to bind both purified LPG and secreted AcPase from wild-type *L. donovani* 1S2D promastigotes but unable to recognize secreted AcPase epitopes from the LPG-deficient mutant *L. donovani* R2D2. Neither of the anti-LPG core mAbs were able to bind *L. donovani* 1S2D AcPase (data not shown).

The data from Figure 7 suggested that not only did mAb CA7AE bind the secreted form of LPG in parasite-conditioned culture supernatants (i.e. mPG; see Figure 6), but also bound secreted AcPase. That mAb CA7AE did indeed bind a secreted protein (AcPase) was demonstrated by using an antigen-capture ELISA to trap $^{35}$S-methionine biosynthetically-labelled molecules from *L. donovani* 1S2D promastigote-conditioned culture medium (Figure 8A). The same assay using spent medium from $^{35}$S-methionine labelled C3PO mutants showed that no counts were bound (Figure 8B). No binding was observed for either parasite when tritiated sugars were used for biosynthetic labelling. It has been established that these radioactive tracers are incorporated into LPG during biosynthesis (Turco et al. 1984; S.J. Turco personal communication). It is likely that AcPase would be preferentially bound over media PG by mAb CA7AE in an antigen-capture ELISA of
parasite supernatants (based on quantities of each molecule) explaining the results seen in Figure 8.
Figure 6. Binding of anti-LPG repeat mAb CA7AE to purified antigens or molecules in promastigote-conditioned culture medium in ELISA. A. Antigen capture ELISA. B. Inhibition ELISA. Antigens/Culture supernatants: •, L. donovani LD3 culture supernatant; △, L. donovani LD3 media PG; ○, L. donovani LD3 cellular PG; ■, L. donovani 1S2D LPG; □, human transferrin; ▲, no antigen. The data points represent single well values from a single experiment. Results from only one of three experiments are shown since the standard deviations from all points for the three assays were less than 0.05 OD units (Fig. 6A) or 0.07 OD units (Fig. 6B) from the mean.
Figure 7. Binding of anti-LPG repeat mAb CA7AE to purified *L. donovani* LPG or AcPase in indirect ELISA. Antigens: ■, *L. donovani* 1S2D LPG; †, *L. donovani* 1S2D AcPase; □, *L. donovani* R2D2 AcPase (LPG-deficient mutant); □, human transferrin.
Figure 8. Detection of antigens excreted into conditioned culture medium as determined using an antigen capture ELISA and mAb CA7AE. Results shown are for samples removed 24 hr after the radiolabels were added to the parasite cultures. A. *L. donovani* LD3 promastigotes. B. *L. donovani* C3PO promastigotes (LPG-deficient mutant). Radioactive substrates: ■, [³⁵S]-Methionine; ○, [³H]-2,3-Mannose; □, D-[6-³H(N)]-Glucosamine Hydrochloride.
Determination and isolation of protein in LPG preparations.

Indirect ELISA using the three anti-LPG mAbs on enzyme-digested LPG samples was performed to determine if protein components associated with the LPG glycan structure were responsible for any of the anti-LPG mAb binding to LPG. Both anti-LPG core mAbs L157 and L98 showed decreased binding after digestion of LPG with *Streptomyces griseus* pronase (compare Figures 9A and 9B). While the binding of mAb L98 was significantly reduced after pronase digestion, the binding of mAb L157 was essentially eliminated (Figure 9B). Binding of the anti-repeat mAb CA7AE was unaffected by pronase digestion of LPG. Neither trypsin nor proteinase K treatment of LPG caused a reduction in anti-LPG mAb binding (data not shown).

When LPG was purified by solvent extraction and column chromatography as previously described (Turco et al. 1984), protein was undetectable when the LPG preparation was tested by BioRad (Bradford 1976) and Lowry protein detection assays. In addition, no protein could be detected following SDS-PAGE and silver staining (data not shown). However, amino acid microanalysis after acid hydrolysis showed an amino acid composition which remained relatively constant from batch to batch of LPG, with only background levels of amino acids detected in unhydrolyzed samples. However, Edman degradation with a gas-phase sequenator provided no sequence, indicating blocked N-termini in material from LPG preparations. Exhaustive attempts to liberate the protein component, including alkaline pH and high salt, urea and guanidinium HCl solubilization and ion-exchange and gel-permeation chromatography, resulted in only a slight decrease in amino acid levels. In addition, pronase treatment of the LPG was similarly unsuccessful at reducing protein levels, as determined by amino acid microanalysis.

Anhydrous TFMSA is routinely used to remove synthetic peptides from solid supports in the last step of a synthesis methodology and is therefore known to preserve peptide bonds (Tam et al. 1986). This reagent is used under the same conditions to deglycosylate glycoproteins by cleavage of glycosidic linkages (Edges et al. 1981). It was therefore possible to utilize this organic acid to degrade the LPG carbohydrate structure, leaving only the associated protein material intact. After TFMSA digestion, the remaining protein material was subjected to reverse phase HPLC and fractions were analyzed for immunoreactivity by ELISA. Results of the indirect ELISA assay of column fractions are shown in Figure 10. A single major component from the elution profile was highly reactive with the anti-LPG core mAbs L157 and L98. Based on the assumption that the purified LPG was essentially homogeneous with respect to the described phosphoglycan structure and was free of observable protein contaminants the anti-LPG core mAbs had until this point been characterized as specific for the LPG carbohydrate core (see above). This new data
clearly indicated that while the epitopes bound by these mAbs were indeed associated with the LPG carbohydrate core (see Figure 5), they specifically bound a protein component tightly complexed with that core. This new protein moiety was termed LPG-associated protein (LPGAP). Significant quantities of LPGAP were then purified from mg quantities of LPG and used in immunoblotting and T cell stimulation assays (see Chapter 3). SDS-PAGE and Coomassie blue staining of the anti-LPGAP mAb L98 reactive material showed an intense doublet band of approximately 14 kDa running with the dye front, along with a number of less intensely staining bands (Figure 11A). However, immunoblotting of the SDS-PAGE resolved proteins, using mAb L98, showed a surprising result. The mAb detected essentially all of the above molecular species, as well as others ranging in apparent molecular mass of 40-55 kDa that were not obvious on the SDS-PAGE profile (Figure 11B, lane A). Results with the other anti-LPGAP mAb L157 were similar, except that the major 14 kDa species was not detected. Treatment of LPGAP with proteinase K prior to immunoblott ing (Figure 11B, lane B) confirmed that these results were not due to a common carbohydrate component on different proteins, substantiating the earlier TFMSA experiments (see Figure 10). MAb L98 showed no non-specific reactivity with MW standards or human transferrin and the control mAb HT1-3 (anti-human transferrin) showed no reactivity against the blotted LPGAP (data not shown).

Indirect and antigen-capture ELISAs using the anti-LPG mAbs failed to detect LPGAP epitopes in promastigote-conditioned or unconditioned culture medium, suggesting that LPGAP was not a component from the medium incorporated into the LPG sample during the purification protocol (data not shown). That LPGAP constituents were parasite-derived was further shown by growing *L. donovani* promastigotes in the presence or absence of added serum and then screening parasite lysates in indirect ELISA using the anti-LPG mAbs. These results are shown in Table 4. Anti-LPGAP mAb L98 bound to molecules in lysates from both *L. donovani* LD3 (wild-type) and C3PO (LPG-deficient mutant) promastigotes when the parasites were grown in either SM medium containing 10% FBS or in serum-free MEDIUM 199. Although the serum-free medium contained hemin as a necessary growth requirement for the parasites, mAb L98 did not bind hemin alone. Similar results were observed for the other anti-LPGAP mAb, L157 (data not shown).
Figure 9. Binding of anti-LPG mAbs to *L. donovani* 1S2D LPG or enzyme-treated LPG in indirect ELISA. A. Intact LPG. B. Pronase-digested LPG. Antibodies were: ■, anti-LPG repeat mAb CA7AE; □, anti-LPG core mAb L157; ○, anti-LPG core mAb L98; ●, HT1-3 (anti-human transferrin mAb).
Figure 10. ELISA detection of immunoreactivity in HPLC fractions after treatment of LPG with anhydrous TFMSA. Fraction numbers 30 and 31 are controls using *L. donovani* 1S2D LPG (10 μg/ml) and human transferrin (10 μg/ml) respectively. Antibodies were: ■ , anti-LPG repeat mAb CA7AE; ●, anti-LPG core mAb L157; □, anti-LPG core mAb L98; △, HT1-3 (anti-human transferrin mAb). The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 0.04 OD units from the mean.
Figure 11. A. SDS-PAGE of octyl Sepharose purified LPGAP. Coomassie blue stain. B. Immunoblot of octyl Sepharose purified LPGAP (a, LPGAP; b, LPGAP after proteinase K digestion). The mAb L98 was used in both a and b. A non-relevant anti-human transferrin mAb (HT1-3) showed no reactivity with either antigen (not shown).
Table 4. Binding of anti-LPG mAbs in ELISA to lysates of *L. donovani* promastigotes grown in the presence or absence of serum.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>CA7AE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>L98&lt;sup&gt;b&lt;/sup&gt;</th>
<th>HT1-3&lt;sup&gt;c&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>1. Parasite lysates&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. donovani</em> LD3 (SM + 10% FBS)</td>
<td>0.72*</td>
<td>0.47</td>
<td>0.04</td>
</tr>
<tr>
<td><em>L. donovani</em> LD3 (M199 + hemin)</td>
<td>0.77</td>
<td>0.41</td>
<td>0.06</td>
</tr>
<tr>
<td><em>L. donovani</em> C3PO (SM + 10% FBS)</td>
<td>0.10</td>
<td>0.38</td>
<td>0.04</td>
</tr>
<tr>
<td><em>L. donovani</em> C3PO (M199 + hemin)</td>
<td>0.10</td>
<td>0.44</td>
<td>0.04</td>
</tr>
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<td>2. Antigens</td>
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<td></td>
</tr>
<tr>
<td>LPG (10 ug/ml)</td>
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<td>0.80</td>
<td>0.05</td>
</tr>
<tr>
<td>LPGAP (10 ug/ml)</td>
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<td>0.93</td>
<td>0.08</td>
</tr>
<tr>
<td>BSA (100 ug/ml)</td>
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<td>0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>none</td>
<td>0.05</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>3. Medias</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM + 10% FBS&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.06</td>
<td>0.08</td>
<td>0.06</td>
</tr>
<tr>
<td>M199&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.09</td>
<td>0.09</td>
<td>0.05</td>
</tr>
<tr>
<td>hemin&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.10</td>
<td>0.10</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 0.02 OD units from the mean.

<sup>a</sup> anti-LPG repeat mAb CA7AE

<sup>b</sup> anti-LPGAP mAb L98

<sup>c</sup> anti-human transferrin mAb HT1-3.

<sup>d</sup> promastigote lysates at 5 X 10<sup>6</sup> parasites/ml. Parasites were grown in the indicated medium.

<sup>e</sup> diluted 1:10 in dH<sub>2</sub>O

<sup>f</sup> at 5.0 ug/ml
DISCUSSION

Monoclonal antibodies have proven to be extremely useful tools for the study of parasites and their antigens. The original intent of this research was to derive mAbs to surface membrane antigens of *L. donovani* promastigotes and then to use these antibodies to identify, isolate and characterize the molecules to which these mAbs were directed. Once identified and isolated, the involvement of these antigens in the immunobiology of *Leishmania*-host interactions could be examined - with emphasis on their potential as vaccine candidates for leishmaniasis. To this end, mice were injected with *L. donovani* promastigotes and mAbs derived that bound *L. donovani* promastigote antigens in immunofluorescence and ELISA. Both wild-type *L. donovani* 1S2D promastigotes and an LPG-deficient *L. donovani* 1S2D mutant, *L. donovani* R2D2 (King and Turco 1988) were used for screening. The strategy was that the R2D2 parasites, by lacking the major surface glycoconjugate LPG, might have different epitopes accessible to antibody binding. Thus a wider variety of surface molecules should be detected by surface-directed mAbs. In addition to a number of other *Leishmania*-specific hybridomas (see Table 2) two hybridomas secreted antibodies which bound to surface epitopes on living 1S2D promastigotes but failed to bind to the surface of living R2D2 cells. These antibodies were selected as being potentially LPG-specific. It was decided to focus further work primarily on these prospective anti-LPG mAbs.

Since the salient structural features of *L. donovani* LPG were known (Orlandi and Turco 1987; Turco et al. 1987; Turco et al. 1989) it was possible to use subfractions and fragments of LPG to localize the anti-LPG mAb epitopes to distinct regions of the LPG molecule. One mAb (CA7AE) raised against heat-killed *L. donovani* 1S2D promastigotes bound to the phosphoglycan portion of LPG (repeating phosphorylated disaccharide units + phosphosaccharide core) in ELISA but not to purified phosphosaccharide core or the 1-0-alklyglycerol moiety. Thus this mAb recognized structures in the repeating phosphorylated disaccharide unit. Indeed, P04-6Gal(61-4)Man was the only disaccharide to strongly inhibit binding of this mAb in an inhibition ELISA, indicating that the epitope was composed of one or more of these phosphorylated disaccharides. The phosphate group comprises part of this epitope since the non-phosphorylated disaccharide Gal(81-4)Man did not inhibit binding in the ELISA even at a concentration greater than 100-fold higher than that required for the phosphorylated disaccharide to effect significant inhibition.

In the experiments reported here, it is noteworthy that *Leishmania* glycolipids appeared to be highly immunogenic since a high proportion of mAbs specific for LPG and other glycolipids were obtained in all fusions. Whether induction of anti-glycolipid mAbs...
antibodies is a natural phenomenon during a *L. donovani* infection is unknown. It is possible that anti-glycolipid antibodies were artificially selected for by the immunization and/or screening protocol. Three additional attempts to produce anti-LPG mAbs of a different isotype than IgM isotype were unsuccessful. When either whole promastigotes or purified LPG were used as the immunizing antigen only anti-LPG phosphorylated disaccharide repeat antibodies of the IgM isotype appeared to be induced. The reason for this is unknown. It may be that LPG acts as a T lymphocyte-independent antigen; that is, an antigen capable of activating B lymphocytes for antibody production independent of helper T lymphocytes. T-independent antigens are all large polymeric molecules with repeating antigenic determinants some of which are able to act at high concentrations as B cell mitogens (Roitt et al. 1989). The most common example of these antigens is the lipopolysaccharides of gram-negative bacteria. While LPG does not seem to possess B cell mitogenic activity (see Chapter 3) by its inherent structure (long carbohydrate repeat polymer) it may act as a T-independent antigen. Since, in general, T-independent antigens predominantly give rise to IgM responses (Roitt et al. 1989), this could explain why attempts to generate anti-LPG repeat mAbs of IgG isotypes were unsuccessful.

The two mAbs (L98 and L157) derived by immunisation with pure LPG bound only to intact *L. donovani* LPG and to the phosphosaccharide core portion of the molecule. As these mAbs were originally believed to bind to carbohydrate epitopes of LPG, experiments using chemically-derived LPG fragments were performed to further define the epitopes. Dephosphorylation of the core residues resulted in loss of binding by each anti-core mAb, indicating that the epitope required the presence of the core's inner phosphate group. Treatment of LPG with trifluoroacetic acid which hydrolyses the galactofuranosylmannose bond also resulted in loss of antibody binding in ELISA. This suggested that the galactofuranosylmannose bond was necessary for recognition by mAb L157. Both anti-core mAbs recognized periodate-oxidized LPG in which the terminal and penultimate galactose residues of the core and C-6 of the galactofuranose were oxidized, and nitrous acid-treated LPG in which the glucosaminylinositol unit of the glycoconjugate was cleaved. Taken together these results suggested that the epitope bound by the anti-core mAbs minimally required the presence of the inner phosphate and an intact galactofuranosylmannose linkage.

In a different set of experiments, LPG was used as a potentially immunosuppressive agent in T cell stimulation assays (see Chapter 3). Surprisingly, it was found that the *L. donovani* LPG molecule was able to stimulate primed murine T cells *in vitro*. This was not expected since glycolipids are not believed to function as T cell stimulatory epitopes (other than as mitogens), a property reserved for protein antigens.
Consequently, further analysis was performed to determine if the LPG provided by Dr. S. Turco was indeed free of protein. Although no free amino acids were present in unhydrolyzed samples of LPG or PG, amino acid microanalysis of acid hydrolyzed samples revealed measurable quantities of amino acids, the relative ratios of which stayed fairly constant between different LPG or PG batches. The protein component was found to be very tightly associated with LPG as exhaustive attempts to remove the protein by solubilization in base, strong denaturing agents, organic solvents and various salts, as well as by a variety of HPLC procedures, were unsuccessful. Selective degradation of oligosaccharides with anhydrous TFMSA resulted in a protein preparation bound by both the anti-LPG mAbs L98 and L157. This protein was termed LPG-associated protein (LPGAP) and it was this moiety that was bound by the anti-LPG carbohydrate core mAbs L98 and L157.

One concern involved an explanation for the large number of LPG-derived polypeptides detected using mAbs L98 and L157 (Figure 11). Since the first step in LPG isolation involves chloroform-methanol extraction (Orlandi and Turco 1987) it was considered unlikely that proteolytic degradation occurred during LPG purification. This was substantiated by isolation of the same array of proteins after lysis of L. donovani promastigotes in 0.1% trifluoroacetic acid (TFA) (data not shown). Moreover, since reactivity with mAbs L98 and L157 persisted after TFMSA digestion, a common carbohydrate epitope was also excluded as an explanation for the large number of cross-reacting species. Similarly, proteinase K digestion of purified LPGAP eliminated mAb binding in immunoblots (Figure 11). However, when LPG (containing LPGAP) was treated with proteinase K the binding of the anti-LPGAP mAbs was unaffected (see Figure 9A). It may be that the LPG molecule interfered with enzyme access to LPGAP or that after digestion of LPGAP the LPG molecule was able to maintain the LPGAP fragments in a complex suitable to anti-LPGAP mAb recognition. It is reasonable to expect that these tightly complexed proteins were derived from a common precursor via a proteolytic process occurring prior to isolation. The most likely candidate is a protein of ~50kDa which dominates the SDS-PAGE electropherogram of material isolated in the presence of 0.1% TFA. The high concentration of low molecular weight species isolated from LPG (with apparent molecular mass near 14kDa) may be explained by preferential partitioning of these extraordinarily hydrophobic molecules during the organic solvent extraction step for LPG isolations.

An obvious concern was that LPGAP was merely a serum or medium component that had an unexpectedly high association with LPG and was co-isolated during LPG purification. The results in Table 4 demonstrated that LPGAP was in fact parasite-derived
as promastigotes grown in medium essentially free of exogenous protein were still able to produce the LPGAP molecule as detected by mAb binding. The specific function of LPGAP and the reason for its association with cellular LPG is another area of conjecture. The possible functions of LPGAP are discussed in Chapters 2 and 3.

Surprisingly, in contrast to the anti-repeat mAb CA7AE, only the anti-LPGAP mAbs L157 and L98 were able to bind purified LPG in immunoblots (Figure 3). However, mAb CA7AE was able to bind, in immunoblots, secreted AcPase purified from promastigote-conditioned culture medium (A. Sigurdson, University of Victoria, personal communication), suggesting that the carbohydrate moiety of LPG was not being retained on the nitrocellulose membrane during the electrophoretic transfer procedure. It is likely that during SDS-PAGE denaturation procedures, the LPG and LPGAP molecules are separated, resulting in LPGAP retention on nitrocellulose membranes and subsequent anti-LPGAP mAb binding.

In ELISA and immunofluorescence procedures mAb CA7AE was shown to bind both cell surface LPG and the epitope found on the secreted form of the molecule (mPG). Why this mAb was not inhibited by mPG in the inhibition ELISA is unknown. It has been reported that approximately half of the at least 40 different proteins found in conditioned culture medium obtained from growing L. donovani promastigotes are glycosylated (Bates et al. 1988). Experiments, using 35S-methionine biosynthetic labelling and the antigen-capture ELISA demonstrated the ability of mAb CA7AE to bind secreted glycoproteins. Indeed, these labelling experiments suggested that the predominant molecules trapped by mAb CA7AE in ELISA were glycoproteins and not mPG. mAb CA7AE was demonstrated to bind specifically to L. donovani secreted AcPase. These data indicate that like L. tropica promastigotes (Jaffe et al. 1990a) L. donovani promastigotes's secreted form of LPG (mPG) and secreted AcPase share a common carbohydrate epitope, the P04-6Gal(β1-4)Man moiety. Interestingly, the LPGAP molecule was not detected in preparations of mPG or secreted AcPase.

A variety of monoclonal antibodies which are specific for LPG (in the past, also called excreted factor) have been reported previously (de Ibarra et al. 1982; Handman and Hocking 1982; Greenblatt et al. 1983; Sacks et al. 1987; Jaffe and Sarfstein 1987; Pimenta et al. 1989; Jaffe et al. 1990a; Handman 1990). A list of these is presented in Table 5. Some of these mAbs are species-specific and most recognize epitopes on both cellular and secreted forms of LPG. However, the specific epitopes for these antibodies have not been defined. Here, are described the first defined mAb epitopes of LPG; the P04-6Gal(β1-4)Man unit recognized by mAb CA7AE and the LPGAP moiety recognized by mAbs L98 and L157.
Table 5. Summary of documented anti-Leishmania LPG monoclonal antibodies and their binding specificities.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Iso-type</th>
<th>Binds to the surface of:</th>
<th>Species-specific</th>
<th>Epitope defined</th>
<th>Binds to</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pros(^a) Amas(^b) Macros(^c)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WIC79.3</td>
<td>G1</td>
<td>+ - +</td>
<td>+ (L. major) repeat</td>
<td>-</td>
<td>1, 2</td>
<td></td>
</tr>
<tr>
<td>WIC79.7</td>
<td>G1</td>
<td>+ - +</td>
<td>+ (L. major) repeat</td>
<td>nd(^f)</td>
<td>1, 2</td>
<td></td>
</tr>
<tr>
<td>WIC108.3</td>
<td>G1</td>
<td>+ - +</td>
<td>- repeat</td>
<td>+</td>
<td>1, 2</td>
<td></td>
</tr>
<tr>
<td>L-5-16</td>
<td>G3</td>
<td>+ + +</td>
<td>repeat</td>
<td>nd (^d)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>3F12</td>
<td>G1</td>
<td>+(^g) nd nd</td>
<td>+ (L. major) repeat</td>
<td>+</td>
<td>4, 6</td>
<td></td>
</tr>
<tr>
<td>T11</td>
<td>M</td>
<td>+ - nd</td>
<td>+ (L. tropica) repeat</td>
<td>+</td>
<td>5, 7</td>
<td></td>
</tr>
<tr>
<td>T15</td>
<td>G3</td>
<td>+ - nd</td>
<td>+ (L. tropica) repeat</td>
<td>+</td>
<td>5, 7</td>
<td></td>
</tr>
<tr>
<td>4A2-A2</td>
<td>nd</td>
<td>+ + +</td>
<td>+ (L. major) repeat</td>
<td>nd(^e)</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>5E6-B4</td>
<td>nd</td>
<td>+ - +</td>
<td>+ (L. major) repeat</td>
<td>nd(^e)</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>CA7AE</td>
<td>M</td>
<td>+ - +</td>
<td>-</td>
<td>P-Gal-Man(^h)</td>
<td>+</td>
<td>9-13</td>
</tr>
<tr>
<td>L98</td>
<td>G1</td>
<td>- - -</td>
<td>-</td>
<td>LPGAP(^i)</td>
<td>-</td>
<td>9-13</td>
</tr>
<tr>
<td>L157</td>
<td>G1</td>
<td>- - -</td>
<td>-</td>
<td>LPGAP</td>
<td>-</td>
<td>9-13</td>
</tr>
</tbody>
</table>

\(^a\) *Leishmania* promastigotes.
\(^b\) *Leishmania* amastigotes.
\(^c\) *Leishmania*-infected macrophages.
\(^d\) A moiety of the LPG molecule has been defined as the epitope recognized by the mAb.
\(^e\) Excreted Factor; includes both media phosphoglycan and secreted AcPase.
\(^f\) not done; or, at least, not reported in the literature
\(^g\) binds specifically to metacyclic *L. major* LPG but not to log-phase LPG.
\(^h\) first defined against the P0\(_4\)-Gal(Bl-4)Man epitope from the LPG repeat of *L. donovani* promastigotes.
\(^i\) LPG-associated protein.

References:
1. de Ibarra et al. 1982
2. Handman and Hocking 1982
3. Greenblatt et al. 1983
4. Sacks et al. 1987
5. Jaffe and Sarfstein 1987
6. Pimenta et al. 1989
7. Jaffe et al. 1990a
8. Handman 1990
9. Tolson et al. 1989
10. Tolson et al. 1990
11. Tolson et al. 1991a
12. Tolson et al. 1991b
Conclusions

1. Twenty-five anti-*L. donovani* mAbs were derived. Four of these mAbs specifically bound *L. donovani* LPG.

2. The epitopes recognized by the anti-LPG mAbs were characterized. MAbs CA7AE and BF9CC recognized the P04-6Gal(β1-4)Man unit of the repeat polymer of the LPG molecule.

3. A parasite-derived protein component (Li-GAP) tightly associated with the LPG carbohydrate structure was discovered. This moiety was recognized by the other anti-LPG mAbs L157 and L98.

4. *L. donovani* promastigote's membrane-bound and secreted forms of LPG (mPG) and secreted AcPase share a common carbohydrate epitope, the P04-6Gal(β1-4)Man moiety which is recognized by mAb CA7AE. The LPGAP molecule was not detected in parasite-conditioned culture medium.

Most of the work outlined in this chapter has been published or submitted for publication:


CHAPTER 2. EXPRESSION OF LPG IN DIFFERENT *LEISHMANIA* SPECIES AND LIFE CYCLE STAGES.

Introduction

2.1 Life Cycle of the *Leishmania* parasite.

*Leishmania* spp. are protozoans of the family *Trypanosomatidae* which can be divided into two groups: those confined to invertebrate hosts and the more advanced group, which includes the genera *Leishmania* and *Trypanosoma* and which has both invertebrate and vertebrate hosts. The *Leishmania* parasites are transmitted to vertebrate hosts by female sandflies of the genera *Phlebotomus* (Old World) and *Leuromyzon* (New World).

a) Life cycle overview.

The life cycle of the leishmanial parasite in the sandfly vector and mammalian host is illustrated in Figure 1 (steps 1 through 16). The motile, flagellated promastigote form of the parasite is introduced into the skin by the bite of a feeding sandfly (1). Once inside the bloodstream of the mammalian host, the promastigote attaches to and is engulfed by cells of the mononuclear phagocyte system (2). The promastigote-containing phagosome fuses with lysosomal vesicles (3) and within this resulting phagolysosome the promastigote differentiates into the non-motile, non-flagellated amastigote form and multiplies (4, 5, 6). The heavily parasitized macrophage eventually ruptures and free amastigotes are released into the bloodstream (7) where they are disseminated throughout the host reticuloendothelial system (8). The parasitized macrophages or free amastigotes are taken up by a sandfly during a blood meal (9). The ingested macrophages rupture and released amastigotes migrate to the sandfly gut (10) where they differentiate into promastigotes (11). The promastigotes multiply by binary fission (12) and attach to gut epithelial cells (13). The attached promastigotes eventually migrate to the insect foregut (14, 15) where they partially obstruct the digestive tract. When the infected sandfly takes a second blood meal, it regurgitates infectious promastigotes from its pharynx into the mammalian bloodstream (16) thus completing the parasite life cycle.

b) Metacyclogenesis of *Leishmania* in the sandfly.

Following ingestion of a *Leishmania*-infected blood meal by the sandfly, the parasites attach to the epithelial cells of the midgut and undergo a process called metacyclogenesis in which the promastigotes undergo development into infectious forms (reviewed in Sacks 1989). During this period the parasites detach from the epithelial cells and migrate to the mouthparts, culminating in free-swimming, highly infectious
Figure 12. Leishmanial life cycle in the sandfly vector and mammalian host (from Chang et al. 1985).
promastigotes in the fly proboscis. The midgut promastigotes, 3 days after fly infection, are relatively avirulent for BALB/c mice (Sacks and Perkins 1984) whereas midgut promastigotes 4-7 days after infection become progressively more virulent. Optimally infective promastigotes appear shortly after bloodmeal passage, coinciding with the time at which another meal is sought by the fly (Sacks and Perkins 1984; Sacks and Perkins 1985). The mechanisms by which the parasites are induced to attach or detach and migrate through the sandfly gut are unknown. However, promastigotes have been demonstrated to migrate along chemical gradients in response to sugars normally stored in high concentration in the sandfly crop and pharynx (Bray 1983a). Therefore, it has been suggested that lectin-mediated interactions facilitate this migratory process (Sacks 1989).

While the role, if any, of LPG during this migration is unknown, recent evidence has shown that dividing log-phase promastigotes in the insect midgut excrete large amounts of LPG (Davies et al. 1990). Though not proven, this LPG has been suggested to be one component of a carbohydrate-based gel-like matrix in which the promastigotes are found embedded in the fly foregut during one phase of metacyclogenesis (Davies et al. 1990).

c) Entry into the mammalian host.

Ten to 100 promastigotes enter the host mammal through a sandfly bite (Theodos et al. 1991b). In the bloodstream, the promastigotes encounter complement, antibodies, and phagocytic cells, all of which can kill them (Pearson et al. 1983). Depending on the parasite species, it has been reported that as many as 80% of the promastigotes are effectively eliminated by such mechanisms (Franke et al. 1985). However, the survivors are inevitably found within host phagocytic cells, primarily neutrophils and mononuclear phagocytes, although long term survival is only possible in cells of the macrophage family (Wright and El Amin 1989). It has also been proposed that sandfly saliva contains an immunosuppressive substance(s) which exacerbates infection by initially enhancing parasite survival through its ability to interfere with H$_2$O$_2$ production and antigen presentation by macrophages (Theodos et al. 1991b). In fact, low number inocula of *L. major* promastigotes did not survive in mice unless they were co-injected with sandfly salivary gland lysates (Titus and Ribiero 1988).

Promastigotes activate complement essentially through the alternative pathway which not only creates a C5A gradient that chemotactically attracts macrophages but also produces the hemolytically active C3 which binds to the parasite surface (Puentes et al. 1988). While log-phase promastigotes are susceptible to lysis by normal serum, stationary-phase (metacyclic) promastigotes are far more resistant (Puentes et al. 1988). It should be noted that the complement system appears to interact with different species and
growth phases of *Leishmania* in quite distinctive manners. For example, while nearly 75% of the C3 bound to *L. donovani* promastigotes is present as C3b which binds to macrophage receptor CR3 (Blackwell et al. 1985), 80-85% of the C3 bound to *L. major* promastigotes is in the form C3b, which binds to macrophage receptor CR1 but not CR3 (Puentes et al. 1988). In addition, *L. major* metacyclics appear to require complement opsonization to facilitate their binding to host macrophages since their binding to macrophages is negligible in the absence of deposited C3b (da Silva et al. 1989). The facilitated attachment and entry of serum-opsonized metacyclic promastigotes into macrophages via complement receptors (Blackwell et al. 1985; Mosser and Edelson 1987) may be a key event in the *Leishmania* parasite's strategy for survival, since receptor-mediated phagocytosis via these receptors fails to trigger the cellular activation pathways that produce the leishmanicidal agent $H_2O_2$ (Wright and Silverstein 1983).

The LPG of *L. major* metacyclic promastigotes has been demonstrated to serve as an acceptor for C3b. Therefore, it has been suggested that LPG may play an important role during metacyclogenesis. This differentiation process involves the transformation of the parasite from an actively dividing (log-phase) non-infective stage to a non-dividing (stationary-phase) infectious "metacyclic" stage (reviewed in Sacks 1989). It has been demonstrated that LPG is subject to developmental modification during this transition period (Sacks and da Silva 1987). During metacyclogenesis of *L. donovani* and *L. major* promastigotes LPG undergoes considerable structural modifications including an approximate doubling in the number of repeating phosphorylated saccharide units and a structural change in these units (Sacks et al. 1990). In contrast, *L. mexicana* promastigotes show a structural change in the repeat units and do not elongate the LPG molecule (Sacks 1989). Electron microscopic ultrastructural studies have shown that the thickness of the *L. major* cell surface glycocalyx increases in metacyclic promastigotes coincident with an increase in the average molecular weight of LPG and that this thickening of the surface coat could be specifically labelled with an antibody against the metacyclic form of LPG (Pimenta et al. 1989). Although not shown specifically for LPG, there is also evidence (Jacobson and Schnur 1990) that the carbohydrate configuration on surface membrane-bound and excreted antigens of *L. major* promastigotes is extremely variable even within a defined population. This variability can be regulated by the availability of culture medium components and, therefore, presumably by the different host environments encountered by the organism.

Because the site of C3 deposition determines the site of C5 cleavage initiating C5b-9 formation, it is postulated that the developmental change in LPG (i.e. larger/longer) during metacyclogenesis sterically hinders access of large molecules to the promastigote
membrane (Sacks 1989). This in turn prevents cell lysis by the C5b-9 membrane attack complex (Sacks 1989; Puentes et al. 1990). In addition, *L. major* metacyclics have recently been shown to spontaneously shed the C5-9 complex from their surface (Puentes et al. 1990). Therefore, the metacyclic LPG provides a barrier against complement-mediated lysis but because it activates complement efficiently resulting in extensive deposition of C3 it also promotes the attachment to and uptake by macrophages via appropriate receptors. The efficient acquisition of C3 fragments by metacyclic promastigotes may be the key event which ultimately establishes *Leishmania* as intramacrophage parasites.

### d) Parasite-macrophage surface interactions.

*Leishmania* promastigotes enter macrophages by a conventional cytochalasin-sensitive phagocytic event (Bray 1983b) although the signals that trigger internalization have not been elucidated. As there does not seem to be a specialized parasite orientation required for interaction between the promastigote and macrophage to occur, interiorization of the promastigotes appears to occur through random promastigote-macrophage interactions (Chang 1979). Progress in the understanding of promastigote-macrophage binding has been complicated by several problems: 1) many studies have been conducted in the absence of serum in order to identify parasite molecules that are able to bind directly to macrophage receptors. However, as was discussed earlier, recent studies have demonstrated that for *L. major* at least, complement plays a central role in infectivity (Puentes et al. 1988); 2) there are inherent differences in the various experimental target cells used (e.g. human versus mouse); 3) since promastigotes are a heterogeneous population (Sacks et al. 1985), parasite metamorphosis probably has relevance to macrophage-promastigote interactions especially if infectivity is a function of receptor-ligand pairing; 4) *Leishmania* is a complex of several different species that are responsible for a spectrum of disease syndromes and show markedly different tissue tropism (see earlier). Therefore, there are probably both qualitative and quantitative differences in the way which these parasites bind to and enter macrophages, although they undoubtedly do share some common mechanisms.

Two promastigote glycoconjugates have been specifically implicated in the attachment and uptake of promastigotes to macrophages. The role of the major surface protease, GP63, was described earlier. Involvement of LPG in macrophage binding was first shown by Handman and Goding (1985) who demonstrated that the attachment of *L. major* promastigotes to macrophages could be inhibited by anti-LPG antibodies. In the absence of serum opsonins, using LPG-coated beads to restrict attachment to a single ligand, it was shown that LPG binds directly to the lectin-like lipopolysaccharide binding.
site present on CR3 and p150,95 - members of the CD18 family of macrophage surface receptors (Talamas and Russell 1989; Talamas-Rohana et al. 1990). In the presence of non-immune serum *L. major* metacyclic promastigotes bind C3b through LPG and attach primarily through CR1 to the macrophage (see above). Interestingly, beads bearing either GP63 or LPG alone attach to macrophages but are not ingested suggesting that more than one signal must be involved in parasite entry (Russell and Wright 1988). Beads bearing both GP63 and LPG are ingested, indicating that a combination of these two signals is sufficient to mediate uptake (Russell and Wright 1988). In addition, although they do not survive (see later), *Leishmania* promastigote mutants deficient in LPG are still readily phagocytized by macrophages (Handman et al. 1986; McNeely and Turco 1990) suggesting there may be other parasite molecules that play a role in phagocytic uptake.

Several other macrophage receptors have been implicated as promastigote attachment sites. These include the mannosyl/lucosyl receptor (Blackwell et al. 1985; Wilson and Pearson 1986), the fibronectin receptor (Wyler et al. 1985; Ouaisi 1988), and the receptor for advanced glycosylation endproducts (Mosser et al. 1987). Attachment and entry of the promastigote into the macrophage may also involve macrophage Fc receptors (Bray 1983b). This would require the presence of specific antibodies to opsonize the parasite and is therefore of little relevance to the naive host.

e) The role of LPG in intracellular survival.

Macrophage activation has been described as a cascade effect which proceeds through several functional stages (reviewed in Roitt et al. 1989). During infection, resting tissue or newly differentiated monocyte-derived macrophages recruited as part of an inflammatory reaction eventually become fully primed for cytotoxicity by cytokine exposure. Subsequently they develop into fully activated cytotoxic effector cells following further stimulation with cytokines or other co-signals. The different macrophage stages produce a variety of secretory products that have been implicated in microbicidal activity (reviewed in Adams and Hamilton 1984). Whether these mediators function as cytotoxic effector molecules depends largely on the susceptibility of the particular microbe.

One unique characteristic of leishmanial parasites is their ability to survive and multiply in macrophage phagolysosomes. With the exception of *Mycobacterium leprae*um*rum* (reviewed in Gaylord and Brennan 1987) other intracellular parasites such as *Toxoplasma gondii* (Jones et al. 1972) and *Trypanosoma cruzi* (Nogueira and Cohn 1979) circumvent the problem of intralysosomal degradation by inhibiting phagosome-lysosome fusion or by escaping from the phagosome, respectively. Leishmanial parasites possess mechanisms that enable them to evade macrophage oxygen-dependent and oxygen-independent microbicidal mechanisms. Reactive oxygen intermediates, such as H₂O₂, are
toxic for log-phase *Leishmania* promastigotes. In fact, H$_2$O$_2$ alone appears to be both necessary and sufficient to achieve this particular leishmanicidal activity (Murray and Nathan 1988). However, *Leishmania* amastigotes appear to be susceptible only to effector molecules produced by fully activated macrophages and are killed by lymphokine-activated macrophages but not by resident or inflammatory macrophages and killing is not dependent on products of the respiratory burst (James and Hibbs 1990). It has recently been reported that interferon-γ-activated macrophages kill intracellular amastigotes *in vitro* by means of a nitrogen-based mechanism (Liew et al. 1990a; Green et al. 1990). However the relative roles of oxygen metabolites and nitrogen intermediates in leishmanicidal activity *in vivo* are still unclear. Although the enzymatic pathways leading to the synthesis of reactive oxygen intermediates and reactive nitrogen intermediates are distinct, there is evidence that the end products can influence each other. For example, superoxide dismutase can enhance the half-life of nitric oxide while catalase can inhibit nitric oxide synthesis in activated macrophages (Liew and Cox 1991).

*Leishmania* metacyclics not only avoid complement-mediated killing but ligation of CR1 and CR3 through promastigote-bound C3 facilitates parasite internalization that does not elicit an oxidative burst (Wright and Silverstein 1983) presumably allowing invading promastigotes to bypass this important host defense mechanism. In addition, *Leishmania* LPG has been proposed to provide other mechanisms for circumventing the respiratory burst. When added to human monocytes purified *L. donovani* LPG has been shown to impair the generation of the respiratory burst (McNeely and Turco 1990) possibly through an inhibitory effect on protein kinase C (McNeely and Turco 1987; McNeely et al. 1989). In addition, the repeating phosphorylated disaccharide moiety of *L. donovani* LPG has been demonstrated to scavenge toxic oxygen radicals *in vitro* (Chan et al. 1989) and both the repeating phosphorylated disaccharide and phosphosaccharide core were able to selectively inhibit signal transduction and subsequent immunologic responses in macrophages (Frankenburg et al. 1990; Descoteaux et al. 1991). Furthermore, *Leishmania* amastigotes, but not promastigotes, show a high activity of enzymes (glutathione peroxidase, superoxide dismutase, catalase) that are known to degrade toxic oxygen products (Murray 1981; Bogdan et al. 1990) and promastigotes possess an acid phosphatase that has been shown to inhibit the oxidative burst in neutrophils (Remaley et al. 1984; Remaley et al. 1985a).

Finally, the unique structure of LPG itself may afford protection against phagolysosomal enzymatic attack by providing a highly anionic impediment, by forming a structural barrier that is not susceptible to hydrolysis by phagolysosomal glycohydrolases and lipases and/or by specifically inhibiting the action of one or more of the hydrolytic
enzymes. One lysosomal enzyme clearly inhibited by LPG was the β-glactosidase of murine peritoneal macrophages although there was no demonstrable effect on several other enzymes (El-On et al. 1980). Table 6 summarizes the activities and functions for *Leishmania* LPG that have been demonstrated or at least suggested.

f) **Amastigotes.**

*Leishmania* amastigotes are obligate intracellular parasites. Indeed, amastigotes exhibit enhanced metabolism and multiplication under acidic conditions reflecting their adaptation to intracellular growth (Mukkuda et al. 1985). Amastigotes are also more resistant than promastigotes to cytokine-induced oxygen-dependent antimicrobial mechanisms (Hall and Joiner 1991). In general, however, studies regarding the amastigote are still in their infancy.

It has been speculative whether or not amastigotes express LPG. While the presence of LPG on amastigotes had not been directly demonstrated its expression has been inferred from the results of immunofluorescence studies detecting LPG on *L. major*-infected macrophages (Handman and Hocking 1982; de Ibarra et al. 1982; Handman 1990). In these studies the infections were initiated with promastigotes which express and release large amounts of LPG. Therefore the possibility that the LPG detected on infected cells was promastigote-derived could not be excluded. Recently it has been demonstrated that *L. major* amastigotes do indeed express a lipophosphoglycan (Turco and Sacks 1991; Glaser et al. 1991). A number of similarities were reported between the amastigote and promastigote forms of the LPG molecule, including: 1) similar solubility properties since identical extraction protocols were used for the two molecules; 2) similar susceptibilities to chemical and enzymatic cleavage processes indicating certain conserved structural residues within the molecule (i.e. a PI-PLC-sensitive lipid anchor and an unacetylated hexosamine); and 3) similar unique mild acid-labile phosphosaccharide repeat polymer (Turco and Sacks 1991). However, the mild acid-generated phosphoglycan fragments of amastigote LPG, in addition to being larger in size, were not as anionic as their promastigote counterparts (Turco and Sacks 1991). The amastigote glycan was reported to be antigenically distinct from the promastigote LPG as determined by binding studies using a rabbit antiserum and mAbs (Glaser et al. 1991; Pimenta et al. 1991) further indicating that the promastigote LPG is a stage-specific structure. The finding that the carbohydrate of the amastigote LPG is distinct from the promastigote LPG may not be too surprising given the previously described developmental polymorphisms of this structure accompanying metacyclogenesis (see above).

Whether the amastigote lipophosphoglycan has a specific role in the intracellular survival of the parasite is unknown. However, amastigotes released from host
macrophages into the bloodstream must rapidly gain entry into adjacent host cells. As *L. donovani* amastigotes are resistant to non-immune serum and are able to fix C3 efficiently (Mosser et al. 1985) it is possible that amastigote LPG is utilized in a manner similar to that already discussed for its promastigote counterpart (see above).

2.2 Leishmanial antigens expressed on macrophages.

Early studies by Farah et al. (1975), Handman et al. (1979), Berman and Dwyer (1981), Handman and Hocking (1982) and de Ibarra et al. (1982) demonstrated the presence of leishmanial antigens on the surface of infected macrophages. The identities of these molecules were never established. More recently, using mAbs and indirect immunofluorescence assays, other research groups showed that leishmanial glycoconjugates were expressed on the surface of *Leishmania*-infected macrophages (Handman and Goding 1985; Williams et al. 1986). In one study (Williams et al. 1986), the authors stated that the *L. braziliensis panamensis* major surface glycoconjugate was not expressed on the surface on murine peritoneal macrophages infected *in vitro* until at least 6 hours post infection, suggesting that the molecules had an intracellular origin and that the surface-exposed antigen represented a breakdown product generated via phagolysosomal degradation and recycling events. The other study demonstrated that a macrophage cell line bound an extracellular form of the predominant surface glycoconjugate of *L. braziliensis* promastigotes (Handman and Goding 1985). In addition, Handman (1990) showed that *L. major* LPG epitopes were expressed on the surface of infected macrophages. These LPG epitopes were also suggested to originate from the intracellular amastigote. *L. major* LPG is one of the few examples of a well-characterized parasite antigen that is displayed on the surface of infected macrophages and which should be available for immune recognition by sensitized T lymphocytes (see Chapter 3).

2.3 Serotyping as the basis for *Leishmania* species identification.

Factors excreted *in vitro* by promastigotes and amastigotes have allowed different leishmanial strains to be assigned to defined serotypes (Schnur et al. 1972) and has proven to be a valuable tool in diagnostic, demographic and ecological studies of leishmaniasis. This classification is based on the interaction of rabbit antisera generated against living promastigotes with carbohydrate antigenic determinants (primarily the secreted forms of LPG and AcPase, see Chapter 1) released by the actively growing parasites (Schnur et al. 1972). This system allows the general classification of parasites into large groupings which have been designated An, Bn and AnB2 (Schnur et al. 1972). For example, polyclonal rabbit antibodies to promastigotes of *L. donovani* (type B
serotype) or *L. major* (type A serotype) differentiate between the excreted factors of these parasites and show no demonstratable cross-reactions (Schnur 1982). Using this simple typing system, *L. tropica* and *L. major* have been assigned to the same group A serotype but segregate into distinct nonoverlapping serotypes since all *L. tropica* species produce excreted factors of the A2 subserotype (Jaffe et al. 1990a). However, serotype designation should not automatically assign a parasite to a clinically defined species as serotypes defined in other test systems have cut across the boundaries of species based on clinical grounds (Schnur et al. 1972).

2.4 Diagnosis of Leishmaniasis.

Human leishmaniasis is caused by at least 14 different species and subspecies of the genus *Leishmania*. Disease diagnosis was initially made on clinical and geographical criteria and thereafter on epidemiological and parasitological grounds (Wirth et al. 1986). More recently, *Leishmania* species have been distinguished by such methods as the buoyant density of nuclear and kinetoplast DNA, by analysing sequence homologies in kinetoplast DNA, by the use of DNA probes, and by electrophoretic mobility of isoenzymes (reviewed in Alexander and Russell 1985; Wirth et al. 1986). In addition, *Leishmania* species and subspecies-specific mAbs have been used to identify parasites, suggesting the potential for an effective and convenient method of rapid diagnosis (reviewed in Alexander and Russell 1985). Until these techniques have been adapted to operate reliably in the field, current diagnosis of leishmaniasis is generally achieved by direct examination of a tissue biopsy or by means of a delayed-type hypersensitivity test referred to as the Montenegro assay (Wirth et al. 1986). Unfortunately, neither of these methods is able to distinguish *Leishmania* species or subspecies and the Montenegro test cannot distinguish current from previous infections.
Table 6. Activities and functions for *Leishmania* LPG that have been demonstrated or at least suggested.

<table>
<thead>
<tr>
<th>Activity or Function</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td><strong>A. Demonstrated functions:</strong></td>
<td></td>
</tr>
<tr>
<td>Associated with metacyclogenesis</td>
<td>1, 2</td>
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<tr>
<td>Acceptor for complement component C3</td>
<td>1, 3-5</td>
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<tr>
<td>Promastigote attachment to macrophages</td>
<td>6-9</td>
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<tr>
<td>Chelator of intracellular calcium</td>
<td>10</td>
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<tr>
<td>Inhibitor of β-galactosidase</td>
<td>11</td>
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<tr>
<td>Inhibitor of protein kinase C</td>
<td>12, 13</td>
</tr>
<tr>
<td>Selective impairment of macrophage signal transduction</td>
<td>14</td>
</tr>
<tr>
<td>Scavenger of oxygen free radicals</td>
<td>15</td>
</tr>
<tr>
<td>Absolutely required for intracellular survival</td>
<td>16, 17</td>
</tr>
<tr>
<td>Stimulation of host Th cells*</td>
<td>18, 19</td>
</tr>
<tr>
<td>Activator of T suppressor cells*</td>
<td>20</td>
</tr>
<tr>
<td>Inhibitor of immunoproliferative responses*</td>
<td>21</td>
</tr>
<tr>
<td>Vaccine candidate for cutaneous leishmaniasis*</td>
<td>22</td>
</tr>
<tr>
<td><strong>B. Suggested functions:</strong></td>
<td></td>
</tr>
<tr>
<td>Migration in sandfly gut</td>
<td>23</td>
</tr>
<tr>
<td>Protection from complement-mediated lysis</td>
<td>2</td>
</tr>
</tbody>
</table>

* discussed in Chapter 3.

References:

1. Sacks and da Silva 1987  
2. Sacks et al. 1990  
3. Puentes et al. 1988  
4. Puentes et al. 1990  
5. Mosser and Edelson 1984  
6. Handman and Goding 1985  
7. Talamas and Russell 1989  
8. Talamas-Rohana et al. 1990  
9. Russell and Wright 1988  
10. Elam et al. 1985  
11. El-On et al. 1980  
12. McNeely and Turco 1987  
13. McNeely et al. 1989  
15. Chan et al. 1989  
16. Handman et al. 1986  
17. McNeely and Turco 1990  
18. Moll et al. 1989  
20. Mitchell and Handman 1986  
21. Frankenburg et al. 1990  
22. Handman and Mitchell 1985  
23. Davies et al. 1990
Materials and Methods

To avoid duplication, Materials and Methods previously outlined in Chapter 1 will not be repeated here.

Parasites. *L. donovani* RT5 promastigotes were derived from *L. donovani* IS2.0 parasites (Dwyer 1977) after mutagenesis and selection for resistance to killing by ricin agglutinin and their inability to synthesize LPG (I'ing and Turco 1988). Promastigotes of *L. donovani* NLB-065, an isolate from a Kenyan patient (Githure et al. 1986a), were obtained from J.I. Githure, Kenya Medical Research Institute, Nairobi, Kenya. *L. major* NIH (Scidman strain) promastigotes were obtained from Dr. Neil Reiner, The University of British Columbia School of Medicine, Vancouver, Canada and were doubly cloned by micromanipulation in our laboratory - the clone being designated A2. *L. major* V121 promastigotes (Handman and Mitchell 1985) were obtained from Dr. E. Handman, The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia and are a cloned population produced from the human isolate LRC-L137 (Handman et al. 1983).

Promastigotes of *L. mexicana amazonensis* M2269 (WHO reference strain #MHOM/BR/73/M2269) and *L. braziliensis panamensis* LS94 (WHO reference strain #MHOM/PA/71/LS94) were purchased from the American Type Culture Collection, Rockville, MD, U.S.A.; the ATCC numbers are 50131 and 50158 respectively.

Promastigotes of *L. donovani* DD8 (WHO reference strain #MHOM/IN/80/DD8), *L. donovani infantum* LEM235 (WHO reference strain #MHOM/TN/80/LEM235), *L. major* 5-ASKH (WHO reference strain #MHOM/SU/73/5-ASKH), *L. tropica* K27 (WHO reference strain #MHOM/SU/74/K27), *L. aethiopica* L100 (WHO reference strain #MHOM/ET/72/L100), *L. m. mexicana* BEL21 (WHO reference strain #MHOM/BZ/82/BEL21), *L. major* LRC-L137, *L. d. infantum* IPT1, *L. major* Jericho II, and *L. b. braziliensis* M2903 (WHO reference #MHOM/BR/75/M2903) were obtained from Dr. L. Schnur, Hebrew University-Hadassah Medical School, Jerusalem Israel.

*L. donovani* amastigotes were isolated and purified from the spleens of *L. donovani*-infected hamsters according to previously described methods (Dwyer 1977; Channon et al. 1984).

All promastigotes were grown at 26°C in SM medium (Cunningham 1973) containing 10% or 15% heat-inactivated FBS. The *L. donovani* promastigotes were transferred to M1640 (see below) at 37°C for the mononuclear phagocyte-infection experiments (see below). For the parasite transformation studies *L. donovani* amastigotes were cultured in SM medium containing 20% FCS (see below).
**Murine peritoneal macrophages.** Five ml of RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS), 5x10^{-2} M 2-mercaptoethanol, and 0.1% gentamycin sulphate - this medium will be referred to as M1640 - were injected into the peritoneal cavity of 8-12 week old female BALB/c mice. After gentle peritoneal massage, the peritoneal exudate was removed and the body cavity was then washed twice with PBS containing 0.34 M sucrose to remove residual membrane-attached macrophages. The cells were pelleted by centrifugation at 400 x g for 10 min, washed once, and then resuspended at 2 x 10^5 cells/ml in M1640. Macrophage monolayers were then prepared by incubating the macrophage suspension on acid-cleaned 12 mm glass coverslips in 24-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ). Each coverslip received 10^5 macrophages in 0.5 ml of M1640. Cells were allowed to adhere for 2 h at 37°C and were then washed twice with M1640 to remove non-adherent cells.

**Other cells.** A doubly cloned hybridoma cell line (TC45/491) which secretes a mAb specific for a procyclin-like molecule on *Trypanosoma congolense* PCF (R.P. Beecroft, and T.W. Pearson, unpublished) was used as the experimental hybridoma cell line in LPG binding experiments. Thymocytes were obtained from 6 week old female BALB/c mice. A Gibbon lymphoma line (MLA 144) (Rabin et al. 1981) was obtained from Dr. Larry Anthony, University of Victoria. Mouse connective tissue fibroblasts (NTC 929, a clone of strain L) were originally obtained from the American Type Culture Collection and were also provided by Dr. L. Anthony. All cell types were maintained in M1640 as described above.

**Antigens.** Amastigote "LPG" (aLPG) was prepared by the same method used for purification of promastigote LPG. Briefly, cells were extracted as previously described (Orlandi and Turco 1987) and the E soak soluble material was passed over a phenyl Sepharose column. That material which bound to the column was eluted with E soak, dried down, and assayed by ELISA using the anti-LPG mAbs.

The procedure used for preparing LPG-coated reverse-phase beads has also been outlined previously (McNeely and Turco 1990).

**Serotyping.** The use of rabbit antisera and mAbs for serotyping *Leishmania* strains has been described elsewhere (Schnur et al. 1972; Greenblatt et al. 1983). Serotyping of the different *Leishmania* species and strains listed in Table 7, was performed by Dr. L. Schnur.
Immunofluorescence. Acetone-permeabilized parasites or murine macrophages were prepared by air-drying cells onto glass slides and fixing for 20 min in acetone which had been pre-chilled to -20°C. Indirect immunofluorescence of these samples was performed as outlined in Chapter 1.

Indirect immunofluorescence of living or acetone-treated murine macrophages infected with *L. donovani* promastigotes or incubated with LPG-coated beads was performed as described in Chapter 1. Ascites fluids containing the different mAbs were used as the first antibody at a dilution of 1/2000 at 4°C for 30 min. After three washes, the cells were incubated for 30 min at 4°C with a 1/200 dilution of affinity-purified goat anti-mouse IgG/IgM conjugated to fluorescein isothiocyanate (Caltag, South San Francisco, CA). The cells were observed using a Zeiss standard binocular microscope fitted with an epifluorescence attachment.

Indirect immunofluorescence using the anti-LPG repeat mAb CA7AE was used to detect LPG epitopes on macrophages and other cell types that had been incubated with *L. donovani* LD3 promastigotes, LPG or PG. Similarly, *L. donovani*-infected macrophage populations with or without various treatments to affect macrophage functions (see below) were assayed using indirect immunofluorescence.

For photography, cells were washed 3 times prior to making permanent slides with polyvinyl alcohol (MW=10,000, Sigma) (Freer 1984) containing a fluorescence-fading inhibitor, p-phenylenediamine (Sigma) (Johnson and Araujo 1981). Photomicrographs were taken using a Zeiss standard binocular microscope fitted with an epifluorescence attachment, Planoproc 100X/1.3 oil immersion objective and a 35 mm photomicrographic camera.

Enzyme-linked immunosorbent assays. Indirect ELISA was performed as described in Chapter 1 with the exception that *L. donovani* amastigote lysates were prepared by diluting the parasites to an initial concentration of 2 X 10^7 parasites/ml in distilled water.

An antigen-capture ELISA (as described in Chapter 1) was used to detect molecules containing the phosphorylated disaccharide repeat epitope in supernatants of *L. donovani* amastigotes during their transformation from amastigotes to promastigotes. The amastigotes were cultured at either 26°C or 37°C immediately after their isolation from hamster spleens.

Infection of macrophages. Promastigotes, harvested in the late-log to stationary growth phases, were used to infect cultures of adherent macrophages on glass coverslips at a ratio of 10 parasites per macrophage. Infection was performed at 37°C and at several intervals
all non-attached or extracellular organisms were removed by washing with ice-cold PBS containing 5% FBS and 1% D-glucose. The cells were then assayed for surface LPG epitopes by indirect immunofluorescence using anti-LPG MAbs. Alternatively, macrophages were infected with promastigotes or incubated with LPG-coated beads at a 3:1 parasites/beads to macrophage ratio. The macrophages were assayed for surface LPG epitopes (living cells) or surface or internal (acetone-treated cells) LPG epitopes by indirect immunofluorescence.

**Inhibition of macrophage phagocytic and phagolysosomal functions.** Adherent macrophages were treated in several different ways in attempts to inhibit or eliminate their phagocytic and phagolysosomal degradation functions. All methods involved the pre-treatment and/or simultaneous treatment of the macrophages as outlined below. Promastigotes were added at a ratio of 10 parasites per macrophage and the cultures were allowed to incubate for 0.5, 2, 7, or 29 hours post infection (p.i.) without removal of the parasite population. At these times, macrophages were analysed for LPG epitope expression using indirect immunofluorescence. All experiments were repeated at least 3 times.

The effects of proteases (50 μg/ml trypsin or 250 μg/ml proteinase K; Sigma) on the ability of macrophages to bind and internalize promastigotes were examined. Macrophages were washed extensively with PBS containing 1% D-glucose and were then incubated in 0.5 ml of the same buffer containing either trypsin (50 μg/ml) or proteinase K (250 μg/ml) for 15 min at room temperature. The macrophages were washed to remove all residual protease and were immediately incubated with promastigotes in M1640 for various times.

For assays using formaldehyde-fixed macrophages or promastigotes the cells were washed twice in PBS, resuspended in 0.5 ml of a 0.5% formaldehyde solution and incubated at room temperature for 30 min. The fixative was removed by extensive washing in PBS and then in M1640. The infection protocol was performed as outlined above.

To examine the effects of inhibiting the polymerization of cytoskeletal components on membrane transport events and thus parasite attachment and uptake, macrophages were incubated in M1640 containing 10 μg/ml cytochalasin B or D (in a final concentration of 1% dimethyl sulfoxide; Sigma) for 30 min prior to the addition of parasites and during the designated period of infection. As a negative control, macrophages were also incubated under the same conditions in M1640 containing 1% dimethyl sulfoxide.

Colchicine (10 μg/ml; Sigma) was used to study the effects of an inhibitor of
microtubule polymerization on parasite attachment and uptake. The procedure used was identical to that used for the cytochalasin treatment.

The lysosomotropic agents chloroquine (0.1 mM; Sigma) and ammonium chloride (10 mM; Fisher Scientific Co., Fair Lawn, NJ) were also used in a protocol identical to that used for the cytochalasin experiments.

To see if an anti-LPG mAb could inhibit parasite attachment and/or the expression of LPG epitopes on the macrophage cell surface, macrophages were incubated with a 1/40,000 dilution of the anti-LPG mAb CA7AE in conditions identical to that used for the cytochalasin experiment. The anti-procyclin mAb TBRP1.247 was used as a negative control at the same dilution. The 1/40,000 dilution of mAb was chosen because it still gave positive binding without causing extensive agglutination of promastigotes.

**LPG epitope expression on the surface of different cell types.** A selection of different living cells was incubated with 50 μg LPG or PG at 4°С or 37°С, either as adherent populations (macrophages, fibroblasts) or as suspension cultures (hybridomas, lymphomas, thymocytes) for 2 hours. The cells were then washed extensively with PBS containing 5% FBS and subsequently assayed for the presence of LPG or PG epitopes on the cell surface using indirect immunofluorescence. Alternatively, cells were incubated with *L. donovani* LD3 promastigotes or *T. b. rhodesiense* ViTat 1.1 PCF at a 10:1 parasite:cell ratio as described above.

**Sera.** Human sera from patients positive or negative for Kala-azar (visceral leishmaniasis caused by *L. donovani*) were obtained from Dr. J.B.O. Were, Kenya Medical Research Institute, Nairobi, Kenya.

Human sera from patients manifesting cutaneous (Oriental) sores due to an active infection with *L. major* or *L. tropica* parasites, or convalescent sera from patients recovered from *Leishmania* infection, were obtained from Dr. L. Schnur, Hebrew University-Hadassah Medical School, Jerusalem, Israel.
Results

Species and strain distribution of LPG-associated epitopes.

To determine if the LPG-associated epitopes recognized by the anti-LPG mAbs were conserved in other *Leishmania* species, indirect immunofluorescence on living or acetone-treated parasites and antigen-capture ELISA of parasite-conditioned culture medium were used. The results are shown in Table 7.

No immunofluorescence was seen when the anti-LPGAP mAbs L157 and L98 (data not shown) were incubated with living parasites. However, indirect immunofluorescence using mAb L157 on acetone-treated parasites showed strong fluorescence associated with the flagella and anterior portion of all *Leishmania* promastigotes tested, including the LPG-deficient mutants R2D2 and C3PO. Weak fluorescence was also observed with the procyclic trypanosomes. With all parasites the mAb L157 fluorescence was distinct, showing a string-like pattern along the flagella which ended with two fluorescent spots at the base of the flagella. This fluorescence pattern is illustrated in Figure 13 (panels B1-3). The other anti-LPGAP mAb, L98, showed no fluorescence with any of the acetone-treated parasites.

The distribution of the epitope bound by mAb CA7AE on living and acetone-treated parasites was more variable than that seen for mAb L157 (see Table 7). With mAb CA7AE most of the live promastigotes showed strong fluorescence over the entire surface and flagella of the cells. This is illustrated in Figure 13 (panel A1). However, the mAb CA7AE repeat epitope was not detected on the surface of the living *L. donovani* LPG-deficient mutants R2D2 and C3PO, *L. major* V121, or *L. major* LRC-L137 promastigotes whereas variable expression of the mAb CA7AE epitope was observed on *L. major* 5ASKH, *L. mexicana amazonensis*, and *L. braziliensis braziliensis* promastigotes (see Table 7 footnotes). In the *Leishmania* species where strong homogeneous fluorescence attributable to mAb CA7AE was observed, extensive agglutination of the promastigotes was also seen. After acetone-treatment, all parasites except promastigotes of *L. tropica* and the LPG-deficient mutant *L. donovani* C3PO and the procyclic trypanosomes expressed the mAb CA7AE surface membrane epitope. It is interesting that while no fluorescence was observed on the living *L. donovani* LPG-deficient mutant R2D2 promastigotes, after acetone-treatment and incubation with mAb CA7AE a localised point of fluorescence was observed in approximately 20% of the variant R2D2 promastigotes at the anterior end of the cells (Figure 13, panel A2).

Immunofluorescence was also performed using formaldehyde-fixed parasites. The results obtained were identical to those seen with living parasites (data not shown).
However, there was slightly less agglutination than that observed with living parasites when using mAb CA7AE.

All parasites, with the exception of promastigotes of *L. major* LRC-L137, *L. tropica*, and the LPG-deficient mutant *L. donovani* C3PO and the trypanosome PCF, secreted the mAb CA7AE epitope into culture medium (Table 7).
Table 7. *Leishmania* and *Trypanosoma* species and strain distribution of LPG epitopes determined using mAb binding in ELISA and immunofluorescence.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain Designation</th>
<th>Serotype</th>
<th>Antigen Trapping ELISA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Immunofluorescence&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>L&lt;sup&gt;+&lt;/sup&gt; A&lt;sup&gt;+&lt;/sup&gt;</td>
<td>L&lt;sup&gt;+&lt;/sup&gt; A&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td><em>L. d. donovani</em></td>
<td>1S2D</td>
<td>B2</td>
<td>5+ 5+ 5+ 5+ 5+</td>
<td>3+</td>
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<tr>
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<td>LD3</td>
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<td><em>L. d. donovani</em></td>
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<td>A</td>
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<td>IPT1</td>
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<td><em>L. d. infantum</em></td>
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<td>B2</td>
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<td>3+</td>
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<td><em>T. b. rhodesiense</em></td>
<td>ViTat 1.1</td>
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<td>5+ 5+ 5+ 5+ 5+</td>
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</table>

<sup>a</sup>WHO International reference strains
<sup>b</sup>*Living parasites, **Acetone-treated parasites
<sup>c</sup>determined by L. Schnur using a serotyping assay previously described (Schnur et al. 1972; Greenblatt et al. 1983)
<sup>d</sup>a positive value signifies binding comparable to the reference strain *L. donovani* 1S2D
<sup>e</sup>anti-LPG phosphorylated disaccharide repeat mAb CA7AE
<sup>f</sup>anti-LPG-associated protein mAb L157
<sup>g</sup>no reaction, LPG-deficient mutant was derived from *L. d. donovani* 1S2D parent strain
<sup>h</sup>10% of parasites 5+; 90% of parasites -
<sup>i</sup>50% of parasites 5+; other 50% of parasites negative for immunofluorescence
<sup>j</sup>75% of parasites 2-4+; other 25% of parasites negative for immunofluorescence
Figure 13. Immunofluorescence patterns on acetone-permeabilized *Leishmania* promastigotes and *Trypanosoma* PCF using anti-LPG mAbs. A. Anti-LPG repeat mAb CA7AE. B. Anti-LPGAP mAb L157. Panels: (1) *L. donovani* 1S2D; (2) *L. donovani* R2D2; (3) *T. b. rhodesiense* ViTat 1.1 PCF.
Expression of LPG epitopes by *L. donovani* amastigotes.

The stage-specificity of the three anti-promastigote LPG mAbs was determined by ELISA using lysates of promastigotes and amastigotes and purified promastigote LPG and amastigote LPG (Figure 14). MAb CA7AE (Figure 14A) bound to lysates of *L. donovani* LD3 and RT5 promastigotes and purified *L. donovani* promastigote LPG (lanes 1, 2, and 7 respectively) but did not bind to lysates of *L. donovani* C3PO promastigotes or *L. donovani* amastigotes (lanes 3, 4, and 5 respectively) or purified amastigote LPG (lane 6). Both mAb L98 (Figure 14B) and rabbit anti-promastigote membrane serum (Figure 14C) bound to lysates from all three promastigote clones (lanes 1-3) and purified promastigote LPG (lane 7) but not to the trypanosome PCF lysate (lane 4). Where mAb L98 did not recognize the amastigote lysate or purified amastigote LPG (lanes 5 and 6 respectively) the rabbit antiserum bound to both antigens, albeit more weakly than to the promastigote antigens. The anti-procyciin mAb bound only the trypanosome lysate (Figure 14D; lane 4).

Indirect immunofluorescence was also performed on *L. donovani* LD3 promastigotes, amastigotes and trypanosome PCF to further delineate the stage-specificity of the anti-LPG mAbs (Table 8). The anti-repeat mAb CA7AE labelled the surface of the living LD3 promastigotes and showed equally strong staining of the same cells treated with acetone. In contrast, the anti-LPGAP mAbs L98 and L157 did not label the surface of the living LD3 promastigotes and only mAb L157 stained a structure at the flagellar pocket of acetone-treated cells (see Figure 13, panel B1). None of the three anti-promastigote LPG mAbs bound to either living or acetone-permeabilized *L. donovani* amastigotes. The control mAb 247 (anti-procyclin) bound only to the trypanosome PCF.

The kinetics of CA7AE epitope expression on *L. donovani* LD3 parasites during transformation *in vitro* from amastigotes to promastigotes was also examined using indirect immunofluorescence (Table 9). MAb CA7AE did not bind to the surface of living amastigotes tested immediately after parasite isolation from infected hamster spleens. As early as 5-10 hours after incubation at 26°C, clear surface fluorescence was observed on the amastigotes. Strong fluorescence was observed over the entire surface of the parasite by 16 hours and was maintained until, and after, the parasites had completely transformed into promastigotes.

*Leishmania* promastigotes are known to release forms of LPG and AcPase, both of which contain the CA7AE epitope (see Chapter 1). To test the possibility that *L. donovani* promastigote CA7AE epitopes are secreted by the amastigote stage of the parasite, an antigen-capture ELISA was performed on culture supernatants from *L. donovani* parasites taken during their transformation from amastigotes to promastigotes *in vitro*.
As shown in Figure 15, CA7AE epitopes were detected in the culture supernatants after approximately 20 hours incubation of the parasites at 26°C, at which time the amastigotes had differentiated into the promastigote form of the parasite (see Table 9). Amastigotes incubated at 37°C produced only marginal amounts of CA7AE epitope and only after 40 hours incubation in vitro. Under these conditions differentiation to the promastigote form did not occur. The amount of CA7AE epitope released at 37°C did not increase significantly over time - perhaps due to the inability of the parasite to proliferate at this temperature in vitro. Promastigote control cultures showed that the CA7AE epitopes could be detected as early as 15-20 min after initiating the cultures at 26°C (data not shown). No epitope was detected in SM media alone, at either temperature.
Figure 14. Binding of anti-LPG mAbs to parasite lysates and purified LPGs in ELISA. Antigens tested were as follows: 1, *L. donovani* LD3 promastigote lysates; 2, *L. donovani* RT5 promastigote lysates; 3, *L. donovani* C3PO promastigote lysates; 4, *T. b. rhodesiense* ViTat 1.1 PCF lysates; 5, *L. donovani* amastigote lysates; 6, *L. donovani* amastigote LPG; 7, *L. donovani* promastigote LPG. (A) Anti-promastigote LPG repeat mAb CA7AE. (B) Anti-promastigote LPGAP mAb L98. (C) Rabbit anti- *L. donovani* promastigote membrane serum. (D) Anti-procyclin mAb 247. Results from only one of two experiments are shown since the standard deviations from all points for the two assays were less than 0.06 OD units from the mean.
Table 8. Indirect immunofluorescence on living or acetone-permeabilized *Leishmania* promastigotes and amastigotes and *Trypanosoma* PCF.

<table>
<thead>
<tr>
<th>MAb</th>
<th>Parasites</th>
<th>Living</th>
<th>Acetone-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LD3(^a) Amas.(^b) Tryp.(^c)</td>
<td>LD3(^a) Amas.(^b) Tryp.(^c)</td>
</tr>
<tr>
<td>CA7AF</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1.157</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1.98</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>247</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\) *L. donovani* LD3 promastigotes.

\(^b\) *L. donovani* LD3 amastigotes.

\(^c\) *Trypanosoma brucei rhodesiense* ViTat 1.1 PCF.

\(^d\) Fluorescence intensity was much weaker than with the *Leishmania* promastigotes.
Table 9. Expression of CA7AE epitopes on the surface of *L. donovani* parasites during their transformation from amastigotes to promastigotes at 26°C in vitro.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>No. of parasites expressing fluor.</th>
<th>Relative strength of fluorescence</th>
<th>Parasite morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>0</td>
<td>-</td>
<td>rounded amastigotes</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>-</td>
<td>rounded amastigotes</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>1-2</td>
<td>rounded amastigotes</td>
</tr>
<tr>
<td>11</td>
<td>31</td>
<td>2-4</td>
<td>rounded amastigotes</td>
</tr>
<tr>
<td>16</td>
<td>43</td>
<td>4</td>
<td>rounded amastigotes</td>
</tr>
<tr>
<td>19</td>
<td>45</td>
<td>5</td>
<td>rounded amastigotes</td>
</tr>
<tr>
<td>22</td>
<td>50</td>
<td>5</td>
<td>rounded amastigotes</td>
</tr>
<tr>
<td>35</td>
<td>57</td>
<td>5</td>
<td>some elongation, short flagella present</td>
</tr>
<tr>
<td>39</td>
<td>114</td>
<td>5</td>
<td>some elongation, medium length flagella</td>
</tr>
<tr>
<td>44</td>
<td>180</td>
<td>5</td>
<td>elongated parasites</td>
</tr>
<tr>
<td>59</td>
<td>300</td>
<td>5</td>
<td>elongated promastigotes</td>
</tr>
<tr>
<td>67</td>
<td>TNTC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5</td>
<td>elongated promastigotes</td>
</tr>
<tr>
<td>86</td>
<td>TNTC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5</td>
<td>elongated promastigotes</td>
</tr>
<tr>
<td>110</td>
<td>TNTC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5</td>
<td>elongated promastigotes</td>
</tr>
</tbody>
</table>

<sup>a</sup> Length of time after removal of amastigotes from *L. donovani*-infected hamster spleen.

<sup>b</sup> Number of parasites exhibiting positive surface fluorescence in 20 fields of view at 1000X magnification.

<sup>c</sup> Based on a subjective visual scale of 1-5; where 1-2 was weak, patchy fluorescence over some/most of the parasite surface, and 4-5 was strong, homogeneous fluorescence over the entire parasite surface and flagellum.

<sup>d</sup> Too numerous to count, due, in part, to extensive agglutination of the parasites.
Figure 15. Measurement of CA7AE epitope in tissue culture medium during transformation of *L. donovani* LD3 from amastigotes to promastigotes *in vitro*. Antigen-capture ELISA was performed using mAb CA7AE. ■ , *L. donovani* amastigotes incubated at 26°C; □ , *L. donovani* amastigotes incubated at 37°C; ▲ , media alone at 26°C; △ , media alone at 37°C. The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 0.04 OD units from the mean.
Expression of LPG epitopes on the surface of macrophages and other cell types.

Murine macrophages were incubated *in vitro* with *L. donovani* promastigotes or with purified *L. donovani* LPG or PG. At 20 hours post-infection/incubation (p.i.) the macrophage cell surface was probed for the presence of LPG epitopes using the anti-LPG repeat mAb CA7AE. MAb CA7AE bound strongly to the entire surface of macrophages incubated with *L. donovani* promastigotes (Figure 16D). Similar binding was seen when the macrophages were incubated with purified LPG or PG (Figure 16B and 16C). Neither anti-LPGAP mAb nor the anti-procyclin mAb TBRP1.247 bound to the *L. donovani*-infected or LPG/PG-pulsed macrophages. None of the mAbs bound to the uninfected control macrophages (Figure 16A).

To examine whether or not LPG epitope expression was restricted to the surface of *L. donovani*-infected macrophages, several cell types were incubated with living promastigotes, LPG or PG at 37°C or 4°C (in order to inhibit membrane flow events) (Table 10). Promastigote-derived LPG epitopes were expressed only on parasite-infected macrophages and only at 37°C. None of the other cell types examined expressed LPG epitopes after incubation with living parasites. However, all of the cell types tested strongly bound LPG or PG and at either 4°C or 37°C. Strong antibody-induced agglutination was exhibited by cells that were tested as suspension cultures; i.e. hybridomas, thymocytes, and lymphomas. None of the cells tested with the anti-procyclin mAb TBRP1.247 showed any fluorescence. Neither of the anti-LPGAP mAbs bound to any of the cells.
Figure 16. Indirect immunofluorescence of Leishmania-infected or LPG/PG-pulsed murine macrophages using the anti-LPG repeat mAb CA7AE. Macrophages were incubated with promastigotes for 2 hrs and then assayed at 20 hrs post-infection, or alternatively, were incubated with purified LPG or PG for 20 hrs and then assayed. (A) uninfected macrophages; (B) macrophages incubated with purified LPC; (C) macrophages incubated with purified PG; (D) macrophages incubated with L. donovani LD3 promastigotes. The magnification is 1000 times.
Table 10. Expression of LPG epitopes on the surface of different cell types incubated with *L. donovani* promastigotes or purified LPG or PG determined using indirect immunofluorescence with mAb CA7AE.

<table>
<thead>
<tr>
<th>ANTIGEN</th>
<th>Macrophages</th>
<th>Fibroblasts</th>
<th>Hybridomas</th>
<th>Thymocytes</th>
<th>Lymphomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LPG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PG&lt;sup&gt;d&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

A. at 37°C<sup>a</sup>

B. at 4°C<sup>a</sup>

<sup>a</sup> temperature at which cells and antigen were incubated together.

<sup>b</sup> Strong immunofluorescence was observed over the entire cell surface. For the suspension cultures (hybridomas, thymocytes, lymphomas) extensive agglutination occurred.

<sup>c</sup> *L. donovani* LD3 promastigotes.

<sup>d</sup> Phosphoglycan (repet phosphorylated disaccharide + phosphosaccharide core).
Kinetics of expression of LPG epitopes on the surface of *Leishmania*-infected macrophages.

The initial experiments involved incubation of *L. donovani* promastigotes with macrophages for 2 hours and then examination of the macrophage surface for LPG epitope expression at 20 hours p.i. (see Figure 16). In addition, macrophages were incubated with *L. donovani* promastigotes for 2 hours and were then immediately tested for LPG epitope expression. The fluorescence intensity exhibited at 2 hours p.i. was as strong as that exhibited at 20 hours p.i. Therefore the macrophage surface was assayed at 5 minute intervals over a 2 hour period after infection with *L. donovani* promastigotes (Figure 17).

The CA7AE epitope was detected on the surface of infected macrophages as early as 5-10 minutes p.i. (Figures 17B and 17C). Initially, fluorescence was specifically localized to the area at the site of promastigote attachment to the macrophage (Figures 17B-D) and subsequently was seen to be evenly distributed over the entire surface of the macrophage within 20-25 minutes p.i. (Figures 17E and 17F).

It is clearly shown in Figures 17B, E and F, that only those macrophages with attached parasites exhibited expression of the LPG epitope, in contrast to neighbouring uninfected macrophages which did not. Similar results were observed at all time points, even at 20 hours p.i. These results also clearly demonstrated that mAb CA7AE did not react with the surface membrane of uninfected macrophages even when they were co-cultured with *Leishmania*-infected cells.

Expression of LPG epitopes on the macrophage cell surface was also apparent within 2 hours of incubation with purified LPG or PG but was never localized as was observed when macrophages were infected with promastigotes. Rather, LPG epitopes appeared to be distributed evenly over the entire macrophage surface. The immunofluorescence intensity increased with time (data not shown).

To examine the duration of expression of LPG epitopes on infected cells, murine macrophages were infected with *L. donovani* promastigotes or incubated with *L. donovani* LPG-coated beads and at intervals the macrophage cell surface was probed for the presence of the CA7AE epitope (see Figure 18A). In addition, infected or antigen-pulsed macrophages were treated with acetone prior to assaying CA7AE epitope expression (Figure 18B). Macrophages infected with *L. donovani* LD3 (wild-type) promastigotes clearly expressed CA7AE epitopes on their surface at 4 hours p.i. (Figure 18A). The fluorescence intensity and percentage of macrophages expressing CA7AE epitopes increased until approximately 50 hours p.i. and then began to decline rapidly. From 4 to 50 hours p.i. strong fluorescence was observed over the entire macrophage surface. At approximately 88 hours p.i. all surface epitope expression had disappeared. CA7AE
epitopes were also expressed on the surface of macrophages infected with the LPG-deficient mutant *L. donovani* RT5. However, the fluorescence pattern observed in this case was very weak and essentially limited to the area of promastigote attachment to the macrophage membrane during parasite internalization. In addition, surface epitope expression had essentially disappeared after approximately 20 hours p.i. Like the promastigotes the LPG-coated beads were also phagocytized by murine macrophages. The fluorescence pattern was very similar to that demonstrated by the cells infected with *L. donovani* RT5-promastigotes. No fluorescence was seen on macrophages infected with the other LPG-deficient mutant *L. donovani* C3PO or in the absence of antigen.

Macrophages were also treated with acetone to allow antibody access to the cell interior. *L. donovani* LD3-derived LPG epitopes could be detected until at least 132 hours p.i. and the percentage of macrophages exhibiting fluorescence was 2-3 times that observed on untreated, living cells (Figure 18B). While the fluorescence observed over the first 75 hours p.i. was distributed evenly over the entire macrophage, subsequent LD3 LPG epitope expression was more localized. Figure 19A illustrates the more punctate epitope expression seen at 132 hours p.i.

The fluorescence pattern seen at 20 hours p.i. for the macrophages infected with *L. donovani* RT5 promastigotes or pulsed with LPG-coated beads was essentially identical to that observed for living macrophages (data not shown). However, for both these antigens, at approximately 40 hours p.i. there was a marked increase in the number of macrophages expressing LPG epitopes. The fluorescence intensity of the *L. donovani* RT5-infected cells was much weaker than that demonstrated by cells infected with wild-type promastigotes. The cells pulsed with LPG-coated beads showed strong fluorescence at 40 hours p.i. From approximately 75 hours p.i., both the RT5-infected and bead-pulsed macrophages showed fluorescence patterns similar to that observed for the LD3 promastigotes. Again, no fluorescence was observed in acetone-treated macrophages infected with *L. donovani* C3PO promastigotes or incubated in the absence of antigen.
Temporal expression of LPG CA7AE epitope on the surface of *L. donovani*-infected murine macrophages as detected by immunofluorescence. (A) 0 min P.I.; (B) 5 min P.I.; (C) 10 min P.I.; (D) 15 min P.I.; (E) 20 min P.I.; (F) 25 min P.I.; (G) 30 min P.I.; (H) 35 min P.I. The magnification is 1000 times.
Figure 18. Kinetics of expression of LPG epitopes on murine macrophages as detected by indirect immunofluorescence. One hundred macrophages were examined at each time point. (A) Living macrophages. (B) Acetone-treated macrophages. ■, Macrophages infected with *L. donovani* LD3 promastigotes (wild-type); ▲, macrophages infected with *L. donovani* RT5 promastigotes (LPG-deficient mutant); △, macrophages infected with *L. donovani* C3PO promastigotes (LPG-deficient mutant); ○, macrophages incubated with *L. donovani* LPG-coated beads; □, uninfected macrophages. The data points represent single sample values from a single experiment done in duplicate, since the values for each point deviated less than 2% from the mean.
Figure 19. Distribution of mAb CA7AE epitopes within acetone-treated murine macrophages. Macrophages were infected with *L. donovani* LD3 promastigotes and assayed at 132 hours post-infection using indirect immunofluorescence with mAb CA7AE. (A) Macrophages incubated with *L. donovani* LD3 promastigotes. (B) Uninfected macrophages or macrophages incubated with *L. donovani* LD3 promastigotes and probed with the anti-trypanosome mAb 247. Magnification, X 660.
The effect of inhibition of macrophage function on LPC epitope expression.

Macrophages were treated under conditions which affect phagocytic and phagolysosomal processing events. The results are shown in Table 11. Macrophages incubated with L. donovani promastigotes in the absence of inhibitors showed an increase over time in the number of parasites bound and the number of macrophages subsequently expressing LPG epitopes.

Reagents such as ammonium chloride or chloroquine had no effect on LPG epitope expression on L. donovani-infected macrophages. These chemicals interfere with phagolysosomal antigen degradation processes by increasing lysosomal pH resulting in the depression of acid hydrolase activity. Similar results were seen with colchicine treatment which inhibits microtubule polymerization.

In contrast, treatments which inhibited membrane flow and thus phagocytosis, including: (1) decreased temperature, (2) the addition of cytochalasins B or D, or (3) mild fixation of macrophages with formalin, resulted in the abolition or drastic reduction of LPG epitope expression. When incubated with normal macrophages, formalin-fixed promastigotes were still able to transfer LPG epitopes to the macrophage surface. The incubation of formalin-fixed promastigotes with macrophages resulted in immunofluorescence patterns similar to those observed using untreated promastigotes.

Pretreatment of the macrophage population with trypsin or proteinase K significantly reduced or abolished promastigote binding and LPG epitope expression up to and including 30 minutes p.i. From 2 hours p.i. the proteinase-treated macrophages expressed LPG epitopes at levels similar to those seen in untreated infected macrophages. No fluorescence was seen when uninfected, treated macrophages were assayed using mAb CA7AE nor when infected, treated macrophages were assayed using the anti-procyclin mAb TBRP1.247. That the proteases were active was confirmed by treating trypanosome PCF with trypsin or proteinase K. The treated trypanosomes did not show fluorescence after incubation with mAbs that detect surface epitopes on the trypanosome glycoprotein procyclin (data not shown) (Richardson et al. 1988). L. donovani promastigotes treated with either trypsin or proteinase K bound the anti-LPG mAb CA7AE as well as untreated parasites did when assayed using indirect immunofluorescence (data not shown).
Table 11. The effect on CA7AE epitope expression of agents which inhibit macrophage phagocytotic or phagolysosomal functions.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>30 min p.i.</th>
<th>2 hrs p.i.</th>
<th>7 hrs p.i.</th>
<th>20 hrs p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>14*</td>
<td>45</td>
<td>64</td>
<td>86</td>
</tr>
<tr>
<td>Uninfected MOs</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MOs at 4°C</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Trypsinized MOs</td>
<td>6</td>
<td>45</td>
<td>65</td>
<td>88</td>
</tr>
<tr>
<td>Proteinase K + MOs</td>
<td>0</td>
<td>36</td>
<td>68</td>
<td>86</td>
</tr>
<tr>
<td>Formalin-fixed MOs</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Formalin-fixed promastigotes</td>
<td>20</td>
<td>48</td>
<td>70</td>
<td>90</td>
</tr>
<tr>
<td>Colchicine + MOs</td>
<td>13</td>
<td>39</td>
<td>53</td>
<td>60</td>
</tr>
<tr>
<td>NH4Cl + MOs</td>
<td>11</td>
<td>32</td>
<td>59</td>
<td>87</td>
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<td>Chloroquine + MOs</td>
<td>16</td>
<td>49</td>
<td>56</td>
<td>73</td>
</tr>
<tr>
<td>Cytochalasin D + MOs</td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Cytochalasin B + MOs</td>
<td>0</td>
<td>5</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>anti-^-PG MAb CA7AE + MOs^c</td>
<td>16</td>
<td>52</td>
<td>73</td>
<td>96</td>
</tr>
</tbody>
</table>

* The data points represent single sample values from a single experiment done in triplicate, since the values for each time point deviated less than 4% from the mean.

a Three hundred cells were examined in each assay at each time point.

b MOs; macrophages.

c In this assay, promastigotes were pre-incubated with mAb CA7AE prior to their addition to the macrophage cultures.
LPG epitopes in the sera of *Leishmania*-infected patients.

Antigen-capture ELISA was used to detect the presence of mAb CA7AE epitopes in the sera of individuals exhibiting an active *Leishmania* infection. The results are shown in Table 12. The mAb CA7AE epitope was detected in 50% of sera assayed from *L. donovani*-infected patients with visceral leishmaniasis (Kala-azar). The epitope was not found in the sera of patients without Kala-azar or from patients exhibiting Oriental sores, indicative of an active *L. major* or *L. tropica* infection. The epitope was not detected in sera from convalescent patients.
Table 12. Presence of mAb CA7AE epitopes in the sera of *Leishmania*-infected patients as determined using an antigen-capture ELISA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>OD (405 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
</tr>
<tr>
<td><em>L. d. donovani</em> LD3 culture supernatant (positive control)</td>
<td>0.97*</td>
</tr>
<tr>
<td><em>L. d. donovani</em> C3PO culture supernatant (negative control)</td>
<td>0.05</td>
</tr>
<tr>
<td>no antigen</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Kala-azar positive sera</strong></td>
<td></td>
</tr>
<tr>
<td>16 sera</td>
<td>0.40-1.05</td>
</tr>
<tr>
<td>16 sera</td>
<td>0.04-0.07</td>
</tr>
<tr>
<td><strong>Kala-azar negative sera</strong></td>
<td></td>
</tr>
<tr>
<td>10 sera</td>
<td>0.04-0.08</td>
</tr>
<tr>
<td><strong>Oriental sore positive sera</strong></td>
<td></td>
</tr>
<tr>
<td>6 sera</td>
<td>0.06-0.08</td>
</tr>
<tr>
<td><strong>Convalescent sera</strong></td>
<td></td>
</tr>
<tr>
<td>3 sera</td>
<td>0.03-0.06</td>
</tr>
</tbody>
</table>

*The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 0.03 OD units from the mean.

*a* All sera/culture supernatants were assayed at three different dilutions. The results shown are for a 1:4 dilution.

*b* Supplied by L. Schnur and J.B.O. Were. Patients were confirmed positive by spleen biopsy.

*c* Supplied by J.B.O. Were.

*d* Supplied by L. Schnur. Patients showed cutaneous sores due to *L. major* or *L. tropica* infections.

*e* Supplied by L. Schnur. Patients cured after an *L. donovani* (1 sera) or *L. major* (2 sera) infection.
Discussion

The species distribution of LPG and LPGAP epitopes was examined in nineteen different *Leishmania* strains using the defined anti-LPG mAbs. The epitope recognized by the anti-LPGAP mAb LI57 was detected in all *Leishmania* strains examined after acetone-permeabilization of the cells. Similarly, this epitope was located in *Trypanosoma brucei* and *T. congolense* PCF suggesting a conservation of this epitope amongst at least these two genera of the family Trypanosomatidae. An LPG-like molecule has been found in African trypanosomes (Hublart et al. 1988). This LPG is biochemically different from the *Leishmania* molecule yet has similar chromatographic and electrophoretic properties. The presence of an immunochemically cross-reactive molecule over such a wide range of *Leishmania* (and *Trypanosoma*) species which demonstrate different life-cycles, tissue tropisms and pathologies, implies that the LPGAP molecule may have a conserved function (see below and Chapter 3).

Immunofluorescence experiments using the living and formaldehyde-fixed promastigotes showed clearly that the anti-repeat mAb CA7AE recognized epitopes on the promastigote surface membrane. In contrast, neither of the anti-LPGAP mAbs bound to the surface of living or formaldehyde-fixed promastigotes. Since LPGAP is associated primarily with the carbohydrate core region of the LPG molecule (see Chapter 1), these observations allowed a schematic model for *L. donovani* LPG to be proposed. Figure 20 illustrates the hypothesized orientation of the LPG molecule on the promastigote cell surface and the accessibility of repeating phosphorylated disaccharide and phosphosaccharide core (and therefore presumably LPGAP) to antibody binding.

The distribution of the epitope recognized by the anti-LPG phosphorylated disaccharide repeat mAb CA7AE was found to be more variable than that seen for mAb L157. Essentially, the repeat epitope was observed in all *Leishmania* of the non-A2 serotypes tested (see Table 7). With the exception of *L. tropica* promastigotes and the LPG-deficient mutants, the CA7AE epitope was expressed to varying extents by all promastigotes assayed. For example, promastigotes of *L. m. amazonensis* and *L. b. braziliensis* (see Table 7) displayed different surface immunofluorescence patterns with mAb CA7AE within the population, in contrast to the homogeneous surface fluorescence pattern exhibited on the *L. donovani* promastigotes. While the available evidence suggests that the carbohydrate core epitopes are identical in all *Leishmania* species, variability amongst the repeat portions of the molecule has been reported (Turco et al. 1989; McConville et al. 1990a; Turco 1990). *L. tropica* promastigotes express an immunochemically and structurally distinct carbohydrate repeat structure whereas *L. major*
Figure 20. Schematic representation of the structure of *L. donovani* LPG and its arrangement at the promastigote cell surface. (A) \(\text{PO}_4\text{-6Gal(\(\beta\)1-4)Man}\) repeating disaccharide (exposed epitope); (B) phosphosaccharide core (hidden epitope); (C) lyso-alkyl-phosphatidylinositol anchor; (D) glycosyl-phosphatidylinositol-anchored membrane proteins. Taken from Tolson et al. (1989).
and *L. mexicana*, in addition to expressing the phosphorylated Gal-β1,4-Man repeat disaccharide backbone of the *L. donovani* LPG, substitute this moiety with a variety of saccharide chains (McConville et al. 1990a). The presence of a common repeat backbone sequence which is variably substituted with species-specific side chains is consistent with serological studies which indicate the presence of both conserved and species-specific epitopes in *Leishmania* LPGs (McConville et al. 1990a). Therefore, the additional saccharide units in *L. major* and *L. mexicana* repeat moieties would be predicted to mask certain immunological determinants and to create others and could explain the binding patterns observed for mAb CA7AE with the different parasite species and strains. Further variability of CA7AE binding may be influenced by the structural changes in LPG that occur during metacyclogenesis (see Introduction this Chapter). It was interesting that both *L. major* V121, a clone derived from *L. major* LRC-L137 (Handman et al. 1983) and its parent strain exhibited similar immunofluorescence profiles with mAb CA7AE yet differed in their expression of the epitope on secreted glycoconjugates.

The patterns of immunofluorescence observed with the mAb CA7AE indicated that the repeating phosphorylated disaccharide epitope is detectable over the entire surface of wild-type promastigotes and is not on the surface of the LPG-deficient mutant promastigotes. After acetone treatment, the CA7AE epitope was detected in the interior of some of the promastigote mutants however, indicating it is synthesized in these cells. In contrast, though the CA7AE epitope is detectable as a secretory product for the R2D2 and RT5 mutants (but not C3PO parasites), intact LPG is not found in the culture medium of these parasites (King and Turco 1988) indicating that secretion or excretion of the intact molecule does not occur in these mutants. The patterns of fluorescence seen with the anti-LPGAP mAbs were unusual. The thin line of fluorescence along the flagellum and its concentration at the flagellar base suggests that either there is a high concentration of this epitope in these areas or that here the epitope conformation or accessibility to it is unique. It is likely that both are true since the same pattern was seen with both wild-type and mutant parasites and it would be expected that the wild-type should stain evenly because of the presence of LPG over its entire membrane. It is possible that these sites may be involved in LPG-membrane biogenesis and although the flagellar pocket is primarily thought to function in the endo- and exocytosis of nutrients and metabolites, membrane biogenesis or assembly may occur here. Immuno-electronmicroscopy by R. Garduno (University of Victoria) using the anti-LPGAP mAbs, showed membrane vesicles containing accumulations of LPGAP epitopes in the flagellar pocket (data not shown). This localized expression of LPGAP epitopes may suggest a putative role for the LPGAP molecule in the biogenesis, transport, organization and/or anchoring of LPG molecules in the promastigote
membrane. Taken together, these data imply that the externally oriented repeating phosphorylated portion of LPG and the phosphosaccharide core (and LPGAP) are synthesized to varying extents by the *L. donovani* R2D2, RT5 and C3PO LPG-deficient mutants and that the absence of intact LPG in these organisms is a result of incomplete assembly of the molecule.

It has been shown previously that amastigotes of *L. major* express an LPG that is structurally distinct from its promastigote counterpart (Turco and Sacks 1991). Other researchers have substantiated these results and, in addition, showed that the *L. major* amastigote glycan was antigenically distinct from the promastigote LPG (Glaser et al. 1991). Not surprisingly, there is evidence that an LPG for *L. donovani* amastigotes also exists (T. McNeely, University of Kentucky College of Medicine, Lexington, Kentucky, personal communication). The studies reported here demonstrated that promastigotes of *L. donovani* express stage-specific LPG epitopes since none of the anti-LPG mAbs recognized epitopes in *L. donovani* amastigotes.

It has been reported that during transformation of *L. major* amastigotes to promastigotes *in vitro* that promastigote LPG epitopes were not evident until 48 hours in culture (Glaser et al. 1991). In contrast, the experiments reported here showed that *L. donovani* promastigote LPG epitopes were detectable on the parasite surface within 5 hours of incubation of the amastigotes at 26°C. This would suggest that, while the kinetics of expression are different, both *L. major* and *L. donovani* parasites developmentally regulate expression of antigenically distinct LPGs throughout the parasite life cycle.

To follow the expression of LPG epitopes throughout the leishmanial parasite's life cycle, macrophages were infected with *L. donovani* promastigotes and then probed using the anti-LPG mAbs. Murine peritoneal macrophages expressed LPG epitopes on their surface after infection with *L. donovani* promastigotes or after incubation with purified *L. donovani* LPG or PG. The epitope recognized by mAb CA7AE (P04-6Galβ1-4-Man) is the first structurally characterized epitope specifically demonstrated to be expressed on the surface of *Leishmania*-infected macrophages. Since neither of the anti-LPGAP mAbs bound to the surface of infected or antigen-pulsed macrophages (nor to promastigote surfaces) this suggests that the LPG and PG molecules are expressed on the macrophage membrane in an orientation similar to that found on the promastigote cell surface (see Figure 20). Alternatively, it is also possible that the LPGAP epitopes dissociate from LPG during macrophage processing and/or are destroyed during antigen processing events or that LPGAP moieties are inaccessible to mAb binding by their
association with other parasite or macrophage molecules once the parasite has entered the phagocyte or transformed into the amastigote.

Purified LPG or PG, the delipidated form of the LPG molecule, also bound to a variety of other cell types in a temperature-independent manner whereas CA7AE epitope expression originating from the promastigote was dependent on internalization of the parasites by phagocytizing cells (see below). This suggests that passive incorporation into the host cell membrane does not require the lipid anchor of LPG. Recently, the incorporation of *Chlamydia trachomatis* lipopolysaccharide (LPS) into host cell membranes has also been found to be independent of host cell type (Karimi et al. 1989). In contrast, purified membrane-form *Trypanosoma brucei* (VSG (which has a glycolipid anchor) was shown to bind to sheep erythrocytes whereas purified soluble VSG (which has lost the glycolipid anchor) did not (Rifkin and Landsberger 1990).

LPG epitope expression occurred at the initial area of parasite:macrophage attachment within 5-10 minutes p.i. By 35 minutes p.i., LPG epitopes were displayed over the entire macrophage surface. These observations contrast markedly with those reported previously (Williams et al. 1986) which suggested that an *L. braziliensis panamensis* major surface glycoconjugate did not appear on the macrophage membrane until several hours after parasitization. However, the exact identity of this glycoconjugate was not determined. A similar observation was made with *C. trachomatis*-infected cells where the LPS antigen from these microorganisms was not detectable on the macrophage surface until about 24 hours p.i. (Karimi et al. 1989). These results support the idea that the *L. b. panamensis* and *C. trachomatis* glycoconjugates detected on macrophage surfaces were of intracellular origin. Recently, LPG epitopes were shown to be displayed on the surface of *L. major*-infected murine macrophages (Handman 1990). These epitopes appeared relatively slowly on the macrophage surface and were also hypothesized to be of intracellular origin.

A previous review outlined four possible pathways for handling and presentation by accessory cells (including macrophages) of antigens which were membrane-bound, intracellular or excretory/secretory (E/S) molecules (Kaye 1987). These were: 1) that soluble E/S antigens are taken up after specific binding or fluid phase pinocytosis and then "processed"; 2) that host cell proteases cleave cell surface molecules to liberate "soluble antigens" which then follow the first pathway; 3) that degradation within host cell phagolysosomal compartments disrupts parasite membrane integrity allowing parasite antigens to be recycled to the macrophage membrane in a "processed" form; or 4) that cell membrane antigens may be "sloughed off" or directly transferred onto the host cell surface during parasite attachment and internalization. Since LPG epitope expression on the
macrophage cell surface occurred within 5-10 minutes of infection and since it was initially localized to the area of promastigote attachment, this strongly suggests that the *L. donovani* LPG, at least initially, made its way to the surface of infected macrophages via pathway #4. Presumably, LPG epitope accumulation on the macrophage surface occurs as soon as parasite internalization begins.

This theory is further substantiated by the experiments which showed that inhibition of macrophage phagolysosomal degradation processes had no effect on epitope expression while reagents that affected macrophage membrane flow functions drastically reduced or abolished expression. Earlier publications (Chang 1979; Zenian et al. 1979) have clearly indicated that attachment and internalization of *Leishmania* were inhibited by low temperature, cytochalasins and mild fixation of macrophages, demonstrating that parasite uptake was dependent on the integrity of the macrophage phagocytic mechanisms. It has been shown previously that LPG can mediate the attachment of promastigotes to macrophages (reviewed in Russell and Talamas-Rohana 1989). The results in Table 11 showed that treatment of macrophages with trypsin or proteinase K prior to infection significantly reduced or abolished LPG epitope expression in a transient fashion. This appeared to be due to the inhibition of parasite attachment. Attachment, and therefore LPG epitope expression, attained normal levels from 2 hours p.i. onwards suggesting that re-expression and redistribution of the appropriate macrophage *Leishmania* receptors occurred during this period.

Secreted *Leishmania* epitopes, both on media PG and secreted AcPase, are recognized by mAb CA7AE. The possibility that *L. donovani* components containing the CA7AE epitope were secreted into the extracellular fluid and then reabsorbed onto the macrophage plasma membrane was discounted, at least for the experiments presented here. Only parasitized macrophages exhibited the LPG epitope whereas non-parasitized neighbouring macrophages did not. This exclusivity of epitope expression occurred as late as 20 hours p.i. when the amount of CA7AE-reactive material in the culture medium had accumulated to levels easily detectable in a double antibody sandwich ELISA. Previous work (Berman and Dwyer 1981) in which *L. donovani* culture supernatants were incubated with uninfected macrophages produced equivocal results in that in some experiments no antigen was detectable on the macrophage surface while in others the macrophage surface was clearly labelled by their anti-*L. donovani* antiserum. The excreted form of *L. major* LPG has been shown to accumulate on the surface of macrophages (Handman and Goding 1985). However, in these studies the authors incubated the macrophages with glycoconjugate purified from culture medium that was 10-fold more concentrated than the late-log phase growth medium from which it was obtained, a situation unlikely to reflect the
environment surrounding the macrophage during infection. Also, since LPG is thought to be released from the promastigotes in two structurally distinct forms (Turco 1988), one which binds very tightly to serum albumin and appears to be similar in structure to cellular LPG, it may be that the authors merely concentrated this form to levels sufficient to accumulate on the macrophage membrane. Alternatively, it is possible that since *L. donovani* and *L. major* have different LPG carbohydrate structures (see earlier) they may utilize different mechanisms for expression of epitopes on macrophage surfaces.

The results from Figure 17 and Table 11 indicate that *L. donovani* LPG disaccharide repeat epitopes accumulate on the surface of infected macrophages during parasite penetration and at least initially their expression does not require active antigen processing. Epitope incorporation was not limited to lipid-tail interactions with the bilayer of the macrophage plasma membrane since delipidated LPG (PG) bound to the macrophage surface as efficiently as native LPG and in a temperature-independent manner. This may suggest that LPG association with eukaryotic plasma membranes may occur via interactions between the carbohydrate portion of the LPG molecule and host cell receptors, such as the integrins, that are present on many cells, including macrophages. These results do not discount the possibility that "processed" LPG epitopes could also contribute to the expression of LPG epitopes exhibited on the macrophage surface as infection proceeds. It has been suggested that expression of LPG epitopes on the surface of *L. major* promastigote-infected J774 macrophages originated from the intracellular amastigote (Handman 1990). In addition, it has been shown that during *L. major* promastigote-to-amastigote transformation, amastigote LPG epitopes were detected on the parasite surface within 12 hours p.i. of promastigote infection of the J774 macrophages whereas promastigote LPG epitopes gradually diminished over 48 hours (Glaser et al. 1991). Antibodies specific to *L. donovani* amastigote LPG epitopes would reveal the presence of amastigote LPG expressed on the surface of infected phagocytes and whether these epitopes were co-expressed with *L. donovani* promastigote LPG. However, since mAb CA7AE does not recognize amastigote LPG epitopes, the fact that promastigote LPG epitopes remain on the macrophage surface until 88 hours p.i. (see below) suggests that *L. donovani* promastigote LPG is at least initially a dominant parasite antigen expressed by the infected host cell. Indeed, it appeared that there was an accumulation of "residual promastigote LPG" surrounding the amastigotes within the phagolysosomal vacuole as late as 5-6 days p.i.

It was also demonstrated that living murine macrophages, when infected with wild-type *L. donovani* LD3 promastigotes, strongly expressed the LPG repeat epitope on their surface until approximately 50 hours p.i. after which the epitope expression decreased
dramatically and finally disappeared from the macrophage surface by approximately 88 hours p.i. When *L. donovani* LPG mutants were incubated with the phagocyte populations, the presence and extent of LPG surface epitope expression correlated with the ability of the different parasites to synthesize LPG or LPG fragments (King and Turco 1988; McNeely et al. 1990). Expression of surface LPG epitopes was similar whether the phagocyte populations were incubated with LPG-coated beads or infected with the variant *L. donovani* RT5 parasites. Acetone treatment of infected phagocytes was used to reveal internalized epitopes or those epitopes normally inaccessible to antibodies. Treatment with acetone prior to probing with the mAb CA7AE revealed LPG epitope expression on and/or within the phagocytes to at least 160 hours p.i. This was evident when the phagocytes were infected with wild-type or *L. donovani* RT5 promastigotes or when incubated with LPG-coated beads.

It is interesting that LPG epitopes linger on the surface and within *L. donovani*-infected murine macrophages for so many hours after infection. If amastigote LPG epitopes are presented on the surface of infected phagocytes it would be useful to determine whether preferential or co-expression of promastigote and amastigote epitopes is favored by either the parasite or the macrophage. While LPG has been shown to be involved in attachment to and uptake by phagocytic cells (reviewed in Russell and Talamas-Rohana 1989) it has also been established that *Leishmania* parasites deficient in LPG while still subject to host cell uptake, are subsequently destroyed once inside (McNeely and Turco 1990). In fact, leishmanial LPG is required by the parasite for intracellular survival (Handman et al. 1986; McNeely and Turco 1990). The relevance of LPG epitope expression on host macrophages for the host immune response is discussed in Chapter 3.

Human leishmaniasis is caused by at least 14 different species and subspecies of the genus *Leishmania* and identification is based on a variety of ecological, biological, biochemical and immunological criteria (Wirth et al. 1986). LPG, media PG (the secreted form of cellular LPG) and secreted AcPase contain private and common immunogenic epitopes which have been identified by both mAbs and polyclonal rabbit sera. In fact, the antigenic and structural variations in LPG epitopes have been used for taxonomic classification of *Leishmania* species (Schnur 1982). Therefore, it was relevant to test the anti-LPG mAbs for their ability to serve as diagnostic markers in human leishmaniasis. Since the anti-LPG mAbs are not species-specific, the intention was to use these mAbs as a rapid screen mechanism for "general" leishmaniasis rather than to differentiate between various species and subspecies as the causative agents of leishmaniasis. Unfortunately, since the LPGAP epitope does not appear to be secreted by the leishmanial parasites, the
cross-reactive protein(s) epitope recognized by mAbs L98 and L157 did not lend themselves to serum-testing assays via these methods and was therefore ineffective as a diagnostic marker. The epitope recognized by mAb CA7AE was evident in Kala-azar-positive sera but only in 50% of the documented cases. It may be that detection of this epitope in infected sera may be a kinetic phenomena, being detectable in the circulation only at specific times during infection. Studies using a vervet monkey model are currently under way to follow the kinetics of the expression of the CA7AE epitope during the course of an active *L. donovani* infection.
Conclusions

1. The epitope(s) recognized by anti-LPGAP mAbs L157 and L98 appears to be conserved within the Family Trypanosomatidae as it was detected in all Leishmania promastigotes and Trypanosoma PCF tested. In contrast, the mAb CA7AE epitope, while present in most Leishmania spp. and strains assayed, displayed more variable expression presumably due to saccharide substitutions of the PO₄-Gal-β1,4-Man disaccharide repeat backbone by the different Leishmaniae (see Chapter 1).

2. The epitopes bound by the anti-LPG mAbs are life cycle stage-specific. The epitopes are expressed in promastigotes and are not expressed in amastigotes.

3. The CA7AE epitope was transferred to the macrophage surface during L. donovani promastigote penetration of the macrophage membrane. CA7AE epitopes remained on the macrophage surface until approximately 88 hours p.i. and could be detected in the interior of the macrophages to at least 160 hours p.i.

4. Treatments which inhibited macrophage phagolysosomal degradation processes had no effect on epitope expression whereas reagents that affected macrophage membrane flow, and thus phagocytosis, drastically reduced or abolished expression. Purified LPG or de-lipidated LPG were also shown to bind to a variety of different cell types but in a temperature-independent manner.

Most of the work outlined in this chapter has been published or submitted for publication:


CHAPTER 3. THE IMMUNOBIOLOGY OF LPG.

Introduction

3.1 The mammalian immune response and antigen presentation.

The mammalian immune system exhibits an impressive ability to recognize and respond to a multitude of foreign macromolecules. This is accomplished through the function of B and T lymphocytes which express diverse sets of antigen receptors. Unlike B cells which directly interact with antigen, T lymphocytes possess an antigen-specific receptor that recognizes a bimolecular ligand composed of "processed" antigen that is "presented" in conjunction with antigens encoded by the major histocompatibility complex (MHC). This antigen presentation is a central feature of the acquired cell-mediated immune (CMI) response. Antigen presentation is the function of a heterogeneous group of antigen processing/presenting cells (APCs) of which the macrophage is an example. APCs serve to localize immune responses, selectively influence lymphocyte activation, and mediate cellular cooperation.

MHC antigens can be divided into two main types, class I and class II molecules, which share a similar function: both bind immunogenic peptides for presentation to specific T cells. The main distinction between them, besides structural features, is that MHC class I molecules interact with CD8+ cytotoxic T cells whereas MHC class II molecules interact with CD4+ T cells. It is the class II molecules in conjunction with the CD4+ T cells which have been demonstrated to be of primary importance in leishmaniasis.

3.2 Cell-mediated immunity and leishmaniasis.

Anti-leishmanial antibodies have been shown to lyse promastigotes in vitro in the presence of complement (Pearson and Steigbigel 1980) and to promote phagocytosis (Herman 1980) but there is little evidence to suggest a corresponding role in vivo for antibody in determining the outcome of leishmanial infection. However, the possibility still exists that antibodies specific for those structures on the parasite's surface that bind to receptors on the membrane of macrophages might have some protective function by preventing the initial attachment of Leishmania to macrophages and thereby preventing entry of the parasite. MAbs to L. major LPG have been shown to inhibit the binding of promastigotes to macrophages in vitro (Handman and Goding 1985) and mAbs specific for membrane components of L. mexicana promastigotes conferred complete protection when inoculated with L. mexicana promastigotes into the footpad of susceptible BALB/c mice (Anderson et al. 1983).
Available information suggests that CMI rather than humoral antibody plays the dominant role in host responsiveness and recovery to leishmaniasis. The following array of clinical and experimental evidence underlines the importance of CMI in resistance to leishmaniasis: 1) Resistant CBA mice rendered relatively T cell deficient by thymectomy followed by irradiation and reconstitution with syngeneic bone marrow cells are less able to control *L. major* infection (Liew 1986). 2) Athymic mutants of the highly resistant CBA and C57BL mice are totally unable to control *L. major* infection which progresses and visceralizes. Normal resistance, however, can be fully restored by reconstitution with syngeneic T cells (Muller et al. 1989). 3) Acquired immunity against *L. major* and *L. donovani* as a result of recovery from infection or prophylactic immunization can also be transferred by T cells but not by B cells (Liew et al. 1982; 1984). 4) Treatment of resistant C3H mice from birth with anti-IgM antibody rendered them defective in antibody responses and also susceptible to *L. major* infection. However, lesion progression in these treated mice can be arrested and the disease outcome reversed by adoptive transfer of T cells alone from normal C3H donors without any restoration of humoral antibody functions (Scott 1989). It is now generally accepted that T cells of the CD4+ subset confer protective immunity in leishmanial infections (reviewed in Liew 1989). This is supported by experimental evidence using adoptive transfer and replacement studies with the murine *L. major* (Liew et al. 1982; 1984) or *L. mexicana* (Gorczynski 1988) models. In addition, lymphokines such as macrophage activating factor (MAF) and gamma interferon (γIFN) produced by specifically sensitized CD4+ cells are deemed to be essential for the activation of infected macrophages to eliminate intracellular parasites (reviewed in Muller et al. 1989).

There is some evidence to suggest a supporting protective role for CD8+ (Titus et al. 1987; Farrell et al. 1989; Hill et al. 1989) and γδ (Modlin et al. 1989) T cells in murine *L. major* infections. It has been suggested that CD8+ and γδ T cell populations may effect CD4+ subset maturation by the production of cytokines such as γIFN or IL-4 (Locksley and Scott 1991). However, the results for CD8+ and γδ T cells are not as definitive as for the CD4+ T cells and thus far protection via CD8+ and/or γδ T cells by direct elimination of the intracellular parasites through cytotoxic activity has not been convincingly demonstrated in leishmaniasis.

Murine infections with *L. major* mimic the spectral nature of the cutaneous disease. Thus, as has been alluded to above, certain strains of mice (e.g. C3H, C57BL, NZB, A/J) are capable of controlling parasite growth and demonstrate varying levels of resistance to the parasites (Handman et al. 1979). In contrast, BALB/c and DBA/2 mice are extremely susceptible to infection by *Leishmania* parasites and develop a chronic disease that is ultimately fatal (Handman et al. 1979; Mitchell et al. 1981). The ability of
mice to control leishmanial infection is directly related to their ability to mount cellular immune responses. Interestingly, the poor response of BALB/c mice is not due to deletions in the T cell repertoire since a variety of immunomodulatory interventions including prior sub-lethal irradiation (Howard et al. 1981), prior CD4+ cell depletion (Titus et al. 1985) and therapy with anti-IL-4 antibody (Sadick et al. 1990) enable these mice to heal subsequent *Leishmania* infection. Each of these procedures is associated with ablation of the predominant Th2 cell (see below) response that occurs in untreated mice.

Murine CD4+ T cells can be functionally subdivided into two subpopulations. Th1 cells secrete γIFN and IL-2 and mediate classical delayed-type hypersensitivity reactions whereas Th2 cells secrete IL-4, IL-5, and IL-10 (Mosmann et al. 1986; Bottomly 1988; Moore et al. 1990). In experimental murine leishmaniasis it is now clear that Th1 cells are host protective (Mitchell et al. 1980; Liew et al. 1982) whereas Th2 cells can lead to disease exacerbation (Heinzel et al. 1989). The initial events that induce activation of distinct Th1 or Th2 cell populations during *Leishmania* infection is unknown. However, antigen-presentation itself is believed to be of crucial importance as is evident from the fact that different routes of injection of the same antigen (killed parasites or purified antigens) leads to either protective T cells or counter-protective T cells. Intravenous or intra-peritoneal immunization will preferentially induce protective Th1 cells whereas the subcutaneous or intra-muscular route leads to induction of the disease-promoting Th2 cells (Mitchell et al. 1984; reviewed in Liew 1986; 1987). Within 72 hours of infection, the draining lymph node cells of BALB/c mice produce a biased Th2 response characterized by the generation of IL-4 and IL-5 after antigen stimulation *in vitro* (Locksley and Scott 1991). Conversely, cells from previously immunized BALB/c or resistant C3H mice generate γIFN (Liew 1989). The outcome of the infection is determined by the balance of Th1 and Th2 cells as it appears that both subsets of CD4+ T cells are differentially regulated in resistant and susceptible mice infected with *L. major*. It has been shown that, whereas resistant mice produce IL-2 and γIFN in response to infection, susceptible mice produce IL-2 and IL-4 but no γIFN (reviewed in Liew 1989). Similarly, IL-4 produced by Th2 cells can neutralize the macrophage-activating and leishmanicidal effect of γIFN produced by Th1 cells (Liew et al. 1989) and IL-10 has been shown to inhibit cytokine synthesis by Th1 cells (Moore et al. 1990). Therefore, these observations suggest that the number of parasite-specific Th1 and Th2 cells activated in a mouse infected with *L. major* and the interplay between these subsets of CD4+ cells and the lymphokines they produce may determine the outcome of infection with *L. major* in mice. In addition, tumor necrosis factor α (TNFα) alone has been shown to activate macrophages to kill *L. major in vitro* (Liew et al. 1990a; Theodos et al. 1991b). The administration of TNFα during infection
has been shown to increase parasite clearance, but neutralization of TNFα (using antibodies) prevents clearance suggesting a role for TNFα in vivo (Titus et al. 1989; Theodos et al. 1991b). Furthermore, the activity of TNFα is markedly synergized by γIFN (Locksley and Scott 1991) suggesting a possible cooperation of these two cytokines in countering in vivo leishmanial infections. Whether TNFα is generated by macrophages or by CD4+ T cells during leishmaniasis is unknown. An illustration summarizing the development of the host immune response to Leishmania infection is shown in Figure 21.

In summary, the current working hypothesis of the immune regulation in leishmaniasis is as follows. Infection of the mammalian host by promastigotes rapidly leads to the establishment of amastigotes in the macrophages. Depending on the genetic constitution of the host this can lead either to limited multiplication of the parasites or to the uncontrolled proliferation of amastigotes (Liew 1987). The former will lead to the preferential induction of protective T cells whereas the latter will activate the disease-promoting T cells. The protective T cells secrete γIFN upon specific antigenic stimulation. Gamma-IFN is a potent macrophage activator that induces macrophages to kill intracellular parasites. In contrast, the disease-promoting T cells can produce IL-4 and other cytokines which inhibit the activation of macrophages by γIFN. The mechanism(s) by which the exacerbating cytokines modulate the macrophage-activation by γIFN is unknown.

It should be noted that although both cutaneous (L. major) and disseminated (L. donovani) leishmanial infections require effective cell-mediated immune responses for their resolution, the majority of immune system response analysis has been performed using L. major parasites and much less is known regarding immunity during an L. donovani infection. Infection of murine macrophages with L. donovani has been shown to dampen both MHC class II expression and IL-1 secretion (Bogdan et al. 1990). Moreover, L. donovani-infected macrophages are refractory to the activating potential of γIFN (Reiner et al. 1988). These results correlate well with the well-known lack of response of T cells in patients with Kala-azar, as documented by the lack of proliferation and IL-2 or γIFN production (Murray et al. 1987; Bogdan et al. 1990) or the lack of delayed-type hypersensitivity reactions (World Health Organization 1984; Sacks et al. 1987). Hence L. donovani is able to escape the immune response by undermining its initial activation. The mechanism(s) behind this lack of responsiveness is unknown. However, as was discussed earlier, this is certainly not true for all Leishmania species. When macrophages are infected with L. major instead of L. donovani they are fully able to induce a T cell response and to produce significant amounts of IL-1 (Cillari et al. 1989). At present, the molecular basis of these differential features of distinct Leishmania species is unknown.
Development of the host immune response to *Leishmania* infection. Effective Th1 T cell response on the left, ineffective Th2 T cell response on the right. The initial events and factors that induce either Th1 or Th2 proliferation are unknown but are thought to include the type of antigen, host genetic constitution and route of inoculation (i.e. environment). Once induced, the subsequent expansion of Th subsets is facilitated and regulated by subset-specific counter-regulatory cytokines: gamma IFN, by inhibiting the proliferation of Th2 cells, IL-4, by inhibiting IL-2 receptor expression and gamma IFN production, and IL-10, by inhibiting cytokine release from Th1 cells. (from Locksley and Scott 1991).
3.3 Immunological role of leishmanial antigens in leishmaniasis.

As has been described above, the host immune response to *Leishmania* is one of extreme complexity. The variability of the organism itself is self-evident. Antigenically the leishmaniae display a variable repertoire of protein-, carbohydrate-, and lipid-containing molecules. Intense interest has centered on the identification of the leishmanial immunogens that, in addition to inducing CMI, might trigger specific Th1 or Th2 subset expansion.

a) LPG.

Recent studies with murine T cell populations (Moll et al. 1989) and human peripheral blood lymphocytes from patients infected with either Old World (Jaffe et al. 1990d; Kemp et al. 1991) or New World (Mendonca et al. 1991) *Leishmania* species have indicated that LPG strongly stimulates T cells. In addition, earlier reports with *L. major*-infected mice have also shown that LPG immunization could elicit a marked immunoprotection that appeared to be T cell mediated - although direct stimulation of T cells by LPG was not reported (Handman and Mitchell 1985). However, none of these studies has rigorously investigated the relative contribution of LPG carbohydrate epitopes versus protein (as contaminants or otherwise) and therefore the exact role of LPG carbohydrate or lipid epitopes in T cell recognition and responsiveness remains undefined.

Another important question that remains unanswered is how the two subsets of CD4+ T cells are preferentially induced. While the mode of antigen-presentation (through route of injection of antigen) appears crucial (see above) an interesting hypothesis was put forward by Mitchell and Handman (1985) who argued that LPG and the delipidated water-soluble form of LPG derived from *L. major* promastigotes play a central role in murine cutaneous leishmaniasis. They proposed that the lipid-containing or membrane-bound form of LPG when anchored by lipid and properly oriented in relation to MHC class II molecules on infected macrophages would preferentially induce the protective Th1 T cells. On the other hand, the soluble or delipidated glycoconjugate bound to macrophages via specific receptors and unassociated with MHC would activate the disease-promoting Th2 cells. Supporting this is the evidence that prior immunization with *L. major* LPG protects mice against subsequent challenge with cutaneous leishmaniasis (Handman and Mitchell 1985). In contrast, the delipidated glycoconjugate is disease-exacerbating when injected into mice (Mitchell and Handman 1986). In addition, *L. tropica* excreted factor has been shown to inhibit human lymphocyte blast transformation *in vitro* (Londner et al. 1983). However, this hypothesis makes the assumption that the glycolipids are the dominant leishmanial immunogens in their LPG preparations and no definite proof has as yet been provided to fully substantiate this theory.
b) Other antigens.

Studies with protective and exacerbative Th cell lines have demonstrated that they respond to different fractions of partially purified soluble leishmanial antigen extracts (Scott et al. 1988). In one instance, a protective Th1 clone proliferated in response to a low molecular weight antigen that did not stimulate the Th2 line (Scott et al. 1990). While results such as these suggest epitope-dependent induction of Th1 and Th2 subsets, other evidence implies that environmental factors (e.g. route of immunization) are also important.

Leishmanial GP63 has been shown to immunize susceptible mice against re-infection (Russell and Alexander 1988). Jardim et al. (1990) used peptides to identify T cell epitopes within the GP63 molecule that can generate protective Th1 responses. When used as an immunogen with adjuvant one peptide provided immunoprotection against two species of Leishmania. In contrast, use of the peptide without adjuvant led to the exacerbation of subsequent disease. Although the predominant T cell subset responding in this case was not identified, the results obtained suggest that Th2 cells were stimulated and that the presence or absence of adjuvant dictated which type of Th cells would predominate. This data implies that signals in addition to those generated by cognate antigen-receptor interactions play a role in the induction of Th1 or Th2 subset expansion in leishmaniasis. Evidence with other systems also suggests that factors such as MHC gene products may influence the induction of CD4+ subset expansion. 1-A8 mice were found to selectively activate Th1 cells, whereas 1-Ab mice exhibited selective activation of Th2 cells in response to collagen IV (Murray et al. 1989).

Repetitive epitopes from another Leishmania antigen (Wallis and McMaster 1987) were also shown to induce a Th2 cell response when administered to BALB/c mice (Liew et al. 1990b). Interestingly, it was necessary to administer the antigen subcutaneously and in an octameric configuration to generate the T cell response. This suggests that interaction with regional antigen-processing cells is important in the stimulation of particular CD4+ subsets. It is probable that accessory signals such as local cytokines play a role in the eventual differentiation of Th1 and Th2 cells from a common precursor upon stimulation by antigen (Gajewski et al. 1989; Street et al. 1990; Weinberg et al. 1990).

3.4 Rationale for these studies.

Initially, as was outlined in the author's thesis proposal, examination of the immunobiological role of LPG was going to be a minor area of study if "time and circumstances permitted". However, it has in fact turned into a substantial portion of the work comprising this thesis. The reasons for this are as follows. Early experiments by the
author detected the presence of LPG epitopes on the surface of *L. donovani*-infected macrophages (see Chapter 2). This led to the hypothesis that LPG possibly could serve as an immunogen that plays a role in host macrophage activation and/or recognition by responsive T cells. Based on the glycolipid structure of LPG these studies also suggested that the responsible determinants in immune responsiveness would have to be carbohydrate in nature. Since T lymphocyte recognition of carbohydrate epitopes was (and still is) a controversial issue - though there was a growing number of publications citing examples of T lymphocyte recognition of carbohydrate antigens (Baer and Chaparas 1964; Shapiro et al. 1982; Robertson et al. 1982; Powderly et al. 1987; Domer et al. 1989; Podzorski et al. 1990), including those of *Mycobacterium leprae* (Mehra et al. 1984; Molloy et al. 1990) and *Leishmania major* (Moll et al. 1989) - it was decided to postpone examination of LPG’s involvement in CMI unless "time and circumstances permitted".

Then, serendipitously, while examining GP63 peptide induction of murine immune responses, Armando Jardim (a fellow graduate student in the department) used some of the author’s LPG stock (obtained from S. Turco) in the T cell stimulation assays in order to investigate the (possible) role of LPG as an immunosuppressive agent on the T cell responses to the GP63 peptides. Surprisingly, the LPG fractions were able to cause the stimulation of murine T cells primed with crude leishmanial antigen extracts. This was very intriguing since it suggested not only a specific role for LPG in leishmanial immunity but also, since it was thought that protein-free LPG material (Orlandi and Turco 1987) was being used in these assays, that it was in fact carbohydrate epitopes that were responsible for T cell recognition. The latter was especially exciting since this went against current scientific dogma and would be the first definite example of carbohydrate being recognized by T cells.

Therefore a collaboration was established to examine in greater detail the involvement of LPG in host CMI. Our early experiments suggested that carbohydrate epitopes were responsible for the observed stimulation associated with LPG. However, later results determined that a protein moiety (LPGAP) tightly associated with the LPG carbohydrate core (see Chapter 1) was responsible for T cell recognition. The progression of these discoveries is outlined throughout this chapter.
Some of the work presented in this chapter was a collaborative effort by Armando Jardim and D.L.T. The division of labour was as follows:

a) **Stimulation of murine T lymphocytes by LPG-associated epitopes (Results section #1).**

With the exception of the experiments for Figure 24, which were designed and conducted by D.L.T., the planning and performance of the other experiments in this section were performed as a 50:50 partnership between A.J. and D.L.T. A.J. provided the different LPG-related antigens used for the experiments in Figure 26.

b) **Results sections #2-4.**

For some of the experiments reported in these three sections, A.J. provided assistance with immunizations, the purification of T cells from LNC preparations and final harvesting of the lymphocyte stimulation cultures.
Materials and Methods

To avoid duplication, Materials and Methods previously outlined in Chapters 2 and 3 will not be repeated here.

Parasites. *T. congolense* 45/1 PCF have been described elsewhere (Richardson et al. 1988). All parasites were grown at 26°C in SM medium (Cunningham 1973) containing 10 or 15% heat-inactivated FBS. The different promastigote or procyclic species or purified *L. donovani* amastigotes were transferred to M1640 at 37°C for the antigen presentation or lymphocyte stimulation experiments (see below).

Mice. The murine strains A/J (H-2^a^), C57BL/10SnJ (H-2^b^), BALB/cJ (H-2^d^), B10.BR/SgSnJ (H-2^k^), B10.AKM/SnJ (H-2^m^), B10.RIII(71NS)/SnJ (H-2^r^), and SJL/J (H-2^s^) used for the lymphocyte stimulation assays were obtained from The Jackson Laboratory, Bar Harbor, Maine. All mice were eight to ten week old females. Other experiments in this chapter used BALB/c mice obtained from Charles River Laboratories, St. Constant, Quebec, The Jackson Laboratory, or from the University of Victoria's animal care facility.

LPG and LPGAP. LPG from *L. donovani* or *L. tropica* promastigotes was extracted, purified and quantitated by phosphate analysis as described previously (Orlandi and Turco 1987) and provided by S. Turco. After this isolation procedure LPGAP still remains bound to the core region of LPG (see Chapter 1).

Monoclonal antibodies, antisera and complement. Rabbit anti-thy 1 antisera (CL2001), rat anti-CD4 mAb (CL012A) and mouse anti-CD8 mAb (CL8921A) were from Cedarlane Laboratories, Hornby, Ontario. The anti-CD4 and anti-CD8 mAbs are cytotoxic antibodies. Low-Tox rabbit complement was also obtained from Cedarlane Laboratories. The murine mAbs anti-I-A^b^ (#CL8702-A; public for B6 and BALB/c Ia determinants only), anti-I-E^d^ (#CL8706-A; private for I-E^d^), anti-H-2K^d^ (#CL9008-A; private for H-2K^d^), and anti-H-2D^d^ (#CL9009-A; private for H-2D^d^) were obtained from Cedarlane Laboratories. Mouse anti-I-A^k^ alloantiserum (#CL8701; A.TH anti-A.TL) was also obtained from Cedarlane Laboratories. Mouse normal sera were prepared from female BALB/c mice one day prior to use.
**T lymphocyte purification.** All T cell populations were purified from lymph node cell (LNC) populations using a mouse T cell recovery column kit (Biotex Laboratories Inc., Edmonton, Alta.) as outlined by the manufacturers. Immunofluorescence microscopy using an FITC-conjugated goat anti-mouse IgG + IgM antibody (Caltag Laboratories) showed that there were less than 0.1% B lymphocytes remaining in the purified T cell populations. This was confirmed by the lack of any measurable proliferation when the purified T cell populations were incubated with the B cell mitogen LPS. Adherent cells were also removed during the T cell purification as judged by light microscopy after cells were incubated in microwells.

**Antigen presentation assay.** Peritoneal exudates from female BALB/c mice were collected (see Chapter 2). The resident cells were washed once by centrifugation and were resuspended at \(3.4 \times 10^5\) macrophages/ml in M1640. Macrophage monolayers were then prepared by incubating the peritoneal exudate suspensions in 96-well culture plates (Becton Dickinson, Lincoln Park, NJ). Each well received \(6-7 \times 10^4\) macrophages. Cells were allowed to adhere for 2 h at 37°C and were then washed twice with M1640 to remove non-adherent cells. It should be noted, that for this thesis, "macrophages" refers to adherent peritoneal exudate cells. Promastigotes harvested from the late-log to stationary growth phases were used to infect cultures of adherent macrophages at a ratio of 4 parasites per macrophage. Alternatively, living or heat-killed (56°C for 30 min) *L. donovani* amastigotes were incubated with the macrophage populations. Infection was performed at 37°C for 20 h and then all non-adherent or extracellular organisms were removed by washing with M1640. Alternatively, adherent macrophages were incubated under the same conditions with 15 ug of the various purified antigens. The macrophages were subsequently cocultured with antigen/promastigote-primed or naive LNC or antigen/promastigote-primed purified T cells and 96 h later the cells were assayed for lymphocyte proliferation as described below.

For antigen presentation by fixed macrophage populations, formaldehyde treatment was performed either prior to the addition of *L. donovani* promastigotes or purified LPG or 20 hours after infection/incubation. Macrophages were washed twice with 200 ul of PBS and then fixed using 0.5% formalin in PBS for 30 minutes at room temperature. After fixation the macrophages were washed twice with 200 ul of PBS and then twice (with 10 minute incubations at 37°C) with 200 ul of M1640. The addition of the different lymphocyte populations to the infected/pulsed or uninfected macrophages and measurement of the resulting lymphocyte proliferation are as described below.
Lymphocyte stimulation assays. Eight to ten week old female BALB/c (H-2^d_) mice were injected subcutaneously in the hindquarters and shoulder with 50 µg of purified antigen emulsified in complete Freund's adjuvant or with adjuvant alone or, alternatively, with 2 X 10^7 living *L. donovani* promastigotes suspended in PBS. After 8-10 days, the cells from the draining lymph nodes were incubated *in vitro* with a variety of soluble antigens, parasites, or parasite-conditioned culture medium, with promastigote-infected or antigen-pulsed macrophage populations, or with purified, living or heat-killed *L. donovani* amastigotes (see above). Antigen/promastigote-primed LNC (5 x 10^5) or naive LNC (5 x 10^5) or antigen/promastigote-primed purified T cells (3.5 x 10^5) were incubated with the different antigens. Ninety-six hours after establishing the cultures lymphocyte proliferation was measured using a ^3H-thymidine uptake assay, essentially as described elsewhere (Jardim et al. 1990). Triplicate cultures were used in all experiments. The T cell mitogen Con A (Vector Laboratories, Burlingame, CA) at 2.5 µg/ml or 0.5 µg/ml was used as a positive control for T lymphocyte stimulation while *Escherichia coli* 0127:B8 LPS (Difco Laboratories, Detroit, Mg) at 10 µg/ml was used as a positive control for B lymphocyte stimulation.

To investigate whether LPG had co-mitogenic properties, naive LNC were incubated with decreasing concentrations of phytohemagglutinin (PHA) (Gibco) or with the same PHA dilutions containing 25 µg/ml *L. donovani* LPG.

Complement-mediated cell depletion assays were used to determine the responding T cell phenotype. Lysis of T-cell subsets was carried out using specific anti-T cell antibodies and complement just before exposure of the LPG-primed T cells to macrophages presenting LPG epitopes. Purified LPG-primed T cells (3.5 X 10^5) were incubated with the various anti-T cell antibodies (see above) for 60 min at 4°C followed by the addition of 10% rabbit complement for 60 min at 37°C.

Measurement of production of the lymphokines IL-2 and IL-4 in the supernatants of the responding T cells has been described elsewhere (Mosmann 1983; Jardim et al. 1990).

MAb/sera inhibition assays. Macrophages were pre-incubated with the anti-LPG mAbs, the anti-procyclin mAb, the anti-MHC mAbs or antisera, or mouse normal sera (1:30 final dilution) 30-60 min prior to the addition of LNC or purified T cells to the infected/pulsed or uninfected macrophages. Lymphocyte proliferation was subsequently measured as described above.
Results

Stimulation of murine T lymphocytes by LPG-associated epitopes.

Female BALB/c mice were injected subcutaneously with LPG purified by solvent extraction and column chromatography (Orlandi and Turco 1987; see Chapter 1) and eight to ten days later the draining lymph nodes were removed and cell suspensions challenged with the glycan in vitro. A marked increase in $^3$H-thymidine uptake was observed in lymph node populations from LPG-immunized mice but not in cells from unimmunized animals and the response was LPG dose-dependent (Figure 22).

The phenotype of the proliferating cells was investigated and the results are shown in Figure 23. When LNCs were incubated with anti-Thy 1 antisera plus complement (lane 4) prior to LPG challenge, $^3$H-thymidine incorporation was essentially eliminated. Data presented in Figure 23 also show that the T cell subset involved was of the CD4+CD8- phenotype, since LPG-induced proliferation of primed lymphocytes was inhibited when CD4+CD8- cells were depleted (lanes 5 and 7). Additional studies using the same antibodies and complement, prior to incubation of the cells with LPS, verified that proliferation of the B cell population was unaffected (data not shown).

Although these data indicated that the LPG-induced stimulation was not a mitogenic response, it was possible that LPG acted as a co-mitogen. This concern was addressed by showing that LPG did not enhance the proliferation of naive LNCs incubated with sub-mitogenic concentrations of PHA in a co-stimulation assay (Figure 24).

To further delineate the region of the LPG molecule responsible for the T cell stimulatory activity various LPG fragments were tested for their ability to cause proliferation. The results are shown in Figure 25. Phosphoglycan (PG; de-lipidated LPG) (lane 2), the core-PI (lane 4), and the core alone (lane 5) were all able to cause stimulation levels comparable to that observed for the intact LPG (lane 1) indicating that the T cell stimulatory epitope was located within the core moiety of the LPG molecule. It should be noted that these proliferation experiments were conducted by normalizing antigen to carbohydrate rather than protein (see below), providing a reasonable explanation for the variation in proliferation response seen with these fragments.

As was outlined in Chapter 1, a protein component was subsequently found to be very tightly associated with purified LPG. To determine if this moiety played a role in the T cell stimulatory activity associated with LPG, LNCs from mice primed with conventionally prepared LPG (Orlandi and Turco 1987) were incubated in vitro with either LPG, octyl Sepharose purified LPG (i.e. protein-free LPG) or LPGAP (Figure 26). A response equivalent to the LPG proliferative activity was seen when LPG-primed LNCs...
were challenged with the LPGAP (lane 2). The low level of T cell stimulation observed with the octyl Sepharose purified L1–G fraction (lane 4) could be accounted for by the trace residual polypeptide contamination still detectable by amino acid analysis (0.1 ug protein/10 ug LPG). This evidence showed that it was the LPGAP moiety and not LPG carbohydrate epitopes which were responsible for the T cell stimulation. In addition, the oligosaccharide component of LPGAP did not contribute to T cell proliferation since comparable results were obtained with TFMSA deglycosylated LPGAP protein (lane 3).
Figure 22. Stimulation of LPG-primed or naive LNCs by varying concentrations of *L. donovani* LPG. The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 2500 cpm from the mean. Cell populations: ■, LPG-primed LNCs; □, naive LNCs.
Figure 23. The effect of treatment of LNCs from LPG-primed and naive mice with anti-T cell antibodies and complement prior to LPG stimulation. A. Experiment number 1. B. Experiment number 2. The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 1900 cpm from the mean. Treatments: 1, Con A (1.25 ug/ml); 2, L. donovani LPG (50 ug/ml); 3, LPG + complement; 4, LPG + anti-Thy 1 antisera + complement; 5, LPG + anti-CD4 mAb + complement; 6, LPG + anti-CD8 mAb + complement; 7, LPG + anti-CD4 + anti-CD8 + complement; 8, untreated LNCs. Cell populations: ■, LPG-primed LNCs; □, naive LNCs.
Figure 24. Stimulation of naïve LNCs by PHA and *L. donovani* LPG. Antigens: ■, PHA alone; □, PHA + LPG (25 µg/ml). The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 1300 cpm from the mean.
Figure 25. Lymphocyte stimulation by *L. donovani* LPG before and after various enzymatic and chemical treatments. A. Experiment number 1. B. Experiment number 2. The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 2800 cpm from the mean. Stimulating antigen: 1, LPG; 2, PG (delipidated LPG); 3, pronase-digested LPG; 4, core-PI (LPG lacking phosphorylated disaccharide repeats); 5, carbohydrate core; 6, no antigen. Cells were incubated with LPG or LPG fragments at 50 μg/ml. Cell populations: ■, LPG-primed LNCs; ☐, naive LNCs.
Figure 26. Lymphocyte stimulation by LPG, octyl Sepharose purified LPG and LPGAP. The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 2600 cpm from the mean. Stimulating antigens: 1, LPG-protein complex (50 ug/ml); 2, LPGAP (25 ug/ml); 3, TFMSA-treated LPGAP (25 ug/ml); 4, octyl Sepharose purified LPG (50 ug/ml); 5, no antigen; 6, Con A (1.25 ug/ml). Cell populations: ■, LPG-primed LNCs; □, naive LNCs; □, LPG-primed T cells.
Presentation of LPGAP epitopes to T lymphocytes by macrophages.

BALB/c mice were injected subcutaneously with purified LPG or LPGAP and the draining LNC were removed and challenged with various antigens or mitogens. The results are shown in Figure 27. LPG (data not shown) or LPGAP-primed LNC proliferated strongly in the presence of LPGAP (lane 1), Con A (lane 2), and LPS (lane 3) while unprimed LNC only responded to the two mitogens. LPGAP-primed purified T lymphocytes responded strongly to Con A (lane 2) but not to LPS (lane 3) substantiating the immunofluorescence results which demonstrated that the T cell population was free of accompanying B lymphocytes. The purified T lymphocytes also did not respond to purified LPGAP in the absence of antigen processing/presenting cells (macrophages) (lane 1). None of the three cell populations were stimulated in the absence of antigen or mitogen (lane 4). The three cell populations were also incubated with normal macrophages (lane 6), macrophages pulsed with purified L. donovani LPGAP (lane 7), or macrophages infected with L. donovani LD3 promastigotes (lane 8). None of the cell populations responded to normal macrophages nor did unprimed LNCs respond to either of the treated macrophage populations. Both the LPGAP-primed LNCs and LPGAP-primed purified T lymphocytes proliferated when LPGAP epitopes were presented by LPGAP-pulsed or promastigote-infected macrophages. Lane 5 demonstrates that exogenous LPGAP (i.e. LPGAP not presented by macrophages) was not responsible for the lymphocyte stimulation observed when purified LPGAP was incubated with macrophage populations. Macrophages incubated with heat-killed promastigotes (56°C for 30 min) at the same macrophage:parasite ratio as was used with the living promastigotes caused an equivalent stimulation of LPGAP-primed cells as that shown for the living, virulent parasites (data not shown). Neither promastigotes cultured alone (i.e. without lymphocytes and/or macrophages) nor macrophages infected with living promastigotes only (i.e. without lymphocytes) showed a significant increase in 3H-thymidine uptake (data not shown).

*Leishmania* promastigotes are known to release a form of LPG termed "excreted factor" (EF), both in vitro and in vivo (Schnur et al. 1972; see also Chapter 1). To test the possibility that *L. donovani* LD3 EF was secreted into the extracellular fluid and then readsorbed onto the macrophage plasma membrane with subsequent stimulation of LPG-primed T lymphocytes, LPG-primed LNCs were cultured with supernatants from *L. donovani* LD3 promastigotes or LPG-deficient control organisms (*L. donovani* C3PO promastigotes or *T. b. rhodesiense* ViTat 1.1 PCF). The results are shown in Figure 28. No stimulation was seen with any of the parasite-conditioned culture supernatants or for purified *L. donovani* EF.
To determine the phenotype of the T lymphocytes stimulated by macrophage-presented LPG epitopes, cells were depleted from purified T cell populations by treatment with specific anti-T cell antibodies and complement. The results are shown in Table 13. Anti-Thy 1 antisera plus complement markedly decreased stimulation levels. Treatment with the anti-CD4 mAb and complement eliminated \( ^3\)H-thymidine incorporation whereas treatment with the anti-CD8 mAb and complement did not.

To further characterize the proliferating T cell subset, lymphokine production was measured. The results are shown in Table 14. LPG-primed T cells produced IL-2 after stimulation by LPG-presenting macrophages. This was shown by the elimination of stimulatory activity of the T cell supernatants by an anti-IL-2 mAb. No IL-4 activity was detected using a similar method of mAb inhibition of stimulatory activity.

To demonstrate that antigen processing by "healthy" macrophages was required for LPGAP epitope presentation to T lymphocytes, macrophage populations were treated with formaldehyde either prior to the addition of \( L. \) \( donovani \) promastigotes or purified LPG to macrophage populations or 20 hours after infection/incubation with the same antigens. The results are shown in Figure 29. Fixation prior to antigen addition (Figure 29A) completely eliminated the ability of macrophages to present LPGAP epitopes to responsive T lymphocytes whereas T cell stimulation was still observed when fixation was performed at 20 hours p.i. (Figure 29B).
Figure 27. Stimulation of lymphocytes by *L. donovani* LPGAP and by LPGAP-pulsed or *L. donovani* LD3 promastigote-infected macrophages. A. Experiment number 1. B. Experiment number 2. The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 2300 cpm from the mean. Stimulating antigens: 1, *L. donovani* LPGAP (50 µg/ml); 2, Con A (1.25 µg/ml); 3, LPS (10 µg/ml); 4, No antigen; 5, LPGAP (75 µg/ml) incubated in complete medium for 20 h and then wells washed as for LPGAP-pulsing of macrophages (see Materials and Methods); 6, macrophages alone; 7, macrophages + *L. donovani* LPGAP (75 µg/ml); 8, macrophages + *L. donovani* LD3 promastigotes. Cell populations: ■, *L. donovani* LPGAP-primed LNCs; ☐, naive LNCs; ☐, *L. donovani* LPGAP-primed T cells.
Figure 28. Stimulation of lymphocytes with culture supernatants from *L. donovani* LD3 or C3PO promastigotes or *T. b. rhodesiense* ViTat 1.1 PCF or purified *L. donovani* EF. The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 3400 cpm from the mean. Antigen concentration was 20% parasite-conditioned culture medium or 50 ug/ml purified EF. SM medium alone (20%) or *L. donovani* LD3 LPG (50 ug/ml) were used as control antigens. Cell populations: ■, LPG-primed LNCs; □, naive LNCs; ▲, LPG-primed T cells.
Table 13. Stimulation of LPG-primed purified T cells after treatment with anti-T cell antibodies and complement.

<table>
<thead>
<tr>
<th>treatment 1</th>
<th>3(^{3})H-thymidine uptake (cpm \times 1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>macrophages only</td>
</tr>
<tr>
<td>none</td>
<td>4.1*</td>
</tr>
<tr>
<td>c' only</td>
<td>3.8</td>
</tr>
<tr>
<td>anti-thy 1 + c'</td>
<td>1.9</td>
</tr>
<tr>
<td>anti-CD4 + c'</td>
<td>0.7</td>
</tr>
<tr>
<td>anti-CD8 + c'</td>
<td>6.4</td>
</tr>
<tr>
<td>anti-CD4 +</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 2100 cpm from the mean.

\(^1\) T cell populations were treated with anti-T cell antibodies and complement prior to incubation with the stimulating antigens.

\(^2\) macrophages infected with *L. donovani* LD3 promastigotes.

\(^3\) macrophages pulsed with *L. donovani* LPG.
Table 14. Lymphokine production by purified T lymphocytes after stimulation by macrophages pulsed with LPG or infected with *L. donovani* LD3 promastigotes.

<table>
<thead>
<tr>
<th>OD (595 nm)¹</th>
<th>supernatant treatment</th>
<th>macrophages only</th>
<th>macrophages + LD³</th>
<th>macrophages + LPG³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1. LPG-primed T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>0.02*</td>
<td>0.13</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>anti-IL-2 mAb⁴</td>
<td>0.02</td>
<td>0.03</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>anti-IL-4 mAb⁵</td>
<td>0.03</td>
<td>0.14</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>2. naive LNCs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>0.01</td>
<td>0.02</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>anti-IL-2 mAb⁴</td>
<td>0.01</td>
<td>0.02</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>anti-IL-4 mAb⁵</td>
<td>0.01</td>
<td>0.02</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

* The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 0.02 OD units from the mean.

¹ based on a tetrazolium salt colorimetric assay (Mosmann 1983).

² macrophages infected with *L. donovani* LD3 promastigotes.

³ macrophages pulsed with *L. donovani* LPG.

⁴ anti-IL-2 mAb S4B6 (Mosmann 1983; Jardim et al. 1990).

⁵ anti-IL-4 mAb 11B11 (Mosmann 1983; Jardim et al. 1990).
Figure 29. Macrophage presentation of LPG epitopes to LPG-primed T lymphocytes requires antigen processing. The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 1500 cpm from the mean. Fixation of cells with formaldehyde was performed either prior to the addition of *L. donovani* promastigotes or purified LPG to macrophage populations (A) or 20 hours after infection/incubation (B). □, macrophages alone; ■, macrophages + LPG; ☐, macrophages + *L. donovani* LD3 promastigotes. Cell populations: 1, *L. donovani* LPG-primed T cells; 2, naive LNCs.
LPGAP presentation by macrophages is MHC-restricted. Mice of seven different haplotypes (H-2\(^{a,b,d,k,m,r,s}\)) were tested for their ability to respond to LPGAP in T cell stimulation assays. The results are shown in Table 15. T lymphocytes from all seven strains were stimulated when LPG-primed LNCs were challenged with LPG as a soluble antigen \textit{in vitro}. None of the naive LNCs demonstrated significant responsiveness to LPG. Similarly, macrophages from all seven strains were able to (syngeneically) present LPGAP epitopes to purified LPG-primed T cells (Table 16).

Attempts to demonstrate MHC-restriction of LPGAP epitopes by using an allogeneic antigen presentation system (i.e. BALB/c (H-2\(^d\)) macrophages presenting LPGAP to C57BL/10 (H-2\(^b\)) LPG-primed T cells and vice versa) were unsuccessful due to high background stimulation levels due to the resulting mixed lymphocyte reaction (MLR).

To determine the effect of anti-MHC mAbs or antiserum on the presentation of LPG epitopes to LPG-primed T lymphocytes, anti-MHC mAbs or antiserum were preincubated with normal macrophages, \textit{L. donovani} LD3 promastigote-infected macrophages, or macrophage populations pulsed with purified LPG 30 minutes prior to the addition of the lymphocyte populations. The results are shown in Table 17. None of the anti-MHC mAbs or antiserum caused changes in levels of thymidine uptake by normal macrophages when compared with normal macrophages incubated in the absence of antibodies. With promastigote-infected macrophages, only the mouse anti-I\(\lambda^k\) alloantiserum, the anti-I\(\text{A}^b\) mAb, and the anti-I\(\text{E}^d\) mAb significantly decreased (80%, 68%, and 67% respectively) the stimulation levels of the LPG-primed T cells. When the LPG-pulsed macrophages were challenged with LPG-primed T lymphocytes, the anti-I\(\lambda^k\) alloantiserum inhibited stimulation by 80% and the anti-I\(\text{A}^b\) and anti-I\(\text{E}^d\) mAbs decreased stimulation by 63% and 62% respectively. Neither of the anti-class I mAbs nor the mouse normal serum significantly inhibited lymphocyte stimulation in any of the lymphocyte populations by parasite-infected or LPG-pulsed macrophages.

Macrophages were also incubated with the three different anti-LPG mAbs to see whether or not LPG-primed T lymphocyte stimulation was affected. Table 18 illustrates that none of the anti-LPG mAbs nor the anti-procyclin mAb TBRP1.247 inhibited lymphocyte stimulation.
Table 15. Ability of LPG-primed or naive LNCs of various haplotypes to respond to LPG or *L. donovani* promastigotes *in vitro*.

<table>
<thead>
<tr>
<th>haplotype</th>
<th>level of stimulation (cpm X 1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no antigen</td>
</tr>
<tr>
<td></td>
<td>naive</td>
</tr>
<tr>
<td>H-2a&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.4* 4.5</td>
</tr>
<tr>
<td>H-2b&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.5 3.5</td>
</tr>
<tr>
<td>H-2d&lt;sup&gt;5&lt;/sup&gt;</td>
<td>4.2 5.0</td>
</tr>
<tr>
<td>H-2k&lt;sup&gt;6&lt;/sup&gt;</td>
<td>6.8 7.5</td>
</tr>
<tr>
<td>H-2m&lt;sup&gt;7&lt;/sup&gt;</td>
<td>4.1 5.1</td>
</tr>
<tr>
<td>H-2r&lt;sup&gt;8&lt;/sup&gt;</td>
<td>1.9 7.3</td>
</tr>
<tr>
<td>H-2s&lt;sup&gt;9&lt;/sup&gt;</td>
<td>3.5 17.4</td>
</tr>
</tbody>
</table>

* The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 2700 cpm from the mean.

<sup>1</sup> LNC populations were incubated with *L. donovani* LD3 promastigotes.

<sup>2</sup> LNC populations were incubated with *L. donovani* LPG.

<sup>3</sup> murine strain A/J.

<sup>4</sup> murine strain C57BL/10SnJ.

<sup>5</sup> murine strain BALB/cJ.

<sup>6</sup> murine strain B10.BR/SgSnJ.

<sup>7</sup> murine strain B10.AKM/SnJ.

<sup>8</sup> murine strain B10.RIII(71NS)/SnJ.

<sup>9</sup> murine strain SJL/J.
Table 16. Ability of LPG-primed T cells or naive LNCs of various haplotypes to respond to LPG presented by macrophages.

<table>
<thead>
<tr>
<th>haplotype</th>
<th>naive level of stimulation (cpm X 1000)</th>
<th>primed level of stimulation (cpm X 1000)</th>
<th>naive level of stimulation (cpm X 1000)</th>
<th>primed level of stimulation (cpm X 1000)</th>
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</thead>
<tbody>
<tr>
<td>H-2a&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.5* 2.5</td>
<td>3.1 14.8</td>
<td>3.2 30.1</td>
<td></td>
</tr>
<tr>
<td>H-2b&lt;sup&gt;5&lt;/sup&gt;</td>
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<td>2.3 8.3</td>
<td>2.1 18.5</td>
<td></td>
</tr>
<tr>
<td>H-2d&lt;sup&gt;6&lt;/sup&gt;</td>
<td>4.0 3.7</td>
<td>4.2 24.6</td>
<td>5.1 33.7</td>
<td></td>
</tr>
<tr>
<td>H-2k&lt;sup&gt;7&lt;/sup&gt;</td>
<td>6.1 7.4</td>
<td>9.4 23.5</td>
<td>7.3 38.2</td>
<td></td>
</tr>
<tr>
<td>H-2m&lt;sup&gt;8&lt;/sup&gt;</td>
<td>1.6 2.1</td>
<td>2.7 13.4</td>
<td>3.1 30.9</td>
<td></td>
</tr>
<tr>
<td>H-2r&lt;sup&gt;9&lt;/sup&gt;</td>
<td>3.5 8.4</td>
<td>5.7 27.4</td>
<td>6.2 30.6</td>
<td></td>
</tr>
<tr>
<td>H-2s&lt;sup&gt;10&lt;/sup&gt;</td>
<td>7.9 5.4</td>
<td>8.6 24.6</td>
<td>10.3 47.3</td>
<td></td>
</tr>
</tbody>
</table>

* The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 1800 cpm from the mean.
1 Lymphocytes were incubated with normal macrophages.
2 Lymphocytes were incubated with *L. donovani* LD3 promastigote-infected macrophages.
3 Lymphocytes were incubated with *L. donovani* LPG-pulsed macrophages.
4 murine strain A/J.
5 murine strain C57BL/10SnJ.
6 murine strain BALB/cJ.
7 murine strain B10.BR/SgSnJ.
8 murine strain B10.AKM/SnJ.
9 murine strain B10.RIII(71NS)/SnJ.
10 murine strain SJL/J.
Table 17. Inhibition of stimulation of naive LNC or LPG-primed purified T cells using anti-MHC mAbs or sera.a

<table>
<thead>
<tr>
<th>mAb/sera</th>
<th>macrophages&lt;sup&gt;b&lt;/sup&gt; naive</th>
<th>macro. + LD&lt;sup&gt;c&lt;/sup&gt; naive</th>
<th>macro. + LPG&lt;sup&gt;d&lt;/sup&gt; naive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>primed</td>
<td>primed</td>
<td>primed</td>
</tr>
<tr>
<td>none</td>
<td>4.0*</td>
<td>6.0</td>
<td>4.3</td>
</tr>
<tr>
<td>L&lt;sub&gt;a&lt;/sub&gt; sera&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.8</td>
<td>5.5</td>
<td>3.5</td>
</tr>
<tr>
<td>MN sera&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4.2</td>
<td>5.4</td>
<td>5.8</td>
</tr>
<tr>
<td>H-2K mAb&lt;sup&gt;g&lt;/sup&gt;</td>
<td>4.2</td>
<td>6.7</td>
<td>4.2</td>
</tr>
<tr>
<td>H-2D mAb&lt;sup&gt;h&lt;/sup&gt;</td>
<td>7.1</td>
<td>6.9</td>
<td>7.1</td>
</tr>
<tr>
<td>I-A mAb&lt;sup&gt;i&lt;/sup&gt;</td>
<td>3.9</td>
<td>6.5</td>
<td>6.8</td>
</tr>
<tr>
<td>I-E mAb&lt;sup&gt;j&lt;/sup&gt;</td>
<td>3.5</td>
<td>6.2</td>
<td>7.0</td>
</tr>
</tbody>
</table>

* The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 2100 cpm from the mean.

<sup>a</sup> Lymphocyte populations were incubated with the inhibitor mAbs/sera and normal macrophages or promastigote-infected or LPG-pulsed macrophages. The results shown are representative of those obtained in three separate experiments.

<sup>b</sup> normal macrophage populations.

<sup>c</sup> macrophages infected with <i>L. donovani</i> LD3 promastigotes.

<sup>d</sup> macrophages incubated with <i>L. donovani</i> LPG.

<sup>e</sup> mouse anti-L<sub>a</sub> alloantiserum.

<sup>f</sup> mouse normal serum.

<sup>g</sup> anti-H-2K<sub>d</sub> mAb (anti-MHC class I).

<sup>h</sup> anti-H-2D<sub>d</sub> mAb (anti-MHC class I).

<sup>i</sup> anti-I-A<sub>b</sub> mAb (anti-MHC class II).

<sup>j</sup> anti-I-E<sub>d</sub> mAb (anti-MHC class II).
Table 18. Inhibition of stimulation of naive LNC or LPG-primed purified
T cells using anti-LPG mAbs.a

<table>
<thead>
<tr>
<th>mAb</th>
<th>level of stimulation (cpm X 1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>macrophagesb</td>
</tr>
<tr>
<td></td>
<td>naive</td>
</tr>
<tr>
<td>none</td>
<td>4.0*</td>
</tr>
<tr>
<td>CA7AEe</td>
<td>5.0</td>
</tr>
<tr>
<td>L157f</td>
<td>3.8</td>
</tr>
<tr>
<td>L98g</td>
<td>3.7</td>
</tr>
<tr>
<td>247h</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 3100 cpm from the mean.

a Lymphocyte populations were incubated with the inhibitor mAbs and normal macrophages or promastigote-infected or LPG-pulsed macrophages. The results shown are representative of those obtained in three separate experiments.

b normal macrophage populations.
c macrophages infected with L. donovani LD3 promastigotes.
d macrophages incubated with L. donovani LPG.
e anti-LPG phosphorylated disaccharide repeat mAb CA7AE.
f anti-LPG-associated protein (LPGAP) mAb L157.
g anti-LPG-associated procyclin (LPGAP) mAb L98.
h anti-*T. b. rhodesiense* ViTat 1.1 procyclin mAb TBRP1.247.
Vaccine potential of LPGAP epitopes.

To determine if LPGAP epitopes were recognized by T cells during an *in vivo* *Leishmania* infection, mice were injected subcutaneously with *L. donovani* LPGAP or with living, virulent *L. donovani* LD3 promastigotes. Both LPGAP-primed and *L. donovani* LD3 promastigote-primed lymphocytes proliferated strongly in the presence of LPGAP or *L. donovani* promastigotes, either as unprocessed antigens (data not shown) or when antigens were presented by macrophages to purified, primed T cells (Figure 30; lanes 1 and 2 respectively). Purified T cells from both LPGAP-primed and promastigote-primed mice responded approximately equally to macrophages infected with either *L. donovani* LD3 or *L. tropica* K27.3 promastigotes (lanes 2 and 3 respectively). Neither cell population was significantly stimulated in the absence of antigen (lane 4).

To investigate whether LPGAP T cell epitopes were *Leishmania* promastigote stage-specific, killed *L. donovani* amastigotes were incubated with *L. donovani* promastigote LPG-primed or unprimed LNC or LPG-primed T cells in the presence or absence of macrophages. The lymphocyte stimulation results are shown in Figure 31. When amastigotes were added to LNC populations LPG-primed LNC were stimulated in a dose-dependent fashion (Figure 31A; lanes 1 to 4). No stimulation of naive LNC or purified primed T cells was seen. When amastigotes were presented to LPG-primed LNC by macrophages, the levels of stimulation observed (Figure 31B; lanes 1 to 4) were significantly above background levels but not as high as when the amastigotes were incubated directly with primed LNC populations. Purified T cells were also stimulated by the macrophages.

Binding of anti-*L. donovani* LPG mAbs to purified *L. donovani* and *L. tropica* LPGs in ELISA (Figure 32) demonstrated that mAb CA7AE (specific for the repeat portion of the *L. donovani* LPG molecule) did not recognize *L. tropica* LPG whereas the anti-*L. donovani* LPGAP mAbs equally bound both LPG molecules.

To investigate whether LPG from two different *Leishmania* species could cause reciprocal lymphocyte stimulation, LPGs purified from *L. donovani* LD3 and *L. tropica* K27.3 promastigotes were incubated with *L. donovani* LPG-primed or *L. tropica* LPG-primed lymphocytes. LPG molecules from either species were able to induce the stimulation of either *L. donovani* or *L. tropica* LPG-primed lymphocytes when the LPGs were added as soluble antigens (data not shown) or when presented by macrophages to purified, primed T cell populations (Figure 33; lane 1, *L. donovani* LPG; lane 3, *L. tropica* LPG). Both cell populations also proliferated strongly when macrophages were infected with either living *L. donovani* or *L. tropica* promastigotes (lanes 2 and 4 respectively). Neither cell population was significantly stimulated in the absence of antigen (lane 5).
LPG-primed or LPGAP-primed T cells also proliferated when incubated with macrophages infected with either *L. major* 5-ASKH or *L. b. braziliensis* M2903 promastigotes (data not shown).

The species distribution of the LPGAP molecule was examined by indirect ELISA on lysates of 12 New World and Old World *Leishmania* strains and two trypanosome PCF. The results are shown in Table 19. The epitopes within LPGAP recognized by mAbs L157 and L98 were present in all strains tested including the LPG-deficient mutant *L. donovani* C3PO and PCF of both trypanosome species. The binding pattern of the anti-LPG repeat mAb CA7AE was more variable, showing no binding to lysates of *L. donovani* C3PO or *L. tropica* K27.3 promastigotes or the trypanosome PCF. The control anti-trypanosome procyclin mAbs 247 and 150 bound only their specific antigens. All three anti-LPG mAbs bound to LPG and purified LPGAP. It is probable that residual repeat epitopes in the purified LPGAP resulted in the unexpected CA7AE mAb binding to this antigen.

The *Leishmania* and trypanosoma strains Table 19 screened for the presence of LPG-associated epitopes were also tested for their ability to stimulate LNC primed with *L. donovani* LPG, LPGAP or living *L. donovani* LD3 promastigotes. The results are shown in Table 20. All the *Leishmania* strains induced the proliferation of all three primed cell populations. However, the stimulation values observed for the Old World *Leishmania* species (i.e. *L. donovani*, *L. aethiopica*, *L. major* and *L. tropica*) were generally higher than those observed for the New World *Leishmania* species (*L. mexicana* and *L. braziliensis*). Stimulation by the *T. congolense* PCF was only approximately twice background for the LPG or LPGAP-primed LNCs. The *T. b. rhodesiense* PCF drastically inhibited growth/proliferation of all four cell populations by an unknown mechanism. All the parasites (except *T. b. rhodesiense*) stimulated the promastigote-primed LNC. The different primed cell populations were also appropriately stimulated by their immunizing antigens.
Figure 30. Stimulation of LPGAP-primed or *L. donovani* LD3 promastigote-primed lymphocytes by LPGAP-pulsed or *L. donovani* LD3 promastigote-infected macrophages. The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 3800 cpm from the mean. Stimulating antigens: 1, *L. donovani* LPGAP-pulsed macrophages; 2, *L. donovani* LD3 promastigote-infected macrophages; 3, *L. tropica* promastigote-infected macrophages; 4, no antigen. Cell populations: □, naive LNCs; ■, *L. donovani* LPGAP-primed T cells; ■, *L. donovani* LD3 promastigote-primed T cells.
Figure 31. Stimulation of lymphocytes by *L. donovani* amastigotes. A. Stimulating antigens: 1, *L. donovani* heat-killed amastigotes (1 X 10^6/well); 2, 5 X 10^6/well; 3, 1 X 10^7/well; 4, 5 X 10^7/well. B. Stimulating antigens: 1, Macrophages incubated with 1 X 10^6 heat-killed *L. donovani* amastigotes/well; 2, 5 X 10^6 /well; 3, 1 X 10^7 /well; 4, 5 X 10^7 /well. Cell populations: ■ , *L. donovani* LPG-primed LNCs; □ , naive LNCs; □ , *L. donovani* LPG-primed T cells.
Antigen Binding of anti-\textit{L. donovani} LPG-associated mAbs to \textit{L. donovani} LPG or \textit{L. tropica} LPG in ELISA. The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 0.04 OD units from the mean. Antigens (coated at 10 \micro{g/ml}): 1, \textit{L. donovani} LPG; 2, \textit{L. tropica} LPG; 3, human transferrin. Antibodies: ■, anti-LPG repeat mAb CA7AE; □, anti-LPGAP mAb L157; ■, anti-LPGAP mAb L98; □, anti-human transferrin mAb HT1-3.
Figure 33. Cross-stimulation of lymphocytes by LPG-pulsed or promastigote-infected macrophages. The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 2800 cpm from the mean. Stimulating antigens: 1, *L. donovani* LPG-pulsed macrophages; 2, *L. donovani* LD3 promastigote-infected macrophages; 3, *L. tropica* LPG-pulsed macrophages; 4, *L. tropica* promastigote-infected macrophages; 5, no antigen. Cell populations: □, naive LNCs; □, *L. donovani* LPG-primed T cells; □, *L. tropica* LPG-primed T cells.
Table 19. Binding of anti-LPG mAbs to *Leishmania* and *Trypanosoma* whole cell lysates and to *L. donovani* LPG and LPGAP in indirect ELISA.

<table>
<thead>
<tr>
<th>antigen</th>
<th>CA7AE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>L157&lt;sup&gt;b&lt;/sup&gt;</th>
<th>L98&lt;sup&gt;c&lt;/sup&gt;</th>
<th>247&lt;sup&gt;d&lt;/sup&gt;</th>
<th>150&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Parasite lysates&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. donovani</em> LD3</td>
<td>0.8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.3</td>
<td>0.5</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td><em>L. donovani</em> DD8*</td>
<td>0.9</td>
<td>0.3</td>
<td>0.5</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td><em>L. donovani</em> C3PO</td>
<td>0.1</td>
<td>0.3</td>
<td>0.6</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td><em>L. d. infantum</em> LEM235*</td>
<td>0.8</td>
<td>0.4</td>
<td>0.6</td>
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<td>0.04</td>
</tr>
<tr>
<td><em>L. aethiopica</em> L100*</td>
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<td>0.2</td>
<td>0.5</td>
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<td>0.04</td>
</tr>
<tr>
<td><em>L. major</em> S-ASKH*</td>
<td>0.8</td>
<td>0.2</td>
<td>0.4</td>
<td>0.06</td>
<td>0.03</td>
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<td><em>L. major</em> Jericho II</td>
<td>0.6</td>
<td>0.3</td>
<td>0.5</td>
<td>0.06</td>
<td>0.04</td>
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<tr>
<td><em>L. tropica</em> K27.3</td>
<td>0.1</td>
<td>0.3</td>
<td>0.5</td>
<td>0.07</td>
<td>0.04</td>
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<tr>
<td><em>L. m. mexicana</em> BEL21*</td>
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<td>0.2</td>
<td>0.4</td>
<td>0.07</td>
<td>0.03</td>
</tr>
<tr>
<td><em>L. m. amazonensis</em> M2369*</td>
<td>0.9</td>
<td>0.2</td>
<td>0.5</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td><em>L. b. braziliensis</em> M2903</td>
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<td>0.3</td>
<td>0.6</td>
<td>0.07</td>
<td>0.03</td>
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<td><em>L. b. panamensis</em> LS94*</td>
<td>0.9</td>
<td>0.2</td>
<td>0.3</td>
<td>0.07</td>
<td>0.03</td>
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<tr>
<td><em>T. b. rhodesiense</em> ViTat 1.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.9</td>
<td>0.03</td>
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<td><em>T. congolense</em> 45/1</td>
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<td>0.2</td>
<td>0.5</td>
<td>0.07</td>
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<td>2. LPG antigens&lt;sup&gt;g&lt;/sup&gt;</td>
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<td>LPG</td>
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<td>0.9</td>
<td>0.9</td>
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<td>0.9</td>
<td>0.9</td>
<td>0.07</td>
<td>0.04</td>
</tr>
</tbody>
</table>

<sup>+</sup> The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 0.05 OD units from the mean.

* WHO International reference strains

<sup>a</sup> anti-*L. donovani* LPG phosphorylated disaccharide repeat mAb CA7AE

<sup>b</sup> anti-LPGAP mAb L157

<sup>c</sup> anti-LPGAP mAb L98

<sup>d</sup> anti-*T. b. rhodesiense* ViTat 1.1 procyclin mAb TBRP1.247

<sup>e</sup> anti-*T. congolense* 45/1 procyclin mAb 150

<sup>f</sup> parasite lysates at 5 X 10<sup>5</sup> parasites/well

<sup>g</sup> antigens at 1.0 ug/well
Table 20. *In vitro* stimulation of LNC primed with *L. donovani* LPG, LPGAP or promastigotes by different *Leishmania* and *Trypanosoma* strains and by purified *L. donovani* LPG and LPGAP.

<table>
<thead>
<tr>
<th>antigen</th>
<th>LPG&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LPGAP&lt;sup&gt;b&lt;/sup&gt;</th>
<th>LD3&lt;sup&gt;c&lt;/sup&gt;</th>
<th>naive&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Parasites</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. donovani</em> LD3</td>
<td>82.4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>47.0</td>
<td>163.6</td>
<td>6.3</td>
</tr>
<tr>
<td><em>L. donovani</em> DD8*</td>
<td>86.6</td>
<td>51.6</td>
<td>152.6</td>
<td>7.3</td>
</tr>
<tr>
<td><em>L. donovani</em> C3PO</td>
<td>138.6</td>
<td>113.3</td>
<td>168.3</td>
<td>15.0</td>
</tr>
<tr>
<td><em>L. d. infantum</em> LEM235&lt;sup&gt;*&lt;/sup&gt;</td>
<td>146.6</td>
<td>130.0</td>
<td>214.6</td>
<td>21.3</td>
</tr>
<tr>
<td><em>L. aethiopica</em> L100&lt;sup&gt;*&lt;/sup&gt;</td>
<td>79.0</td>
<td>59.6</td>
<td>148.6</td>
<td>8.7</td>
</tr>
<tr>
<td><em>L. major</em> 5-ASKH&lt;sup&gt;*&lt;/sup&gt;</td>
<td>82.3</td>
<td>52.3</td>
<td>161.3</td>
<td>10.0</td>
</tr>
<tr>
<td><em>L. major</em> Jericho II</td>
<td>96.0</td>
<td>47.7</td>
<td>157.7</td>
<td>11.0</td>
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<tr>
<td><em>L. tropica</em> K27.3</td>
<td>93.6</td>
<td>64.2</td>
<td>168.7</td>
<td>5.0</td>
</tr>
<tr>
<td><em>L. m. mexicana</em> BEL21&lt;sup&gt;*&lt;/sup&gt;</td>
<td>48.7</td>
<td>32.7</td>
<td>115.3</td>
<td>5.0</td>
</tr>
<tr>
<td><em>L. m. amazonensis</em> M2269&lt;sup&gt;*&lt;/sup&gt;</td>
<td>41.3</td>
<td>24.3</td>
<td>104.3</td>
<td>2.0</td>
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<tr>
<td><em>L. b. braziliensis</em> M2903</td>
<td>52.0</td>
<td>25.7</td>
<td>146.7</td>
<td>4.7</td>
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<tr>
<td><em>L. b. panamensis</em> LS94&lt;sup&gt;*&lt;/sup&gt;</td>
<td>65.3</td>
<td>35.3</td>
<td>119.7</td>
<td>7.7</td>
</tr>
<tr>
<td><em>T. b. rhodesiense</em> ViTat 1.1</td>
<td>0.2</td>
<td>0.2</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td><em>T. congolense</em> 45/1</td>
<td>26.7</td>
<td>22.0</td>
<td>118.0</td>
<td>7.0</td>
</tr>
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<td><strong>2. LPG antigens</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPG</td>
<td>119.4</td>
<td>91.5</td>
<td>113.0</td>
<td>8.6</td>
</tr>
<tr>
<td>LPGAP</td>
<td>160.5</td>
<td>150.4</td>
<td>137.8</td>
<td>9.7</td>
</tr>
<tr>
<td>no antigen</td>
<td>10.2</td>
<td>15.2</td>
<td>23.6</td>
<td>4.1</td>
</tr>
</tbody>
</table>

<sup>+</sup> The data points represent single well values from a single experiment done in triplicate, since the values for each well deviated less than 6000 cpm from the mean.

<sup>*</sup>WHO International reference strains

<sup>a</sup> LPG-primed LNC
<sup>b</sup> LPGAP-primed LNC
<sup>c</sup> *L. donovani* LD3 promastigote-primed LNC
<sup>d</sup> naive LNC
<sup>e</sup> 3 X 10<sup>5</sup> parasites per 5 X 10<sup>5</sup> LNC
<sup>f</sup> all antigens at 50 μg/ml
Discussion

T lymphocyte-dependent immune responses play a major role in leishmaniasis (reviewed in Liew 1989). Research on the immunology of leishmaniasis has led to the hypothesis that a Th1 subset of the CD4+CD8- T lymphocyte phenotype is required for gamma interferon-induced activation of infected macrophages to become microbicidal (and immunoprotective) and that a Th2 subset may cause disease exacerbation (Hill et al. 1989).

Previous studies (Farah et al. 1975; Handman et al. 1979; Berman and Dwyer 1981; de Ibarra et al. 1982; Williams et al. 1986) have demonstrated the presence of leishmanial antigens on the surface of parasite-infected macrophages. In addition, this has been shown specifically for the LPG of both *L. major* (Handman 1990) and *L. donovani* (see Chapter 2) making LPG one of the few examples of a well-characterized parasite antigen displayed on the surface of infected macrophages. It is thus possible that LPG-associated epitopes could be recognized by *Leishmania*-reactive T lymphocytes. Indeed, recent studies with peripheral blood lymphocytes from patients infected with either Old World (Jaffe et al. 1990d; Kemp et al. 1991) or New World (Mendonca et al. 1991) *Leishmania* species have indicated that LPG strongly stimulates T cells. Moreover, earlier studies with *L. major*-infected mice have also shown that LPG immunization could elicit a marked immunoprotection that appeared to be T cell mediated - although direct stimulation of T cells by LPG was not reported (Handman and Mitchell 1985).

The above studies with LPG have resulted in speculation that the responsible determinants were carbohydrates (Moll et al. 1989). Although T lymphocyte recognition of carbohydrate epitopes has long been a controversial issue, there is a growing number of publications citing examples of T lymphocyte recognition of carbohydrate antigens (Baer and Chaparas 1964; Shapiro et al. 1982; Robertson et al. 1982; Powderly et al. 1987; Domer et al. 1989; Podzorski et al. 1990), including those of *Mycobacterium leprae* (Mehra et al. 1984; Molloy et al. 1990) and *Leishmania major* (Moll et al. 1989). However, these studies did not rigorously examine the relative contribution of carbohydrate and associated protein (as a contaminant or otherwise) and thus the role of carbohydrates in T lymphocyte recognition and stimulation remains equivocal.

The experiments presented here provide evidence that a Th1 subpopulation of murine T cells is involved in the recognition of *L. donovani* LPG isolated using standard procedures (Orlandi and Turco 1987). Since LPG stimulated proliferation of LNCs only from LPG-immunized mice and not from unimmunized control animals, it was clearly not mitogenic. Neither did LPG stimulate proliferation of LNCs incubated with submitogenic concentrations of PHA and thus it was not co-mitogenic. The LPG-induced T cell
proliferation was therefore of an antigen-specific nature. While these data showed that LPG does induce T cell proliferation, no evidence was found to support the suggestion that the stimulation was solely dependent upon carbohydrate epitopes. Separation strategies (see Chapter 1) produced purified LPG and LPGAP components from conventionally purified LPG which were then used in T cell proliferation assays. Results of these experiments clearly showed that the observed T cell stimulation was due to the strongly bound LPGAP. It is important to note that even after two consecutive chromatographic steps, residual protein could still be measured in the LPG as acid hydrolyzable amino acids, explaining how earlier workers might have interpreted the LPG stimulatory activity to be carbohydrate associated. In addition, earlier studies did not use amino acid microanalysis to detect residual protein levels in the purified LPG samples.

In order to investigate further LPGAP-induced T cell stimulation, the ability of macrophages to present LPGAP epitopes to LPGAP-primed lymphocytes was examined. Promastigote-infected macrophages and LPGAP-pulsed macrophages were both able to stimulate LNC and purified T cells primed with LPG (containing LPGAP), LPGAP alone, or living L. donovani promastigotes whereas normal macrophages did not. Since none of the macrophage populations stimulated $^3$H-thymidine uptake in unprimed lymphocytes and only "healthy" (i.e. non-formalin fixed cells) macrophages were able to present LPGAP epitopes, these results together indicate that the T lymphocyte proliferation, in addition to being antigen-specific, also required active antigen processing and accessory cell presentation of LPGAP epitopes. These conclusions were substantiated further by the fact that neither unprimed LNCs nor purified antigen-primed T lymphocytes were stimulated by soluble promastigote LPGAP in the absence of antigen processing/presenting cells. Since promastigote-primed LNC and T cells proliferated in the presence of LPGAP in vitro, this strongly suggests that LPGAP epitopes are recognized during infection with Leishmania in vivo.

It is normally accepted that T lymphocyte recognition of antigens is an H-restricted phenomenon. Therefore, experiments were performed to demonstrate whether or not the macrophage presentation of LPG epitopes was MHC-restricted. Unfortunately, both syngeneic and allogeneic antigen presentation systems failed to indicate MHC-restriction, as all seven murine haplotypes tested were able to respond to LPG-associated epitopes and high background levels made the allogeneic stimulation assays impossible to interpret. Recent experiments by Lopez et al. (1991) demonstrated that there were three different patterns of response, influenced by both H-2 (class II) and non-H-2 genes, in experiments using purified L. mexicana GP63. Congenic mouse strains could be grouped into high responders (H-2$b^q,r$), low responders (H-2$d^j,v,z$), or intermediate responders (H-
Essentially comparable results were seen with the experiments outlined here with LPG. It should be noted that the immune response to GP63 did not correlate with susceptibility of mouse strains to cutaneous infections with \textit{L. mexicana} promastigotes (Lopez et al. 1991). Similar results would be predicted for \textit{L. donovani} murine infections and LPG responsiveness.

MHC-restricted LPGAP presentation was also examined using several anti-MHC mAbs and an alloantiserum in antibody inhibition experiments. When \textit{L. donovani}-infected macrophages were first incubated with anti-Ia alloantiserum or the anti-I\textsuperscript{b} or anti-I-E\textsuperscript{d} mAbs their ability to stimulate LPG-primed purified T cells was markedly inhibited whereas normal mouse serum and anti-class I MHC (anti-H-2K\textsuperscript{d} and anti-H-2D\textsuperscript{b}) mAbs had no effect. These results suggest that the LPG epitopes are presented on the macrophage surface in association with MHC class II molecules. The fact that LPGAP stimulates proliferation of T cells of the CD\textsuperscript{4+}CD\textsuperscript{8−} (Th1) phenotype supports this hypothesis.

None of several well-defined anti-LPG mAbs inhibited the macrophage presentation of LPG epitopes to primed T cells. Since the mAb CA7AE binds to the LPG repeat epitope on the surface of parasite-infected or LPG-pulsed macrophages (see Chapter 2) it implies that the repeating phosphorylated disaccharide epitope had no supporting role in the recognition of LPGAP by the T lymphocytes. The LPGAP epitopes are not accessible to the anti-LPGAP-specific mAbs (L157, L98) either on living \textit{L. donovani} promastigotes or on the surface of parasite-infected or LPG-pulsed macrophages (see Chapter 2). This implies either that LPGAP epitopes are not accessible to antibodies but are to T lymphocytes or that the LPGAP molecule is altered prior to or after insertion into the macrophage membrane. The fact that the anti-LPGAP mAbs were unable to bind to acetone-permeabilized promastigote-infected macrophages (see Chapter 2) and that accessory cell presentation of LPG-associated epitopes requires active antigen processing helps substantiate the latter theory. It is also possible that the relevant LPGAP T cell epitope(s) is bound in the MHC cleft (Bjorkman et al. 1987) in such a conformation that it is not available for binding by these mAbs.

Purified amastigotes were also able to stimulate LPG-primed LNC. These results indicate that the relevant amastigote epitopes are similar to those of the promastigote and that on the amastigotes, these epitopes are in a form recognizable by primed T cells. Primed LNC populations were clearly stimulated when challenged with amastigotes in contrast to the stimulation induced by the addition of amastigotes to macrophages and purified T cells. It may be that amastigote epitopes are presented to responding T cells much better by B cells than by macrophages.
Experiments were also performed to determine whether the LPGAP recognized by the T cells was a species-specific moiety. The observation that *L. donovani* LPG-primed lymphocytes were stimulated by *L. tropica* LPG and *vice versa*, suggested that LPGAP was a molecule shared across species. ELISA (and immunofluorescence - data not shown) using the anti-LPG mAbs showed that the epitopes recognized by the anti-LPGAP mAbs were distributed over a wide range of parasites; the epitopes being present in all 12 New World and Old World *Leishmania* strains (and even the two species of trypanosome PCF) tested. The same parasite strains were able to stimulate LPGAP-primed LNC further suggesting that the LPGAP molecule contains epitopes shared by different species of *Leishmania*.

It seems likely that the epitopes in LPGAP recognized by the anti-LPGAP mAbs are probably not identical to the epitopes recognized by the responding T cell populations. Recent evidence has shown that *L. major* amastigotes express a lipophosphoglycan molecule that is structurally different from its promastigote counterpart (Turco and Sacks 1991; Glaser et al. 1991) and the *L. major* promastigote and amastigote LPG have been shown to be immunochemically distinct. In addition, experiments outlined in Chapter 2 showed that *L. donovani* amastigotes lack the epitopes recognized by the anti-LPGAP mAbs. However, *L. donovani* amastigotes were still able to stimulate promastigote LPGAP-primed lymphocytes. In addition, essentially equal levels of binding of the anti-LPGAP mAbs to the various *Leishmania* and *Trypanosoma* strains tested was observed. In contrast, although all of the *Leishmania* parasites stimulated LPGAP-primed T cells, they varied in the degree of stimulation. The results suggested that the greatest degree of relatedness of the T cell epitope(s) was between the Old World *Leishmania* species. Perhaps there are minor differences in the LPGAP molecule of the New World strains. It is questionable whether the trypanosome PCF, members of the same evolutionary family (Trypanosomatidae), stimulated the LPGAP-primed lymphocytes even though the anti-LPGAP mAb epitopes are undoubtedly present. An LPG-like molecule has been found in African trypanosomes that is biochemically different from the *Leishmania* LPG molecule, yet has similar chromatographic properties (Hublart et al. 1988). It may be that the anti-LPGAP mAbs recognize epitopes associated with this molecule, but the molecule is different enough that it does not induce stimulation of *Leishmania* LPG-primed lymphocytes.

From an prophylactic perspective, since LPG has been shown to be immunoprotective when injected into mice (Handman and Mitchell 1985, Russell and Alexander 1988), it is very likely that these effects can be explained by the presence of LPGAP. Indirect support for this position has been provided by Mendonca et al. (1991)
who showed that peripheral blood lymphocytes from leishmaniasis patients were stimulated by *L. braziliensis* and *L. mexicana* LPG but not by LPG first treated with proteinase K. It was interesting that a similar finding was not obtained in this investigation after incubation of LPG with pronase. Again, these contrasting results could either reflect different specificities of the proteases or differences in the structures of New World and Old World parasite LPG-protein complexes.

The data presented here show that macrophages can present *Leishmania donovani* LPGAP epitopes to LPGAP-primed T lymphocytes and can induce their proliferation. The relevant LPGAP epitopes specifically stimulate CD4<sup>+</sup>CD8<sup>-</sup>, IL-2 secreting (i.e. Th1) T lymphocytes - the cell population shown to be associated with γIFN-induced host protection (Liew 1989). Recognition of the LPGAP epitopes is dependent on processing and presentation of the epitopes by antigen presenting cells, is an MHC-restricted event and, while is not stage- or species-specific, is an antigen-specific event. Since living, virulent *L. donovani* promastigote-primed lymphocytes were able to respond to purified LPGAP *in vitro*, this implies that this can occur *in vivo*. The presence of this immunochemically cross-reactive molecule over such a wide range of *Leishmania* species which demonstrate different tissue tropisms and pathologies suggests that the LPGAP may have a conserved function perhaps playing an important role LPG biogenesis or in the immunobiology of leishmaniasis. Since *L. major* LPG has been used to successfully immunize mice against cutaneous leishmaniasis (Handman and Mitchell 1985; Russell and Alexander 1988), the evidence outlined here strongly suggests the potential of the LPGAP molecule of *Leishmania* LPG as a vaccine candidate for leishmaniasis.
Conclusions

1. Lymph node cells from mice primed with *L. donovani* LPG or LPGAP or with living, virulent *L. donovani* promastigotes were specifically stimulated to proliferate *in vitro* by the LPGAP moiety of the LPG molecule.

2. The LPGAP epitopes specifically stimulated CD4+CD8-, IL-2 secreting T lymphocytes. The T cell response was antigen-specific, dose-dependent, and non-mitogenic.

3. Purified T lymphocytes primed with purified LPG or LPGAP were not stimulated by LPG or LPGAP *in vitro* unless promastigote-infected or LPG-pulsed or LPGAP-pulsed macrophages were added. Recognition of LPGAP epitopes required active antigen processing by the macrophages and the T cell stimulation was an MHC-restricted event.

4. The T cell stimulation caused by LPGAP was not species-specific and the responding T cells recognized epitopes in amastigotes. This data strongly suggests the potential of *Leishmania* LPG and/or LPGAP as a vaccine candidate for leishmaniasis.

Most of the work in this chapter has been published or submitted for publication:


LITERATURE CITED


