

**Immunological and Biochemical Characterization of the Major Surface Membrane  
Proteins: gp63 and the Lipophosphoglycan Associated Protein of *Leishmania***

by

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## ABSTRACT

Leishmaniasis is a disease caused by the protozoan parasite *Leishmania*. It has been estimated that globally up to 12 million individuals are affected by this disease. The lack of a reliable immunoprophylactic agent has made immunization of populations in endemic regions largely unsuccessful. Since recovery from leishmaniasis is dependent on the activation of Th1 subset of helper T cells, the *L. major* gp63 primary structure was screened for putative T cell epitopes using computer based predictive algorithms. Although several immunostimulatory peptides were identified, one peptide PT3, elicited proliferation of a CD4<sup>+</sup> T cell population which secreted IL-2 but not IL-4, attributes ascribed to Th1 cells. More importantly, PT3 injected with the adjuvant Poloxamer 407 protected both BALB/c and CBA mice against a challenge with both *L. major* and *L. mexicana* promastigotes.

In a related study, lymphocytes from mice immunized with *L. donovani* lipophosphoglycan (LPG) were specifically stimulated to proliferate *in vitro* by purified LPG or its delipidated congener, phosphoglycan (PG). The response was dose-dependent and required prior immunization with either LPG or PG. Proliferation was eliminated by specific depletion of Thy1<sup>+</sup> cells and the proliferating T-cell subset was further shown to be CD4<sup>+</sup> secreting IL-2 in response to an LPG challenge. Tests of various LPG fragments indicated that the T-cell stimulation was associated with the core structure of LPG rather than the lipid or phosphoglycan repeat structure. Amino acid analysis of both LPG and T lymphocyte reactive LPG fragments, following acid hydrolysis, showed high levels of amino acids, diagnostic of proteinaceous material in the LPG preparation. Treatment of LPG with either trifluoromethanesulfonic acid or anhydrous hydrazine, revealed the presence of polypeptide material which reacted with mAbs L98 and L157, previously thought to be specific for the LPG core glycan. This novel 11 kDa protein, designated lipophosphoglycan associated protein (LPGAP), was purified by reversed phase

chromatography and subsequently shown to be the T cell proliferative component rather than LPG.

LPGAP was detected in both *L. donovani* promastigotes and amastigotes. The primary structure of this molecule was determined using a combination of Edman degradation and DNA sequencing. In addition, several post-translational modifications were identified on the *L. donovani* promastigote LPGAP. These include a putative O-glycosylation and a N<sup>ε</sup>-monomethyl arginine residue. Subcellular fractionation in conjunction with immunoblot analysis showed this molecule to be associated with a membrane fraction. Immunoprecipitation of <sup>125</sup>I-labeled promastigotes further localized LPGAP to the cell surface where it was expressed at a copy number similar to that of LPG (1-2 x 10<sup>6</sup> molecules per cell), making this a major protein on the parasite cell surface.

Computer-based secondary structure analyses predicted LPGAP to be almost exclusively α helical, with the protein adopting a helix-loop-helix motif. This was verified by circular dichroism measurements of the promastigote LPGAP which indicated a very high helical content estimated to be approximately 86% in trifluoroethanol. Arrangement of the residues located in the putative helical regions on an Edmundson helical wheel showed that this molecule could have a strong amphipathic conformation and thus provided an explanation for how such a highly charged protein might be inserted into the plasma membrane. Evidence in support of LPGAP association with lipid bilayers was provided by showing that LPGAP could mediate carboxyfluorescein release from liposomes. Taken together with the close association with LPG, these findings suggested that LPGAP may function to stabilize the unstable LPG lysophospholipid anchor within the parasite membrane. From a more practical perspective the wide distribution of gp63 and LPGAP on both promastigotes and amastigotes makes these molecules ideal candidates for vaccine development.

**Examiners:**

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**i) History of Leishmaniasis:**

Leishmaniasis embodies a spectrum of diseases with varying clinical manifestations which result from infection by protozoan parasite of the genus *Leishmania*. Reports of this disease in man date back to 1745, when Pizzaro's expedition in South America documented skin sores in the native population which resembled lesions presently associated with cutaneous leishmaniasis. However, the correlation between this illness and a parasitic agent was not established until 1903, when a Scottish physician by the name of Leishman found unusual bodies in liver biopsies taken from troops suffering from "Dum-Dum" fever. A year later these bodies were cultured by Donovan (Vickerman, 1985) demonstrating that leishmaniasis was due to a parasitic infection.

According to the World Health Organization, leishmaniasis affects approximately 80 countries in tropical and subtropical regions with as many as 12 million individuals currently being afflicted by this illness (World Health Organization, 1993). On the basis of clinical symptoms and geographical distribution, this disease has been grouped into three categories; cutaneous, mucocutaneous, and visceral leishmaniasis.

The cutaneous disease is the most common form and is characterized by skin lesions which develop 2-8 months after a sandfly bite. At the site of inoculation a nodule forms which ulcerates. These ulcers spontaneously heal in 1-3 years and impart the host with protective immunity to subsequent infection. The ancient practice of "leishmanization", carried out in the Middle Eastern countries attempted to immunize babies while restricting the site of lesion formation by strategically exposing only the babies buttocks to a sandfly bite.

Mucocutaneous leishmaniasis resembles the cutaneous disease in the initial stages with the formation of a self curing lesion. However, if untreated with chemotherapeutics, the parasites metastasize to the oronasal and pharyngeal mucosa, where parasite proliferation is maintained in check by the immune system for up to several decades after the appearance of the primary lesion. Without medical intervention, however, progressive tissue destruction occurs around the nose, mouth, and ears resulting in a pathology similar to leprosy (World Health Organization, 1984). Fatalities associated with the

mucocutaneous disease are attributed to opportunistic infections, the most common of these being bronchopneumonia (World Health Organization, 1984).

Visceral leishmaniasis, also known as "kala-azar" or "Dum-Dum" fever is the most fatal form of the disease in humans. The impact of this illness on mankind is clearly illustrated by the death of as many as 10,000 people in India, in 1992, and 40,000 individuals in the Sudan between the period of 1988 to 1993 (World Health Organization, 1993). The sector of the population most at risk are young children between 1-9 years of age in countries where malnutrition is a major socio-economic factor. The incubation period for visceral leishmaniasis may vary from 10 days to 1 year, and unlike the cutaneous and mucocutaneous forms of the disease a lesion does not always appear at the site of inoculation. The parasites migrate from the bite site and are harboured by cells of the reticuloendothelial system, with the spleen, liver, and bone marrow being the primary foci of infection. Early stages of visceral leishmaniasis are asymptomatic, however, disease progression is accompanied with fever, malaise, weight loss, and frequent diarrhoea. Clinical hallmark symptoms of this infection are hepatosplenomegaly, lymphadenopathy, and hyperglobulinemia, due to a polyclonal B lymphocyte activation (Campos-Netos & Bunn-Moreno, 1982). In late stages of the disease hematopoiesis is depressed resulting in anemia. This condition is further aggravated by the decreased hepatocyte prothrombin production and bleeding of the intestinal mucosa (Bray, 1985). At this point, the host is severely immunocompromized and extremely susceptible to secondary infections. Leading causes of death are bronchopneumonia, tuberculosis, and dysentery. More recently immunosuppressed AIDS patients have become a serious component of individuals afflicted with leishmaniasis (World Health Organization, 1993).

#### **ii) Distribution of Leishmaniasis:**

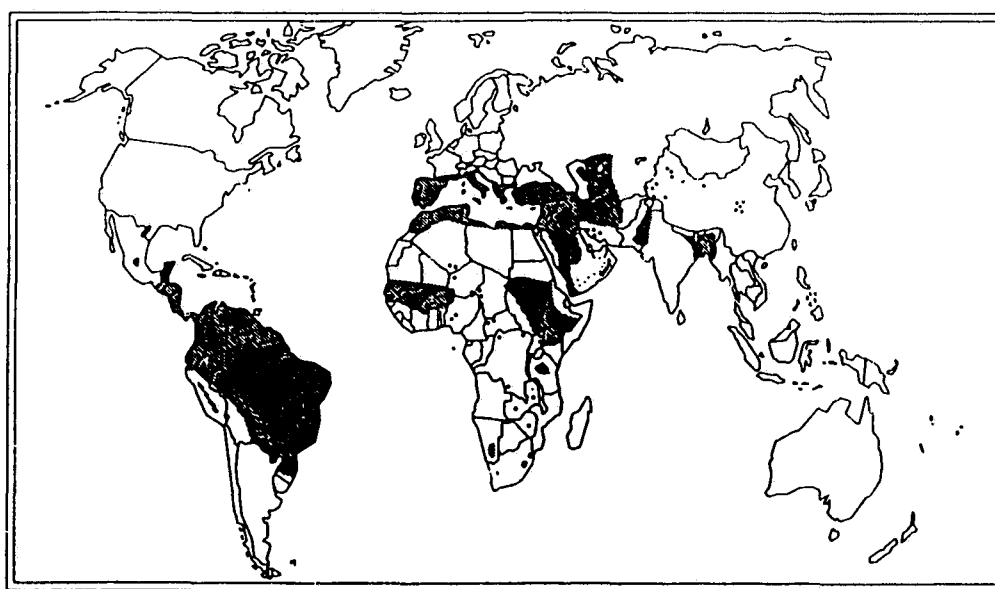
Diseases caused by parasites of the *Leishmania* genus have been divided into two categories, designated Old World and New World leishmaniasis on the basis of geographical distribution, parasite species, and the species of sandfly vector responsible for disease transmission. The Old World group consists of cutaneous and visceral leishmaniasis, whereas the New World maladies includes mucocutaneous disease along

with the former. The cutaneous disease is the most prevalent form of leishmaniasis world wide, and is concentrated in areas of the Middle East, the Mediterranean basin, Africa, and southern Asia, with the major etiological agents isolated from skin lesions being *L. aethiopica*, *L. major*, or *L. tropica* (Figure 1). In endemic areas, man has been implicated as the primary reservoir for the cutaneous disease transmitted via the sandfly (Zuckerman & Lainson, 1977). In non-endemic regions, dogs, rats, and hyrax have been implicated as secondary reservoirs for the above group of organisms (Abranches, 1989). Although rare, cases of cutaneous leishmaniasis have been reported arising from *L. donovani* infections which failed to visceralize (World Health Organization, 1984). Similarly, clinical symptoms resembling cutaneous leishmaniasis have been reported in Latin America and regions of South America in patients infected with *L. braziliensis* and *L. mexicana*, parasites normally associated with mucocutaneous disease. However, skin lesions caused by these New World parasites result in severe chronic disease which requires medical intervention for resolution (Deane & Grimaldi, 1985).

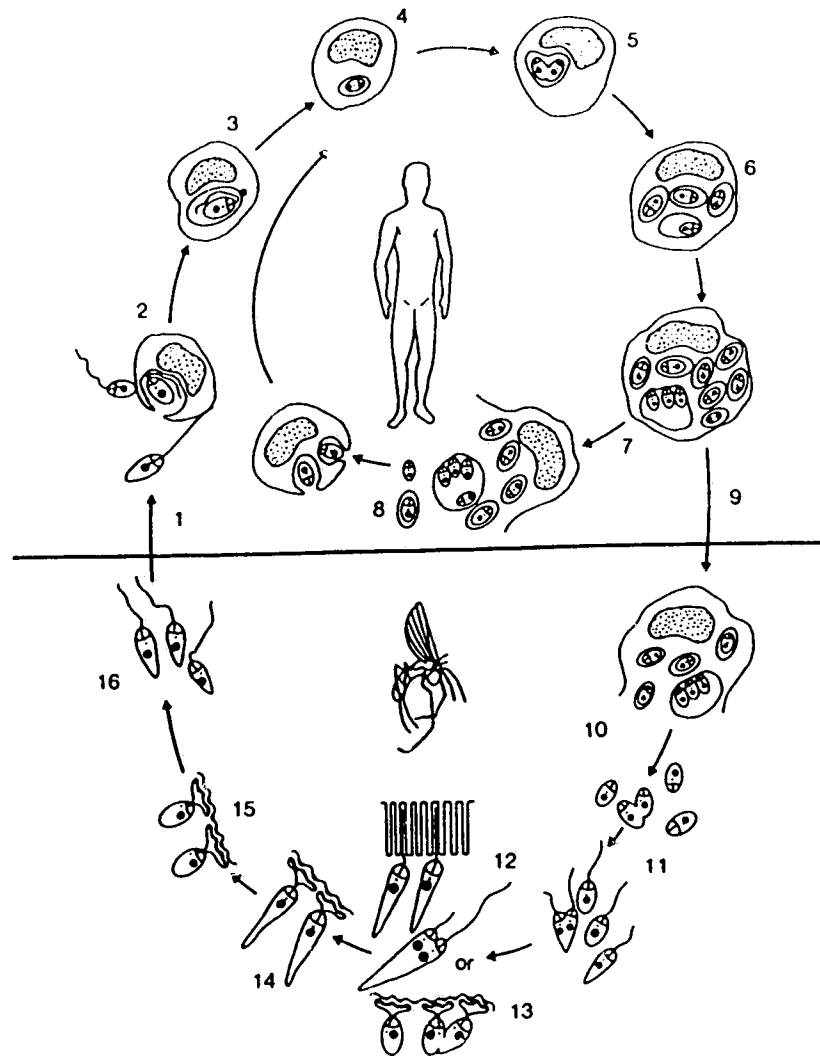
The visceral disease has been reported from three species: *L. donovani donovani*, *L. donovani infantum*, and *L. donovani chagasi*. The latter is the cause of New World visceral leishmaniasis. While the two former organisms are endemic to areas of India, Africa, and parts of China where man and dogs are believed to be the primary reservoir for the parasite (World Health Organization, 1984).

### iii) Life cycle of *Leishmania*:

*Leishmania* is a digenetic organism belonging to the order Kinetoplastida and the family Trypanosomatidae. Members of this family also include *Crithidia*, *Trypanosoma cruzi*, and the African trypanosomes. As shown in Figure 2, *Leishmania* has two life cycle stages, the flagellated and highly motile promastigote form which has evolved to survive the harsh environment encountered in the sandfly gut, and the non-flagellated obligate intracellular amastigote form, which resides in the phagolysosomal vacuoles of phagocytic cells of the reticuloendothelial system. The life cycle is initiated when the sandfly takes a blood meal from an infected host. Although plant sugars constitute the primary sandfly diet, blood meals are essential for stimulation of oogenesis (Schlein, 1986).



**Figure 1:** Global distribution of leishmaniasis. Hatched area indicate areas where leishmaniasis is endemic. The data presented in the map includes cutaneous, mucocutaneous, and visceral disease. The dots represent locations where several cases of leishmaniasis were recorded between the period of 1991-1993. (Map was taken from the WHO, Eleventh Programme Report on Tropical Disease Research, 1991-1992).



**Figure 2:** Life cycle of *Leishmania* in the mammalian and sandfly hosts. This figure was redrawn from Chang *et al.* (1985).

In the gut of the sandfly, amastigotes are triggered to transform into promastigotes by a rapid decrease in temperature on being removed from the mammalian host. *In vitro* this process can be mimicked by dropping the incubation temperature of infected macrophage or purified amastigotes from 37°C to 26°C. Under these conditions there is a greater than 90% conversion to promastigotes within 24-48 h. Although the environmental factors and the exact time period influencing parasite transformation in the sandfly are still unclear, Davies *et al.* (1990) have demonstrated the presence of promastigotes as early as 24 hours after sandflies take a blood meal, and by 5-7 days no evidence of intracellular amastigotes is found in the fly gut. Newly converted promastigotes break through the peritrophic membrane and attach via the flagella to the microvilli of the epithelial cells of the midgut where they proliferate and undergo morphological changes (Killick-Kendrick *et al.*, 1974; Warburg *et al.*, 1989) developing into the highly infectious metacyclic promastigotes. *L. major* promastigotes taken from the gut of a sandfly two days after a blood meal are relatively non-infective, however, by day 3-5 parasites detach from the midgut and move forward to the mouth parts of the insect where cell division ceases (Bates, 1994). These metacyclic promastigotes, unlike the midgut promastigotes, are extremely infectious (Sacks & Perkins, 1984) and have a short and slender shape with a long flagellum and exhibit a high degree of motility. *L. major* promastigotes cultured axenically also undergo a process of metacyclogenesis with log phase promastigotes exhibiting a much lower infectivity when compared to stationary phase parasites. Differentiation and migration of *Leishmania* parasites from the gut of the fly to the pharynx, from which highly infectious promastigotes are isolated, requires at least 6-8 days (Davies *et al.*, 1990). Transmission of *Leishmania* by an infected sandfly requires inoculation of a new host when the skin is pierced in taking a subsequent blood meal.

The second part of the *Leishmania* life cycle is centered upon evasion of the host immune system while gaining entry into macrophage. Internalization of parasites is a receptor mediated process, and several ligands have been characterized on the promastigote and specific receptors on the macrophage surface. As will be discussed below, the heparin receptor and the C3 complement receptors have been implicated in cell to cell spread of amastigotes. Via this receptor mediated process, promastigotes are

internalized into a phagosomal vacuole which subsequently fuses with a secondary lysosome to form a phagolysosome. The rapid increase in environmental temperature, on going from the sandfly to the mammalian host, together with the decrease in pH of the phagolysosome provides the necessary signals needed to trigger the conversion of the promastigotes into amastigotes. This conversion from the sandfly form of the parasite to the obligate intracellular parasite form proceeds by a rounding up of the cell, progressive loss of the flagella, and remodelling of the cell surface molecules such as LPG, gp63 and oligosaccharide structures on membrane proteins. In the phagolysosome the amastigotes multiply, and the infection is promulgated by macrophage rupture and release of amastigotes into the surrounding interstitial fluid, allowing infection of new professional phagocytes.

#### **iv) Immunology of Leishmaniasis:**

##### **Innate Immune Responses to *Leishmania*:**

For many pathogenic organisms the first line of defence encountered in the mammalian host is the cascade of lytic serum proteins of the classical and alternative complement pathways. Depending on the *Leishmania* spp. and source of the serum used, both complement pathways have been demonstrated to have lethal effects on promastigotes. Effective lysis of *L. enrietti*, *L. tropica*, or *L. major* with fresh normal serum or serum deficient in the C2 or C4 component of the complement cascade strongly suggests that the primary mechanism involved in killing *Leishmania* promastigotes proceeds via the activation of the alternative complement pathway (Mosser & Edelson, 1984; Puentes *et al.*, 1988; Franke *et al.*, 1985). In contrast, Pearson and Steigbigel (1980) have implicated the classical pathway showing that human serum depleted of either C2 or antibodies had no lethal effects when added to *Leishmania donovani* promastigotes. However, eukaryotic organisms such as *Leishmania* have evolved mechanisms not only to evade being killed by the host, but to enhance the rate of promastigote internalization into phagocytic cells. *In vitro* experiments have indicated that sensitivity to complement is

directly related to the culture stage of the parasite (Franke *et al.*, 1985). Incubation of log phase *L. major* promastigotes with human serum resulted in complete cell lysis with serum concentrations as low as 3%. In contrast, metacyclic promastigotes purified from pH 7.0 stationary cultures, required at least 8% for an LD<sub>50</sub> and 25% serum for complete killing (Puentes *et al.*, 1988). Similar experiments carried out with *L. mexicana* metacyclic promastigotes cultured in pH 5.5 media, showed an even more striking degree of resistance to lysis by serum factors, with more than 80% parasite survival persisting with serum concentrations as high as 50% (Bates & Tetley, 1993). Likewise, *L. donovani* amastigotes exhibit no detrimental effects when treated with fresh serum (Pearson & Steigbigel, 1980).

Parasite resistance to complement has been correlated with an increase in the number of LPG repeat units (Ilg *et al.*, 1992; McConville *et al.*, 1992). The increased thickness of this surface coat forms a physical barrier which protects the plasma membrane from the complement components C5-9 (Puentes *et al.*, 1989 & 1990) in a process analogous to that described for LPS protection of *E. coli* from complement (Joiner *et al.*, 1986).

#### **The Role of B Lymphocytes in Leishmaniasis:**

Evidence acquired from studies attempting to dissect the immune responses associated with *Leishmania* infections, clearly suggest that the humoral arm of the immune system exerts little influence in either controlling parasite proliferation in the early stages of infection or subsequent elimination of parasites from the mammalian host. In visceral leishmaniasis one of the classical symptoms found in patients in the advanced stages of the disease is a gross enlargement of the spleen as well as a dramatic increase in the serum gamma globulin levels (Manson-Bahr, 1971; Zuckerman, 1975). Using the Syrian golden hamster, an animal model which closely mimics the disease symptoms observed for kala-azar in humans (Veress *et al.*, 1977), Campos-Netos *et al.* (1982) investigated the B cell responses associated with visceral leishmaniasis. As with humans, hamsters infected with *L. donovani* exhibited a dramatic 20 fold increase in the splenic plasma cell population by day 35 post-infection. This polyclonal expansion of B lymphocytes

paralleled the accumulation of large numbers of parasitized histocytes (Veress *et al.*, 1977) accounting not only for the enlargement of the spleen but also for the three fold increase in levels of circulating antibodies (Weintraub *et al.*, 1982). Although these authors suggested that this non-specific event was unique to *L. donovani*, as hamsters infected with either *L. mexicana amazonensis* or *L. braziliensis braziliensis* showed no alterations in immunoglobulin levels. It appears that this phenomenon may be a common feature of parasitic infections, as polyclonal hypergammaglobulinemia has been observed for *L. tropica* (Weintraub *et al.*, 1982), African trypanosomes (Kobayakawa *et al.*, 1979), malaria (Freeman & Parish, 1978), and *Trypanosoma cruzi* (Ortiz-ortiz *et al.*, 1980) infections.

An indication that B lymphocytes can alter the immune response to leishmaniasis was obtained in mice depleted of B cells by treatment with anti-IgM antisera. Normal BALB/c mice infected with either *L. tropica* or *L. mexicana* promastigotes developed non-healing skin lesions which eventually visceralize and become fatal (Alexander & Phillips, 1980; Howard *et al.*, 1984). However, Sacks *et al.* (1986) found that BALB/c mice depleted of B lymphocytes exhibited a cure phenotype in response to cutaneous leishmaniasis. Susceptibility to leishmaniasis correlated with an early development of a Th2 immune response in normal BALB/c mice (Locksley *et al.*, 1987; Scott *et al.*, 1989). However, in B cell deficient BALB/c mice the data suggests that the resulting immunity to *Leishmania* is predisposed to development of Th1 T helper cells. This is in agreement with the work of Gajewski *et al.* (1991) which suggests that B cells preferentially present antigen to Th2, while adherent cells such as macrophage, dendritic, and Kupffer cells present antigen primarily to Th1 cells.

#### **Interactions between Macrophage and *Leishmania* Parasites:**

Regardless of the *Leishmania* spp., the course of events following inoculation of the mammalian host by an infected sandfly can be divided into four stages: a) attachment and internalization of promastigotes by macrophage, b) reduction of the parasite inoculum by macrophage killing of promastigotes, c) proliferation and dissemination of intracellular *Leishmania* parasites, d) clearance of intracellular parasites by macrophage activated by T cell derived cytokines.

**a) Attachment and Internalization:**

The preponderance of evidence now suggests that successful parasitization of macrophage by *Leishmania* is a receptor mediated event involving attachment of promastigotes, usually via the flagellum, followed by engulfment of the bound parasite in an energy dependent process into a parasitophorous vacuole (Alexander, 1975; Alexander & Vickerman, 1975; Blackwell & Alexander, 1981). This bimodal method of ingestion can be distinguished from non-specific phagocytosis by treating macrophage with cytochalasin B or by allowing host-parasite interactions to proceed at 4°C. Under these conditions *L. mexicana* or *L. donovani* promastigotes readily attach to macrophage but are not internalized (Alexander, 1975; Chang, 1981; Pearson, 1981). The involvement of specific promastigote molecules in the attachment process was demonstrated by the ability of crude parasite extracts to saturate the promastigotes binding sites on macrophage (Benoliel *et al.*, 1980). Similar inhibitory effects were also obtained with antisera to promastigote cell surface molecules. In particular, Russell and Wilhelm (1986) blocked ingestion of *L. mexicana* promastigotes by J774 macrophage by up to 70% using antibodies to gp63. Gp63 itself was also demonstrated to be an important ligand for entry of promastigotes into human macrophage, as the purified protein blocked attachment as effectively as the antibodies (Chang & Chang, 1986; Wilson & Hardin, 1988). The importance of gp63 as a mechanism of entry into macrophage was underscored by the increase of gp63 on *L. donovani* and *L. braziliensis* metacyclic promastigote (Kweider *et al.*, 1987; Wilson *et al.*, 1989).

As with gp63, the other major surface molecule of *Leishmania*, the lipophosphoglycan (LPG), has been shown to be an important ligand for promastigote attachment and subsequent ingestion by macrophage. The importance of this molecule in the invasion process was demonstrated by an 80% decrease in promastigote attachment when macrophage were treated with purified LPG (Handman & Goding, 1985). Although these two major surface molecules which play critical roles in facilitating uptake of promastigotes by macrophage, it is clear that the limited expression of these molecules on the amastigote form of the parasite argues for other molecules on amastigotes.

Although numerous macrophage receptors have been implicated in the engulfment

of foreign components (Gordon *et al.*, 1988), only a few are apparently utilized by *Leishmania* to gain entry. On the basis of ligand specificity these receptors can be categorized into two groups: a) lectin-like molecules which include the mannosyl-fucosyl (MFR), the advanced glycosylation end product (AGER), and the heparin receptors and b) the integrin family which contains the fibronectin, CR3, and CR1 receptor. Early experiments by Chang (1981) showed that macrophage infection could be blocked by deglycosylation of membrane glycoproteins or competitively inhibited with monosaccharides, namely, mannose, glucose, and fucose. The sensitivity to mannose suggested that promastigote entry into macrophage may be facilitated by the MFR. This was verified by down modulation of the MFR on mannan coated slides resulting in a 60-65% decrease in *L. donovani* promastigote binding (Channon *et al.*, 1984; Wilson & Pearson, 1985). The utilization of the MFR for macrophage invasion was perplexing as entry by this receptor induces a vigorous respiratory burst (Berton & Gordon, 1983; Channon *et al.*, 1984). However, the relevance of this cannot be dismissed as immature macrophage have impaired leishmanicidal activity (Gorczynski & McRae, 1982; Hoover & Nacy, 1984). The importance of the MFR was further emphasized by the recent elucidation of the oligosaccharides on gp63, which are all high mannose structures with the potential to bind the MFR (Olafson *et al.*, 1990).

A second lectin-like receptor, is the AGER, implicated in clearing molecules or cells which are non-enzymatically modified by reaction with glucose at the epsilon amino group of lysine (Vlassara *et al.*, 1981). The involvement of this receptor in leishmaniasis was inferred from the competitive inhibition of promastigote-macrophage interactions by glucose (Chang, 1981). Using glucosylated bovine serum albumin, Mosser *et al.* (1987) observed a dose dependent inhibition of *L. major* promastigote binding which levelled off at 65%. However, this group found that by treating macrophage with glucosylated-BSA and antibodies to the CR3 receptor, promastigote binding was decreased by 90%. These data illustrate that *Leishmania* utilize several mechanisms for gaining entry into macrophage. The AGE receptor has gained greater biological significance as a virulence factor by the finding that the G11 oligosaccharide structure on *L. mexicana* gp63 contains a terminal glucose residue (Olafson *et al.*, 1990).

Heparin receptors have recently been identified on *Leishmania* promastigotes and amastigotes (Butcher *et al.*, 1990; Butcher *et al.*, 1992; Love *et al.*, 1993). Unlike the MFR and AGER, the heparin receptors mediate infection of macrophage by binding glycosaminoglycans deposited on the surface of macrophage. For amastigotes this receptor may represent a dominant mechanism for invasion of macrophage (Love *et al.*, 1993). It is interesting to note that the binding of heparin to *T. cruzi* has been previously shown to facilitate the internalization of these parasites into mammalian cells (Ortega-Barria & Periera, 1991).

Fibronectin receptors have also been implicated in the phagocytosis of *Leishmania* promastigotes, by the inhibition of the promastigote-macrophage interaction with antisera raised against fibronectin. Moreover, antibodies specific to the tetrapeptide RGDS, the fibronectin binding site, immunoprecipitated a dominant 63 kDa iodinated promastigote protein (Rizvi *et al.*, 1988). These findings were initially difficult to rationalize, as the gp63 primary sequence lacked an RGDS motif. However, Soteriadou *et al.* (1992) have shown that the tetrapeptide SRYD, present in the gp63, can cross react with the RGDS antisera, explaining the above results. But more importantly, the biological relevance of this motif was demonstrated by the ability of the octapeptide IASRYDQYL to block *L. major* infection of murine macrophage by up to 70%, at micromolar concentrations (Soteriadou *et al.* 1992), indicating that the gp63 on the parasite surface may bind the fibronectin receptor. Since amastigotes generally have down regulated levels of gp63 and LPG, the fibronectin receptor may provide an additional mechanism for binding to phagocytes as cells released into the extracellular environment are rapidly coated with fibronectin (Ouaissi *et al.*, 1984).

Good evidence now exists that the C3 complement receptors are the major receptors utilized by promastigotes for uptake by macrophage. *Leishmania* have evolved to utilize these receptors in the presence or absence of complement. In the presence of complement the efficiency of macrophage is much greater since promastigotes actively bind complement C3 and proteolytically activate it on the surface of the promastigote either to C3b, which is the ligand for the CR1 receptor, or to C3bi which is bound by the CR3 receptor. The acceptor molecules which have been characterized on the surface of

the promastigotes include gp63 and LPG. The type of activated C3 and receptor utilized vary with the *Leishmania* species. For *L. donovani* promastigotes incubated with fresh human serum, gp63 is the acceptor molecule which facilitates deposition of C3, primarily as C3bi which is covalent linked to gp63 (Puentes *et al.*, 1989). *L. major* and *L. mexicana* promastigotes both deposit C3 in the form of C3b which utilizes the CR1 receptor for internalization into macrophage. However, these two parasites use different acceptors for the C3 complement component, which is gp63 in the case of *L. mexicana* and LPG for *L. major* promastigotes (Puentes *et al.*, 1988; Russell, 1987). *L. major* exhibit a requirement for complement opsinization which is dependent on the culture stage. Metacyclic promastigotes deposit C3 on their surface primarily as C3b, and thus are taken up by macrophage via the CR1, whereas, log phase promastigotes deposit both C3bi and C3b and utilize the CR1 and CR3 receptors (Da Silva *et al.*, 1989). Similarly, *L. major* amastigotes also deposit both C3b and C3bi and utilize the latter receptors (Mosser *et al.*, 1985). It is important to note that internalization of promastigotes via CR1 or CR3 promotes survival within the macrophage as these receptors do not stimulate a respiratory burst (Da Silva *et al.*, 1989; Wright & Silverstein, 1983).

In the absence of serum factors, only the CR3 receptors appear to mediate the invasion of promastigotes into macrophage (Blackwell *et al.*, 1985). This receptor has been shown to consist of a complex which contains two binding domains, one site is specific for the Arg-Gly-Asp sequence characteristic of the integrin attachment site, while the other binding site is a lectin site and which binds lipopolysaccharide (Wright *et al.*, 1983 & 1989). Using reversed phase beads coated with gp63 the major receptor involved was shown to be CR3. The binding of these particles was eliminated by down modulating this receptor or by competition with synthetic peptide containing an RGD motif (Russell & Wright, 1988). However it was subsequently shown that gp63 does not contain an RGD but rather an RYD sequence which mimics RGD (Soteriadou *et al.*, 1992). That gp63 binds to the RGD site on CR3 was demonstrated by blocking the binding of gp63 coated beads with antibodies to the CR3 receptor (Russell, 1987; Russell & Wright, 1988; Talamas-Rohana *et al.*, 1990). In addition to gp63, CR3 also binds to LPG at a site which was shown to be distinct from the RGD binding site and identical with the LPS binding

site. Soluble delipidated LPG could block the binding of *E. coli* LPS to the CR3 and the p150,95 receptors, which have an alpha chain that shares significant homology with CR3 (Talamas-Rohana & Russell, 1990). Experiments with reversed phase beads coated with *Leishmania* gp63 and LPG also showed that internalization of promastigotes by macrophage requires at least two types of receptors to be triggered, as beads coated with either LPG or gp63 bound to macrophage but were not internalized. On the other hand particles containing both LPG and gp63 were rapidly bound and internalized (Russell & Talamas-Rohana, 1989). These results indicate that at least two receptors are required for parasite uptake. Similar observations have been reported by Wright and Silverstein (1983) showing that promastigote interaction with the CR1 receptor alone was not adequate for entry into macrophage.

**b) T-cell Independent Macrophage Killing of Intracellular Promastigotes:**

It has been estimated that the bulk of the promastigotes injected by the sandfly into the endodermis are rapidly killed by macrophage and polymorphoneutrophils. *In vitro* infection of murine macrophage with *L. donovani* promastigotes triggers a respiratory burst which kills 80-90% of the intracellular promastigotes within several hours after fusion of the phagosome with secondary lysosomes (Alexander, 1975; Chang, 1981; Murray, 1981). The involvement of the respiratory burst in the clearance of *Leishmania* promastigotes was demonstrated in macrophage cell lines with a defect in the molecular systems required for the generation of reactive oxygen intermediates (Pearson *et al.*, 1982). In these cells promastigotes were internalized and transformed into amastigotes without killing (Murray, 1981).

*In vitro* experiments with *L. donovani* promastigotes shows that these cells are highly susceptible to  $H_2O_2$  and are readily killed by peroxide added to promastigote cultures. On the other hand the amastigote form is much more resistant to peroxide which may be of significance for survival in the hostile environment of the phagolysosome (Murray, 1982). The peroxide resistance of the amastigotes has been attributed to the higher levels of catalase and superoxide dismutase, both of which are enzymes that degrade the reactive oxygen intermediates of the respiratory burst (Murray, 1982).

The ability of resting monocytes to kill intracellular parasites appears to be dependent on the origin of the macrophage. Pearson *et al.* (1982) found that macrophages derived from human monocytes were infected with a frequency of about 54%, with each phagocyte containing approximately 2 *Leishmania* parasites per cell. As with the murine macrophage, parasitized human macrophage triggered an oxidative response which failed to protect against promastigote infections (Pearson *et al.*, 1982). The failure of the human cells to protect against *Leishmania* promastigotes has been attributed to the lower levels of reactive oxygen intermediates produced by the human macrophage, which have been shown to decreased levels of the myeloperoxidase complex required for respiratory oxidative burst (Pearson *et al.*, 1982). The involvement of myeloperoxidase was demonstrated by the ability of human polymorphonuclear leukocytes (PMN) to phagocytize promastigotes, in a complement dependent manner, and rapidly destroy these parasites within 3 hours after ingestion (Pearson & Steigbigel, 1981). Unlike macrophage, parasites taken up by polymorphonuclear leukocytes are readily killed, due to the massive respiratory burst which accompanies the uptake of foreign particles. The efficiency with which the PMNs kill promastigotes is likely associated with the action of myeloperoxidase, an enzyme present at lower levels in macrophage.

**c) *Leishmania* Mechanisms for Intracellular Survival:**

Numerous mechanisms have evolved in *Leishmania* parasites to subvert the hosts immunosurveillance systems or to promote parasite survival within the hostile environment of the phagolysosome. These are proposed to include parasite derived molecules, such as the membrane acid phosphatase and LPG, as well as the induction of lymphokines which result in decreased macrophage killing. The importance of promastigote acid phosphatase as a virulence factor was illustrated by the rapid destruction of avirulent *L. donovani* promastigotes which did not express this enzyme (Katakura & Kobayashi, 1988). Remaly *et al.* (1984) have provided evidence indicating that treatment of PMN with the membrane acid phosphatase results in the inhibition of superoxide anion production thereby decreasing the killing potential of phagocytes. That these same effects were also observed

with the *E. coli* alkaline phosphatase suggests that the phosphatase may be acting by dephosphorylating membrane receptors on the phagocyte.

A second multifunctional molecule which has been demonstrated to be essential for the survival of promastigotes within the macrophage host is LPG. Macrophage infected with either *L. major* or *L. donovani* promastigote mutants unable to synthesize mature LPG are destroyed by macrophage within 16 h of infection (Handman *et al.*, 1986; McNeely & Turco, 1990). However, if the mutant promastigotes were coated with wild type LPG they survived (Handman *et al.*, 1986). Similar effects have also been reported showing that the delipidated form of LPG inhibited the lysis of red blood cells by macrophage (Eilam *et al.*, 1985). The ability of LPG to protect promastigotes from macrophages may be attributed to several factors: a) NMR and molecular modelling studies have suggested that LPG covers 25-60% of the parasite surface which could protect the plasma membrane from hydrolases (Homans *et al.*, 1992; Pimenta *et al.*, 1991); b) the phosphodissacharide repeat (PO<sub>4</sub>-Gal-Man) has recently been found to attenuate the respiratory burst by scavenging the superoxide anion and the hydroxyl radical, which may provide a rationale for the doubling in the repeat structure observed in metacyclic promastigotes (Chan *et al.*, 1989; Ilg *et al.*, 1992; McConville *et al.*, 1990); c) the lipid moiety of LPG has also been implicated as a mechanism for the down regulation of the respiratory burst by inhibiting protein kinase C required in the activation of the pentose phosphate shunt and subsequent generation of NADPH utilized by the myeloperoxidase (McNeely *et al.*, 1989).

*Leishmania*, once transformed into the amastigote form, can combat the reactive oxygen intermediates produced by non-activated macrophage with an increased expression of catalase, superoxide dismutase, and glutathione reductase (Murray, 1982), enzymes which degrade H<sub>2</sub>O<sub>2</sub> and superoxide anion. In humans the ability of amastigotes to survive and multiply has been linked to the reduced respiratory burst response which is insufficient to kill intracellular amastigotes (Berman *et al.*, 1979; Pearson *et al.*, 1983).

The type of phagocytes which sequester the *Leishmania* promastigotes inoculated into the endodermis by the sandfly will determine whether or not a successful infection is established. Locksley *et al.* (1988) have shown that Langerhans cells obtained from the

skin primates are not readily parasitized since they lack functional C3 complement receptors. On the other hand, dermal cells, which are macrophage like and express CR3 receptors, rapidly phagocytize and support promastigote transformation to amastigotes as they are incapable of killing intracellular parasites due to the lack of a respiratory burst.

Studies have shown that IL-3 exacerbates *Leishmania* infections (Feng *et al.*, 1988; Lelchuk *et al.*, 1988). IL-3 has been proposed to mediate these effects by stimulating proliferation and differentiation of hematopoietic progenitor cells and the subsequent attraction of immature macrophage to the site of infection. These immature macrophage promulgate the disease by providing a safe environment for *Leishmania* parasites since these macrophage have been demonstrated to have an impaired ability to generate a respiratory burst required to kill intracellular parasites (Fortier *et al.*, 1982; Mendonça *et al.* 1990).

**d) Cell Mediated Activation of Macrophage for Killing of Intracellular *Leishmania*:**

As indicated above, *Leishmania* promastigotes are well adapted to survive within both murine and human macrophage (Murray, 1982; Pearson *et al.* 1983). Clearance of these intracellular parasites could be facilitated by activation of infected macrophage with soluble factors present in Con A stimulated splenocyte culture supernatants. This increased leishmaniacidal activity was correlated with the stimulation of a potent respiratory burst which could be ablated by the addition of anti-IFN- $\gamma$  antibodies (Belosevic *et al.*, 1988; Murray *et al.*, 1985). These findings implicated IFN- $\gamma$  as a critical factor in regulating macrophage resistance to infection by *Leishmania* amastigotes. Indeed, addition of recombinant IFN- $\gamma$  to infected resident peritoneal macrophage resulted in amastigote destruction within 72 h (Belosevic *et al.*, 1988; Hoover *et al.*, 1985; Nacy *et al.*, 1985).

IFN- $\gamma$  has also been shown to be a key factor in mediating resistance to leishmaniasis *in vivo*. Treatment of resistant mouse strains such as C3H/HeN with anti-IFN- $\gamma$  neutralizing antibodies prior to or at the time of infection inhibited the ability of mice to control the development of cutaneous leishmaniasis. These experiments also indicated that IFN- $\gamma$  has a temporal effect, as administration of anti-IFN- $\gamma$  antibodies one

week post-infection had no effect on the disease outcome (Belosevic *et al.*, 1989). Conversely, susceptible mouse strains such as BALB/c could be converted to a resistant phenotype by administration of recombinant IFN- $\gamma$  (Murray *et al.*, 1987). However, this protective effect of IFN- $\gamma$  was time dependent and required that this lymphokine be injected concomitant with the promastigotes or within 3-4 days after infection (Scharton & Scott, 1993).

Although the findings of several laboratories have indicated that IFN- $\gamma$  mediates its leishmanicidal effects by stimulation of the respiratory burst, Scott *et al.* (1985) found that macrophage cell lines lacking a functional respiratory burst could be activated with lymphokines to kill intracellular amastigotes. An alternative mechanism by which murine macrophage killed intracellular amastigotes involved the production of nitric oxide (NO) in an arginine dependent mechanism which could be inhibited either by depleting culture supernatants of arginine or by the addition of the competitive inhibitor L-N<sup>G</sup>-monomethyl arginine (Green *et al.*, 1990; Liew *et al.*, 1990; Roach *et al.*, 1991). Stimulation of the nitric oxide leishmanicidal activity by macrophage *in vitro* requires at least two signals, the first being IFN- $\gamma$  which synergizes with either LPS, IL-1, or IL-2 to elicit the second signal which is the necrosis factor alpha (TNF- $\alpha$ ) produced by macrophage (Belosevic *et al.*, 1990; Green *et al.*, 1990 & 1990b, Liew *et al.*, 1990; Roach *et al.*, 1991). In addition, the TNF- $\alpha$  can also be replaced by infecting macrophage with either Bacillus Calmette-Guerin (BCG) or *Leishmania* amastigotes (Green *et al.*, 1990 & 1990b; Roach *et al.*, 1991). That nitrogen oxidation in this system involved TNF- $\alpha$  was demonstrated by the inhibition of NO production and the loss of the ability of macrophage to kill intracellular amastigotes when anti-TNF- $\alpha$  antibodies were added to macrophage cultures (Belosevic *et al.*, 1989).

The significance of nitric oxide in controlling cutaneous leishmaniasis in mice has recently been investigated using the nitric oxide synthase inhibitor, L-N<sup>G</sup> monomethyl arginine (LNMMA). CBA mice treated with LNMMA and infected with *L. major* promastigotes were found to develop significantly larger lesions than control animals, but more importantly, treated animals were found to have a parasite burden which was 4 log units higher than the control mice (Liew *et al.*, 1990). These findings indicated that nitric

oxide was the dominant macrophage product involved in controlling the growth of *Leishmania* parasites in mice.

Evidence for the importance of TNF- $\alpha$  in conferring resistance to *Leishmania* infections in mice was demonstrated by Titus *et al.* (1989) who found that high levels of TNF- $\alpha$  production by LNC harvested from infected C3H mice was correlated with the healing of cutaneous lesions. Although BALB/c mice showed no differences in the pattern of disease when treated with anti-TNF- $\alpha$  antibodies, in C3H or CBA mice these antibodies resulted in disease exacerbation (Kossodo *et al.*, 1994; Titus *et al.*, 1989). In humans, tumor necrosis factor does not appear to exhibit ameliorating effects as this lymphokine is present in high levels in the serum of patients with active visceral leishmaniasis (Barral-Netto *et al.*, 1991). This may in part be due to the fact that human macrophage have not been demonstrated to possess the nitric oxide killing mechanism observed in mice.

#### **Cell mediated Immune Responses to Leishmaniasis:**

The involvement of T lymphocytes in protecting susceptible mouse strains against leishmaniasis was initially demonstrated by the transfer of immunoprotection to naive recipients with the splenic T cells populations obtained from recovered donors immunized with sublethally irradiated promastigotes (Alexander & Phillips, 1980; Howard *et al.*; 1981). Immunization of BALB/c mice with crude *Leishmania* antigen was found to have a dual effect, dependent upon the route of immunization. If antigen was injected via an i.v. route, mice developed resistance to a promastigote challenge, whereas administration of antigen s.c. resulted in disease exacerbation (Howard *et al.*, 1982; Liew *et al.*, 1985a; Titus *et al.*, 1984). The phenotype of cells mediating both immune responses was found to be CD4<sup>+</sup> (Liew *et al.*, 1985; Titus *et al.*, 1984). These observations were further complicated by the finding that CBA mice depleted of CD4<sup>+</sup> cells *in vivo*, developed a susceptible phenotype when infected with *L. major* promastigotes (Titus *et al.*, 1987), while BALB/c mice exhibited resistance to cutaneous leishmaniasis (Howard *et al.*, 1981; Titus *et al.*, 1985). This immunological dichotomy was rationalized by the findings of Mosmann *et al.* (1986) showing that CD4<sup>+</sup> cells could be divided into two subsets, Th1

and Th2 according to the secreted cytokine profiles. Th1 cells were characterized by the production of IL-2 and IFN- $\gamma$  but not IL-4 or IL-5. Conversely, Th2 cells secreted IL-4 and IL-5 but not IL-2 or IFN- $\gamma$ . The impact of these T helper cells subsets in leishmaniasis was addressed by Heinzl *et al.* (1989) who demonstrated that draining lymph node cells from resistant mice contained high levels of IFN- $\gamma$  with minor levels of IL-4, whereas susceptible mice contained low levels of IFN- $\gamma$  and high levels of IL-4. The ability of Th1 cells to protect mice against leishmaniasis was further verified by Scott *et al.* (1989), who showed that BALB/c mice immunized by adoptive transfer of Th1 T helper cell clones, specific for an 11 kDa *Leishmania* protein, were protected against *L. major* infection.

Development of Th2 T helper cells which promote the disease process in leishmaniasis is regulated primarily by the lymphokine IL-4. BALB/c mice treated with anti-IL-4 mAbs several days prior to infection with *L. major* promastigotes, to deplete endogenous IL-4, developed only small lesions which healed within 6 weeks of post-infection, indicating that in the absence of high IL-4 levels the BALB/c immune system was predisposed to differentiation to Th1 (Coffman *et al.*, 1991). The above observation has recently been substantiated by experiments showing that *in vitro* priming of lymph node cells in the presence of IL-4 yielded T cells which secreted high levels of IL-4, but not IL-2 or IFN- $\gamma$ , when stimulated with antigen (Seder *et al.*, 1993).

A second soluble factor promoting a Th2 response is IL-10. However, unlike IL-4, this lymphokine acts at the level of the accessory cells, impairing the ability to produce leishmanicidal factors, such as nitric oxide, and antigen presentation to Th1 cells which is required for cytokine release (Fiorentino *et al.*, 1991; Hsieh *et al.*, 1992; Romani *et al.*, 1994).

IFN- $\gamma$  has been implicated as one of the factors regulating the expansion of Th1 T helper cells, since healer mice (C3H/HeN) treated with anti-IFN- $\gamma$  monoclonal antibodies exhibited a marked decrease in their ability to control lesion size and subsequent healing (Belosevic *et al.*, 1990). IFN- $\gamma$  alone was not sufficient to trigger Th1 development in non-healer mice (BALB/c) as administration of this lymphokine with *Leishmania* antigens afforded only minimal protection against cutaneous leishmaniasis. However, protection could be achieved by including the adjuvant *C. parvum*, suggesting that additional factors

provided signals favouring a Th1 immune response (Scott, 1991). One such factor is interleukin-12, and indeed immunization of BALB/c mice with *Leishmania* antigen plus IL-12 induced CD4<sup>+</sup> lymphocytes which secreted high levels of IFN- $\gamma$  but only minor amounts of IL-4 when stimulated in vitro (Afonso *et al.*, 1994). IL-12 has been proposed to activate the immune response by stimulating IFN- $\gamma$  production by natural killer (NK) cells within 3-4 days after infections.

The requirement for NK cells in conferring host resistance to *Leishmania* was illustrated by the finding that C3H/HeN mice depleted of NK cells, with antibodies specific for the asialoGM1 ganglioside, developed larger lesions which contained significantly higher parasite burdens. However, elimination of the NK T cell population did not appear to diminish the ability of mice to resolve the cutaneous lesions (Scharton & Scott, 1993). An apparent correlation between the NK cells populations and murine resistance to cutaneous leishmaniasis has recently been observed. In these studies, BALB/c mice exhibited the lowest NK cytotoxicity activity and the highest degree of susceptibility to *L. major* infection, while C3H/HeN mice had the highest NK cytotoxicity activity and were also the most resistant to cutaneous disease (Scharton & Scott, 1993).

Based on the data from laboratories of Scharton & Scott (1993) and Hsieh *et al.* (1993), a preliminary model for the development of a Th1 T helper cell response to leishmaniasis has recently emerged. These researchers propose that infection of resistant mouse strains with promastigotes causing cutaneous leishmaniasis stimulates IL-12 secretion by macrophages within 2-3 days after inoculation, which in turn triggers NK cells to release high levels of IFN- $\gamma$ , thereby predisposing the differentiation of Th0 cells into Th1 (Gajewski, *et al.*, 1989, Scharton & Scott, 1993). Although macrophage production of IL-12 could be induced by *Listeria* (Hsieh *et al.*, 1993), infection of macrophage with either log phase or metacyclic promastigotes failed to elicit IL-12, which would tend to compromise the above model (Reiner *et al.*, 1994). However, IL-13 production, which synergizes with IL-2 to induce production of IFN- $\gamma$  by NK cells, was detected in macrophage parasitized by *L. major* promastigotes (Reiner *et al.*, 1994). It is interesting to note however, that Reiner *et al.* (1994) found that once transformed into amastigotes, the *L. major* parasites were capable of eliciting IL-12 from macrophage. Although head

way has been made in understanding the signals involved in T cell activation, an area of leishmaniasis which is critical to the development of either protective or exacerbative immunity, the interactions between the macrophage and *Leishmania* parasites, is still not well understood.

In leishmaniasis CD8<sup>+</sup> cells are generally believed to play only a minor role in the resolution of cutaneous disease. However, Hill *et al.* (1989) have implicated these cytotoxic cells as the active cells involved in the resolution of the cutaneous disease in BALB/c mice depleted of CD4<sup>+</sup> cells. Activation of *Leishmania* specific CD8<sup>+</sup> cells with synthetic peptide derived from *L. major* gp63 failed to protect BALB/c mice against progressive disease. Similar experiments with  $\beta$ -2 microglobulin deficient mice which do not have functional CD8<sup>+</sup> cells showed no differences in disease kinetics when compared to mice with functional cytotoxic activity (Wang *et al.*, 1993). These experiments illustrate that in general the CD8<sup>+</sup> cells participate only in a minor role in the elimination of *Leishmania* parasites. This response is most evident when the CD4<sup>+</sup> cell are eliminated. Recent evidence obtained in both humans and mice indicate that CD8<sup>+</sup> cells have a more prominent role in conferring resistance to secondary infections. Muller *et al.* (1994) have shown that re-infection of mice recovered from cutaneous leishmaniasis resulted in a 50-fold increase in CD8<sup>+</sup> T cell population in both the spleen and lymph nodes, moreover this response was accompanied with a production of high levels of IFN- $\gamma$  which activate macrophage to rapidly kill intracellular parasites. Similar results have also been obtained in humans. By monitoring the percentage of T cells according to CD4<sup>+</sup> or CD8<sup>+</sup> phenotypes in American cutaneous leishmaniasis patients, Da-Cruz *et al.* (1994) have shown that disease convalescence, arising spontaneously or by drug intervention resulted in a substantial increase in both the percentage of CD8<sup>+</sup> cells and IFN- $\gamma$  levels when peripheral blood monocytes were stimulated *in vitro* with *Leishmania* antigens. Surprisingly, this group also found a decrease in the *Leishmania* specific CD4<sup>+</sup> cells. In brief, the immunological data amassing for leishmaniasis strongly suggests that CD4<sup>+</sup> cells, in particular, Th1 T helper cell are critical for resolution of primary infections, while CD8<sup>+</sup> cells are necessary for protection against re-infection.

### **Thesis Objectives**

- a) To identify putative T cell epitopes from the *L. major* gp63 primary sequence using either the Rothbard and Taylor or the Garnier secondary structure predictive algorithms, and to test selected synthetic T cell epitopes as immunoprotective agents against murine cutaneous leishmaniasis.
- b) To characterize the immunological responses to the *L. donovani* lipophosphoglycan and to identify the nature of the epitope inducing the proliferative and immunoprotective effects reported for this glycolipid.
- c) To purify and characterize the lipophosphoglycan associated protein co-isolating with the *L. donovani* lipophosphoglycan.

## METHODS AND MATERIALS

### Peptide Synthesis:

Peptides were synthesized on an Applied Biosystems 430A automated peptide synthesizer with Fmoc protected amino acids (Novabiochem, La Jolla, CA). HPLC grade dichloromethane, dimethylformamide, and N-methyl pyrrolidone, were obtained from Burdick & Jackson (Muskegon, MI). MBHA resins, piperidine, dicyclohexylcarbodiimide, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate, and trifluoroacetic acid were purchased from Applied Biosystems Inc. (Foster city, CA). Trifluoromethanesulfonic acid (TFMSA) was obtained from Sigma Chemical Co. (St. Louis, MO). Gp63 and LPGAP peptides corresponding to potential T cell epitopes were synthesized on MBHA resin to give a final product with a C-terminal amide. Amino acids were dissolved in dichloromethane, added to the reaction vessel of an Applied Biosystems 430A automated peptide synthesizer and activated *in situ* with HBTU according the protocol of the instrument manufacturer. Peptides were simultaneously removed from the solid support and deprotected with anhydrous TFMSA (Tam *et al.*, 1986) and recovered from the cleavage mixture by diethyl ether precipitation followed by filtration using a sintered glass funnel. Peptides were dissolved in 8 M urea and purified by reversed phase chromatography on an Applied Biosystems prep-10 C<sub>18</sub> column (10 x 250 mm) equilibrated with 0.1% TFA and developed with a 0.5% linear gradient of acetonitrile at a flow rate of 5 ml/min. Two millilitre fractions were collected and assessed by chromatography on an Aquapore RP-300 C<sub>8</sub> column (2.5 x 100 mm) using the above mobile phases. Homogeneous fractions were pooled, lyophilized, and the peptide compositions confirmed by amino acid analysis.

### Animals:

BALB/c mice were obtained from the University of Victoria Animal care facility while A/J, C57BL/6, and CBA/Ca strains were purchased either from Jackson Laboratories (Bar

Harbor, ME) or Charles River Breeding Laboratories (St. Constant, Quebec). Animals in these experiments were all 8-10 week old female mice .

#### **Culture Media and Buffers:**

RPMI 1640 tissue culture medium , for T-cell proliferation assays, was (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT),  $5 \times 10^{-5}$  M tissue culture grade  $\beta$ -mercaptoethanol, and 50  $\mu$ g/ml gentamycin (both from Sigma) and was designated complete RPMI 1640. This medium was used for all lymphocyte tissue culture procedures. *Leishmania* promastigotes were grown at 26°C in M199 media supplemented with 1000 U/ml penicillin, 50  $\mu$ g/ml streptomycin, 5 mg/l hemin (all from Sigma Chemical Co.), Eagle's basal medium vitamin solution (Gibco, Grand Island, NY), and 5% heat inactivated fetal bovine serum (Hyclone) The 20X SSC buffer used for DNA hybridization assays, consisted of 3 M NaCl and 300 mM sodium citrate pH 7.0. DNA samples were dissolved in TE buffering containing, 10 mM Tris HCl, pH 7.4, 1 mM EDTA. Phenol:chloroform was prepared by equilibrating equal volumes of liquid phenol (90%), (Fisher Scientific, Ottawa, Ont.) and chloroform (Burdick-Jackson, Muskegon, MI) three times with 100 mM Tris HCl pH 7.0, to extract trace carboxylic acid contaminants from the phenol. The phenol:chloroform was stored at 4°C in a brown bottle in the presence of 1% 8-hydroxyquinoline (Sigma). A stock Denhardt's solution (100X) was prepared by dissolving 2 g Ficoll type 400, 2 g polyvinylpyrrolidone, and 2 g of bovine serum albumin in 100 ml of H<sub>2</sub>O (all from Sigma Chemical Co.).

#### **T Lymphocyte Proliferation Assays:**

Antigens (100  $\mu$ g) were emulsified in a mixture of PBS and Complete Freund's adjuvant (100  $\mu$ l 1:1, PBS:CFA) (Gibco, Grand Island, NY), and 50  $\mu$ l was injected at the base of the tail and the nape of the neck. Draining lymph nodes (inguinal, axillary, and periaortic) and spleen were removed 7-9 days post-immunization and single cell suspensions were prepared by macerating the organs with forceps in RPMI 1640 medium. Connective tissue debris was removed by drawing the cell suspension gently through a 26 gauge needle. Lymph node cells and splenocytes were harvested by centrifugation at 500 x g for 10

minutes, and washed once with complete RPMI 1640. Viable cells were enumerated with a hemocytometer (American Optical Corp., Buffalo NY) using 1% Trypan blue in PBS (Sigma) and the cell density was adjusted to  $5 \times 10^6$  /ml in complete RPMI 1640. Lymphocytes ( $5 \times 10^5$  / well) were stimulated in triplicate in 96 well microculture plates in a final volume of 200  $\mu$ l using either synthetic peptides over a concentration range of 3.1 nM to 150  $\mu$ M or 7-250  $\mu$ g/ml LPG/LPGAP. Stimulated cultures were incubated for 76 hours at 37°C in a 95% humidity, 5% CO<sub>2</sub> atmosphere and pulsed for an additional 20 hours with 1  $\mu$ Ci/ well of [<sup>3</sup>H]thymidine (Dupont Canada Inc., Mississauga, Ont.) in 50  $\mu$ l of complete RPMI 1640 medium. Cells were harvested onto glass fiber filters and the radiolabel incorporated into the DNA was determined by liquid scintillation counting using Dupont 963 scintillation cocktail (Dupont Canada Inc.). Proliferation results were expressed as cpm  $\pm$  1 standard deviation.

#### **Complement Depletion of T Lymphocytes:**

Lymph node cell suspensions were adjusted to  $1 \times 10^7$  cells/ml in complete RPMI 1640 medium and incubated with either rabbit anti-mouse Thy-1 antisera (1:40 dilution) (CL2001), rat anti-mouse L3/T4 mAb (1:500 dilution) (CL012A), or rat anti-mouse Lyt-2.1 mAb (1:1000 dilution) (CL8921A) for 60 minutes at 4°C. Unbound antibody was removed by centrifugation and the cells were re-suspended to  $1 \times 10^7$ /ml with 10% Low-Tox rabbit complement (CL3051) in medium and incubated for 1 hour at 37°C. Lymphocytes were pelleted at 500 x g, washed once with medium and cultured in the presence or absence of antigen. All reagents were purchased from Cedarlane (Hornby, Ont.).

#### **Quantitation of Interleukins 2 and 4 in Culture Supernatants:**

Lymph node cells ( $1 \times 10^6$ ) harvested from mice immunized with either peptides or LPG/LPGAP were cultured in 1 ml of complete RPMI 1640 medium in 24 well microculture plates at 37°C, in 5% CO<sub>2</sub> atmosphere. Lymphocytes were stimulated *in vitro* with either synthetic peptides or LPG/LPGAP at a concentration of 100  $\mu$ g/ml and culture supernatants, harvested 48 h after stimulation, were filtered through a 0.2 micron filter and stored at 20°C. Interleukins 2 and 4 (IL-2 and IL-4) were determined in culture

supernatants using a bioassay based on the IL-2 and IL-4 dependent CTLL2 cell line (Gillis *et al.*, 1978) from the American Type Culture Collection. CTLL2 cultures were expanded for 3 days in medium supplemented with 10% rat splenocyte concanavalin A supernatant (Taylor *et al.*, 1987). Prior to use in the assay, cells were washed four times with complete RPMI 1640 medium, and  $1 \times 10^4$  cells were dispensed into each flat bottom well in 140  $\mu$ l of medium. Lymphokine levels in lymph node cell conditioned culture supernatants were determined by adding 60  $\mu$ l of this supernatant to the CTLL2 culture and incubating for 20 h. Cells were pulsed with 1  $\mu$ Ci of [ $^3$ H] thymidine for an additional 4 h and harvested on glass fiber filters prior to liquid scintillation counting. Each supernatant was assayed in quadruplicate and the concentration of interleukin extrapolated from a standard curve generated by culturing the CTLL2 cells with either recombinant murine IL-2 (mIL-2) or recombinant murine IL-4 (mIL-4) (Genzyme Corporation, Boston, MA) over a concentration range of 10 U/ml to 0.31 U/ml for both lymphokines. To determine the portion of CTLL2 cell proliferation stimulated specifically by IL-2 in culture supernatants, cells were co-cultured with the anti-IL-4 mAb 11B11 (1  $\mu$ g/ml) (Chara, 1985) to neutralize this lymphokine. Conversely, the CTLL2 proliferation due to IL-4 was assessed by neutralizing the effect of IL-2 in the culture supernatants with the mAb S4B6 (1  $\mu$ g/ml) (Zurawski *et al.*, 1986). Alternatively, the levels of IL-2 or IL-4 secreted by primed lymph node cells following *in vitro* stimulation, was determined using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (MTT) according to the method of Mosmann (1983). This procedure entailed culturing CTLL2 cells for 20 h, with the conditioned culture medium, then measuring the cell proliferation by adding MTT to each well to a final concentration of 1 mg/ml and incubating for an additional 6 h. The formazan crystals, formed as a result of reduction of MTT by metabolically active cells, were solubilized overnight in the dark at 37°C with 1% SDS and the plates read on a Dynatech microplate (Chantilly, VA) reader at 595 nm.

#### **Quantitation of Interleukin-3 in Culture Supernatants:**

Peptide primed lymph node cells were incubated at a density of  $1 \times 10^6$  /ml in complete RPMI 1640 medium in 24 well microculture plates. Lymphocytes were stimulated with

either PT3, PT4, or PT6 at a concentration of 100 µg/ml and after 48 h the culture supernatants were removed and filtered through a 0.2 µm filter. Interleukin 3 secreted by primed lymph node cells stimulated with synthetic T cell epitopes was measured in a bioassay with the IL-3 dependent DA-1 cell line obtained from Dr. J. Schrader, Biomedical Research Center, UBC, Vancouver. The DA-1 cell line was expanded in complete RPMI 1640 medium containing 1% recombinant IL-3 prior to use in the assay (Genzyme) for three days. To quantitate the IL-3 secreted by the lymph node cell culture, the DA-1 cells were washed 4 times and  $1 \times 10^4$  cells were dispensed into 140 µl of media in a 96 well microculture plate. Quadruplet wells received 60 µl of the conditioned culture supernatant and were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 20 h. Cells were pulsed with 1 µCi of [<sup>3</sup>H] thymidine, incubated for an additional 4 h prior to harvesting onto glass fiber filters, and counted in a Beckman LS 8100 liquid scintillation counter. Levels of IL-3 in the culture supernatants were extrapolated from a standard curve generated with recombinant IL-3 over a concentration range of 0.31-10 U/ml.

#### **Monoclonal Antibodies:**

Monoclonal antibodies were a generous gift from Dr. T.W. Pearson (University of Victoria). The mAb CA7AE (IgM) has been previously characterized and shown to be specific for the Galβ1-4 Man PO<sub>4</sub> repeat disaccharide present on the *L. donovani* LPG and secreted acid phosphatase (Tolson *et al.*, 1989 & Tolson, 1992). As will be discussed below, mAbs L98 (IgG<sub>1</sub>) and L157 (IgG<sub>1</sub>) were originally thought to bind to the LPG core (Tolson *et al.*, 1989) but were subsequently shown to be specific for the lipophosphoglycan associated protein (LPGAP). MAb HT-1 (Pearson, T.W., unpublished), which is specific for human transferrin, was used as a negative control for ELISA and immunoblot assays.

#### **Culturing of *Leishmania* Promastigotes:**

*Leishmania donovani* LD3 promastigotes, obtained from Dr. S. Turco, (University of Kentucky) were derived from the 1S2D clone (Dwyer, 1977) by needle passage in hamsters. *L. major* A2 (Neva *et al.*, 1979) promastigotes were from a clone provided by

Dr. T. W. Pearson, (University of Victoria). *L. mexicana amazonensis* M2269 (WHO reference strain MHOM/BR/73/M2269), *L. tropica* K27.3 (WHO reference strain MHOM/SU/74/K27), *L. major* LV39 (WHO reference strain MRHO/SU/59/P), and *L. major* (WHO reference MHOM/SU/73/5-ASKH) were obtained from the American Type Culture Collection (Rockville, MD). *Leishmania* promastigotes were grown to late log-stationary phase in M199 media (Gibco, Grand Island NY) and harvested by centrifugation at 2000 x g, washed twice with PBS and were used immediately to infect mice or were stored as pellets at -20°C, for purification of LPG/LPGAP.

**Isolation of *Leishmania donovani* Amastigotes:**

*Leishmania donovani* LV9 amastigotes were obtained from Dr. J. Blackwell, (Cambridge University, Cambridge, England) and maintained in Syrian golden hamsters by interperitoneal needle passage of  $2 \times 10^8$  amastigotes approximately every two months. Amastigotes were isolated according to the method of Channon *et al.* (1984). Briefly, infected hamster spleens were macerated in 10 ml of RPMI 1640, 2 mM EDTA, and the connective tissue debris was removed by gently drawing the single cell suspension through a 26 gauge syringe. Infected macrophage were lysed with ten strokes in a tight fitting Potter-Elvehjem homogenizer and the homogenate was centrifuged at 200 x g for 5 minutes in a Beckman TJ-6 centrifuge to remove macrophage nuclei and red blood cells. The supernatant was transferred to a 15 ml conical tube and centrifuged at 3,000 x g for 30 min. to obtain a crude preparation of *L. donovani* amastigotes. Residual red blood cells were lysed by suspending the cell pellet in 10 ml of 140 mM  $\text{NH}_4\text{Cl}$  buffered with 17 mM Tris HCl pH 7.4: PBS (10:1 v/v) for 5 min at 37°C. The lysate was diluted three fold with cold medium and the amastigotes pelleted by centrifugation at 3,000 x g for 30 minutes. Amastigotes were purified, free of red blood cell and phagolysosomal membranes, by density centrifugation. The pellet was suspended in 2.5 ml of RPMI 1640 medium, and overlaid onto a 5 ml Percoll (Pharmacia Canada Inc.) solution adjusted to a density of 1.037 g/ml with RPMI 1640 medium and was centrifuged at 2000 x g for 45 min.

**Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of LPGAP:**

Crude or purified LPGAP along with the control protein, human transferrin were coated onto ELISA plates (Costar, Cambridge, MA) by drying 1 µg/well in 100 µl of H<sub>2</sub>O for indirect ELISA (Engvall & Perlman, 1971; Tolson *et al.*, 1989). Free sites on the plate were blocked with 200 µl of 3% skimmed milk powder in PBS for 1 h at 37°C. The blocking solution was removed and the wells were washed twice with 100 µl of PBS containing 0.05% Tween 20 (Sigma). This wash buffer was used throughout the ELISA assay. Ascites fluid containing either mAbs L98, L157, CA7AE, or HT-1 (anti-human transferrin mAb used as a negative control) were diluted 1:1,000 in buffer containing 0.5% BSA and 100 µl of each antibody solution were added. Plates were then incubated at 37°C for 1 h. Excess mAb was removed by washing wells 6 times with 100 µl buffer. The bound antibody was detected by incubating 100 µl of a goat anti-mouse IgG/IgM horseradish peroxidase conjugated second antibody (Pierce Chemical Co., Rockford, IL) diluted 1:2,000 in buffer containing 0.5% BSA, and at 37°C for 1 h. After washing wells 6 times with 100 µl of buffer to remove unbound antibody, ELISAs were developed using 20 mM 2,2'-azino-bis(3-ethylbenzthiazolinesulphonic acid and 0.008% H<sub>2</sub>O<sub>2</sub> (Sigma Chemical Co.) in 50 mM sodium citrate buffer, pH 4.0, as substrate. Plates were read on a Dynatech microplate reader at 405 nm.

**Immunoblot Detection of LPGAP:**

LPGAP separated by SDS-PAGE electrophoresis on a 15% slab gel was transferred to nitrocellulose membrane (BioRad, Richmond, CA) at 90 volts for 20 min in a 12.5 mM Tris, 170 mM glycine, pH 8.2 buffer containing 10% methanol as described by Aebersold *et al.* (1986). Free sites on the membrane were blocked with 10% heat inactivated bovine calf serum in PBS containing 0.5% Tween 20 for 1 h at 37°C with constant agitation. Blots were probed with either mAbs L98 or HT-1 diluted 1:1,000 in PBS containing 10% bovine calf serum, 10% glycerol, 0.1% NP-40 and 0.5% Tween 20 for either 1 h at 37°C or overnight at 4°C. Blots were washed for 1 h at room temperature with 100 ml of PBS/0.5% Tween 20 with shaking. The first antibody was detected by incubating blots for 1 h at 37°C with a goat anti-mouse IgG/IgM alkaline phosphatase conjugate (Pierce,

Chemical Co., Rockford, IL) diluted 1:2,000 with 1% BSA in the previous buffer. The membranes were washed 4 x 10 min with the PBS/0.5% Tween 20 buffer, then 2 x 5 min with 100 mM NaHCO<sub>3</sub>, 1 mM MgCl<sub>2</sub>, 1 μM ZnSO<sub>4</sub>, pH 9.8. Blots were developed without shaking in 10 ml of 100 mM NaHCO<sub>3</sub>, 1 mM MgCl<sub>2</sub>, pH 9.8 containing 1.5 mg 5-bromo-4-chloro-3-indoxyl phosphate p-toluidine salt and 3.3 mg of nitro blue tetrazolium chloride (both from Boehringer-Mannheim). To stop development, blots were washed in 100 ml dH<sub>2</sub>O and air dried in the dark.

#### **Extraction of the Lipophosphoglycan/Lipophosphoglycan-Associated Protein Complex (LPG/LPGAP):**

Promastigotes or amastigotes were extracted as previously described by Turco *et al.*, 1984; Orlandi & Turco, 1987. Briefly, 2 x 10<sup>11</sup> *L. donovani* parasites were delipidated by sequential extraction with 3 x 25 ml of chloroform:methanol:water (3:2:1), 3 x 25 ml of chloroform:methanol:water (1:1:0.3), and 3 x 25 ml of 4 mM MgCl<sub>2</sub>. Finally, the LPG/LPGAP complex was extracted with 4 x 25 ml of solvent E (water:ethanol:diethyl ether:pyridine:NH<sub>4</sub>OH (15:15:5:1:0.017)). Extraction efficiency was enhanced by sonic dispersion of the delipidated residue. Solvent E supernatants were pooled and taken to dryness under reduced pressure at 37°C on an Eyela roto-evaporator (Rikakikai Co. Ltd., Tokyo, Japan). The residue was suspended in 5 ml of 40 mM NH<sub>4</sub>OH, 1 mM EDTA and the insoluble material was removed by centrifugation at 15,000 x g for 10 min. The supernatant was applied to a Sephadex G-100 column (2.7 x 34 cm) developed with 40 mM NH<sub>4</sub>OH, 1 mM EDTA. Fractions (2 ml) were assayed for LPG and LPGAP by indirect ELISA with the mAbs CA7AE and L98 (Tolson *et al.*, 1989). Positive fractions eluting in the void volume were pooled, dialyzed against H<sub>2</sub>O, and lyophilized. The LPG/LPGAP complex was resuspended in 4 ml of solvent E and precipitated with an equal volume of methanol for 16 h at -20°C to remove trace levels of phospholipid.

#### **Reversed Phase Chromatographic Purification of LPGAP:**

The LPG/LPGAP complex was dissolved in 3 ml of 40 mM NH<sub>4</sub>OH, 1 mM EDTA and applied to an octyl Sepharose column (1.0 X 30 cm) (Pharmacia Canada Inc.) equilibrated with 100 mM ammonium acetate, pH 6.7 containing 10% 2-propanol and developed with

a 240 ml linear gradient from 10% to 70% 2-propanol 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 indirect ELISA with mAbs CA7AE and L98 to detect LPG and LPGAP, respectively (Tolson *et al.*, 1989 & McNeely *et al.*, 1990). Fractions with both 280 nm absorbance and L98 reactive material were pooled, lyophilized, and re-chromatographed on the same column to remove trace amounts of LPG detected by the mAb CA7AE.

#### **Preparative SDS-PAGE Purification of LPGAP:**

Prior to preparative SDS-PAGE electrophoresis the LPG was depleted from these preparations by depolymerization with 40 mM trifluoroacetic acid at 100°C for 10 min (Turco, 1982) and delipidation by precipitation of the protein component with 20 volumes of cold acetone. The crude LPGAP was dissolved in Laemmli sample buffer (Laemmli, 1970) and fractionated at a constant voltage (20 V/cm) on a 2.5 x 100 mm 7% denaturing tube gel on an Applied Biosystems model 230 preparative gel electrophoresis system (Applied Biosystems Inc., Foster city, CA), collecting 150 µl fractions. The purity of each fraction was assessed on a 15% SDS-PAGE gel stained with Coomassie blue dye and verified by Western blotting with mAb L98. Fractions containing a homogeneous 11 kDa immunopositive protein were pooled, dialyzed, concentrated and acetone precipitated to remove SDS.

#### **Preparation of Lipophosphoglycan Fragments:**

The LPG used to generate fragments used in the T-cell proliferation assays was purified according to the methods of Orlandi and Turco (1987) and was generously provided by Dr. Turco, University of Kentucky. Phosphoglycan (PG), the delipidated form of LPG, was produced by treating 500 µg LPG with 150 mU of *B. cereus* phosphatidylinositol-specific phospholipase C (PIPLC) (Boehringer-Mannheim) dissolved in 100 µl of 50 mM HEPES buffer, pH 7.4, containing 1 mM EDTA, for 16 h at 37°C (Ellman, 1961). The digest was both terminated and the lysophosphatidylinositol lipid was extracted with chloroform (Orlandi & Turco, 1987). The phosphatidylinositol-core fragment (PI-core),

containing the lipid moiety, was obtained by dissolving 200 µg of LPG in 200 µl of 20 mM HCl and incubating at 100°C for 10 min to hydrolyze the phosphodiester linkage in the repeat structure (Turco, 1982). The Galβ1-4ManPO<sub>4</sub> disaccharide was removed from the hydrolysate by chromatography on an octyl Sepharose (Sigma) column (0.5 x 3 cm) equilibrated with H<sub>2</sub>O. After sample application the column was washed with 3 bed volumes of H<sub>2</sub>O and the PI-core was eluted with 4 bed volumes of 60 % 2-propanol. To prepare the fragment encompassing the carbohydrate core, the PI-core was treated with PIPLC and purified on octyl Sepharose as indicated for the PG fragment. Cleavage of the LPG at the galactofuranose residue within the carbohydrate core was accomplished by incubating 200 µg of LPG for 3 h at 100°C in 40 µl of 50 mM trifluoroacetic acid (de Lederkremer *et al.*, 1980). This acid hydrolyzed material was lyophilized and used directly in T cell proliferation assays.

#### **Periodate Oxidation of LPG:**

Lipophosphoglycan (200 µg) was dissolved in 150 µl of 200 mM sodium formate pH 4.6, containing 10 mg periodic acid and incubated at room temperature for 18 h (Spiro, 1972). To reduce the aldehyde groups generated by oxidation of the sugar residues to secondary alcohols, the mixture was made alkaline by the addition of 50 µl of 1 M Tris pH 10.5 followed by the addition of 20 mg sodium borohydride dissolved in 40 µl of 100 mM NaOH and incubation for 1 h at 37°C. The modified glycoconjugate was recovered in the void volume after desalting on a Sephadex G-10 (1 x 50 cm) column eluted with H<sub>2</sub>O.

#### **Chemical Deglycosylation of the LPG/LPGAP complex:**

To isolate the protein component co-purifying with the LPG the glycolipid was selectively degraded by treatment with anhydrous trifluoromethanesulfonic acid (Sigma). This procedure cleaves all glycosidic linkages while leaving peptide bonds intact (Edge *et al.*, 1981; Tam *et al.*, 1986). LPG/LPGAP (500 µg) was dissolved in 400 µl of anhydrous TFMSA: anisole (2:1), sparged with nitrogen and incubated at 4°C for 2 h with constant stirring. To recover the LPGAP the reaction mixture was cooled to -40°C with dry ice and the TFMSA was neutralized by the addition of solid sodium carbonate (Beeley, 1985).

Excess salts were removed by exhaustive dialysis against H<sub>2</sub>O with a 1000 molecular weight cut off membrane (Spectrum, Houston, TX).

#### **Hydrazinolysis of LPG/LPGAP:**

*L. donovani* LPG/LPGAP (125 µg) was lyophilized 4 days in a screw cap Kimax tube (1.5 x 15 cm). The sample was dissolved in 300 µl anhydrous hydrazine (Oxford Glycosystems, Rosedale, NY) and incubated at 85°C for 10 h, then dried under reduced pressure. To remove residual hydrazine the LPG sample was suspended in 200 µl of toluene and dried under vacuum, four times. The LPG was dissolved in H<sub>2</sub>O and the amino acid hydrazides removed by applying the material to an octyl Sepharose column (0.5 x 5 cm) equilibrated with H<sub>2</sub>O, washed with 3 column volumes of H<sub>2</sub>O and the LPG eluted with 4 column volumes of solvent E.

#### **Protease Digests of the LPG/LPGAP Complex:**

Pronase treatment of LPG and PG was performed by incubating 200 µg of each preparation dissolved in 200 µl of 40 mM N-ethylmorpholine pH 8.0 containing 4 µg *Streptomyces griseus* pronase (Sigma) for 4 h at 37°C. Proteinase K digestion of LPG-associated protein was carried out in SDS-PAGE sample buffer at 55°C for 3 h (100:1 w:w substrate to protease ratio) prior to electrophoresis and immunoblotting with the L98 mAb.

#### **Cyanogen bromide digest of LPGAP:**

Purified LPGAP (100 µg) was dissolved in 100 µl of 70% TFA containing 10 mg of cyanogen bromide (CNBr) (Eastman Kodak, Rochester, NY). The solution was nitrogen sparged and incubated at room temperature in the dark with stirring for 36 h (Gross, 1962). The reaction was terminated by diluting the digest mixture with 3 ml H<sub>2</sub>O and taken to dryness in a Speed Vac concentrator (Savant Instruments, Hicksville, NY). CNBr peptides were dissolved in 100 µl of 0.1% TFA and chromatographed on a PRP-1 reverse phase HPLC column (Hamilton, Reno, Nevada) equilibrated with 0.1% TFA and developed with a linear gradient of acetonitrile at a rate of 0.5%/min. Fractions were collected in 1.5 ml Eppendorf tubes and dried in a Speed-Vac concentrator.

**Proteolytic Digests of LPGAP with Endoproteinase Lys-C:**

Purified LPGAP (100 µg) was dissolved in 150 mM ammonium acetate/4 M urea, pH 8.0 and incubated at 100°C for 5 min to aid denaturation. Two µg of endoproteinase Lys-C (*Lysobacter enzymogenes*, EC 3.1.4.10) (Boehringer-Mannheim) was added to the LPGAP and the digested at 37°C for 8 h as described by Jekel *et. al.* (1985). Peptides were separated on an Aquapore RP-300 C<sub>8</sub> reverse phase column (Applied Biosystems Inc.) equilibrated with 0.1% TFA and developed with a 0.5%/min linear gradient of acetonitrile containing 0.1% TFA at a flow rate of 0.5 ml/min. Fractions were collected in 1.5 ml Eppendorf tubes and dried in a Speed-Vac concentrator.

**Proteolytic Digests of LPGAP with Endoproteinase Asp-N:**

LPGAP (50 µg) was dissolved in 100 µl of 100 mM ammonium acetate pH 8.0/1 M urea and digested overnight with 1 µg Asp-N protease (*Pseudomonas fragi*) (Boehringer-Mannheim) (Drapeau, 1980). The digest mixture was separated as described above for the Lys-C digests.

**Peptide Microsequencing:**

CNBr or protease derived peptides were dissolved in 20 µl of 20% methanol and applied to 4 mm<sup>2</sup> pieces of Problot membrane (Applied Biosystems Inc.). Membranes were washed twice with 500 µl of H<sub>2</sub>O, air dried, and proteins were either sequenced directly on a model 473 pulsed liquid sequenator (Applied Biosystems Inc.) or membranes were stored at -20°C.

**Determination of the LPGAP Isoelectric Point:**

Crude promastigote LPGAP (400 µg) was depleted of LPG and dissolved in 60 ml of 1% pH 3-10 ampholyte (BioRad, Richmond, CA) solution and focused on a BioRad Rotoфор at 12 watts for 5 h. Three ml fractions were collected and assayed for LPGAP by ELISA with the mAb L98. The pI of LPGAP was determined by measuring the pH of each fraction with a Radiometer pH meter.

**Amino Acid Analysis:**

LPGAP was dried as a film in a 4 x 25 mm borosilicate tube and subjected to gas phase hydrolysis with 6 M HCl at 165°C for 1 h under argon. Hydrolysates were dissolved in 20  $\mu$ l H<sub>2</sub>O and analyzed on an Applied Biosystems 420 amino acid analyzer (Applied Biosystems Inc.).

**Monosaccharide Composition of LPGAP:**

LGPAP (20  $\mu$ g) dissolved in 200  $\mu$ l of 2 M HCl was incubated at 100°C for 4 h in a 1.5 ml screw top polypropylene tube. Hydrolysates were thoroughly dried in a Speed Vac concentrator to remove the HCl and subsequently dissolved in 25  $\mu$ l of H<sub>2</sub>O. The monosaccharide composition was determined on a carbohydrate dedicated Dionex Bio-LC system equipped with a CarboPacA-1 column (Dionex Corporation, Sunnyvale, CA) and developed isocratically with 16 mM sodium hydroxide. Sugars were detected with a pulsed amperometric detector with the following settings  $E_1 = 0.05$  V ( $t_1 = 300$  ms),  $E_2 = 0.6$  V ( $t_2 = 120$  ms),  $E_3 = 0.6$  V ( $t_3 = 60$  ms).

**Mass Spectroscopy Analysis of LPGAP CNBr-2 Peptide:**

The parent ion of the CNBr-2 peptide was determined in the laboratory of Dr. R. Caprioli, University of Texas, by continuous-flow FAB mass spectroscopy on a Finnigan MAT-90 high field mass spectrometer. The peptide was dissolved in 0.1% TFA containing 5% glycerol and injected at a flow rate of 5  $\mu$ l/min.

**Preparation of Promastigote Plasma Membranes:**

Cytoplasmic and plasma membrane fractions were prepared from *L. donovani* promastigotes according to the method of Dwyer (1981). Briefly,  $2 \times 10^{10}$  cells were suspended in 30 ml of 20 mM Tris pH 8.0, 1 mM EDTA and incubated at 0°C for 30 minutes and lysed in tight fitting Potter-Elvehjem homogenizer with 15 strokes. The lysate was centrifuged at 27,000 x g for 1h. The crude membrane pellet was suspended in 20 ml of 1.52 M sucrose in 20 mM Tris HCl, pH 8.0, 5 mM MgCl<sub>2</sub> (TM buffer) and layered over 6 ml of 1.75 M sucrose in TM buffer. The membrane suspension was in turn overlaid with

12 ml of 1.23 M sucrose in TM buffer and centrifuged at 82,000 x g in a Beckman SW 28 rotor for 1 h. The plasma membrane fraction was harvested from the 1.23 M and 1.52 M sucrose interface and dialyzed exhaustively against PBS.

**Immunoprecipitation of  $^{125}\text{I}$  Labeled Promastigotes with anti-LPGAP MAb L157:**

Mid-log phase promastigotes ( $5 \times 10^7$ ) were suspended in 500  $\mu\text{l}$  of cold PBS containing 1% glucose and radiolabeled with 500  $\mu\text{Ci}$  [ $^{125}\text{I}$ ] (Dupont Canada Inc., Mississauga, Ont.) in a borosilicate tube coated with Iodogen (Sigma) as previously described (Fraker & Speck, 1978). Cells were washed 4 times with 1 ml cold PBS-1% glucose to remove free [ $^{125}\text{I}$ ] and lysed by boiling in 30  $\mu\text{l}$  of 1% SDS for 3 min. and diluted with 300  $\mu\text{l}$  1% NP-40 in PBS. Cleared lysates were obtained by centrifugation at 15,000 rpm in a microfuge for 20 min. and preabsorbed with 50  $\mu\text{l}$  of settled protein G Sepharose (Pharmacia Canada, Baie d'Urte, Que.). Immunoprecipitation was affected by adding 10  $\mu\text{l}$  of either mAb L157 or anti-human transferrin ascites fluid to 150  $\mu\text{l}$  of lysate with end-over-end rotation room temperature for 1h. Antigen-antibody complexes were precipitated with 20  $\mu\text{l}$  settled protein G Sepharose beads treated with unlabeled *L. donovani* promastigote lysates to reduce non-specific binding of radiolabeled parasite proteins. Immunoprecipitates were characterized by electrophoresis on a 15% SDS-PAGE. The gel was dried and autoradiographed at  $-70^\circ\text{C}$  for 16 h.

**Circular Dichroism (CD) Measurements:**

The CD spectra were recorded in the laboratory of Dr. C. Kay, University of Alberta, on a Jasco J-700 spectropolarimeter (Jasco Inc., Easton, MD) calibrated with ammonium (1S)-(+)-10-camphorsulfonate (Yang, 1986) using a cell pathlength of 0.02 cm. Octyl Sepharose purified LPGAP was dissolved in either 20 mM Tris HCl, pH 7.8 or 10 mM Tris HCl, pH 7.8/50% trifluoroethanol (TFE) at a concentration of 320  $\mu\text{g}/\text{ml}$ . The CD spectra of LPGAP were analyzed for secondary structure elements using the Contin program version 1.0 (Provencher, 1981).

**Preparation of Carboxyfluorescein Loaded Small Unilamellar Vesicles:**

Unilamellar liposomes were prepared according to the method of Weinstein *et al.* (1977).

Briefly, 12 mg of distearoyl phosphatidylcholine (Sigma) was dissolved in 2 ml of chloroform and dried as a thin film in a 50 ml round bottom flask and lyophilized overnight. The film was rehydrated in 2 ml of 240 mM carboxyfluorescein (Eastman Kodak) with vigorous mixing then sonicated for 40 min on ice with a microtip sonicator (Heat Systems Ultrasonics) with 5 min 20 Watt pulses. The translucent liposome suspension was annealed for 1 h at 55°C and free carboxyfluorescein was removed by gel permeation chromatography on a Sephadex G-75 column (2 x 25 cm) equilibrated with 100 mM Tris HCl, pH 7.5, 150 mM NaCl. Carboxyfluorescein loaded liposomes eluted in the void volume while the unencapsulated dye eluted in the column bed volume. For leakage assays, purified liposomes were diluted 1:40 with 100 mM Tris HCl, pH 7.5, 150 mM NaCl and octyl Sepharose purified LPGAP was added to a final concentration of 20 µg/ml. Control assays, for potential glycolipid contaminants in LPGAP preparations, were conducted with protease K digested LPGAP (1:1 (w:w) substrate to protease ratio). The fluorescence dequenching was recorded on a G. K. Turner associates fluorometer with an excitation wavelength of 490 nm and emission wavelength of 520 nm.

#### **Preparation of the LPG/LPGAP for Electron Microscopy:**

The *L. donovani* promastigote LPG/LPGAP complex eluting in the void volume of a Sephadex G-100 column, was coated onto copper grids (200 mesh) by floating the grids, polished side down, on 50 µl of sample for 5 min at room temperature. Excess solvent was removed by blotting the grids with Whatman 3MM filter paper and air drying for 5 min. The LPG/LPGAP micelles were negatively stained by floating the grids, sample side down, on 100 µl of 1% ammonium molybdate, 0.1% glycerol for 1 min at room temperature. After drying, the samples were analyzed on a Hitachi transmission electron microscope at a potential of 75 kV.

#### **Isolation of Promastigote Genomic DNA:**

Isolation of high molecular weight genomic DNA used in the production of the cosmid library was prepared according to the method of Blin and Stafford (1976). Log phase promastigotes ( $1 \times 10^{10}$ ) were harvested by centrifugation (3,000 rpm for 10 min.) and

washed twice with 50 ml phosphate buffered saline. Cells were resuspended in 3 ml of 0.5 M EDTA in PBS containing 0.5% SDS, and digested with 300 µg of proteinase K (Boehringer-Mannheim) by incubating the mixture at 50°C for 12 h with occasional mixing by inversion. The digest was extracted twice with an equal volume of phenol:chloroform (1:1), with mixing and gentle inversion of the tubes to remove residual proteins. Excess phenol was removed by a single chloroform extraction. Genomic DNA was precipitated with an equal volume of cold 95 % ethanol followed by centrifugation at 10,000 rpm for 20 min. The supernatant was carefully decanted and the tubes were inverted to drain residual ethanol. The DNA was suspended in 3 ml Tris EDTA (TE) buffer and dissolved by gently inverting while warming at 50°C and contaminating RNA was removed by RNase A (100 µg/ml) digestion at 37°C for 2 h. Purified DNA was obtained by repeating the phenol:chloroform extraction and ethanol precipitation as described above. The genomic DNA was dissolved in 2 ml TE buffer and the concentration was approximated at 260 nm from DNA sheared through a 30 gauge needle. One O.D.<sub>260 nm</sub> for double stranded DNA was assumed to be equal to 50 µg/ml (Sambrook *et al.*, 1989).

#### **Construction of *L. donovani* Promastigote gDNA Cosmid Library:**

This library was a gift from Drs. S. Hanson and B. Ullman, Oregon Health Science University. *Leishmania* gDNA fragments were cloned into the Stratagene SuperCos 1 cosmid vector as described by Stratagene (La Jolla, CA). A time course digest of genomic DNA was obtained by digesting 10 µg of DNA with 0.5 U of *Mbo* I (Gibco/BRL Canada, Burlington, Ont.) in 100 µl of buffer at 37°C. Fifteen microliter aliquots were removed at 0, 5, 10, 20, 30, and 45 minute intervals and analyzed on a 0.5% agarose gel to determine an optimal digest time of 30 min was required to generate 30-50 kb fragments. The above digest was scaled-up to 100 µg and the reaction was terminated by addition of 20 µl of 0.5 M EDTA, pH 8.0. The restriction endonuclease was extracted with phenol:chloroform (1:1), chloroform and the DNA was precipitated with 10 volumes of cold 95% ethanol. The DNA pellet was suspended in 100 µl of TE buffer and dephosphorylated with 60 U of calf intestine alkaline phosphatase (CIAP) (Boehringer-Mannheim) at 37°C for 1 h. The reaction was stopped with 3 µl of 0.5 M EDTA and incubated at 68°C for 10 min,

followed by phenol:chloroform extraction and DNA precipitation with ethanol. The DNA was dissolved in TE buffer at a concentration of 1 µg/ml.

SuperCos 1 DNA (20 µg) was linearized with *Xba* I (180 U) in 200 µl buffer at 37°C for 1 h, then extracted with phenol:chloroform and ethanol precipitated. The vector DNA was dephosphorylated with 12 U of CIAP as outlined above and then digested with *Bam* HI (100 U) in 200 µl at 37°C for 1 h. After extraction with phenol:chloroform and ethanol precipitation the *Xba* I/*Bam* HI double digested SuperCos 1 was dissolved at a concentration of 1 µg/µl in TE buffer.

The cosmid library was generated by ligating 2.5 µg *Mbo* I *Leishmania* DNA (30-50 kb fragments), with 1 µg *Xba* I/CIAP/*Bam* HI treated SuperCos 1 DNA, in a 20 µl volume containing 2 µl of 10X ligation buffer, 2 µl of 10 mM ATP, and 1 µl of T4 DNA ligase (2 Weiss U (BRL, Gaithersburg, MD)). The ligation reaction was incubated overnight at 14°C and packaged into λ phage using the Stratagene Gigapak II XL kit (Stratagene) according to the suppliers instructions. The library was titered using the NM554 *E.coli* strain (Stratagene, La Jolla, CA) on LB plates containing 50 µg/ml ampicillin (Sigma), according to Stratagene protocol.

#### **Cosmid Library Screening for the *lpgap* Gene:**

From the LPGAP protein sequence (residues 45-68) a 45 base pair oligonucleotide probe (ATGATCAAGGAGCACAC<sup>G</sup>/<sub>C</sub>GAGAAGTTCAACAAGAAGATGCACGAG) was synthesized (Calgary Oligonucleotide Service, Calgary, Alta.) with a G/C bias in the wobble position. The probe was labelled enzymatically at the 5' end with γ <sup>32</sup>P-ATP (Dupont Canada Inc.) and T4 polynucleotide kinase (Pharmacia Canada Inc.), then purified by gel permeation chromatography (Sambrook, 1989). Approximately 2000 cosmid clones were plated on a 150 mm nylon filter (Schleicher & Schuell, Keene, NH) overlaid on LB plates containing 100 µg/ml ampicillin and 50 µg/ml tetracycline (LBAT). Plates were incubated overnight at 37°C, and the filter and agar were marked with pin holes in three non-symmetrical points. The master filter was removed and two lifts prepared by sandwiching sterile nylon filters and the master filter between two sheets of Whatman 3 MM filter paper. All three filters were placed colony side up on fresh LBAT

plates and incubated at 37°C for 5 h. The master filter from which positive clones were picked was stored at 4°C. The two duplicate filters were alkali denatured, neutralized, and baked at 80°C for 2 h and prehybridized at 42°C for 1 h. The prehybridization solution consisted of: 7.5 ml of 20X SSC, 5 ml H<sub>2</sub>O, 7.5 ml formamide, 2.5 ml 100X Denhart's solution, 0.5 ml 10% SDS, 1 ml 0.5 M sodium phosphate pH 6.5, and 0.5 ml of 10 g/ml salmon sperm DNA. The labeled probe was added to the hybridization solution at an activity of  $1.2 \times 10^6$  cpm/ml (specific activity  $4 \times 10^7$  cpm/ $\mu$ g) and hybridized overnight at 42°C with constant shaking. Filters were washed 3 times with 100 ml 2X SSC at 42°C, then air dried and autoradiographed. By aligning the orientation marks on the autoradiogram with those on the plate an area around the positive clone was picked and re-screened using the above procedure. Clones of interest were purified until all colonies hybridized with the LPGAP probe.

#### **Isolation of Cosmid DNA for Restriction Digest Analysis:**

Cosmid DNA was isolated using the alkali lysis method as outlined by Sambrook *et al.* (1989). Cultures were prepared by inoculating 2 ml of LB broth containing 100  $\mu$ g/ml ampicillin and 50  $\mu$ g/ml tetracycline with a single bacterial colony containing the *lpgap* cosmid DNA. The overnight culture (1.5 ml) was pelleted in a microfuge and the supernatant carefully removed by aspiration. The cells were resuspended in 100  $\mu$ l of 25 mM Tris HCl, pH 8.0, 10 mM EDTA, 50 mM glucose and incubated on ice for 10 min. Bacterial cells were lysed with 250  $\mu$ l of 0.2 M NaOH, 1% SDS, then mixed gently and incubated on ice for another 10 min. Chromosomal DNA was precipitated with 200  $\mu$ l of 3 M potassium acetate, 2 M acetic acid solution and centrifuged at 15,000 rpm for 15 min. The supernatant was carefully removed and digested with RNase A for 30 min at 37°C at a final concentration of 100  $\mu$ g/ml to eliminate contaminating RNA. The DNA was de-proteinated by sequential phenol:chloroform (1:1), chloroform extractions and precipitated by adjusting the aqueous phase to 0.3 M ammonium acetate (pH 7.0) and adding 3 volumes of cold ethanol (-70°C). The sample was then centrifuged at 15,000 rpm for 15 min. in a 4°C microcentrifuge and the pellet washed with 70% ethanol, air dried and suspended in 20  $\mu$ l of TE buffer. Plasmid DNA was also isolated from *E. coli* strains

transformed with pBluescript constructs using the above protocol. Plasmid DNA (1 µg) was digested with a variety of restriction endonucleases using 5-10 units of enzyme for 2 h at 37°C. Digests were separated on a 0.8% agarose gel at 5 V/cm and blotted onto nitrocellulose membrane by capillary action (Sambrook, 1989). The membrane was baked at 80°C for 2 h and probed with the LPGAP 45mer oligonucleotide probe as described above.

**Southern Blot Analysis of the Cosmid Clones Hybridizing with the LPGAP Probe:**

Cosmid DNA (2 µg) from each of the two clones were singly or doubly digested with a variety of restriction endonuclease (10 units) in 10 µl volumes, for 2 h at 37°C. Digests were separated on 0.8 % agarose gel at 5 V/cm and blotted onto a nitrocellulose membrane by capillary action with 200 mM NaOH overnight (Sambrook *et al.*, 1989). The membrane was sequentially rinsed for 5 min with 20X SSC and H<sub>2</sub>O to remove excess salt and air dried at room temperature. The DNA was irreversibly bound to the membrane by sandwiching the blot between squares of Whatman 3 MM paper and baked at 80°C for 2 h. The blots were hybridized with the LPGAP 45mer oligonucleotide probe labeled at the 5' end with [<sup>32</sup>P] as described above in the cosmid library screening protocol.

**Subcloning the *lpgap* Gene Fragment into the pBluescript Vector:**

The *Sac* I digest of the cosmid clone 1 yielded a 3.4 kb fragment which hybridized with the LPGAP 45 bp probe. This fragment was purified from agarose gel slices by the freeze-thaw method and ethanol precipitated. To subclone the *Sac* I 3.4 kb fragment into the blunt end *Sma* I site of the pBluescript vector, it was necessary to fill in the cohesive ends. This was accomplished by dissolving 2 µg of DNA in 20 µl of Klenow buffer containing 33 µM of each deoxynucleotide triphosphate (Pharmacia Canada Inc.), 1 unit of DNA polymerase I the Klenow fragment (New England Biolabs, Beverly, MA) and incubating the mixture at 25°C for 15 min. The reaction mixture was diluted to 100 µl with H<sub>2</sub>O, was sequentially extracted with phenol:chloroform, chloroform then ethanol precipitated. Two micrograms of pBluescript II Ks +/- vector, purchased from Stratagene, was digested with 10 units of *Sma* I (New England Biolabs) at 37°C for 2 h, then extracted as above with phenol:chloroform, chloroform and ethanol precipitated. The

linearized vector DNA was dephosphorylated with CIAP as described above for the SuperCos 1 vector, and the DNA fragment encoding the LPGAP gene was ligated into the linearized pBluescript vector by mixing 1 µg of insert DNA with 100 ng of vector DNA in 20 µl of T4 ligase buffer (New England Biolabs) containing 400 µM of ATP and 4 units of *E. coli* T4 ligase (New England Biolabs) and incubated for 20 h at 14°C. The *lpgap* construct (2 µl of the ligation mixture) was transformed into DH5α competent cells (Gibco/BRL, Gaithersburg, MD) according to the vendors protocol, and 50, 100, 200 µl of the transformation mixture were plated on LB plates containing 100 µg/ml ampicillin (Sigma Chemical Co.) and 50 µg/ml X-gal (Boehringer-Mannheim). White colonies were picked onto duplicate nylon filters and the clones with the *lpgap* insert were identified by Southern blotting with the [<sup>32</sup>P] labeled LPGAP 45 bp probe as outlined above for the screening of the cosmid library. A single clone designated pBAJ22 was isolated and used for the characterization of the LPGAP gene.

#### **Generation of Nested Deletions for Sequence Analysis of the *lpgap* Gene:**

Deletion mutations of the pBAJ22 construct were prepared using Exonuclease III (Sambrook, 1989). Briefly, the construct (10 µg) was doubly digested with *Sac* I and *Bam* HI to generate a linear plasmid containing a recessed 3' terminus on one end and a 3' overhang terminus on the other end. The DNA was extracted with phenol:chloroform, ethanol precipitated and air dried to remove traces of ethanol. The DNA was dissolved in 60 µl of 1X exonuclease III buffer and 75 units of exonuclease III (New England Biolabs) enzyme added per picomole of DNA. After mixing the sample was incubated at 37°C and at 30 second intervals, 2.5 µl aliquots of the digest was added to a series of tubes pre-warmed to 37°C containing 23 mU of S1 nuclease in 7.5 µl of buffer. After sampling was completed all tubes containing the S1 digest mixture were incubated at 30°C for 30 min, followed by a 10 min incubation at 70°C to inactivate the enzymes. Two microliters of each fraction was analyzed on a 0.8% agarose gel and those fractions containing DNA fragments between 3.0 and 6.4 kb were pooled and treated with the Klenow fragment of DNA polymerase I to fill-in 2 to 3 base pair overhangs not removed by S1 nuclease. The mixture of nested deletions was extracted with phenol:chloroform and ethanol

precipitated. The DNA pellet was dissolved in ligation buffer, treated with *E. coli* T4 ligase ( New England Biolabs) for 16 h at 14°C, transformed into DH5 $\alpha$  competent cells and plated on LB plates containing 100  $\mu$ g/ml ampicillin and 50  $\mu$ g/ml X-gal (both from Sigma). Details for the fill-in reaction, ligation and transformation procedures are the same as described above. Prior to sequence analysis of the transformed cells *lpgap* genes, twenty-five transformed clones were selected and the plasmid preparations characterized by an 0.8% agarose gels according to size, after linearizing the plasmid with *EcoR* I. In addition, these clones were screened for the LPGAP insert by dot blot hybridization with the LPGAP 45 bp probe and positive clones were subjected to Sanger dideoxy sequence analysis.

#### **DNA Sequence Analysis:**

The *lpgap* gene was sequenced using plasmid DNA prepared by the alkali-lysis protocol (Sambrook *et al.*, 1989) in conjunction with the Sequenase version 2.0 sequencing kit (United States Biochemical, Cleveland, OH) as outlined by the manufacturer. The primers used for sequencing double strand were the LPGAP 45 bp oligonucleotide and the 276 bp forward universal primer supplied with the sequencing kit.

**CHAPTER 1**

**Synthetic T cell Epitopes Derived from the *L. major* Surface  
Glycoprotein gp63**

## Introduction

Attempts to develop a subunit vaccine against leishmaniasis, which would selectively activate a Th1 response, have focused on surface membrane molecules.

Immunoprotective effects against visceral and cutaneous leishmaniasis in mice have been reported with several different promastigote derived molecules which include; the *L. mexicana* gp46 (Lohman *et al.*, 1990), *L. donovani* p80 (White & McMahon-Pratt, 1990) and dp70/72 (Jaffe *et al.*, 1990), and the *L. major* LPG and gp63 (Mitchell & Handman, 1985; Russell & Alexander, 1988), with the protease gp63 being the most intensively studied.

Gp63 is a dominant dimeric surface structure found across the *Leishmania* genus at an estimated abundance of  $5 \times 10^5$  molecules/cell (Bouvier *et al.*, 1986, 1987; 1989). This protein is a metalloprotease which is dependent on a zinc cofactor for activity and treatment of the purified enzyme with the chelator 1,10 phenanthroline or the heavy metals such as  $Hg^{2+}$ ,  $Co^{2+}$ , and  $Cd^{2+}$  rapidly inhibits the proteolytic activity of this enzyme (Bouvier *et al.*, 1989; Chaudhuri *et al.*, 1989). Primary sequence analysis indicates that this *Leishmania* protease shares substantial sequence homology with the zinc binding sites motif (HE<sub>xx</sub>H) of other protease such as thermolysin, subtilisin, and fibroblast collagenase (Bouvier *et al.*, 1989; Chaudhuri *et al.*, 1989). The pH optimum reported for gp63 varies from pH 4 for *L. mexicana* to pH 7-8 for the *L. major* counterpart (Bouvier *et al.*, 1990; Chaudhuri *et al.*, 1989). Since both enzymes have a highly conserved catalytic site, the difference observed for these two enzymes is probably attributed to the substrate utilized in proteolytic assays (Tzinia & Soteriadou, 1991). Gp63 is an endoproteinase showing preferential cleavage of synthetic peptides at sites with a hydrophobe-hydrophobe-basic-(basic) structural motif, with cleavage occurring at the peptide bond between the two hydrophobic residues. This enzymatic specificity has been postulated to be responsible for the autoactivation of gp63 by cleavage of the prosequence on the parasite surface (Bouvier *et al.*, 1990).

Aside from facilitating the entry of promastigote invasion of macrophage via the C3 complement receptor, no biological role has been established for the proteolytic activity of gp63. One role proposed for gp63 is to protect promastigotes against degradation by the hydrolytic enzymes in the phagolysosome. This idea has been advanced on the basis that the *L. mexicana* gp63 exhibits an acidic pH optimum together with the observation that liposomes coated with gp63 were not degraded in the macrophage phagolysosome (Chaudhuri *et al.*, 1989; Bouvier *et al.*, 1989). However, the drastic down regulation of gp63 in amastigotes does not favour this hypothesis. Quantitative analysis of the protease activity indicates that *L. major* amastigotes contain only 1% of the gp63 activity present on promastigotes (Medina-Acosta *et al.*, 1989; Schnieder *et al.*, 1992). It is conceivable, however, that the high levels of gp63 may play an essential role in promastigote survival within the sandfly host.

Structural characterization of gp63 indicated that this molecule was inserted into the plasma membrane via a dimyristoyl glycosylphosphatidylinositol anchor and could be liberated from the membrane with phosphatidylinositol specific phospholipase C (Bordier *et al.*, 1986). In *L. mexicana* amastigotes the gp63 is differentially processed and is inserted into the plasma membrane with an anchor which is not sensitive to PIPLC, most likely via a transmembrane protein domain (Medina-Acosta *et al.*, 1989). Analysis of the carbohydrate structures indicated that *L. mexicana* gp63 contained three N-linked high mannose oligosaccharide. However, one of these structures was found to contain a unique terminal glucose residue which has been proposed to facilitate macrophage ingestion of promastigotes via the AGE receptor (Olafson *et al.*, 1991; Mosser *et al.*, 1987).

The gene for the *L. major* gp63 was initially cloned and sequenced by Button and McMaster (1988) and subsequently corrected by Miller *et al.* (1990). Subsequently DNA sequences for gp63 have been obtained for *L. mexicana*, *L. chagasi*, *L. guyanensis*, and *L. donovani* all sharing approximately 80-90% sequence identity at the protein level (Inverso *et al.*, 1993). Interestingly, gp63 is not specific to the *Leishmania* genus as a homologue of this protease has been characterized in *Crithidia fasciculata* (Inverso *et al.*, 1993). Southern blot analysis of the *Leishmania* DNA indicates that gp63 is encoded by multiple

genes found in a tandemly repeating arrangement with the number of gene copies varying from 4 copies for *L. major* (Button *et al.*, 1989) to 12 gene copies organized in three distinct clusters in the *L. mexicana* genome (Medina-Acosta *et al.*, 1989).

Immunoprotection studies conducted by Russell and Alexander (1988) demonstrated that the *L. major* promastigote surface glycoprotein, gp63, could initiate protective immunity against cutaneous leishmaniasis in BALB/c and CBA mice, and that this protection could be transferred to naive recipients with T cell populations. In these studies, immunoprotection was observed when gp63 was incorporated into liposomes and administered intravenously. However, when the mice were immunized with these preparation via a subcutaneous route no protection was afforded. Numerous other vaccine studies have also demonstrated that subcutaneous immunization with either attenuated whole promastigotes or *Leishmania* antigens, resulted in disease exacerbation (Kahl *et al.*, 1990; Liew *et al* 1985, Titus *et al*, 1984), suggesting that a Th2 response had been preferentially induced. These findings pose a considerable obstacle for the development of an immunoprophylactic agent for the immunization of populations in regions where leishmaniasis is endemic. In an alternate approach, the study presented here was initiated to identify T cell epitopes from the *L. major* gp63 sequence which would activate a Th1 response and provide protection against a subsequent challenge by *Leishmania*.

Development of a T helper lymphocyte cell mediated immune response to an exogenous protein antigen requires uptake by antigen processing cells (APC) which digest the protein into small peptides within the endosomes, where it binds to the major histocompatibility class II molecules and is presented on the cell surface for recognition by T cells (Unanue, 1984). These antigenic peptides are recognized in the context of the MHC by a T cell membrane complex which includes the T cell receptor and other accessory molecules including the CD4 molecule. This T cell-APC interaction stimulates T cell division and production of lymphokines which modulate immunity (Livingstone & Fatham, 1987). In addition, memory T cells are generated which can mount a rapid and more heightened immune response upon re-exposure to antigen, resulting in protection

from re-infection. In addition, to using whole protein molecules, a T cell response can also be stimulated with synthetic peptides which can bind to the MHC molecule.

In this connection, attempts to develop a molecularly defined vaccine against leishmaniasis focused on gp63 which was previously demonstrated to be immunoprotective in mice. Using the Rothbard and Taylor algorithm (1988) or the Garnier (1978) secondary structure predictive algorithm, gp63 was screened for putative T cell epitopes. Those epitopes inducing a Th1 response were then screened for their protective effects against cutaneous leishmaniasis in mice. Another aspect of this study was to investigate the adjuvant potential of block copolymer Poloxamer 407, which has been shown to be safe for human use.

## RESULTS

### 1.1 Identification of T Cell Epitopes of *L. major* gp63:

The *L. major* gp63 protein sequence was analyzed with the Rothbard and Taylor algorithm (1988) for putative T cell epitopes. As shown in Figure 1.1, eight peptides containing the tetra or pentapeptide motif were identified. However, only seven of these peptides were synthesized, as the peptide designated PT5 contained a potential N-linked glycosylation site. Based on the MHC class II-T cell receptor interaction hypothesis (Allen, 1987), it was argued that glycopeptides would pose steric constraints to this interaction and would therefore be poor T cell epitopes, although recent publications using T cell clones have demonstrated that glycopeptides can bind the MHC class II receptor and stimulate T cell proliferation (Ishioka *et al.*, 1992 & Harding *et al.*, 1993). In order to attain the minimum peptide length required to form a stable complex with the MHC class II molecules, the Rothbard and Taylor T cell epitopes containing only 4-5 residues were extended on the flanks to 12-15 residues (Rothbard & Geftter, 1991). (In all cases, the putative T cell epitopes were synthesized by extending the predicted sequence by 5 residues at both the amino and carboxy termini). The resulting peptides PT1 - PT4 and PT6 - PT8 were the most extensively characterized epitopes in terms of T cell proliferation, T cell phenotyping, and immunoprotection trials. A second group of peptides, PT9 - PT15, was synthesized from helical regions of the *L. major* gp63 (Figure 1.1 & Table 1-1) based upon the work of DeLisi and Berzofsky (1985) who proposed that immunodominant T cell epitopes could be identified from the propensity of a peptide to form an amphipathic structure. Although most epitopes would adopt an  $\alpha$  helical conformation, it has been suggested that the MHC class II binding site can accommodate amphipathic peptides with a  $\beta$  sheet structure (Miles *et al.*, 1989). These peptides were themselves detected using the Garnier algorithm (1978), which predicts the secondary structure of proteins from the amino acid sequence. All putative T cell epitopes were synthesized using the Merrifield tBoc chemistry, cleaved from the solid support with

20 40 60  
VRDYNWGALRIAVSTEDLTDPAYHCARVGOHVKDHAGAIVTCTAEDILTNEKRDILVKHLIPQA  
 PT1 PT2 PT9

80 100 120  
VQLHETERLKVQQVQGGKWKVTD MVGDI CGDFKVPQAHITEGFSNTDFVMYVASVPSEEGVLAWAT  
 PT10

PT9

140 160 180  
TCQTFSLGHPAVGVINIPAANIASRYDQLVTRVVTHEMAHALGFSGPFEDARIVANVPNVRGK  
 PT3

PT4 PT11

200 220 240  
NFDVPVINSSTAVAKAREQYGC DTLEYLEVEDQGGAGSAGSHIKMRNAQDELMA PAAAAGYYTA  
 PT12 PT14

PT13

260 280 300 320  
LTMAIFODLGFYOADFSKAEVMPWGDNAGCAFLTNKCMEQSVTQWPAMFCNESEDAIRCPTSRL  
 PT15

340 360 380  
SLGACGVTRHPGLPPYWQYFTDPSLAGVSAFMDYCPVVVPYSDGSCTQRASEAHASLLPFNVFS  
 PT6

PT7

400 420 440  
DAARCIDGAERPKATDGI VKS YAGLCANVQCDTATR TYSVQVHGSNDYTNCTPGLRVELSTVSNAF  
 PT6

PT8

PT7

460 480  
EGGGYITCPPYVEVCQGNVQAAKDGGNTAAGRRGPRAAATALLVAALLAVAL

**Figure 1.1:** The *Leishmania major* gp63 protein sequence showing putative T cell epitopes (Button & McMaster, 1988; Miller *et al.*, 1990). Peptides were selected and synthesized using either the Rothbard and Taylor algorithm (1988) which identifies potential T cell epitopes (PT1-PT8) or the Garnier *et al.* (1978) algorithm which predicted peptides with a propensity to adopt helical conformations (PT9-PT15).

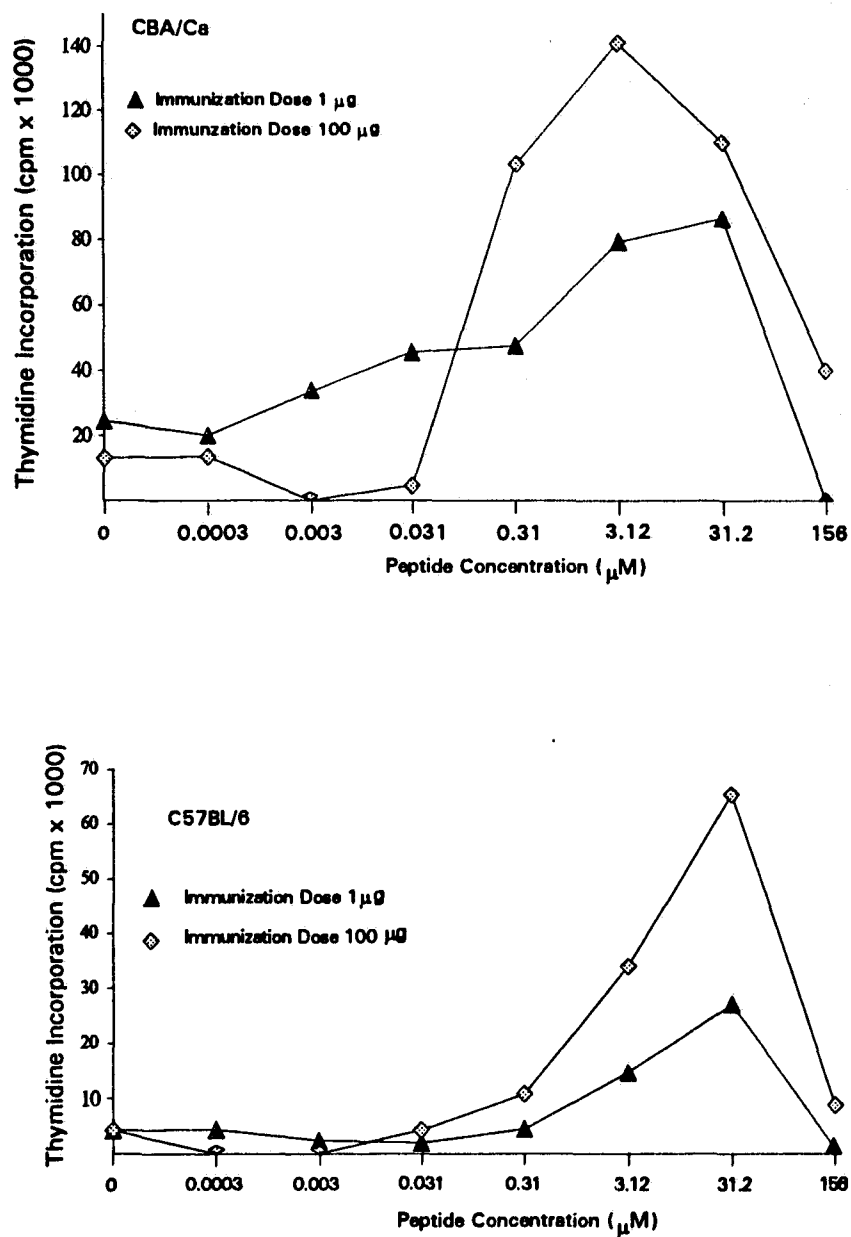
TABLE 1-1  
 Putative T cell epitopes synthesized from the *L. major* A2  
 metalloproteinase gp63 sequence

Peptide Designation	Primary Sequence	Residue Number
PT1	VRDVNWGALRIAVS	1-14
PT2	LTNEKRDILVKHLIP	48-62
PT3	YDQLVTRVVTHEMAHA	154-169
PT4	TRVVHEMAHALGFSG	159-174
PT6	PFNVFSDAARCIDGAF	379-394
PT7	AARCIDGAFRPKATDG	386-401
PT8	RPKATDGIVKSYAGLC	395-410
PT9	IPQAVQLHTERLKVQQVQG	61-79
PT10	VPSEEGVLAWATTCQ	116-130
PT11	GFSGPFFEDARIVANVP	171-187
PT12	INSSTAVAKAREQYGC	199-214
PT13	QYGCDTLEYLEVEDQGG	212-227
PT14	QDELMAPAAAAGYYTALMA	240-260
PT15	FGDLGFYQADFSKAEV	262-277

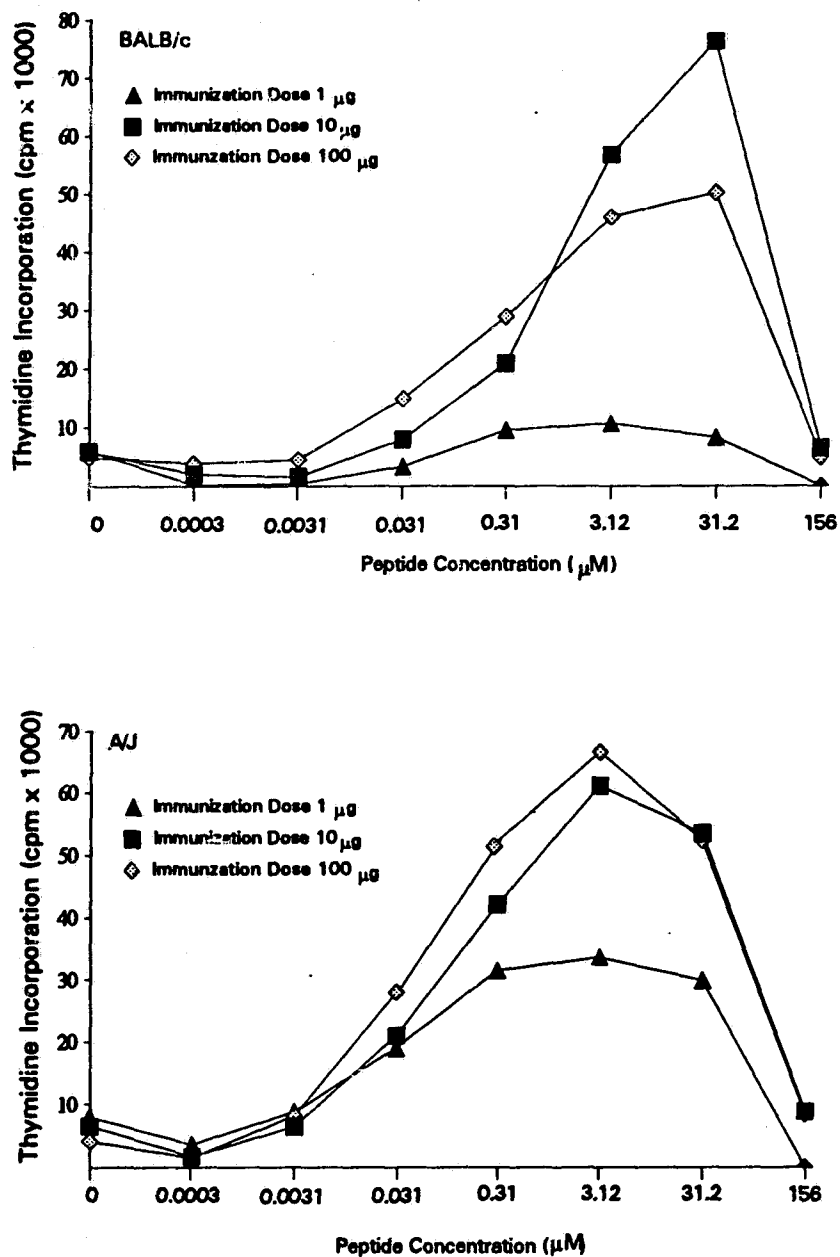
trifluoromethanesulfonic acid, purified by reversed phase chromatography and analyzed by amino acid composition.

### **1.2 Screening Synthetic Peptides for T cell Proliferation Activity:**

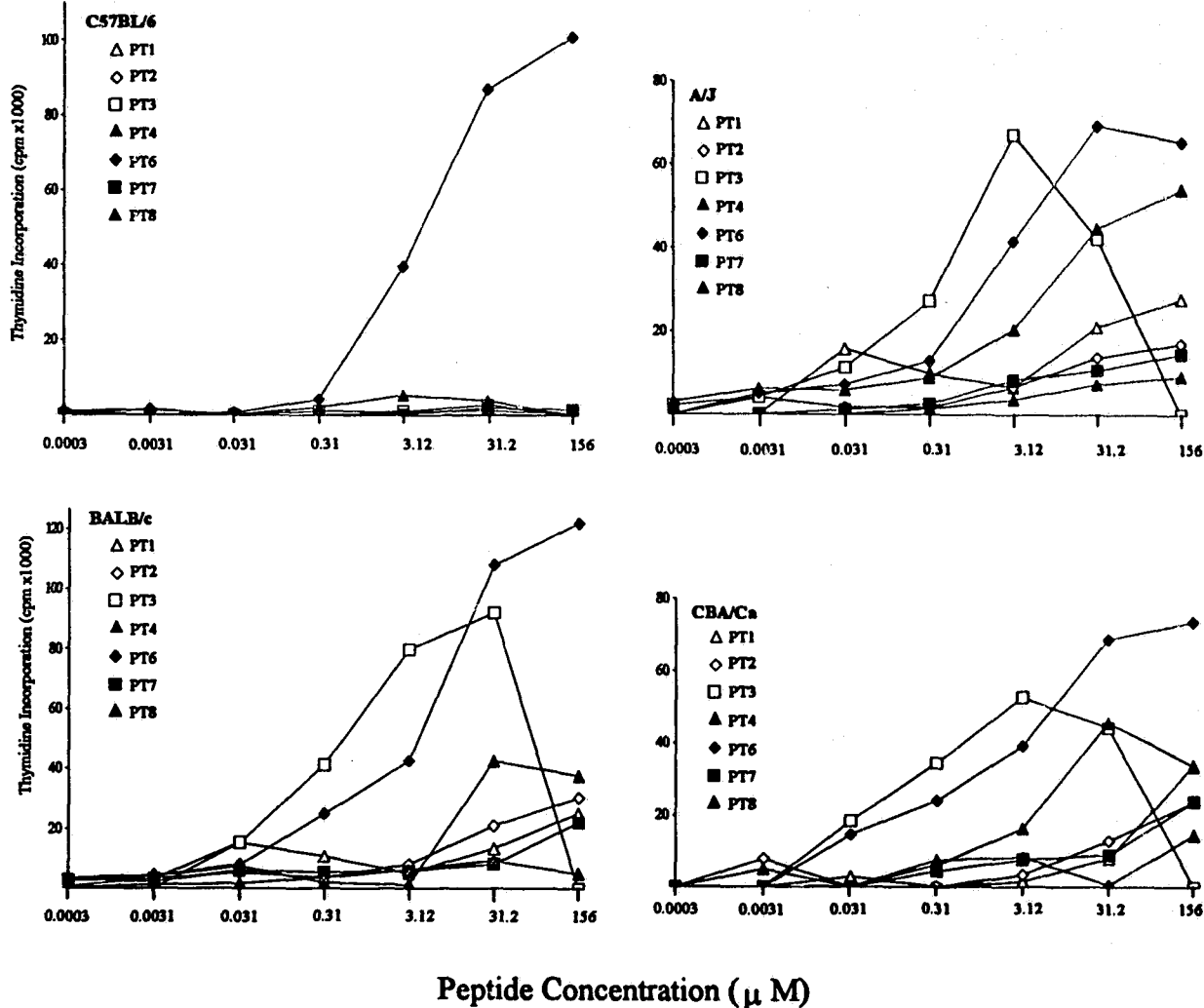
The protocol employed for evaluating the T cell activation potential of gp63 synthetic peptides was the method developed by Corradin *et al.* (1977). This procedure provides a quantitative assessment of an antigens stimulatory potency by measuring the level of radiolabeled [<sup>3</sup>H]-thymidine incorporated into the newly synthesized DNA of T cells stimulated to proliferate *in vitro*. In the initial experiments, peptides PT1-PT8 were used together in a cocktail to establish the T cell proliferation assay. This study was carried out using four strains of mice with different MHC class II haplotypes to assess the extent of MHC restriction. The mice tested included: A/J (H-2<sup>k</sup>), BALB/c (H-2<sup>d</sup>), CBA/Ca (H-2<sup>b</sup>), and C57BL/6 (H-2<sup>b</sup>). To determine an effective immunizing peptide dose, mice were immunized subcutaneously at the base of the tail with the peptide cocktail containing either 1 µg, 10 µg, or 100 µg of each peptide emulsified in complete Freund's adjuvant. The animals were rested for 7-10 days and the lymph node cells removed and stimulated *in vitro* with the peptide cocktail over a concentration range of 3.1 nM to 156 µM. Figures 1.2 and 1.3 shows that all three priming doses of peptide were sufficient to activate a T cell response *in vivo*. Animals receiving 10 or 100 µg of peptide showed almost identical proliferative responses *in vitro*. However, mice immunized with 1 µg of peptide, regardless of H-2 haplotype, showed lower responses *in vitro* over the entire range of peptide concentrations tested. This may reflect either a more limited T cell expansion *in vivo* as a result of rapid antigen clearing or alternatively, that the peptide concentrations administered were below the threshold required to effectively displace resident peptides from MHC class II molecules on the surface of antigen presenting cells (Adorini *et al.*, 1991). In all subsequent experiments mice were immunized with 50 µg of peptide/animal. As shown in Figures 1.2 & 1.3, the optimal peptide concentration stimulating proliferation of lymph node populations ranged from 0.31 to 31.2 µM when peptide cocktails were used. In all cases, peptide concentrations of 156 µM exhibited diminished T cell proliferation, which may be attributed to either a cytotoxic effect



**Figure 1.2:** Effects of immunization dose on the proliferative responses of C57BL/6 and CBA/Ca lymphocytes stimulated *in vitro* with a cocktail of synthetic peptides. Mice were immunized subcutaneously with either 1 or 100  $\mu\text{g}$  of each peptide (PT1-PT8) in 100  $\mu\text{l}$  of complete Freund's adjuvant. Eight days post-immunization the draining LNC were removed and stimulated with increasing concentrations of a peptide cocktail containing PT1-PT8.



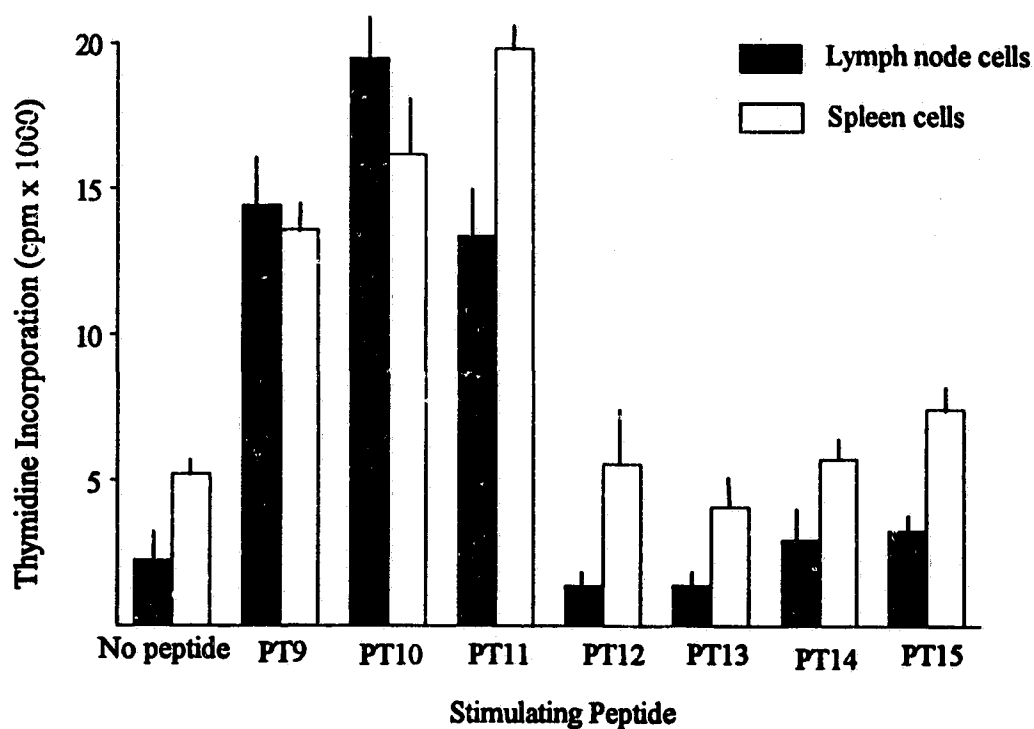
**Figure 1.3:** Effects immunization dose on the proliferative responses of BALB/c and A/J lymphocytes stimulated *in vitro* with a cocktail of synthetic peptides. Mice were immunized subcutaneously with either 1, 10, or 100  $\mu\text{g}$  of each peptide (PT1-PT8) in 100  $\mu\text{l}$  of complete Freund's adjuvant. Eight days post-immunization the draining LNC were removed and stimulated with increasing concentrations of a peptide cocktail containing PT1-PT8.



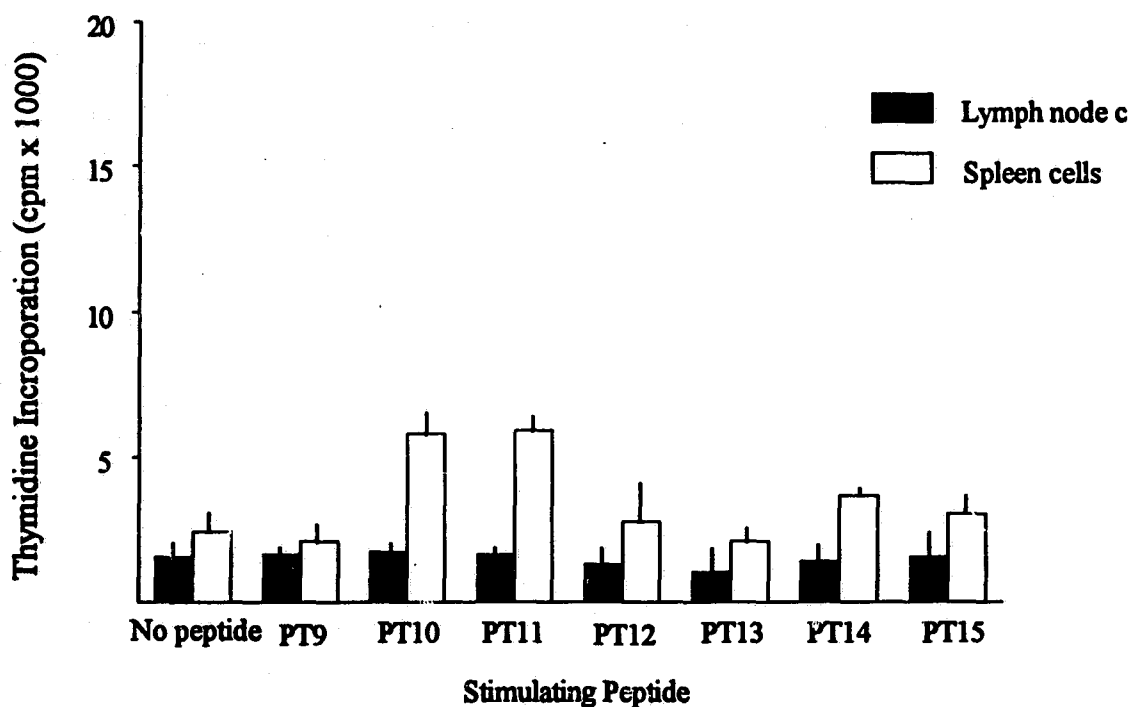
**Figure 1.4:** T cell proliferation dose response curves for seven *L. major* gp63 synthetic peptides PT1-PT8 screened against four mouse strains with differing MHC haplotypes. Data represent averages of triplicate proliferation experiments after subtraction of average control proliferative responses. The average control values were: C57BL/6,  $6.8 \times 10^3$  cpm; BALB/c,  $11.6 \times 10^3$  cpm; CBA/Ca,  $21.8 \times 10^3$  cpm; A/J,  $8.4 \times 10^3$  cpm.

associated with high levels of amphipathic peptides or a rapid proliferation event which peaked prior to the addition of the reporter radionucleotide [ $^3\text{H}$ ]. Lymphocytes of all four mouse haplotypes responded to the *L. major* synthetic peptide cocktail. To further dissect the response to individual peptides mice were immunized and challenged *in vitro* with individual peptides (PT1 - PT8). LNC of three of the four mouse strains, BALB/c, CBA/Ca, and A/J were found to proliferate when challenged with PT3, PT4, and PT6. However, the C57BL/6 lymphocytes recognize only PT6, as shown in Figure 1.4.

Similarly, peptides selected from predicted helix forming sequences of the *L. major* gp63 were screened using the above procedure to identify peptides that stimulated T cells. Unlike the former group of peptides, PT9 - PT15 were screened solely in BALB/c mice. Figure 1.5 shows that three peptides, PT9, PT10, and PT11 stimulated strong T cell proliferation in BALB/c lymph node cells. More importantly, the immune response stimulated by a subcutaneous immunization was not limited to the draining lymph nodes, since splenic T cells responded equally as well when challenged *in vitro* with these peptides (Figure 1.5). In the BALB/c cutaneous leishmaniasis model and human visceral leishmaniasis, this stimulation of splenocytes by synthetic peptides is absolutely essential, since the *Leishmania* parasites responsible for these diseases home primarily to the spleen. Therefore the presence of lymphocytes in this organ might confer protective advantages for the mouse. The route of antigen administration was also shown to be a critical factor in inducing an immune response. As shown above, subcutaneous immunization of BALB/c mice with peptides PT9, PT10, and PT11 emulsified in complete Freund's adjuvant, stimulated a systemic response. However, administration of peptides by an intravenous route, in the absence of adjuvant, failed to prime lymph node cell populations (Figure 1.6). Nonetheless, although eliciting only a weak response (stimulation indices of 2.0), intravenous administration of PT10 and PT11 was found to prime T cell within the spleen. Antigens injected into the lateral tail veins of a mouse drain directly into the spleen. It is possible that high transient concentrations of synthetic T cell epitopes administered into the tail veins may associate with a limited set of antigen presenting cells and would induce a weak cell mediated response.



**Figure 1.5:** Screening putative T cell epitopes (PT9-PT15) identified in the gp63 primary structure by the Garnier *et al.* algorithm (1978). BALB/c mice were immunized subcutaneously with a peptide cocktail containing 100  $\mu$ g of each peptide (PT9-P15) emulsified in complete Freund's adjuvant (100  $\mu$ l). Lymphocytes from the spleen and draining lymph nodes were harvested from mice and stimulated in triplicate with individual peptides at a final concentration of 50  $\mu$ g/ml. Cultures were incubated at 37°C for 96 h. and proliferative responses were measured by pulsing cells with [ $^3$ H]-thymidine for 20 h prior to harvesting cells. Incorporated radiolabel was determined by scintillation counting.

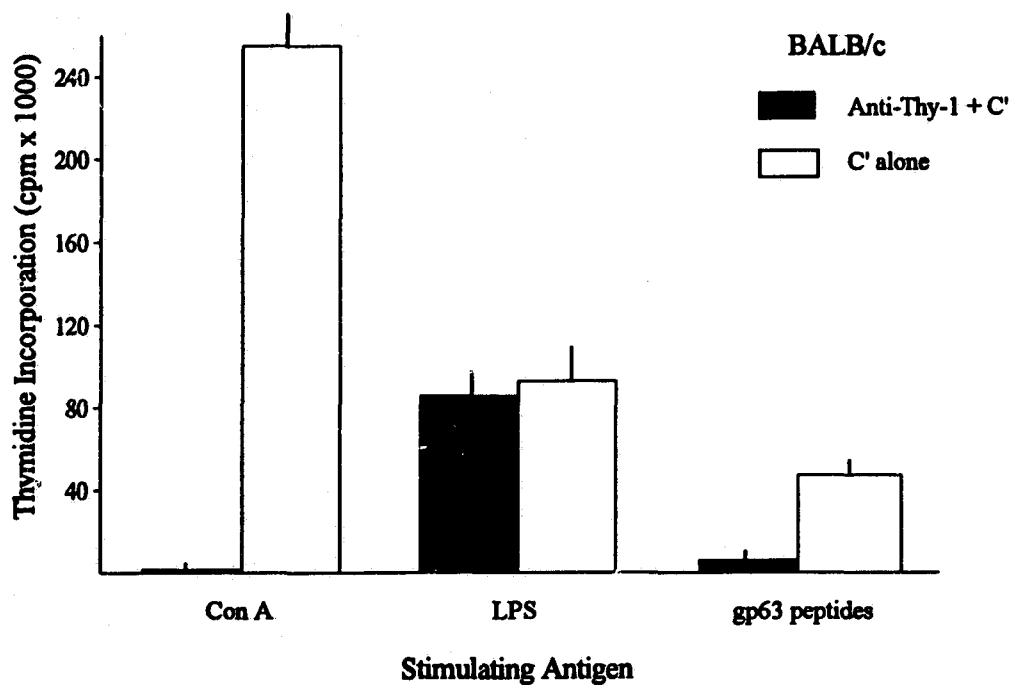


**Figure 1.6:** Proliferative responses of lymph node and splenic lymphocytes obtained from BALB/c mice immunized with synthetic T cell epitopes in the absence of adjuvant. Mice were injected with a cocktail of peptides containing 100  $\mu\text{g}$  of each peptide (PT9-PT15) dissolved in 100  $\mu\text{l}$  of PBS. Eight days later lymph node cells and splenic cells were removed and stimulated separately with individual peptides, PT9 to PT15, at a final concentration of 50  $\mu\text{g}/\text{ml}$ . Cultures were incubated for 96 h and the responses to the peptide stimuli were measured by pulsing cells with 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ]-thymidine 20 h prior to harvesting. Each peptide was used in triplicate wells.

### **1.3 Analysis of T cell Subsets Stimulated by Synthetic Peptides:**

Although the immunization protocols employed in these experiments were designed to preferentially prime T lymphocytes (Corradin *et al.*, 1979), it was essential to verify the phenotype of the cells responding to a peptide challenge, by specifically depleting T cell populations with anti-Thy-1 antisera and rabbit complement. The effectiveness of this procedure was assessed in BALB/c mice by stimulating treated LNCs with either the T cell mitogen concanavalin A (Con A) or the B lymphocyte specific mitogen lipopolysaccharide (LPS) from *E. coli*. Figure 1.7 demonstrates that responses to Con A were completely ablated following antibody treatment without impairing B cell responses to LPS. In addition to the Con A stimulation, the anti-Thy-1 antisera also significantly eliminated all antigen specific proliferations when LNCs were challenged with a cocktail of gp63 peptides (Figure 1.7). This latter observation confirmed that the synthetic epitopes derived from the *L. major* gp63 were activating T cells *in vivo*.

Lymph node cells harvested from mice primed with either a single peptide (PT6), in the case of C57BL/6 mice, or a cocktail of immunostimulatory peptides (PT3, PT4, PT6), for BALB/c and A/J mice, were further characterized to determine the T cell subset stimulated by the individual peptides. LNCs were treated with monoclonal antibodies specific for the murine L3/T4 (CD4<sup>+</sup>) helper T cell marker or the murine Lyt-2.1 (CD8<sup>+</sup>) cytotoxic T cell marker together with Low-Tox rabbit complement to deplete the whole lymph node cell populations of these specific T cell sets. As shown in Figure 1.8, incubation of primed LNC in the absence of antigen resulted in minimal [<sup>3</sup>H]-thymidine incorporation for all three mouse strains, whereas cells treated with complement alone proliferated to the same extent as LNC cultured without prior manipulation. Treatment of cells with anti-CD8 mAbs produced variable responses. For example, BALB/c and A/J mice showed significant decreases in tritiated thymidine incorporation when challenged with either PT3, PT4, or PT6; while PT9 and PT11 in the BALB/c and PT6 in the C57BL/6 mice showed no difference in proliferation in LNC treated with and without anti-CD8 mAbs. The diminished responses for BALB/c and A/J may be attributed to two possibilities: 1) complement lysis due to non-specific binding of antibody or 2) the



**Figure 1.7:** Phenotypic analysis of lymph node cell populations proliferating *in vitro* in response to gp63 peptides. Peptide primed LNC from BALB/c mice were treated with rabbit complement (C') alone or anti-Thy-1 antisera plus rabbit complement (anti-Thy-1 + C') to deplete T cell populations. Cells were then stimulated with the T cell mitogen concanavalin A (2.5  $\mu\text{g/ml}$ ), the B cell mitogen *E. coli* lipopolysaccharide (20  $\mu\text{g/ml}$ ), or a cocktail of gp63 peptides (PT1-PT8) at a concentration of 3.1  $\mu\text{M}$  for each peptide.

presence of a CD8<sup>+</sup> set of T cells which recognize the PT3, PT4, and PT6 (Figure 1.8) (Wang *et al.*, 1993).

A more striking result, illustrated in Figure 1.8, was the complete abrogation of proliferation for all peptides in the three mouse strains after incubation with anti-CD4 antibodies. These data support the contention that the five epitopes derived from the *L. major* gp63, when injected in the presence of complete Freund's adjuvant, activated almost exclusively a CD4<sup>+</sup> T helper cell subset in the draining lymph nodes of mice. Prior to screening these T cell epitopes as potential immunoprotective agents in the murine cutaneous leishmaniasis model, it was essential to characterize the proliferating T cells as Th1 or Th2 according to the lymphokine profiles secreted (Mosmann *et al.*, 1986). The lymphokines secreted by BALB/c, CBA/Ca, and C57BL/6 primed lymph node cells were quantitated with bioassays using the CTLL2 cell line, which is dependent on IL-2 or IL-4 for growth and the DA-1 cell line which requires IL-3. For CTLL2 proliferation due to IL-4, IL-2 was neutralized in the culture supernatant with the mAb S4B6 (Gillis *et al.*, 1978). Similarly, IL-2 levels were measured by neutralizing the IL-4 activity with the mAb 11B11 (Ohara & Paul, 1985). The 48 h culture supernatants of peptide primed BALB/c LNC all contained measurable levels of IL-3 after stimulation with either PT3, PT4, or PT6 (Table 1-2). Whereas, C57BL/6 PT6 cultures supernatants contained no detectable IL-3. As well, no IL-4 was found in the culture supernatants of any of the mouse strains tested in response to the stimulatory peptides. However, stimulation of BALB/c and CBA/Ca LNC with PT3 produced significant levels of IL-2, 0.8 and 1.9 units/ml, respectively (Table 1-2). These data, together with the fact that the proliferating T cells were of the CD4<sup>+</sup> phenotype was diagnostic of the BALB/c and CBA/Ca cells stimulated by PT3 belonging to the Th1 subset. The sustained ability of these PT3 specific cells to secrete lymphokines *in vitro* was examined by assaying BALB/c culture supernatants for the above lymphokines after a secondary peptide challenge. As in the primary stimulation, all three peptides resulted in the production of IL-3, the levels of which were only slightly increased (5.1, 0.3, and 8.3 U/ml for PT3, PT4, and PT6, respectively). In comparison to the primary challenge the restimulation of the LNC populations with PT3 produced a 5 fold increase in IL-2 (4.4 U/ml) above background.

TABLE 1-2

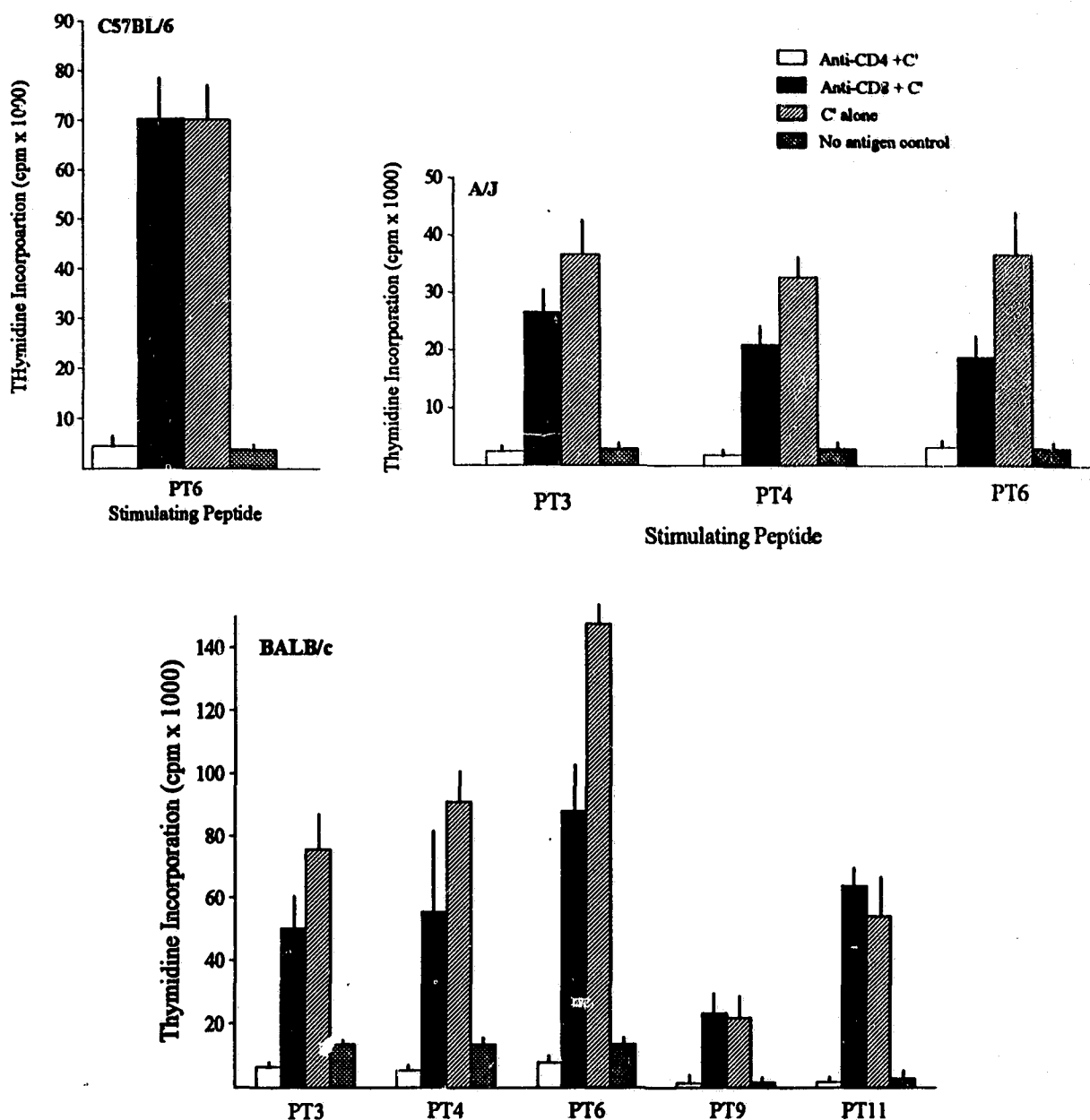
Lymphokine production by lymph node cells stimulated *in vitro* with  
*L. major* gp63 synthetic peptides<sup>a,b</sup>

Challenge Peptide	Lymphokines (U/ml)*								
	IL-2			IL-3			IL-4		
	BALB/c	CBA/Ca	C57BL/6	BALB/c	CBA/Ca	C57BL/6	BALB/c	CBA/Ca	C57BL/6
PT3	0.81	1.81	-	3.8	ND	-	0	0	-
PT4	0	0	-	0.3	ND	-	0	0	-
PT6	0	0	0	7	ND	0	0	0	0

\* Values represent the difference in cytokine production between non-stimulated and peptide stimulated lymph node cell populations.

*a* Values are an average of lymphokines produced in two separate experiments.

*b* Primed LNC were harvested and stimulated with 50 µg/ml of individual peptides and the culture supernatants were collected 48 h after challenge and then assayed.



**Figure 1.8:** Determination of T cell subsets stimulated by synthetic T cell epitopes *in vitro*. Groups of mice were primed subcutaneously with either individual or a cocktail of immunostimulatory peptides emulsified in complete Freund's adjuvant. Seven days later the draining lymph nodes removed and cells were either treated with low-tox rabbit complement, anti-CD4 mAb plus complement, anti-CD8 mAb plus complement, or put directly into culture without treatment. Cells were stimulated in triplicate with the appropriate peptide at a concentration of 50  $\mu\text{g}/\text{ml}$  for 96 h. Proliferation was assessed by incorporation of [ $^3\text{H}$ ]-thymidine for 20 h prior to harvesting cells.

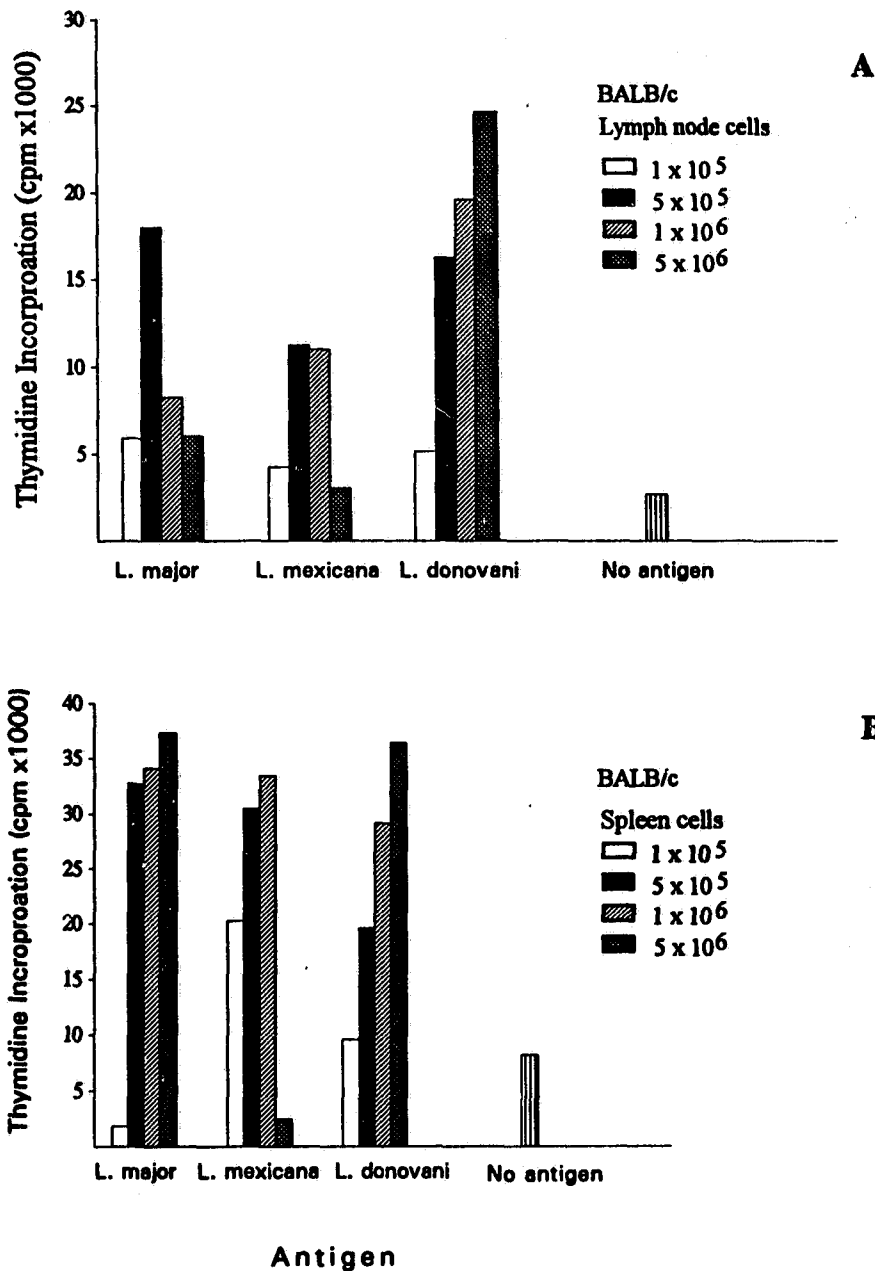
These results indicate that the production of IL-2 in the primary challenge was not transient and that the expansion of the PT3 specific T cell clones was continuing.

#### **1.4 *In vitro* Stimulation of Peptide Primed T Lymphocytes with *Leishmania***

##### **Promastigotes:**

Although six synthetic peptides derived from the *L. major* gp63 sequence were found to be immunogenic in mice, in order to evaluate their potential as peptide vaccines it was essential to demonstrate that APCs could process parasite associated gp63 and stimulate peptide primed lymphocytes. To assess the processing efficiency of APC, BALB/c mice were immunized subcutaneously with a cocktail of synthetic peptides (PT1-15) emulsified in complete Freund's adjuvant and both the draining lymph nodes and splenic lymphocytes were subsequently challenged *in vitro* with a range of  $1 \times 10^5$  to  $5 \times 10^6$  whole heat killed promastigotes. Lymph node populations stimulated with *L. major* promastigotes showed a narrow peak of proliferation with  $5 \times 10^5$  promastigotes giving an optimal stimulation index (SI) of 7.5 (Figure 1.9A). In the case of *L. mexicana*, the proliferative responses observed for the peptide primed lymph node cells were over a broader range and  $5 \times 10^5$  and  $1 \times 10^6$  parasites/ well gave maximum incorporation of tritiated thymidine. A notable difference between results for *L. mexicana* and those of the *L. major* challenge was the lower proliferative responses for *L. mexicana* (SI values of 4.5 and 4.3). However, unlike *L. major* and *L. mexicana*, stimulation of LNC by *L. donovani* promastigotes showed a progressive increase in [ $^3\text{H}$ ] thymidine incorporation which correlated with the number of promastigotes used to challenge the lymphocytes. The maximal response had an SI value of 10.

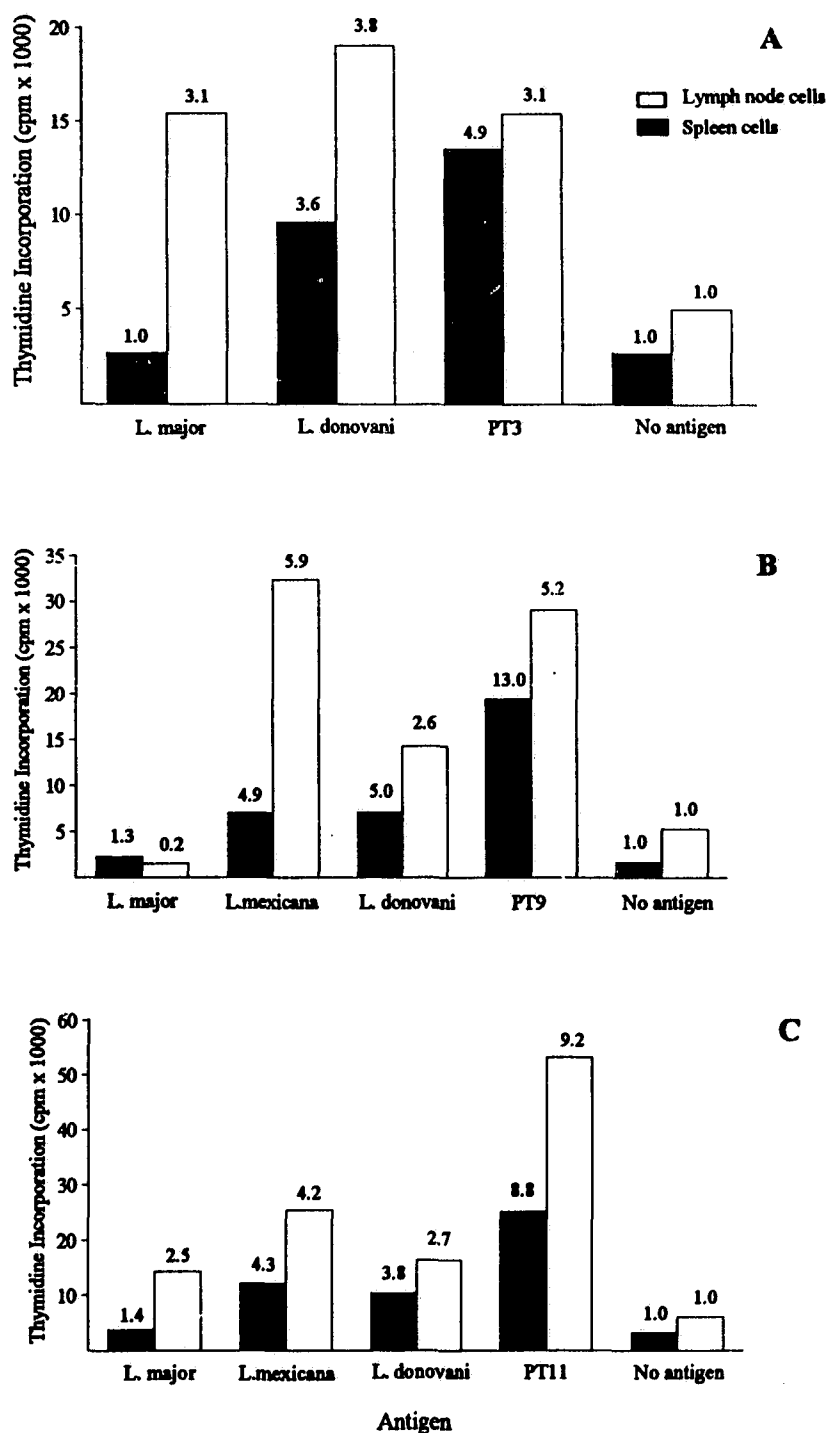
Priming mice subcutaneously with these peptides not only initiated an immune response in the draining lymph nodes, but the spleen was also found to contain lymphocytes which proliferated when challenged with all three species of promastigotes. However splenic populations showed subtle differences in proliferation which may affect a differential ability of lymph node and splenic macrophage to present antigen. For example, *L. major* promastigotes, used at densities of  $5 \times 10^5$  to  $5 \times 10^6$  resulted in a near equivalent response in the spleen, whereas in the LNC, significant proliferation was



**Figure 1.9:** Stimulation of peptide primed BALB/c lymphocytes with *Leishmania* promastigotes. Mice were immunized subcutaneously with a cocktail of synthetic peptides (PT1-15) emulsified in complete Freund's adjuvant. Eight days post-immunization the draining lymph nodes (A) and the spleen (B) were removed and the cells challenged with live *Leishmania* promastigotes.

observed with only  $5 \times 10^5$  promastigotes/well. The response observed in lymph node cells following a challenge with either *L. donovani* or *L. mexicana* was mirrored by the splenocytes (Figure 1.9B).

The response to the peptide cocktail was further dissected by determining if whole promastigotes could be processed *in vitro* to generate T cell epitopes recognizable by T cells stimulated *in vivo* with synthetic peptides. To this end, LNC and splenocytes from BALB/C mice were immunized subcutaneously with PT3, PT9, or PT11 in complete Freund's adjuvant and challenged again *in vitro* with late log phase *L. major* A2, *L. mexicana mexicana* or *L. donovani* LD3 promastigotes. Results are shown in figure 1.10. Both lymph node and splenic lymphocytes proliferated *in vitro* to PT3 with stimulation indices of 4.9 and 3.1 respectively. It should be noted that these responses were lower in this particular experiment than previously observed. Lymph node and splenic populations challenged with heat killed *L. donovani* promastigotes responded with SIs of 3.6 and 3.8 respectively (Figure 1.10A). Unexpectedly, when these primed cells were challenged with *L. major*, promastigotes only the LNC were stimulated to divide. This was perplexing as the PT3 sequence was derived from the *L. major* gp63 protein sequence. A similar result was found with peptide PT9. As shown in Figure 1.10B, PT9 primed lymphocytes not only responded strongly to a self challenge but also to *L. donovani* and *L. mexicana mexicana* promastigotes in both lymph node and splenic populations. But once again, the PT9 primed BALB/c cells, stimulated with *L. major* promastigotes gave only background responses. On the other hand, results of studies with PT11 primed cells differed somewhat from the above findings. Vigorous proliferation was observed when lymph node and splenic lymphocytes were challenged with PT11 (Figure 1.10C) while splenic T cells were only moderately stimulated when challenged with *L. mexicana mexicana* (SI of 4.2) whereas, both *L. donovani* and *L. major* promastigotes gave only marginally significant responses (SIs of 2.7 and 2.5). In LNC populations, moderate responses were obtained with *L. mexicana mexicana* and *L. donovani* promastigotes (SIs 4.3 and 3.8 respectively), while LNC T cells showed only background levels of [ $^3\text{H}$ ]- thymidine incorporation in the presence of *L. major*.

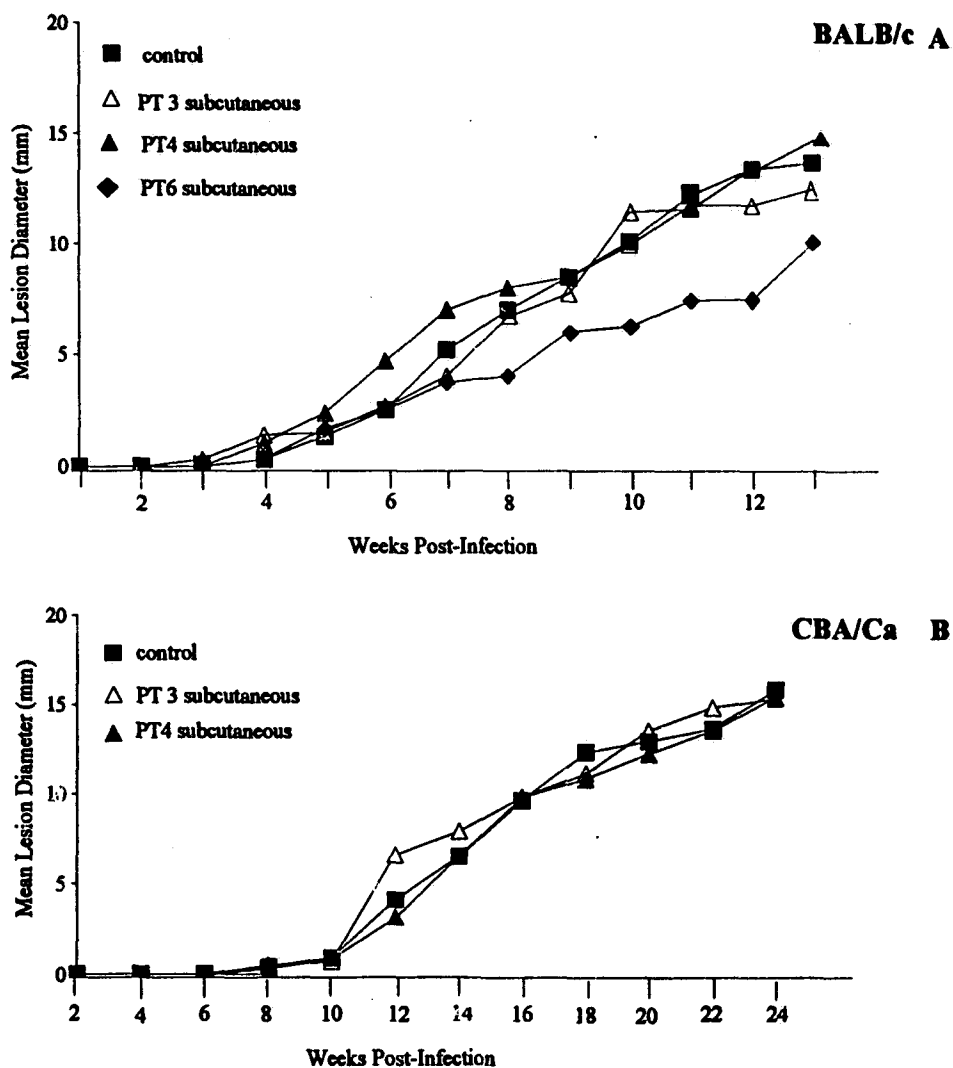


**Figure 1.10:** Stimulation of BALB/c lymphocytes primed with individual synthetic T cell epitopes with *Leishmania* promastigotes. Mice were primed with either PT3 (A), PT9 (B), or PT11 (C), eight days post-immunization the draining lymph node and the spleens were stimulated  $5 \times 10^5$  live promastigotes/well. Values over each bar indicate the stimulation index, which is the ratio of [ $^3\text{H}$ ]-thymidine incorporation of the antigen stimulated lymphocyte cultures over the unstimulated cultures.

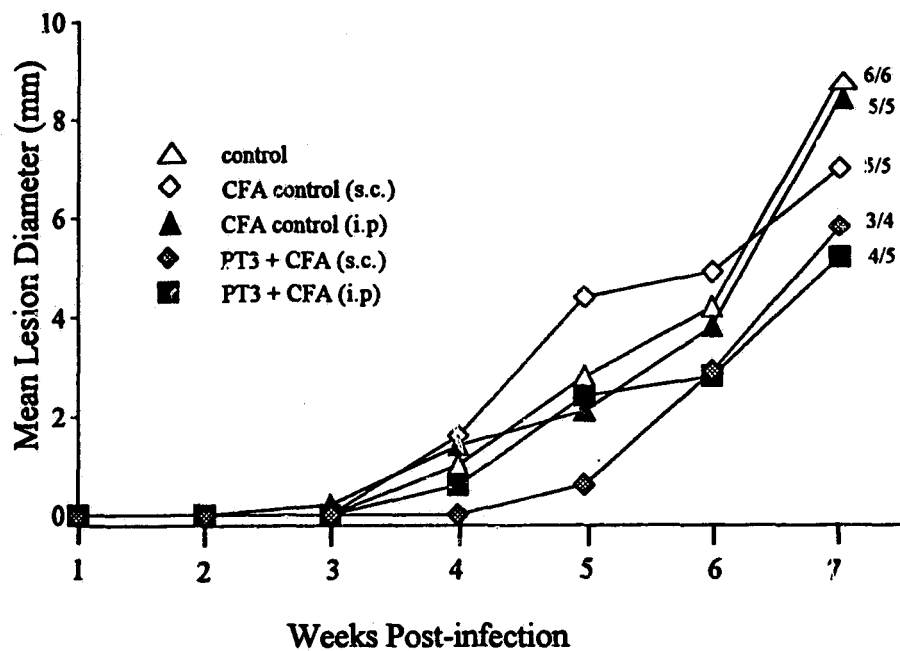
## **1.5 Immunoprotection of BALB/C and CBA/Ca Mice Against Cutaneous**

### **Leishmaniasis by Vaccination with Synthetic T Cell Epitopes:**

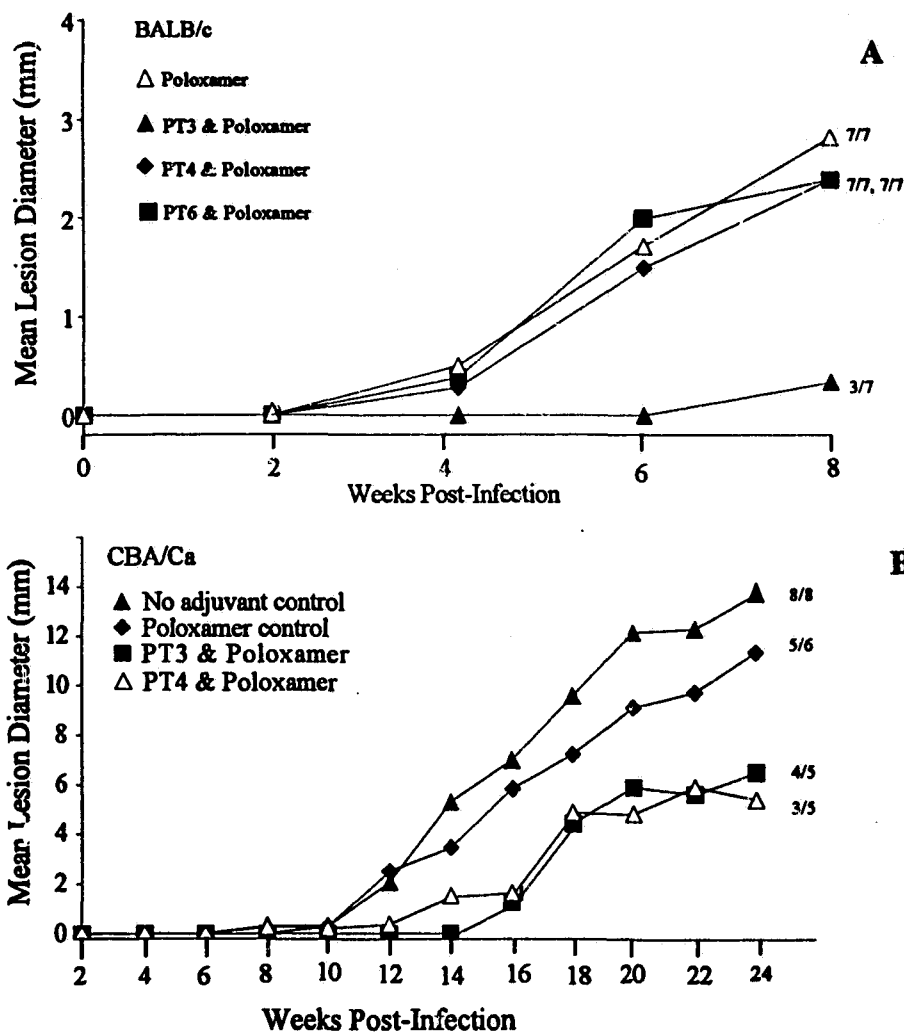
From the initial group of seven peptides selected using the Rothbard and Taylor algorithm (1988) peptides PT3, PT4, and PT6 were found to stimulate proliferation of a CD4<sup>+</sup> T cell subset *in vitro*. Furthermore, one of these peptides, PT3, activated a Th1 like response, as indicated by the secretion of the lymphokine IL-2 (Mosmann *et al.*, 1986). This subset of lymphocytes had been demonstrated to be essential in the protection of mice against cutaneous leishmaniasis (Heinzel *et al.*, 1991; Locksley, 1987; Scott *et al.*, 1988). The ability of these synthetic epitopes to protect mice against a *Leishmania* challenge was evaluated in both BALB/c and CBA/C mouse strains which are susceptible to *L. major* and *L. mexicana* promastigotes respectively and develop cutaneous leishmaniasis upon infection. The following set of experiments were undertaken to clarify two key points: 1) are chemically defined synthetic T cell epitopes sufficient to initiate protective immunity against *Leishmania*? 2) what role does adjuvant play in potentiating a protective immune response? To address the latter question, BALB/c or CBA/Ca mice immunized subcutaneously (s.c.) with individual synthetic peptides (PT3, PT4, and PT6) in PBS, were then challenged with either *L. major* or *L. mexicana* 4 weeks after immunization. As shown in Figure 1.11, BALB/c or CBA/Ca mice receiving peptide in the absence of adjuvant showed no difference in lesion development when compared to control animals injected with PBS alone. However, BALB/c mice immunized s.c. with PT3 in complete Freund's adjuvant (CFA) showed only marginal immunoprotective effects as shown by a 2 week delay in the development of cutaneous lesions (Figure 1.12). Once erupted, lesion size increased dramatically with 3 of 4 mice showing a mean lesion diameter of  $5.2 \pm 1.6$  mm, which was nonetheless significantly smaller than the control group injected with PBS alone. The minimal control of the *L. major* promastigote infection was not due to adjuvant alone as mice receiving CFA showed a disease pattern similar to the PBS controls. Although no conclusive statements can be drawn from this experiment alone, the data presented in Figure 1.12, together with previous reports in the literature (Liew *et al.*, 1985a; Russell & Alexander 1988), suggested that the route of vaccination may influence the nature of the elicited immune response. Administration of PT3 together with complete



**Figure 1.11:** Immunoprotective effects against cutaneous leishmaniasis in BALB/c mice immunized with synthetic T cell epitopes in PBS. Groups of BALB/c or CBA/Ca mice were immunized subcutaneously at the base of the tail with 100  $\mu$ g of each peptide in phosphate buffered saline. After resting the animals for four weeks, mice were challenged in the hind flank with (A)  $2 \times 10^5$  *L. major* LV39 or (B)  $2 \times 10^5$  *L. mexicana mexicana* promastigotes. The disease process was monitored by measuring the resulting lesions at the site of inoculation with Vernier callipers.



**Figure 1.12:** Immunoprotective effects against cutaneous leishmaniasis in BALB/c mice immunized with synthetic T cell epitopes in complete Freund's adjuvant. Groups of BALB/c mice were immunized subcutaneously in the flank with 100  $\mu$ g of the peptide, PT3, emulsified in complete Freund's adjuvant or adjuvant alone. Animals were rested for 4 weeks after immunization then challenged with  $2 \times 10^5$  *L. major* LV39 promastigotes in the hind quarters. The disease process was monitored by measuring diameter of lesions which erupted at the site of inoculation.



**Figure 1.13: Immunoprotective effects of synthetic T cell epitopes administered in Poloxamer 407 against cutaneous leishmaniasis.** Groups of BALB/c or CBA/Ca mice were immunized subcutaneously at the base of the tail with a single 100  $\mu$ g dose of peptide in 100  $\mu$ l of 8% Poloxamer 407 as the adjuvant. After a six week rest period, mice were challenged in the hind flank with (A)  $2 \times 10^5$  *L. major* LV39 or (B)  $2 \times 10^5$  *L. mexicana mexicana* promastigotes. The diameter of the cutaneous lesions which developed at the site of infection were measured with Vernier callipers to monitor disease development.

Freunds adjuvant, via an intraperitoneal route, resulted in lesions developing at the same rate as the PBS or CFA intraperitoneal (i.p.) control animals early in infection. It should be noted, however, that by week 7 in BALB/c mice receiving PT3 plus CFA, only 3/4 of mice immunized subcutaneously (s.c.) and 4/5 of mice immunized i.p. had measurable lesions, which were significantly smaller than the control groups (Figure 1.12).

To further investigate the potential role of adjuvants in modulating the immune response for protection against *Leishmania*, the synthetic T cell epitopes were administered s.c. mixed with the phase transition gel Poloxamer 407 (Gilbert *et al.*, 1986). After a single 100 µg dose of peptide, animals were rested for minimum of 6 weeks prior to challenge with either *L. major* or *L. mexicana* promastigotes. The data presented in Figure 1.13A shows that BALB/c mice immunized with either Poloxamer 407 alone, Poloxamer 407 and PT4, or Poloxamer 407 and PT6 could not control the *L. major* infection, with all three groups of animals developing measurable lesions 4 weeks after challenge. Conversely, mice immunized with Poloxamer 407 and PT3 were highly competent in attenuating the onset of cutaneous leishmaniasis. The appearance of small lesions in a few mice did not occur until 8 weeks after infection with *L. major*, and one month after the control group. At the time this experiment was terminated, 3 of the 7 mice showed small skin lesions of a mean diameter of  $0.3 \pm 0.1$  mm, whereas 7/7 animals in the other three groups had large lesions ranging from  $5.6 \pm 0.7$  mm to  $4.9 \pm 0.45$  mm. Similar results were also obtained for the CBA/Ca and *L. mexicana* cutaneous leishmaniasis model, Figure 1.13B. In this system, lesions did not appear in control groups receiving either PBS or Poloxamer alone until approximately 12 weeks post infection with *L. mexicana* promastigotes. As in the BALB/C model, PT3 administered in conjunction with Poloxamer 407 was successful in controlling lesion eruptions until 16 weeks post-infection. Unexpectedly, PT4 and Poloxamer 407 also exhibited protective effects in CBA/Ca mice (Figure 1.13B), although this epitope failed to stimulate a Th1 phenotype *in vitro* in this mouse strain. Immunoprophylactic treatment of mice with either PT3 or PT4 resulted in development of smaller lesions peaking in size by week 18 with no appreciable change, even after 24 weeks of infection. These results demonstrate that synthetic peptides are capable of activating an antigen specific immune response against

***Leishmania.*** However the choice of adjuvant was a key factor in achieving an immunoprotective effect.

## DISCUSSION

Recent immunoprotection trials conducted by Russell and Alexander (1988) and Yang *et al.* (1990) have shown that gp63 induces protective immunity against cutaneous leishmaniasis in CBA mice caused by either *L. major* or *L. mexicana mexicana* promastigotes. More importantly, gp63 was also shown to stimulate proliferation of human peripheral blood monocytes (PBMC) obtained from leishmaniasis patients, recovered patients, or from subjects immunized with a polyvalent mixture of promastigotes (Mendonça *et al.*, 1991; Nascimento *et al.*, 1990; Russo *et al.*, 1991). In one of these studies (Russo *et al.*, 1991), PBMCs were found to secrete IL-2 and IFN- $\gamma$  in response to a gp63 challenge. Both of these lymphokines have been found to be essential in the clearance of *Leishmania* parasites (Nacy *et al.*, 1985; Murray *et al.*, 1985; Murray *et al.*, 1987). In light of the fact that complex antigens, such as attenuated whole promastigotes have been shown to exacerbate murine cutaneous leishmaniasis (Liew *et al.*, 1985a, 1985b; Titus *et al.*, 1984), the focus of the present study was to identify and characterize synthetic T cell epitopes, from the *L. major* gp63 sequence, capable of inducing development of a Th1 CD4<sup>+</sup> T cell subset capable of protecting susceptible mice against leishmaniasis.

By employing the Rothbard and Taylor algorithm (1988), seven peptides were identified, three of which PT3, PT4, and PT6 stimulated T cell proliferative activity in an array of different mouse H-2 haplotypes. An additional set of eight peptides were also synthesized based upon the secondary structure of gp63, as proposed by the Garnier *et al.* algorithm (1978). Again, three of these peptides were found to be immunostimulatory in BALB/c mice. The rationale underlying the latter approach in identifying T cell epitopes, was based on the fact that the preponderance of immunodominant epitopes characterized to date adopt an amphipathic helical conformation with a hydrophobic epitope which binds in the cleft of the MHC class II molecule and a hydrophilic epitope domain which binds the T cell receptor (Allen, 1987; Bjorkman *et al.*, 1987; DeLisi & Berzofsky, 1985; Waltho *et al.*, 1989; Rothbard & Taylor, 1988; Spouge *et al.*, 1987). Indeed, placement

of the immunoactive peptides selected by both Rothbard and Taylor motif (1988) and the Garnier *et al.* algorithm (1978) on a helical wheel (Schiffer & Edmundson, 1967) showed a strong propensity to form amphipathic structures.

Subsequent to the publication of these findings, Yang *et al.* (1991) using the AMPHI algorithm (Margalit *et al.*, 1987) synthesized a series of 24 peptides covering 75% of the gp63 sequence. However, when these putative T cell epitopes were screened in BALB/c mice, only one peptide (p10-28) stimulated a T cell response. In contrast, 6 of the 15 peptides identified in this study, some of which shared significant sequence identity with peptides reported by Yang *et al.* (1991), were found to be potent stimulators of BALB/c lymph node cells primed *in vivo* with synthetic peptides. Of the 24 peptides screened by Yang *et al.* (1991) only 6 peptides were found to be potent T cell immunogens and only one peptide, spanning the residues p146-171, coincided with epitopes identified in this lab (PT3 and PT4). A further noteworthy observation was that the peptides p378-396 and p395-414 synthesized by Yang *et al.* (1991) had sequences almost identical to PT6 and PT8 respectively, yet opposite results were obtained. P395-414 was found to be immunostimulatory, whereas PT8 was not and PT6 was found to be a strong stimulator, not only of the CBA mouse strain but also of the A/J, BALB/c, and C57BL/6, whereas Yang *et al.* (1991) found p378-396 lacking in activity. The discrepancies observed between these studies may be attributed to a number of differences in the experimental design. For example, the amino acid sequences used in the two studies differed slightly, and more importantly different adjuvants were employed in both studies.

For the synthetic T cell epitopes derived from the *L. major* gp63 primary sequence to be of immunological significance as a protective vaccine it is essential that T cell epitopes generated from whole promastigotes by antigen processing cells cross react with T cells primed with synthetic peptides. To this end, lymph node and splenic lymphocytes from BALB/c mice primed subcutaneously with PT3, PT9, or PT11 were challenged with either *L. major*, *L. mexicana*, or *L. donovani* promastigotes known to be the causative agents of cutaneous, mucocutaneous, or visceral leishmaniasis, respectively. *In vitro*, *L. donovani* and *L. mexicana* promastigotes stimulated a significant T cell response in both spleen and lymph node cells of animals primed with synthetic peptides, indicating that

APCs processed and presented peptides derived from native gp63 which were analogous to the epitopes predicted by Rothbard and Taylor (1988) and the Garnier et al. (1978) algorithms. Although the peptides used to prime BALB/c mice corresponded to the *L. major* gp63 sequence, it was surprising to find that save the PT3 primed LNC, the *L. major* promastigotes failed to stimulate a significant T cell response in peptide primed lymph node lymphocytes. This effect was even more pronounced with splenic T cells which incorporated only background levels of [<sup>3</sup>H]-thymidine. It is possible that the impaired proliferative response to the *L. major* promastigotes may be due to a parasite dependent down regulation in Ia molecules on the antigen presenting cells as previously reported by others (Reiner *et al.*, 1988). Alternatively, the high levels of gp63 expressed by *L. major* A2 strain may result in a decreased proliferative response due to a tolerance phenomena associated with high levels of antigen. These proposed immunosuppressive effects alone cannot account for the lack of stimulation, since LNC and splenic lymphocytes from BALB/c mice immunized with a cocktail containing the 15 synthetic peptides proliferated strongly *in vitro* when challenged with *L. major* promastigotes.

Recent studies characterizing the cell mediated immunity of human patients recovered from leishmaniasis or subjects immunized with a polyvalent mixture of *Leishmania* promastigotes have implicated gp63 as an immunodominant antigen recognized by the human immune system (Nascimento *et al.*, 1990; Russo *et al.*, 1991). These data, together with the fact that mice immunized with gp63 in liposomes or recombinant gp63 expressed in either *Salmonella typhimurium* or BCG were protected against cutaneous leishmaniasis, make gp63 an ideal candidate for the development of a molecularly defined vaccine (Connell *et al.*, 1993; Russell & Alexander, 1988; Yang *et al.*, 1990). In this connection, the immune response of human peripheral blood lymphocytes (PBMC) to the putative gp63 epitopes, were investigated in a collaborative study with Dr. S.G. Reed of the Seattle Biomedical Institute. The 15 synthetic peptides were screened against PBMCs from patients recovered from either cutaneous, mucocutaneous, or visceral leishmaniasis, all of New World origin (Russo *et al.*, 1993). The panel of stimulatory peptides observed in this study overlapped, but were not identical with those found in the murine system. In mice PT3, PT4, PT6, PT9, PT10, and PT11 induced

strong proliferation. In humans, the responses were more varied, presumably due to the outbred populations. However peptides PT4, PT7, and PT8 induced a proliferative response in most of the subjects examined, regardless of the clinical form of leishmaniasis. Other peptides exhibiting a more limited stimulatory activity included PT9, PT11, and PT13. It is curious, however, that although PT6 and PT7 share a 9 residue overlap, only the former peptide stimulated murine T cells while the latter peptide, PT7, induced a response only with human PBMC. It is possible that the sequences unique to each peptide differentially control either the binding to the major histocompatibility antigens or the T cell receptor recognition of the epitopes.

Analysis of the PBMC culture supernatants from patients with different forms of leishmaniasis stimulated with PT4, PT7, or PT8 were found to contain high levels of IFN- $\gamma$ . FACS analysis of a PT7 specific T cell line derived from recovered patients indicated the presence of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. This observation was intriguing since it indicated that a single epitope could be recognized by cytotoxic and T helper cells. These results have implications in our understanding of the immunological events attending disease resolution. In mice, a body of evidence has been amassed indicating that CD4<sup>+</sup> helper T cells are essential for primary resistance to leishmaniasis, while CD8<sup>+</sup> cells function in a more limited capacity in controlling these *Leishmania* infections (Farrell *et al.*, 1989; Locksley *et al.*, 1987; Scott *et al.*, 1989; Titus *et al.*, 1987). However, recent studies in both humans and mice have shown that clearance of the initial parasite burden correlates with an increase in the percentage of CD8<sup>+</sup> cells recognizing *Leishmania* antigens (Müller *et al.*, 1994; Da-Cruz *et al.*, 1994; Wang *et al.*, 1993). These cells have been implicated in the production of high levels of IFN- $\gamma$  which confers resistance to secondary *Leishmania* infections.

A noteworthy observation regarding stimulatory activity of peptides PT3, PT4, and PT6 in mice and PT7 in humans was the promiscuous nature of these synthetic epitopes which could activate a T cell response in several different MHC class II haplotypes. The possibility that these peptides exhibited mitogenic activation of T cells was discounted, since addition of the above peptides to naive lymph node cells failed to elicit T cell division. Similar pan proliferative responses have been reported with synthetic

T cell epitopes derived from the malaria circumsporozoite protein (378-398) and tetanus toxin peptides p2 and P30 (Panina-Bordignon *et al.*, 1989; Sinigaglia *et al.*, 1988). Although the reason for this MHC class II tolerance is unknown, it has been suggested that these peptides interact primarily with the conserved DR $\alpha$  or I-E $\alpha$  chains of the MHC molecule (Sinigaglia *et al.*, 1988). The promiscuous phenomena exhibited by these synthetic epitopes is dependent on peptide length, as removal of 3-4 residues from the amino or carboxy termini of the circumsporozoite peptide limited its binding to a more restricted group of HLA-DR alleles (Sinigaglia *et al.*, 1988). It is tempting to speculate that the tendency for a peptide to bind to a variety of MHC class II types may be associated with structural features of the peptide itself. A sequence comparison of PT3, PT4, tetanus toxin peptide p2, and the circumsporozoite peptide (378-398) shows a preference for basic residues with the T cell epitopes predicted by the Rothbard and Taylor algorithm (1988) (bold sequence illustrated below).

Circumsporozoite peptide (378-398)	<b>DIEK<b>K</b>IA<b>K</b>ME<b>K</b>ASSVF<b>N</b>V<b>V</b>NS</b>
PT3	<b>YDQLVTRV<b>V</b>THE<b>M</b>HA</b>
PT4	<b>VTRV<b>V</b>THE<b>M</b>HALGFSG</b>
Tetanus toxin p2	<b>QYIKAN<b>S</b>K<b>F</b>IG<b>I</b>E<b>K</b></b>

Analysis of the murine LNC stimulated by the gp63 synthetic epitopes showed that a CD4<sup>+</sup> helper T cell population was consistently activated by all peptides tested. That these immunogenic peptides failed to prime T lymphocytes *in vivo* in the absence of adjuvant, indicated that the resultant cell mediated response to these synthetic peptides was in part modulated by the complete Freund's adjuvant (CFA), which has been previously shown to preferentially elicit a Th1 like response (Grun & Maurer, 1989). Although it is well accepted that adjuvants can potentiate the outcome of an immune response, Liew *et al.* (1990) have recently obtained evidence showing that the structure of the synthetic epitope can in itself direct a Th2 response, even when administered in CFA. The precise role of adjuvants in modulating the immune system is not well understood,

however, it is proposed that these immunoactive agents act by: 1) providing a depot for slow release and increase the exposure of antigen to the immune system (Bomford, 1989), 2) absorbing or entrapping peptides or proteins into a particulate form which facilitates the uptake and subsequent presentation of antigen by macrophage, 3) stimulating the release of co-stimulatory factors (Virelizier *et al.*, 1989), 4) and up regulating the expression of MHC class II molecules by antigen presenting cells (Howerton *et al.*, 1990; Virelizier *et al.*, 1989).

When PT3 is administered with CFA it induces a Th1 response which affords BALB/c mice partial protection against a challenge with *L. major* LV39 promastigotes (This study; Wang *et al.*, 1993; Yang, *et al.*, 1991). However, in parallel experiments where immunized BALB/c mice were infected with the *L. major* 173 strain of promastigotes, no protection was observed (Wang *et al.*, 1993). This result may be attributed to several factors. First, the *L. major* 173 strain rapidly visceralize after inoculation, whereas the LV39 parasites visceralize and immunosuppress the host only in the advanced stage of the disease (Alexander, 1993). Secondly, the *L. major* 173 amastigotes fail to express detectable levels of gp63 (Funk *et al.*, 1994) which would preclude the activation of PT3 specific memory T cells required to control the infection.

Since complete Freund's adjuvant is not recommended for most human or veterinary applications it was essential to identify an alternative adjuvant which could be employed together with synthetic peptide for vaccination of humans. One such candidate, was Poloxamer 407, which is a block copolymer of polypropylene oxide and ethylene oxide (average M.W. 12,600) with a gel forming property at temperatures above 10°C (Gardener & Jones, 1984). This surface active agent had been previously used as a delivery vehicle for pharmaceuticals in humans, with no toxic side effects (Gibbs *et al.*, 1976). In addition, other copolymers which are structurally related to Poloxamer 407 have been shown to be potent stimulators of humoral immunity (Hunter & Bennett, 1984). These adjuvants were also found to increase the expression of the MHC class II molecules on macrophage thereby enhancing antigen presentation to T cells (Howerton *et al.*, 1990). Immunoprotection trials with PT3 and Poloxamer 407 injected via a s.c. route induced a significant degree of protection against cutaneous leishmaniasis in both

susceptible and moderately resistant mouse strains. In BALB/c mice PT3/poloxamer delayed the onset of the disease by 6 weeks with only 3 of 7 animals showing measurable lesions. In contrast all control animals receiving PBS alone had large lesions at the site of inoculation. This protective effect could not be attributed to adjuvant alone as the control group injected with Poloxamer 407 developed skin ulcerations comparable to the PBS controls. In CBA mice, the effects of Poloxamer 407 were even more dramatic, as this immunopotentiating agent together with the T cell epitopes PT3 or PT4, not only delayed the disease process by 6 weeks, but resulted in the development of a cell mediated immunity capable of keeping the lesion size in check. In a similar experiment conducted by Williams (1993), in which the Poloxamer concentration was increased from 8 to 15%, CBA mice immunized with PT3/poloxamer were completely protected against a challenge with *L. mexicana* promastigotes, with no detectable lesions being observed up to 18 weeks after infection.

To date, subcutaneous immunizations of resistant or susceptible mice with crude *Leishmania* antigens has resulted in disease exacerbation. Here it is demonstrated that by the judicious selection of T cell epitopes and adjuvants which stimulate a Th1 response that protection via the s.c. route can be achieved. Although the mechanism of action of Poloxamer 407 is unknown, it is clear that the vesicles formed by the gel phase at physiological temperatures, may facilitate a slow antigen release which continually primes the immune system (Fults & Johnston, 1990). Recent reports have also documented that liposomes or micropolystyrene beads coated with Poloxamer 407 were preferentially phagocytized by bone marrow macrophage (Moghimi *et al.*, 1991). The latter observation has significant implications since the work by Kahl *et al.* (1989, 1990) indicates that antigen loaded distearoyl phosphatidylcholine/cholesterol liposomes are targeted to bone marrow macrophage which induce a persistent host protective immune response. Alternatively Poloxamer 407 may modulate the development of a Th1 protective immunity by inducing interleukin-12 production in tissue macrophage. This hypothesis is supported by the findings of Hsieh *et al.* (1993) showing that macrophage cultured with heat killed *Listeria* induced IL-12 production which correlated with a subsequent development of a Th1 T helper cells specific for *Listeria* antigens. Afonso *et al.* (1994) have expanded on

these recent findings and demonstrated that subcutaneous immunization of BALB/c mice with soluble *Leishmania* antigen together with IL-12 completely protected mice against promastigotes.

In conclusion, this work was the first to show that the use of molecularly defined T cell epitopes which stimulate a Th1 like response *in vitro*, can protect mice against cutaneous leishmaniasis when administered with an appropriate adjuvant. In addition, the strong T cell proliferative responses obtained from PBMCs isolated from humans (Mendonça *et al.*, 1991; Russo *et al.*, 1991 & 1993) or vervet monkeys (Curry *et al.*, 1994) recovered from leishmaniasis, indicated that these peptides may be ideal vaccine candidates for human use. However, due to the outbred nature of human populations it will likely be essential to provide a cocktail of immunodominant Th1 T cell stimulatory peptides for such a subunit vaccine to be effective. Epitopes could be derived not only from gp63 but also from other recently discovered proteins, such as *L. donovani* dp72 (Jaffe *et al.*, 1990) and p80 (White & McMahon-Pratt, 1990) as well as from the *L. mexicana* gp46 (Lohman *et al.*, 1990), all of which have recently been shown to protect mice against leishmaniasis.

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**CHAPTER 2**

**T Lymphocyte Proliferative Responses to the *Leishmania*  
Lipophosphoglycan-Associated Protein**

## INTRODUCTION

*Leishmania donovani* parasites are protozoans which cause a severe, debilitating and often fatal disease called visceral leishmaniasis. In the search for a leishmaniasis vaccine, several parasite surface molecules have been intensively studied (Handman & Mitchell, 1985; Jaffe *et al.*, 1990; Lohman *et al.*, 1990; Russell & Alexander, 1988). One of these, the lipophosphoglycan (LPG) has been shown to be the major glycoconjugate found on all *Leishmania* promastigotes examined to date; it is estimated to be expressed at a level of  $1-5 \times 10^6$  molecules/promastigote cell (Ilg *et al.*, 1992; Jaffe *et al.*, 1990; McConville *et al.*, 1990; Turco *et al.*, 1984). Although this unique glycolipid is expressed by *L. major* amastigotes, it is structurally large and antigenically distinct from the promastigote form and in addition, is expressed only at low levels (Glaser *et al.*, 1991; Turco & Sacks, 1991). Similar studies with *L. donovani* amastigotes failed to indicate the presence of LPG (Turco, 1992).

Using a combination of enzymatic and physico-chemical techniques the structure of LPG has been deduced for three different *Leishmania* species and all have a tripartite composition consisting of a 1) lysolipid anchor, 2) a core glycan portion, and 3) a structurally variable  $\text{PO}_4\text{-Gal}\beta 1\text{-4Man}$  repeating disaccharide linked via a phosphodiester bond. The LPG is inserted into the plasma membrane via a lysophosphatidylinositol moiety containing either a  $\text{C}_{24}$  or  $\text{C}_{26}$  alkyl chain in approximately equal amounts attached to the core glycan via an inositolglycerolphosphate linker which is susceptible to PIPLC (Ilg *et al.*, 1992; McConville, *et al.*, 1987; Orlandi & Turco, 1987). This mode of anchoring the LPG to the parasite membrane is relatively unstable and is reflected in the large quantities of LPG found in the culture media, with the majority of the species containing the shorter  $\text{C}_{24}$  alkyl chain (Ilg *et al.*, 1992). Although the mechanisms by which the LPG is liberated are unclear, the process shows a requirement for proteins with lipid binding activity such as ovalbumin or bovine serum albumin (King *et al.*, 1987).

The phosphosaccharide core is conserved in all *Leishmania* species characterized to date, and consists of a  $\text{Gal}\alpha 1\text{-6Gal}\alpha 1\text{-3Gal}\beta 1\text{-3Man}\alpha 1\text{-3Man}\alpha 1\text{-4GlcN}$  structure

attached to the inositol of the lipid anchor through an  $\alpha$ 1-6 bond (Ilg *et al.*, 1992; McConville *et al.*, 1990; Turco *et al.*, 1987). In addition, this structure contains a glucose side branch linked  $\alpha$ 1-6 through a phosphate to a mannose residue distal to the lysolipid anchor (Thomas *et al.*, 1992). A unique feature of this glycan structure is a galactose found in furanose conformation, which in *Leishmania* has to date been detected only in LPG.

The third component of LPG is the repeat structure which is highly conserved between *Leishmania* species and life cycle stages. The basic repeat unit is  $\text{PO}_4\text{-6Gal}\beta$ 1-4Man disaccharide, polymerization of these units through a phosphodiester linkage generates the simplest repeating sequence of all the LPGs and is found on *L. donovani* promastigotes (Turco *et al.*, 1987). The *L. mexicana* LPG is slightly more complex in that every fifth repeat unit is modified with a glucose side branch linked  $\beta$ 1-3 to the galactose residue of the disaccharide repeat (Ilg *et al.*, 1992). Analysis of the phosphorylated repeat unit isolated from *L. major* promastigote LPG indicated a complex side branch linked to the 3 position of the galactose in the disaccharide repeating unit. The dominant side chain modifications found on the *L. major* promastigote LPG are: hydrogen, Gal $\beta$ 1-, Gal $\beta$ 1-3Ar $\alpha$ 1-2Gal $\beta$ 1 and Gal $\beta$ 1-3Gal $\beta$ 1-. The relative distribution of these structures is 7, 52, 9, and 25 mole percent for log phase promastigotes and 15, 31, 45, 6 mole percent for metacyclic promastigotes respectively (McConville *et al.*, 1990). Changes in the metacyclic LPG structure have been proposed to regulate the development of the promastigote in the midgut of the fly and may account for the decreased respiratory burst triggered by metacyclic organisms (McConville *et al.*, 1992). The transformation of promastigotes from the log phase to the metacyclic phase is paralleled by an increase in the molecular weight of the LPG structure for all *Leishmania* species and is associated with a doubling of the repeat unit from approximately 16 to 30 (McConville *et al.*, 1992).

The multiple functions ascribed to this glycolipid have been reviewed by Turco (1990) and include: a) the formation of an anionic barrier which protects the promastigote from lysis by serum factors and hydrolytic enzymes in the phagolysosome, b) activation of the C3 complement component to facilitate promastigote entry into macrophage, c) scavenging of the reactive oxygen intermediates thereby impairing the killing effects of the

respiratory burst d) inhibition of the protein kinase C by the lipid portion of LPG, a second messenger enzyme involved in activation of the hexose phosphate shunt which produces the NADPH required for the respiratory burst. More recently, studies by Pimenta *et al.* (1992) have shown that LPG in *L. major* promastigotes regulates the accumulation of highly infective metacyclic parasites within the mouth parts of the sandfly by anchoring the uninfected log phase promastigotes to the epithelial cells of the midgut through a galactose specific lectin until metacyclogenesis is complete (Pimenta *et al.*, 1992).

The preponderance of LPG on the promastigote surface, together with its appearance on the surface of infected macrophage (Tolson *et al.*, 1990) has suggested that LPG may play an important role in the immunobiology of the host-parasite relationship in leishmaniasis. Indeed, immunoprotection trials conducted by Handman and Mitchell (1985) indicated that BALB/c mice immunized with LPG purified from *L. major* promastigote together with *C. parvum*, induced an immune response which protected mice against cutaneous leishmaniasis. That this disease resistance was associated with a cell mediated immunity was inferred by the transfer of immunoprotection to naive recipients with splenic T cell populations from immunized donors. Further support demonstrating that the LPG preparations prime T lymphocytes *in vivo* was provided by the development of a strong delayed type hypersensitivity response to both LPG and whole promastigotes and the presence of IgG antibodies to LPG (Moll *et al.*, 1989). However, this latter study failed to give evidence that the T cells activated *in vivo* could recognize and proliferate *in vitro* to LPG. In addition, a number of investigators have reported LPG was also capable of stimulating a vigorous proliferative response in peripheral blood monocytes obtained from patients recovered from leishmaniasis (Kemp *et al.*, 1991; Mendonca *et al.*, 1991). Although these findings would suggest that LPG is a T cell epitope, it was difficult to rationalize the interaction between this highly anionic glycolipid and the MHC class II molecule.

In contrast to the protective effects observed with LPG, mice immunized with the delipidated form of LPG were not only unprotected but showed disease exacerbation and larger skin lesions which failed to heal (Mitchell & Handman, 1986). This water soluble form of LPG has also been shown to inhibit blastogenesis of human lymphocytes from

immune human donors stimulated with leishmanial antigens or mitogens, (Londner *et al.*, 1983). Although the mechanisms underlying these responses are unknown, it is clear that these suppressive effects may in part explain the depression of the immune system associated with the advanced stages of leishmaniasis.

In this connection, this particular study was undertaken to characterize the murine immunological responses to the *L. donovani* LPG and the delipidated phosphoglycan, and to delineate the epitope(s) responsible for the immunoprotective or exacerbative effects previously reported with the *L. major* LPG. Evidence is provided here demonstrating that the T cell responses observed with the *L. donovani* LPG and presumably, the *L. major* LPG, were not due to a glycolipid but rather a small molecular weight protein which is associated tightly with the lipid portion of LPG and co-purified with the LPG by solvent extraction and gel permeation chromatography. In addition, this protein epitope was found to be conserved across *Leishmania* species as indicated by the T cell proliferative assay.

## RESULTS

### 2.1 Lipophosphoglycan Stimulation of Murine Lymph Node Cells:

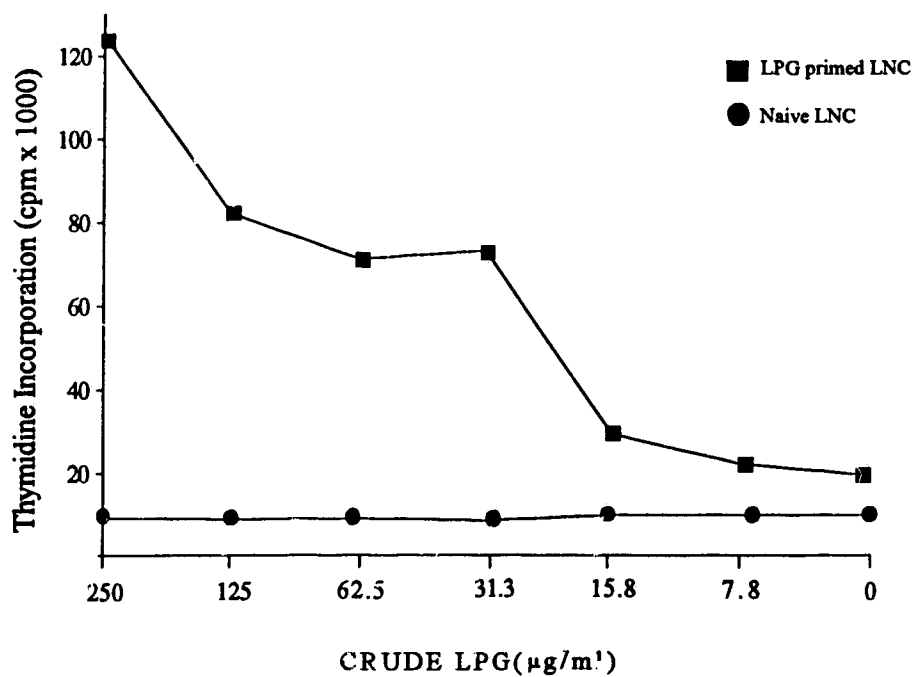
The protective immunity reported to be associated with *Leishmania* lipophosphoglycan was intriguing as carbohydrate T cell epitopes were previously undescribed (Russell & Alexander, 1988; Handman & Mitchell, 1985). Therefore an investigation of the *L. donovani* promastigote LPG was therefore undertaken to isolate and characterize the protective epitopes associated with this glycoconjugate. Lymph node cells obtained from BALB/c mice immunized subcutaneously (s.c.) with organic solvent E extracted and gel permeation purified LPG (Orlandi & Turco, 1987) in complete Freund's adjuvant (CFA), were challenged *in vitro* with LPG over a range of 7.8 to 250 µg/ml. As shown in Figure 2.1, the incorporation of [<sup>3</sup>H]thymidine diminished progressively with serial dilution of the LPG. This dose response curve strongly argued for the presence of an immunogenic component which specifically stimulated the proliferation of LNC. The possibility that the stimulation elicited by the *L. donovani* LPG was due to a mitogenic effect was dismissed as naive lymphocytes failed to proliferate even with LPG concentrations as high as 250 µg/ml.

### 2.2 Characterization of T Cell Epitopes in *L. donovani* LPG Preparations:

To define the LPG fragment associated with the proliferative activity, BALB/c mice were immunized s.c. with intact *L. donovani* promastigote LPG in CFA. Eight days post immunization the draining lymph node lymphocytes were challenged *in vitro* with various glycolipid fragments derived from crude LPG<sup>1</sup> using a combination of chemical and enzymatic procedures (Figure 2.2). In all experiments, no significant difference in tritiated thymidine incorporation was observed when BALB/c LNC were stimulated with either LPG or the delipidated phosphoglycan (Figure 2.3), indicating that the immunostimulatory epitope of this unique parasite glycolipid was not the lysolipid

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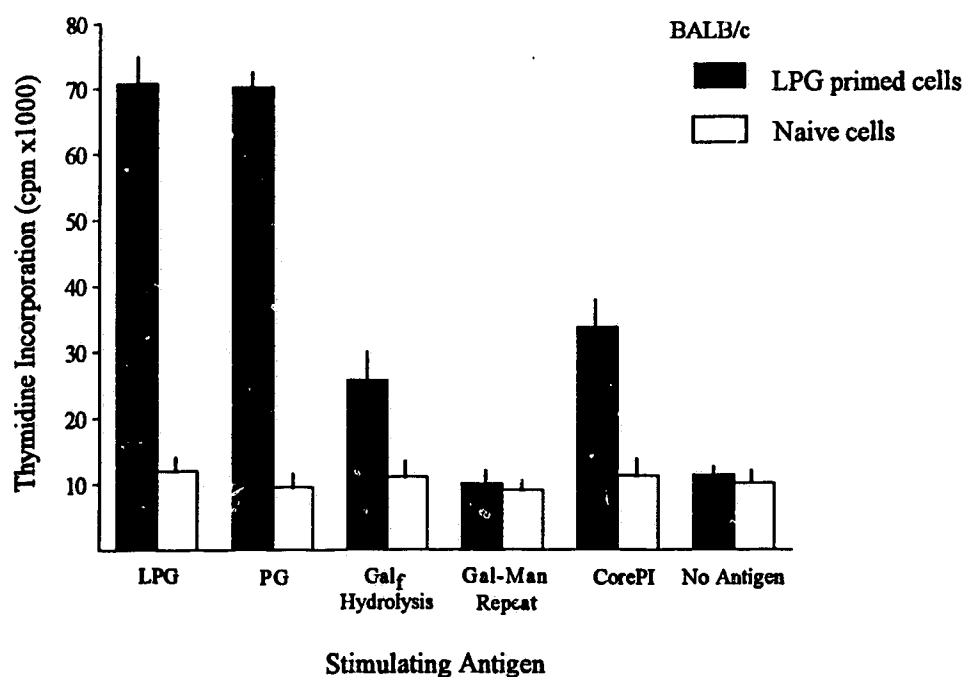
1- "crude LPG" refers to LPG purified according to the method of Orlandi and Turco (1987). These preparations contained significant levels of lipophosphoglycan associated protein detected by amino acid analysis, hitherto LPG will be designated as LPG/LPGAP to reflect the presence of the LPGAP protein.



**Figure 2.1:** Lymph node cell proliferative dose response curve with lipophosphoglycan. Stimulation of LPG primed or naive murine lymph node cells by varying concentrations of *L. donovani* LPG. The results shown are representative of those obtained in two separate experiments.

**1) Lipophosphoglycan (LPG):****2) Phosphoglycan (PG):****3) Phosphatidylinositol-core (Core-PI):****4) Gal, acid hydrolysis fragment (Gal, fragment):****5) Dissacharide repeat unit (Gal-Man repeat):**

**Figure 2.2:** Structures for the *I. donovani* lipophosphoglycan and fragments resulting from chemical or enzymatic digests.



**Figure 2.3:** Stimulation of draining lymph node cells from BALB/c mice immunized with crude *L. donovani* LPG. Lymphocytes were challenged *in vitro* with various fragments derived from LPG by enzymatic or chemical treatments. All antigens were used at a concentration of 50  $\mu\text{g/ml}$  as determined by the phenol/sulfuric acid assay (Beeley, 1985). Structures of the carbohydrate or glycolipids fragments are illustrated in Figure 2.2. The line over each bar represents the standard deviation for triplicate values obtained for each experiment.

structure. The LPG core hexasacchride (PI-core) generated by weak acid depolymerization of the PO<sub>4</sub>-Gal-Man (Orlandi & Turco, 1987) also elicited lymphocyte proliferation, although the response was diminished by approximately 50% in comparison to intact LPG. Similarly, the fragment generated by partial acid hydrolysis at the Gal<sub>4</sub> residue (de Lederkremer *et al.*, 1980) also exhibited immunogenic activity. However, the incorporation of [<sup>3</sup>H]-thymidine by T cells was reduced to one third the levels obtained with LPG or PG (Figure 2.3). LNC cultures challenged with the repeat disaccharide PO<sub>4</sub>-Gal-Man exhibited no proliferative activity. The progressive loss in the stimulatory activity which accompanied the fragmentation of the LPG structure, suggested that the T cell epitope was associated with the core oligosaccharide. However, as protein was detected in all stimulatory fractions (Figure 2.3), and because no carbohydrate epitopes have yet been shown to bind to the MHC molecule, these data were interpreted to indicate that the proliferation was probably due to the protein component.

### **2.3 Immunological Characterization of the Lipophosphoglycan-Associated Protein in LPG preparations:**

Although LPG purified by the method of Orlandi and Turco (1987) was shown to be devoid of protein by both the Bradford assay and silver stained SDS-PAGE, significant levels of proteinaceous material were detected by amino acid analysis. This discrepancy between analytical techniques was attributed to the LPG and the co-purifying proteinaceous material, designated lipophosphoglycan associated protein (LPGAP), forming a complex which inhibited the Bradford reagent from binding effectively to the protein component. This resulted in an artificially low levels of protein being detected in the LPG preparations. Since gel filtration chromatography was the final step in the LPG purification, it was reasoned that the contaminating amino acids were of a protein or peptide origin. This was substantiated by the fact that only background levels of amino acids were detected, unless samples were hydrolyzed prior to analysis. The amino acid composition of at least five different batches of LPG gave similar results, with a characteristically high mole percent (approximately 31%) of acidic or amide residues

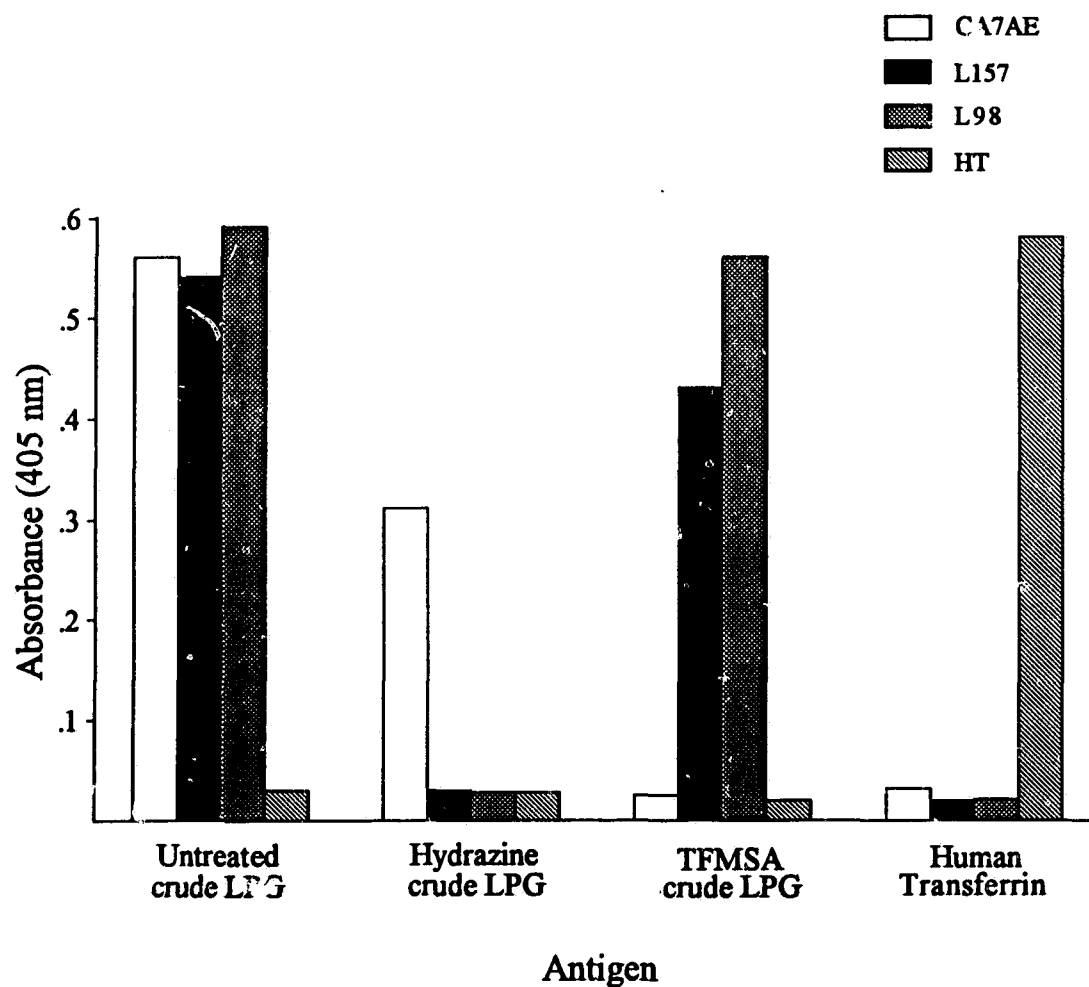
(Table 2-1). The ratio (w/w) of LPG to protein in preparations obtained by gel permeation chromatography was calculated to be 2:1 using the phenol/sulfuric acid assay and amino acid compositions. It is possible that the LPGAP was not detected by previous researchers as the dominant 11 kDa molecules migrates ahead of the bromophenol blue dye front on 10% polyacrylamide gels used to characterize the LPG (Handman *et al.*, 1985; Orlandi & Turco, 1987; Turco *et al.*, 1982).

The monoclonal antibodies CA7AE, L98, and L157 developed against *L. donovani* promastigote LPG by Tolson *et al.* (1989) were initially characterized and reported to be specific for carbohydrate determinants on this glycolipid. The minimum epitope recognized by CA7AE was the phosphorylated disaccharide PO<sub>4</sub>-Gal-Man which is present on both LPG and the secreted acid phosphatase of *L. donovani* (Jaffe *et al.*, 1990; Lovelace & Gottlieb, 1987; Tolson, 1992). Similarly, the cross-reactivity of mAbs L98 and L157 with *L. donovani* LPG fragments and the LPG deficient *L. donovani* C3PO and R2D2 mutants (King *et al.*, 1987; McNeely *et al.*, 1990) suggested that the determinants for these antibodies were localized to the core hexasaccharide. This was based on the knowledge that the latter two mutants could not synthesize the repeat units. However, amino acid analysis of LPG purified by solvent E extraction and gel permeation chromatography (Orlandi & Turco, 1987) showed significant levels of protein. To verify the specificity of these mAbs, LPG was degraded with anhydrous trifluoromethanesulfonic acid (TFMSA) which selectively cleaves all glycosidic linkages without hydrolyzing peptide bonds (Beeley, 1985; Tam *et al.*, 1986). The protein component on the other hand was selectively degraded with anhydrous hydrazine which cleaves amide bonds without affecting glycosidic linkages (Schroeder, 1972; Takasaki *et al.*, 1982). As shown in Figure 2.4, treatment of the LPG/LPGAP with hydrazine eliminated all L98 and L157 mAb reactivity, while significant CA7AE activity was retained confirming previous observations (Tolson *et al.*, 1989). In contrast, the TFMSA destroyed all CA7AE determinants without affecting the L98 and L157 epitopes indicating that these latter mAbs recognized protein. The initial oversight in the characterization of these antibodies was made based on the assumption that the LPG preparations used in the immunization and screening of the

**TABLE 2-1**  
**Amino Acid Composition Of Proteinaceous Material in LPG Prepared**  
**by Gel Permeation Chromatography**

<b>Residue</b>	<b>Mole %</b>	<b>Residue</b>	<b>Mole%</b>
<b>Asx*</b>	<b>12.45</b>	<b>Ala</b>	<b>9.04</b>
<b>Glx*</b>	<b>19.12</b>	<b>Pro</b>	<b>4.67</b>
<b>Ser</b>	<b>4.98</b>	<b>Tyr</b>	<b>2.31</b>
<b>Gly</b>	<b>5.2</b>	<b>Val</b>	<b>3.99</b>
<b>His</b>	<b>2.48</b>	<b>Met</b>	<b>3.82</b>
<b>Arg</b>	<b>3.69</b>	<b>Ile</b>	<b>2.93</b>
<b>Thr</b>	<b>4.16</b>	<b>Leu</b>	<b>6.67</b>
<b>Phe</b>	<b>5.88</b>	<b>Lys</b>	<b>8.61</b>

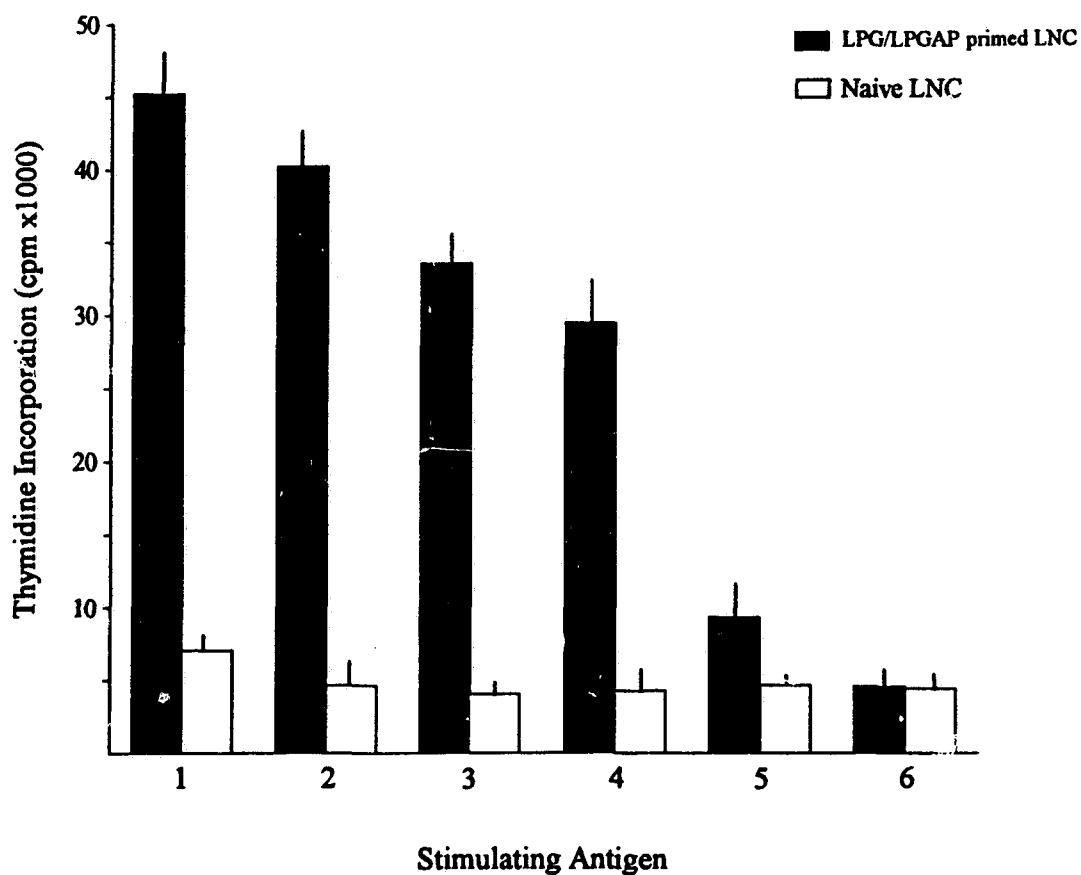
\* Asx and Glx refers to Asp plus Asn and Glu plus Gln respectively



**Figure 2.4:** Characterization of the immunoreactive determinants recognized by monoclonal antibodies raised against *L. donovani* LPG preparations. Crude LPG was treated with TFMSA to selectively degrade carbohydrates or hydrazine to selectively degrade protein then assayed by ELISA to determine the epitopes recognized by mAbs CA7AE, L98, and L157. Treated and untreated LPG (2  $\mu\text{g}/\text{well}$ ) was dried in triplicate wells. Human transferrin (1  $\mu\text{g}/\text{well}$ ) was used as a negative control.

monoclonal antibodies were homogeneous. The observation that these antibodies were specific to the LPGAP was fortuitous as these immunological reagents were used as an assay for this protein in its subsequent purification.

To evaluate the potential of LPG as a unique carbohydrate T cell epitope, it was essential to purify this glycoconjugate free of the protein. Numerous attempts to separate the LPG and the proteinaceous material using either  $C_8$  or phenyl based reversed phase HPLC columns were unsuccessful and moreover, resulted in irreversible binding of both components to these columns as judged by ELISA with mAbs CA7AE and L98. LPG eluted as a symmetrical 280 nm absorbing stable protein-glycolipid complex on anion exchange chromatography (see chapter 3 Figure 3.4A). Furthermore, amino acid analysis of the LPG before and after ion-exchange chromatography showed no significant reduction in the total protein content, suggesting that the protein component was tightly associated with the LPG. It was postulated that this bimolecular interaction may be stabilized by a divalent cation bridge between the acidic residues of the protein and the  $PO_4$ -Gal-Man repeat of LPG as previously proposed by Eilam *et al.*, (1985). However, treatment of this complex with 10 mM EDTA followed by anion exchange chromatography afforded no separation of the two molecular species. Similarly, incubation of the LPG-protein complex with the proteases endoproteinase Glu-C or chymotrypsin followed by ion exchange purification was also ineffective at removing the protein from the LPG as indicated by an invariant amino acid composition. As the protein component co-purifying with the LPG proved difficult to eliminate by either chromatographic or enzymatic methodologies, the role of LPG in stimulating proliferation of lymph node cells was initially addressed in part by chemical degradation of the glycolipid with either periodic acid oxidation or anhydrous trifluoromethanesulfonic acid (TFMSA). As shown in Figure 2.5, the modification or complete destruction of the carbohydrate structures in the LPG preparations resulted in only minor variations in the proliferative responses. This hypothesis was further verified by conducting parallel experiments with the octyl Sepharose purified LPGAP or the glycoconjugate LPG (refer to chapter 3 for purification). The data presented in Figure 2.5 shows that purified LPGAP, TFMSA treated LPGAP, and LPG/LPGAP were all highly stimulatory antigens. In



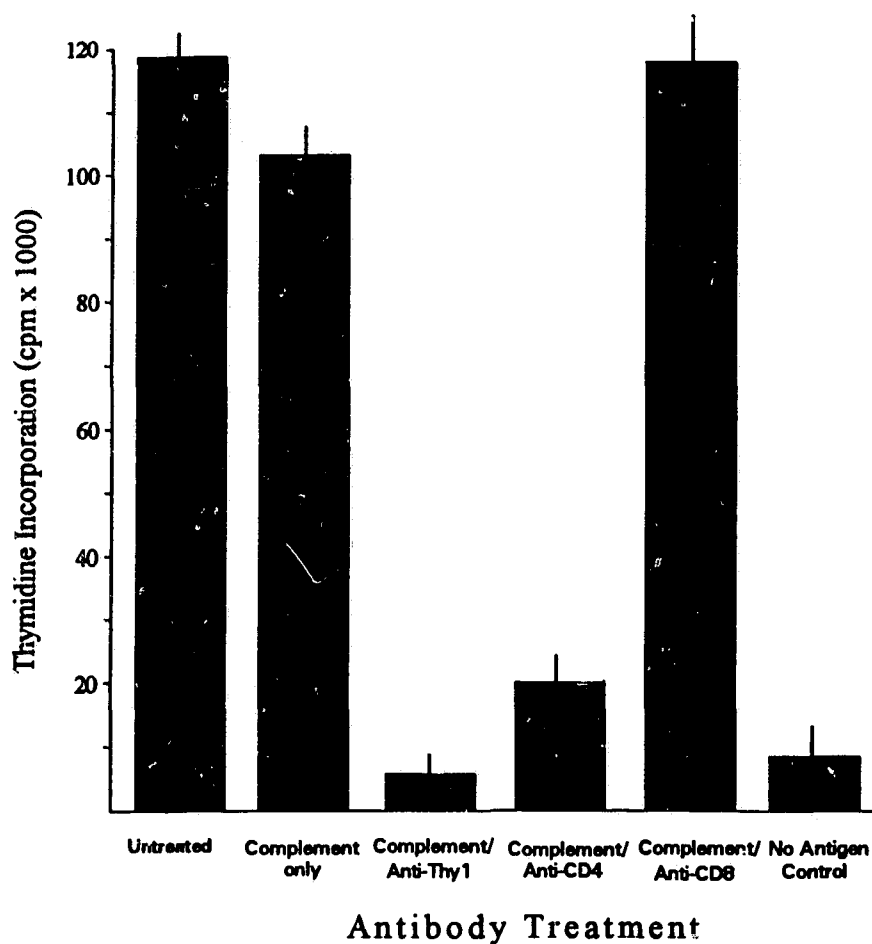
**Figure 2.5:** Stimulation of BALB/c LNC primed with 50 µg crude *L. donovani* LPG emulsified in 100 µl complete Freund's adjuvant. 1, 50 µg/ml crude LPG treated with sodium periodate; 2, 25 µg/ml crude LPG deglycosylated with trifluoromethanesulfonic acid; 3, 50 µg/ml crude LPG treated with *S. griseus* pronase; 4, 25 µg/ml octyl Sepharose purified LPGAP; 5, 50 µg/ml octyl Sepharose LPG; 6, no antigen control.

contrast, addition of octyl Sepharose purified LPG to primed LNC at a concentration of 50 µg/ml resulted in only a two fold stimulation. By comparison a stimulation index of 11 was obtained with 25 µg/ml LPGAP. It is interesting to note, that treatment of the LPG/LPGAP preparations with pronase failed to decrease the proliferative responses associated with the proteinaceous material. This may be due to the tight interaction between the LPG and the LPGAP which protects the latter from proteolytic degradation.

To further investigate the immunogenicity of the *L. donovani* LPGAP, BALB/c mice were primed with octyl Sepharose purified LPGAP. Draining lymph node cells stimulated with 50 µg/ml purified LPGAP or LPG/LPGAP responded with SIs of 9.9 and 6.0 respectively. These data strongly suggested that the antigen stimulating the BALB/c LNC was a protein constituent and not carbohydrate.

#### **2.4 Phenotypic Analysis of T Lymphocytes Stimulated by LPGAP:**

To determine the phenotype of T cells proliferating *in vitro* to LPG/LPGAP, primed BALB/c LNC were treated with antibodies to T cell surface markers together with complement to deplete specific T cell populations. The results are shown in Figure 2.6. Whole lymph node cell suspensions incubated with anti-Thy-1 antisera and low-tox rabbit complement resulted in complete abolition of tritiated thymidine incorporation, confirming that the cells primed *in vivo* were indeed T lymphocytes. The loss of proliferation was due to specific lysis, as LNC treated with complement alone exhibited only a minor decrease in proliferative response (Figure 2.6). To further delineate these lymphocytes into T helper or T cytotoxic subsets, parallel experiments were conducted with either anti-CD4 or anti-CD8 mAbs. Removal of the CD8<sup>+</sup> population prior to *in vitro* challenge with LPG/LPGAP had no effect on the proliferative response. Conversely, thymidine incorporation was dramatically decreased to near background levels with the anti-CD4 mAb, thus establishing that the cells recognizing the LPGAP epitopes were CD4<sup>+</sup> helper T cells. Culture supernatants from naive and LPG primed LNCs stimulated with LPG/LPGAP



**Figure 2.6:** Phenotype of T cell subsets in lymph node populations responding to crude *L. donovani* LPG/LPGAP stimulation *in vitro*. BALB/c mice were immunized s.c. at the base of the tail with crude LPG/LPGAP emulsified in complete Freund's adjuvant. Eight days post immunization draining lymph node cells were treated with either anti-Thy1, anti-CD4, or anti-CD8 antibodies in the presence of Low-Tox rabbit complement to deplete LNC suspensions of specific T cell populations. Lymphocytes were cultured for 96 h in the presence of crude *L. donovani* promastigote LPG/LPGAP at a concentration of 50  $\mu$ g/ml.

TABLE 2-2

Assay for Interleukin 2 and 4 production by primed whole lymph node cells stimulated *in vitro* with crude *L. donovani* Lipophosphoglycan <sup>a b</sup>

Supernatant <sup>c</sup>	CTLL-2 cell division measured by MTT reduction (O.D. <sub>595</sub> nm)		
	no mAb control	anti-IL-2 (S4B6)	anti-IL-4 (11B11)
Primed LNC stimulated with crude LPG	0.17	0.03	0.18
Naive LNC stimulated with crude LPG	0.03	0.03	0.04

*a* Lymph node cells were primed s.c. with crude *L. donovani* LPG emulsified in complete Freund's adjuvant and draining lymph nodes removed eight days later.

*b* This work was conducted in collaboration with D.L. Tolson.

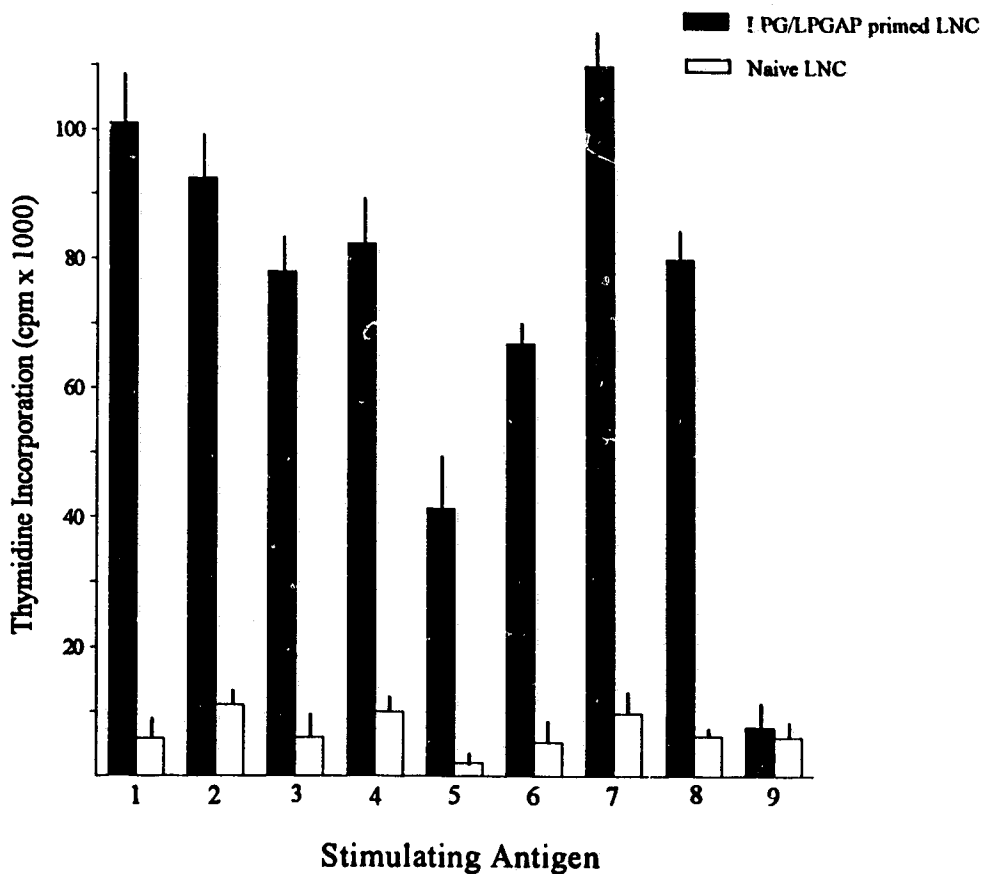
*c* Supernatants were harvested 48 h after stimulation with antigen.

*d* Monoclonal antibodies were added to the CTLL-2 cultures to a final concentration of 1 µg/ml.

were assayed for IL-2 and IL-4 using the IL-2/IL-4 dependent CTLL-2. Proliferation stimulated by each of these the interleukins was distinguished by addition of the neutralizing anti-IL-4 mAb 11B11 (Ohara & Paul, 1985) or the anti-IL-2 mAb S4B6 (Zurawski *et al.*, 1986). As shown in Table 2-2, supernatants obtained from LPG stimulated LNC were found to support the growth of the CTLL-2 cell line. Inhibition of cell growth with the inclusion of the mAb S4B6 indicated that the LPG primed LNC released only IL-2 as addition of the mAb 11B11 had no effect on proliferation. The above data, demonstrated that the LPGAP epitopes activated a CD4<sup>+</sup> T cell population which produced IL-2 when stimulated *in vitro*. Taken together, these observations strongly suggested that LPGAP stimulated a Th1 like immune response in mice.

### **2.5 Distribution of the LPGAP Antigen Across *Leishmania* Species and Life Cycle Stages:**

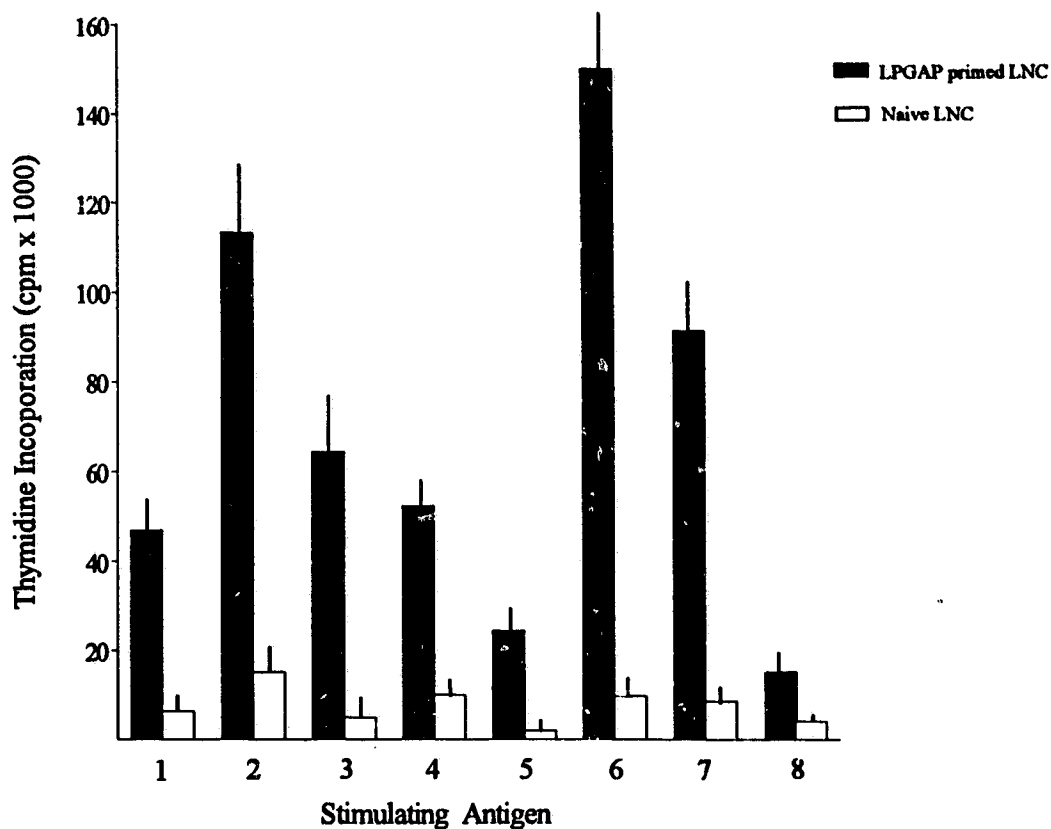
BALB/c LNC primed with crude *L. donovani* LPG were stimulated with *L. donovani*, *L. tropica*, *L. major*, and *L. mexicana amazonensis* promastigotes, representing the cutaneous, mucocutaneous, and visceral leishmaniasis complexes. The results are illustrated in Figure 2.7. A strong proliferative response was observed with all promastigote species tested, indicating that the LPGAP protein is not unique to *L. donovani* but is conserved across the genus *Leishmania*. Since the degree of T cell proliferation, as measured by tritiated thymidine incorporation, is in part a reflection of the antigen concentration, it is possible to infer from the data in Figure 2.7 that the levels of LPGAP are similar in these *Leishmania* promastigotes (Louis *et al.*, 1979; Corradin *et al.*, 1977). In the initial studies aimed at describing the nature of the T cell epitopes associated with the LPG, the *L. donovani* mutant C3PO devoid of mature LPG (King *et al.*, 1987; McNeely *et al.*, 1990), was found to be a potent stimulator of T lymphocytes primed *in vivo* with *L. donovani* LPG/LPGAP. The proliferative response obtained with the C3PO mutant indicated that synthesis of LPGAP was not dependent upon synthesis of full length LPG. Moreover, this data lends further support to the previous findings which showed that LPG was not a T cell epitope.



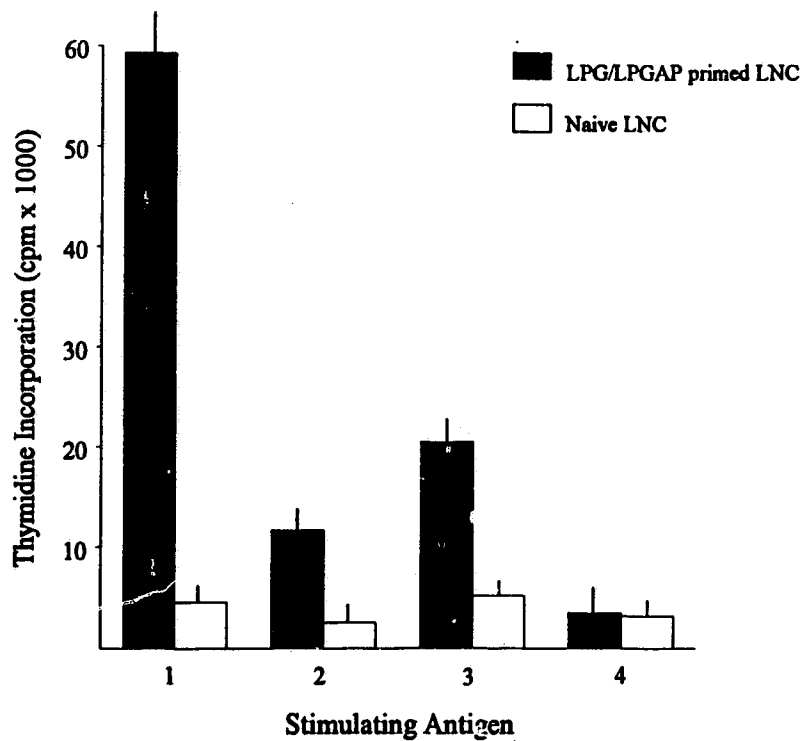
**Figure 2.7:** Stimulation of crude LPG primed BALB/c LNC with live *Leishmania* promastigotes or *Leishmania* crude membranes. 1, *L. donovani* (LD3); 2, *L. donovani* (C3PO); 3, *L. tropica* K27.3; 4, *L. major* 5-ASK; 5, *L. mexicana amazonensis* M2269; 6, *L. donovani* (LD3) membrane fraction; 7, octyl Sepharose purified LPGAP; 8, crude LPG; 9, no antigen control. Lymphocytes ( $5 \times 10^5$ /well) were stimulated with  $3 \times 10^5$  live promastigotes or 50  $\mu\text{g/ml}$  soluble antigens. The results are expressed as the mean value for LNC cultures stimulated in triplicate for each antigen.

The ability of the LPGAP alone to induce a cell mediated immunity in mice was investigated by immunizing BALB/c mice with octyl Sepharose purified LPGAP and once again challenged with a variety of *Leishmania* promastigote species. As shown in Figure 2.8, LNC proliferated vigorously *in vitro* when stimulated with either LPGAP (SI 15.5) or LPG/LPGAP (SI 10.6). The responses in these experiments were generally lower in comparison to the LPG/LPGAP primed LNC. This result was unexpected as the LPG/LPGAP and the octyl Sepharose purified LPGAP elicited similar T cell responses regardless of the antigen used to immunize (Figures 2.7 & 2.8). It should be emphasized that only background levels of [<sup>3</sup>H]-thymidine were incorporated when naive LNC were co-cultured with live *Leishmania* promastigotes or soluble antigens, indicating that the stimulation of T cells was a specific event which required prior exposure to antigen *in vivo*. The latter control also demonstrated that radiolabel incorporated into newly synthesized DNA was attributed solely to T cell proliferation, as growth of live promastigotes was arrested when the culture temperature was upshifted from 26 to 37°C.

The cross stimulation of *L. donovani* primed LNC with a variety of *Leishmania* promastigotes was further verified by repeating the *in vitro* challenges with LPG isolated from *L. major* and *L. tropica* promastigotes by Drs. E. Handman and S. Turco respectively. As shown in Figure 2.9, both the *L. major* and *L. tropica* glycoconjugate stimulated T cell proliferation, however the responses were significantly lower than those observed for the *L. donovani* LPG/LPGAP. Since these investigations were undertaken prior to establishing that the immunogenic component co-purifying with the LPG was a protein rather than a glycan, antigens used in the T cell proliferation assays were normalized to carbohydrate using the phenol/sulfuric acid assay. In light of the fact that *L. donovani*, *L. major*, and *L. tropica* promastigotes gave similar proliferative responses (Figure 2.7 & 2.9), it is likely that the amount of protein associating with the LPG during the isolation protocol may have varied as a function of the complexity of the LPG repeat structure (Turco *et al.*, 1987; McConville *et al.*, 1990). Nonetheless, the proliferation of *L. donovani* LPG/LPGAP primed lymphocytes following a challenge with *L. tropica* and *L. major* LPG indicated that the LPGAP is also found to co-purify with LPGs from other *Leishmania* species and very likely accounts for the immunogenic responses reported for



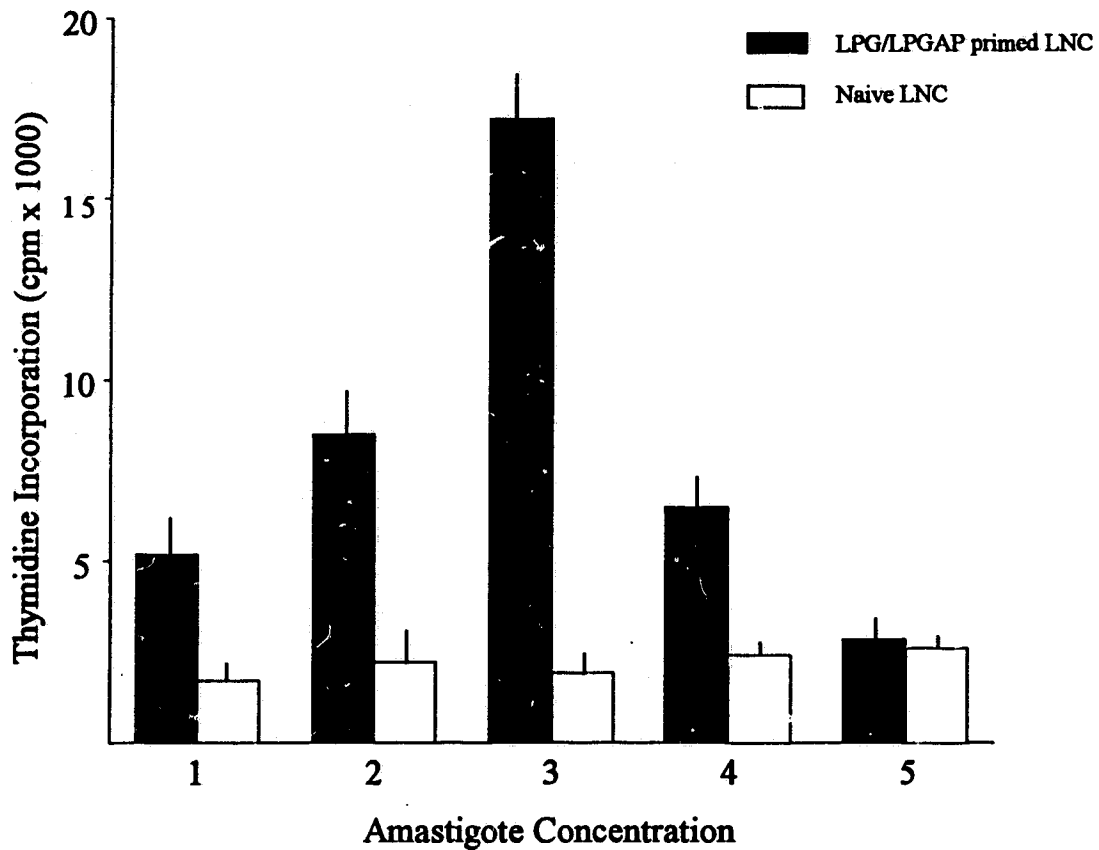
**Figure 2.8:** Stimulation of LPGAP primed BALB/c LNC with live *Leishmania* promastigotes. 1, *L. donovani* (LD3); 2, *L. donovani* (C3PO); 3, *L. tropica* K27.3; 4, *L. major* 5-ASK; 5, *L. mexicana amazonensis* M2269; 6, octyl Sepharose purified *L. donovani* LPGAP; 7, crude LPG; 8, no antigen control. Lymphocytes ( $5 \times 10^5$ /well) were co-cultured with  $3 \times 10^5$  live promastigotes or 50  $\mu$ g/ml soluble antigens for 96 h and the resulting proliferative responses were measured by [ $^3$ H]-thymidine incorporation for a period of 20 h prior to harvesting LNC. Data represents the mean of triplicate values.



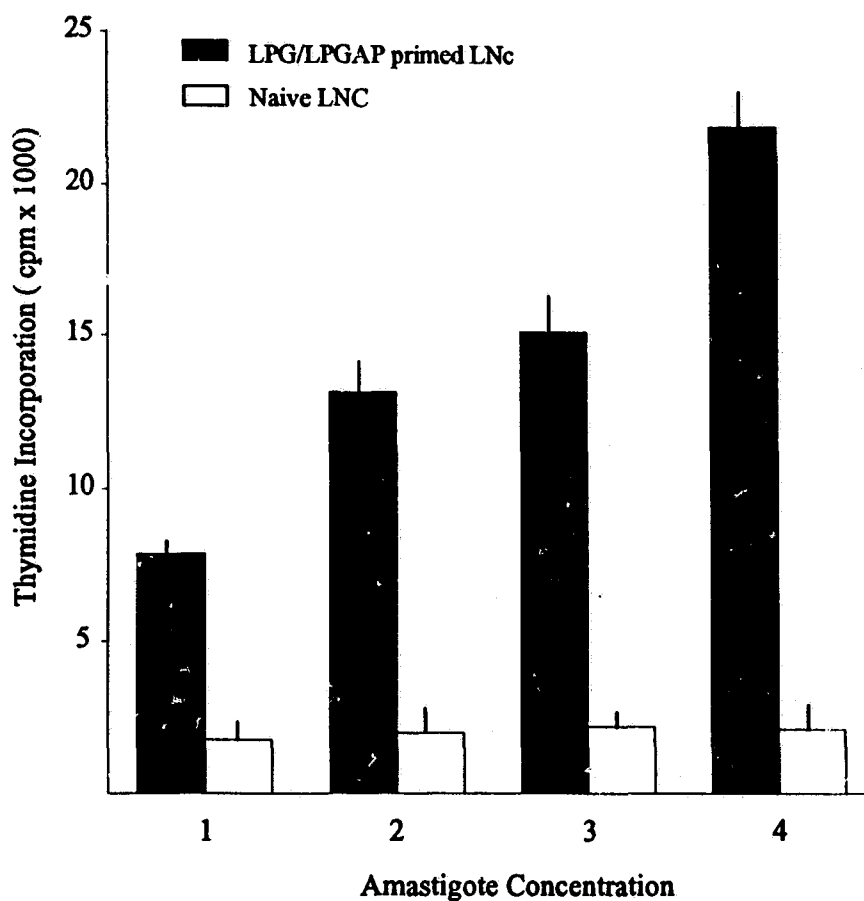
**Figure 2.9:** Proliferation of *L. donovani* LPG/LPGAP primed BALB/c LNC with *L. major* and *L. tropica* promastigote LPG. LNC were cultured with 50  $\mu\text{g/ml}$  LPG from each *Leishmania* species in triplicate for 96 h at 37°C. Antigen concentrations were determined based on carbohydrate content determined by the phenol/sulfuric acid assay (Beeley, 1985). The *L. major* LPG was purified according to the method of McConville *et al.* (1987), and the *L. tropica* LPG was prepared as described by Orlandi and Turco (1987). 1, *L. donovani* LPG; 2, *L. major* LPG; 3, *L. tropica* LPG; 4, no antigen.

this glyconjugate (Handman & Mitchell, 1985; Russell & Alexander, 1988, Moll *et al.*, 1991).

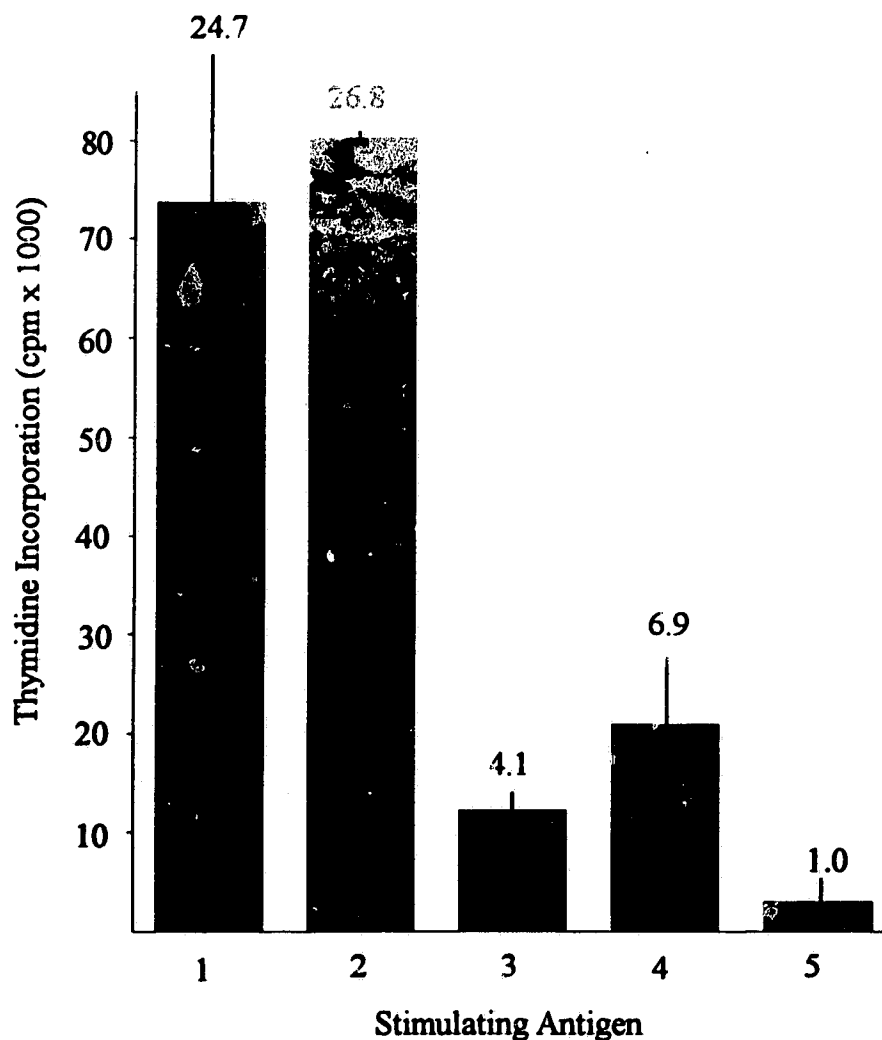
To assess if the LPGAP was expressed during the amastigote life stage of *Leishmania*, draining lymph node cells, primed with *L. donovani* LPG/LPGAP, were challenged with live *L. donovani* LV9 amastigotes isolated from Syrian golden hamster spleens. A maximal response was observed when  $5 \times 10^5$  lymphocytes were cultured with  $1 \times 10^7$  amastigotes (Figure 2.10). When primed LNC were pulsed with heat killed amastigotes ( $56^\circ\text{C}$  for 30 min) a gradual increase in [ $^3\text{H}$ ]-thymidine incorporation was observed over the range of  $1 \times 10^6$  to  $5 \times 10^7$ , with the latter parasite concentration giving the optimal proliferative response (Figure 2.11). Since *L. donovani* amastigotes do not synthesize LPG (Turco, 1993), it is reasonable to assume that the immunogenic component in the amastigotes, is the LPGAP. That the LV9 amastigotes express LPGAP was further substantiated by ELISA and immunoblot analysis of solvent E extracts with mAbs L98 and L157 (see chapter 3). In support of these findings, the same solvent E extracts were also found to induce a T lymphocyte response in BALB/c LNC populations primed with octyl Sepharose purified *L. donovani* promastigote LPGAP (Figure 2.12). Together, these data confirm that LV9 amastigotes also synthesize LPGAP. From the quantitative T cell proliferative data (Corradin *et al.*, 1977; Louis *et al.*, 1979) it is tempting to suggest that the levels of LPGAP are significantly lower in the amastigote form of *Leishmania*. A comparison of the results in Figure 2.8 and 2.10 shows that the optimal T cell response was observed with  $3 \times 10^5$  promastigotes, whereas maximal LNC stimulation required  $1 \times 10^7$  amastigotes. On a per cell basis these data suggested that transformation from the amastigote to the promastigote form was accompanied by a 30 fold increase in LPGAP synthesis. It is important, however, that these interpretations be tempered with caution as some lines of evidence have shown that *L. donovani* amastigotes can down regulate Ia expression in macrophage (Reiner *et al.*, 1988).



**Figure 2.10:** Stimulation of *L. donovani* LPG/LPGAP primed BALB/c lymph node cells with live *L. donovani* LV9 amastigotes. Primed or naive LNC were cultured in triplicate for 96 h at 37°C with varying concentrations of LV9 amastigotes isolated from infected Syrian golden hamster spleens: 1,  $1 \times 10^6$  amastigotes/ml; 2,  $5 \times 10^6$  amastigotes/ml; 3,  $1 \times 10^7$  amastigotes/ml; 4,  $5 \times 10^7$  amastigotes/ml; 5, no antigen controls. Proliferation was measured by pulsing cultures with [ $^3\text{H}$ ] thymidine 20 h prior to harvesting cells.



**Figure 2.11:** Stimulation of *L. donovani* LPG/LPGAP primed BALB/c lymph node cells with heat killed *L. donovani* LV9 amastigotes. Primed or naive LNC were cultured in triplicate for 96 h at 37°C with varying concentrations of LV9 amastigotes isolated from infected Syrian golden hamster spleens: 1,  $1 \times 10^6$  amastigotes/ml; 2,  $5 \times 10^6$  amastigotes/ml; 3,  $1 \times 10^7$  amastigotes/ml; 4,  $5 \times 10^7$  amastigotes/ml; 5, no antigen controls. Proliferation was measured by pulsing cultures with [ $^3$ H]-thymidine 20 h prior to harvesting cells.



**Figure 2.12:** Cross stimulation of BALB/c LNC primed with LPGAP with *L. donovani* LV9 amastigote solvent E extracts. 1, 50  $\mu\text{g}/\text{ml}$  octyl Sepharose purified *L. donovani* LD3 LPGAP; 2, 25  $\mu\text{g}/\text{ml}$  octyl Sepharose purified *L. donovani* LD3 LPGAP; 3, 50  $\mu\text{g}/\text{ml}$  solvent E extract from *L. donovani* LV9 amastigotes; 4, 25  $\mu\text{g}/\text{ml}$  solvent E extract from *L. donovani* LV9 amastigotes; 5, no antigen control. Antigen concentration for the solvent E extracts was determined gravimetrically. LNC cultures were incubated for 96 h at 37°C in triplicate and the proliferative responses were measured by pulsing cells with [ $^3\text{H}$ ]-thymidine 20 h prior to harvesting cells.

## DISCUSSION

Recent studies with peripheral blood lymphocytes from patients infected with either Old World (Jaffe *et al.*, 1990; Kemp *et al.*, 1991) or New World (Mendonça *et al.*, 1991) *Leishmania* species have indicated that LPG strongly stimulates T cells. Moreover, 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 & Mitchell, 1985; Russell & Alexander, 1988). These observations have resulted in the speculation that the responsible determinants were carbohydrates (Moll *et al.*, 1989). However, this hypothesis was difficult to rationalize in light of the work reported by Unanue and colleagues (1989) showing that the hydrophilic polysaccharides could not be accommodated in the hydrophobic cleft of the MHC class II molecule. In this connection an investigation was undertaken to delineate not only the immunostimulatory epitope(s), but also to characterize the lymphocyte population activated by this unique parasite glycolipid. Here, direct evidence was provided showing that a CD4<sup>+</sup> subpopulation of murine T cells was involved in the recognition of *L. donovani* LPG isolated using published procedures (Turco *et al.*, 1984; Turco & Orlandi, 1987). In addition, IL-2 production was elicited when primed whole lymph node cell populations were stimulated *in vitro* with crude LPG. These data are consistent with the responding lymphocytes being Th1-like T helper cells which is in agreement with the immunoprotective effects of LPG previously reported by others. Since LPG stimulated proliferation of LNC only from LPG-immunized mice, it was clearly not mitogenic. The LPG-induced T lymphocyte proliferation was therefore of an antigen-specific nature requiring an *in vivo* priming event. While these data showed that LPG does induce T cell proliferation, no evidence could be found to support the suggestion that the stimulation was solely dependent upon carbohydrate epitopes.

Although free amino acids could not be detected in unhydrolyzed samples of LPG or PG, amino acid analysis of hydrolyzed samples revealed significant quantities of amino acids, the relative ratios of which stayed fairly constant between different LPG or PG batches. This polypeptide component was found to be very tightly associated with LPG as exhaustive

attempts to remove the peptide by gel permeation under strong denaturing conditions (6 M guanidinium hydrochloride), organic solvent extraction or reversed phase HPLC chromatography (Aquapore C<sub>8</sub> column), were unsuccessful. It was therefore important to determine the individual contribution of both carbohydrate and protein in the lymphocyte stimulation. Selective degradation of the LPG glycan with anhydrous TFMSA resulted in a polypeptide preparation recognized by both the anti-LPG core mAbs L98 and L157, providing a detection method for LPGAP. That these antibodies did not recognize carbohydrate as previously proposed (Tolson *et al.*, 1989) was further corroborated by depletion of the immunoreactive determinant with hydrazine, which selectively cleaves peptide bonds without affecting glycosidic linkages (Schroeder, 1972). Using these monoclonal antibodies in an ELISA assay, it was possible to monitor the separation of the LPGAP and the LPG by octyl Sepharose reversed phase chromatography and subsequently test them in T cell proliferation assays. Results of these experiments clearly showed that the observed LPG T-cell stimulation was due to the strongly bound LPGAP. These findings were further substantiated by studies showing that primed murine T lymphocytes readily responded to native gp63 but failed to recognize highly purified LPG containing traces of contaminating protein, as determined by amino acid analysis (López *et al.*, 1991). It is important to note that even after two consecutive reversed phase 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 T-cell stimulatory activity to be carbohydrate associated.

Recently, Scott *et al.* (1987; 1988) developed a T cell line which produced high levels of IL-2 and IFN- $\gamma$  in response to stimulation with a dominant 11 kDa *Leishmania* protein. Adoptive transfer of these T cells protected BALB/c mice against an *L. major* promastigote infection. Although the immunoreactive protein identified by this group has not been structurally characterized or shown to react with the LPGAP specific mAbs L98 or L157, several lines of evidences indicate that this protein may belong to the LPGAP family. For example, this low M<sub>r</sub> protein co-purifies with *L. major* LPG (Scott *et al.*, 1987) suggesting an association similar to that seen with *L. donovani* LPG/LPGAP. Second, these T cell lines respond to a conserved protein expressed by *L. major*, *L. donovani*, *L. braziliensis*, and *L. donovani* R2D2 (Scott *et al.*, 1990).

The significance of LPGAP as a potent stimulator of a cell mediated immune response and more importantly, its potential role as an immunoprotective antigen was underscored by studies which have expanded the above findings in mice to the human system. Kemp *et al.* (1993) and Russo *et al.* (1992) have demonstrated that PBMCs from patients recovered from either New World or Old World cutaneous, mucocutaneous, or visceral leishmaniasis proliferated strongly to LPGAP but not LPG. In both of these studies the activation of T lymphocytes was accompanied by the production of IFN- $\gamma$ , essential for macrophage activation and killing of intracellular *Leishmania* parasites (Murray *et al.*, 1985; Nacy *et al.*, 1985). Although IFN- $\gamma$  was the abundant cytokine elicited by T cell lines stimulated with LPGAP, it was also possible to isolate LPGAP reactive T cell clones which secreted IL-4 from patients recovered from visceral leishmaniasis (Kemp *et al.*, 1993). These data suggest that different epitopes within a given antigen may be capable of inducing either a Th1 or Th2 subset of T helper cells (Evavold *et al.*, 1992).

From a pragmatic perspective, since LPG has been shown to be immunoprotective when injected into mice (Handman & Mitchell, 1985; Russell & Alexander, 1988) and since evidence provided here indicates that these preparations invariably contained LPGAP, it is very likely that the observed immunization was due to the presence of this protein. Indirect support for this position was provided by Mendonça *et al.* (1991) who showed that peripheral blood lymphocytes from leishmaniasis patients were stimulated with either *L. braziliensis* or *L. mexicana* LPG but not by LPG first treated with proteinase K.

Other findings lend further support to the proposal that the LPGAP has vaccine potential. The mAbs L98 and L157 are known to be cross-reactive with *L. tropica*, *L. major*, *L. mexicana amazonensis* and *L. donovani* promastigotes (Tolson *et al.*, 1994), suggesting that this protein is conserved throughout the genus *Leishmania*. In addition, T-cell proliferation assays have shown a similar cross-stimulation between *L. tropica* and *L. donovani* promastigote LPG preparations, and *L. donovani* amastigote LPG preparations. Thus the strong proliferative responses observed with both murine and human lymphocytes stimulated with LPGAP, together with the fact that this protein is found all life cycle stages of *Leishmania* makes LPGAP an ideal vaccine candidate.

Finally, the physiological function of LPGAP remains an area of conjecture. While the present data might be interpreted to indicate that LPGAP is merely an anomaly caused by an unexpectedly high affinity of this protein to LPG, it is equally possible that this protein is involved in membrane associated functions, such as regulating membrane pressure or stabilizing lysoglycolipids within the parasite plasma membrane. The presence of LPGAP in *L. donovani* amastigotes which do not express LPG (Turco, 1993), suggests a more fundamental biological role for LPGAP than just association with the glycoconjugate LPG. As part of our investigations to clarify this question, the LPGAP was purified and physico-chemically characterized and experiments to determine the cellular localization were performed. Results from these studies are discussed in the following chapter.

## ACKNOWLEDGEMENTS

It should be indicated that the studies, dealing with the *Leishmania* promastigote cross-species stimulation and the T cell proliferative response to *L. tropica* and *L. major* LPG was carried out as collaborative effort with a fellow graduate student Dr. D.L. Tolson working with Dr. T.W. Pearson. I would like to thank Dr. S.J. Turco, University of Kentucky, for the initial *L. donovani* LPG samples, for the purification of the *L. tropica* LPG and for providing the LPG negative mutants R2D2 and C3P0 used in this study. The author also wishes to gratefully acknowledge the technical assistance provided by Dr. Hung Siah Teh and his laboratory in setting up the MTT colorimetric assay for determination of IL-2 and IL-4.

**CHAPTER 3**

**Isolation and Structural Characterization of the *Leishmania*  
Lipophosphoglycan-Associated Protein**

## INTRODUCTION

*Leishmaniasis* is a disease caused by protozoan parasites of the genus *Leishmania*. Depending upon the species, the disease symptomology may range from a self healing skin lesion to the fatal visceral form commonly known as kala-azar. Recent attempts to develop molecularly defined vaccines for these maladies have focused on major parasite cell surface molecules, including the metalloproteinase gp63 (Russell & Alexander, 1988; Yang *et al.*, 1990) and a lipophosphoglycan (LPG). The latter glycoconjugate has been implicated as a key molecule in the internalization and subsequent survival of parasites within the macrophage (Turco, 1990) and its abundance on the promastigote surface has made it an attractive vaccine candidate (King *et al.*, 1987; McConville & Bacic, 1990). Whereas protective immunity was reported with LPG in BALB/c mice (Handman & Mitchell, 1985; McConville *et al.*, 1987) and circulating lymphocytes from human leishmaniasis patients were shown to proliferate on exposure to LPG (Kemp *et al.*, 1991; Mendonça *et al.*, 1991) the mechanism by which a glycan could affect such a response was difficult to rationalize in view of the present structural understanding of MHC-T cell epitope interactions. While several reports have indicated that LPG could protect against cutaneous disease and that this immunity could be adoptively transferred with L3T4<sup>+</sup> splenic T cells (Russell & Alexander, 1988; Handman & Mitchell, 1985), the possibility that this could be attributed to contaminating proteins in LPG preparations was not rigorously tested. Indeed, Mendonça *et al.* (1991) have recently shown that treatment of LPG with protease K eliminated all human circulating lymphocyte proliferation in cells from American cutaneous leishmaniasis patients. At the same time an 11 kDa protein co-purifying with LPG, was first isolated in this laboratory and designated as a lipophosphoglycan associated protein (LPGAP). This protein was shown to be a strong stimulator of T cell proliferation and reacted with monoclonal antibodies, L98 and L157, raised against LPG preparations (see chapter 2; Tolson *et al.*, 1989). Results communicated in this chapter show that LPGAP to be a major molecule found on the surface of *Leishmania* promastigotes. This protein was also detected on

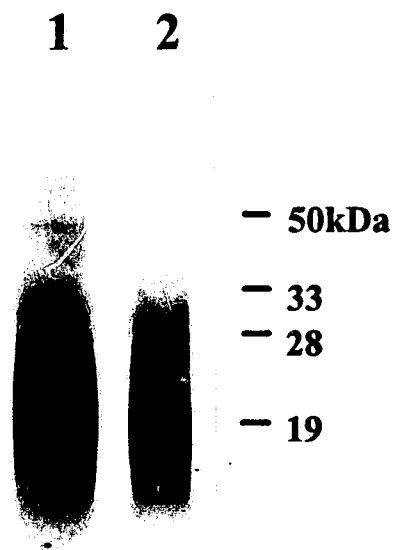
*L. donovani* amastigotes by ELISA with mAb L98. Aside from a small N-terminally blocked amino terminal region, the amino acid sequence of the protein was determined by Edman degradation, revealing the position of an N-methylarginine post-translational modification and facilitating the determination of epitopes reacting with two monoclonal antibodies. Further characterization of the LPGAP was carried out by cloning and sequencing the *lpgap* gene. The complete primary structure of the LPGAP, together with a computer predicted secondary structure, allowed the suggestion of a hypothetical role for LPGAP involving modulation of the stability of LPG in the parasite membrane.

## RESULTS

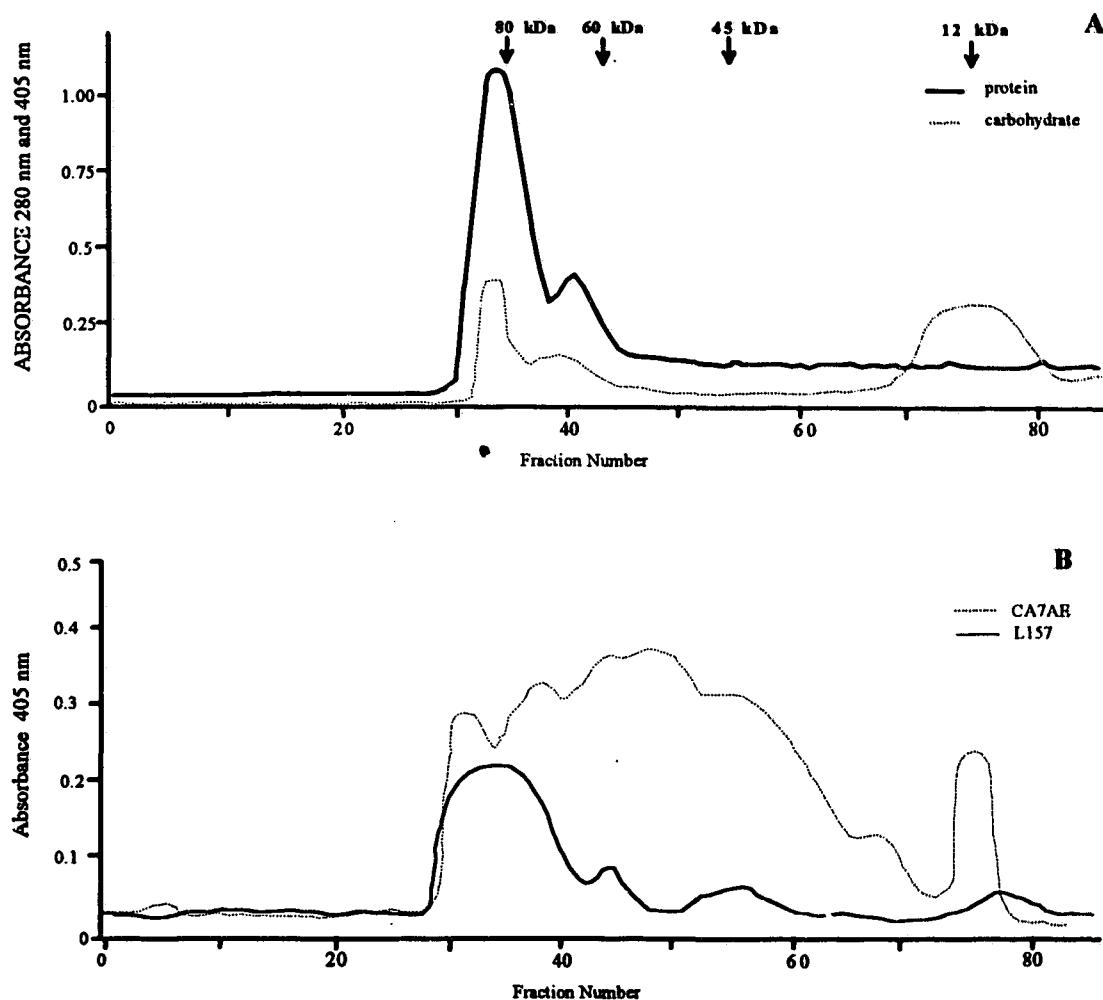
### 3.1 Characterization of the Interaction Between the Lipophosphoglycan and the Lipophosphoglycan Associated Protein:

Electropherograms of solvent E extracts from whole delipidated promastigotes showed a major Coomassie blue staining protein component which migrated as a broad band with  $M_r$  of 11,000 (Figure 3.1, lane 1). Fractionation of this material by Sephadex G-100 gel permeation chromatography yielded two 280 nm absorbing components eluting with a  $M_r$  in excess of 80,000. Both fractions also contained significant levels of carbohydrate as determined by the phenol/sulfuric acid colorimetric assay (Figure 3.2A). ELISA assays using the LPG specific mAbs CA7AE and the LPGAP specific mAb L157, showed that these molecules co-eluted in fractions 29-39 suggesting bimolecular complex (Figure 3.2B). In addition, the detection of LPG over the entire gel permeation profile indicated that this glycan combine to form aggregates with a wide array of molecular weights (Figure 3.2B). Due to the amphipathic structure of the LPG, it was rationalized that in polar solvents the most favorable conformation adopted by this glycolipid would be a micelle structure to minimize the interaction of the alkyl chain with the aqueous milieu. Moreover, the 280 nm absorbance associated with fractions containing LPG was indicative of the inclusion of LPGAP into micelles. Indeed, analysis of the GPC void volume fractions by negatively stained electron microscopy showed the presence of spherical structures with an approximate mean diameter of  $20 \pm 4$  nanometers (Figure 3.3). Experiments to demonstrate the presence of LPGAP in these micelles by immunogold affinity labelling with mAbs L98 and L157 were not undertaken as immunofluorescence labelling of promastigotes with these antibodies required prior permeabilization with saponin or acetone (McNeely, *et al.* 1990; Tolson, *et al.* 1992).

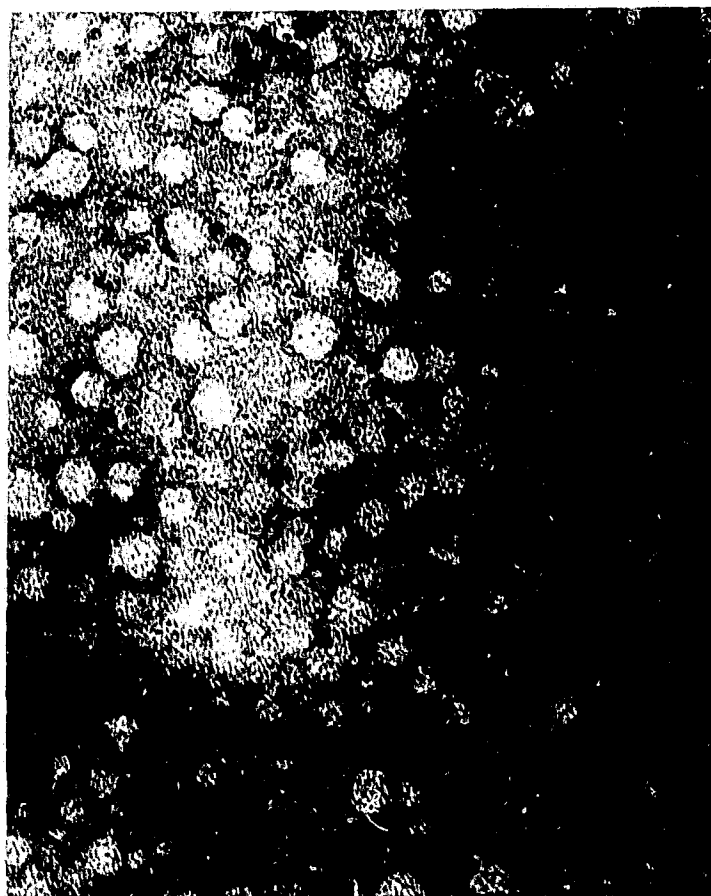
The nature of the interaction facilitating the formation of a complex by LPG and LPGAP was further investigated by ion exchange chromatography. The crude solvent E extract was applied to a Mono Q column and developed with a 0-1 M linear sodium chloride gradient and one millilitre fractions were collected for analysis by indirect ELISA



**Figure 3.1:** SDS-PAGE electropherogram of the *L. donovani* promastigote LPGAP. Solvent E extracts were concentrated to a 2 ml and the LPG/LPGAP was precipitated with acetone and the pelleted was analyzed on a 15% Laemmli gel (lane 1). The precipitated was chromatographed on a Sephadex G-100 column and the void fraction s containing LPG/LPGAP (Figure 3.2) were electrophoresed on the above SDS-PAGE gel (lane 2).



**Figure 3.2:** Gel permeation chromatography of *L. donovani* promastigote solvent E extracts. After evaporation of solvent E the residue was dissolved in 40 mM  $\text{NH}_4\text{OH}$ , 1 mM EDTA (4 ml) and chromatographed on a Sephadex G-100 (2.7 x 34 cm) developed with the above solvent at a flow rate of 0.5 ml/min. Panel (A) shows the 280 nm profile superimposed upon a profile of carbohydrate as determined by the phenol/sulfuric acid colorimetric assay. Panel (B) shows the ELISA data for each fraction with the monoclonal antibodies CA7AE and L157. The Sephadex G-100 column was calibrated with a protein standard containing: human transferrin (80 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and cytochrome c (12 kDa).

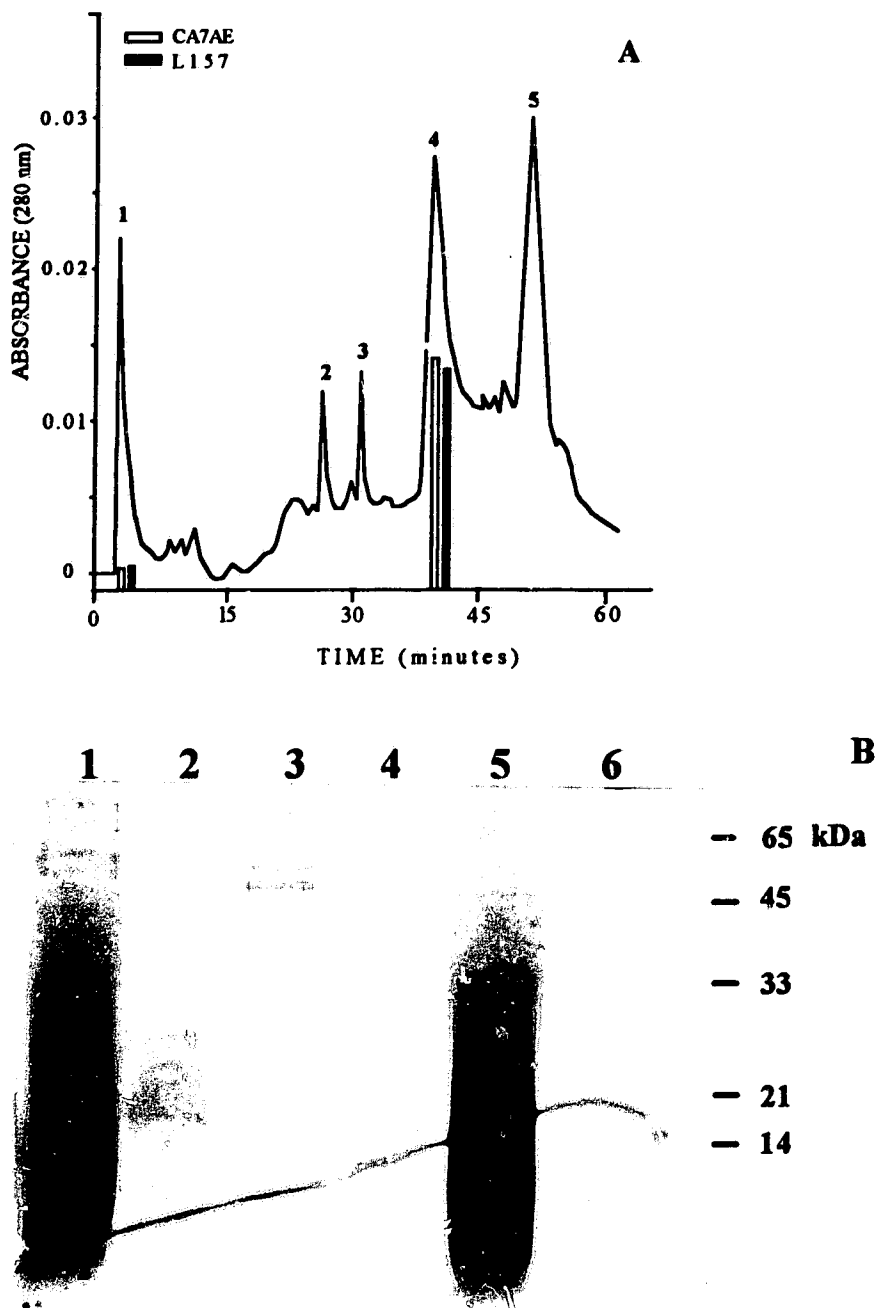


**Figure 3.3:** Electron micrograph of an LPG/LPGAP complex isolated by gel permeation chromatography. The LPG/LPGAP was coated onto copper grids and negatively stained with molybdate and analyzed on a Hitachi transmission electron microscope at a potential of 75 kV. Magnification 100,000X.

with mAbs L157 and CA7AE. Using this chromatographic technique the LPG and LPGAP co-eluted in a symmetrical peak with approximately 0.5 M NaCl, as illustrated in Figure 3.4A. Only those fractions corresponding to peak 4 gave ELISA responses above background for both L157 and CA7AE and SDS-PAGE analysis of the peaks marked 1 to 5 showed that only peak 4 contained an 11 kDa protein. The latter observation provided further evidence for the presence of a proteo-glycolipid micelle complex.

Finally it should be noted that the lack of Coomassie blue staining material in the 280 nm absorbing peak 5 was probably due to hemin which had been previously detected in solvent E extracts analyzed by mass spectroscopy and spectrophotometrically. It should also be noted, that this low level hemin contamination persisted with the LPG/LPGAP after gel permeation and anion exchange chromatography, as evidenced by its faint green colour.

Insight into the molecular interactions involved in stabilizing the LPG/LPGAP association were obtained by treating the complex with phosphatidylinositol specific phospholipase-c (PIPLC), which cleaves the lysolipid anchor from LPG (Orlandi & Turco, 1987 & 1989). Chromatography of the digest on a Mono Q anion exchange column (Figure 3.5A) resulted in the disappearance of the 280 nm absorbance corresponding to the LPG/LPGAP (peak 4), this was accompanied by an increase in the L157 positive material eluting in the void volume. More importantly, this latter fraction was devoid of CA7AE activity confirming the effective cleavage of the phosphoglycan structure by PIPLC. The presence of residual CA7AE and L157 activity eluting at the position of the LPG/LPGAP complex indicated the sensitivity and the non-linear response of the indirect ELISA assay. This was verified by amino acid analysis which indicated substantial amounts of LPGAP in the void fraction (nanomole) and only background levels of amino acids were detected in the L157 reactive material eluting at 0.5 M NaCl. The shift in LPGAP elution suggested that the interaction between this protein and the LPG is hydrophobic in nature rather than electrostatic, as the latter would not be disrupted by PIPLC treatment. Supporting evidence for this non-polar interaction between the lysolipid anchor of LPG and the LPGAP was provided by anion exchange chromatography of the LPG/LPGAP in the presence of nonionic detergent CHAPS. Figure 3.5B, shows that



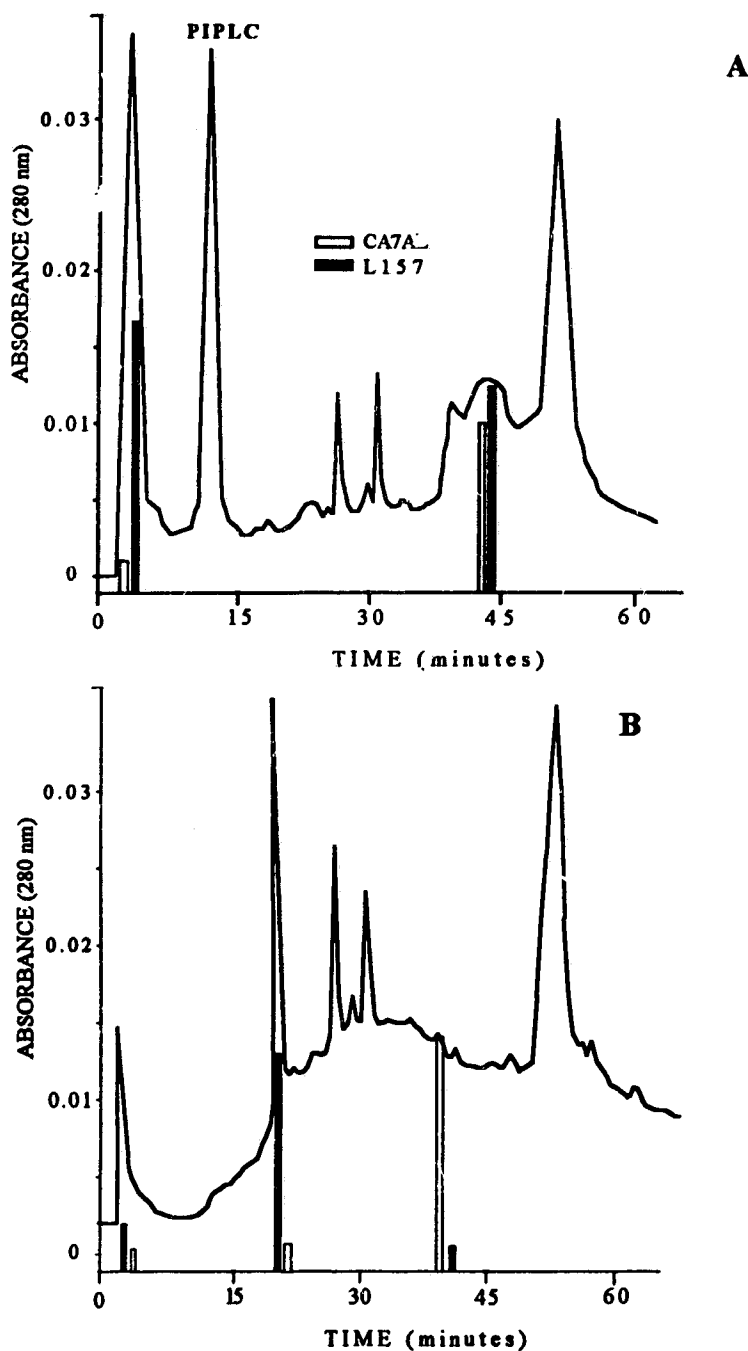
**Figure 3.4:** Anion exchange chromatography of the LPG/LPGAP complex. (A) the residue from solvent E extracts were dissolved in buffer A and applied to a Mono Q column equilibrated in 50 mM Tris HCl pH 8.0 (buffer A) and developed with a 50 ml gradient from 0-1 M NaCl in buffer A at a flow rate of 1 ml/min. (B) peaks denoted by numbers were collected, dialyzed exhaustively, against H<sub>2</sub>O, lyophilized, and analyzed by SDS-PAGE on a 15% gel and stained with 0.1% Coomassie blue. Lane 1, crude solvent E extract injected on to the Mono Q; lane 2, peak 1; lane 3, peak 2; lane 4, peak 3; lane 5, peak 4; lane 6, peak 5.

dissolution of the LPG/LPGAP complex in 0.5% CHAPS resulted in dissociation of the complex with the LPGAP eluting with approximately 0.25 M NaCl on the Mono Q, while the LPG interacted more tightly with the Mono Q support and was eluted with 0.5 M NaCl. Together these data would argue that LPGAP possesses a hydrophobic domain which associates with the lipid portion of the LPG forming a micelle structure in aqueous solutions.

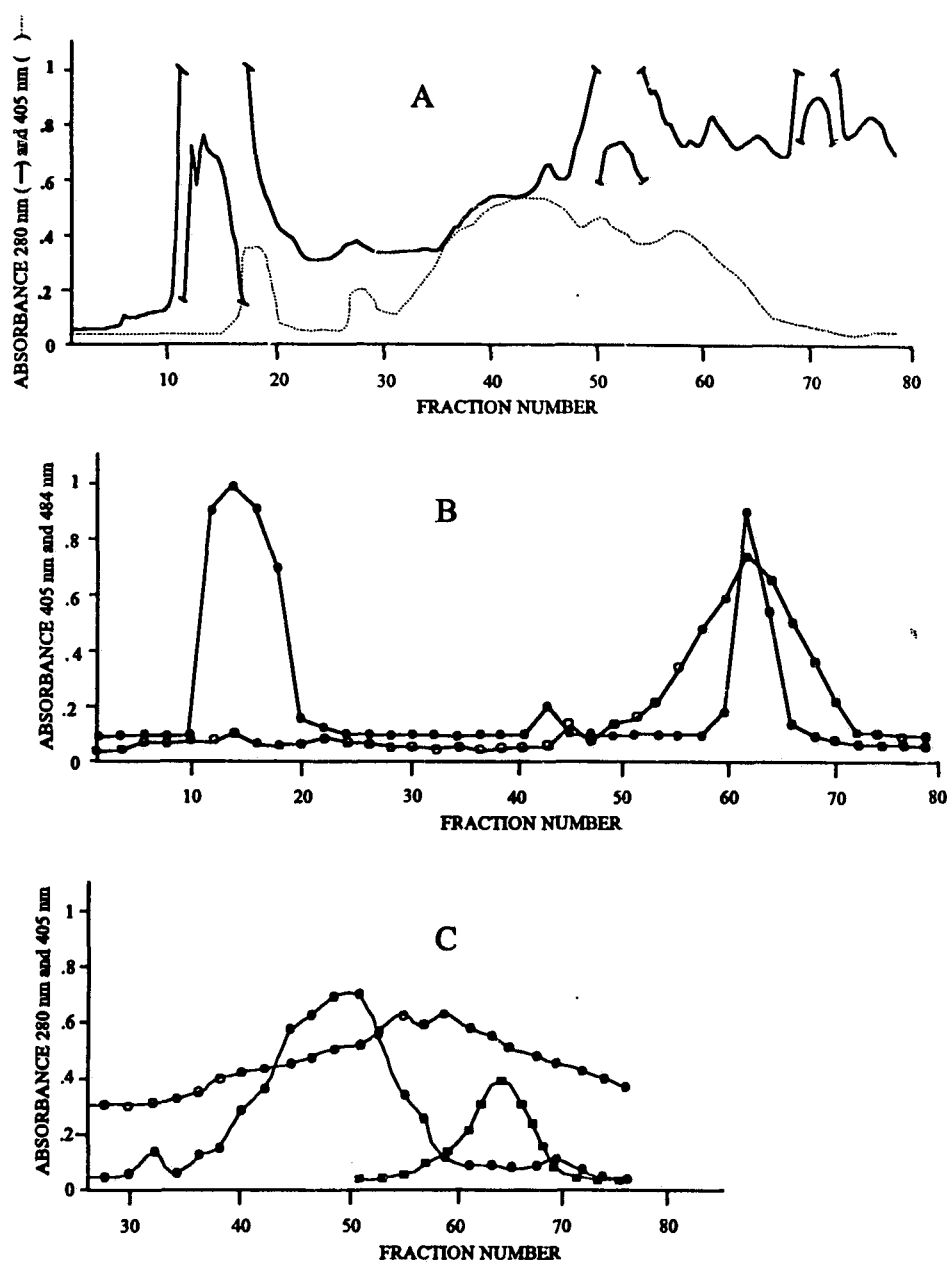
### **3.2 Purification of *L. donovani* Fromastigote LPGAP:**

Demonstration that the LPG/LPGAP complex involved a hydrophobic interaction suggested that the separation of these two components could be obtained by reversed phase chromatography. Indeed, McConville had used this approach earlier to purify *L. major* LPG (McConville *et al.*, 1987). A modification of this procedure was employed using an octyl Sepharose column developed with a linear gradient of 2-propanol. By monitoring the column effluent spectrophotometrically (280 nm) and assaying the fractions with the mAb L98 on an indirect ELISA, the LPGAP was localized to fraction 35-65 (Figure 3.6A) while the quantitative phenol/sulfuric acid assay indicated that significant levels of carbohydrate were found in fractions 1-15 and 62-75. The latter peak was confirmed to contain LPG using the mAb CA7AE (Figure 3.6B). Thus it was possible to resolve the LPGAP and the glycolipid LPG. Since LPGAP eluted as a broad peak, the L98 positive fractions were pooled and re-chromatographed on the above octyl Sepharose column to eliminate trace levels of LPG. Once again, monitoring the fractions with L98 and phenol/sulfuric acid facilitated pooling the LPGAP containing fractions free of LPG (Figure 3.6C).

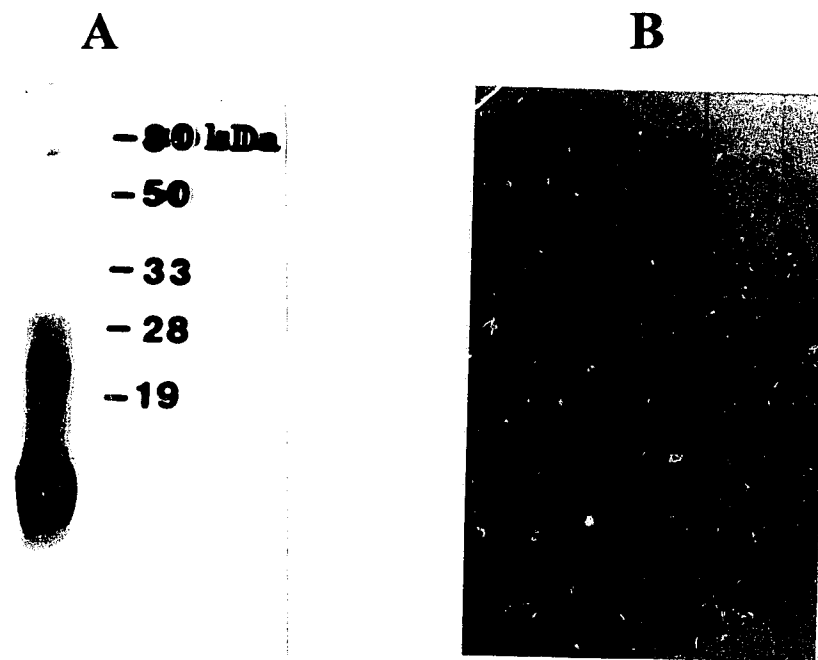
Analysis of the L98 reactive material by SDS-PAGE showed a major band with a  $M_r$  of 11,000 and approximately 20 minor species ranging in  $M_r$  from 11-40,000. All cross-reacted with L98 and L157 on immunoblots (Figure 3.7 A and B). These data suggested that the 11 kDa LPGAP species was derived from a parent 40 kDa molecule by a post-translational proteolytic maturation event. However, as will be discussed below, the isolation and sequencing of the *lpgap* gene showed that the multiple bands were due to aggregation and not due to proteolysis.



**Figure 3.5:** Characterization of the LPG and LPGAP interaction Mono Q by anion exchange chromatography. (A) Crude LPG/LPGAP was incubated with phosphatidylinositol specific phospholipase-C overnight at 37°C. The hydrolysate was analysis on a Mono Q column equilibrated in 50 mM Tris HCl, pH 8.0, (buffer A) and developed with 0-1 M NaCl in buffer A at a flowrate of 1 ml/min. (B) crude LPG/LPGAP was dissolved in buffer A containing 0.5% CHAPS and chromatographed on the Mono Q with the buffer system described for panel containing 0.5% CHAPS.



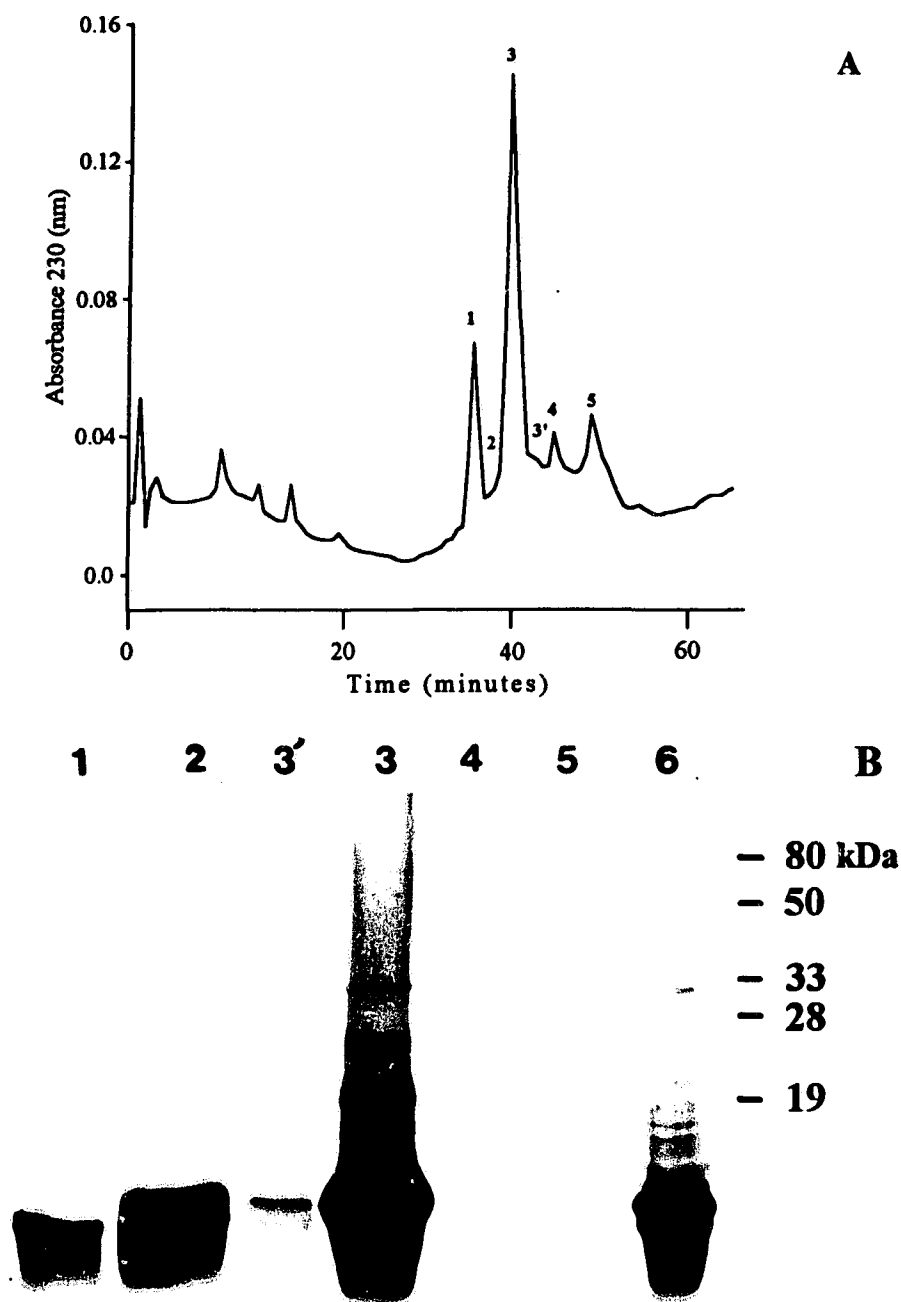
**Figure 3.6:** Reverse phase chromatography of LPG/LPGAP. The LPG/LPGAP complex was chromatographed on an octyl Sepharose column equilibrated with 0.1 M ammonium acetate/10% 2-propanol and developed with linear 2-propanol gradient to 70% over 12 h at a flowrate of 0.3 ml/ min. (A) 280 nm (---); ELISA reaction with L98 mAb, 405 nm (—); (B) phenol/sulfuric acid colorimetric assay for carbohydrate, 484 nm (closed circles); ELISA reaction with anti-LPG repeat mAb CA7AE, 405 nm (open circle); (C) fractions 32 to 50 from trace A were pooled and re-chromatographed under identical conditions used in A. ELISA reaction with L98 mAb, 405 nm (closed circles); 280 nm (open circles); phenol/sulfuric acid colorimetric assay for carbohydrate (closed squares).



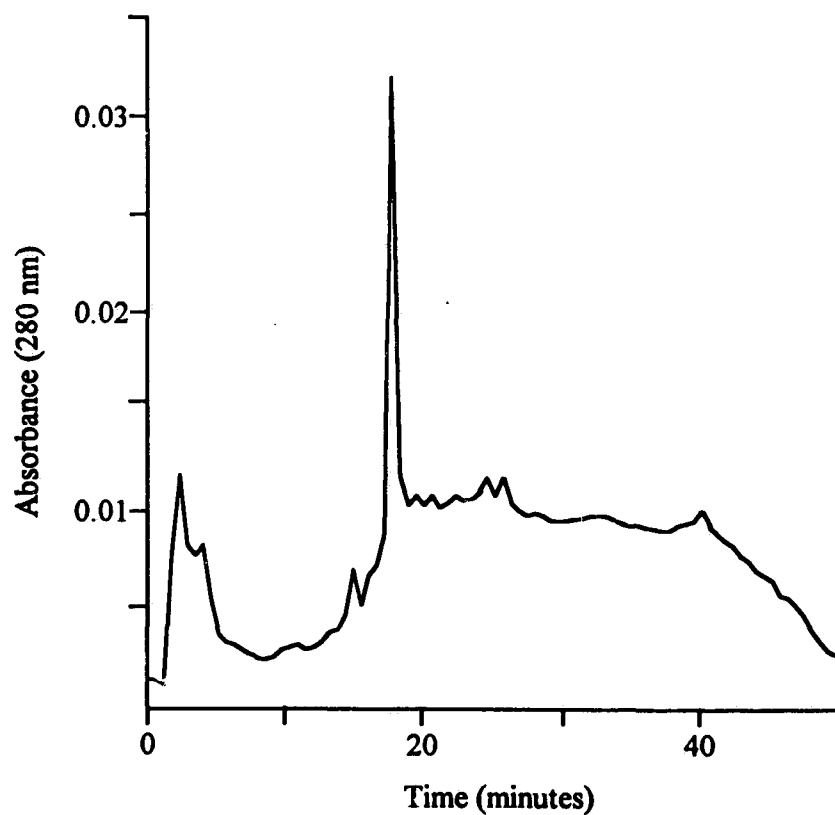
**Figure 3.7:** Analysis of L98 positive material purified by octyl Sepharose chromatography. Immunopositive material was separated by SDS-PAGE electrophoresis on a 15% gel and stained (A) Coomassie blue stain (B) Immunoblot probed with the anti-LPGAP mAb L98.

Efforts to purify the dominant 11 kDa protein from either crude solvent E extracts or the octyl Sepharose purified LPGAP using silica based C<sub>8</sub> reversed phase columns were ineffective and resulted in dramatic losses of material due to irreversible binding of the protein to the solid support. However, employing a PRP-1 polymer based reversed phase media derivatized with C<sub>1</sub> alkyl groups, it was possible to resolve the crude LPGAP preparation into 4 major components (Figure 3.8A) all exhibiting L98 and L157 reactivity. The presence of a common 11 kDa protein species in these fractions (Figure 3.8B), together with the ELISA data, indicated that all 4 peaks contained closely related proteins which differentially eluted on reversed phase HPLC presumably as a function of either post-translational modifications or variations in the tertiary conformation. This hypothesis was further substantiated by amino acid analysis of peaks 1 and 3 (Table 3-1) which showed similar compositions, including a diagnostic high mole percent for aspartic, glutamic and lysine residues. To investigate the possibility that the multiple peaks observed for the LPGAP on the PRP-1 column were due to conformational differences, the L98 positive material obtained by octyl Sepharose chromatography was dissolved in 50 mM Tris HCl pH 8.0 containing 8 M urea, 0.5% CHAPS and incubated at 100°C for 10 min. Examination of the denatured LPGAP by anion exchange HPLC yielded a major component eluting with 0.24 M NaCl (Figure 3.9). Analysis of the Mono Q fractions by ELISA confirmed that this material was recognized by both L98 and L157 mAbs. In addition, a peak eluting at the void volume contained a substantial 280 nm absorbance which also cross reacted with the above mAbs. However, to further demonstrate that the octyl Sepharose LPGAP preparations contained a single protein species was provided by identical cyanogen bromide peptide maps for peaks 1 and 3, obtained by reversed phase chromatography on the PRP-1 column.

An alternative purification scheme which afforded a high recovery of the dominant 11 kDa LPGAP protein from crude solvent E extracts, with fewer chromatographic steps, utilized preparative SDS-PAGE electrophoresis. Prior to electrophoresis, the LPG PO<sub>4</sub>-Gal-Man repeat was depolymerized by mild acid hydrolysis (Turco *et al.*, 1982), and the remaining LPG core glycolipid extracted with acetone. Treatment with TFA had no detrimental effect on the protein backbone, as judged by amino acid composition



**Figure 3.8:** Analysis of octyl Sepharose purified LPGAP by reversed phase HPLC. (A) LPGAP (50  $\mu$ g) purified free of LPG by octyl Sepharose chromatography was dissolved in 100  $\mu$ l of 0.1% TFA containing 8 M urea and applied to a PRP-1 HPLC column equilibrated with a 0.1% TFA and developed with a 0-60% linear gradient of 2-propanol at a flowrate of 1 ml/min. (B) Fractions with 230 nm absorbance were collected, electrophoresed on 15% SDS-PAGE gel and stained with 0.1% Coomassie blue dye. Lanes 1-5 correspond to the peak labels in panel A and lane 6 shows the crude material loaded onto the PRP-1 column.



**Figure 3.9:** Anion exchange analysis of LPGAP purified by octyl Sepharose chromatography. Fifty micrograms of LPGAP was dissolved in 100  $\mu$ l of 50 mM Tris HCl, pH 8.0 containing 8 M urea, 0.5% CHAPS and incubated at 100°C for 10 min. The mixture was analyzed on a Mono Q anion exchange column equilibrated with buffer A containing 0.5% CHAPS (buffer A) and developed with a 50 ml 0-1 M linear NaCl gradient in buffer A at a flowrate of 1 ml/min.

**TABLE 3-1**  
**Amino Acid Composition of Lipophosphoglycan associated proteins**  
**Chromatographed on the PRP-1 HPLC column<sup>a</sup>**

Amino Acids	Peak 1 <sup>b</sup>	Peak 3 <sup>b</sup>
Aspartic	6	9
Glutamic	22	22
Serine	5	5
Glycine	3	2
Histidine	7	5
Arginine	3	4
Threonine	3	4
Alanine	6	8
Proline	4	3
Tyrosine	3	3
Valine	1	1
Methionine	3	4
Isoleucine	1	1
Leucine	5	6
Phenylalanine	6	8
Lysine	15	13

*a* Refer to Figure 3.8A

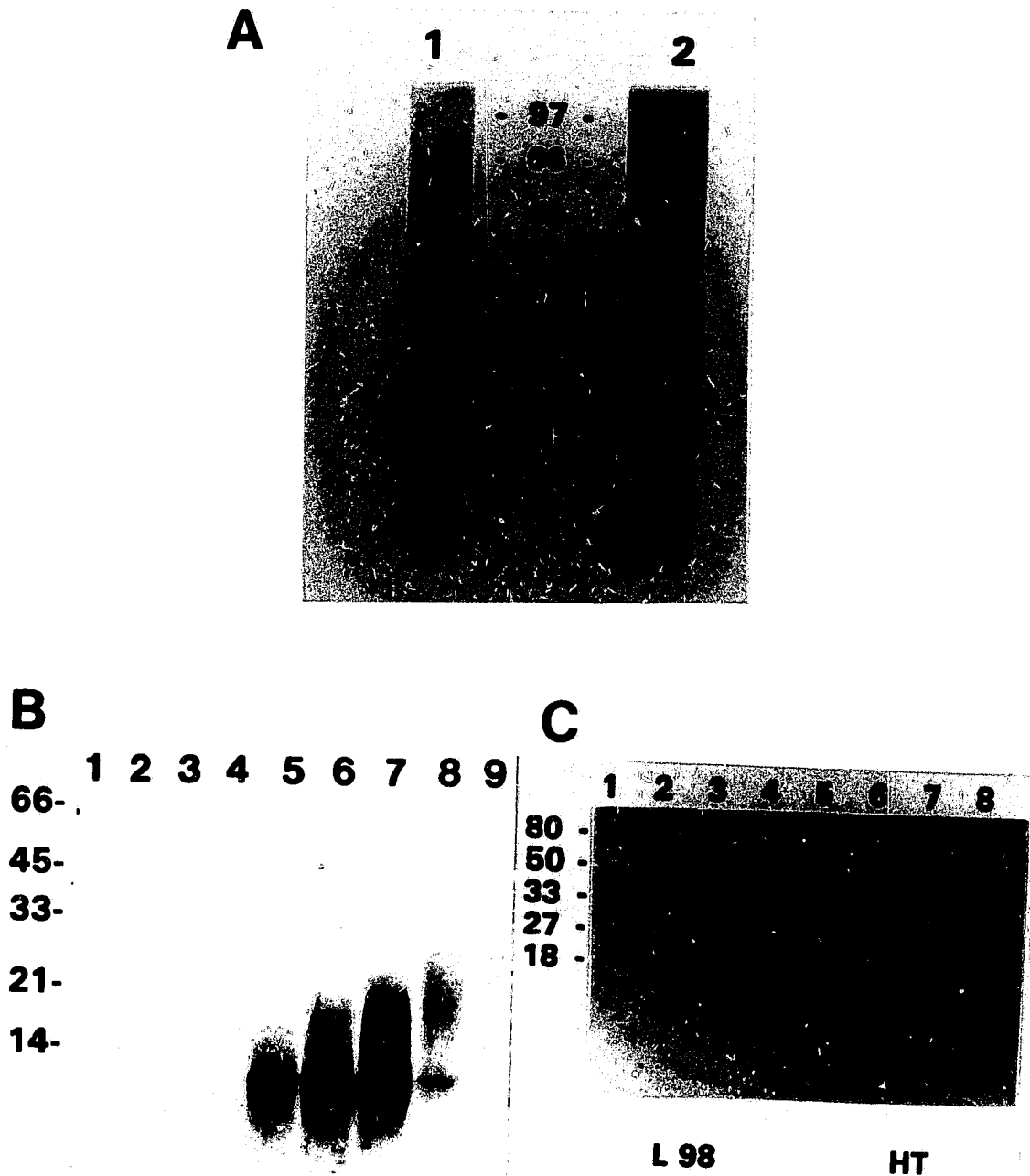
*b* amino acid compositions were calculated assume a molecular weight of 11 kDa for LPGAP.

of Western blotted samples, but caused the LPGAP to migrate as a more compact band on SDS-PAGE (Figure 3.10). As shown in Figure 3.10B and 3.10C, fractions 5 and 6 contained a single protein by Coomassie blue staining which immunostained with the LPGAP specific mAb L98. These fractions were pooled, dialyzed, concentrated in a Speed Vac and acetone precipitated to recover the protein free of detergent.

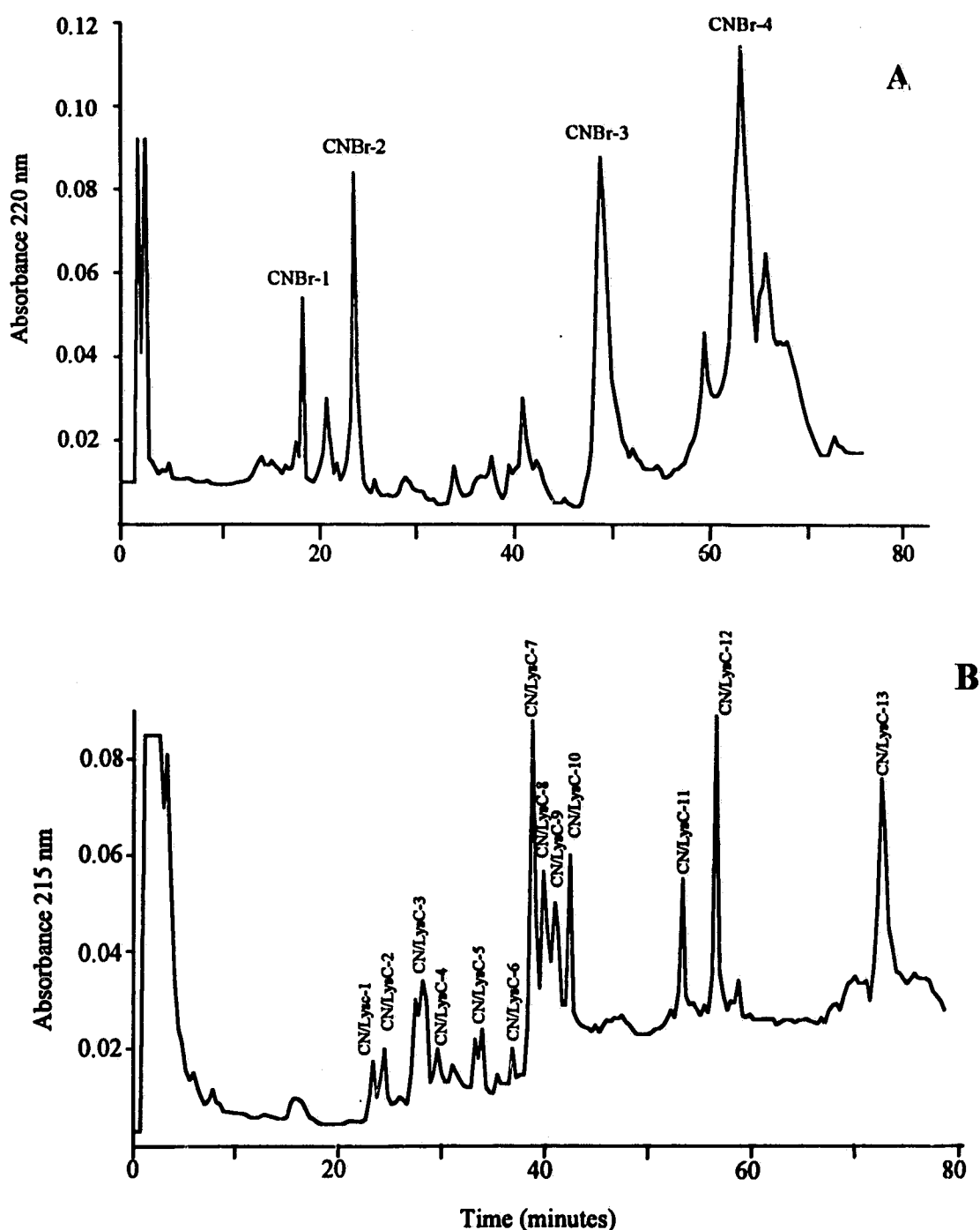
### **3.3 Primary Structure Analysis of *I. eishmania donovani* Promastigote LPGAP:**

Several attempts to obtain sequence data directly from SDS-PAGE purified LPGAP were unsuccessful due to a modification at the N-terminus. Treatment of the LPGAP with cyanogen bromide yielded 4 peptides resulting from cleavages at methionine residues 44, 54, and 66 (Figure 3.11A). Using microsequencing data, verified by amino acid compositions (Tables 3-2 and 3-3), it was possible to deduce the complete primary structure for three of these peptides, CNBr-1, CNBr-2, and CNBr-3 as shown in Table 3-3. A partial cyanogen bromide digest of LPGAP also afforded the peptide CNBr-5, encompassing residues 45-84, this fragment confirmed the contiguous arrangement of the cyanogen bromide peptides in the following order: CNBr-2, CNBr-1 followed by CNBr-3 (Figure 3.12). Digestion of the N-terminal blocked CNBr-4 peptide with the protease Lys-C yielded 13 peptides (designated CN/LysC). However only 3 overlapping peptides were found to have unmodified amino termini (Table 3-3) (Figure 3.11B). Comparison of the amino acid composition of CNBr-4 with the sequence afforded by CN/LysC peptides 7, 10, and 11 indicated that only a partial sequence for the CNBr-4 fragment had been deduced. These findings together with the failed sequence analysis of the intact LPGAP molecule suggested that CNBr-4 corresponded to the N-terminal fragment of LPGAP. This was later corroborated by the isolation and sequencing of the gene encoding LPGAP.

It was noteworthy, that the first Edman degradation cycle for the peptide CNBr-2 did not co-elute with any of the standard PTH amino acids. Amino acid analysis of this peptide indicated a composition with 2 Arginine residues, although the primary sequence analysis of CNBr-2 indicated only a single Arg, at position 53. As will be shown below, this post-translational modification was determined to be a N<sup>ε</sup>-monomethylarginine by mass spectroscopy.



**Figure 3.10:** Purification of *L. donovani* LPGAP. A) Denaturing SDS-PAGE gel of *L. donovani* promastigote LPGAP; lane 1) crude LPGAP depleted of LPG by mild acid hydrolysis; lane 2) crude LPGAP extracted with solvent E. B) Coomassie blue stained gel of preparative electrophoresis fractions 1-9 (lanes 1-9); C) Western blot analysis of preparative gel fractions with LPGAP specific mAb L98 and human transferrin specific HT (negative control): lanes 1 & 5) fraction 9, lanes 2 & 6) fraction 8, lanes 3 & 7) fraction 7, lanes 4 & 8) fraction 6.



**Figure 3.11:** Fragmentation of *L. donovani* promastigote LPGAP purified by octyl Sepharose chromatography. (A) LPGAP CNBr digests were separated by reversed phase chromatography on a PRP-1 column developed with a linear gradient of acetonitrile and sequenced by Edman degradation. (B) Endoproteinase Lys-C digest of the N-terminally blocked CNBr-4 peptide. Digest was separated by reversed phase chromatography on an Aquapore 300 C<sub>2</sub> column, developed with a linear gradient of acetonitrile.

TABLE 3-2

Amino acid Composition of Intact *L. donovani* LPGAP and CNBr Peptides

	Intact <sup>a</sup>	CNBr-1 <sup>b</sup>	CNBr-2 <sup>c</sup>	CNBr-3 <sup>c</sup>	CNBr-4 <sup>d</sup>
Aspartic <sup>e</sup>	7.1	1.11			6.38
Glutamic <sup>e</sup>	19.7	2.23	3.38	7.83	10.4
Serine	4.8			1.82	2.62
Histidine	4.9	0.97	0.99	2.77	1
Arginine	3.5		1.83		4.04
Threonine	3.6	0.97			3.6
Alanine	7			3	4.79
Proline	3.3			0.97	0.83
Tyrosine	2.5		0.92	0.91	2.04
Methionine	4.1				
Isoleucine	1.2	0.72			
Leucine	5.1			1.95	4.28
Phenylalanine	6.4	1	1	2.04	4.71
Lysine	12.5	3.77	1.17	4.27	4.77

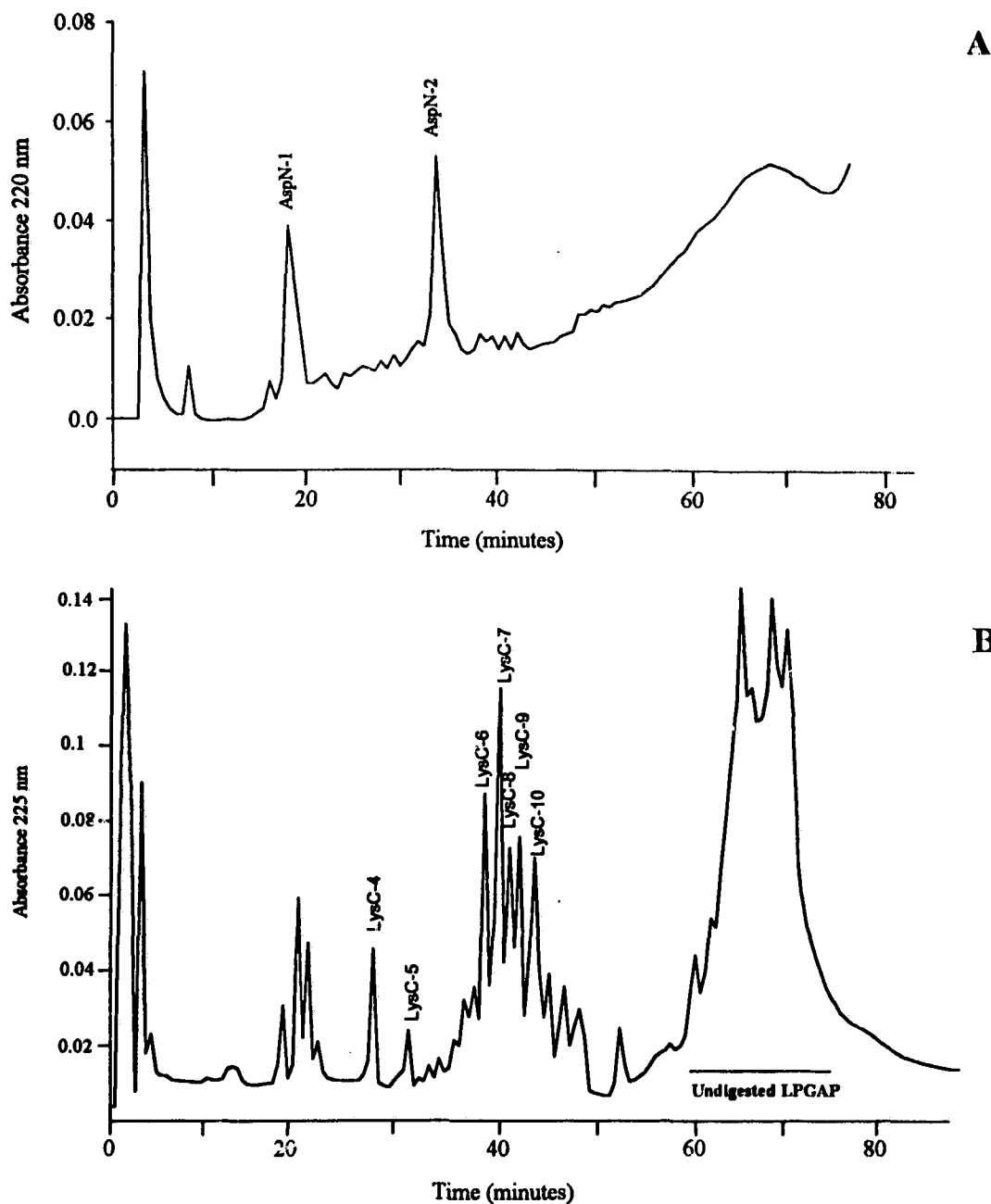
<sup>a</sup> Values are an average of four different compositional analysis and are based on a molecular weight of 11 kDa.

<sup>b</sup> values were calculated assuming 1 phenylalanine residue

<sup>c</sup> values were calculated assuming 3 alanine residues.

<sup>d</sup> values were calculated assuming 1 histidine residue.

<sup>e</sup> Aspartic acid values represent both the aspartic acid and asparagine composition, likewise the glutamic acid values include both the glutamic acids and glutamine values.



**Figure 3.12:** Chromatographic profiles of the lipophosphoglycan associated protein digested with the endoproteases Asp-N and Lys-C. LPGAP was digested either with endoprotease Asp-N (A) or endoprotease Lys-C (B) for 8 h at 37°C, then applied to a C<sub>8</sub> Aquapore 300 (10 x 100 mm) reversed phase HPLC column equilibrated with 0.1% TFA. The column was developed at a flowrate of 0.5 ml/min with a 0.5%/min linear acetonitrile gradient to a final acetonitrile concentration of 60%. Peaks were manually collected and subjected to protein sequence analysis.

TABLE 3-3

Amino Acid Sequence of LPGAP Fragments Determined by Edman Degradation of CNBr, Endoproteinase Lys-C, or Endoproteinase Asp-N Peptides

Digest	Peptide Designation	Peptide Sequence <sup>a,b</sup>
CNBr LPGAP	CNBr-1	IKEHTEKFNNK
	CNBr-2 <sup>c</sup>	ZEHYEKFER
	CNBr-3	HEHSEHFKQKFAELLEQQKAAQYPsk
	CNBr-5 <sup>c</sup>	ZEHYEKFERMIKEHTEFNKKMHEhSeHFKQkfAELLEqQ
LysC/CNBr-4	CN/LysC-7	ttYEEFsaKLDRLle
	CN/LysC-10	LDRLDDEEFN
	CN/Lys-C 11	LDRLDDEEFN
LysC LPGAP	LysC-4	FAELLEQQ
	LysC-5	QKFAELLEQQ
	LysC-6	ADKPDESTLSPEM
	LysC-7	KKMHEHSEHFKQKFAELLEQQ
	LysC-8	ADKPES
	LysC-9	FFADKPDEST
	LysC-10 <sup>c</sup>	ADKPDESTLSPEMZEHYxKFxRMIKEHTh
Asp-N LPGAP	AspN-1	EQQKAAQYPSK
	AspN-2 <sup>c</sup>	DEEFNRKZQEQNakf
<i>in vivo</i> cleavage	IVP peptide	ADKPDESTLSPEMxE

<sup>a</sup> lower case indicated equivocal residue assignment.

<sup>b</sup> x- denotes an unidentified amino acid residue.

<sup>c</sup> z- indicates amino acid residues not co-eluting with standard 20 PTH-amino acids.

Cyanogen bromide fragmentation of LPGAP facilitated the determination of approximately 50% of the primary structure by Edman degradation. However, as indicated above, a blocked N-terminal precluded direct analysis of the intact molecule. Further proteolytic digests were, therefore, employed to acquire additional N-terminal amino acid sequence information. Cleavage of intact LPGAP with the protease Asp-N generated two peptides (Figure 3.13A), the first of these, peptide AspN-1 had an unexpected glutamyl N-terminus and mapped to the C-terminal region of LPGAP confirming the previous sequence data obtained from the CNBr-3 (Table 3-3). Although the endoproteinase Asp-N shows a high degree of specificity for aspartic and cysteic acid residues, it has been shown to cleave at glutamyl residues but with a rate 1000 times slower than that observed for aspartic acids (Drapeau, 1980).

The second peptide AspN-2 provided new sequence data corresponding to the amino terminal portion of the protein not obtained by CNBr or the endoproteinase Lys-C digest of the CNBr-4 fragment. More importantly, during the sequence analysis of the AspN-2 peptide a second modified residue, co-eluting in the approximate position of the PTH-Met sulfoxide, was found at position 22 (Table 3-3). This amino acid was subsequently confirmed to be a methionine from the gene sequence and may explain the resistance of Met at position 22 to cleavage with cyanogen bromide. The oxidation of the thioether group on the methionine side chain decreases the partial negative charge located at the sulfur which in turn eliminates the formation of the cyanosulfonium bromide reaction intermediate required for cleavage of methionyl peptide bonds (Gross & Witkop, 1962). In the case of LPGAP, this unusual modification was apparently due to a facile oxidation to methionine sulfoxide, as subsequent cleavage attempts were successful providing the molecule was first reduced with mercaptoethanol (Houghten *et al.*, 1979).

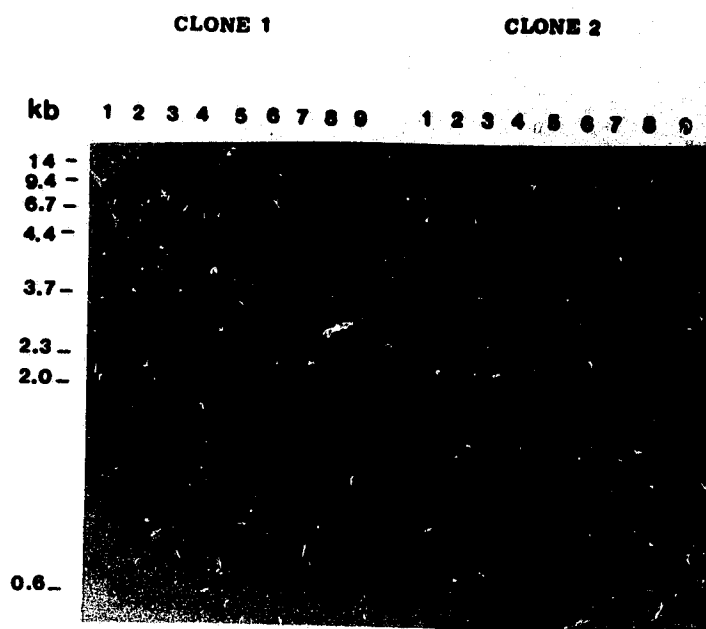
As a final attempt to complete the primary structure of LPGAP by protein microsequence analysis, octyl Sepharose preparations were digested with endoproteinase Lys-C, in hopes of isolating peptides spanning the sequences derived from CNBr and the Asp-N fragmentation. By this method a single peptide, LysC-10, provided essential residues which overlapped with the CNBr peptides 2, 3, and 5 allowing the N-terminal portion of LPGAP to be extended by an additional 13 amino acids (Table 3-3). A limited

population of the remaining peptides from this digest were also subjected to Edman degradation, however, the resulting sequences served only to confirm data obtained by cyanogen bromide and endoproteinase Asp-N fragmentation. One interesting observation stemming from the Lys-C treatment of the LPGAP was the fact that the structures ascertained for several peptides began with the sequence ADKP. From these results it was originally thought that these peptides arose from the cleavage of LPGAP at a putative lysine residue located upstream of the N-terminal Ala. But this postulate was dismissed with the isolation and characterization of the peptide LysC-9 (Table 3-3) which had the following sequence FFADKPDEST. Based on the strict requirements of the protease Lys-C for lysyl bonds (Jekel *et al.* 1983) it was concluded that cleavage of the LPGAP between the phenylalanine and the alanine must have occurred *in vivo* by a parasite protease exhibiting a cleavage specificity for the amino acid motif FFADKP.

In addition to the full length LPGAP, a truncated fragment was routinely found to co-purify with LPGAP on octyl Sepharose. N-terminal characterization of at least four different preparations gave a single protein sequence for this *in vivo* processed fragment (IVP), as shown in Table 3-3. Sequence analysis suggested that although this peptide constituted only a minor population of the LPGAP present on the surface of the parasite, the regularity with which this sequence was detected strongly supported the contention that the truncated fragment resulted from a specific proteolytic event.

#### **3.4 Isolation and Characterization of the *lpgap* gene:**

As a consequence of the blocked N-terminal, further protein structural analysis of the LPGAP was halted and the available sequence information utilized to clone and sequence the gene. This alternative approach not only allowed completion of the LPGAP sequence but also facilitated elucidation of the parent molecule in knowledge of apparent proteolytic processing. Immunoblots of SDS-PAGE separation using the monoclonal antibodies L98 and L157 had indicated that the 11 kDa LPGAP protein may be derived from a larger parent molecule of approximately 40 kDa. Therefore it was necessary to isolate the LPGAP gene and determine the DNA sequence to clarify this question.



**Figure 3.13:** Southern blot analysis of *L. donovani* gDNA cosmid clones hybridizing with the LPGAP 45mer oligonucleotide probe. Each clone was subjected single or double digests with a series of restriction endonucleases, separated on a 0.8% agarose gel, clotted by capillary action onto nitrocellulose membrane and hybridized with a 45 bp oligonucleotide probe specific for the LPGAP protein. Lane 1) *EcoR* I; 2) *BamH* I; 3) *Sal* I; 4) *Sma* I; 5) *EcoR* I/*BamH* I; 6) *EcoR* I/*Sal* I; 7) *Sac* I; 8) *BamH* I/*Sal* I, 9) *Sac* II.

Under moderate stringency conditions two thousand *L. donovani* genomic DNA cosmid clones were screened using a low degeneracy 45 base pair oligonucleotide (Langford *et al.*, 1992) corresponding to the protein sequence, MIKEHTEFNKMKMHEH two positive clones were isolated. These LPGAP positive cosmid clones were initially characterized by restriction and Southern blot analysis. As shown in Figure 3.13, digests of clones 1 and 2 with either *EcoR* I or *BamH* I yielded a 10 kb fragment which hybridized with the LPGAP 45mer probe. The LPGAP insert in cosmid clone 1 was further narrowed to 3.4 kb by a *Sac* I digest and similar digests of clone 2 afforded two fragments of 3.4 and 4.2 kb, both hybridizing with the LPGAP oligonucleotide probe. Additional restriction digests of clones 1 and 2 with *Sal* I gave either two (0.9 and 2.0 kb) or three (0.9, 2.0, and 3.1 kb) fragments respectively, all cross hybridizing with the LPGAP 45mer (Figure 3.13). The *Sac* I and *Sal* I bands common to both clones were diagnostic of the *Leishmania* insert DNA being derived from overlapping fragments generated by the *Mbo* I restriction of the *L. donovani* promastigote gDNA. The presence of multiple cross hybridizing *Sal* I fragments in both clones suggested that the LPGAP was encoded by multiple gene which were closely linked in the genome. The possibility that multiple hybridizing bands arose from a *Sal* I restriction event within the hybridization site was discounted, as inspection of the LPGAP 45 mer probe did not reveal a *Sal* I site.

Prior to subcloning the *Sac* I 3.4 kb fragment into a sequencing vector, the presence of the *lpgap* gene with this fragment was verified by a partial DNA sequence using cosmid clone 1 as the template and the LPGAP 45mer probe as the primer. Although limited sequence data was obtained at this stage, we observed a single open reading frame which corresponded precisely to residues 82-91 (Figure 3.14) of the LPGAP protein sequence.

The complete gene sequence was deduced by inserting the 3.4 kb *Sac* I fragment into the pBluescript phagemid vector, followed by the generation of a series of nested deletion mutations (Sambrook *et al.*, 1989) used in conjunction with the T7 DNA polymerase based Sequenase version 2.0 sequencing kit. A 375 base pair sequence was generated (Figure 3.14) with a single open reading frame of 276 bp. The translated gene sequence had a calculated  $M_r$  of 11,263 and aligned perfectly with the protein sequence

deduced by Edman degradation techniques. Three base pairs upstream of the initiation codon in frame with the LPGAP sequence was a stop codon which confirmed LPGAP to be 92 amino acids in length. The nature of the N-terminal methionine modification in the translated product remains to be investigated. It was significant that an in-frame termination codon was present three bp 5' to the ATG initiation codon, indicating that the small size of LPGAP cannot be ascribed to the proteolytic degradation of a larger polypeptide as originally suggested by immunoblots.

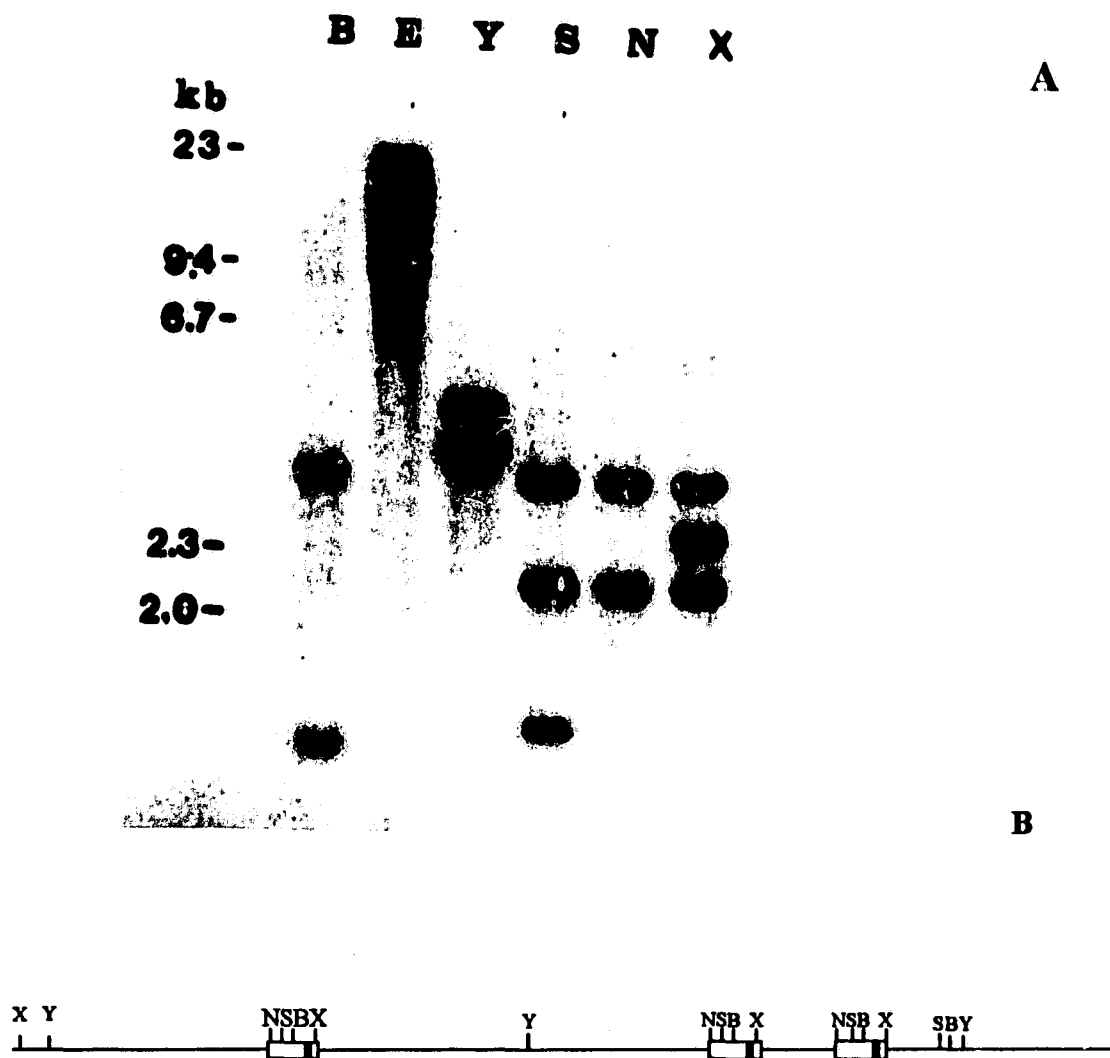
Experiments were next undertaken to elucidate not only the *lpgap* gene copy number found in the *L. donovani* promastigotes but to also establish the genomic organization. Previous restriction analysis of the cosmid clone suggested LPGAP was encoded by at least three gene copies located within a 10 kb fragment (Figure 3.13). To address the question of whether or not these genes were organized in a tandemly repeating scheme, the genomic DNA was digested with the restriction endonucleases *Bcl* I, *Nco* I, *Sac* I, *Sal* I, and *Xho* I, all possessing a single cleavage site within the *lpgap* gene. By this approach, tandemly linked genes would yield a single DNA fragment hybridizing with the LPGAP 45 bp probe representing the minimal repeat unit (Button *et al.*, 1989 & Murray *et al.*, 1990 & Webb *et al.*, 1991). Conversely, multiple genes which are separated by intergenic regions of varying length, would give rise to multiple hybridizing fragments of different sizes. As shown in Figure 3.15, the *L. donovani* LD3 promastigote genome encoded 3 *lpgap* genes arranged with intergenic regions of 1.9 and 3.5 kb and were thus not tandemly repeated genes.

### **3.5 Subcellular Distribution of LPGAP on *L. donovani* Promastigotes:**

The co-isolation of LPG and LPGAP as a stable complex suggested a close relationship between these two molecules on the parasite cell surface. However, the possibility existed that the strong binding of these two molecules was an artefact of preparation. Cellular fractionation of promastigotes (Dwyer, 1980) coupled with immunochemical detection techniques showed the LPGAP to be predominantly distributed in the plasma membrane, along with the cell surface marker LPG (King *et al.*, 1987) (Table 3-4). These

GCGCTTTCTGTTTGCCTTCGATCCTCGCTCAACGTGCGCATAACACACTCTCCT  
 \*  
 TTCACGCGCTCGACTTTTTCTTCCACCCCTCCCGTGTAACCATGGCCACC  
 1  
 M A T  
 ACGTACGAGGAGTTTTCGGCGAAGCTGGACCGCCTGGATCAGGAGTTCAACA  
 10 20  
 T Y E E F S A K L D R L D E E F N  
 GGAAGATGCAGGAGCAGAACGCCAAGTTCTTTGCGGACAAGCCGGATGAGTC  
 30  
 R K M Q E Q N A K F F A D K P D E S  
 GACGCTGTCGCCCAGATGAGAGAGCACTACGAGAAGTTCGAGCGCATGATC  
 40 50  
 T L S P E M R E H Y E K F E R M I  
 AAGGAACACACAGAGAAGTTCAACAAGAAGATGCACGAGCACTCGGAGCACT  
 60 70  
 K E H T E K F N K K M H E H S E H  
 TCAAGCAGAAGTTCGCCGAGCTGCTCGAGCAGCAGAAGGCTGCGCAGTACCC  
 80 90  
 F K Q K F A E L L E Q Q K A A Q Y P  
 \*  
 GTCCAAGTAAGATCAGGACCAC  
 S K

**Figure 3.14:** Primary structure of LPGAP deduced by Edman degradation and Sanger dideoxynucleotide sequencing. Residues in bold were determined by protein microsequencing and confirmed by the gene sequence. The remaining amino acids were derived from the DNA sequence alone. The arrow at position 45 denotes a modified arginine residue and the asterisks indicate termination codons at the 5' and 3' ends of the *lpgap* open reading frame.



**Figure 3.15:** Restriction analysis of the *L. donovani* LPGAP gene locus. (A) Autoradiograph of a DNA Southern blot hybridized with the LPGAP 45 mer probe 5' end labelled with [ $^{32}$ P]. Each lane contained 20  $\mu$ g of DNA digested with the following restriction endonuclease having a single cleavage site in the *lpgap* gene. B, *Bcl* I; E, *EcoR* I; N, *Nco* I; S, *Sal* I; X, *Xho* I, Y, *Sac* I. (B) Schematic representation of the data obtained from panel A.

data confirm earlier immunofluorescences and immunogold labelling studies which suggested that the LPGAP was localized to the *Leishmania donovani* promastigote flagellar pocket plasma membrane (Garduno *et al.*, 1989 & Tolson, 1992 & McNeely *et al.*, 1992). However, to obtain these results it was essential to treat fixed parasites with acetone or saponin, agents known to disrupt membrane integrity, making it difficult to conclude that the LPGAP was a surface oriented protein. Neither mAb L98 nor L157 recognized LPGAP on whole or broken cell preparations without first treating the membranes with these reagents. Furthermore, treatment of purified membranes with either 10 mM HCl or 1 M NaCl, conditions commonly used to solubilize peripheral membrane proteins, released only trace levels of LPGAP suggesting that the LPGAP association with the membrane was hydrophobic in character. Immunoblot analysis of the *L. donovani* plasma membrane and cytoplasmic fractions with L98 showed that LPGAP was present only in the membrane fraction (Figure 3.16). The presence of more than one species is consistent with the known propensity for LPGAP aggregation - even in the presence of SDS. The strongest evidence for surface membrane association, however, was the demonstration that radiolabeled LPGAP could be immunoprecipitated from <sup>125</sup>I-labeled promastigotes with the mAb L157. These data are shown in Figure 3.17.

*L. donovani* promastigote LPGAP accounts for a significant fraction of the total plasma membrane protein. Quantitative amino acid analysis estimates of LPGAP obtained from membrane preparations indicated a copy number of  $1-2 \times 10^6$  molecules /cell, which is comparable to the levels of LPG found on *Leishmania* promastigotes (Orlandi & Turco, 1987; McConville & Bacic, 1990; Ilg *et al.*, 1992).

### **3.6 Distribution of LPGAP in Life Cycle Stages *L. donovani* :**

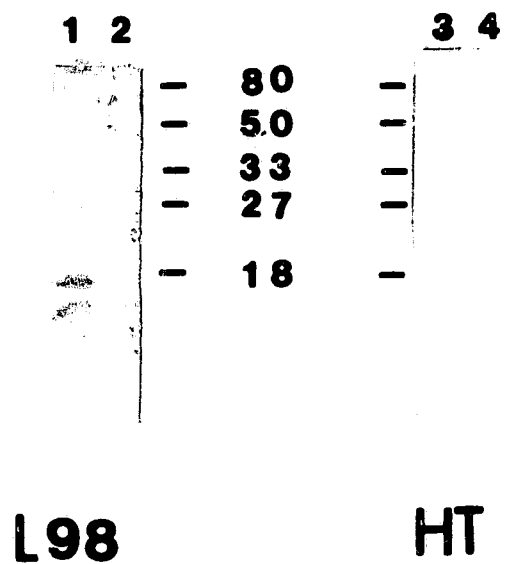
LPGAP has been detected on a wide range of New and Old world *Leishmania* promastigote species using both ELISA (Tolson *et al.*, 1994) and T cell proliferation assays. The cross-reactivity of mAbs L98 and L157 with LPGAP from such a diverse range of *Leishmania* species suggests that this molecule is highly conserved in this genus. Figure 3.18 shows that LPGAP is not only expressed on promastigotes, but that solvent E

**TABLE 3-4**  
Subcellular Distribution of LPGAP in *L. donovani* Promastigotes

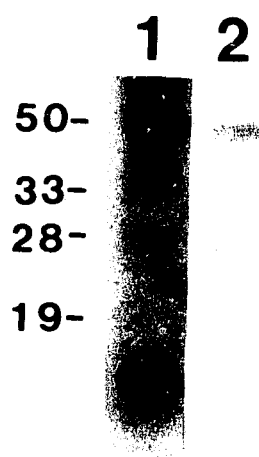
	LPG <sup>a</sup> O.D. <sub>405</sub>	LPGAP <sup>a</sup> O.D. <sub>405</sub>
Cytoplasmic fraction <sup>b</sup>	0.25	0.08
Membrane fraction <sup>b</sup>	0.67	0.44

<sup>a</sup> ELISA assay: LPG and LPGAP were detected with mAbs CA7AE and L98 respectively (Engvall & Perlman, 1971).

<sup>b</sup> Promastigotes were fractionated according to the method of Dwyer (1980).



**Figure 3.16:** Western blot of *L. donovani* subcellular fractions to localize LPGAP. *Lanes 1 & 3*) plasma membrane purified by density gradient centrifugation, *lanes 2 & 4*) 100,000 x g cytoplasmic fraction. An equivalent of  $2 \times 10^6$  promastigotes was loaded in each lane. Identical Western blots were probed either with the LPGAP specific mAb L98 (L98) or the control monoclonal antibody specific to human transferrin (HT).



**Figure 3.17:** Autoradiogram of  $^{125}\text{I}$ -labeled immunoprecipitated LPGAP. Promastigote lysates were reacted with either the LPGAP specific mAb L157 (*lane 1*) or the control mAb HT specific for human transferrin (*lane 2*) and the antibody-antigen complex precipitated with protein G Sepharose. The band observed at approximately 50,000  $M_r$  in both control and experimental lanes is immunoglobulin heavy chain arising from fetal calf serum in the growth medium (Handman, 1984).

extractable LPGAP is also present on *L. donovani* amastigotes. Coomassie blue stained SDS-PAGE gels of the solvent E extracts showed an 11 kDa protein which immunostained with the LPGAP specific mAbs L157. Although LPGAP appears to be constitutively expressed between species and across life stages, the data presented here is qualitative and does not provide information about variation in protein levels which may be associated with the morphological changes which the parasite experiences upon moving from the sandfly to the mammalian host.

### **3.7 Mapping mAb L98 and L157 Epitopes:**

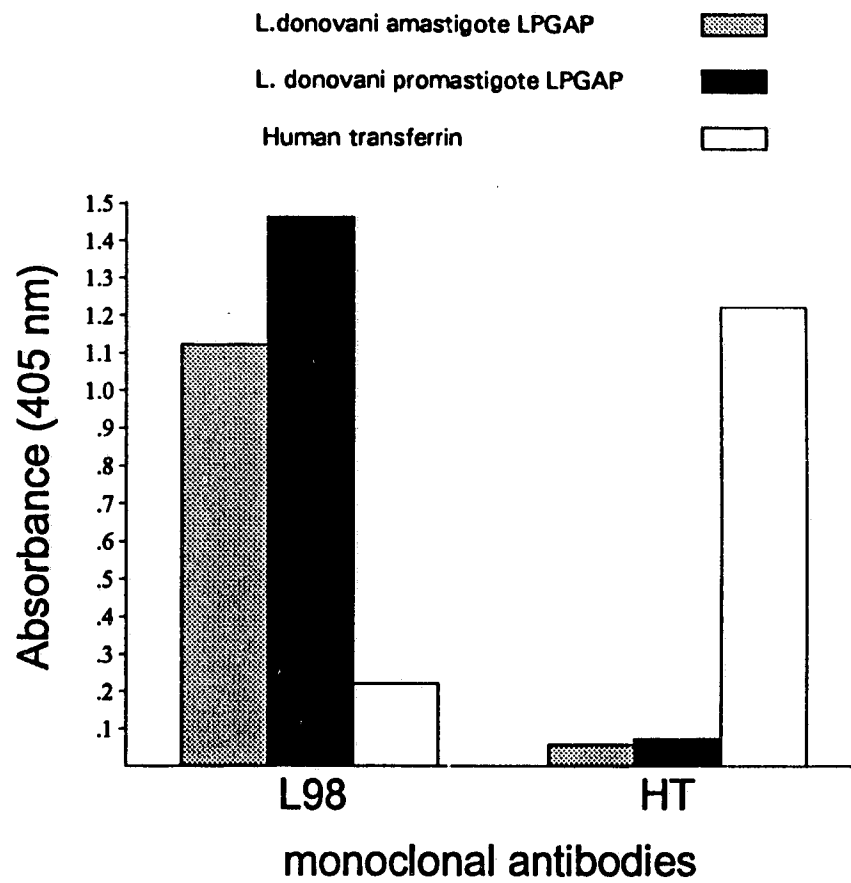
To confirm a previous finding which indicated that the monoclonal antibodies L98 and L157 recognized protein determinants in the LPGAP rather than LPG, a set of nested peptides spanning residues 32-92 of the LPGAP sequence were synthesized. Figure 3.19 shows L98 reacted with all three peptides which localized the binding site to the C-terminal portion (residues 72-92) of the molecule. MAb L157 on the other hand, was found to react only with the full length peptide, thereby narrowing its determinant to residues 32-45.

### **3.8 Post-Translational Modifications of *L. donovani* Promastigote LPGAP:**

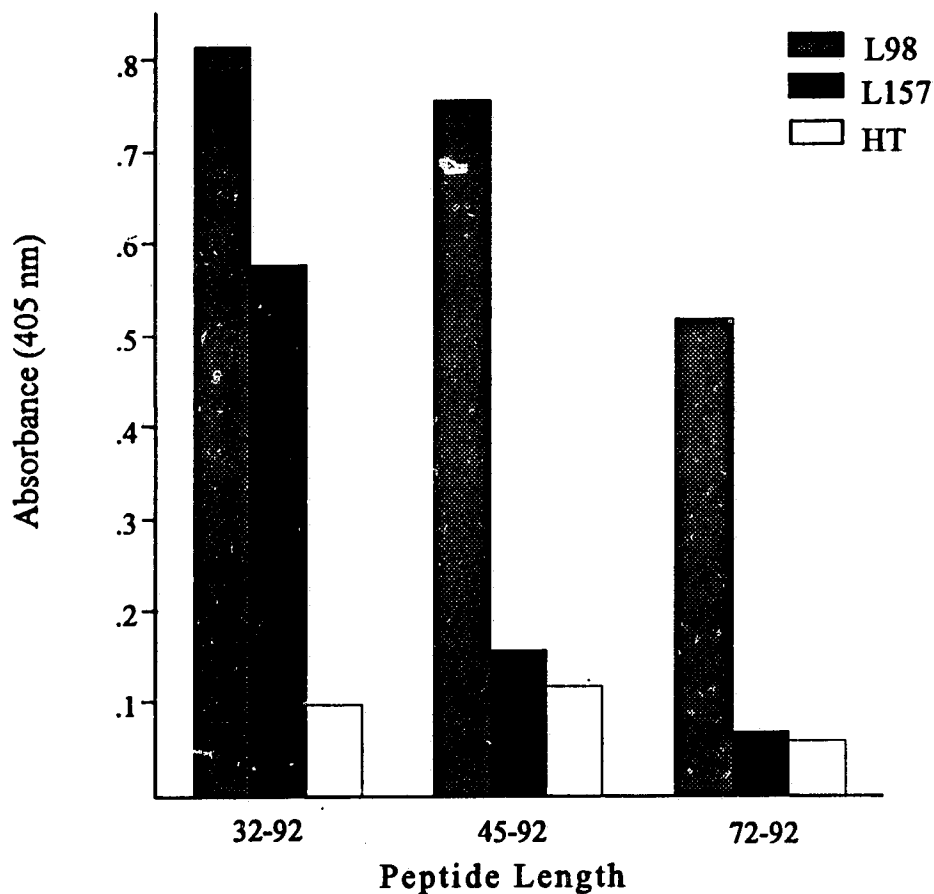
As Edman degradation of CNBr-2 indicated the presence of a modified amino acid at the N-terminal, to deduce the nature of the post-translational modification this peptide was subjected to mass spectroscopic analysis. Based on amino acid composition data, the theoretical mass calculated for CNBr-2 was 1376. However, the M+1 parent ion observed by fast atom bombardment mass spectroscopy was 1391 (Figure 3.20). This difference of 14 mass units was consistent with the modified amino acid at position 45 being an N<sup>o</sup>-monomethyl-arginine (Paik, 1984).

### **3.9 Glycosylation of LPGAP:**

Ion-exchange chromatography of acid hydrolyzed LPGAP on a Dionex Bio-LC system indicated the presence of galactosamine, galactose, glucose, mannose, and an unidentified monosaccharide residue in the glycoprotein structure (Figure 3.21A). Moreover, as



**Figure 3.18:** ELISA screening of *L. donovani* amastigotes for the expression of LPGAP. Wells were coated with 5  $\mu\text{g}$  of promastigote or amastigote LPGAP. Reported values are the mean of triplicate assays.



32            42            52            62  
 ADKPDESTLSPEMREHYEKFERMIKEHTEK  
  
 63            72            82            92  
 FNKKMEHSEHFKQKFAELLEQQKAAQYPSK

**Figure 3.19:** Epitope mapping of the LPGAP specific monoclonal antibodies L 98 and L157. Peptides (10  $\mu$ g/well) were dried and free sites were blocked with 100  $\mu$ l of 3% skimmed milk powder in PBS and triplicate wells were probed with either mAbs L98, L157, or the control mAb HT.

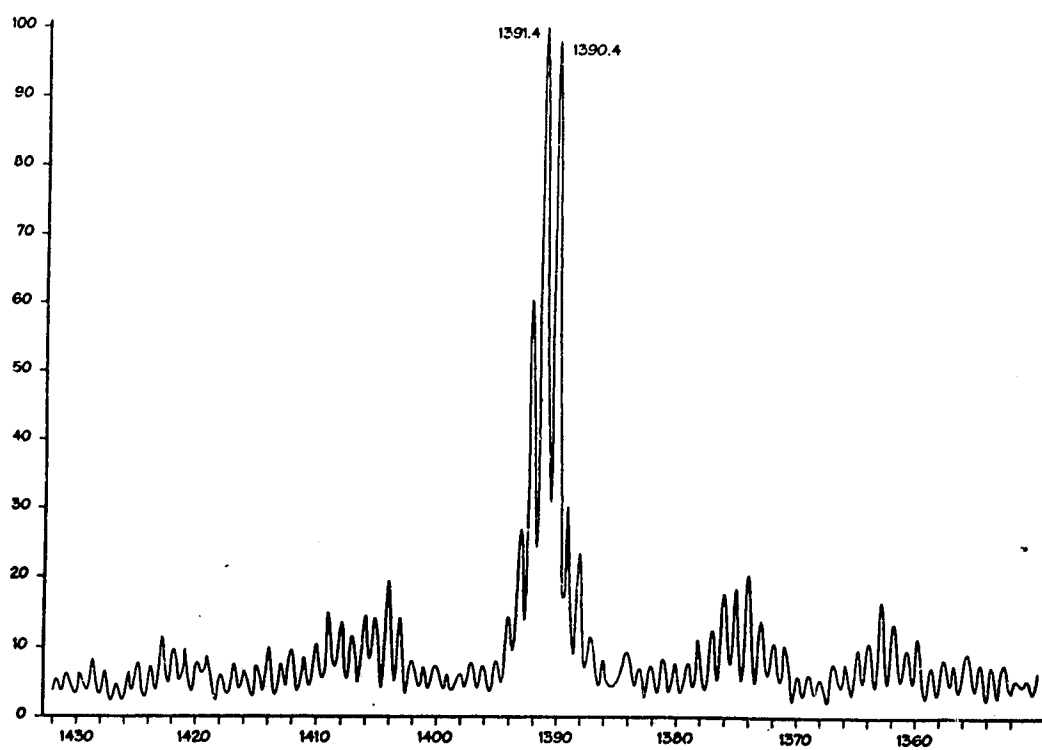
shown in Figure 3.21B, it was significant that only galactosamine could be detected following acid hydrolysis of LPGAP previously treated with anhydrous trifluoromethanesulfonic acid (TFMSA). Since it is known that TFMSA readily cleaves all glycosidic linkages, save the bond between the terminal sugar and the protein (Beeley, 1985), these findings implicated galactosamine in attachment - very likely as GalNAc in an O-glycosidic linkage. This conclusion is given further support by the absence of both a consensus sequence for an N-linked sugar and the absence of glucosamine in the compositional analysis. Preliminary Bio-gel P-4 chromatography results, obtained by Ms. V. Funk in this laboratory, revealed that the hydrazine released oligosaccharide eluted with the same hydrodynamic volume as a polyglucose tetrasaccharide standard (Kobata *et al.*, 1987). These data are consistent with the conclusion that LPGAP is a surface membrane molecule. Although intracellular glycoproteins are known to occur in eukaryotes, glycosylation is a typical post-translational modification of cell surface proteins.

### **3.10 Determination of the Isoelectric Point of LPGAP:**

Further characterization of the physiochemical properties of the lipophosphoglycan associated protein was undertaken by measuring the isoelectric point of the protein in crude solvent E from *L. donovani* promastigotes. Preparations were depleted of LPG by mild acid hydrolysis and subjected to isoelectric focusing on a Bio-Rad Rotofor system (Sunnyvale, CA). Spectroscopic measurement of the Rotofor fractions at 280 nm indicated that a single peak with L98 activity (fractions 10-13) contained a dominant 11 kDa protein on SDS-PAGE (Figure 3.22). Since the isoelectric focusing was conducted in the absence of chaotropic agents it was possible that the protein found in the broad shoulder (fractions 4-9) containing L98 reactive material may be due to an aggregated form of LPGAP. With this procedure the isoelectric point shown in Figure 3.22 was determined to be 4.8.

### **3.11 Prediction of LPGAP Protein Secondary Structure:**

The secondary structure determination of LPGAP was initially undertaken by analyzing the protein primary structure with the empirical relations of both Garnier *et al.* (1978) and



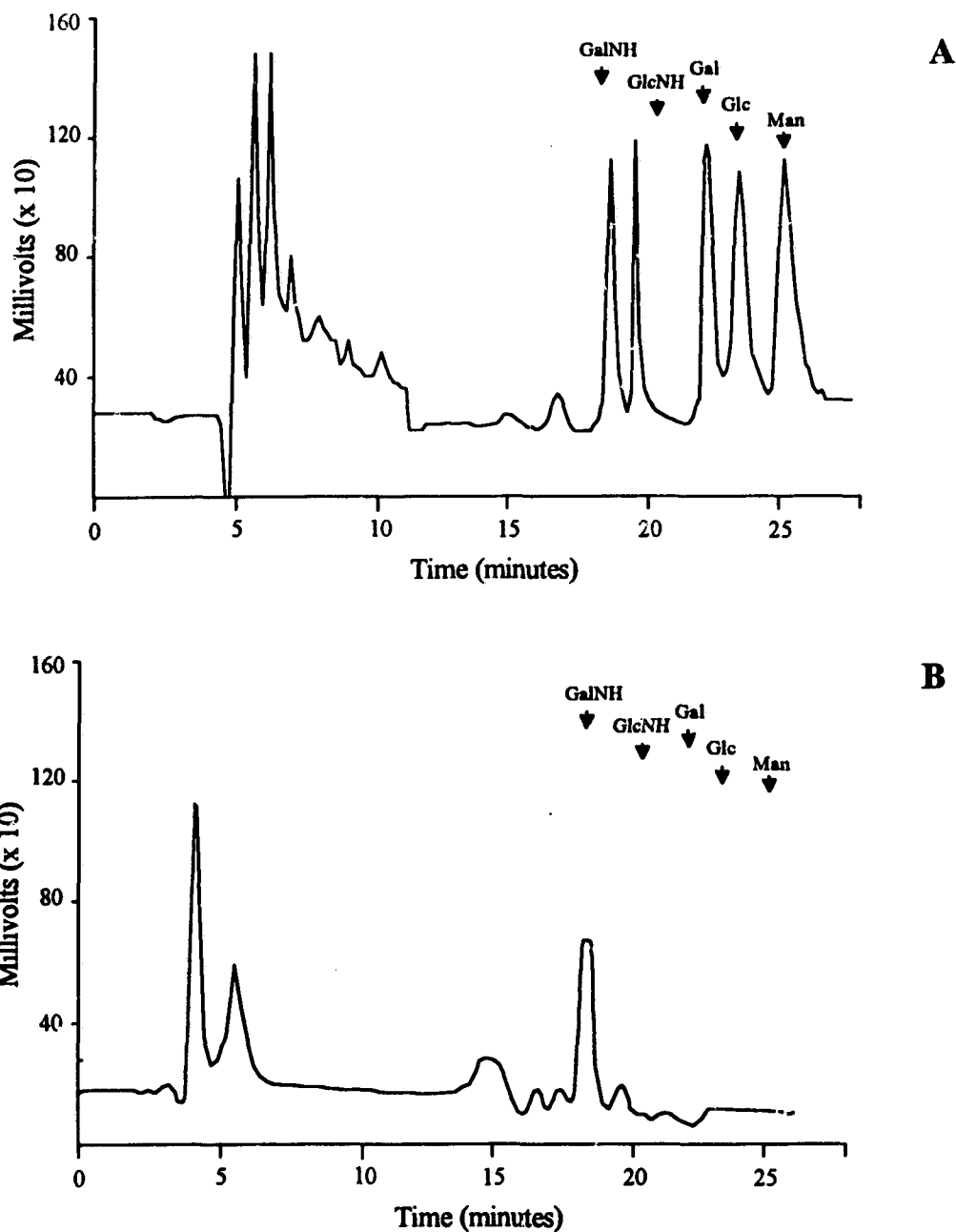
**Figure 3.20:** Continuous flow FAB-mass spectroscopy of CNBr-2. Based on the amino acid composition data the theoretical value calculated for this peptide was 1376 Daltons. The observed parent ion (M+1) at 1391 this difference of 14 mass units is consistent with the modified amino acid in peptide CNBr-2 being a N<sup>ω</sup>-monomethyl arginine.

Chou and Fasman (1974). Both methods predicted an unusually high  $\alpha$  helical content (91%) with two long helices containing 33 and 41 residues respectively. A random coil segment was predicted to lie between both helical regions as well as at the C-terminal, producing a helix-turn-helix motif (Figure 3.23A) (Garnier *et al.*, 1978). When the helical segments of this hairpin-like structure were plotted on an  $\alpha$  helical wheel (Schiffer & Edmundson, 1967), both were shown to have amphipathic character with approximately one third of the surface of each helix being hydrophobic and the remainder highly polar. A structure consistent with these findings is shown in figure 3.23B. More importantly, this folding model proposed for LPGAP places basic residues at the hydrophobic-hydrophilic interface of both helices. In analogous systems, lysine residues in these positions have been demonstrated to be essential, not only potentiating insertion of amphiphilic peptide into membranes, but also stabilizing peptide-lipid association through electrostatic interactions between the cationic lysine side chains and the negatively charged phosphates in phospholipid head groups (Mchaourab *et al.*, 1994; Mirsha *et al.*, 1994).

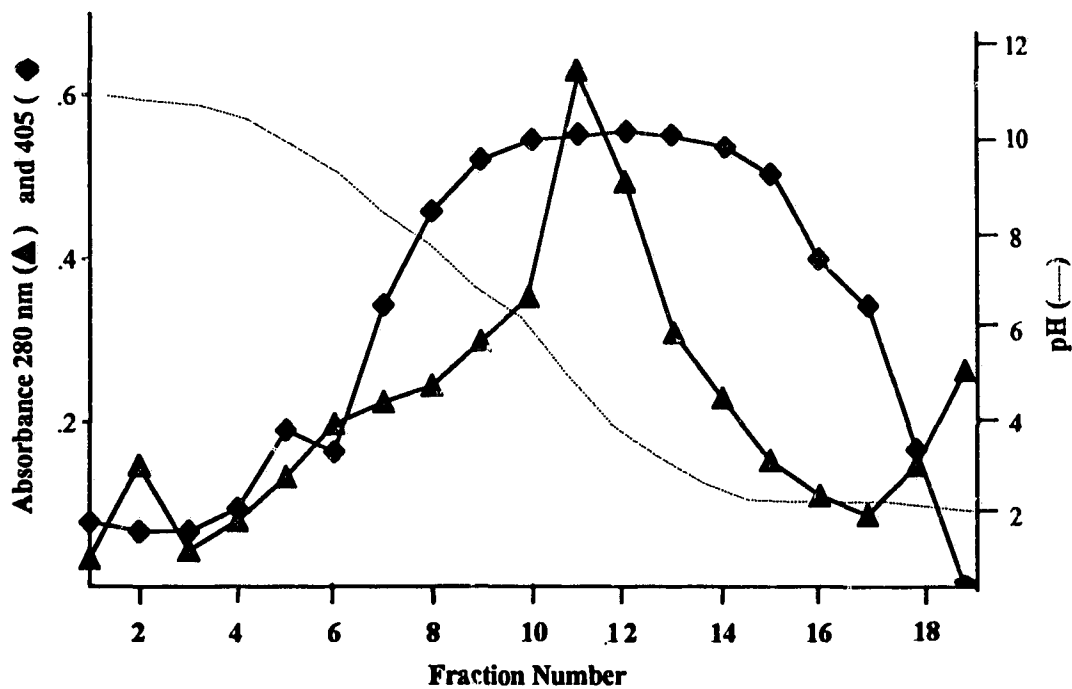
Circular dichroism measurements of the *L. donovani* promastigote LPGAP indicated that the octyl Sepharose purified protein adopted primarily  $\alpha$ -helix (53%) and random coil (36%) conformations in aqueous buffers (Table 3-5). A similar helical content was observed not only for the promastigote LPG/LPGAP complex isolated by Mono Q ion exchange chromatography, but also for LPGAP prepared by solid phase peptide synthesis (Table 3-5). However, the CD spectra of these LPGAPs recorded in 50% trifluoroethanol, an  $\alpha$ -helix potentiating solvent, yielded results in close agreement with the structures predicted by both the Garnier and Chou-Fasman algorithms. In this organic solvent the  $\alpha$ -helical content of LPGAP was dramatically increased to 86-91% when the spectral data was subjected to the Contin analysis (Provencher & Glockner, 1980).

### **3.12 Protein Sequence Database Search for LPGAP Homologous Proteins:**

A database search of the NBRF and the Swissprot databases with the NCBI Blast Network Service program (Altschul *et al.*, 1990) and the FASTA algorithm (Lipman & Pearson, 1987) verified LPGAP to be a unique molecule. Although the *Leishmania*



**Figure 3.21:** Monosaccharide composition for LPGAP isolated from *L. donovani* promastigotes. (A) Monosaccharide composition of LPGAP purified by reversed phase chromatography on a PRP-1 column (Peak 3, Figure 3.8). (B) monosaccharide composition of LPGAP following deglycosylation with trifluoromethane sulfonic acid. Prior to analysis monosaccharides were released from the protein backbone by hydrolysis with 2 M HCl at 100°C for 4 h then applied to a Dionex Bio-LC system equipped with a CarboPacA-1 column. Sugars were specifically detected with a pulsed amperometric detector.



**Figure 3.22:** Determination of the isoelectric point of *L. donovani* promastigote LPGAP. Crude LPGAP (400  $\mu$ g) depleted of LPG was added to 60 ml of a 1% ampholytes (pH 3-10) and focused at 12 Watts for 5 h on a Bio-Rad Rotofor system. Three millilitre fractions were collected, the protein content was determined spectrophotometrically at 280 nm, and assay for LPGAP by indirect ELISA with mAb L98. The pH of each fraction was measured with a Radiometric pH meter.

**Table 3-5**

**Secondary Structure of *Leishmania donovani* Promastigote  
LPGAP Determined by Circular Dichroism Measurements<sup>a</sup>**

**Secondary structure Elements (%)**

Sample	Solvent system	$\alpha$ -helix	$\beta$ -sheet	$\beta$ -turn	Remainder
Octyl Sepharose	20 mM Tris pH 7.8	53	9	2	36
Purified LPGAP	50% TFE	86	12	0	2
Mono Q Purified	20 mM Tris pH 7.8	55	16	0	29
LPG/LPGAP	50% TFE	91	9	0	0
Synthetic (92 mer)	20 mM Tris pH 7.8	42	42	13	3
LPGAP	50% TFE	79	19	2	0

<sup>a</sup> Percentage of secondary structure elements was calculated by the Contin program (Provencher & Glockner, 1981)

protein was not highly homologous with other proteins in the database, two interesting alignments were found. Figure 3.24 shows the alignment between LPGAP and the optimal scoring proteins, bovine apolipoprotein A-I (score 72) and apolipoprotein A-IV (score 60). Although these proteins had reasonably high scores, it was possible that they were the result of fortuitously similar amino acid compositions and thus the significance of these homologies required statistical assessment. Subjecting the rat phosphodiesterase query sequence to the Lipman and Pearson (1987) RDF analysis gave a value of 2.6 standard deviations above the mean value for 20 randomly permuted sequences, which was slightly below the value accepted as significant. Similar calculations comparing LPGAP with apolipoprotein A-I and A-IV gave optimized values of 9.6 and 11.7 standard deviations, respectively, providing statistical evidence that both apolipoproteins were significantly homologous with LPGAP.

### **3.13 LPGAP Association with Small Unilamellar Vesicles:**

The predicted amphipathic nature of LPGAP, together with the apolipoprotein A-I and A-IV sequence homologies, suggested that the molecule may exhibit functionally important surface active properties. The carboxyfluorescein technique (Weinstein *et al.*, 1977), previously used in studies of apolipoprotein A-I synthetic peptides (Kanellis *et al.*, 1980 & Segrest *et al.*, 1983), was employed to evaluate the potential of LPGAP insertion into a lipid bilayer. Distearoyl phosphatidylcholine vesicles loaded with carboxyfluorescein were extremely stable, with less than 1% of the entrapped dye escaping after a 1 h incubation at room temperature. Lysis of the vesicles with 1% SDS was equivalent to 100% fluorescence. When LPGAP was added to the liposome suspension, to a final concentration of 20  $\mu\text{g/ml}$ , a rapid release of carboxyfluorescein was noted with a  $t_{1/2}$  of 12-15 s (Figure 3.25). To verify that the perturbation of the vesicle membranes was induced by protein and not a contaminating glycolipid, the LPGAP was digested with proteinase K. Pretreatment of LPGAP for 1 h with protease K increased the observed  $t_{1/2}$  to 120-130 s and was accompanied by a decrease in the total fluorescence from 75% to 50% over a period of 5 minutes. Digestion of LPGAP for 20 h eliminated all dequenching activity. This observation confirmed that LPGAP and not lipid, induced disruption of the



**liposome bilayer with subsequent release of carboxyfluorescein, supporting the predicted amphipathic structure for LPGAP and highlighting a unique property of this molecule.**

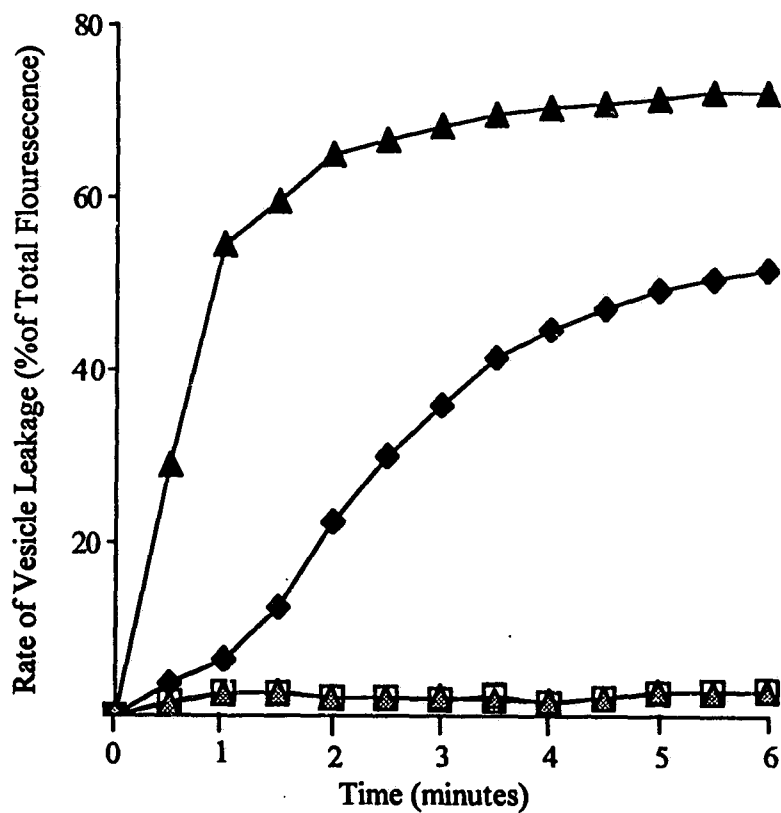
```

LPGAP          LSPEMREHYEKFERMIKEHTEKFNKKMHEHSEHFQK
HUMAN          L  + +  E+F R ++ + E FNKK+ +  E ++QK
APO A-IV      LGGHLDQQVEEFRRRVEPYGENFNKALVQQMEQLRQK

LPGAP          DESTLSPEMREHYEKFERMIKEHTEKFNKKMHEHSEHFQKFAELLEQ
BOVINE         + +L  EM+  +E          +  + F+KK+HE  E ++QK+A L E+
APO A-I       ETASLRQEMHKDLEEVKQKVQPYLDEFQKKWHEEVEIYRQKVAPLQEE

```

**Figure 3.25:** Sequence alignment of shared homologous region between LPGAP, bovine apolipoprotein A-I and human apolipoprotein A-IV as identified by the FASTA algorithm. Plus signs indicate homologous residues or amino acid changes which arise from a single point mutation.



**Figure 3.26:** LPGAP mediated release of carboxyfluorescein from loaded distearoyl phosphatidylcholine vesicles. The rate of carboxyfluorescein release was monitored by fluorescence dequenching; ▲, untreated LPGAP; ◆, LPGAP digested for 1 h with protease K; ◻, LPGAP digested for 20 h with protease K; ▲, addition of protease K alone.

## DISCUSSION

The *L. donovani* LPGAP which co-isolates with the glycoconjugate LPG has been shown to be the antigenic component responsible for the stimulation of both murine T cells (Chapter 2 & Handman & Goding, 1985 & Kemp *et al.*, 1991) and peripheral blood lymphocytes from patients recovered from New World mucocutaneous or visceral leishmaniasis (Mendonça *et al.*, 1991; Russo *et al.*, 1992). On SDS-PAGE the purified protein migrates as multiple bands ranging in  $M_r$  from 11 to 40 kDa, all sharing common determinants recognized by mAbs L98 and L157. This unusual migration of LPGAP on SDS-PAGE was initially ascribed to proteolytic degradation of a larger parent molecule to yield the dominant 11 kDa protein species. However, results presented here clearly indicate that the *lpgap* genes only encode an 11 kDa protein. Evidently, the polydisperse nature observed on electrophoresis was due to an aberrant electrophoretic behaviour resulting from aggregation or alternatively, a non-quantitative binding of SDS giving rise to multiple SDS-LPGAP populations exhibiting differential electrophoretic mobilities as a function of variable charge to mass ratio. The oversight of such an abundant membrane molecule by previous workers can therefore be attributed to the apparent stability of these multimeric forms in the presence of detergent. No doubt the asymmetric charge density in this amphipathic molecule plays a role, as the polydisperse behaviour is eliminated at acidic pH, allowing for a single band on SDS polyacrylamide gels. This effect may be rationalized by the fact that at low pH the glutamic and aspartic acid residues are protonated thereby reducing the anionic repulsion and facilitating the interaction of SDS with LPGAP (Jirgensons, 1976; Wu *et al.*, 1981).

Initial attempts to obtain N-terminal sequence information from the intact LPGAP were unsuccessful due to a modification at the N-terminus. The chemical nature and origin of this blocking moiety remains unknown, precluding complete characterization of the molecule by Edman degradation. However, elucidation of approximately 80% of the internal sequence was facilitated using chemical and proteolytic fragmentation. The remaining structural information was determined by cloning and sequencing of the *lpgap*

gene isolated from a gDNA library using an oligonucleotide probe generated from internal protein sequence. This gene encoded a 276 base pair open reading frame with the translated amino acid sequence agreeing perfectly with the data derived from protein microsequencing. Two notable features of this protein were the absence of valine, glycine, tryptophan and cysteine residues, together with a high charge density, giving the protein a calculated acidic isoelectric point of 5.0 which was in close agreement with the experimentally determined value of 4.8.

In addition to the 11 kDa protein, a minor proteolytic fragment (IVP fragment) was consistently present in different LPGAP preparations isolated from *L. donovani* promastigotes. Sequence analysis showed that the IVP fragment corresponded to residues 32-92 of the LPGAP C-terminal. The cleavage specificity of the LPGAP parent molecule, together with its position on the cell surface, made the GPI-anchored metalloprotease gp63 (Etges *et al.*, 1986) a likely candidate for this proteolytic event. Bouvier *et al.* (1990) demonstrated that gp63 recognized cleavage sites which contain a hydrophobe at position P1' and basic residues at positions P2' and/or P3' where P1' refers to the residue immediately adjacent to the cleaved bond on the N-terminal side while P1 refers to the residue on the C-terminal side of the cleavage site. In addition, a preference for bulky residues such as tyrosine at P1 was also exhibited. The proposed cleavage site between residues 31 and 32 conforms to this motif with Ala-32 at P1', Lys-34 at P3' and the bulky Phe-31 at P1. Furthermore, the Garnier algorithm predicted the above sequence to be situated in a random coil configuration making it relatively susceptible to proteolytic attack.

Although the protein backbone remained faithful to the gene sequence, LPGAP did undergo post-translational modification involving methylation of Arg-45 to N<sup>G</sup>-monomethyl-arginine. The exact role of this modification is unclear as the positive character of the side chain is retained. One possible benefit could be an increase in the hydrophobic moment allowing the arginine to be more thermodynamically stable in a non-polar environment. Monomethylated and dimethylated arginines have been detected in a number of eukaryotic proteins including histones, actin, myosin, and ribosomal proteins

(Paik, 1984) but the biological relevance of this modified residue has not been established. Recently, Lawrence *et al.* (1993) have demonstrated, using [<sup>3</sup>H-methyl]-S-adenosyl-methionine as a label, that one third of all methylated proteins in *L. donovani* promastigotes were base stable N-methylations of membrane associated proteins. Moreover, highly infectious stationary phase promastigotes exhibited elevated levels of methylation activity with the N-methyl proteins displaying the most dramatic increases.

A speculative role which could be proposed for monomethyl arginine in *Leishmania* membrane proteins relates to the survival of parasites within the phagolysosome. Reports by Liew *et al.* (1990) and Roach *et al.* (1991) have implicated nitric oxide as the central leishmanicidal agent in activated murine macrophage. Treatment of macrophage with monomethyl-arginine, a competitive inhibitor of the nitric oxide synthase, resulted in inhibition of the leishmanicidal effect allowing parasites to proliferate intracellularly. It is conceivable that in the acidic phagolysosomal environment, with a pH equal to the isoelectric point of LPGAP, the protein is released from the parasite surface and degraded generating free monomethyl arginine. Further experimentation will be necessary to help elucidate the inhibitory potential of monomethyl-arginine containing peptides on nitric oxide synthase.

In addition to methylation, a glycosylation was also detected on LPGAP. The absence of an Asn-X-Ser/Thr N-linked consensus sequence for glycosylation, together with the lack of glucosamine and presence of galactosamine in compositional analyses, suggested that this sugar was covalently attached via an O-linked structure. Further evidence that this was the case was obtained by showing that the only sugar found after treatment of LPGAP with anhydrous trifluoromethanesulfonic acid was galactosamine, as expected for an O-linked glycoprotein (Edges *et al.*, 1981). While the precise position of glycosylation has not been formally demonstrated, the potential attachment sites include Thr-3, Thr-4, and Ser-9 which, due to the blocked N-terminus, were the only hydroxylated residues not sequenced by Edman degradation. To date no cell surface proteins with O-linked sugars have been reported for *Leishmania* promastigotes making this a unique post-translational modification.

Since a secondary structure prediction based upon both the Garnier *et al.* (1978) and the Chou and Fasman (1974) algorithms clearly predicted a helix-coil-helix motif, we were encouraged by the unusually low pI to consider the relative positions of polar and apolar residues in the predicted helical sequences. This analysis indicated a strongly amphipathic compound and suggested that LPGAP had the potential of forming a hairpin-like structure with both hydrophilic and hydrophobic surfaces. The latter would facilitate a strong interaction with lipid bilayers.

Evidence in favour of the above hypothetical folding model was provided by circular dichroism analysis of LPGAP, which indicated a high propensity for  $\alpha$ -helical structure in an aqueous environment. This was significantly enhanced by addition of the organic solvent trifluoroethanol (Sonnichsen *et al.*, 1992; Jasanoff & Fersht, 1994). It has been suggested that the latter solvent more closely approximates the apolar lipid bilayer environment expected for membrane protein-bilayer association, resulting in an increase in helicity (Gierasch, 1989). That phospholipids may be responsible for such an increase in the helical secondary structure of proteins can be extrapolated from CD experiments demonstrating that other amphipathic molecules such as SDS also potentiates helix formation (Jasanoff & Fersht, 1994; Waterhous & Johnson, 1994; Wu *et al.*, 1981). Finally, the asymmetric distribution of polar and nonpolar residues on LPGAP strongly suggested that this protein could be considered a Class A amphipathic peptide. Recently, Mishra, *et al.* (1994) have put forth the "Snorkel hypothesis" which proposes that amphiphilic proteins can be accommodated in membranes by inserting the hydrophobic face of the peptide into the acyl chains of the lipid bilayer, while the charged polar domain of the helix ion pairs with surface phospholipid groups.

Further indications of the nature of the LPGAP membrane interaction may be inferred from homologies detected by protein sequence alignment analysis with the available protein database. Bovine apolipoprotein A-I and apolipoprotein A-IV were shown to be homologous with LPGAP. Apolipoprotein A-I is the major component of the high density lipoprotein (HDL) cholesterol transport particle and has been suggested to enhance packing by filling spaces on the particle monolayer created by the phospholipid polar head groups and cholesterol (Shen *et al.*, 1977). Unlike apo A-I, apolipoprotein

A-IV has been less extensively studied, however, a structural feature shared by these two lipoproteins is a 22 amino acid segment repeated six times with a strong potential for amphipathic  $\alpha$  helix formation (Fitch, 1977) similar to that predicted for LPGAP. Work by Yokoyama *et al.* (1980) has shown that synthetic peptides corresponding to one or two tandemly repeating apolipoprotein sequences, not only favour helical conformations but also closely approximate the surface active properties observed for native apolipoprotein A-I on egg yolk lecithin monolayers or unilamellar vesicles (Yokoyama *et al.*, 1980). The regions of homology between the LPGAP and the apolipoproteins align with these amphipathic domains. It is notable that with the exception of helix length, the predicted conformation adopted by LPGAP mirrors the synthetic peptide model derived for the apolipoprotein-lipid interaction (Yokoyama *et al.*, 1980). This folding pattern for LPGAP is consistent with the experimental data which shows LPGAP to be an extremely polar, membrane associated,  $^{125}\text{I}$ -surface labelling protein.

The ability of LPGAP to bind lipid was initially suggested by the tight association with LPG *in vitro*. Subsequent studies have helped to corroborate this supposition. For example, in an aqueous environment LPG would favour aggregation into mixed micelles containing LPGAP. Preliminary investigations have shown that the LPG/LPGAP complex elutes from gel permeation chromatography columns with an apparent molecular weight in excess of 80 kDa and that treatment of this complex with either phospholipase-c or the detergent CHAPS allows both molecules to be resolved independently. Further evidence demonstrating the affinity of LPGAP for lipids was obtained from an LPGAP mediated release of carboxyfluorescein loaded liposomes. As yet the precise mechanism by which surface active proteins insert into bilayer is not fully understood, it is clear from this study that the protein perturbs the lipid bilayer sufficiently to allow carboxyfluorescein to escape (Weinstein *et al.*, 1977).

This affinity of LPGAP for lipid bilayers could have a bearing upon the observed association with LPG and may underlie the physiological function of this molecule. The number of LPG molecules on the promastigote surface has been approximated to be  $1-5 \times 10^6$ /cell (Orlandi & Turco, 1987; McConville *et al.*, 1989) forming a protective anionic barrier shielding the parasite membrane from harsh enzymatic environments. It has been

previously demonstrated that the stability of the lipid bilayer is sensitive to the charge of ionizable phospholipid or glycolipid head groups, which can cause destabilization of the membrane as reflected in a decrease in the transition phase temperature (Cevc, 1987; Yao *et al.*, 1987). In this connection, it is plausible that the repulsive forces presented by the highly negatively charge LPG molecule would induce a lateral expansion and destabilization of the membrane causing an increase in the fluidity of the phospholipid bilayer (Trauble *et al.*, 1976). Although, some of these distortions may be mitigated by the long C<sub>24</sub> to C<sub>26</sub> alkyl chains of LPG (Ilg *et al.*, 1992; McConville & Bacic, 1988; Orlandi & Turco, 1987), this may well be offset by the further instability inherent in the LPG lysophospholipid structure (Op den Kamp, 1979). It seems possible, therefore, that the expected LPG induced perturbations of the lipid bilayer may be ameliorated by LPGAP in a role analogous to that of apolipoprotein A-I. Thus LPGAP may function to regulate the lipid bilayer pressure.

A striking feature of axenically cultured promastigotes is the release of vast amounts of LPG from the promastigote surface by a process which is dependent on extracellular proteins such as bovine serum albumin or ovalbumin (Handman *et al.*, 1984; King *et al.*, 1987). To date no adequate mechanism has been presented for the partitioning of LPG from the parasite membrane into the bulk solution. It is clear, however, that the mechanism appears to be specific to LPG, as the other major glycosyl phosphatidylinositol lipid anchored molecule, gp63, is not detected in the culture medium. This selectivity may be in part attributed to the preference of lysophospholipids, such as LPG, to form micelles rather than bilayers (Boggs *et al.*, 1993; Op den Kamp, 1979). It is of interest, in this connection, that LPG has also been implicated in the attachment of promastigotes to the epithelial cells of the sandfly gut until completion of metacyclogenesis (Davies *et al.*, 1990 & Sacks, 1989). The latter morphological change is accompanied by an increased expression of both LPG and the protease gp63 (Glaser *et al.*, 1991). Based on the observations made here and its homology with the apolipoprotein, it is reasonable to speculate that LPGAP may function to stabilize the LPG lysophospholipid within the parasite membrane and in addition, provide a mechanism for LPG release at a pre-programmed stage in the parasite life cycle. The consistent appearance of the

previously mentioned IVP peptide during LPGAP purification, suggests that LPGAP may be specifically hydrolyzed in the non-helical loop section of the protein by gp63. Since isolation is initiated with highly denaturing solvent extraction conditions known to destroy gp63 enzymatic activity, the proposed cleavage could not have transpired during purification. This proteolytic event could result in a decreased helical cooperativity similar to that already described for the apolipoprotein model (Yokoyama *et al.*, 1980), a decrease in membrane bilayer pressure and thereby a facilitated partitioning of LPG from the parasite plasma membrane. Such a function would offer a novel mechanism for alteration of the physicochemical properties of *Leishmania* parasite membranes, providing a selective change in the surface LPG which has been correlated with increased pathogenesis (Pimenta *et al.*, 1992; Sacks *et al.*, 1984). Immunofluorescence studies have localized LPGAP predominantly to the flagellar pocket, the site proposed for membrane turnover in parasites (Webster & Russell, 1993), lending support to the contention that this protein be involved in remodelling the parasite surface.

Finally, endonuclease restriction and Southern blot analysis of genomic DNA indicated that the LPGAP gene locus consists of three gene copies separated by variable intergenic regions. LPGAP is estimated to be present at  $1-2 \times 10^6$  copies/cell on *L. donovani* promastigotes, a number comparable to that reported for both LPG (Orlandi and Turco, 1987) and the major surface glycoprotein, gp63. The latter has been found to be encoded by as many as seven gene copies (Webb *et al.*, 1991). Although further investigation of the transcriptional regulation of LPGAP in both the promastigote and amastigote forms is required, an obvious explanation for the multiple gene copies is that it provides a mechanism for high level protein expression. Since this molecule has been detected in high levels in wide range of kinetoplastid organisms examined to-date and is present in all *Leishmania* life cycle stages, it is evidently a molecule of considerable significance to protozoan parasites (Tolson *et al.*, 1994; Stebeck *et al.*, 1994). This recent observation would thus suggest that LPGAP may have a more general function, such as regulating the physical character of the plasma membrane. As LPGAP has been found on organisms which do not have LPG like structures the designation of lipophosphoglycan

associated protein has been recently changed to the more encompassing name of "kinetoplastid membrane protein-11" (KMP-11).

Finally, since the discovery that KMP-11 was a major component of membrane preparations, several laboratories have shown that circulating lymphocytes from both New (Russo *et al.*, 1992; Mendonça *et al.*, 1991) and (Kemp *et al.*, 1993) Old World leishmaniasis patients proliferated strongly in the presence of this molecule. Earlier work using LPG contaminated with LPGAP had also demonstrated protection from leishmaniasis in vaccinated mice (Russell & Alexander, 1988; Handman & Mitchell, 1985). Thus KMP-11 appears to be an excellent candidate for vaccine studies which are presently underway.

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# CURRICULUM VITAE

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**Publications in Refereed Journals and Proceedings:**

**Jardim, A., Hanson, S., Ullman, B., McCubbin, W.D., Kay, C.M. and Olafson, R.W.**  
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**Manuscripts in Preparation:**

**Stebeck, C.E., Beecroft, R.P., Singh, B.N., Jardim, A., Olafson, R.W., Prenevost, K.D. and Pearson, T.W. 1994. Kinetoplastid membrane protein 11 (KMP-11) is differentially expressed throughout the life cycle of African trypanosomes and is found in a wide variety of kinetoplastid protozoan parasites. Mol. Biochem. Parasitol. (submitted)**

**Lectures and Addresses:**

**1993 Olafson, R.W., Hanson, S., Ullman, B. Caprioli, R.M., Jardim, A. Structural Characterization of the *Leishmania donovani* Lipophosphoglycan-associated Protein - A Major Membrane Glycoprotein. Gordon Conference, New London, NH.**

**1993 Jardim, A. and Olafson, R.W. Characterization of a Novel *Leishmania donovani* Membrane Glycoprotein Protein. West Coast Kinetoplastid Conference, UCLA, Los Angeles, CA.**

**1993 Jardim, A. Specimen preservation for future biochemical analysis. Short Course in Natural History. Royal British Columbia Museum.**

**1990 Jardim, A. and Olafson, R.W. Specific stimulation of CD4<sup>+</sup> T<sub>H</sub><sup>1</sup> T-cells by an immunoprotective *L. major* synthetic T-cell epitope. West Coast Kinetoplastid Conference, Fort Worden, Washington.**

**1990 Jardim, A. and Olafson, R.W. Stimulation of murine cell mediated immunity with the *Leishmania donovani* lipophosphoglycan associated protein. Department of Biochemistry, University of Dundee, Scotland. (Invited lecture).**

