LOCALIZATION OF LIPOPHOSPHONOGLYCAN ON BOTH SIDES

OF *ACANTHAMOEBA* PLASMA MEMBRANE

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INTRODUCTION

Recent studies have shown that approximately 30% of the plasma membrane of *Acanthamoeba castellanii* consists of a complex carbohydrate termed lipophosphonoglycan (6, 11, 13). Lipophosphonoglycan contains about 14% long chain saturated and 2-hydroxy fatty acids, 10% aminophosphonic acids, 3% esterified phosphate, 26% neutral sugars, and 3% amino sugars. The neutral sugars are glucose, mannose, galactose, and xylose in the mole ratio of 5:4:1:1, and the amino sugars are galactosamine and glucosamine, 3:1. Because of this chemical composition it was expected that lipophosphonoglycan would react with stains that are selective for complex carbohydrates. By use of selective stains that can be visualized in the electron microscope we hoped to obtain information about the distribution of lipophosphonoglycan in the ameba plasma membrane. The results with three such stains, concanavalin A, ruthenium red, and phosphotungstic acid-chromic acid, indicate that the sugars and acidic groups of lipophosphonoglycan are exposed at both sides of the *Acanthamoeba* plasma membrane.

MATERIALS AND METHODS

Acanthamoeba castellanii (Neff strain) was cultured axenically in 1.5% proteose peptone-1.5% glucose solution with added vitamins in rotating low-form culture flasks (13). Plasma membranes were isolated according to procedures described previously (27).

The procedure of Bernhard and Avrameas (3) was used to stain membranes with concanavalin A-horseradish peroxidase (Con A-HRP). Approximately 1 mg of unfixed plasma membranes was incubated in 20 ml of 0.01 M Tris-HCl buffer, pH 7.4, containing 25 μg/ml of Con A for 25 min followed by two buffer rinses. The membranes were resuspended in the same buffer with 1 mg/ml HRP for 15 min, washed once in buffer, and fixed in 3% glutaraldehyde for 30 min. The fixative was washed out and the membranes were incubated in 3,3'-diaminobenzidine solution for 15 min (8). The membranes were then pelleted, refixed in 3% glutaraldehyde for 15 min, postfixed in OsO$_4$ for 1 h, and embedded in Epon (14). The incubations, fixations, and embedments were carried out at room temperature. Control preparations were treated identically except for the addition of 0.02 M α-methyl mannoside to the Con A solution 10-20 min before the incubation of the membranes.

Whole cells were rinsed twice in 0.1 M sodium phosphate buffer, pH 6.8, then once in 0.02 M NaCl
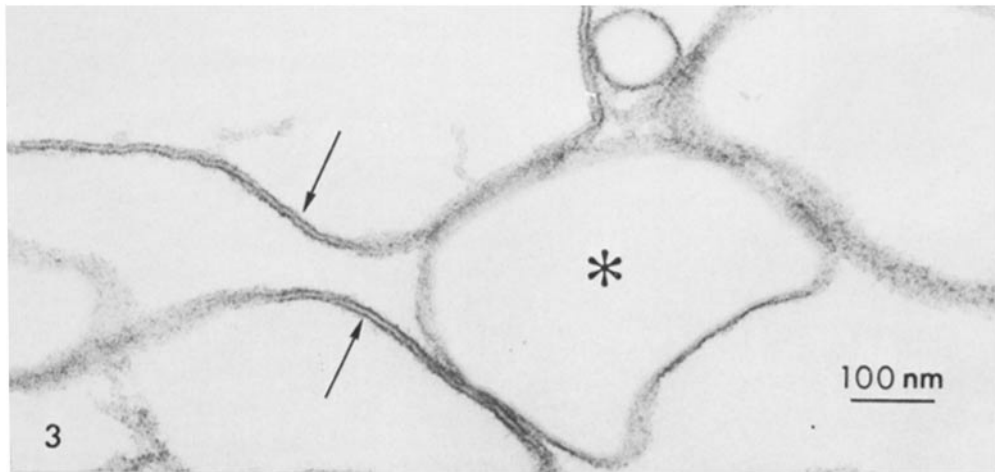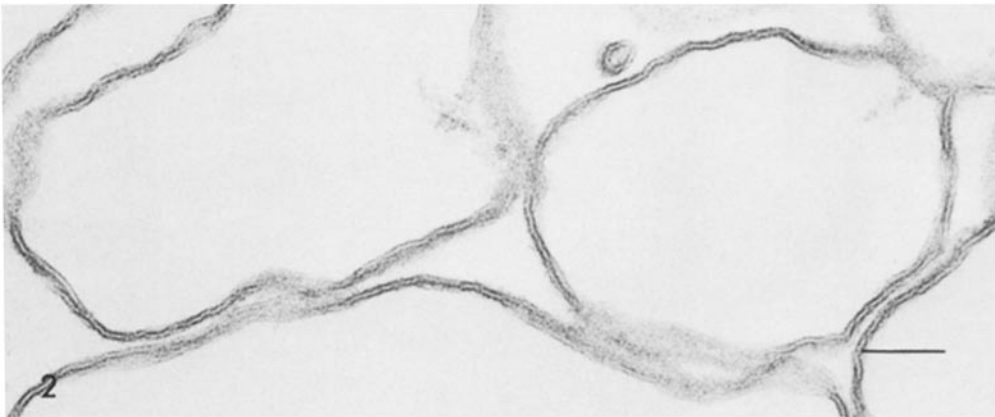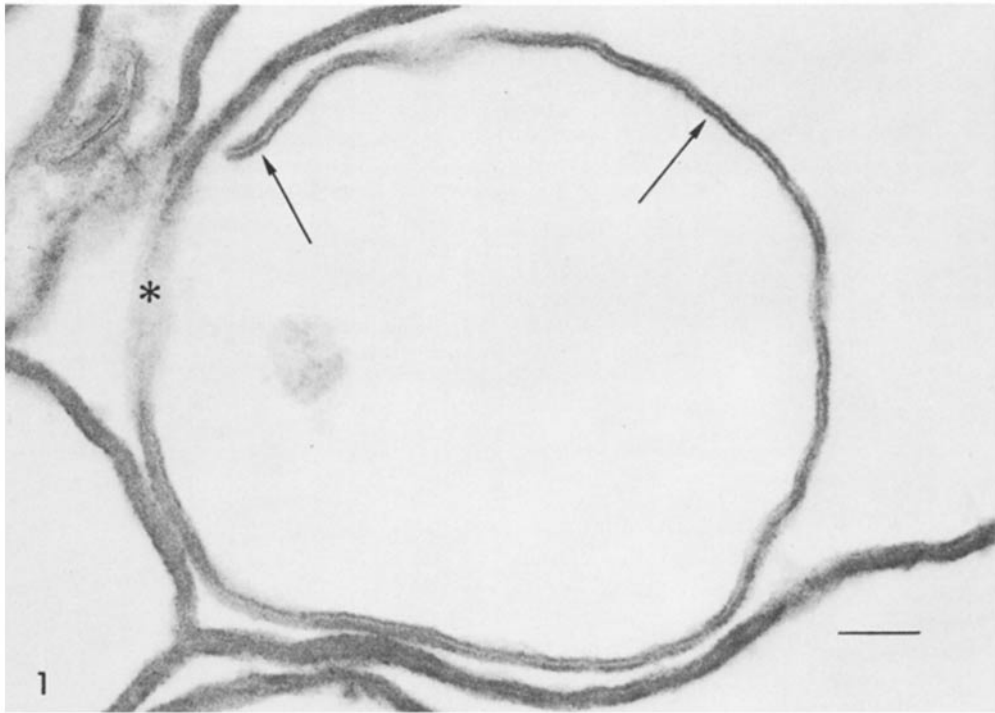
containing 0.1 M cellobiose. The cells were suspended in the NaCl-cellobiose solution containing 100 μg/ml of Con A for 1 h at 4°C, followed by two rinses in cold NaCl-cellobiose solution, and were resuspended in the same solution with 1 mg/ml HRP for 1 h at 4°C. The cells were then fixed in glutaraldehyde, incubated in diaminobenzidine, and carried through to embedment as above.

Membranes and cells were stained with ruthenium red exactly as described by Luft (15). The protocol of Roland, Lembi, and Morré (23) was used for staining sections of Epon-embedded cells and membranes with phosphotungstic acid-chromic acid.

RESULTS

Isolated plasma membranes of *Acanthamoeba* were incubated in the presence of concanavalin A, a ligand that binds to oligosaccharides with terminal glucose and mannose residues. Subsequent binding of horseradish peroxidase to the Con A allows visualization of the Con A-HRP complex. It was found that both sides of the *Acanthamoeba* plasma membrane stained with this technique (Fig. 1). The staining is specific since it is blocked by the addition of α-methyl mannoside to the Con A solution (Fig. 2). As expected from its sugar content, purified lipophosphonoglycan interacts with, and is precipitated by, Con A in a specific reaction that is inhibited by α-methyl mannoside (Table I). Since lipophosphonoglycan accounts for all the neutral sugars of the *Acanthamoeba* plasma membrane (11) these results show that glucose and/or mannose residues of the lipophosphonogly-can are exposed at both surfaces of the isolated plasma membrane.

Ruthenium red is an inorganic dye that complexes readily with compounds of high anionic charge density. It, too, stains both sides of the

TABLE I
Reaction of Purified Lipophosphonoglycan with Concanavalin A

LPG	Con A	α-methyl mannoside	Soluble P
mg	*mg*	*μmol*	*μmol*
0.2	0	0	0.24
0	1	0	0
0	0	40	0
0.2	1	0	0
0.2	1	10	0.19
0.2	1	40	0.21

All in 1 ml of 0.1 M sodium acetate, pH 7.0, with 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$. Tubes were let stand at room temperature for 1 h, and centrifuged for 15 min, and the supernate was assayed for phosphorus (4).

isolated plasma membrane (Fig. 3). While ruthenium red does not precipitate purified lipophosphonoglycan from solution, it does stain lipophosphonoglycan immobilized in polyacrylamide electrophoretic gels (Fig. 4). The interaction of ruthenium red and lipophosphonoglycan is further demonstrated by the observation that, when applied simultaneously with the sample to the gel, ruthenium red prevents the electrophoretic migration of lipophosphonoglycan. Therefore, the staining of membranes with ruthenium red suggests that the acidic groups of lipophosphonoglycan are also accessible at both sides of the isolated plasma membrane.

Since neither Con A nor ruthenium red penetrates the membrane of intact cells, these stains had to be applied to isolated plasma membranes in order to demonstrate staining of both sides of the membrane. The possibility exists, however, that the manipulations required for membrane isola-

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FIGURE 1 Unfixed plasma membrane was incubated in Con A and stained with HRP, subsequently postfixed, and embedded in Epon. In favorable orientations the electron-lucent space of the plasma membrane is seen between two layers of HRP reaction product (arrows). Fuzzy areas of the membrane (asterisk) are regions in which the membrane twists in the plane of section. Sections stained with uranyl acetate and lead citrate. × 109,000.

FIGURE 2 Control membranes prepared as for Fig. 1, except that α-methyl mannoside was added specifically to block Con A binding. Note that there is no binding of HRP to the membrane when Con A binding is blocked. Sections stained with uranyl acetate and lead citrate. × 113,000.

FIGURE 3 Isolated plasma membrane stained with ruthenium red during fixation showing that open sheets of membrane are stained on both sides (arrows). In closed membrane vesicles (asterisk), only the outer side of the membrane is stained. The unstained inner leaflet, visible as a faint gray line, indicates the contrast of unstained material. The sections were not stained with uranyl acetate and lead citrate. × 110,000.

tion and purification might have altered either or both surfaces. Whether the outer surface stains with these two reagents before isolation can be tested with intact cells that have been gently rinsed

FIGURE 4 Scans of purified lipophosphonoglycan after electrophoresis on acrylamide gels and staining with ruthenium red (top scan) or periodic acid-Schiff reagent (bottom scan). 10 μg of lipophosphonoglycan were analyzed on 7% acrylamide-7% butanol gels as described by Korn and Wright (12). The PAS-stained gel was fixed in 12.5% TCA before staining; the ruthenium red-stained gel was not fixed before staining.

free of culture medium. Both Con A and ruthenium red stain the outer surface of intact cells, albeit somewhat more intensely than isolated membranes (Figs. 5, 6).

The inner and outer aspects of membranes are, of course, equally accessible in plastic-embedded thin sections. Phosphotungstic acid at low pH is a simple stain that can be applied to sectioned material and that appears to be selective for polysaccharides. Phosphotungstic acid stains material on both sides of the plasma membrane of deosmicated thin sections of Epon-embedded *Acanthamoeba* cells (Fig. 7), as well as both sides of isolated membranes (Fig. 7, inset). Therefore, staining of intact cells appears to be similar to that of the isolated membranes.

DISCUSSION

The *Acanthamoeba* plasma membrane is sufficiently well characterized chemically to allow correlation of the observed staining with the presence in the membrane of lipophosphonoglycan. Concanavalin A is a stereospecific reagent that binds most avidly to α-D-mannosyl and α-D-glucosyl residues (7). About 30% of the mass of lipophosphonoglycan is accounted for by neutral sugars, of which mannose and glucose comprise 81%, and concanavalin A reacts with purified lipophosphonoglycan. Since all of the glucose and mannose in the membrane are contained in lipophosphonoglycan, the staining of both sides of the plasma membrane by concanavalin A is convincing evidence that sugar residues of lipophosphonoglycan are found on both sides of the isolated plasma membrane.

Ruthenium red is a less specific stain and complexes with a number of molecules that have in common a high anionic charge density (15). This
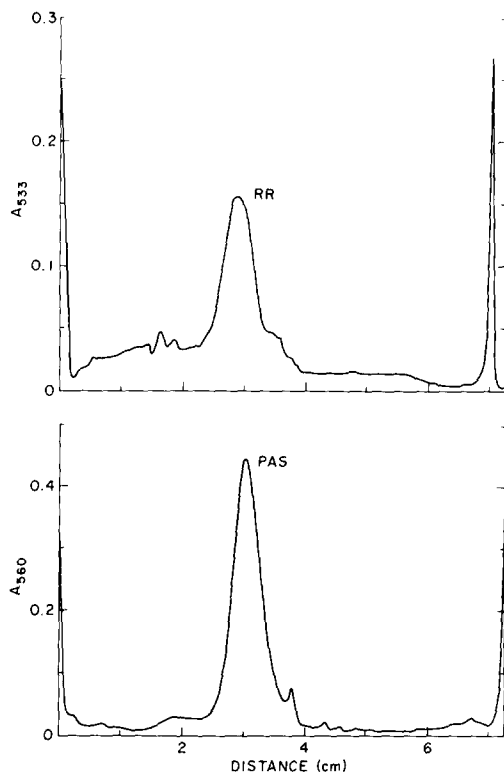
FIGURE 5 Intact *Acanthamoeba* stained with ruthenium red during fixation. The stain does not penetrate the membrane and stains only the outer leaflet. The inner leaflet of the membrane is visible as a faint gray line. The cytoplasm (C) lacks contrast because the sections were not stained.

FIGURE 6 Intact *Acanthamoeba* incubated with Con A and HRP before fixation. The outer leaflet of the plasma membrane shows reaction product. Inset: control cell carried through the same procedure except that α-methyl mannoside was added to block Con A binding. Sections stained with uranyl acetate and lead citrate. × 91,000.

FIGURE 7 Conventionally fixed, Epon-embedded *Acanthamoeba* stained in section only with 1% phosphotungstic acid in 10% chromic acid. There is stainable material on both sides of the plasma membrane (arrows). In addition, some glycogen granules are stained (g). Membranes of internal vesicles (asterisks) are not stained. × 113,000. Inset, isolated plasma membrane fraction fixed only in glutaraldehyde and stained in section as above. × 175,000.
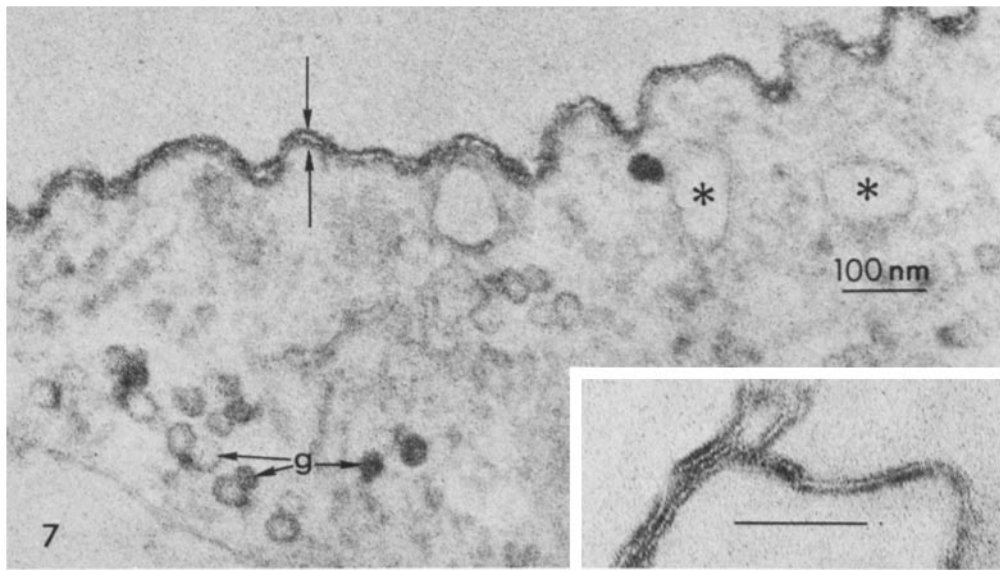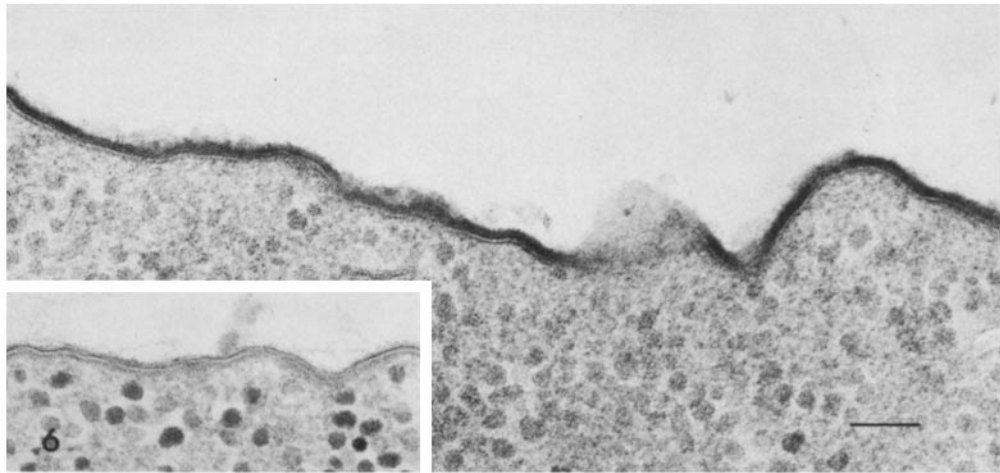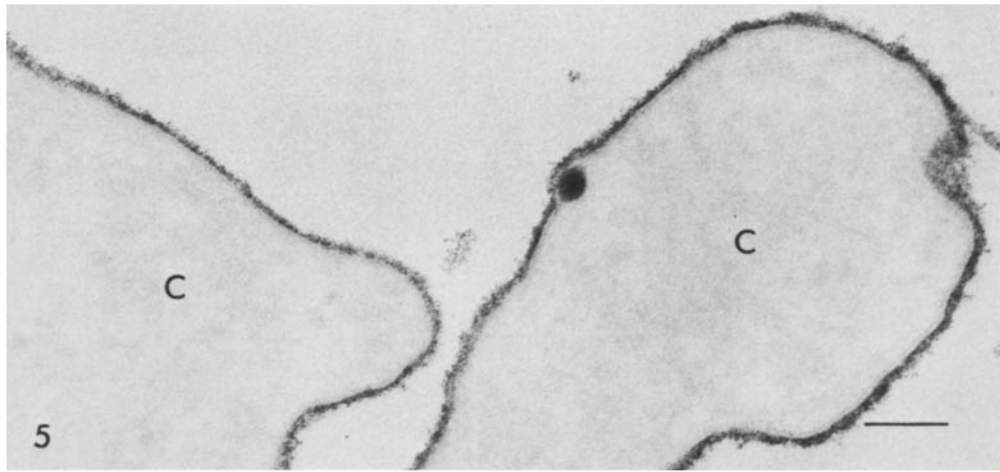
raises the question of whether some component of the membrane other than lipophosphonoglycan might account for the staining with this reagent. The lipids and proteins of *Acanthamoeba* plasma membranes have relatively low percentages of acidic groups (12, 27) and thus it is unlikely that either has sufficient charge density to be stained by ruthenium red. Purified lipophosphonoglycan, on the other hand, has an isoelectric point at pH 3 (Dearborn and Korn, unpublished observations) because of its phosphonic and phosphoric acid content, and is therefore highly charged around neutrality. Moreover, ruthenium red also reacts with purified lipophosphonoglycan. It is therefore most probable that the staining of membranes by ruthenium red demonstrates the presence of acidic groups of lipophosphonoglycan on both surfaces of the plasma membrane.

Although there is some disagreement about the chemical specificity of phosphotungstic acid, numerous cytochemical studies have shown that acidic solutions of phosphotungstic acid selectively stain PAS-positive material in cell sections (19–22). In its present application, phosphotungstic acid is particularly useful in confirming the presence of stainable material on both sides of the plasma membranes of fixed, intact cells, thus providing a control for possible artifacts that might be introduced by isolating plasma membranes.

Purified lipophosphonoglycan is highly aggregated in water, partially dissociated in water saturated with 1-butanol, and completely dissociated by dodecyl sulfate into two polymers that can be separated electrophoretically (11). We know nothing about the extent of aggregation of lipophosphonoglycan molecules within the ameba plasma membrane. Nor do we know whether it is equally distributed on the two sides of the membrane. The stains used in the present study are probably unsuitable for determining quantitative differences in distribution between two sides of the membrane. The visualizations of Con A-HRP and ruthenium red apparently depend on catalytic reactions with OsO_4 (9, 15) that would tend to obscure any quantitative differences that might exist. Considerable morphological asymmetry is evident when the isolated *Acanthamoeba* plasma membrane is examined by freeze-etching (Bowers and Olszewski, unpublished observations). When the frozen membranes are fractured and deep-etched, portions of the true inner and outer surfaces are revealed by the etching. The inner surface of the plasma membrane is relatively smooth whereas the outer surface shows many particles. Although the freeze-etch technique probably does not visualize highly hydrated carbohydrate moieties, this morphological asymmetry is surely a reflection of an underlying chemical asymmetry in the plasma membrane which may also involve the lipophosphonoglycan in some way. It is hoped that these ambiguities will be resolved by studies currently under way.

The localization of lipophosphonoglycan in the *Acanthamoeba* plasma membrane is clearly unrelated to the well-developed carbohydrate-containing surface coat that extends 100–200 nm beyond the plasma membrane of some large amebas (1, 18). The present studies confirm earlier evidence for the absence of such a surface coat in *A. castellanii* as observed either by transmission electron microscopy (4) or by light microscopy after staining with periodic acid-Schiff reaction. It is most probable that lipophosphonoglycan is an integtral component of the *Acanthamoeba* plasma membrane with its fatty acids interdigitated among the fatty acids of phospholipids on both sides of the membrane bilayer. The ameba lipophosphonoglycan has many chemical features similar to the well-characterized bacterial lipopolysaccharide. There is chemical evidence for such an integration of the fatty acid moieties of bacterial lipopolysaccharide into the phospholipid bilayer component (outer membrane) of gram-negative bacterial walls (24). Other studies using ferritin-tagged antibodies to lipopolysaccharide have localized it on both sides of this outer membrane but only on the outer surface of the inner or plasma membrane of the bacterium (25). A number of studies have reported the presence of sugars of glycoproteins and glycolipids on the external surfaces of mammalian cells (10, 16), but in the few cases studied, there is no evidence for carbohydrate moieties on the *inside* surface of mammalian plasma membranes (2, 17, 26). Mammalian cell plasma membranes are not known to contain a component analogous to the ameba lipophosphonoglycan, and the *Acanthamoeba* membrane appears not to have any glycoproteins. It may be that the significant functional fact for the cell is the display of oligosaccharides at the surface regardless of the chemical nature of the moiety that anchors them in the membrane. That is to say, presumably the sugars exposed on the exterior of

the ameba cell will have some functional similarities to those on mammalian cells. The ameba cells, for example, have functional characteristics similar to those of mammalian macrophages. The data at present are insufficient to determine whether the presence of exposed sugars on both sides of the *Acanthamoeba* plasma membrane, thus far a unique observation, is a consequence of unusual biosynthetic pathways for this membrane or of some as yet undiscovered novel functional properties.

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