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Is lipophosphoglycan a virulence factor? A surprising diversity between *Leishmania* species

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Lipophosphoglycan is a prominent member of the phosphoglycan-containing surface glycoconjugates of *Leishmania*. Genetic tests enable confirmation of its role in parasite virulence and permit discrimination between the roles of lipophosphoglycan and related glycoconjugates. When two different lipophosphoglycan biosynthetic genes from *Leishmania major* were knocked out, there was a clear loss of virulence in several steps of the infectious cycle but, with *Leishmania mexicana*, no effect on virulence was found. This points to an unexpected diversity in the reliance of *Leishmania* species on virulence factors, a finding underscored by recent studies showing great diversity in the host response to *Leishmania* species.

Leishmania parasites assemble an abundance of distinctive glycoconjugates that are expressed on their surface or secreted^{1–3} (Fig. 1). *Leishmania* produce a characteristic family of glycoconjugates containing phosphoglycan (PG) that includes membrane-bound lipophosphoglycan (LPG) and proteophosphoglycan (PPG), as well as secreted PG,

PPG and acid phosphatase (sAP). In addition, the surface of *Leishmania* contains an array of molecules attached to glycosylphosphatidylinositol (GPI) anchors (Fig. 1). These include free entities such as glycosylinositolphospholipids (GIPLs), LPG itself and proteins including the promastigote surface protease (also known as leishmanolysin, PSP or gp63).

The abundance, location and uniqueness of these *Leishmania* structures have led to the suggestion that these glycoconjugates, particularly LPG, have one or more important functions for the parasite throughout its life cycle^{2,4,5}. These include the procyclic and metacyclic promastigotes carried by the sand fly vector, and the amastigote stage, which resides within the phagolysosome of macrophages (Fig. 2). Most of the roles proposed for the glycoconjugates have been deduced using purified molecules and examining their effects on mouse macrophage function and interactions with the sand fly vector. From these *in vitro* experiments, there is substantial evidence that LPG is required for survival during the initial stage of establishment in the macrophage, when the parasite is most vulnerable. For example, LPG (or purified portions thereof) has been shown to bind macrophages and to inhibit macrophage signal transduction and cytokine production^{5,6}. However, the distribution of PG units and GPI anchors across a large number of different molecules (Fig. 1) raises questions about the correct assignment of functions for the individual glycoconjugates. Assays of LPG function using purified LPG might mimic effects that normally arise through other PGs or GPI-anchored molecules. This problem is compounded by the fact that LPG (or portions thereof) is often tested outside the biological milieu, at concentrations that might exceed that attained during parasite invasion.

The use of parasite mutants provides an opportunity to test the role of LPG in a biologically relevant setting. Examples of these include the

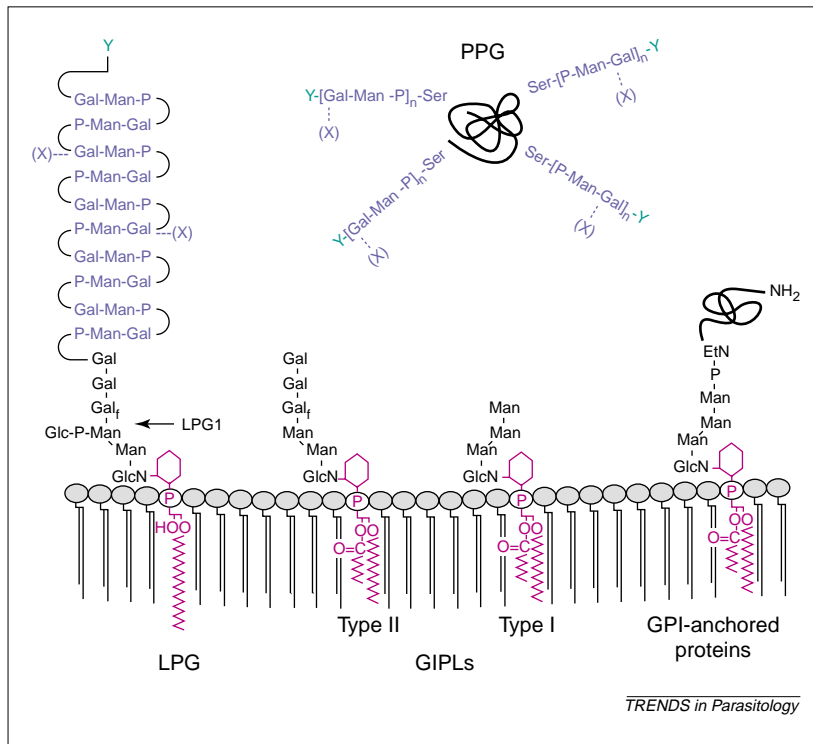


Fig. 1. The major surface and secreted glycoconjugates of *Leishmania*. The lipid anchors of these glycoconjugates (purple) are either 1-alkyl-2-acyl-phosphatidylinositol [for proteins anchored to glycosylphosphatidylinositol (GPI) and most glycosylinositolphospholipids (GIPLs)] or 1-alkyl-2-lyso-phosphatidylinositol [for lipophosphoglycan (LPG) and some GIPLs]. The structure and number of phosphoglycan repeating units (blue) in LPG and the secreted proteophosphoglycan (PPG) vary, and depend on the growth conditions, stage of differentiation and the species of *Leishmania*. Additional variant substituents to the phosphoglycans are shown as 'X' and 'Y' (green), such as the mannose-containing cap of LPG. Detailed information on these glycoconjugates is available elsewhere¹⁻³.

chemically induced *Leishmania donovani* Star-Wars mutants *R2D2*, *C3PO*, *OB1* and *JEDI*, which display alterations in LPG often accompanied by alterations in related glycoconjugates¹. By characterizing and properly selecting mutants, functional separation might be achieved by identifying LPG-specific genes.

To conclude that a particular gene or glycoconjugate (or any other molecule) is involved in virulence, the putative factor must satisfy 'molecular Koch's postulates'; that is, its absence in a cell must result in an attenuated/avirulent phenotype and its restoration at proper levels must restore virulence⁷. This is a key point affecting previous studies of *Leishmania*, as many mutants (including those mentioned above) were obtained after heavy mutagenesis and extensive laboratory culture, conditions that frequently give rise to a non-specific loss of virulence in *Leishmania* and other pathogens.

Episode I: LPG is a virulence factor for *Leishmania major* promastigotes

A genetic approach was taken by implementing the homozygous disruption of LPG-specific genes in *Leishmania major*⁸. Unlike *L. donovani*, this species has the advantage of retaining virulence during the periods of time required for the gene-knockout and -restoration procedures. For these studies, we chose the *LPG1* gene, which was identified by complementation of the *L. donovani* *R2D2* mutant. *LPG1* encodes a putative galactofuranosyltransferase (Gal_f transferase) that functions within the biosynthetic pathway required to generate the LPG glycan core⁹. Although recent studies have shown that *R2D2* contains a mutated version of *LPG1* (R. Zufferey and S.M. Beverley, unpublished), it had been generated by heavy mutagenesis in an *L. donovani* background, making it unsuitable for

detailed studies of virulence. Thus, we used two rounds of homologous gene replacement (because *Leishmania* is typically diploid) to generate a null mutant of *L. major* lacking *LPG1*, which we called *lpg1*⁻ (Ref. 8).

Biochemical characterizations of *lpg1*⁻ showed that only LPG was affected, with GPI anchored proteins, secreted PGs and GIPLs being unaffected⁸. Restoration of *LPG1* expression to the mutant (*lpg1*⁻/*LPG1*) resulted in normal LPG expression. Notably, the Gal_f-mannose (Gal_f-Man) linkage normally present in both LPG and GIPLs was lost from the LPG but not the GIPLs of the *lpg1*⁻ strain of *L. major* (Fig. 1). This implied the existence of a GIPL-specific Gal_f transferase, which is now under study in our laboratories. Thus, these data revealed that the *lpg1*⁻ parasites were the first 'clean', LPG-specific mutant⁸. The virulence of the *lpg1*⁻ mutant was then assessed in three separate venues.

Survival within the sand fly vector

In the sand fly vector, the dense glycocalyx formed by LPG has been implicated in the protection of the parasite from the action of midgut digestive enzymes. For the first few days after ingestion, *Leishmania* are found within the bloodmeal encased by the peritrophic matrix (PM). After the PM degenerates, parasites escape and bind to the midgut wall, thereby preventing loss through excretion as the bloodmeal is eliminated. During metacyclogenesis, LPG undergoes structural modifications that result in loss of midgut binding and migration of the parasite anteriorly in anticipation of the next time the sand fly bites a new host.

Although the *lpg1*⁻ parasites were only slightly deficient in their ability to survive in the early stages within the PM-enclosed bloodmeal, their ability to persist in the midgut after bloodmeal excretion was completely lost¹⁰. As expected, this defect was associated with their inability to bind to midgut epithelial cells *in vitro*. Importantly, the *lpg1*⁻/*LPG1* parasite behaved like the wild-type controls.

Survival within mammalian macrophages in vitro

A second venue was survival in mammalian macrophages⁸. Following opsonization with complement (because deposition of C3 occurs during normal *Leishmania* infections and has been implicated in parasite uptake and virulence¹¹), the *lpg1*⁻ *L. major* promastigotes entered macrophages normally but were mostly eliminated within two days. Again, restoration of *LPG1* expression resulted in full parasite survival. In contrast to promastigote infections, *lpg1*⁻ amastigotes infected and replicated in macrophages normally. This was expected as LPG is synthesized only at trace levels in amastigotes. In addition, this confirmed that *lpg1*⁻ mutants are specifically compromised in LPG biosynthesis.

Ability to induce lesions in susceptible mice

The last venue was infection of susceptible BALB/c mice⁸. The *lpg1*⁻ parasites had a greatly delayed

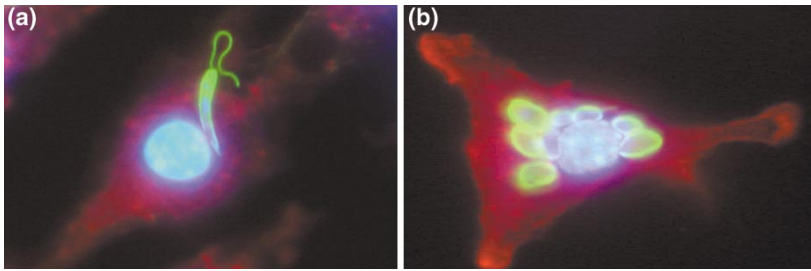


Fig. 2. *Leishmania major* developmental stages interacting with cultured mouse peritoneal macrophages.

(a) Stationary-phase promastigotes invading macrophages. These parasites express high levels of surface lipophosphoglycan and other phosphoglycans. (b) Amastigotes residing within macrophages; these parasites do not express lipophosphoglycan but do express other phosphoglycans. The parasite is stained with an anti-tubulin antibody (green), the macrophage is stained with an anti-actin antibody (red) and the nuclei of both are stained with Hoechst dye (blue).

formation of lesions compared with wild-type controls, whereas lesions produced by the *lpg1*^{+/+}*LPG1* parasites showed minimal delay and thereby resembled wild-type controls. Importantly, the *lpg1*⁻ parasites that did emerge, after a considerable delay, were 'escapees' rather than revertants: after recovery and differentiation back to promastigotes, a second mouse infection yielded the same delayed lesion phenotype.

Episode II: *Leishmania mexicana* strikes back

Thus, in three different assays probing different aspects of the *Leishmania* infectious cycle, loss of LPG conferred by loss of *LPG1* led to a dramatic reduction in virulence in *L. major*. It was therefore surprising to learn of seemingly contradictory results obtained with *Leishmania mexicana* in the laboratory of Thomas Ilg¹². In these studies, knockout *lpg1*⁻ *L. mexicana* failed to show decreased virulence following mouse infections and appeared to replicate normally in macrophages (sand fly studies were not performed). It was justifiably concluded that LPG is not a virulence determinant in *L. mexicana*, which raised the possibility that other related glycoconjugates, such as the membrane-bound form of PPG, performed this function.

The different results obtained with *L. major* and *L. mexicana* were a surprise to the workers involved as well as to the *Leishmania* glycobiology community, who mostly anticipated that LPG would carry out similar functions in all *Leishmania* species^{1,2}. It seems unlikely that trivial technical explanations can account for the different findings. In the *L. mexicana* macrophage studies, infections were performed for 24 h and then replication of the surviving parasites were scored¹². However, after 24 h, any surviving parasites are expected to have differentiated into amastigotes, which lack LPG and so normal replication was expected (and indeed observed in the *L. major lpg1*⁻ line). This emphasizes the importance of how 'virulence' tests are applied and evaluated.

Small biochemical differences in the parasites were also noted: in *L. major*, the membrane-bound form of PPG was not detectable⁸ and, in the *L. mexicana lpg1*⁻ parasites, upregulation of PPG was observed¹², which complicates assessment of the role of LPG alone. In the mouse assays, a potential factor arises from a saturation phenomenon revealed in the *L. major* studies: at high parasite inocula (10⁷), the difference between control and *lpg1*⁻ parasites was minimized, but, at lower inocula (≤10⁶) differences in virulence progressively increased (in Nature, sand flies probably deposit <100 parasites). However,

several parasite doses were tested in *L. mexicana* with similar outcomes, making this explanation less likely (barring species-specific changes in the dose–infectivity relationship).

Thus, it appears that, in the mouse model, *L. mexicana* and *L. major lpg1*⁻ mutants show different behaviours. This discrepancy will not end with *LPG1*: at a recent meeting (*Gene action and cellular function in parasitic protozoa*, Manchester, UK, July 2000), an even greater discrepancy was reported between these species for knockout mutants in *LPG2*, which was identified by complementation of the *C3PO* mutant and encodes a Golgi GDP–mannose transporter that is required for the synthesis of all PGs¹³. Comparison of the *lpg2*⁻ knockouts shows that the *L. mexicana* mutant remains virulent¹⁴, whereas the *L. major* knockouts show a much more pronounced loss of virulence than the *lpg1*⁻ parasites, in sand fly, mouse and macrophage assays¹⁰ (G.F. Späth *et al.*, unpublished). This extends the species differences to include all *Leishmania* PGs, not just LPG.

Episode III: The revenge of the mouse response

Despite the confident predictions of glycobiologists, could *Leishmania* species differ so dramatically in their reliance upon LPG and related glycoconjugates for virulence? Several lines of evidence suggest that this might be the case. In recent years, it has become clear that the murine response to *L. major* can differ considerably from that to other *Leishmania*. Although *L. major* is beloved by immunologists for its role in the discovery of the Th1–Th2 paradigm of immune cell subsets mediating resistance and susceptibility, other *Leishmania* species (including members of the *L. mexicana* species complex, such as *Leishmania amazonensis*) often do not give as simple an association^{15,16}.

Moreover, the consequences of genetic inactivation of host immune-response genes implicated in the control of *L. major* give radically different outcomes with other *Leishmania*. For example, immunodeficient mice are susceptible to *L. major* but remain resistant to *L. amazonensis*¹⁵. Because there is a substantial immune response to LPG and related molecules during mouse infections, it seems reasonable that this somehow translates into different outcomes of infection, with LPG playing a more critical role in *L. major*. Alternatively, the overall biology of infection by *L. mexicana* might place less emphasis on LPG and/or related glycoconjugates separate from the immune response; perhaps it arises from differences in the rate of replication in the host, for example.

Differences between species have surfaced previously in murine tests of *Leishmania* virulence factors: underproduction of the *L. mexicana* gp63 surface protease has been implicated in macrophage survival¹⁷ but, in *L. major*, gp63-deficient mutants show remarkably normal virulence¹⁸. In fact, gene knockout tests of a number of putative virulence

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genes affecting molecules belonging to the LPG 'family' in *L. mexicana* have failed to reveal the expected roles in virulence. These include the heavily phosphoglycosylated sAPs *SAP1* and *SAP2* (which are expressed at very low levels in *L. major*^{19,20}), all GPI-anchored proteins²¹, and the amastigote proteophosphoglycan *PPG2* (Ref. 22; Fig. 1). In combination with the previously mentioned results with *LPG1* and *LPG2*, one might question the ability of the *L. mexicana*-mouse system (or, conversely, the *L. major*-mouse system) to assess the role of candidate virulence factors. However, several *L. mexicana* complex mutants have shown decreased virulence in mice, including ones lacking cysteine proteinases²³ and free GIPLS²⁴.

In the future, the role (or lack thereof) of prospective virulence genes will require assessment with mechanistic studies focusing on relevant steps within the infectious cycle. Chief among these is the use of the infective metacyclic form of the *Leishmania* parasite, which is a challenge in the case of the *lpg*⁻ parasites because developmental changes in LPG itself are currently the basis for metacyclic identification and purification¹⁰. To meet this challenge, we developed a method for purifying metacyclics independently of their LPG state; the metacyclic *lpgT*⁻ parasites show decreased virulence, exactly as before (G.F. Späth and S.M. Beverley, unpublished).

Mechanistic studies of pathogenesis offer the potential to identify, confirm and test exactly how proposed virulence factors function in the infectious cycle. Preliminary studies have shown the importance

of LPG for virulence of the *lpgT*⁻ *L. major* at several key steps, including complement and oxidant resistance, and transient inhibition of phagolysosomal fusion (G.F. Späth and S.M. Beverley, unpublished). Thus, both mechanistic and biological assays point to the importance of LPG in the infectious cycle of *L. major*. It will be interesting to learn how *L. mexicana lpgT*⁻ mutants fare in similar biological and mechanistic analyses in the future, and whether LPG will be implicated in these seemingly universal pathogenic processes.

Perspective

Thus, instead of being monolithic, *Leishmania* seem to exhibit considerable diversity in their emphasis on virulence factors, which now appear to differ between species and possibly between hosts. This might be associated with the evolutionary antiquity of *Leishmania*, as divergences within this genus can exceed 80 million years and approach those separating their various mammalian hosts. These observations now place the burden on investigators to assess the role of virulence factors in different species and in biologically relevant mammalian and sand fly hosts. The human host is of particular interest, as is whether the differences in requirements for virulence factors that is now evident in murine models can account for differences in the diseases caused by different *Leishmania* species. There is little doubt that future episodes ("The Phantom Menace?") promise to yield exciting new insights into the diversity and role of LPG and related molecules in *Leishmania* virulence.

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