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Leishmania infantum: Lipophosphoglycan intraspecific variation and interaction with vertebrate and invertebrate hosts

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ABSTRACT

Interspecies variations in lipophosphoglycan (LPG) have been the focus of intense study over the years due its role in specificity during sand fly-*Leishmania* interaction. This cell surface glycoconjugate is highly polymorphic among species with variations in sugars that branch off the conserved Gal(β1,4)Man(α1)-PO_4 backbone of repeat units. However, the degree of intraspecies polymorphism in LPG of *Leishmania infantum* (syn. *Leishmania chagasi*) is not known. In this study, intraspecific variation in the repeat units of LPG was evaluated in 16 strains of *L. infantum* from Brazil, France, Algeria and Tunisia. The structural polymorphism in the *L. infantum* LPG repeat units was relatively slight and consisted of three types: type I does not have side chains; type II has one β-glucose residue that branches off the disaccharide-phosphate repeat units and type III has up to three glucose residues (oligo-glucosylated). The significance of these modifications was investigated during in vivo interaction of *L. infantum* with *Lutzomyia longipalpis,* and in vitro interaction of the parasites and respective LPGs with murine macrophages. There were no consequential differences in the parasite densities in sand fly midguts infected with *Leishmania* strains exhibiting type I, II and III LPGs. However, higher nitric oxide production was observed in macrophages exposed to glucosylated type II LPG.

1. Introduction

Visceral leishmaniasis (VL) is caused by *Leishmania* (*Leishmania*) *infantum* (syn. *Leishmania* (*Leishmania*) *chagasi*) and *Leishmania* (*Leishmania*) *donovani*. This disease has a broad range of manifestations from asymptomatic or subclinical infection to acute, subacute or chronic (Herwaldt, 1999). *Lutzomyia longipalpis* (Diptera: Psychodidae) is the main vector of VL in Latin America (Grimaldi et al., 1989). In the Mediterranean Western Basin, VL is mainly transmitted by *Phlebotomus perniciosus* (Ready, 2010). Despite being a rural disease, VL is urbanising as a result of environmental changes and vector adaptation to human habitats and domestic reservoirs (Ashford, 2000).

The dominant cell surface glycoconjugate of *Leishmania* is lipophosphoglycan (LPG), which has been implicated in a wide range of functions, both in vertebrate and invertebrate hosts. In the vertebrate host, this virulence factor's main functions include: attachment and entry into macrophages, modulation of nitric oxide (NO) production (Brittingham and Mosser, 1996), inhibition of protein kinase C (Giorgione et al., 1996) and induction of neutrophil extracellular traps (NETs) (Guimarães-Costa et al., 2009). In the invertebrate host, variations in LPG have been implicated in the specificity of different sand fly spp. to *Leishmania* (Pimenta et al., 1992, 1994), where attachment of the parasite to a midgut receptor is a crucial event during the interaction of parasite and vector (Kamhawi et al., 2004).

LPG structures have already been described for several dermotropic and viscerotropic *Leishmania* spp. (McConville et al., 1992, 1995; Sacks et al., 1995; Mahoney et al., 1999; Soares et al., 2002, 2004, 2005). However, there is no available information on the degree of variability in the LPG structure from Old and New World strains of *L. infantum*. Basically, LPGs have a conserved glycan core region of Gal(α1,6)Gal(α1,3)Gal_f(β1,3)[Glc(α1)-PO_4]-Man(α1,3)Man(α1,4)-GlcN(α1) linked to a 1-*O*-alkyl-2-*lyso*-phosphatidylinositol anchor. The salient feature of LPG is another conserved domain consisting of the Gal(β1,4)Man(α1)-PO_4 backbone of repeat units ($n = \sim 15–30$). The distinguishing feature of LPGs responsible for the polymorphisms among *Leishmania* spp. is in the variability of sugar composition and sequence of branching sugars attached to the repeat units and cap structure (Turco and Descoteaux, 1992). For example, *L. infantum* (strain

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PP75) LPG contains β-glucoses attached to the C3 hydroxyl of the repeat unit galactose (Soares et al., 2002).

Intracellular parasitism by *Leishmania* and the involvement of LPG has previously been the subject of investigation (Alexander et al., 1999; Descoteaux and Turco, 2002). As an example, LPGs of *Leishmania* were identified as potent agonists of Toll-like receptor 2, (TLR2) in human natural killer (NK) cells and murine macrophages, triggering the production of TNF-α and IFN-γ via a functional MyD88 adaptor protein. Importantly, the integrity of the lipid anchor is necessary for activity (Becker et al., 2003; de Veer et al., 2003). Early studies by Proudfoot et al. (1996) showed that *Leishmania major* LPG synergized with IFN-γ for the induction of inducible NO synthase (iNOS) expression in murine macrophages in vitro. Also, this regulatory activity of LPG was contained within the phosphoglycan (PG) moiety. Since the lipid moiety is conserved, it is still unknown whether intraspecific variations in the PG carbohydrate portion will trigger different responses in the New World *L. infantum*.

Intraspecific variation in LPGs is poorly understood. In *L. donovani* (Sudan) the LPG is devoid of side-chains (Sacks et al., 1995) whereas the Indian strain possesses 1–2 β-glucose residues in the repeat units (Mahoney et al., 1999). In *L. major*, the LV39 strain has longer poly-galactosylated side-chains, whereas the Friedlin (FV1) strain has shorter galactosylated side-chains, often capped with α-arabinose residues (McConville et al., 1992; Dobson et al., 2003). In *Leishmania tropica*, intraspecific LPG variability was assessed in the Israeli strains L810/L863 and L747, where the presence of β-galactose residues was a determinant in preventing their development in *Phlebotomus sergenti* (Soares et al., 2004). In this study, we report the intraspecific LPG variability of *L. infantum* (Brazil, Europe and Africa). Those polymorphisms were evaluated during interaction with *L. longipalpis* and murine macrophages.

2. Materials and methods

2.1. Materials

Materials were obtained as follows: Medium 199 from Gibco Life Technologies (USA); FCS from Atlanta Biologicals (USA); AG50W-X12 cation-exchange resin and AG1-X8 anion-exchange resin from Bio-Rad (Hercules, CA, USA); phenyl-Sepharose CL-4B, alkaline phosphatase (*Escherichia coli*) from Sigma.

2.2. Parasites

Leishmania strains are listed in Table 1. Starter cultures of promastigotes were grown in Medium 199 supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (50 μg/ml), 12.5 mM glutamine, 0.1 M adenine, 0.0005% hemin and 40 mM HEPES, pH 7.4 at 25 °C (Soares et al., 2002).

2.3. Extraction and purification of LPG

LPGs from parasites in the early stationary phase were extracted in solvent E (H_2O/ethanol/diethyl ether/pyridine/NH_4OH; 15:15:5:1:0.017). The extract was dried by N_2 evaporation, resuspended in 0.1 N acetic acid/0.1 M NaCl, and applied to a column of phenyl-Sepharose (2 ml), equilibrated in the same buffer. LPG was eluted using solvent E (Orlandi and Turco, 1987).

2.4. Immunoblotting

Purified LPGs were transferred to nitrocellulose paper. The membrane was blocked (1 h) in 5% milk in PBS and probed for 1 h with monoclonal antibody (mAb) CA7AE (1:1,000), which recognises the unsubstituted Gal(β1,4)Man repeat units (Tolson et al., 1989). After three 5 min washes in PBS, the membrane was incubated for 1 h with anti-mouse IgG conjugated with peroxidase (1:10,000) and the reaction was visualised using luminol.

2.5. Preparation of repeat units

Purified LPG was subjected to mild acid hydrolysis (0.02 N HCl, 5 min, 100 °C) to depolymerise the repeat units and cap structures. Water-soluble fractions were partitioned using 1-butanol and repeat units were desalted by passage through a two-layered column of AG50W-X12 (H^+) over AG1-X8 (acetate) (Mahoney et al., 1999).

2.6. Enzymatic treatments

Phosphorylated repeat units were treated with alkaline phosphatase in 15 mM Tris buffer, pH 9.0 (1 U, 16 h, 37 °C). Neutral oligosaccharides were treated with *E. coli* β-galactosidase in 80 mM Na_3PO_4, pH 7.3 (4 U, 16 h, 37 °C) (Soares et al., 2002).

2.7. Preparation of monosaccharides

The purified repeat units were subjected to strong acid hydrolysis (2 N trifluoroacetic acid, 3 h, 100 °C). Samples were desalted as described above and subjected to fluorophore-assisted carbohydrate electrophoresis (FACE) and HPLC.

2.8. FACE

For repeat units, samples were fluorescently labelled with 0.05 N ANTS (8-aminonaphthalene-1,3,6-trisulfate) and 1 M cyanoborohydride (37 °C, 16 h). Monosaccharides were fluorescently labelled with 0.1 M AMAC (2-aminoacridone) in 5% acetic acid and 1 M cyanoborohydride. Sugars were subjected to FACE and the gel was visualised by a UV imager (Soares et al., 2004).

2.9. Capillary electrophoresis (CE)

Dephosphorylated repeat units were labelled with 0.02 M APTS (8-aminopyrene-1,3,6-trisulfonic acid trisodium salt) in 15% acetic acid in sodium cyanoborohydride buffer and incubated overnight at 37 °C. Samples were run on CE at 25 kV for 20 min under pressure injection of 5 psi (Soares et al., 2004).

2.10. HPLC

Monosaccharides were separated using a DX-500 HPLC (Dionex Corp., USA) with ED40 electrochemical detection. The samples were run on a CarboPac PA10 column (4 × 250 mm) in the presence of 18 mM NaOH (flow rate 1 mL/min, 2000 psi). Glucose, galactose and mannose (100 μg/mL) were used as controls.

2.11. Purification of murine peritoneal macrophages, cell culture and nitrite measurements

Thioglycollate-elicited peritoneal macrophages were removed from C57BL/6 mice by peritoneal washing and enriched by plastic adherence. Cells (5×10^5 cells/well) were cultured at 37 °C in 5% CO_2 in DMEM supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 U/mL of penicillin and 50 μg/mL streptomycin. Cultures were kept in 96-well plates, primed with IFN-γ (100 IU/mL) (Kolodziej et al., 2008), incubated with parasites (10:1) and LPG (0.5 mM) purified from procyclic *Leishmania*. Culture supernatants were collected after 72 h and nitrite concentrations were determined by Griess reaction (Drapier et al., 1988).