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CHARACTERIZATION OF JABBA, A RICIN-RESISTANT MUTANT OF LEISHMANIA DONOVANI

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CHARACTERIZATION OF JABBA, A RICIN-RESISTANT MUTANT OF
LEISHMANIA DONOVANI

DISSERTATION

A dissertation submitted in the partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
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ABSTRACT OF DISSERTATION

CHARACTERIZATION OF JABBA, A RICIN-RESISTANT MUTANT OF LEISHMANIA DONOVANI

The abundant cell-surface lipophosphoglycan (LPG) of Leishmania parasites plays a central role throughout the eukaryote’s life cycle. A number of LPG-defective mutants and their complementing genes have been isolated and have proven invaluable in assessing the importance of LPG and related glycoconjugates in parasite virulence. While ricin agglutination selection protocols frequently result in lpg- mutants, one L. donovani variant we isolated, named JABBA, was found to be lpg+. Procyclic (logarithmic) JABBA expresses significant amounts of a large-sized LPG, larger than observed from procyclic wild-type but similar in size to LPG from wild-type from metacyclic (stationary) phase.

Structural analysis of the LPG from logarithmically-grown JABBA by capillary electrophoresis protocols revealed that it averaged 30 repeat units composed of the unsubstituted Gal(β1,4)Man(α1)-PO4 typical of wild-type L. donovani. Analysis of JABBA LPG caps indicated that 20% are the disaccharide Glc(β1,2)Man, trisaccharide Gal(β1,4)[Glc(β1,2)]Man, and tetrasaccharide Gal(β1,4)[Glc(β1,2)Man(α1,2)]Man in addition to wild-type Gal(β1,4)Man and Man(α1,2)Man and Gal(β1,4)[Man(α1,2)]Man terminating caps. These glucose containing isoforms were absent in stationary parasites.

Consistent with these structural observations, analyses of the relevant glycosyltransferases in JABBA microsomes involved in LPG biosynthesis showed a two-fold increase in elongating mannosylphosphoryltransferase activity and up-regulation of a β-glucosyltransferase activity. The β-glucosyltransferase in both JABBA and wild-type in vitro produced a β-glucosidase sensitive and β-galactosidase sensitive trisaccharide, indicative of the mannose of repeating units and caps being used in substrate, novel in comparison to other Leishmania species. Furthermore, the caps of JABBA LPG are cryptic in presentation as shown by the loss of binding by the lectins ricin, peanut agglutinin and concanavalin A and reduced accessibility of the terminal galactose residues to oxidation by galactose oxidase.
These results indicate that LPG from JABBA is intriguingly similar to the larger LPG in wild-type parasites that arises following the differentiation of the non-infectious procyclic promastigotes to infectious, metacyclic forms, and has a unique β-glucosyltransferase not active \textit{in vivo} in wild-type parasites.

KEYWORDS: Parasites, Glycosyltransferases, Ricin Agglutinin, \textit{Leishmania}, Lipophosphoglycan.

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Chapter I
Introduction

The World Health Organization lists leishmaniasis as one of its top six tropical diseases for research and control efforts. Approximately 350 million people (Kaye, P. and Scott, P. 2011) in 88 countries are at risk, with 1-2 million new cases a year, making leishmaniasis the second most common parasitic disease in the world behind malaria. There are three primary forms of leishmaniasis, which range from self-healing ulcers to disfiguring or deadly infections. The host’s immune system and the species of parasite have been shown to cause the varying state of illness. Drug treatments are few, most are toxic, and resistance to those drugs is showing up in areas where leishmaniasis is endemic. Vaccination development is slow, and the focus has been on vaccination of non-human reservoirs.

*Leishmania* parasites lead a complex life cycle, and have a complex glycocalyx to protect them. They have two forms in this cycle, the promastigote, and an amastigote. The promastigote is ingested by a sand fly when it consumes a blood meal from an infected mammal, it will then reside in the sand fly’s midgut for approximately a week, undergoing metacyclogenesis to become the infectious parasite. When the parasites release from the midgut they migrate to the mouth parts to be injected at the next blood meal. In the newly infected mammal, the parasites travel to the macrophage, where they reside in the phagolysosome change into amastigotes, the form during the course of infection.

In order to survive the sand fly midgut, the mammalian bloodstream, and the mammalian macrophage, *Leishmania* have an extensive glycocalyx containing a variety of glycolipids, GPI-anchored proteins, and secreted glycoproteins. Lipophosphoglycan (LPG) is the dominant molecule coating the surface of promastigotes, found in excess of one million copies. The basic structure of LPG is a conserved glycan core and a phospholipid anchor, a developmentally regulated number of disaccharide repeating units, and a neutral oligosaccharide cap. LPG plays a role in midgut attachment, parasite virulence, and host immune response.
Current research in this lab has focused on identifying critical molecules in the LPG biosynthesis pathway. The unique structures found on LPG have made it a potential drug target with potentially fewer toxic side effects compared to most anti-protozoal drugs. Parasites were exposed to mutagens in order to generate a selection of mutant strains, and parasites with abnormal LPG structures were selected for further study. This dissertation introduces JABBA, the first over-expressing LPG mutant, and analyzes the structure of its mutant LPG and the biochemical causes for the changes. Lastly, we utilize lectins to determine how the altered LPG structure could affect the parasites life cycle.

1. Leishmaniasis

Leishmaniasis is caused by multiple species, found in multiple countries, and presents a variety of clinical manifestations. Cutaneous leishmaniasis is found predominantly in Africa, the Mediterranean, the Middle East, and Asia and parts of India. The species that cause the bulk of cutaneous leishmaniasis are *L. major*, *L. tropica*, *L. mexicana* (western hemisphere), and *L. aethiopica* (Berman, J.D. 1997; Grimaldi, G., Jr., et al. 1989). The disease can develop within a few days or over years after the bite from an infected sand fly, presenting with a lesion that is covered by a thin crust and slowly spreads. These sores can heal on their own leaving unpigmented scars. A secondary case can occur called diffuse cutaneous leishmaniasis, causing nodules and plaques to form under the skin of extremities months or years after the initial infection. Another unique form of cutaneous leishmaniasis is mucocutaneous leishmaniasis (Berman, J.D. 1997), localized from Mexico to Argentina, and caused by *L. braziliensis* and *L. panamaensis*. After an initial cutaneous infection has ran its course, the parasites metastasize to the mucus membranes, forming ulcers which destroy the mucus membranes of the nose and mouth.

Visceral leishmaniasis, or “kala azar” in India, is found in the Mediterranean, Russia, China, India, and East Africa and caused by parasites of the *L. donovani* complex. In the western hemisphere, the disease is caused by *L. tropica*, *L. amazonesis*, and *L. chagasi* (Berman, J.D. 1997; Grimaldi, G., Jr., et al. 1989). Symptoms include fever, weight-loss, anemia, and swelling of the spleen and liver; untreated, mortality is guaranteed. As with cutaneous leishmaniasis, the incubation period is a few days to a
year after bite from infected sand fly. Currently, co-infection with HIV has become a major concern with visceral leishmaniasis (Montalban, C. 1991).

The best treatment option available currently is miltefosine, a drug given orally, rather than intravenously (Coelho, A.C., et al. 2014). Miltefosine is a derivative of phosphatidylcholine, and was approved by the FDA for the treatment of all leishmaniasis in 2014. However, during 2013, in Asia Minor and India approximately 20% of patients given miltefosine relapsed within a year (Vanaerschot, M., et al. 2014a; Vanaerschot, M., et al. 2014b)

Other treatments for leishmaniasis rely on pentavalent antimonial drugs which inhibit glycolytic enzymes and fatty acid oxidation in amastigotes (Markell, 1999). Sodium stibogluconate and N-methylgucamin antimonials require a 20 day course of medication (Berman, J.D. 1997; Grimaldi, G., Jr. and Tesh, R.B. 1993) but the side effects of these treatments require monitoring and in locations affected by leishmaniasis the overall course is often not completed.

Pentamidine and Amphotericin B are possible forms of treatment, with the latter proving extremely effective. Both of these drugs however are toxic to humans at the necessary therapeutic levels (Berman, J.D. 1997; Herwaldt, B.L., et al. 1992; Melby, P.C. 2002), with side effects intense enough that patients must be medically monitored. Cutaneous leishmaniasis does not require treatment, and antibodies developed from untreated cutaneous leishmaniasis are effective against future cases in most people.

Vaccinations against leishmaniasis have been proposed in a variety of forms. Recently a number have focused on different proteases in New World species (Zahedifard, F., et al. 2014), some that could even be administered intra-nasally (de Matos Guedes, H.L., et al. 2014). A uniform problem has been found in translating vaccine designs to humans, as most vaccines with favorable results in mice do not prove successful in humans (de Matos Guedes, H.L., et al. 2014). The newest technique attempts to use live attenuated parasites to produce an immune response, with moderate success (Dey, R., et al. 2014). The biggest leap forward overall in working to control leishmaniasis is the development of two commercially available vaccines for dogs (Fernandes, C.B., et al. 2014), a frequent mammalian host for the parasite.
2. Life Cycle

*Leishmania* sp. are members of the order Kinetoplastida, in the family Trypanosomatida. A number of infectious parasites reside in the same family, including the highly prevalent Chagas Disease. Characteristic of the Kinetoplastid family, the parasite has a kinetoplast, a single tubular mitochondrion of condensed DNA consisting of roughly 20,000 minicircles of DNA and 20-50 maxicircles, at the base of the flagellum. The parasites have a single nucleus and either an elongated body with a long protruding flagellum or a short body with a nonprotruding flagellum. *Leishmania* and some other trypanosomes are heteroxenous, others, such as *Herptomonas*, are monoxenous.

Leishmania have a digenetic life cycle (Turco, S.J. and Descoteaux, A. 1992), a model of which is illustrated in Figure 1. Parasites are ingested by the sand fly during its blood meal, and they proceed to differentiate in the alimentary tract of the sand fly. In the midgut the parasites attach to the epithelium using insect lectins (Kamhawi, S., *et al.* 2004; Sacks, D.L., *et al.* 1995), and over the course of approximately a week the parasites undergo metacyclogenesis and transition to the metacyclic promastigote (de Assis, R.R., *et al.* 2012; Sacks, D.L. 1989). While it has not been seen in the wild, co-infection of a sand fly can result in DNA exchange between species (Akopyants, N.S., *et al.* 2009; Inbar, E., *et al.* 2013). Promastigotes, the life cycle form in the sand fly, are spindle shaped, 10-15 µM in length, and 2-3 µM in diameter with the flagellum at the basal body of the anterior pole and highly motile for its migration to the proboscis. Approximately 10 different forms have been described, varying by species and sand fly vector (Lawyer, P.G., *et al.* 1990).

A variety of systems are used to culture promastigotes. They grow as a heterogenous population in liquid culture, with population percentages varying based on culture conditions and growth phase. The parasites prefer temperatures about 24-26 ºC and a neutral to slightly acidic pH, similar to the conditions found in the sand fly midgut. The actual infectivity of a liquid culture varies, it is estimated that 10% of the stationary phase culture manages to fully differentiate into metacyclic parasites. Lectins are used to select for the unique cell surface antigens found on metacyclics, isolating that population for further study (Sacks, D.L. and da Silva, R.P. 1987; Sacks, D.L. and Perkins, P.V.)
1985). For example, peanut agglutinin is used to identify metacyclic *L. donovani* parasites due to their resistance to agglutination. Recently algorithms have been developed to identify metacyclic parasites based on the slight changes in cell architecture, including an extreme narrowing of the cell (Berg, M., *et al.* 2013).

In the mammalian host, the aflagellated amastigote develops in the parasitophorous vacuole contained in the macrophage (Turco, S.J. and Descoteaux, A. 1992), where it continues to replicate until it lyses the macrophage and spills out to infect other cells. The amastigotes are round, approximately 2-3 μM in diameter and lack a visible flagellum, as it does not extend past the cell circumference. Amastigotes can be grown axenically (Bates, P.A., *et al.* 1988; Doyle, P.S., *et al.* 1991), a low pH and high temperature are required to trigger the transformation (Zilberstein, D. and Shapira, M. 1994).

### 3. Structure of Lipophosphoglycan

*Leishmania* parasites encounter a number of hostile environments between the sand fly and host, and thus require an extensive, dense, glycocalyx (seen in Figure 1.2) for survival and protection. The glycocalyx consists of lipophosphoglycan (LPG), glycoinositolphospholipids (GIPLs) (McConville, M.J. and Blackwell, J.M. 1991; Orlandi, P.A., Jr. and Turco, S.J. 1987; Turco, S.J., *et al.* 1987), excreted proteophosphoglycans (PPG) (Ilg, T., *et al.* 1994a; Ilg, T., *et al.* 1996; Ilg, T., *et al.* 1994b) and secreted acid phosphatase (sAP) (Lovelace, J.K. and Gottlieb, M. 1986). LPG is one of the dominant molecules in the promastigote phase, found all over the cell surface including the flagellum with approximately one million copies per cell.

LPG consists of four domains: (i) a lipid anchor, (ii) glycan core, (iii) Gal(β1,4)Man(α1)-PO₄ repeating units, and (iv) an oligosaccharide cap structure (Descoteaux, A. and Turco, S.J. 1999; Turco, S.J. and Descoteaux, A. 1992). The lipid is a 1-O-alkyl-2-lyso-phosphatidyl(myo)inositol, containing an aliphatic C24 or C26 unbranched, saturated hydrocarbon. The glycan core contains an unacylated glucosamine, 2 mannopyranoses, 2 galactopyranoses, and a unique galactofuranose residue. *L. donovani* and *L. mexicana* contain a Glc(α1)-PO₄ attached to the C-6 hydroxyl of the proximal mannose via a phosphodiester bond (Ilg, T., *et al.* 1992; Turco, S.J., *et al.* 1989).
The core, lipid, and repeating unit backbone are highly conserved among species, while the distinguishing variations between species are found in the side chains attached to the repeating units and the caps (Figure 1.3), with the C-3 of the galactosyl residue the most common location for modifications. *L. donovani* isolated from Sudan contains no side-chain modifications (Turco, S.J., *et al.* 1987), while the Indian isolate contains one or two β-glucosyl residues linked every few repeating units (Mahoney, A.B., *et al.* 1999). *L. major* contains 1-4 β-galactosyl chains terminated with arabinosyl (Dobson, D.E., *et al.* 2003b; Sacks, D.L., *et al.* 1990), *L. mexicana* contains β-glucosyl chains, and *L. tropica* is the most complex with over 19 different types of glycans (Soares, R.P., *et al.* 2004). *L. aethopica* is the only LPG found that contains α-mannosyl side chains linked to the C-2 of the mannosyl residue (McConville, M.J., *et al.* 1995). The caps are frequently varied, the common structure found in *L. donovani* is the trisaccharide Gal(β1,4)[Mana1,2]Man, and *L. major* has a dominant Man(α1,2)Man cap.

LPG is the primary ligand for binding to the midgut epithelium of the sand fly. The developmentally regulated changes are known to help in both attachment and eventual detachment from the midgut. This includes an increase in repeating units from approximately 15 to 30 as the parasite undergoes metacyclogenesis and potentially changes in side chains, in the case of *L. major*, there is a downregulation in the number of galactosyl side chains and capped with an arabinosyl residue. This, along with the changes in the number of repeating units, helps facilitate its release from the sand fly gut (Pimenta, P.F., *et al.* 1992; Volf, P., *et al.* 2014). *L. donovani* uses the increase in repeating units alone, and this increase has been shown to hide the terminal cap from lectin accessibility *in vitro* (Sacks, D.L., *et al.* 1995).

LPG molecules are still expressed in the amastigote stage, but are extremely downregulated, with no more than 1000 molecules per cell (Schneider, P., *et al.* 1993). In *L. major*, these LPG molecules are biochemically distinct from the promastigote stage, including about 36 repeating units, with 70% of them unsubstituted. The substitutions include either glucosyl or galactosyl chains, with a maximum of 11 residues attached to a repeating unit and a predominantly Gal(β1,4)Man cap (Moody, S.F., *et al.* 1993).

There are some excreted molecules of LPG (Slutzky, G.M., *et al.* 1979) both with and without the lipid anchor. Those without the lipid are called phosphoglycans (Greis,
K.D., et al. 1992). While the reason for this variation is not quite understood, those with the lipid are thought to arise through the interaction of albumin in the media with LPG on the cell surface causing this turnover.

4. Other Cell Surface Glycoconjugates

*Leishmania* produce a number of other glycoconjugates which will be discussed here.

A. Glycoinositolphospholipids

Glycoinositolphospholipids (GIPLs) are low molecular weight glycolipids that are expressed on the cell surface; they can resemble protein anchors, but do not explicitly anchor proteins (McConville, M.J., et al. 1993; McConville, M.J., et al. 1994; Turco, S., et al. 1994). They are expressed in extremely high copy number, and have three basic types: Type 1 resembles protein GPI anchors, Type 2 resembles those of LPG glycan core, and a third type that is expressed as a hybrid of the two. Type I and the hybrid differ in the lipid composition, as they contain an alkyl-acyl-PI with shorter chains than LPG, while Type 2 produce a heterogeneous mixture. Little is known about the functions of GIPLs, but they are a major constituent of the amastigote cell surface, likely conferring protection to some capacity. They have been demonstrated to modulate signal events in the macrophage such as nitric oxide synthesis and the oxidative burst (McNeely, T.B., et al. 1989; Proudfoot, L., et al. 1995b; Schofield, L., et al. 1996; Tachado, S.D., et al. 1997). The enzymes in GPIL biosynthesis have been demonstrated to be essential for virulence (Ilgoutz, S.C., et al. 1999)

B. gp63

With approximately 500,000 copies per cell, gp63 is the major protein found on the surface of promastigote parasites, where it makes up a total of 1% of all cellular protein. The production of gp63 is downregulated in the amastigote stage of the life cycle and is found only in the flagellar pocket.

The importance of the molecule is not well understood. It is seen to be proteolytically active against a number of molecules (Schlagenhauf, E., et al. 1998), and is considered a possible ligand for complement components. The high-mannose oligosaccharides found on its surface could be used for cell invasion using the mannose-
fucose receptor. Overall, there is conflicting data as *L. mexicana* lacking gp63 suffers no apparent detriment in macrophages or in a mouse model of infection (Hilley, J.D., *et al.* 2000).

C. **Secreted acid phosphatase**

All promastigotes, save *L. major*, release secreted acid phosphatase (sAP) from the flagellar pocket (Bates, P.A., *et al.* 1989; Lovelace, J.K. and Gottlieb, M. 1987; Stierhof, Y.D., *et al.* 1994) and can be found in cellular media. Old World strains of *Leishmania*, including *L. donovani*, release mono or oligomeric sAPs, while New World strains release a filamentous sAP (Ilg, T., *et al.* 1999; Ilg, T., *et al.* 1994a). *L. donovani* sAPs are heavily glycosylated on the C-terminal serine or threonine domains via phosphodiester linkages, and have about 32 Gal(β1,4)Man(α1)-PO4 repeating units. In contrast, *L. mexicana* is oligomannosylated.

D. **Proteophosphoglycan**

There are a number of types of proteophosphoglycan (PPG): filamentous PPG (fPPG), GPI-anchored form (mPPG), and an amastigote, non-filamentous form (aPPG) (Ilg, T., *et al.* 1994b; Stierhof, Y.D., *et al.* 1994). fPPG is 95% phosphoglycans, while the protein component is primarily serine, alanine, and proline. The serine molecules are phosphoglycosylated with the repeating units Gal(β1,4)Man(α1)-PO4 terminated with small cap structures. There is no obvious evidence for its function, but hypotheses suggest that it traps the parasites in the midgut or deters the ingestion of a second blood meal (Killick-Kendrick, R., *et al.* 1988).

aPPG has a defined polypeptide backbone with modified phosphoglycans, both common and novel structures, present on the serine residues (Ilg, T., *et al.* 1998). In the parasitophorous vacuole, the concentrations of aPPG can be as high as mgs/ml (Ilg, T., *et al.* 1995) and contributes to the overall size of the vacuole. These have been shown to activate the complement cascade.

5. Functions of LPG

A. **In the sand fly vector**

*Leishmania* species use the sand flies of two primary genera and many species as its vector. Old world strains of *Leishmania* use *Phlebotomus spp.* and New World strains
use *Lutzomyia*. As of this writing, two different capabilities of binding (LPG dependent and LPG independent) have been established, non-permissive and permissive vectors (Jecna, L., *et al.* 2013). In the case of *L. major*, the primary vector is *P. papatsi*, and is referred to as the non-permissive vector; *P. perniciosus* is the permissive vector.

Using *L. major* mutants lacking LPG it has recently been shown that the non-permissive, or primary, vector has an explicitly LPG dependent mechanism of binding to the mid-gut epithelium. This had been demonstrated in other capacities over the years using purified LPGs (Butcher, B.A., *et al.* 1996; Pimenta, P.F., *et al.* 1992). At the same time, a non-permissive species binding has been established that exhibits a non-LPG dependent mechanism for binding to the midgut. Parasite attachment is critical as it allows the parasite to undergo the life cycle progression of metacyclogenesis without being excreted (Sacks, D.L. and Perkins, P.V. 1984; Sacks, D.L. and Perkins, P.V. 1985).

The lengthening of LPG from 15 repeating units to 30 repeating units results in detachment from the epithelium (Sacks, D.L., *et al.* 1995). Data has shown that in *L. major* and nonpermissive binding, in addition to the increase in repeating units, the capping of β-galactosyl chains with an arabinosyl residues obscures the motif from the insect lectins to aid in detachment. In *L. donovani* (Sudanese isolate) the caps lose accessibility to lectins, which most likely allows for the detachment from the midgut and migration to the mouth parts. The *L. donovani* (Indian isolate) has been shown to rely on the glucose side chains *in vitro* for the binding of the parasite to the insect midgut, and that the decrease in glucose as it progresses through metacyclogenesis aids in detachment (Mahoney, A.B., *et al.* 1999). The three dimensional structure of LPG is critical to the parasite’s attachment in the sand fly and subsequent release for the continuation of its life cycle.

**B. In the mammalian bloodstream**

*Leishmania* parasites must use the host immune cells to gain access to the macrophage which is normally used to eliminate pathogens. Upon initial infection in the bloodstream, the promastigotes are exposed to complement mediated lysis by complement factors. Logarithmic phase procyclic promastigotes are sensitive to fresh blood serum and lysis, while stationary phase promastigotes are increasingly resistant to lysis (Franke, E.D., *et al.* 1985; Puentes, S.M., *et al.* 1988). When compared to
logarithmic-phase *L. major*, peanut agglutinin purified metacyclics show that complement lysis does not affect binding.

Metacyclic parasites manage to activate the complement cascade via the classical pathway, as C3b is bound on the surface. The membrane attack complex of traditional cascade occurs by cleavage of the factors. *L. major* cannot have C5b-9 inserted on its surface, essentially forming a protective barrier (Puentes, S.M., *et al.* 1990; Puentes, S.M., *et al.* 1989; Puentes, S.M., *et al.* 1988). In the case of *L. donovani* (Sudanese isolate), only inactive C3b is bound to LPG, which does not result in activation of the C5 convertase (Puentes, S.M., Dwyer, D.M., *et al.* 1989), which will later help with a neutral phagocytosis by the macrophage. Bound C3 can then be released by proteolytic cleavage. Gp63 can cleave active C3b to its inactive form. In addition to complement, mannan-binding protein can bind to the caps and trigger the complement cascade (Descoteaux, A. and Turco, S.J. 1999).

**C. In the mammalian macrophage**

LPG and gp63 are the primary parasite receptors for binding to the macrophage; CR1, CR3, and the mannose-fucose receptor are the primary receptors for the macrophage. Inactive C3b allows for opsonization through CR1 and CR3 (Mosser, D.M. and Edelson, P.J. 1985), though *L. donovani* can bind in the absence of these through the mannose-fucose receptor with a mannose-terminating cap (Mosser, D.M., *et al.* 1992). Binding through CR3 triggers phagocytosis without the oxidative burst (Wright, S.D. and Silverstein, S.C. 1983) allowing for easy entry into the macrophage while also inhibiting IL-12 induced cell-mediated immunity (Descoteaux, A. and Turco, S.J. 1999). The multiple ways *Leishmania* parasites can enter immune cells allows for initial entry to Langerhans cells in the skin, where inducible nitric oxide synthase allows for the parasite to outwit the immune system (Alexander, J., *et al.* 1999).

After endocytosis the parasite enters a phagosome, referred to as the parasitophorous vacuole, where a series of fusion events occur that allow the vacuole to become the mature phagolysosome. This is a highly acidic and hydrolase rich environment that allows for amastigote development. The promastigotes have survival techniques to allow for existence prior to this conversion, primarily LPG, secondarily secreted acid-phosphatase and proteophosphoglycan, all of which include the same basic
repeating unit structure. As amastigotes bear little LPG, these other molecules are thought to protect the parasite in this stage. For example, *L. mexicana* produces a vacuole approximately 70% of the size of the host, which is full of copious amounts of these materials (Peters, C., *et al.* 1997).

As the primary mechanism for protection for promastigotes, LPG has been investigated using LPG defective mutants that are unable to establish infection in the macrophage phagolysosome. Mutants of *L. donovani* with LPG shorter than 5 repeating units are unable to inhibit phagosome-endosome fusion (Desjardins, M. and Descoteaux, A. 1997). Mutants lacking repeating units have also been shown to have the phagolysosome rapidly mature and acquire rab7 and LAMP1, markers for this maturation, while wild-type promastigotes do not (Scianimanico, S., *et al.* 1999).

The importance of LPG in establishing infection within the macrophage has also been shown by immunofluorescence and anti-LPG antibodies appearing on the cell surface within 5-10 minutes post-infection (Tolson, D.L., *et al.* 1990). *L. donovani* promastigotes are thought to inhibit the phagosome-endosome fusion mentioned above through steric hindrance. During endocytosis, LPG molecules are shed, and they intercalate into the outer leaflet of the plasma membrane of the phagosome (Miao, L., *et al.* 1995).

**D. Inhibition of hydrolytic enzymes**

While there is a dramatic decrease in the amount of LPG produced as the parasite transforms into the amastigote stage, the relatively small amount produced may still be important. The conversion results in a decrease of LPG from a few million molecules per cell to less than 1000 in the amastigote. Some data have suggested that LPG blocks lysosomal hydrolases. LPG coated erythrocytes are resistant to cytolysis compared to uncoated erythrocytes (Eilam, Y., *et al.* 1985), though no exact understanding of this mechanism is known.

**E. Chelation of Calcium**

The phosphate of LPG molecules can chelate calcium, which may have important impacts on the survival of the amastigote in the macrophage. The endocytosis of LPG coated blood cells, resistant to cytolysis, show an increase in intracellular calcium, possibly through LPG binding. The binding of calcium to LPG does not disturb the
structure, and this would impair signal transduction, critically through protein kinase C (Eilam, Y., et al. 1985; Homans, S.W., et al. 1992; Kane, M.M. and Mosser, D.M. 2000). Other evidence has shown LPG can chelate other ions as well, particularly divalent cations, preventing production of radicals during the oxidative burst (Turco, S.J. and Descoteaux, A. 1992).

F. Inhibition of Host Signaling Pathways

Leishmania parasites have many unique strategies to disrupt the function of the macrophages and turn off the microbicidal functions. Leishmania parasites and their LPG have been shown to interrupt the oxidative burst and radical synthesis, modulate macrophage apoptosis, inhibit antigen presentation and T-cell stimulation, nitric oxide production, and interfere with IL-12, INF-γ, and TNF-α. LPG is selective for the regulatory domain only. The regulatory domain allows for binding of diacylglycerol, calcium, and phospholipids; the 1-O-alkylglycerol moiety of LPG is the most effective inhibitory factor. In vitro LPG inhibits protein kinase C (McNeely, T.B. and Turco, S.J. 1987). There is some inhibition of PKC from the repeats, but LPG is required to be intact for this, individual repeating units do not work (McNeely, T.B., et al. 1989). An alternative hypothesis has been presented that LPG disturbs the lipid bilayer and alters the properties, preventing PKC insertion and subsequent activity (Giorgione, J.R., et al. 1996). This is a possibility as it inhibits PKC from an opposing monolayer. LPG mediated inhibition may disrupt other PKC-dependent events, including c-fos gene expression and macrophage chemotaxis (Descoteaux, A. and Turco, S.J. 1999). GIPLs also inhibit PKC, but as they have no repeating units, they most likely act through a different mechanism.

The nitric oxide produced by macrophages is a key regulator in pathogen destruction through the induction of inducible nitric oxide synthase in response to signals from invaders such as lipopolysaccharide. LPG-like treatment inhibited nitric oxide synthesis in a time and dose dependent manner (Proudfoot, L., et al. 1995a). Full length LPG had no effect, but repeating units alone diminished the production of nitric oxide (Proudfoot, L., et al. 1996). LPG associated protein kinetoplast membrane protein 11 decreased iNOS activity due to its similarity to L-arginine, a known inhibitor of iNOS
Production of nitric oxide is directly correlated with inhibition of leishmanicidal activity.

G. Oxidative Burst Metabolite Scavenging

Macrophages use the synthesis of arachidonic acid metabolites and oxidative metabolism as its key regulators for killing foreign organisms. NADPH oxidase is the primary source as it produces super-oxide and hydrogen peroxide (Nathan, C. and Shiloh, M.U. 2000). The Gal(β1,4)Man(α1)-PO4 repeat units of LPG may protect the promastigote from the oxidative burst by scavenging the cytocidal products (Chan, J., et al. 1989; el-On, J., et al. 1990).

H. Cytokines

Manipulation of cytokine production alters the immune response to the invasion of *Leishmania* parasites, and protects the parasite by altering antigen presentation. *Leishmania* infected macrophages do not produce IL-12, without which there is no INF-γ, and no helper T-cell differentiation. Leishmania selectively inhibits IL-12 production, thought to be a response as excess IL-12 may cause tissue damage detrimental to the parasite and its survival. LPG selectively suppresses cytokine production as inhibition of IL-1β gene transcription by LPG as a gene silencer through a specific sequence within the IL-1β promoter (Hatzigeorgiou, D.E., et al. 1996).

6. LPG Biosynthesis

A. Core-Lipid Biosynthesis

Biosynthesis of the lipid portion of LPG is not well understood, though it is proposed to begin with the formation of a 1-O-alkyl-2-acyl-phosphatidylinositol. Early data (Hart, D.T. and Opperdoes, F.R. 1984) suggest this begins with the use of dihydroxy acetone phosphate acyltransferase (DHAP) which acylates the C-1 position of the lipid. Reduction by NADPH and acylation at the C-2 results in the formation of 1-O-alkyl-2-acyl phosphatidic acid. On the cytoplasmic side of the endoplasmic reticulum, a glucosamine unit is added and a flippase transfers it to the luminal leaflet where a mannosyltransferase adds a Man(α1,4) unit. It has been suggested that up until this point, the synthesis of GPI anchors for proteins and LPG is the same (Ralton, J.E. and McConville, M.J. 1998). LPG synthesis continues in the Golgi where the second
mannose is added to the core. A unique galactosylfuranose residue is then added (Ilgoutz, S.C., *et al.* 1999), and synthesis is continued with the addition of two more galactose molecules to give a final core structure of Gal(α1,6)Gal(α1,3)Gal(β1,3)[Glcα1-PO₄6]Man(α1,3)Man(α1,4)GlcN(α1,6). At some stage during this process, fatty acid remodeling occurs, losing the C-2 fatty acid to yield the mature 1-O-alkyl-2-lyso-phosphatidyl(myo)inositol anchor. While having structures similar to this point, GIPLs are synthesized via a different pathway, where the intermediates are controlled and isolated from the copious production of LPG (Proudfoot, L., *et al.* 1995b).

**B. Repeating Unit Biosynthesis**

The start of repeating unit biosynthesis begins with an initiating mannosylphosphoryltransferase (iMPT). The iMPT recognizes the Gal(α1,6)Gal of the core, the same motif found in stachyose. Mannose-phosphate transfer has been shown to be most likely localized to the golgi, as all studies have shown the necessary GDP-Mannose transporter is localized in the golgi (Figure 1.4). The activity was first characterized by Mengeling and Turco (1997), and has been shown to be downregulated in amastigotes. An *in vitro* assay performed by Carver and Turco (1992) established that repeating unit biosynthesis continues by alteration of two enzymes; the mannosylphosphoryltransferase and a galactosyltransferase.

The continuing synthesis of repeating units requires the use of an elongating mannosylphosphoryltransferase (eMPT), shown to be different than an iMPT activity through the use of the ricin-resistant mutant JEDI (Descoteaux, A., *et al.* 1998). The eMPT recognizes Gal(β1,4)Man (Figure 1.4). There is a third mannosylphosphoryltransferase present responsible for the addition of the Man-P to the serine of proteoglycans (Moss, J.M., *et al.* 1999). The eMPT has been shown in a number of species, including *L. donovani*, to require the negative charge of a phosphodiester, the C-6 hydroxyl of the α-mannosyl residue and C-6′-hydroxyl of β-galactosyl residue for substrate recognition (Brown, G.M., *et al.* 1996; Routier, F.H., *et al.* 2000). While there is a consensus that there are 3 MPTs, there is a running debate over the existence of two separate eMPTs: one required for the addition of repeating units to form the procyclic length of 15 units and a separate enzyme that adds the repeating units required for metacyclic production. If there is not a second eMPT, there is an extensive regulatory
mechanism of the single eMPT controlling the number of repeating units during the life cycle.

C. Cap Biosynthesis

The terminating repeating unit of the LPG molecule is referred to as the cap, the creation of which involves the same enzymes, and can range from a single mannose to a full repeating unit. In the *L. donovani* (Sudanese isolate) there can be a oligo-mannosyl cap produced, linked α1,2 to the terminal repeating unit mannosyl residue. Other strains of *Leishmania* produce different cap variants based on the side-chain glycosyltransferases the parasite uses in the production of its LPG, which are discussed below.

D. Side Chain Biosynthesis

As mentioned previously there are three types of LPG: Type I has no side chains, such as *L. donovani*, Type II has side chains on the C-3 of the galactose, and Type III has side chains on the C-2 of the mannose. The only Type III LPG characterized to date is found on *L. aethiopica* which adds α-mannosyl chains. The only side chain glycosyltransferases characterized are those of Type II, adding side chains to the C-3 of the galactosyl residue. Side chain glycosyltransferases are critically regulated, as the eMPTs, their presence or absence affects vector choice and competence. Recently in the case of *L. major*, it has been identified that in certain vectors where the insect lectins aren’t directly recognizable to the side chains of *L. major*, there is an entirely different mechanism for mid-gut binding used by the parasite. In most vectors, the LPG must match insect mid-gut lectins.

The best studied group of side-chain glycosyltransferases are those involved in the addition of the side chains in *L. major* (Ng, K., *et al.* 1996). These have been shown to act on nascent LPG after synthesis of the primary glycan chain, as *L. donovani* LPG is used as the acceptor in the *in vitro* assays. A family of 14 genes from *L. major* have been identified, and only a number of these will be active when transferred into *L. donovani* (Dobson, D.E., Scholtes, L.D., *et al.* 2003). *L. major* has an additional arabinosyltransferase for side chain synthesis (Dobson, D.E., *et al.* 2003a; Goswami, M., *et al.* 2006) to add arabinosyl caps to the side chains as they proceed to stationary phase. In non-permissive vectors, the addition of this arabinose allows for the release of the parasite for galectins in the insect gut to continue infection. The regulation of both of
these are critical. *L. donovani* (Indian isolate) also produces type II LPGs, containing one or two β-linked glucoses added to the C-3 of the galactose (Mahoney, A.B., *et al.* 1999). These are developmentally regulated, as it has been shown that the activity and presence of glucose is downregulated in the progression to stationary phase, and affects the binding of the parasite to the midgut of the sand-fly.

7. *lpg*- mutants, their selection and complementation

The *L. donovani* genome was only recently sequenced and many difficulties were encountered attempting to create knockout parasites due to the life cycle (Gueiros-Filho, F.J. and Beverley, S.M. 1997), thus identification of genes in the lipophosphoglycan biosynthesis pathway was accomplished using mutagenesis (Figure 1.5). Parasites were harvested and incubated with N-methyl-N-nitroso-N’nitroguanidine (MNNG) (Iovannisci, D.M. and Ullman, B. 1984; King, D.L. and Turco, S.J. 1988). Mutants were then selected for using RCA 120, ricin agglutinin, which recognizes terminal β-galactosyl residues. The parasites were subjected to multiple rounds of selection with ricin, and isolated as single cells. Parasites not bound by ricin agglutinin were assumed to be deficient in LPG synthesis, or *lpg*- . Structural and enzymatic analysis of these mutants was performed to determine their suspected defect. A suitable cosmid was developed a 30kB extrachromosomal vector containing a selectable marker termed cLHYG for transformation of *Leishmania* parasites via electroporation (Ryan, K.A., *et al.* 1993a). Cosmid libraries could be placed into this vector, which holds approximately 40kbp of DNA, allowing for the entire genome to be screened using only 1000 cosmids total. Hygromycin was used to select for parasites that contained the cosmid and mutants that had corrected LPG structures could then be selected. The cosmids are then removed and shuttled back into *E. coli* for propagation and screening for open reading frames. Later variations allowed for creations of single knock out mutants and add backs in *L. major* after manipulation of the mutations in the *L. donovani*.

R2D2 was the first of the mutants selected after mutagenesis with MNNG to be studied. The structure of the LPG was found to terminate just prior to the addition of the galactosylfuranose to the core. The parasites still survived in macrophages, did not produce any full-length LPG (King, D.L. and Turco, S.J. 1988). Further studies of these
parasites identified LPG1, a galactosylfurasyltransferase unique to the parasites (Huang, C. and Turco, S.J. 1993; Ryan, K.A., et al. 1993b).

C3PO was the second mutant studied. C3PO lacked the addition of any repeating units to the core. Complementation identified LPG2, a GDP-Mannose transporter (Descoteaux, A., et al. 1995; Ma, D., et al. 1997). While initial studies suggested LPG2 might be a single subunit of the GDP-Man transporter, further studies established that the GDP-Mannose transporter was a hexameric complex of LPG2 subunits capable of not only transferring GDP-Mannose into the golgi complex, but capable of transferring GDP-Arabinose and GDP-Fucose as well (Hong, K., et al. 2000). This was a critical identification, as C3PO was not deficient in N-glycosylation, but only in LPG synthesis. While LPG2 was found in *L. donovani*, other strains of *Leishmania* can incorporate fucose and arabinose into their LPG.

OB1 was the third mutant characterized. The structure of OB1 LPG consisted of only the first half of a repeat unit, Man(1)-PO4 attached to the core. Complementation identified OB1 to be defective in LPG3, a homolog to the mammalian chaperone GRP94 (Descoteaux, A., et al. 2002). Like GRP94, it localized to the endoplasmic reticulum, but unlike anything known to have a function with mammalian GRP94, it showed a defect in production of all glycoconjugates, particularly that of LPG and GPI-anchored proteins. There were no growth defects, but unlike anything seen with a mammalian chaperone, GRP94 mRNA was developmentally regulated, but not regulated through heat or stress. LPG3 might be a GRP94 homolog, but has a completely unique function compared to its mammalian counterpart.

JEDI was the mutant with the last gene characterized. While originally JEDI had been selected by using ricin, it became quickly obvious that JEDI ended in a terminal galactose. JEDI has a single complete repeating unit added to the core, but not any others, and thus further studies with JEDI used CA7AE, an antibody that recognizes the LPG repeating unit to further purify JEDI out of any potential revertants prior to analysis (Descoteaux, A., et al. 1998). JEDI was complemented using LPG4A, and was the first mutant to concretely establish that the iMPT and eMPT activities were in fact discrete entities. LPG4A complementation of JEDI lead to a further understanding of the genes in the context of MPT activities. Further analysis of JEDI identified that it wasn’t just
defective in one gene, but it was defective in another later to be identified as LPG4C. LPG4A and LPG4C were located on the chromosome next to each other, and are fused together.

8. Current studies on LPG4A, LPG4B, and LPG4C

LPG4A, LPG4B, and LPG4C have since been individually knocked out in *L. major*. Work on these mutants has shown LPG4A to have no defects in LPG synthesis, but defects in other phosphoglycans. LPG4C produces LPG similar to JEDI, and LPG4B can not add the first repeating unit to the core. Enzymatic analysis show LPG4B is most likely the iMPT, as iMPT activity using stachyose as the acceptor is completely obliterated in that mutant. Surprisingly, LPG4A and LPG4C knockouts, while retaining iMPT activity, show a marked decrease in iMPT activity. These mutants show no difference in the galactosyltransferase activity, suggesting this is likely do to the absence of each of these individual enzymes suspected to be in a complex. LPG4C knockouts have an absence of eMPT activity, while there is no obvious change in eMPT activity in LPG4A knockout. All of these are consistent with their suspected functions based on their structures. A mutant lacking LPG4C but with an additional copy of LPG4A rescues LPG synthesis based on LPG structure (Beverley et.al, personal communication), similar to the rescuing of JEDI by LPG4A alone, even though LPG4A is not outright involved in LPG synthesis. A summary of all mutants is found in Table 1.1.

9. Overview of Dissertation

The repeating units of *Leishmania* LPG are a structural motif not found in humans. The number of repeating units on LPG is highly regulated and controlled, and is critical to the parasites life cycle, critical to the infectivity of the parasite, and can alter the innate immune response. The importance and uniqueness of the repeating units makes them a key chemotherapeutic target, and stresses the importance on the understanding of the biosynthesis of LPG.

There are a number of critical enzymes in LPG biosynthesis, but MPTs are key as they add the unique Man(α1)-PO₄. There are 3 MPTs minimum to the formation of LPG, an initiating, an elongating, and a serine-specific. Another key regulator in the life cycle
are the side chain glycosyltransferases which can affect vector specificity and immune response.

Mutagenized parasites and their functional rescue have been key to understanding LPG biosynthesis, and the mutants themselves are critical to understanding the structural formation LPG takes while on the cell surface to cause ricin resistance. JABBA, a parasite selected for resistance to ricin after mutagenesis with MNNG, in preliminary LPG analysis showed an increase in the number of repeating units in logarithmic phase and an increase in cap isoforms. This dissertation aims to fully characterize the LPG, analyze the enzymes responsible for these changes, and examine the causes of ricin resistance. The central hypothesis is an upregulation of key enzymes resulting in the increase in repeating units and additional cap isoforms, and that addition of repeating units affects ricin resistance.

JABBA shows two key differences in the structure of LPG from wild-type; the number of repeating units and the cap structures. Wild-type *L. donovani* (Sudanese) has 15 repeating units in logarithmic phase, and 30 repeating units in stationary phase, while JABBA LPG has 30 repeating units in logarithmic phase, and 47 in stationary phase, with a quantifiable length shift in mid-logarithmic phase. JABBA LPG has 3 unique cap structures found only in logarithmic phase: Glc(β1,2)Man, Gal(β1,4)[Glcβ1,2]Man, and Gal(β1,4)[Glcβ1,2Manα1,2]Man.

The potential enzymes causing these changes, including the iMPT, eMPT, and β-glucosyltransferase were investigated. JABBA had no change from wild-type in iMPT activity, but showed a doubling in both eMPT and β-glucosyltransferase activity. Product characterization of the β-glucosyltransferase activity of both JABBA and wild-type parasites revealed a Gal(β1,4)[βGlc]Man product on *L. donovani* LPG in both parasites, resembling the structure of the unique trisaccharide cap in JABBA parasites. Wild-type has no *in vivo* activity, but this reveals the presence of a post-translationally regulated glucosyltransferase that differs from the *L. donovani* (Indian isolate) β-glucosyltransferase.

While terminating in galactose, the LPG was still ricin resistant. Logarithmic and stationary phase parasites were checked for agglutination by ricin, peanut agglutinin, and concanavalin A and susceptibility to galactose oxidase. While parasites in all phases
showed increased resistance compared to their wild-type counterparts, stationary phase parasites showed the greatest resistance, showing the number of repeating units was causing the cap to go cryptic, masking the terminal galactose. This work furthers previous research in this area, as the caps are most likely to be inaccessible are in parasites with LPG longer than 30 repeating units.

The work in this dissertation furthers the studies on regulation of the biosynthesis of *Leishmania* LPG. Regulation of the eMPTs is critical to developing chemotherapeutics, and JABBA may hold the key to understanding the differences between Sudanese and Indian isolates of *L. donovani*. Overall, JABBA allows for a unique view into the parasites life cycle not seen previously.
Table 1.1. Summary of mutants and their identified gene mutations.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>LPG Structure</th>
<th>Gene</th>
<th>Mutation</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2D2</td>
<td>Man-Man-GlcN-PI</td>
<td>LPG1</td>
<td>Point</td>
<td>Gal(\text{\textsubscript{f}}) Transferase</td>
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<td>LPG4A</td>
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<td>-</td>
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<tr>
<td>LPG4\textsubscript{A\textsuperscript{-/-}}</td>
<td>No Change</td>
<td>-</td>
<td>K.O.</td>
<td>PPG eMPT</td>
</tr>
<tr>
<td>LPG4\textsubscript{B\textsuperscript{-/-}}</td>
<td>Core-PI</td>
<td>-</td>
<td>K.O.</td>
<td>iMPT</td>
</tr>
<tr>
<td>LPG4\textsubscript{C\textsuperscript{-/-}}</td>
<td>Gal-Man-PO\textsubscript{4}-Core-PI</td>
<td>-</td>
<td>K.O.</td>
<td>eMPT</td>
</tr>
</tbody>
</table>
Figure 1.1. Life cycle of *Leishmania* species: adopted from (Bray, A. 1999)
Figure 1.2 Structures of the *Leishmania* glyocalyx. Large circles represent proteins, small clear circles represent phosphoglycans. The triangles are caps, and the rectangles represent a conserved glycan core attached to the representative phospholipid.
Figure 1.4. The reactions to synthesize the major carbohydrate component of LPG. These reactions take place in the golgi apparatus. The eMPT and GalT trade off until the molecule is complete. Some species, such as the Indian strain of *L. donovani* have an addition of side chains, which are believed to be added after the repeating units are added to the molecule. iMPT: initiating mannosylphosphoryltransferase, eMPT: elongating mannosylphosphoryltransferase, GalT: galactosyltransferase, β-GlcT: β-glucosyltransferase
Figure 1.5 A diagram representing the methodology for mutagenesis of parasites and identify those deficient in LPG synthesis.
Chapter II
Materials and Methods

1. Materials and Equipment

A. Materials

Chemicals and reagents were obtained as follows: Bacto- Brain Heart Infusion. (Difco). Alkaline Phosphatase (E.coli), Beta-glucosidase (Almond), Beta-galactosidase (E.coli), Alpha Mannosidase (Jack Bean), ANTS (8-Aminonaphthalene-1,3,6-trisulfonic acid) from Sigma, feta bovine serum (Atlanta Biologicals), APTS (8-aminopyrene-1,3,6-trisulfonate) labeling and capillary electrophoresis columns (Ab-Sciex), C-18 Sep-Pak columns (Waters), BCA Reagent Kit (Pierce), and Protease Inhibitor (Roche).

B. Equipment


2. Parasites and Mutagenesis

A. Parasites

L. donovani (MHOM/SD/00/1S-2D), L. donovani (MHOM/IN/83/Mongi-142), and JABBA were maintained in M199 with 10% FBS as described previously (Kapler, G.M., et al. 1990).

B. Mutagenesis

Parasites were selected and mutagenized as described previously (King, D.L. and Turco, S.J. 1988). The cell concentrations used in ricin selection were between 2 x 10^6 and 2 x 10^7 cells/ml.

3. Purification and analysis of LPG

A. Purification of LPG

Logarithmic phase JABBA promastigotes were extracted at 5.0 x 10^5 to 1.0x 10^6 parasites per milliliter from brain heart infusion supplemented with heme and adenosine (Orlandi, P.A., Jr. and Turco, S.J. 1987). LD4 and Mongi were extracted at 3.0 x 10^6 to
6.0 x 10^6 parasites per milliliter for logarithmic phase. All strains were extracted at greater than 2.0 x 10^7 parasites per milliliter for stationary phase.

Logarithmic parasites were extracted using the Ferguson method as detailed previously (Proudfoot, L., et al. 1996) using two 2 mL 4 h washes in methanol/chloroform/water (2:1:0.8) to remove various glycolipids followed by two 0.5 mL washes in 9% 1-butanol collected after 30 min of water bath sonication. Purification of these fractions on octyl-sepharose chromatography, using a 60% propanol gradient. Fractions were spotted on silica thin layer chromatography plates and sprayed with orcinol and sulfuric acid and subsequently charred in a 100°C oven for approximately 8 min to identify the LPG containing fractions.

Stationary parasites were extracted by the Folch method (McConville, M.J., et al. 1990; Orlandi, P.A., Jr. and Turco, S.J. 1987). After centrifugation, pellets were washed twice with PBS, twice with chloroform/methanol/water (3:2:1), three times with 4 mM MgCl₂ and three times with solvent E (water/ethanol/diethylether/pyridine/NH₄OH, 15:15:5:1:0.017) to purify and collect LPG, then dried under N₂. Dried material was resuspended in 0.1 N acetic acid/ 0.1 N NaCl (running buffer) and purified using phenyl-sepharose chromatography. The column was equilibrated with 6 column volumes running buffer, then crude LPG was applied. The column was then rinsed with 6 column volumes running buffer, 4 column volumes 0.1 N acetic acid and 1 column volume water. The LPG was eluted with 6 column volumes solvent E and dried under a stream of N₂.

**B. Mild Acid Hydrolysis**

Dried LPG was resuspended in 150 µL 0.02 N HCl and incubated at 60°C for 15 min. After the mixture was dried under a stream of N₂, 150 µL toluene was added and evaporated three times. This treatment cleaves at the phosphate of the repeating units.

**C. Butanol Partition and C18 lipid removal**

To remove the core-lipid from samples treated with mild acid hydrolysis, dried samples were treated with water-saturated 1-butanol (McConville, M.J., et al. 1987). The top (organic layer) was removed and set aside for further analyses of the core and lipid. The aqueous layer was dried for anionic chromatography to collect caps and repeats. If the lipid was not being saved, the aqueous sample could be applied to a C18 sep-pak column and carbohydrate collected with water.
D. Nitrous Deamination

To separate the lipid from carbohydrate, purified core-lipid or whole LPG was dried and resuspended in 300 µL 0.25 M sodium acetate (pH 4.0) and 300 µL 0.5 M sodium nitrite (Ferguson, M.A., et al. 1985). The mixture was left in a water bath for 16 h at 37ºC.

E. Strong Acid Hydrolysis

To cleave between the glycosidic bonds, oligosaccharides were treated at 100ºC for 3 h with 2 N trifluoroacetic acid. After this treatment, desalting is required (see section G).

F. Enzymatic Treatments

Alkaline phosphatase treatment was performed using 1U in 1 mM Tris-HCl (pH 9) overnight at 37ºC. β-glucosidase treatment was performed overnight using 1U in 200 mM ammonium acetate at 37ºC. α-mannosidase treatment was performed overnight using 0.1 M sodium acetate pH 4.5 with 1 U at 37ºC. β-galactosidase treatment was performed overnight with 1U in sodium phosphate buffer at 37ºC.

G. Desalting and Anionic Exchange Chromatography

Desalting was performed with a mixed bed of equal parts AG-50WX10 and AG1-X8. For most small samples, a total column volume of 0.5 mL was used. For desalting prior to capillary electrophoresis, a 1 mL mixed bed column was used. For anionic exchange chromatography, a 1 mL bed of AG1-X8 was used and samples were applied and allowed to sit for ten min. 6 mL of water was used to collect caps, then 6 mL of 0.3 M NaCl was used to collect repeats from samples.

H. Stains-All

Approximately 20 µg of LPG was run for 1 h at 120 V on 12% SDS-PAGE. The gel was rinsed three times for 10 min in 25% isopropanol and left immersed for approximately 16 h. The isopropanol was poured off, and the gel was incubated in stains-all solution (64.5% water, 25% isopropanol, 5% formamide, 5% stains-all, 1% water) for 2 h (Bahr, V., et al. 1993). The gel was destained in 40% ethanol until visualization.

I. Fluorophore Assisted Carbohydrate Electrophoresis.

Oligosaccharide fluorophore assisted carbohydrate electrophoresis was used following the method of Lehrman and Gao and the subsequent modifications (Gao, N., et
The resolving gel stock was 38% acrylamide/2% bis-acrylamide with a buffer of 1.5 M tris-chloride (pH 8.9). The stacking gel was 10% acrylamide, 2.5% bisacrylamide, with a buffer of 1.0 M tris-chloride (pH 6.8). Samples were labeled with 0.15 M ANTS in 15% acetic acid and 1 M sodium cyanoborohydride in DMSO overnight at 37°C. The gel was run at 20 mAmps and 4°C for approximately 3 h until it reached the bottom. A glucose standard ladder of oligosaccharides was used.

J. Separation of Monosaccharides via Capillary Electrophoresis

Samples were labeled using 2 µL monosaccharide APTS and 2 µL 1M sodium cyanoborohydride in THF for 90 min at 55°C. Before loading into the unit, 196 µL of water was added to the samples. The capillary was conditioned for 2 min with 0.1 N NaOH and then 0.1 N HCl with a 1 min water rinse between. The capillary was coated for 2 min with a running buffer of 30 mM sodium borate with 5% methanol. Samples were injected at a rate of 0.4 psi for 4 sec, and voltage separated for 20 min. Standard curves of anhydromannose, created by nitrously deaminating glucosamine, and mannose were used for quantification. The sensitivity ratio between mannose and anhydromannose was found to be 1.5 to 1, consistent with previous results (Barron, T.L. and Turco, S.J. 2006). Galactose and glucose are roughly equal to mannose in sensitivities.

K. Separation of Oligosaccharides via Capillary Electrophoresis

Neutral desalted oligosaccharides were run through a carbohydrate coated silica column. They were labeled overnight using oligosaccharide APTS containing acetic acid and sodium cyanoborohydride in the dark at room temperature. The capillary was conditioned using 10 min rinse of water at 30 psi followed by a 10 min 30 psi rinse of carbohydrate buffer. The column was coated with gel buffer for 5 min at 20 psi before applying samples. The ladder was injected for 0.4 sec at 0.4 psi and separated at a maximum of -25 kV for 20 min with reverse polarity. For product characterization of the glucosyltransferase assay, the sample injection rate was 5 psi for 5 sec. For every other visualization, 0.4 psi for 4 sec was used. (Soares, R.P.P., et al. 2004)

L. Partially Methylated Alditol Acetates

The structure of the cap isoforms was determined using partially methylated alditol acetates, adapted from the previously published protocol (Pettolino, F.A., et al. 2013; Lehrman, M.A. and Gao, N. 2003).
2012). Material was dried in a speed vacuum concentrator then resuspended in methanol and evaporated twice. 50 µL of DMSO was applied to the sample in a screw top tube and placed in a sonication water bath for 30 min. Approximately 50 µL of slurry of NaOH in DMSO was applied via capillary pipette to the sample and sonicated again for 30 min in the water bath. Three applications of iodomethane were applied with two 10 µL, 10 min, sonication applications and a 20 µL, 20 min, application. The methylated product was extracted using dichloromethane and water. The dichloromethane soluble portion was dried and resuspended in 250 µL 2 N TFA in a 100°C oven for 90 min. The sample was dried and resuspended in 50 µL 2 M ammonium hydroxide. A 1 M solution of sodium borodeuteride in 2 M ammonium hydroxide was freshly prepared and 50 µL applied to the sample and allowed to incubate at room temperature for 2.5 h. The samples were then evaporated with methanol in 5% acetic acid twice, followed by two rinses of methanol. 250 µL of acetic anhydride was applied to the samples in a 100°C oven for 2.5 h. After removal from the oven, the sample was allowed to cool and 2 mL of water was added. The samples were then extracted with 1mL of dichloromethane and cleaned with 2 mL of water twice. The dichloromethane was then collected and dried under a stream of N₂ carefully. The sample was resuspended in an appropriate amount of dichloromethane and sent to the UK Mass Spectrometry facility for analysis via gas chromatography-mass spectrometry.

The GC-MS was performed by the UK Mass Spectrometry facility on a Shimadzu GCMS-QP5000 (quadrupole mass spectrometer) equipped with a GC-17A gas chromatograph. Electron ionization (EI) mass spectra were recorded at 70eV, scanning m/z 45-550 at ~1 scan/sec. The GC column was a DB-5ms (Agilent/J&W) 30m x 0.25mm (0.25 um df) with helium as the carrier gas. The column oven temperature program was 50°C (1 min initial hold) to 280°C at 10°C/min, with an injector temperature of 250°C and an interface temperature of 280°C.

M. Lipid Analysis

For purification of the lipid for thin layer chromatography, whole LPG was treated with mild acid. A butanol partition was then performed collecting the butanol and drying it under a stream of N₂. Collected product was nitrously deaminated to cleave the lipid overnight at 37°C. Product was subject to another butanol partition, dried, then
resuspended in 0.1N HAc/NaCl and purified via phenyl sepharose chromatography, collecting the final solvent E fraction. Thin layer chromatography of the nitrously deaminated lipid was performed as described previously using silica gel 60 TLC plates and developing with CHCl₃: CH₃OH: 4.2 N NH₄OH (9:7:2) system. Additional controls used on the plate included phosphatidylinositol, phosphatidylcholine, and previously purified alkyl-lyso-phosphatidylinositol in addition to *L. donovani* and JABBA purified lipids. (Mahoney, A.B., *et al.* 1999; Orlandi, P.A., Jr. and Turco, S.J. 1987).

4. Enzymatic Assays

A. Preparation of Microsomal Membranes.

Microsomal membranes were prepared as previously described (Mengeling, B.J., *et al.* 1997b), with the following modifications: Cultures of parasites (200 m) were centrifuged at 2700 rpm for 7 min, and resuspended in 10 mL of lysis buffer containing 100 mMol HEPES (pH 7.4), 50 mMol KCl, 1 mMol EDTA (pH 8.0), 10% glycerol, and protease inhibitor. The suspension was subjected to nitrogen cavitation using a Parr bomb for 30 min at 1500 psi on ice. Subsequent centrifugations took place at 4°C for 3000 x g, 10000 x g for 10 min, and 100000 x g for 1 h. The supernatant was removed, and the pellet was resuspended in membrane buffer containing 100 mMol HEPES (pH 7.4), 50 mMol KCl, 1 mMol TLCK, and 1 µg/ml leupeptin using a homogenizer. The final protein concentration was determined with a BCA reagent kit.

B. Initiating Mannosylphosphoryltransferase (iMPT) Assay

The iMPT *in vitro* assay was performed as previously reported (Mengeling, B.J., *et al.* 1997b) with the following modifications. The assay mixture contained 1 mg membrane protein, 15% glycerol, 2.5 mMol thioglycerol, 10 mMol MnCl₂, 5 mMol MgCl₂, 4 mMol dithiothreitol, 800 µMol GDP-Man, and 27 nCi per4 mMol stachyose in 100 µL. The mixture was incubated for 4 h at 30°C and the reaction was terminated on ice. Product purification was performed as previously detailed (Mengeling, B.J., *et al.* 1997b) and radioactivity was counted via scintillation.

C. Elongating Mannosylphosphoryltransferase (eMPT) Assay

The eMPT assay involved the preparation of soluble proteins from the microsomal preparations. These soluble microsomal proteins were prepared with final concentrations of 50 mMol HEPES (pH 7.5), 150 mMol KCl, 10 mMol MnCl₂, 5 mMol
MgCl₂, 20% glycerol, 1 µg/ml leupeptin, and 0.5% dodecyl-maltoside and 3 mg of microsomes. The microsomal mixture was rotated for 1 h at 4°C and then centrifuged at 100,000 x g for 1 h. The supernatant collected was used for the eMPT assay.

The eMPT activity was quantified as done previously (Carver, M.A. and Turco, S.J. 1991) with the following modifications using an assay mixture of 1 mMol ATP, 0.5 mMol dithiothreitol, 2 mMol GDP, 10 mMol MnCl₂, 5mMol MgCl₂, 225 nCi of [³H]-GDP-Man at 200 µMol and 75 µL solubilized microsomal protein in a total of 150 µL. An exogenous acceptor (25 µg) of JEDI LPG (Descoteaux, A., et al. 1998), possessing a single Gal-Man-PO₄ repeat on the glycan core-PI, was used. The assay mixture was incubated for 1 h at 28°C. The reaction was terminated with 0.5 M EDTA and the radioactive product was purified via chromatography on phenyl-sepharose and product containing fractions were determined by scintillation counting.

D. β-Glucosyltransferase Assay

The glucosyltransferase assay was quantified using 0.5 µCi of 34 µMol UDP-Glc in the same final volume as previously described (Mahoney, A.B. and Turco, S.J. 1999). LPG was extracted, and total radioactivity was counted via scintillation.

E. Product Characterization of β-Glucosyltransferase Assay

For product identification, the product from nonradioactive β-glucosyltransferase assays, along with assays lacking UDP-Glc and exogenous LPG were desalted on a mixed bed column (2 mL) of AG-50W and AG1-X8 then dried. One aliquot of the product was treated with mild acid as described previously, then desalted. Another aliquot was treated with mild acid hydrolysis and dephosphorylated with alkaline phosphatase, then desalted. The samples were then reductively labeled overnight with APTS and sodium cyanoborohydride in THF in the dark at room temperature. Product obtained after mild acid and alkaline phosphatase was also analyzed by exoglycosidase treatments. For analysis by oligosaccharide capillary electrophoresis, injection pressures were 5 psi for 5 sec (Soares, R.P., et al. 2004) unless otherwise stated.

5. Resistance Assays

A. Lectin Assays

For 30 min, 5.0x10⁶ cells were rocked in 5 mL of M199 containing 100 µg of peanut agglutinin, RCA 120 (ricin agglutinin), or concanavalin A. 10 µL of cells were
placed on a hemacytometer and free cells were counted. The parasites were selected in logarithmic phase at a culture density of less than $1.0 \times 10^6$ cells and in stationary phase at a minimum of $1.0 \times 10^7$ cells.

**B. Galactose Oxidase Treatment.**

$1.0 \times 10^9$ logarithmic and stationary cells from *L. donovani* and JABBA were treated with 3U galactose oxidase in 500 µL PBS at 25°C for 1 h (Sacks, D.L., et al. 1995). LPG was collected and purified using the Folch method. Caps were isolated after mild acid hydrolysis, butanol partition, and separation of AG1X-2 using the charge, then dried in a speed vacuum concentrator. Caps were then treated with 2 N TFA in a 100°C oven for 3 h, and desalted as described above. The caps were dried and labeled as described for monosaccharide capillary electrophoresis. A 75 µM bare fused silica capillary was run at 20 kV using a 15 mM sodium borate, 2.5% methanol buffer for 10 min. The percent of galactose available for reaction with galactose oxidase was calculated using caps collected in the same fashion, and normalizing to the areas of mannose. The percentage galactose available was calculated as the total area of galactose found in galactose-oxidase treated cells to those that were not.
Chapter III

The Structure of the Lipophosphoglycan of JABBA parasites

1. Introduction

*Leishmania* parasites have a unique life cycle that requires an extensive glycocalyx for protection from the sand-fly gut, complement in the bloodstream, and the mammalian phagolysosome (Kaye, P. and Scott, P. 2011). Lipophosphoglycan, one component of the *Leishmania’s* glycocalyx, is critically regulated throughout this life cycle as it progresses from procyclic promastigote through metacyclic promastigote to amastigote (de Assis, R.R., *et al.* 2012). Lipophosphoglycan is the dominant glycocalyx molecule in the promastigote stage, and most frequently studied there.

The *Leishmania* genome has only recently been sequenced (Ivens, A.C., *et al.* 2005), so earlier attempts to identify genes involved in biosynthesis of LPG involved chemical mutagenesis of parasites by MNNG and selection by ricin exposure to identify mutants defective in LPG (Iovannisci, D.M. and Ullman, B. 1984; King, D.L. and Turco, S.J. 1988). Critical mutants selected have been defective in LPG synthesis and identified four genes (Descoteaux, A., *et al.* 2002; Descoteaux, A., *et al.* 1998; Hong, K., *et al.* 2000; Ma, D., *et al.* 1997; Ryan, K.A., *et al.* 1993b): LPG1, a galactosylfuranosyltransferase, LPG2, the GDP-Mannose transporter, LPG3, a heat shock protein chaperone, and LPG4A, a mannosylphosphoryltransferase. LPG4B and LPG4C were also identified from studies with these mutants (Beverley, personal communications). JABBA is the first extensively studied ricin resistance parasite not defective in LPG synthesis, and is critical to determining the complete structure of LPG and how it differs from wild-type.

Early data suggests that the structure of the parasites have abnormal cap isoforms, most likely with the addition of a β-glucose reminiscent of the β-glucose containing LPG of the Indian strain of *L. donovani*. The increase in repeating units, while not having been quantified, suggests the parasite may be “stuck” in metacyclogenesis, and will not necessarily elongate in once in stationary phase. The nature of these hypotheses will be discussed alongside the complete structure.
2. Results

The structure of lipophosphoglycan consists of a conserved core and unique 1-O-lyso-alkyl-phosphatidylinositol, a stage specific number of Gal(β1,4)Man(α1)-PO₄ repeating units, and a terminal cap. LPG in wild-type *L. donovani* Sudan (LD4) averages 15 unsubstituted repeating units in logarithmic phase and 30 unsubstituted repeating units in stationary phase (Barron, T.L. and Turco, S.J. 2006), while the terminal cap structure can vary from a single terminal mannose to a full repeating unit, occasionally with an additional α-mannose (Greis, K.D., *et al.* 1992). A schematic of *L. donovani* LPG is found in Figure 3.1.

A. JABBA produces a much larger LPG than wild-type.

JABBA was selected for being ricin-resistant after mutagenesis with MNNG. Most parasites selected through this technique produce much shorter LPG than wild-type. Initial quantification of total LPG estimates that JABBA produces approximately 1/3rd the LPG of *L. donovani* (LD4). Through visualization by stains-all on a 12% SDS gel, logarithmic phase JABBA was found to produce LPG roughly twice the size of wild-type, similar in size to LPG in stationary wild-type. Surprisingly, in JABBA stationary phase LPG appears to increase in size relative to logarithmic phase. (Figure 3.2)

B. The structure and number of the repeating units of JABBA LPG

An increase in the size of LPG is due to either the number of repeating units or the addition of side chains to the repeating units. Using fluorophore assisted carbohydrate electrophoresis to visualize the repeating units, *L. donovani* was shown to contain a disaccharide as expected, as did JABBA (Figure 3.3). The purified disaccharides were treated with strong acid hydrolysis and the individual components were verified to be galactose and mannose in both *L. donovani* and JABBA by monosaccharide capillary electrophoresis. (Figure 3.4)

After verifying that the composition of JABBA’s repeating units is the same as *L. donovani*’s, it was necessary to determine the number of repeating units present. In order to quantify the repeating units using monosaccharide capillary electrophoresis, the fluorescence ratio of anhydromannose to mannose was found to be 1.5 to 1 as previously published (Barron, T.L. and Turco, S.J. 2006). Standard curves were used to determine the total amounts of anhydromannose and mannose in a sample. An example of the CE
chromatogram found from analyses to determine the repeating units is found in Figure 3.5. The number of *L. donovani* repeating units was determined to be 15 in logarithmic phase and 30 in stationary phase, matching previously published data (Sacks, D.L., *et al.* 1995). Logarithmic phase JABBA LPG has 30 repeating units, 47 in stationary phase. Over the course of data collection, a mid-logarithmic LPG size was found to be 42. (Table 3.1)

**C. The structure of the caps of JABBA Lipophosphoglycan**

As JABBA LPG structure is larger than wild-type, it was critical to establish if the caps contained a galactose. In other mutants, ricin-resistance was conferred by shorter LPG structures that without a terminal galactose (Descoteaux, A., *et al.* 2002; Huang, C. and Turco, S.J. 1993). Using fluorophore assisted carbohydrate electrophoresis, the cap structures of the *L. donovani* and JABBA were compared. *L. donovani* showed a disaccharide and monosaccharide, with a faint trisaccharide, reminiscent of the published cap structures of *L. donovani* (Greis, K.D., *et al.* 1992). JABBA showed the same 3 bands, plus an additional trisaccharide and tetrasaccharide (Figure 3.6).

As FACE gels require large quantities of material to visualize JABBA caps, oligosaccharide capillary electrophoresis was used to view the enzymatically reacted cap structures due to its sensitivity. In *L. donovani* caps, one disaccharide was β-galactosidase sensitive, while the other disaccharide was α-mannosidase sensitive; the trisaccharide was sensitive to both. No *L. donovani* caps were β-glucosidase sensitive. This matches previously published data for *L. donovani* caps (Greis, K.D., *et al.* 1992), and the exact linkages were not pursued further.

Capillary electrophoresis of reacted JABBA caps revealed the presence of two trisaccharides, one of which aligned with the trisaccharide from *L. donovani*. The CE chromatogram of JABBA caps are found in Figure 3.7. These two structures were both β-galactosidase and β-glucosidase sensitive, but α-mannosidase resistant. Parasites collected at a density greater than 3.0 x 10⁶ parasites/ml lacked both the unique glucose-containing trisaccharide and tetrasaccharide, matching the wild-type cap profiles.

Monosaccharide capillary electrophoresis was used to calculate the relative ratios of substituents of bulk cap samples shown in Figure 3.8. The chromatograms are adjusted to the elution times to view the glucose. Mannose was used for normalization, as every
cap isoform contains a mannose in both *L. donovani* and JABBA caps. The comparison showed a similar amount of galactose in logarithmic cap samples of *L. donovani* and JABBA, but stationary JABBA cap samples showed approximately half the amount of galactose. Equivalent amounts of glucose and galactose were found in JABBA logarithmic cap samples. Combined with oligosaccharide capillary electrophoresis, the relative amounts of each cap isoform were calculated in Table 3.2.

These cap structures were predicted to be Gal-(Glc)Man and Gal-(Glc-Man)Man. It is important to note that after both β-galactosidase and β-glucosidase treatment there was a decrease in the total amount of disaccharide present in the sample, suggesting there was a glucose containing disaccharide present. Predicted disaccharide structures include Man-Man, Gal-Man, and Man-Glc.

Partially methylated alditol acetates were used to determine the linkage as the composition and order were already known. *L. donovani* has Gal (β1,4)Man, Man(α1,2)Man, Man, and Gal(β1,4)[Man(α1,2)]Man caps. Besides a 2,4-linked mannose, JABBA showed no other hinge mannose, suggesting that the linkage (Figure 3.9) between the glucose and mannose is Glc(β1,2)Man, consistent with type-III LPG. Approximately 20% of the sample was terminal glucose, verifying the capillary electrophoresis calculations on total cap isoforms. The unique cap structures found on JABBA LPG were Glc(β1,2)Man, Gal(β1,4)[Glc(β1,2)]Man and Gal(β1,4)[Glc(β1,2)Man(α1,2)]Man. The percentages of all structures are found in Table 3.3.

**D. Core and Lipid Structure of JABBA LPG**

For analysis of the core, both *L. donovani* and JABBA LPG glycan cores were separated from the lipid by nitrous deamination. The primary components of each were identified as galactose, mannose, and anhydromannose (Orlandi, P.A., Jr. and Turco, S.J. 1987; Turco, S.J., *et al.* 1989). The oligosaccharide analysis showed both as a hexasacharide. The monosaccharide compotents and oligosaccharide size of both JABBA and *L. donovani* matched previously published data.

The lipid was purified on phenyl-sepharose after nitrous deamination. Thin layer chromatography was performed developing in a chloroform/methanol/ammonium hydroxide system observing *L. donovani* and JABBA purified lipids from LPG along
with standards: phosphatidylinositol, and phosphatidylcholine. The migration times were consistent with the published data (Orlandi, P.A., Jr. and Turco, S.J. 1987) classifying the structure of the lipophosphoglycan lipid. TLC plates showed approximately 8.5cm migration time for the lipid in \textit{L. donovani} (LD4) and JABBA. Migration times are found in Table 3.4.

### 3. Discussion

The structure of LPG throughout the life cycle of the parasite is critically regulated (Sacks, D.L., \textit{et al.} 1995). The glycan core and alkyl-lyso-phosphatidylinsoitol lipid anchor are conserved in the structure (McConville, M.J., \textit{et al.} 1990; Orlandi, P.A., Jr. and Turco, S.J. 1987; Turco, S.J., \textit{et al.} 1989) and is frequently found as the basis for other glycolipids on the cell surface. The procyclic promastigote LPG has 15 repeating units consisting of Gal(β1,4)Man(α1)-P and ends in a terminal cap that can be a mannose, a full repeating unit, Man(α1,2)Man or a repeating unit substituted with α-mannose on the mannose of the repeating unit. This is all present on the C-2 of the mannose as LPG. Metacyclic LPG has approximately 30 repeating units, the increase in units causing the release from the parasite gut (Sacks, D.L., \textit{et al.} 1995), and the number of repeating units effects many functional attributes (Gaur, U., \textit{et al.} 2009).

Besides the number of repeating units, species of Leishmania that have LPG with side chains are also developmentally regulated (Coelho-Finamore, J.M., \textit{et al.} 2011; de Assis, R.R., \textit{et al.} 2012; Dobson, D.E., \textit{et al.} 2003b). While \textit{L. donovani} Sudan doesn’t possess any side chains, \textit{L. donovani} India has β-Glucose substituted on the C-3 of galactose (Mahoney, A.B., \textit{et al.} 1999). The percentage of glucose decreases as the parasite progresses from procyclic to metacyclic form. Other species, like \textit{L. major}, cap their side chains with other sugars as they proceed from procyclic to metacyclic phase allowing for release of the parasite from the sand-fly gut (Dobson, D.E., \textit{et al.} 2010; Dobson, D.E., \textit{et al.} 2003a).

JABBAs LPG size increase is reminiscent of a parasite that has undergone metacyclogenesis in logarithmic phase. There is no obvious explanation for why it continues to stationary phase, contrary to the initial hypothesis that it would not elongate. The repeating units are the same composition as wild-type and the increase to 30 units in
logarithmic phase and to 47 in stationary is the primary difference between the strains. JABBA also shows a mid-logarithmic length of 42, a change that has not been previously noted in *L. donovani*. Depending on the assay and the total density of the parasites used, an increase in the average number of repeating units can be found in cultured logarithmic phase *L. donovani*. If this phenomenon is a by-product of culturing the parasite outside of the sand-fly is unknown. For this reason, we have defined our phases to avoid mid-logarithmic length: logarithmic is less than $3.0 \times 10^6$ parasites/ mL, stationary is greater than $1.0 \times 10^7$ parasites/mL.

In regards to the cap structure, there is the inclusion of a β-glucose similar to the Indian strain of *L. donovani*, but the acceptor has changed. Whether this is a separate mutation within JABBA, or the result of a novel β-glucosyltransferase inactive in LD4 (Sudanese *L. donovani*) will be examined later in this work.

The apparent down-regulation of glucose in JABBA cap isoforms is also similar to *L. donovani* India, as it decreases the number of glucose side chains present on its LPG as it progresses from procyclic to metacyclic phase, as this decrease is critical for the release of the parasite from the sand fly gut (Mahoney, A.B., *et al.* 1999; Mahoney, A.B. and Turco, S.J. 1999). This makes it the most likely explanation for the origin of the glucose present on the caps of JABBA, as *L. donovani* Sudan, the parasite that was originally mutagenized to create JABBA, does not have an β-glucosyltransferase active *in vivo*.

An interesting statistic is the dramatic decrease of galactose in JABBA LPG cap during the progression from logarithmic phase to stationary phase. Ratios between mannose, galactose and glucose were calculate in wild-type *L. donovani*, JABBA logarithmic caps, and JABBA stationary caps. As mentioned above, there was no glucose in JABBA stationary caps, which was demonstrated by a change in the isoforms back to wild-type, no glucose in themonosaccharide analysis, and the caps resistantance to treatment with β-glucosidase. Looking at the relative ratios of mannose to galactose, JABBA stationary phase caps contain half the galactose of JABBA logarithmic caps and wild-type *L. donovani*. While this would be an abnormal statistic in any normal strain, this decrease does lead to one hypothesis on the formation of JABBA LPG. In stationary phase, the parasite is continuing to try to elongate its LPG; it does not have a stopping
point. In other words, the eMPT is essentially unregulated, the possibility exists that the glucose is the only regulator, as modification to the terminal-galactose molecule has been shown to prevent action of the eMPT (Routier, F.H., et al. 2000).

Based on our defined points of logarithmic and stationary phase, the glucosyl residue is lost at approximately $3.0 \times 10^6$ parasite/ml, where we start to see the initial increase in size. One might believe the glucose is the only reason we see any apparent regulation of the eMPT in JABBA. The 47 repeating units is likely to be a stopping point where the parasite is physically unable to continue to elongate any further at this point. There is also a definite possibility that the parasite can not move LPG beyond that length to the surface. We know formation of the LPG takes place in the golgi, and moving large LPG to the surface could cause transport problems.

There are quite a number of questions that still exist in our understanding of metacyclogenesis. Recently an algorithm was formulated that would allow to show there is a notable difference in morphology of the parasite once it has undergone metacyclogenesis (Berg, M., et al. 2013). While not shown here, there is a notable visual difference between logarithmic phase *L. donovani* and logarithmic phase JABBA parasites. The overall behavior of the parasites more closely resembles those of stationary *L. donovani* parasites; they have a greater tendency to form rosettes or stick together like stationary *L. donovani*. This behavior, combined with the structure of JABBA’s LPG, suggests further analyses of JABBA could lead to an understanding of the critical regulatory events that are required for Leishmania species to undergo metacyclogenesis.
Table 3.1. Average number of repeating units per molecule of LPG in *L. donovani* (LD4) and JABBA parasites based on the growth-phase. Logarithmic phase samples n>8, stationary phase samples n=3 separate LPG preparations for analysis.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Growth Phase</th>
<th>Average Repeats</th>
<th>Parasite</th>
<th>Growth Phase</th>
<th>Average Repeats</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD4</td>
<td>Logarithmic</td>
<td>15</td>
<td>JABBA</td>
<td>Logarithmic</td>
<td>30</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>Stationary</td>
<td>30</td>
<td></td>
<td>Stationary</td>
<td>47</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2: The ratio of mannose to glucose to galactose of cap samples based on capillary electrophoresis area calculations. The calculations were normalized to mannose. Note the decrease in galactose found in stationary JABBA LPG caps. Data was averaged from three separate runs of each sample.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Mannose</th>
<th>Glucose</th>
<th>Galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD4</td>
<td>1</td>
<td>0</td>
<td>.7</td>
</tr>
<tr>
<td>Jabba Logarithmic</td>
<td>1</td>
<td>.8</td>
<td>.8</td>
</tr>
<tr>
<td>Jabba Stationary</td>
<td>1</td>
<td>0</td>
<td>.42</td>
</tr>
</tbody>
</table>
Table 3.3. **Cap isoforms and relative percentages found on JABBA LPG.** Calculations were based on area percentages from capillary electrophoresis and permethylation experiments

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Cap Structures</th>
<th>Percentage of total sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disaccharides</td>
<td>Gal((\beta 1,4))Man</td>
<td>50-80%</td>
</tr>
<tr>
<td></td>
<td>Man((\alpha 1,2))Man</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glc((\beta 1,2))Man</td>
<td></td>
</tr>
<tr>
<td>Trisaccharides</td>
<td>Gal((\beta 1,4)[Man(\alpha 1,2)])Man</td>
<td>10-46%</td>
</tr>
<tr>
<td></td>
<td>Gal((\beta 1,4)[Glc(\beta 1,2)])Man</td>
<td></td>
</tr>
<tr>
<td>Tetrasaccharide</td>
<td>Gal((\beta 1,4)[Glc(\beta 1,2)Man(\alpha 1,2)])Man</td>
<td>0-4%</td>
</tr>
</tbody>
</table>
Table 3.4: Thin layer chromatography lipid migration distances. Distances were measured from the initial spot to the start of the final spot upon completion.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Distance Migrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylinositol</td>
<td>9.5cm</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>10.5cm</td>
</tr>
<tr>
<td>LD4 LPG Lipid</td>
<td>8.5cm</td>
</tr>
<tr>
<td>Jabba LPG Lipid</td>
<td>8.5cm</td>
</tr>
</tbody>
</table>
Figure 3.1. Diagram of LPG from LD4 (wild-type) and JABBA LPG. The structure of the lipid anchor, is 1-O-alkyl-2-phosphatidylinositol. The percentages of the varying cap isoforms in JABBA are found in Table 3.3.
Figure 3.2. Representative SDS-PAGE of purified LPG from logarithmic phase and stationary phase wild-type LD4 and JABBA parasites. Following electrophoresis the gel was stained with Stains-all solution. Each panel is from a different gel, but representative of the migratory differences.
Figure 3.3. Fluorophore assisted carbohydrate electrophoresis of the dephosphorylated repeat units of LPG. The band from JABBA co-migrating with the Gal(β1,4)Man disaccharide from wild-type LD4 repeat unit was determined to be identical based on β-galactosidase digestion and the linkage was confirmed by methylated alditol acetates by GC-MS.
Figure 3.4. Monosaccharide capillary electrophoresis of dephosphorylated, strong acid treated repeat units from LD4 and JABBA. Mannose and galactose are found in equal proportions in both species, indicative of being found in equal percentage in both repeating units. Peaks at 11 and 13 min (unlabeled) are artifacts from the core and glucose.
Figure 3.5 Representative chromatogram from monosaccharide capillary electrophoresis for repeating unit quantification. The critical LPG derived peaks are labeled. Peaks at 11 and 13 min are a core associated artifact and exogenous glucose.
Figure 3.6. Fluorophore assisted carbohydrate electrophoresis of LD4 and JABBA caps collected from the logarithmic stage. LD4 has the standard monosaccharide, disaccharide, and trisaccharide, while JABBA has the presence of an additional trisaccharide and tetrasaccharide. The gel front is present just under the G1 marker in all 3 lanes.
Figure 3.7 Oligosaccharide capillary electrophoresis of caps collected from logarithmic JABBA LPG. Disaccharides elute at 7 min, trisaccharides elute at 8 min, and tetrasaccharides elute at approximately 9 min. These bulk samples were treated with β-galactosidase, β-glucosidase, and α-mannosidase to determine their relative structures. Gal-Man, Man-Man, and Gal-(Man)Man elution times were similar in LD4. Injection pressure was 5 psi, 5sec to allow for better visualization of the tetrasaccharide.
Figure 3.8. Monosaccharide capillary electrophoresis of strong-acid hydrolyzed caps collected from logarithmic and stationary phase JABBA LPG. There is an absence of glucose in stationary phase JABBA LPG caps and wild-type LD4 (not shown), compared to logarithmic phase JABBA LPG caps. Ratios of each sugar are found in Table 3.2.
Figure 3.9. GC-MS of partially methylated alditol acetates from a bulk sample of JABBA caps from LPG. GC retention times were determined using Gal(β1,4)Man, Man, Gal, and Cellobiose standards. 2-Man and 2,4-Man were identified matching wild-type GC retention times and MS spectra. T-Gal: terminal galactose, T-Man: terminal mannose, T-Glc: terminal glucose, 2-Man: 2-linked mannose, 4-Man: 4-linked mannose, 2,4-Man: 2,4-linked mannose.
Chapter IV

Characterization of the initiating mannosylphosphoryltransferase, the elongating mannosylphosphoryltransferase, and β-glucosyltransferase activities of JABBA and L. donovani

1. Introduction

Many enzymes are responsible for the synthesis of LPG in the parasite. For the synthesis of the repeating units, an initiating mannosylphosphoryltransferase (iMPT) is required for addition of the mannose-phosphate (Man-P) to the core (Mengeling, B.J., et al. 1997b), a galactosyltransferase which adds the galactose to complete the first repeating unit (Carver, M.A. and Turco, S.J. 1991; Mengeling, B.J., et al. 1997a). An elongating mannosylphosphoryltransferase (eMPT) adds the next Man-P group, and the galactosyltransferase adds the next galactosyl unit to complete the second repeating unit (Descoteaux, A., et al. 1998).

In some Leishmania species and strains, side chains are added to the repeating units at either the C-3 of the galactose or the C-2 of the mannose residues. L. donovani Sudanese isolates do not have any side chains on the repeating units, but do have an additional α-mannose on the C-2 of the cap’s mannosyl unit (Greis, K.D., et al. 1992; Thomas, J.R., et al. 1992). L. donovani Indian isolate has a β-glucosyltransferase that adds β-glucose to the C-3 of the galactose, and it is subsequently downregulated as the parasite progresses from logarithmic to stationary phase (Mahoney, A.B., et al. 1999; Mahoney, A.B. and Turco, S.J. 1999). Based on the structural changes in JABBA LPG, one would expect an increase in the eMPT activity and an increase in β-glucosyltransferase activity. The iMPT, eMPT, and β-glucosyltransferase are explored here as potential causes for the modifications found on the LPG of JABBA, and are compared to both LD4 (Sudanese L. donovani, wild-type) and Mongi (Indian L. donovani) for a greater understanding of JABBA in the context of other L. donovani strains.
2. Results

The iMPT is required to add the first Man-P to the galactose of the core, with the rate never expecting to vary, with the exception of the amastigote stage (Mengeling, B.J., et al. 1997b). Two hypotheses exist to the nature of the eMPT; either there are two that are stage specific or one that is regulated in an unknown manner (Routier, F.H., et al. 2000). *L. donovani* Indian isolate is used as a control for assaying the β-glucosyltransferase activity. The assays for both the iMPT and eMPT with product characterization are well documented (Carver, M.A. and Turco, S.J. 1992; Descoteaux, A., et al. 1998; Mengeling, B.J., et al. 1997b), so the only product characterization included here is that for the β-glucosyltransferase. Schematics of reactions produced in each assay are found in Figure 4.1

A. Analysis of the iMPT and eMPT

To account for the increased number of repeating units, there are two critical enzymes to consider that could cause this increase; the iMPT applying the first Man-P, and the eMPT applying every subsequent Man-P (Descoteaux, A., et al. 1998). Figure 4.2 shows both iMPT and eMPT activity. The iMPT activity of LD4 (Sudanese *L. donovani*) had a rate of 250 pMol mannose-phosphate transferred and JABBA had a rate of 375 pMol mannose-phosphate transferred per milligram protein/h; there was no statistical difference between the two. The activity of Mongi was no different from LD4 and JABBA. The eMPT activity was measured using JEDI LPG. LD4 (Sudanese *L. donovani*) had 35 pMol mannose-phosphate transferred and JABBA had 70 pMol mannose-phosphate transferred per milligram protein/h. The activity of the eMPT in Mongi had not previously been measured, and was shown to be very close to that of JABBA. Only 3 counts had been taken, so no statistics were calculated using Mongi. While not shown here, the galactosyltransferase responsible for adding the galactose to the repeating units was initially surveyed, and no difference was observed.

B. Analysis of the β-Glucosyltransferase

The β-glucosyltransferase assay was adapted from the publication on *L donovani* Indian isolate (Mahoney, A.B. and Turco, S.J. 1999) and measures the last reaction found in Figure 4.1. JABBA showed an activity rate of 9 pMol glucose transferred per
milligram protein/h, compared to 4 for LD4 (L. donovani Sudanese isolate) (Figure 4.3). The Mongi (L. donovani India) control matched published data. The β-glucosyltransferase is well characterized in Mongi (L. donovani Indian isolate), but has never been analyzed in LD4 (L. donovani Sudanese isolate) due to issues with the methodologies available at that time. To identify the origin of the β-glucosyltransferase in JABBA, it was critical to determine if the apparent activity in vitro in LD4 (Sudanese L. donovani) was from the β-glucosyltransferase or was a strict artifact.

C. Partial product characterization of the β-glucosyltransferase assay

Product characterization for the glucosyltransferase from L. donovani Sudan and JABBA was critical since there is no β-glucosyl chains on the LPG of the Sudanese isolate of L. donovani; it had been assumed that the activity from Sudanese L. donovani was an artifact (Mahoney, A.B. and Turco, S.J. 1999). Descending paper chromatography did not allow for product characterization, as the total radioactivity produced was 1/10th the amount required, so more sensitive methods were adapted. If measuring β-glucosyltransferase activity, a Gal-(Glc)Man or Glc-Gal-Man trisaccharide would be seen after mild acid hydrolysis alone or with dephosphorylation, if the activity was not present only a Gal-Man disaccharide would be evident. Capillary electrophoresis allowed for separation of any trisaccharide product present in the sample from the overwhelming amount of disaccharide repeating units. A number of conditions had to be met to determine if any present trisaccharide came from LPG. A trisaccharide was first found in both L. donovani and JABBA after mild acid hydrolysis and dephosphorylation using alkaline phosphatase. This suggested the trisaccharide was coming from the repeating units. Before continuing to analyze the trisaccharide, a number of conditions were tested that found the trisaccharide to be UDP-Glucose dependent, exogenous LPG dependent, and mild acid hydrolysis dependent (Data not shown). A small trisaccharide was found after mild acid treatment alone, suggesting the trisaccharide was also coming from the caps in addition to the repeats. From this it was concluded that the β-glucosyltransferase was acting on both the caps and repeating units in vitro in L. donovani and JABBA.

While L. donovani having in vitro β-glucosyltransferase activity explains the presence of a β-glucose present on the cap of JABBA LPG, it was important to determine
if the galactosyl or the mannosyl unit was the acceptor for this β-glucose residue in Sudanese *L. donovani*. The trisaccharide was subjected to both β-galactosidase and β-glucosidase treatment (Figure 4.4) and run on capillary electrophoresis for visualization to address this question. In both *L. donovani* and JABBA, the trisaccharide was sensitive to both treatments: β-glucosidase sensitivity indicates the trisaccharide is formed by the addition of a β-glucose, β-galactosidase sensitivity indicates a product of Gal-(Glc)Man instead of Glc-Gal-Man. This was expected in JABBA due to the mannose as the attachment point for the β-glucose in the caps, but unexpected in *L. donovani* Sudanese isolate due to the β-glucosyltransferase adding to the galactose in *L. donovani* Indian isolate.

3. Discussion

The techniques to assay and solubilize LPG synthesizing enzymes have existed for years (Carver, M.A. and Turco, S.J. 1991; Carver, M.A. and Turco, S.J. 1992; Descoteaux, A., *et al.* 1998; Mengeling, B.J., *et al.* 1997b) but two of the critical enzymes have only recently been identified. The LPG4 family consists of 3 enzymes, LPG4A, LPG4B, and LPG4C which have been identified as a proteophosphoglycan-specific eMPT, the iMPT, and a LPG specific eMPT, respectively (Beverley *et al.*, unpublished observations). The eMPTs are related enough that functional rescue of both *L. major* knockouts of LPG4C, and the *L. donovani* mutant JEDI can be accomplished by complementation with LPG4A. With JABBA being a larger LPG mutant, it would be difficult to employ traditional techniques to identify the causal mutation.

Regulation of these enzymes is poorly understood, though there is an understanding that a number of these enzymes are stage specific; particularly those for side-chains. The glucosyltransferase(s) in the Indian isolate of *L. donovani* (Mahoney, A.B. and Turco, S.J. 1999) responsible for the addition of β-glucose side chains show an 80% decrease in activity in the progression from procyclic to metacyclic promastigotes. The arabinosyltransferase responsible for the capping of the side chains in *L. major* functions primarily in meta cyclic phase. There are two hypotheses as to the nature of the regulation of eMPT activity, either one enzyme that is regulated differentially depending
on the stage, or two enzymes, one that functions during each phase (Routier, F.H., et al.
2000). It has been proposed that a single eMPT should eventually be able to stop
elongating, as it has less affinity for longer structures. The changes in JABBA suggest
that not only is this not the case, there is a similar mode of regulation between the eMPT
and the β-glucosyltransferase, as both are equally upregulated in comparison to wild-
type, but the iMPT is regulated in a different manner.

JABBA did have a slight increase in iMPT compared to LD4 and Mongi, which
can be explained by the increase in eMPT activity. When LPG4A or LPG4C is knocked
out, there is a significant decrease in iMPT activity (Beverley et. al, unpublished data). If
these enzymes are working as a complex, or were to have a similar regulatory
mechanism, the increase in eMPT activity could correlate with an increase in iMPT
activity, even if there is less LPG on the cell.

JABBA had twice the eMPT activity and β-glucosyltransferase activity of wild-
type LD4, in line with initial hypotheses. Surprisingly, Mongi eMPT activity more
resembled that of JABBA than LD4. This would suggest that the eMPT and the β-
glucosyltransferase were regulated in the same fashion on some level: whether it be
transcriptional or translational. One could potentially rule out regulatory factors as none
required for activation in Mongi (L. donovani India) (Mahoney, A.B. and Turco, S.J.
1999). As Leishmania genetic regulation is similar to that of bacteria (De Gaudenzi, J.G.,
et al. 2011), one would believe it is more likely due to post-translational regulation.

The β-glucosyltransferase product characterization was critical to understanding
the addition of the glucosyl residue to the caps in JABBA, and identifying potential
mutations to cause this addition. While the product was partially characterized, it should
be noted normal oligosaccharide CE has an injection rate of 0.4 psi for 4 sec, but for
analysis of the trisaccharide 5 psi for 5 sec was required due to such low activity in LD4.

The β-glucosyltransferase in LD4 (L. donovani Sudan) and subsequently JABBA
(Figure 4.1, bottom reaction) differs in acceptor specificity from the β-
glucosyltransferase in Mongi (L. donovani India) (Figure 4.1, top reaction), but based on
total cap data, appears to be stage-specific similar to the β-glucosyltransferase from
Mongi (L. donovani India). This suggests the two enzymes are related, and the change in
acceptor is likely to be due to drift from the lack of in vivo use in LD4 (L. donovani
Sudan). As other *L. donvani* complex species such as *L. infantum* maintain a β-glucosyl residue linked to the galactosyl unit, it is unlikely for the shift to have changed from the mannosyl unit as the acceptor to the galactosyl unit, but through lack of use, the gene drifted. There is also a possibility another strain of *L. donovani* exists with a highly active β-glucosyltransferase attaching a glucosyl residue to the mannosyl residue.

There is a possibility that the β-glucosyltransferase only adds one glucose per 1000 LPG molecules in LD4 *in vivo*, where it would never be seen from structural carbohydrate techniques, or acquisition of membranes actually results in activation. The only conclusive statement that can be made is that there is a β-glucosyltransferase in the genome of LD4 (*L. donovani* Sudan) and is transcribed and translated into protein. Based on the nature of its position in JABBA, it is likely to have a greater preference for terminal repeating units and the increase in material created by the *in vitro* assay allowed for it to add a glucose to the mannosyl of repeating units.

The base levels of the enzyme are low, and are most likely regulated at the transcriptional or translational level, while the increase in both the glucosyltransferase and the eMPT in JABBA suggests they are regulated in the same fashion. As Mongi (*L. donovani* India) has an increase in the eMPT activity compared to LD4 (*L. donovani* Sudan), one could hypothesize that the mutation in JABBA is activating a key regulatory difference between the two strains, leading to LPG that in structure and biosynthesis is more closely related to Mongi (*L. donovani* India) than the parent LD4 (*L. donovani* Sudan). This could be a single regulatory difference, or changes at multiple regulatory points.
**iMPT Reaction**

\[ \text{GDP-Man} + \text{Stachyose} \rightarrow \text{Man-P-Stachyose} \]

\[ \text{Stachyose} = \text{Gal(\(\alpha1,6\))Gal(\(\alpha1,6\))Glc(\(\beta1,2\))Fru} \]

**eMPT Reaction**

\[ \text{GDP-Man} + \text{GaL-Man-P-Core-PI} \rightarrow \text{Man-P-GaL-Man-P-Core-PI} \]

**\(\beta\)-Glucosyltransferase Reaction**

\[ \text{UDP-Glc} + \text{Cap-[GaL-Man-P]}_{15}-\text{Core-PI} \rightarrow \text{Cap-[GaL-Man-P]}_{15}-\text{Core-PI} \]

---

**Figure 4.1. Schematic of the reactions measured in individual enzymatic assays comparing JABBA and LD4.** The Gal(\(\alpha1,6\))Gal linkage in stachyose makes it an appropriate substrate for the iMPT activity (Mengeling, B.J., et al. 1997b). The \(\beta\)-GlcT assay was developed for Glc(\(\beta1,3\))Gal linkage in the Indian isolate of \(L.\) \(donovani\) (Mahoney, A.B. and Turco, S.J. 1999), but also allows for measurement of the Glc(\(\beta1,2\))Man linkage seen in JABBA LPG caps.
Figure 4.2  Comparative iMPT and eMPT activities in microsomal preparations of wild-type LD4, JABBA, and Mongi. Microsomal preparations from wild-type LD4, JABBA, and Mongi were assayed for iMPT and eMPT activities as described in Materials and Methods. Top Panel, iMPT activity, N=3; Bottom Panel, eMPT activity, N=8 microsomal preparations with the exception of Mongi, N=3. Error bars represent standard deviation.
Figure 4.3 Comparative $\beta$-glucosyltransferase activities in microsomal preparations of wild-type LD4, JABBA, and Mongi. Microsomal preparations from wild-type LD4, JABBA, and Mongi were assayed for $\beta$-glucosyltransferase activities as described in Materials and Methods. N= 8 microsomal preparations for LD4 and JABBA, 3 for Mongi. Statistics were not calculated for Mongi. Error bars represent standard deviation.
Figure 4.4. Partial characterization of the product produced by JABBA β-glucosyltransferase assays using whole LPG. Disaccharides run at 7 minutes elution time; trisaccharides run at 8 minutes. Top Panel: Mild Acid Hydrolyzed, dephosphorylated product. * Gal-(Glc)Man, **Gal-Man. Middle Panel: β-glucosidase treated after mild acid hydrolysis and dephosphorylation. ** Gal-Man. Lower Panel: β-galactosidase treated after mild acid hydrolysis and dephosphorylation. *** Glc-Man. LD4 profiles (not shown) look the same.
Chapter V
Determining the cause of Ricin Resistance

1. Introduction

Ricin, specifically RCA120, has been used to identify LPG-null Leishmania mutants (Descoteaux, A., et al. 2002; Descoteaux, A., et al. 1998; King, D.L. and Turco, S.J. 1988; Ma, D., et al. 1997) for the identification of critical LPG biosynthesis pathways and genes. Prior to JABBA, the mutants presented were all LPG null, expressing truncated LPG that often did not terminate in a galactose molecule. As RCA 120 is highly reactive with terminal β-galactosyl units, more so than other terminal β-galactosyl binding lectins like peanut agglutinin, these truncated LPG structures clearly explain the cause of ricin-resistance (Andrade, A.F. and Saraiva, E.M. 1999). JABBA is the first mutant with large LPG published to date, leaving the resistance to ricin a mystery.

There are two options to explain ricin resistance in JABBA parasites, either the unique cap structures containing a β-glucosyl residue could block the ability for ricin to bind to the LPG even if a galactose is present, or the number of repeating units blocks access to the caps, causing the caps to become “cryptic”. The “cryptic caps” hypothesis is supported by both nuclear magnetic resonance imaging showing LPG to have a helical structure and from assays showing that metacyclogenesis and the elongation of LPG blocks access of lectins to the caps (Homans, S.W., et al. 1992; Sacks, D.L., et al. 1995) allowing for release from the sand-fly gut. These two hypotheses are modeled in Figure 5.1. Understanding why JABBA is ricin-resistant will help to explain the critical events required for release of the parasite from the sand-fly gut to continue its life-cycle.

2. Results

Peanut Agglutinin (PNA), Concanavalin A (ConA), and Ricin (RCA 120) are considered to have equal abilities to agglutinate logarithmic parasites when used at appropriate concentrations (Andrade, A.F. and Saraiva, E.M. 1999). Selection was originally performed with multiple rounds of ricin at 100 µg/ml (King, D.L. and Turco, S.J. 1988). As mentioned previously, L. donovani produces roughly 100 pMol of LPG / 10^7 parasites, while JABBA produces only 30.
A. Lectin Resistance

Traditionally, lectins have been used to determine if the LPG cap is accessible (Sacks, D.L., et al. 1995). Logarithmic *L. donovani* has been shown to be agglutinated by both ConA, being able to bind the Man(α1,2)Man found on the terminus of some caps, and PNA which recognizes the terminal galactose found on a number of caps. ConA, PNA, and RCA 120 were all tested. Previously published data shows approximately 10 µg of ricin, or 100 µg of ConA or PNA, is sufficient to agglutinate *L. donovani* (Andrade, A.F. and Saraiva, E.M. 1999). The previously mentioned critical density designations for logarithmic and stationary phase were maintained throughout the entire experiment.

When exposed to 100 µg/ml of ConA, 7% of logarithmic phase *L. donovani* parasites and 25% of logarithmic phase JABBA parasites showed resistance. In stationary phase, 14% of *L. donovani* and 30.73% of JABBA parasites were resistant to ConA (Figure 5.2, top panel). The *L. donovani* resistant percentages are similar to those published (Sacks, D.L., et al. 1995). The difference between *L. donovani* and JABBA in both logarithmic and stationary phases was found to be significant, but the difference between logarithmic and stationary phases of JABBA was not.

After treatment with 100 µg/ml of PNA only 1% of logarithmic and 6.27% of stationary *L. donovani* parasites showed resistance, similar to published data. Of treated JABBA parasites, 8.5% showed resistance in logarithmic phase, and 16% showed resistance in stationary phase (Figure 5.2, bottom panel). The difference in resistance values between *L. donovani* and JABBA in both logarithmic and stationary phases was found to be significant, as was the difference between logarithmic and stationary JABBA. Additionally, both *L. donovani* and JABBA parasites were grown in the presence of 100 µg/ml of PNA and the LPG was extracted. The total amount of LPG extracted from both parasites doubled, showing selection with PNA in both parasites increases the total apparent amount of surface LPG.

Selection experiments were repeated with 10 µg/ml RCA 120, which is considered to have similar agglutination properties as PNA at 100 µg/ml concentration. Approximately 2.4% of logarithmic *L. donovani* parasites showed ricin resistance, as did about 3.5% of stationary *L. donovani*. Approximately 7.05% of logarithmic JABBA parasites showed ricin resistance, as did 15.5% of stationary JABBA parasites (Figure
A significant difference between *L. donovani* and JABBA was found in both phases, as well as between logarithmic and stationary JABBA with a $<0.005$ p-value.

### B. Galactose Oxidase Accessibility

In previous publications, the accessibility of terminal galactose to the caps of LPG on whole cells to galactose oxidase has been critical in making the observation on the accessibility of the caps to molecules (Sacks, D.L., *et al.* 1995). Unfortunately, measuring this required reduction with tritiated sodium borohydride, and the reaction’s byproduct: tritiated hydrogen gas. We attempted to make use of the sensitivity of capillary electrophoresis to avoid use of tritiated sodium borohydride. By comparing the reduction of total galactose in caps treated with galactose oxidase and comparing it to caps from untreated parasites, one could calculate the relative amount of galactose available to galactose oxidase. Greater than 85% of galactose on logarithmic phase *L. donovani* is accessible to galactose oxidase, while only 35% is available on stationary phase parasites. This 3-fold change is similar to the change in radioactivity between *L. donovani* logarithmic phase and stationary phase in previously published data. In logarithmic phase JABBA had approximately 31% terminal galactose to galactose oxidase, in stationary phase only 8% of its terminal galactose was accessible (Table 5.1).

### 3. Discussion

Previously, ricin-resistance mutants were characterized if they were defective in LPG synthesis. To date the four mutants characterized have been R2D2, C3PO, OB1, and JEDI (Descoteaux, A., *et al.* 2002; Descoteaux, A., *et al.* 1998; King, D.L. and Turco, S.J. 1988; Ma, D., *et al.* 1997) The LPG of two of these mutants did not possess a terminal galactose, and only JEDI’s LPG contained a full repeating unit. Despite possessing a terminal galactose, JEDI demonstrated ricin-resistance, leaving the exact mechanisms of ricin selection unknown. Of the entire set of recovered mutants, two were over-expressing mutants: JABBA and HUTT. HUTT was abandoned due to abnormally low levels of LPG synthesis. JABBA is the first large LPG mutant characterized, allowing for the investigation of its specific mechanism of ricin resistance.

The most significant lectin data was found using peanut agglutinin and ricin, both of which recognize terminal galactose. In both cases, there is a significant difference in
resistance between *L. donovani* and JABBA at both logarithmic and stationary phases, but also between the logarithmic and stationary JABBA parasites. These differences suggest that the number of repeating units was the greatest influence on ricin resistance. Previous data has shown that LPG is helical in nature (Homans, S.W., *et al.* 1992), and that the only major LPG structural change in *L. donovani* from procyclic phase to metacyclic phase is the number of repeating units. This change in LPG length is potentially responsible for the cap becoming “cryptic” or otherwise inaccessible to lectins (Sacks, D.L., *et al.* 1995). This is further supported by the stark increase in resistance of JABBA from logarithmic to stationary phases. The presence of a significant difference in resistance between JABBA logarithmic and *L. donovani* stationary parasites suggests that the addition of glucose to the caps confers some additional resistance to terminal galactose binding lectins.

While only 10 µg/ml of ricin was used compared to the original selection levels of 100 µg/ml, the densities for mutagenesis suggest the parasites were selected using what we now define as stationary parasites. Original work states that parasites were selected when grown to densities of 2.0 x 10⁶ to 2.0 x 10⁷ parasites/mL (Iovannisci, D.M. and Ullman, B. 1984; King, D.L. and Turco, S.J. 1988), and JABBA shows the repeating unit increase and loss of cap glucosyl residues starting at 3.0 x 10⁶ parasites/mL. As originally selected the parasites had an average repeating unit length of 42, substantially higher than the logarithmic phase repeating unit length of 30.

ConA most often recognizes Man(α1,2)Man, a structure found in *L. donovani* caps. The Man(α1,2)Man cap is not as dominant an isoform in JABBA cap structures compared to its prominence in *L. donovani*, thus explaining JABBA’s phase independent resistance to ConA.

Total LPG collected nearly doubled in both *L. donovani* and JABBA when exposed to peanut agglutinin. *L. donovani* binds to the insect gut via galectins: lectins that recognize galactose (Kamhawi, S., *et al.* 2004). If that is the case, selection via terminal galactose lectins during stationary phase would identify parasites which successfully release from the insect gut. An increase in the number of molecules of LPG on the cell surface would only be relevant to the survival of the parasite once it has entered the mammalian bloodstream (Franke, E.D., *et al.* 1985; Green, P.J., *et al.* 1994). LPG protects against
complementation and other factors that would cause inevitable parasite death if they reached the cell surface.

Using galactose oxidase, the relative percentage of surface galactose accessible can be calculated in order to determine just how cryptic the cap is. Capillary electrophoresis was used in lieu of radioactivity. In theory, capillary electrophoresis could have quantified total moles of product, but this proved difficult as the galacto-hexodialdose product does not necessarily label as a single peak; the labeling scheme for capillary electrophoresis relies the monosaccharides having only one reducing end, it has two. Due to this fact, direct analysis of the dialdose product was not possible, so the quantification of accessible galactose was done against untreated caps and normalizing to the total amount of mannose in the sample. This did not appear to affect the data for LD4 (Sudanese *L. donovani*), but decrease in galactose in stationary JABBA caps could’ve helped skew the data.

Two hypotheses can be made to put all of the ricin and other lectin resistance data in perspective in regards to the life cycle of the parasite. At 15 repeating unit LPG, the procyclic promastigote binds to lectins in the sand fly gut, we can surmise from the resistance data the parasite has the best chance of survival with a greater number of LPG molecules on the cell surface and with greater than 30 repeating units on LPG molecules. It remains unclear exactly why parasites resistant to PNA have an apparent increased amount of LPG on the cell surface, but one possibility is that parasites with fewer molecules agglutinate as the lectins reach the layers of the glycocalyx closer to the cell surface and binding to terminating galactosyl residues present. These differences could be investigated using LPG collected from parasites using the ability for red blood cells to be opsonized, or how parasites survive complement if they’ve been exposed to PNA.
Table 5.1. The percentage galactose available to galactose oxidase based on capillary electrophoresis quantification. Averages were determined using seven experiments.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Growth Stage</th>
<th>Galactosyl Residues oxidized (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD4</td>
<td>Logarithmic</td>
<td>&gt;85</td>
</tr>
<tr>
<td></td>
<td>Stationary</td>
<td>35</td>
</tr>
<tr>
<td>JABBA</td>
<td>Logarithmic</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Stationary</td>
<td>8</td>
</tr>
</tbody>
</table>
Figure 5.1: Two potential models for ricin-resistance of JABBA parasites. A. This model relies on the use of abnormal cap structures in logarithmic phase to block binding of ricin to terminal $\beta$-galactose. B. The entire cap becomes inaccessible due to curvature of the LPG due to the number of repeats. This model could result in increased binding in logarithmic or stationary phase parasites.
Figure 5.2: Agglutination profiles of logarithmic and stationary parasites exposed to Concanavalin A and Peanut Agglutinin. Top panel: Logarithmic and stationary parasites of both LD4 and JABBA were exposed to Concanavalin A for 30 minutes, and the free cells were counted. The p-values between parasites in the same phase are <.01. Bottom panel: Logarithmic and stationary parasites were exposed to Peanut Agglutinin for 30 minutes and free cells were counted. The p-values between parasites in the same phase were <.01. Minimum of 4 separate counts was used for each average. Error bars are representative of standard deviation.
Figure 5.3: Agglutination profiles of logarithmic and stationary parasites exposed to Ricin. Parasites grown in logarithmic and stationary phase were exposed to ricin (RCA-120) at 10 µg/ml concentration for 30 min. Free cells were counted. The p-value comparing parasites of the same phase was <.005. Averages were a minimum of 4 separate runs. Error bars are representative of standard deviation. Stationary phase JABBA was most likely the structure found on the parasites originally selected after mutagenesis.
Chapter VI
Conclusions and Future Directions

The creation of \textit{lpg-} mutants in our lab using mutagenesis with N-methyl-N'-nitroso-N-nitroguanidine and selection by ricin agglutinin has resulted in the direct identification of the genes LPG1, LPG2, LPG3, and LPG4A (Descoteaux, A., \textit{et al.} 2002; Descoteaux, A., \textit{et al.} 1998; Hong, K., \textit{et al.} 2000; King, D.L. and Turco, S.J. 1988; Ma, D., \textit{et al.} 1997; Ryan, K.A., \textit{et al.} 1993b) and aided in the identification of LPG4B and LPG4C. LPG4A has a primary function in the elongation of glycan chains on proteophosphoglycans, while the other five genes are directly involved in the synthesis of LPG. While selection with ricin agglutinin was designed to identify parasites with truncated LPG; the identification of hyper-phosphoglycosylated mutants from this project was completely unexpected. The goals of this project were to determine the structure of JABBA’s LPG, determine the cause of its ricin resistance, and identify any changes in glycosyltransferases involved in LPG biosynthesis.

JABBA has a unique larger LPG structure; in all phases, JABBA LPG has a higher molecular weight than wild-type. The repeating unit structure is the same as wild-type, being identified as 6Gal(β1,4)Man(α1)PO$_4$ (Figures 3.3 and 3.4), but JABBA LPG has an increase in the number of repeating units compared to wild-type. Logarithmic phase JABBA LPG has 30 repeating units, stationary phase LPG has 47, in comparison to wild-type’s 15 repeating units in logarithmic phase and 30 in stationary phase (Table 3.1). Logarithmic phase JABBA starts as “metacyclic length” LPG and continues to increase in size as it would during a normal life cycle.

JABBA LPG also differs in its cap isoforms. JABBA has a total of six cap isoforms, the three dominant isoforms found in wild-type and three containing a Glc(β1,2)Man linkage (Table 3.3). The unique isoforms are Glc(β1,2)Man, Gal(β1,4)[Glc(β1,2)]Man, and Gal(β1,4)[Glc(β1,2)Man(α1,2)]Man. The Glc(β1,2)Man linkage is not found in any other parasite. The β-glucose is stage specific, and the glucose-containing cap isoforms are downregulated as the parasite progresses from
logarithmic to stationary phase, starting at a relative density in culture of 3.0 x 10^6 parasites/ml (Figure 3.8).

Differences in the eMPT and β-glucosyltransferase activity are responsible for the structural differences in JABBA LPG (Figures 4.1 and 4.2), as the activity of both of these enzymes in JABBA is double that in wild-type. No statistically significant difference in the iMPT activity of JABBA and wild-type was found, but a slight increase in mean activity is evident. Analyzing the product of LD4 (wild-type Sudanese *L. donovani*) β-glucosyltransferase assay helped explain how a mutagenized parasite from a species that does not have an *in vivo* active β-glucosyltransferase seemingly had an active one.

The β-glucosyltransferase product from LD4 (wild-type Sudanese *L. donovani*) and JABBA characterized as a βGal-(βGlc)-Man trisaccharide, similar to the trisaccharide found on JABBA caps. This activity in both the parent and JABBA differed from Mongi (*L. donovani* Indian isolate) that has a β-glucosyltransferase which adds to the galactose of repeating units and cap, and explains that the enzyme, which only active *in vitro* in the wild-type, when upregulated would explain the unique cap structures.

Results of agglutination with peanut agglutinin, ricin agglutinin, and concanavalin A, along with the reduced accessibility of terminal galactose to galactose oxidase, suggests that the primary mechanism for resistance comes from the number of repeating units causing the caps to become cryptic, not the unique cap structures. Stationary parasites, which do not contain the unique cap isoforms, showed the greatest resistance to agglutination by lectins and the least accessibility of terminal galactose to galactose oxidase. The structure of the LPG found at this density was most likely selected after mutagenesis based on the densities the parasites at which ricin selection occurred.

The initial structural and biosynthetic analysis of JABBA LPG allows for a few important conclusions to be drawn. The significant increase in unagglutinated parasites after exposure to ricin or peanut agglutinin, along with a decrease in the amount of galactose available to galactose oxidase between logarithmic and stationary phase JABBA parasites, shows that every repeating unit over 30 increases the likelihood that the cap is going to become “cryptic” and inaccessible to lectins. Those parasites resistant to lectins have an increased amount of LPG collected from the cell surface, even in wild-
type, while the structure of LPG is not overtly changed. The cause is unknown, but a denser glycocalyx would provide greater protection as the parasite attempts to infect a mammalian host. Lastly, LD4 (wild-type *L. donovani* Sudanese isolate) contains a \( \beta \)-glucosyltransferase active *in vivo* that is regulated at some point post-translationally to inhibit its activities. This \( \beta \)-glucosyltransferase differs from Mongi (Indian isolate), as it uses mannose as the acceptor instead of galactose.

Further studies of JABBA are complicated compared to the original *lpg*-mutants as up regulation of enzymes does not allow for identification of the mutation in JABBA parasites using the traditional cosmid approach. A diagram of the ways JABBA can be studied is found in Figure 6.1. To rule out any transcriptional up-regulation in JABBA, quantitative PCR could be used to look at transcript levels of LPG4C, the eMPT. This may not be conclusive due to the polycistronic transcripts produced by *Leishmania* parasites. The best mechanism to further study JABBA would be to identify the putative \( \beta \)-glucosyltransferase from the *L. donovani* genome, and trace the difference from each strain and identify the differences. Putative \( \beta \)-glucosyltransferases could also be identified using bioinformatics data from other trypanosomes.

The other, simpler, question to tackle is if it is truly an upregulation resulting in an increased amount of enzyme found in JABBA, or if it is point mutations found in the eMPT. Data from the LPG4A, LPG4B, LPG4C project could allow for sequencing of LPG4C from JABBA to eliminate the possibility of a point mutation in LPG4C affecting the rate. Antibodies from LPG4A created from previous work could potentially be used to total the amount of all three enzymes and compare, but will not elucidate the two eMPTs and the iMPT. Little work has been done on the genetic regulation of *Leishmania* parasites, particularly involving metacyclogenesis, but JABBA has the potential to increase understanding.

JABBA provides a unique method for identifying how certain changes affect the parasite throughout its life cycle. JABBA parasites should be studied with both the logarithmic and stationary phases in the sand fly and mouse models of infectivity. In the sand fly, it should be identified if the parasite can bind at all to the sand fly gut and, if so, does the glucose containing cap structure affect binding. We saw some minor, but not significant evidence it had an effect on lectin binding, as in many cases the logarithmic
JABBA parasites were more resistant to lectins than stationary *L. donovani* parasites. Stationary phase parasites should also be checked for their ability to bind to the sand fly gut, but based on the lack of galactose oxidase sensitivity one would find it unlikely.

In the mouse model of infectivity, the required parasite burden to produce an infection should be measured in both logarithmic and stationary phase, with the overall immune response compared to that of normal metacyclic parasites. There is a distinct possibility in both phases that there is a stronger or altered immune response as either the glucose present in logarithmic phase caps or the increase in repeating units could change the innate immune response. Current data have shown the responses from TLR2 and TLR4 could vary based on these changes (Ibraim, I.C., *et al.* 2013). With these studies on infectivity and the immune response, the ability to trigger the complement cascade along with the response of other blood serum factors should be examined as it could be comparatively altered. JABBA provides a unique view that we have not had for studying the parasite and its life cycle, since many hypo-phosphoglycosylated LPG mutants exist but JABBA is the first hyper-phosphoglycosylated LPG mutant.
Figure 6.1: Flow-chart of ways to pursue future study of JABBA.
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Glycosylation mutants of *Leishmania donovani* (Phillips, Megan and Turco, S.J) American Society for Biochemistry and Molecular Biology, San Diego, California, April 2012 (poster presentation)

Abstracts:


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