Inhibition of Phagolysosomal Biogenesis by the \textit{Leishmania} Lipophosphoglycan

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Summary

Whereas amastigotes of the protozoan parasite \textit{Leishmania} proliferate inside acidic phagolysosomal vacuoles of the macrophage, vacuoles induced by \textit{Leishmania donovani} promastigotes during initiation of infection are poorly characterized. Here, evidence is presented that interaction of these parasitophorous vacuoles with endocytic organelles is very limited. In contrast, vacuoles formed around \textit{L. donovani} mutants lacking the cell surface lipophosphoglycan (LPG) fuse extensively with endosomes and lysosomes. The role of LPG repeating units in the inhibition of phagosome–endosome fusion was demonstrated using two different approaches. First, genetic complementation of the LPG-defective C3PO mutant restored its ability to inhibit phagosome–endosome fusion to a degree similar to that of wild-type promastigotes. Second, opsonization of C3PO mutant cells with purified \textit{L. donovani} LPG also conferred to this mutant the ability to inhibit phagosome–endosome fusion. Inasmuch as LPG is essential for infecting macrophages, these results suggest that inhibition of phagolysosomal biogenesis by LPG repeating units represents an intramacrophage survival strategy used by promastigotes to establish infection.

During their life cycle, protozoan parasites of the genus \textit{Leishmania} alternate between the midgut of their insect vector, where they exist as promastigotes, and a macrophage phagolysosomal compartment, where they proliferate as amastigotes. Over the past several years, evidence was provided that \textit{Leishmania} parasites succeed in avoiding host immune defenses and destruction by expressing specialized stage-specific molecules (1, 2). The dominant cell surface molecule of promastigotes is lipophosphoglycan (LPG)\(^1\), a glycosylinositolphospholipid (GPI)-anchored polymer consisting, in \textit{L. donovani}, of the repeating disaccharide-phosphate unit \([\text{Gal}1\beta1,4\text{Man}(\alpha1-PO_4\rightarrow\text{Gal}4\beta1,3\text{Glc}4\beta1,6\text{Man})]\) (between 16 to 30 units) (1). This molecule, particularly the repeating units moiety, is essential for the interaction of promastigotes with both the insect vector and the mammalian host (1, 3–11). The requirement for LPG in the establishment of infection inside the macrophage was evidenced by the demonstration that LPG repeating units–defective mutants are rapidly destroyed after phagocytosis, and that passive transfer of purified LPG significantly prolonged their survival (7, 8). This role for LPG repeating units in intramacrophage survival has also been genetically proven (11).

Thus, without LPG repeating units promastigotes are unable to withstand the conditions prevailing inside the maturing parasitophorous vacuole. This is in contrast with the amastigotes, which proliferate inside acidic, hydrolase-rich vacuoles (12–17), despite the fact that they synthesize little or no detectable LPG (18–20). Thus, the role of this molecule in intramacrophage survival may be restricted to the establishment of infection, during promastigote-to-amastigote conversion (7–9, 11).

Different properties of LPG repeating units are consistent with their protective role during the establishment of infection, including efficient scavenging of toxic oxygen metabolites generated during the oxidative burst (8, 21), and modulation of the inducible nitric oxide synthase expression (22). In addition, the several million copies of LPG at the promastigote surface form a dense glyocalyx that may provide a physical barrier against the action of hydrolytic enzymes (10, 13). Recent in vitro studies demonstrated that incorporation of full-length LPG in lipid bilayers resulted in reduced fusogenic capacities (23). This finding raised the possibility that LPG repeating units may protect freshly phagocytized promastigotes by limiting the delivery of endosomal contents to the parasitophorous vacuoles, a process requiring multiple fusion events (24, 25). Here, we compared the ability of parasitophorous vacuoles induced by either wild-type \textit{L. donovani} promastigotes or LPG repeating units–defective mutants to interact with endocytic organelles. We show that LPG repeating units enable \textit{Leishmania} to inhibit the phagosome–endosome fusion process efficiently, thereby suggesting a survival strategy during their differentiation into amastigotes.

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\(^1\)Abbreviations used in this paper: LPG, lipophosphoglycan; GPI, glycosylinositolphospholipid; PKC, protein kinase C; WT, wild-type.
Materials and Methods

Cell Culture. The murine macrophage cell line J774 was passaged in DMEM supplemented with 10% FCS, 1% glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO2 atmosphere. J774 cells were plated in 3-cm dishes for electron microscopy studies. All experiments were done with subconfluent cultures.

Parasites and LPG. Leishmania donovani wild-type strain 1S (WT), the LPG-deficient mutants C3PO and RT5 (26), C3PO overexpressing LPG2 (C3PO+LPG2) (27), and the LPG repeating unit–defective line created by targeted disruption of LPG2 (termed lpg2 knockout) (27) were all grown at 26°C in modified M199 medium as described (8). Opsonization of C3PO with purified L. donovani LPG (provided by S.J. Turco, University of Kentucky) was performed as described (8). All lines were used in stationary phase of growth.

Macrophage Infections, Electron Microscopy, and Morphological Studies. Interactions and fusion between endocytic organelles and either L. donovani–containing phagosomes or latex bead–containing phagosomes were studied using a morphological approach at the electron microscope level. This in vivo fusion assay, based on the transfer of electron-dense tracers from endosomes to phagosomes, was used because of its simplicity over in vitro fusion assays and its sensitivity. Endocytic organelles were loaded by internalization of 5- and 16-nm gold particles coated with BSA (BSA–gold) using standard procedures (28). Phagosomes were formed by the addition of stationary-phase promastigotes to a parasite-to-host cell ratio of 10:1 in culture medium at 37°C for 60 min. Excess parasites were removed by four washes with cold PBS. Infected cells were then further incubated in culture medium for 60 min to allow complete internalization of bound promastigotes. Two types of loading procedures were used. In some cases, BSA–gold was internalized first, followed by incubation in culture medium to allow the marker to fill various endosome populations. Cells containing BSA–gold were then infected with L. donovani. This approach allowed us to study the interaction of early endosomes and lysosomes with newly formed phagosomes. The second procedure consisted in infecting macrophages with L. donovani first for 60 min, followed by incubation in culture medium for either 60 min or 4 h to form early or late phagosomes, respectively. These macrophages were then fed BSA–gold particles for 30 min and were either processed immediately or further incubated in culture medium for 60 min. This approach allowed us to study the interaction of preexisting phagosomes of various ages with maturing endosomes. For some experiments, promastigotes and 0.8-μm latex beads (Sigma, St. Louis, MO; diluted 1:200 in culture medium) were cointernalized, to study the interaction of endosomes with different phagosome populations. After all internalization steps, cells were washed thoroughly with cold PBS three times for 5 min. At the end of each experiment, cells were fixed in 1% glutaraldehyde, postfixed in OsO4, processed for embedding in Epon 812 resin as described previously (24), and observed by electron microscopy.

Intermixing of BSA–gold particles and L. donovani was then recorded for each combination of incubations performed. The presence of a single gold particle inside a phagosome was scored as a fusion event. For the analysis of fusion occurrence (Fig. 2), each experiment was done at least twice and a minimum of 25 Leishmania–containing phagosomes per timepoint were recorded, while extreme care was taken to avoid serial sections. For the quantitative analysis of the transfer of BSA–gold particles from endosomes to Leishmania–containing phagosomes (Table 1), the number of 5- and 16-nm gold particles was counted on at least 12–20 of the BSA–gold positive phagosome profiles at the electron microscope.

Figure 1. Fusion of L. donovani–containing phagosomes with newly formed endosomes. J774 macrophages were infected with stationary-phase promastigotes for 60 min and further incubated for 60 min. Cells were then fed a 5- and 16-nm BSA–gold particles mixture for 30 min, followed by a chase period of 60 min, and processed for electron microscopy. (A) Phagosomes containing wild-type Leishmania (P) display low fusogenic properties with endocytic organelles (E). When fusion occurred, only few gold particles were observed in the phagosome (arrow). (B) Phagosomes containing the LPG-deficient C3PO mutant (P) were filled with large number of BSA–gold particles, indicating their acute ability to fuse with endosomes.
Results and Discussion

The transfer of BSA–gold particles from endosomes to phagosomes containing either wild-type promastigotes or the LPG repeating unit–defective C3PO mutant cells (26) was first used to evaluate the interaction occurring between these organelles. Very few gold particles were generally transferred to WT–containing phagosomes despite the close presence of BSA–gold particle–filled endosomes (Fig. 1 A). In contrast, most phagosomes containing the LPG repeating units–defective C3PO mutant were filled with numerous BSA–gold particles (Fig. 1 B). While only ~40% of WT-phagosomes received BSA–gold particles, 90% of phagosomes induced by C3PO mutant cells fused with endocytic organelles (Fig. 2 A). The difference in levels of fusion was more striking when the number of gold particles transferred from endosomes to phagosomes was quantified. Indeed, a sixfold increase in the BSA–gold particle content of C3PO phagosomes over WT-phagosomes was observed (Table 1). High levels of fusion were also observed with phagosomes induced by lpg2 null mutant cells, which are phenotypically identical to C3PO cells (27), and by RT5, a mutant that accumulates truncated forms of LPG containing 3–5 repeating units (26) (Fig. 2 A). Because assembly of the Gal(β1,4)Manα1-PO₄→6) repeating units is defective in these mutants, our observations indicated a role for this LPG moiety in the inhibition of phagosome–endosome fusion. Further, immunofluorescence analysis showed that LPG–expressing promastigotes are contained within a LAMP1–negative compartment, whereas LPG–deficient mutants were found in LAMP1–positive phagosomes (data not shown), indicating their respective levels of fusion with endocytic organelles enriched for LAMP1 molecules.

To demonstrate directly a role for LPG in the inhibition of phagosome–endosome fusion, two distinct approaches were taken. First, we assessed the fusogenic properties of phagosomes induced by C3PO cells transfected with a LPG2 expression construct (C3PO+LPG2) (27). As expected, phagosomes containing C3PO+LPG2 cells behaved similarly to WT–phagosomes with respect to their ability to fuse with endocytic organelles (Fig. 2 A; and Table 1). Thus, restoration of full-length LPG repeating units synthesis by genetic complementation enabled C3PO to inhibit phagosome–endosome fusion. Because LPG2 expres-

<table>
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<th>5-nm BSA–gold particles</th>
<th>16-nm BSA–gold particles</th>
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<tr>
<td>Wild type</td>
<td>57.3 ± 28.0</td>
<td>4.3 ± 2.7</td>
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<tr>
<td>C3PO</td>
<td>323.6 ± 142.5</td>
<td>23.6 ± 11.4</td>
</tr>
<tr>
<td>C3PO+LPG2</td>
<td>86.2 ± 34.4</td>
<td>8.2 ± 3.5</td>
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J774 macrophages were infected as described in the legend to Fig. 1. Cells were then fed a 5-nm and 16-nm BSA–gold particles mixture for 30 min, followed by a chase period of 60 min, and processed for electron microscopy. The 5- and 16-nm gold particles present in the profile section of phagosomes containing the various Leishmania lines were counted. At least 25 phagosomes were analyzed for each cell line. Phagosomes devoid of BSA–gold particles were not included in this quantitative analysis.
The Leishmania Lipophosphoglycan Inhibits Phagolysosomal Biogenesis

In C3PO also restores repeating units addition on secreted molecules, including the secreted acid phosphatase (27), the possibility existed that such repeating unit addition could have accounted for the ability of C3PO + LPG2 cells to inhibit phagosome–endosome fusion. Therefore, repeating unit modification of the secreted acid phosphatase, which is not required for both secretion and activity (27, 30), appears to be without apparent effect on phagosomal fusogenic properties.

We also assessed the ability of newly formed phagosomes to fuse with two distinct endosome populations. Here, BSA–gold particles were first internalized for 30 min, followed by either a 15-min or 4-h chase. The first population is considered as early endosomes and the second as lysosomes. At the end of the chase period, macrophages were infected with either WT promastigotes or C3PO mutant cells for 1 h. Increased fusion between early endosomes and C3PO/phagosomes with respect to WT-phagosomes was observed (see Fig. 2 B). Interestingly, WT-phagosomes consistently displayed lower fusogenicity with lysosomes (35% fusion) than with early endosomes (55% fusion), suggesting that, similar to Mycobacterium phagosomes (31–34), fusion with the various endosomal populations might be selective, albeit to a lesser extent.

To evaluate the specificity of the LPG repeating units–induced alteration(s) of phagosome fusion properties, J774 macrophages were coinfected with latex beads and the various Leishmania lines. Latex beads and promastigotes were present in distinct phagosomal compartments (Fig. 4). Regardless of the coinfectioned Leishmania line, the fusion rate between endocytic organelles and latex bead phagosomes was around 90% (see Fig. 2 C). Thus, in a given macrophage, fusion can occur between endosomes and latex bead phagosomes but not with WT-phagosomes. This observation indicated that (a) inhibition of fusion by LPG repeating unit is selective, (b) the general host cell fusion machinery remains operational during infection, and (c) LPG repeating units selectively alter local phagosomal fusogenic properties. From a mechanistic point of view, the following models can be considered. First, WT promastigotes and the repeating unit–defective mutants use distinct receptors for attachment and entry, resulting in the formation of phagosomes with different biochemical composition and fusion properties (35–37). However, the observation that promastigote-to-amastigote transformation, which is paralleled by the loss of LPG, restores phagosome–endosome fusion argues against this idea (12–20). In the second model, LPG may inhibit activation of phagosome–associated protein kinase C (PKC) (38–40). Although the precise function of PKC in phagosome–endosome interaction remains speculative, it is known to phosphorylate MARCKS, a membrane protein associated with actin-based motility (41) and with membrane trafficking (42). In this regard, PKC-dependent phosphorylation of phagosome–associated MARCKS results in its displacement from the membrane to Lamp1–positive lysosomes (42) and may therefore participate in the movement of both phagosomes and endosomes on microtubules. Inhibition of PKC-dependent MARCKS phosphorylation by LPG (43–44) may block this movement. However, this model is not consistent with the inhibition of PKC-dependent processes by C3PO and amastigotes.
during infection (43, 45, 46), both of which induce phagosomes that fuse freely with endocytic organelles (see Figs. 1 and 2A; references 12–17). In a third model, LPG is transferred within minutes from the promastigote surface to the macrophage membrane at the immediate area of internalization (29). Insertion of LPG in lipid-bilayer membranes stabilizes the bilayer against the formation of an inverted hexagonal structure, resulting in reduced fusogenic properties (23). As a consequence, LPG would give rise to an effective steric repulsion between phagosomal and endosomal membranes or reduce the negative curvature strain in bilayers, increasing the energy barrier for forming highly curved fusion intermediates (23), thereby preventing fusion. Interestingly, truncated forms of LPG containing few repeating units are ineffective in modifying the fusogenic properties of membranes (23). This in vitro finding is in agreement with our observation that RT5, a mutant expressing truncated forms of LPG with three to five repeating units (26), is unable to inhibit phagosome–endosome fusion. Alternatively, the possibility exists that LPG inhibits phagosome–endosome fusion indirectly. Indeed, LPG may prevent parasite destruction by scavenging toxic oxygen radicals generated during the oxidative burst (8, 21), allowing for the production of a yet unidentified factor directly responsible for preventing fusion. Regardless of the nature of the exact underlying mechanism, our data have unequivocally demonstrated that expression of full-length LPG enables promastigotes to inhibit phagosome–endosome fusion. The extent to which inhibition of fusion by LPG contributes to promastigote survival remains to be determined.

In a study aimed at determining the position of *L. mexicana* parasitophorous vacuoles within the endocytic network of the host macrophage, Russell et al. (16) observed that the transfer of endosomal content to parasitophorous vacuoles induced by promastigotes increased in efficiency with respect to the age of the infection. To explain this phenomenon, the authors suggested that these vacuoles had functionally translocated from a lysosomal to a late endosomal compartment, consistent with their increased content in mannose-6-phosphate receptors. In the light of the findings reported herein, we suggest that the reduced fusogenicity of the parasitophorous vacuoles observed in early infections (day 2) by Russell et al. (16) is caused by the presence of promastigote-derived LPG. Indeed, LPG repeating units epitopes are maximally present in the macrophage membrane 1–2 d after infection, and by 5–6 d, these epitopes are no longer detectable (29). Thus, the transformation of promastigotes into amastigotes, associated with the loss of LPG expression, occurs concomitantly with the release of phagosome–endosome fusion inhibition.

Inhibition of phagosome–endosome fusion is an intramacrophage survival strategy used by a variety of intracellular pathogens (35–37). Although the molecular bases of this phenomenon are poorly understood, microbial surface components analogous to the *Leishmania* LPG may alter the fusion properties of the endocytic system (47). In this regard, in vitro studies have shown that Cord factor (α,α-trehalose 6,6′-dimycolate), a cell wall glycolipid of Mycobacteria, inhibits fusion between phospholipid vesicles (48). Although exclusion of the vacuolar proton–ATPase from mycobacteria–phagosomes appears to be closely related with their reduced fusogenicity, it is not known, however, whether this exclusion is the consequence or the cause of the altered fusion properties (31).

The mechanisms and molecules involved in the regulation of phagosome fusion properties remain largely unknown. While proteins of the rab family were found to associate and dissociate from maturing phagosomes, their role in phagosome fusion is still unclear (24). Phagosome maturation is also accompanied by the acquisition of Nramp1, a molecule conferring resistance to a variety of intracellular microbes, including *L. donovani* (49), the sequential phos-
phorylation and dephosphorylation of some of their pro-
teins, as well as modifications of their phospholipid content
(50, 51). Although no direct correlation between phago-
some fusogenic properties and their lipid composition has
been demonstrated, the finding that LPG modifies the mo-
lecular structure of lipid bilayers (23) suggests a role for lip-
ids in the fine tuning of membrane fusion events. In this
regard, we expect that molecular and biochemical charac-
terization of Leishmania-containing phagosomes will yield
novel insights on the mechanistic aspects of membrane fu-
sion.

LPG repeating units are essential for the successful transi-
tion of Leishmania parasites from the sandfly midgit to the
inside of a macrophage phagolysosome (3–11). In the mac-
rophage, LPG repeating units may contribute to promasti-
gote survival through their capacity to scavenge oxygen
radicals (21), to inhibit PKC (40, 44), and to modulate ni-
tric oxide synthase expression (22). Our data suggest a
novel function for LPG repeating units during the early
phase of macrophage infection, where inhibition of phago-
lysosomal biogenesis may protect invading promastigotes
from hydrolytic degradation and provide an environment
propitious for their differentiation into amastigotes. Finally,
the anti-fusogenic properties of LPG repeating units pro-
vide a powerful probe to investigate the regulation of phago-
some–endosome fusion during microbial invasion.

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