INFECTION AND IMMUNITY, Feb. 1997, p. 783–786 Vol. 65, No. 2
0019-9567/97/$04.00+0

NOTES

Proteophosphoglycan Secreted by *Leishmania mexicana* Amastigotes Causes Vacuole Formation in Macrophages

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Received 5 July 1996/Returned for modification 9 September 1996/Accepted 24 October 1996

The amastigote form of *Leishmania mexicana* parasites colonizes macrophage phagolysosomes and induces the enlargement of these compartments to form huge parasitophorous vacuoles. We report here that a purified secreted amastigote product, proteophosphoglycan, is a macromolecule which causes vacuolization of peritoneal macrophages in vitro. Secretion of this glycoconjugate by intracellular parasites may contribute to the expansion of phagolysosomal compartments in infected cells.

Parasitic protozoa of the genus *Leishmania* are the causative agents of a variety of important tropical diseases (1). The mammalian stage of *Leishmania*, the amastigote, resides intracellularly in phagolysosomes of macrophages. In the case of parasites from the *Leishmania mexicana* complex, the initial phagolysosome rapidly expands to form a large parasitophorous vacuole (2, 14). It is currently unknown how the parasites manipulate their host cells to form these large compartments. However, it is well established that high-molecular-mass polyanionic polysaccharides cause vacuole formation in macrophages at low concentrations (5). We have recently characterized a polyanionic proteophosphoglycan (PPG), which is secreted by intracellular *L. mexicana* amastigotes into the parasitophorous vacuole. Amastigote-derived PPG shares some carbohydrate structures with two products of the insect stage of the parasite (promastigote), the major cell surface glycolipid, lipophosphoglycan, and a secreted PPG (8–10). In this study, we demonstrate that amastigote-derived PPG is highly effective in inducing macrophage vacuolization and may, therefore, be involved in the expansion of *L. mexicana*-containing phagolysosomes.

Purification of *L. mexicana* amastigote PPG and determination of its molecular size. *L. mexicana* amastigote PPG was purified from infected mouse tissue: dorsal lesions of CBA mice were excised, weighed, homogenized in 100 mM NaCl–5 mM EDTA–20 mM Tris-HCl (pH 7.5), and sonicated. The pellet obtained after centrifugation (5,000 × *g*, 20 min) was subjected to another round of homogenization, sonication, and centrifugation. The combined supernatants were processed further as previously described (9) except for the use of a Superose 6 column equilibrated in 250 mM ammonium acetate. This resulted in the inclusion of PPG instead of void volume elution, which occurred in the previously used Superose 12 column. After hyaluronidase digestion (9), benzonnuclease treatment (3), and phenol extraction, an additional Superose 6 chromatography was performed. The final product had the same composition and properties as the previously described PPG preparation as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, amino acid analysis, phosphate determination, and monoclonal antibody binding (9). Due to its high phosphate content (>14% [wt/wt] [9]), *L. mexicana* PPG is a polyanionic compound. The purification yielded 43 ± 17 μg of PPG per g of infected mouse tissue ($n = 5$). Size determination on a calibrated Superose 6 gel filtration column indicated a very high, yet heterogeneous, apparent molecular mass ranging from about 2×10^6 to 4×10^5 Da (Fig. 1A). Electron microscopic inspection of purified PPG after negative staining or rotary shadowing (18) confirmed its macromolecular nature and size heterogeneity. The molecules are rod shaped, up to 70 nm in length, and 25 to 30 nm in diameter (Fig. 2A and B) and differ from the antigenically related filamentous promastigote PPG (10). They can be labeled by antioligosaccharide monoclonal antibodies (MAb) (9).

***L. mexicana* amastigote PPG induces vacuole formation in macrophages.** Cells obtained by peritoneal lavage of BALB/c mice with 5 ml of Dulbecco's modified Eagle's medium (DMEM) containing 15% inactivated fetal calf serum (iFCS) were seeded on Lab-Tek Permanox Chamber slides (Nunc, Wiesbaden, Germany) at 10^5 cells/ml and incubated for 3 h at 37°C. Nonadherent cells were removed by three washing steps with DMEM–15% iFCS. After 2 days in culture, the adherent macrophages were incubated with different compounds dissolved in DMEM–15% iFCS. After various incubation times, the cells were washed three times with DMEM, fixed (9), and inspected by phase-contrast microscopy for vacuole formation. Macrophages containing more than five phase-translucent vacuoles were considered vacuolized. The background level of vacuolization was 4 to 6%.

Incubation with dextran sulfate (average molecular weight of 500,000; Sigma, Deisenhofen, Germany) led to the vacuolization of about 90% of the macrophages. This vacuolization was time and concentration dependent and was maximal after 5 to 6 h with 20-μg/ml dextran sulfate (Fig. 1B and C). Dextran 500 (Sigma), the neutral counterpart of dextran sulfate, caused no vacuolization above background levels (Fig. 1C) as previously reported (5). Exposure of macrophages to *L. mexicana* amastigote PPG led to vacuolization with characteristics similar to those of vacuolization with dextran sulfate (Fig. 1B and C). Concentrations of the compound as low as 3 μg/ml were effective (Fig. 1C), and maximal vesicle formation was ob-

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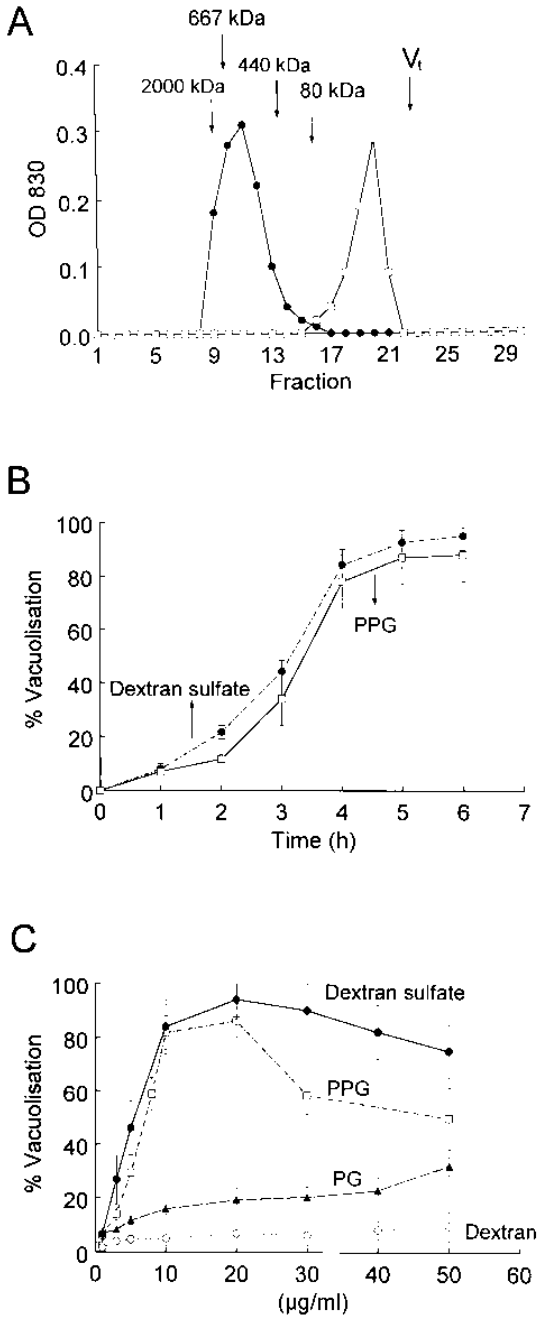

FIG. 1. (A) Superose 6 chromatography. Elution profiles of PPG (●) and PG (□) are shown. Molecular mass markers were blue dextran (2,000 kDa), thyroglobulin (667 kDa), ferritin (440 kDa), and human transferrin (80 kDa). CTP was used for the determination of the inclusion volume (V_t). The optical density (OD) at 830 nm indicates the phosphorus content of the fractions. (B) Time course of vacuolization in macrophages induced by dextran sulfate (20 μg/ml) and PPG (20 μg/ml). (C) Concentration dependence for vacuolization induced by dextran sulfate, PPG, and PG in macrophages (incubation time, 5 h). For every time point shown in panels B and C, 100 macrophages were evaluated. The average of three experiments is presented.

served after 6 h with 20-μg/ml PPG. The effect of suboptimal doses of dextran sulfate and PPG on macrophages was additive. Simultaneous incubation of cells with both compounds at a concentration of 2.5 μg (each) per ml resulted in 35% vacuolization, compared to 14 and 27% with PPG and dextran sulfate alone, respectively (Fig. 1C). The presence of PPG in the newly formed vacuoles was demonstrated by immunofluorescence and immunoelectron microscopy using the anticarbohydrate MAb LT22 (9). PPG clearly colocalized with the phase-translucent vesicles as shown by immunofluorescence (Fig. 3A and B), and electron microscopy of macrophage sections showed PPG in vesicles (Fig. 2C). By double-labeling experiments on ultrathin cryosections (17), it could be demonstrated that PPG-containing vesicles also contained LAMP-1 (rat MAb IG11) (Fig. 2D) and LAMP-2 (not shown), indicating the prelysosomal or lysosomal nature of the vesicles. With cryosections, we consistently detected much less PPG in the vesicles than we did with sections from resin-embedded cells. This was caused by poor fixation of PPG, which contains only very small amounts of lysine residues. Remarkably, most of the vacuoles containing PPG were not more than 2 μm in diameter and obviously did not fuse with each other. For unknown reasons, an increase in polyanion level beyond an optimal concentration can lead to a decrease in the number of vacuolized cells (Fig. 1C; see results for dextran sulfate and PPG) (5). The structurally related *L. mexicana* phosphoglycan (PG), obtained from promastigote lipophosphoglycan by treatment with phosphatidylinositol-specific phospholipase C (8), acted only as a weak vacuolization agent at very high concentrations (Fig. 1C). This result is likely due to the differences between the average molecular mass of PPG (~600 kDa) and that of PG (<80 kDa) (Fig. 1A). Similar results were reported earlier for the vacuolization potency of dextran sulfates with different degrees of polymerization (5).

Kielian and Cohn (12) demonstrated a more-than-100-fold accumulation of dextran sulfate in macrophage lysosomes, where the substance was present in the milligram-per-milliliter concentration range. Although the concentration of PPG in the parasitophorous vacuole has not been accurately measured, it is likely to be present in the milligram-per-milliliter range, because at least 40 μg/g of tissue is found in mouse lesions (see above) and the majority of this antigen is located in parasitophorous vacuoles (9).

We propose that PPG causes or at least contributes to the enlargement of phagolysosomes in *L. mexicana*-infected macrophages. First, purified PPG can induce vacuolization in macrophages. Second, amastigotes of *Leishmania major* apparently do not secrete PPG-like molecules (10a), and this parasite does not induce the formation of large parasitophorous vacuoles in macrophages. Third, there is an interesting correlation between the properties of vacuoles induced by polyanions and those induced by *L. mexicana* amastigotes. Polyanion-induced endosomes can fuse with lysosomes but not with large phagosomes (6, 7, 11). This effect may be due to charge interactions of polyanions with the luminal face of the lysosomal membrane modulating membrane fluidity and fusion (12, 13). Similarly, phagosomes formed after uptake of *L. mexicana* readily fuse with lysosomes (4, 15, 16), while the resulting parasite-containing phagolysosomes fuse inefficiently with vesicles containing latex beads or erythrocytes (19, 20). In contrast, these particles are efficiently transferred to the parasitophorous vacuoles of another intracellular pathogen, *Coxiella burnetii*. Vesicles containing smaller, yeast-derived particles, such as zymosan, do fuse with phagolysosomes harboring either *C. burnetii* or *L. mexicana amazonensis*, possibly involving carbohydrate ligands and host cell receptors (20). Their transfer to the parasite-containing vacuole is again much less efficient in *Leishmania*-infected than in *Coxiella*-infected host cells. The lower competence of *Leishmania*-containing vacuoles to fuse to large phagosomes may be caused by the polyanion PPG secreted by the intracellular parasites. More detailed analysis of PPG and

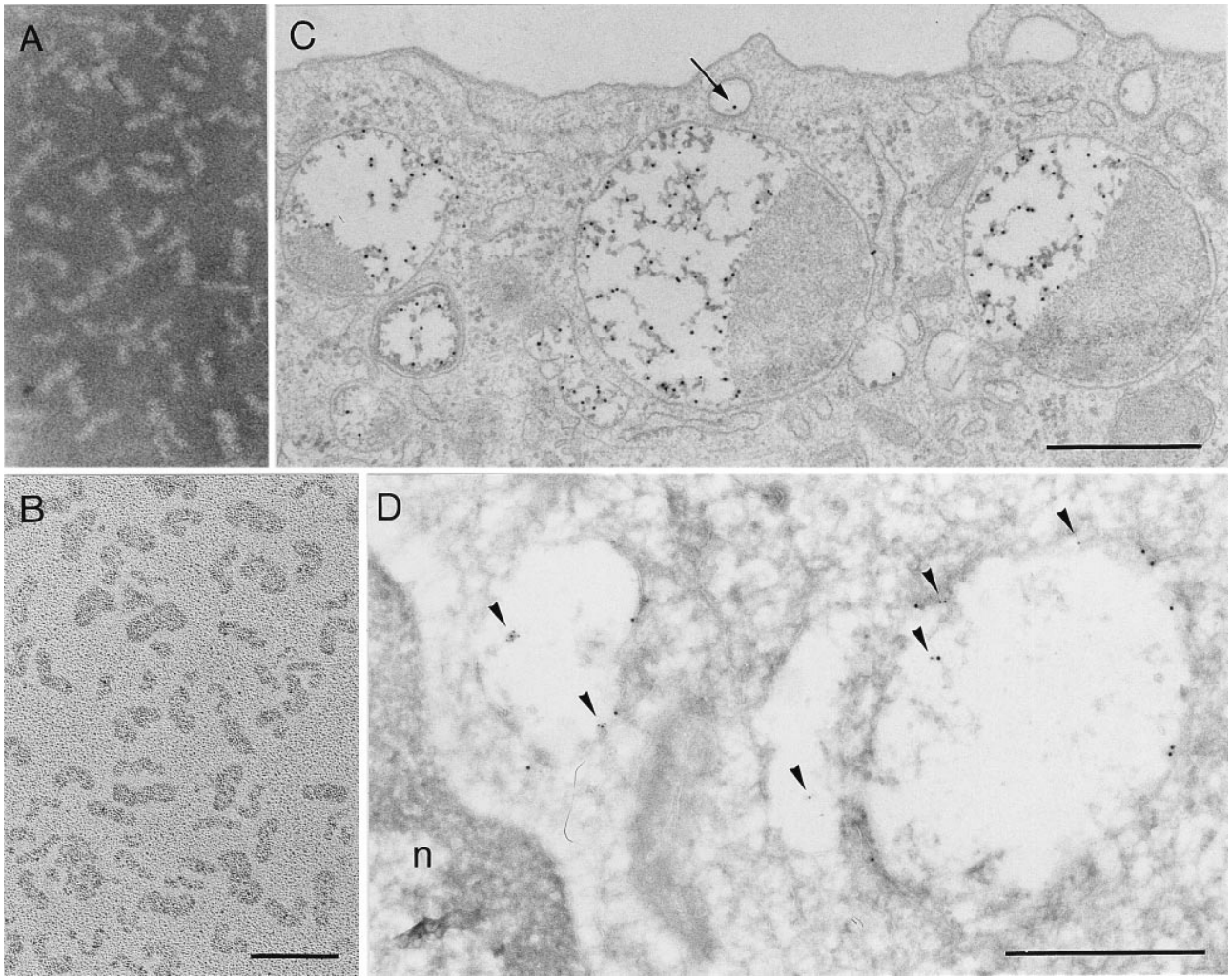

FIG. 2. Electron microscopy of PPG. (A) Purified PPG negatively stained with Nano-W (Nanoprobes, Brookhaven, N.Y.). (B) Purified PPG after glycerol spraying and rotary shadowing with platinum-carbon. (C) Macrophages treated for 5 h with 10-μg/ml PPG and then embedded in resin. Shown is an on-section labeling with MAb LT22 and protein A–15-nm-diameter gold particles. The arrow points to a labeled coated pit, suggesting endocytosis via a receptor. (D) Immunogold double labeling on ultrathin cryosections of PPG (MAb LT22 followed by protein A–6-nm gold) (arrowheads) and LAMP-1 (rat MAb IG11 followed by goat anti-rat IgG–12-nm gold [Aurion, Wageningen, The Netherlands]) in macrophages fed with PPG. n, nucleus. Bars, 100 nm (A and B) (the bar in panel B is for panels A and B) and 500 nm (C and D).

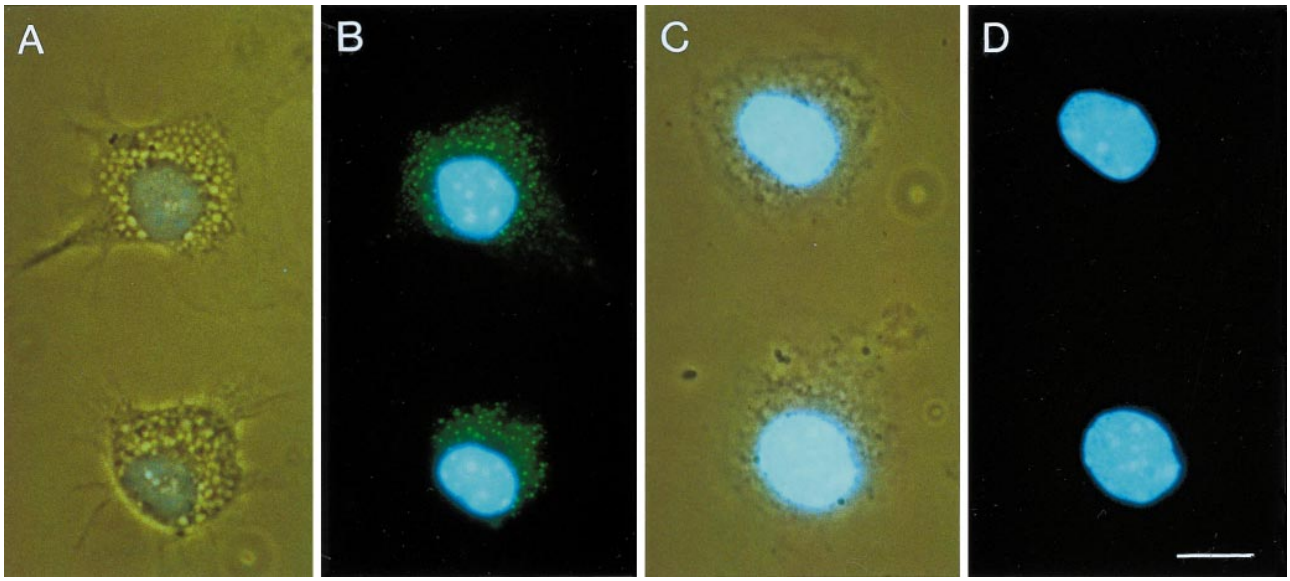

FIG. 3. Light (A and C) and fluorescence (B and D) microscopy of PPG-induced vacuolization in macrophages. After 5 h of incubation with 10-μg/ml PPG (A and B) or without PPG (C and D), cells were fixed and permeabilized and endocytosed PPG was labeled with MAb LT22 and fluorescein isothiocyanate-labeled secondary antibodies and DAPI (4′,6-diamidino-2-phenylindole) for nuclear staining. PPG-treated cells show strong vacuolization (A) and colocalized, spotty fluorescence (B). Bar, 10 μm.

its action on membrane fusion and fission may reveal the biochemical basis for this fascinating modulation of vesicular traffic by *Leishmania* parasites.

We thank T. Aebischer and P. Overath for critically reading the manuscript, G. Griffiths for antibodies against LAMP-1 and LAMP-2, and K. Pohl and G. Müller for electron microscopic and photographic work.

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Editor: J. M. Mansfield