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Leishmania major lipophosphoglycan: Discrepancy in toll-like receptor signaling

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ABSTRACT

Lipophosphoglycan (LPG) is structurally characterized by a series of phosphoglycan repeat units. Cellular LPG, isolated from promastigotes, has a very similar structure to culture supernatant LPG, but differs in the average number of phosphorylated oligosaccharide repeat units and in glycan composition. Comparison of these LPGs with capillary electrophoresis and immunoblotting indicate that these molecules are highly conserved structurally and composed of galactosylated Gal-Man repeats but their size and molecular weight are very different which is due to glycan portion. There are 30 and 20 repeat units in sLPG and mLPG, respectively. Both LPGs induced nitric oxide in macrophages cell line while sLPG had the higher stimulatory effect. In the presence of anti-TLR2 nitric oxide stimulated by LPG was reduced to control levels. In addition, in the presence of anti-TLR4, nitric oxide stimulated by LPGs was not affected. We propose that lipophosphoglycan induces nitric oxide production via TLR2 signaling pathway.

1. Introduction

Protozoan parasites of the genus *Leishmania* are the causative agents of leishmaniasis. The life-cycle of the pathogens alternates between an aflagellated amastigote form in the mammalian macrophage and a flagellated promastigote form in the midgut of the sand fly. Stage-specific adhesion of *Leishmania* promastigotes in the sand fly midgut is mediated by structural variation involving the abundant cell surface lipophosphoglycan (LPG) (Descoteaux and Turco, 2002). The basic LPG structure in all *Leishmania* species consists of a 1-*O*-alkyl-2-lyso-phosphatidyl inositol lipid anchor, a heptasaccharide glycan core, a long phosphoglycan (PG) polymer composed of $(Gal\beta1\text{-}4Man\alpha1\text{-}PO4)_n$ repeat units (n = 10–40), and a small oligosaccharide cap. In different *Leishmania* species the PG repeat units contain additional substitutions that mediate key roles in stage-specific adhesion (Mc Conville et al., 1987, 1992; Mahoney et al., 1999). In addition, promastigotes of the *Leishmania* species, shed LPG into the culture medium through releasing the micelles from the cell surface (Ilg et al., 1994). Like the cell associated LPG, culture supernatant LPG is amphiphilic and composed of a lysoalkylphosphatidylinositolphosphosaccharide core connected to species-specific phosphosaccharide repeats and oligosaccharide caps. Cell associated LPG and culture supernatant LPG have the same structure but their molecular sizes are different (Ilg et al., 1994).

The innate immune system senses the presence of invading microbes by the use of specific receptors that recognize pathogen-associated molecular patterns (PAMPs), and activation of these receptors initiates host responses. Sensing has been shown to be mediated by an ancient family of membrane proteins known as the Toll-like receptors (TLR), which recruit and activate signaling molecules involved in innate immunity. Members of the TLR family are responsible for the recognition of pathogen-associated molecular patterns expressed by a wide spectrum of infectious agents. TLRs activate the NF-κB pathway, which regulates cytokine expression, nitric oxide and reactive oxygen species production through several adaptor molecules including MyD88. Activation of the NF-κB pathway links innate and adaptive immune response by production of inflammatory cytokines such as IL-1, IL-6, IL-8, TNF-α, IL-12, chemokines and induction of costimulatory molecules such as CD80, CD86, and CD40 (Takeda et al., 2002; Van Der Kleij et al., 2002; Aderem and Ulevitch, 2000).

Therefore, we assessed structural and functional properties of parasite membrane isolated LPG (mLPG) and culture supernatant LPG (sLPG). The differences in structural properties was analyzed by immunoblotting and capillary electrophoresis. The effect of LPGs on nitric oxide (NO) production was studied on a murine macrophage cell line, J774.1A. The functional difference between these phosphoglycans in the presence of anti-TLR2 and anti-TLR4 was discussed.

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2. Materials and methods

2.1. Parasite culture and LPG purification

The parasites (strain MRHO/IR/75/ER) were cultured in the RPMI-1640 medium (Sigma, Chemical Co., St. Louis, USA) supplemented with 10% FBS (Sigma), 292 μg/ml L-glutamine (Sigma) and 4.5 mg/ml glucose. The starting parasite inoculation was 1×10^6/ml. Under these culture conditions, the stationary phase of parasite growth was obtained in six days as determined by peanut agglutination assay (Sacks et al., 1985). Membrane LPG (mLPG) was extracted from 10^{11} metacyclic promastigotes by solvent extraction, hydrophobic interaction chromatography and cold methanol precipitation (Turco et al., 1984). Soluble LPG (sLPG) from the culture medium was purified with ion-exchange chromatography, hydrophobic interaction chromatography and cold methanol precipitation (Ilg et al., 1996). The pellet was further analyzed for the presence of LPG by gel electrophoresis and immunoblotting.

2.2. Immunoblotting of Leishmania major LPG

The purified LPGs, as described above was subjected to electrophoresis on a SDS–PAGE gel using 5% and 12% polyacrylamide for the stacking and separating gel, respectively. The transfer was done at 12 V for 1 h onto nitrocellulose. The membrane was blocked with 5% milk in phosphate-buffered saline. The primary mouse monoclonal antibody WIC79.3 that recognizes the galactosylated Gal-Man-P repeat units of LPG was used at a 1:1000 dilution. A goat anti-mouse horseradish peroxidase secondary antibody (Pierce, USA) was used to detect the LPG at a 1:10,000 dilution.

2.3. Preparation of monosaccharide for capillary electrophoresis

Capillary electrophoresis profile was done by Prof. Turco. Briefly, LPG samples were incubated with 0.25 M sodium acetate pH 4.0, and 0.5 M sodium nitrite in a 1:1 ratio at 37 °C. The lipid portion was removed from the samples by C18-Sepharose columns equilibrated in water. The aqueous-soluble material was dried by vacuum centrifugation and subjected to strong acid hydrolysis in 2 N trifluoroacetic acid for 3 h at 100 °C. The multiple PO4-Gal linkages in LPG are refractory to hydrolysis under these strong acidic conditions. The substituents were dried using vacuum centrifugation and then labeled in a volume of 4 μl for 90 min at 55 °C with the fluorophore APTS (0.2 M) containing 1 M sodium cyanoborohydride, which labels the reducing termini of saccharides by reductive amination. The reaction was stopped by adding 46 μl of water, diluting the sample 10-fold. Prior to injection in CE, the samples were further diluted in H_2O at a ratio of 39:1. APTS-derivatized monosaccharides were separated using capillary electrophoresis system. An uncoated capillary was conditioned using each of 0.1 N HNO_3, 0.1 N HCl, and 0.1 N NaOH. Following the washes, the conditioning was completed with a methanol wash for 2.5 min and H_2O wash for 1 min. The samples were injected for 4 s at 0.4 psi and separated for 20 min at 20 kV with the settings of 100 μA maximum current at 25 °C, with a threshold of 2 and peak width of 9. Normal filter settings of the laser used were 488 nm excitation and 520 nm emission (Barron and Turco, 2006).

2.4. Analytical procedures

Phosphate was determined by the Ames method (Ames, 1996) and glycan content was determined by phenol–sulfuric acid method (Rao and Pattabiraman, 1989). Discontinuous SDS–PAGE was performed on 5% stacking and 12% separating gel according to the Lammeli method for 3–4 h using 120 V at room temperature. Following electrophoresis, gels were stained with Sudan Black (200 mg of Sudan Black B in 100 ml of 60% ethanol, overnight). In order to indicate the presence of gram negative bacterial endotoxin (LPS), Limulus Amoebocyte Lysate (LAL) assay was performed based on manufacturer's instruction (LAL Kit, Charles River Endosafe, T2092 CTK7, USA).

2.5. Macrophage culture, TLR blocking and nitric oxide assay

The murine macrophage cell line (J774.1A) was cultured in DMEM medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum. Adherent macrophages were scraped from flask and washed with warm medium (25 °C). Cells were counted and their viability was determined by trypan blue dye exclusion. J774.1A cells were cultured in 24 well plates (10^6 cells/ml, 1 ml/well) and incubated in 37 °C, 5% CO_2 for 18 h to adhere and non-adherent cells were removed. For TLR blocking macrophages pretreated with Anti-TLR2 (10 μg/ml, functional grade, ebioscience, USA) and anti-TLR-4 (10 μg/ml, functional grade, ebioscience, USA) for 2 h pretreated and untreated macrophages stimulated with purified *L. major* LPG (1 μg/ml). After 48 h the supernatants were collected and NO production was determined by Griess reagent (Miranda et al., 2001). Briefly, to 100 μl of culture medium, 100 μl of vanadium chloride (III) and 50 μl of Griess reagents [1:1 (v/v) of 0.1% naphthylethylenediaminedihydrochloride (NED) in H_2O + 2% sulphanilamide in 5% H_3PO_4] was added and incubated at 37 °C for 40 min and the absorbance was read at 540 nm.

2.6. Statistical analysis

Statistical significance ($P < 0.05$) was analyzed by Student's *t*-test using SPSS version 10.

3. Results

The *L. major* mLPG was extracted and purified by solvent extraction, hydrophobic interaction chromatography and cold methanol precipitation. The efficiency of purification was analyzed by Sudan Black staining and immunoblotting and one heterogeneous band was absorved (Fig. 1). Compositional analysis indicated that the ratio of glycan to phosphate is 2.5. The sLPG from culture medium was purified with ion-exchange chromatography, hydrophobic interaction chromatography and cold methanol precipitation. The purified sLPG was also examined by Sudan Black staining and immunoblotting and one heterogeneous band was observed (Fig. 1). Compositional analysis indicated that the ratio of glycan to phosphate is approximately 8. As seen in Western blotting of mLPG and sLPG purified from metacyclic stages of *L. major* promastigotes, both the membrane-bound and excretory LPGs are highly heterogeneous in size. The antibody used in this Western blot was WIC79.3 which recognizes Galα-1-3 side chains that branch of the Gal-Man-PO4 backbone of LPG. Thus, it is likely that the mLPGs and sLPGs are very similar in structure but their molecular weight is different and sLPG has higher molecular weight.

For analysis by capillary electrophoresis, the delipidated LPG was also hydrolyzed to substituents with trifluoroacetic acid and then the substituents were derivatized with the fluorophore APTS. Capillary electrophoresis profile indicate the existence of monosaccharide, disaccharide probably Gal-Man repeats unit, trisaccharide probably galactosylated Gal-Man repeats and tetrasaccharide probably digalactosylated repeat units that are same in two LPG preparation (Fig. 2). For quantitation purposes, response factors were determined for APTS-derivatized mannose and 2,5-anhydro-