Histochemical evidence for lipid A (endotoxin) in eukaryote chloroplasts

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ABSTRACT **Lipopolysaccharide (LPS) (a.k.a., endotoxin) is an essential component of the outer leaflet of the outer membrane of Gram-negative bacteria and is a potent activator of the innate immune system of animals. Lipid A, the glycolipid core of LPS, is the agent responsible for disease and death from Gram-negative sepsis, an important cause of human mortality and morbidity. Although it is generally accepted that lipid A is restricted to the prokaryotes, recent efforts to purify molecules from green algae with structural features unique to lipid A have met with success. Furthermore, the vascular plant *Arabidopsis thaliana* has been found to contain genes that encode all of the enzymes of the biosynthetic pathway for lipid A. It is not known whether vascular plants synthesize lipid A or where lipid A might be located in the tissues. For the present study, we used affinity reagents for lipid A to probe green alga and tissues of the garden pea for a light microscopic localization of lipid A in these eukaryote cells. We find staining for lipid A in free-living and endosymbiotic green algae and in the chloroplasts of vascular plants, indicating that this molecule is not restricted to prokaryotes, but is found also in select eukaryotes.—Armstrong, M. T., Theg, S. M., Braun, N., Wainwright, N., Pardy, R. L., Armstrong, P. B. Histochemical evidence for lipid A (endotoxin) in eukaryote chloroplasts. *FASEB J.* 20, E1506–E1509 (2006)**

Key Words: lipopolysaccaride · E. coli · LALF · prokaryote

Lipopolysaccharid (LPS) constitutes the principal lipid of the outer leaflet of the outer membrane of Gram-negative bacteria and the cyanobacteria (1). Lipid A, the phospholipid core of LPS, is a complex amphipathic glucosamine-based phospholipid with unusual β-hydroxy fatty acids that is derivatized with a disaccharide of the novel sugar, 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) (2). Lipid A is the causative agent of Gram-negative sepsis, a potentially lethal condition claiming more than 100,000 lives annually in the U.S., and with no known cure (3), making it a target for intensive investigation. Lipid A activates the innate immune system of a variety of animals, including arthropods (4;5) and mammals (6), and plays a role in nitrogen-fixing nodules of legumes (7). It is generally thought that lipid A is unique to Gram-negative cells and that this moiety is diagnostic for these bacteria (8). Thus, the discovery of a lipid A-like molecule in unicelluar algae was unanticipated. The algal molecule contains Kdo and β-hydroxy fatty acids and thus is chemically similar to bacterial lipid A (9–11). Furthermore, the vascular plant, *Arabidopsis thaliana* (Angiospermae, Dicotyledonae) has been found to contain genes that encode all of the enzymes of the lipid A biosynthetic pathway (2), although it is not known whether vascular plants synthesize lipid A or where lipid A might be located in the tissues. The object of the present study is to investigate the presence and to determine the location of lipid A-like molecules in green algae and vascular plants.

MATERIALS AND METHODS

Organisms

Escherichia coli (strain DH5α) were harvested from log phase cultures grown in Luria broth. *Chlorella* sp (strain NC64A, American Type Culture Collection (ATCC) 50528) was cultured under axenic conditions (11). *Paramecium busaria* and *Chlorohydra viridis* were obtained from Ward's (Cat 87W1305 87W2120, Rochester, NY, USA). Pea chloroplasts were isolated as in ref. 12.

Affinity labeling

Whole cells and tissues were fixed in freshly prepared 4% paraformaldehyde in PBS (10 min, room T), then quenched with 0.1 M glycine and blocked with 5 mg/ml bovine serum albumin (BSA). Cells and paraffin-sectioned tissues were exposed to recombinant Limulus antilipopolysaccharide factor (LALF) in Tris-buffered saline (TBS), followed by treatment with rabbit anti-LALF and Cy2-goat anti-rabbit IgG (Jackson Laboratories, Baltimore, MD, USA), or to polymyxin

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B labeled with the fluorophore biodipy (Molecular Probes, Eugene, OR, USA; cat P-13235, lot 4481–1). Biodipy-labeled polymyxin B was preincubated with bacterial LPS (Sigma cat. L-3129) for use as a negative control for biodipy-polymyxin B staining. Paraffin sections of pea seedlings and hydra were prepared according to standard methods. Because of the potential presence of contaminating LPS, solutions were prepared in pyrogen-free water (Sigma W3500), and all relevant glassware and microscope slides (Fisher Superfrost/Plus cat 12–550-15) were decontaminated by baking 4 h at 180°C. A Zeiss Axioplan light microscope and a Leica DMRBE confocal microscope were used for microscopy.

RESULTS

We used two lipid A binding agents to identify and localize lipid A in cells: LALF (Limulus antilipopolysaccharide factor), a 12 kDa protein from the secretory granules of the blood cells of the horseshoe crab (13), and polymyxin B, an antibiotic of fungal origin (14). The two probes for lipid A both bind strongly to that molecule (15–18). Polymyxin B also binds certain anionic phospholipids such as phosphatidic acid (19) and phosphatidyl glycerol (20). LALF appears to be more selective for lipid A (21;22). LALF binding was detected by immunohistochemical staining, and the presence of the fluorescent dye conjugate biodipy-polymyxin B identified the binding targets of polymyxin B. We also tested an antilipid A monoclonal antibody (mAb) and human LPS binding protein (LBP), but neither reagent stained our positive control, the Gram-negative bacterium *E. coli*, making them unsuitable as staining probes. Perhaps both molecules were too large to gain access to the lipid A moiety buried in the outer leaflet of the outer membrane of the bacterium.

The positive control, the Gram-negative bacterium *Escherichia coli*, showed affinity labeling of the surface with both LALF and polymyxin B (**Fig. 1*A***). Staining failed to occur when LALF was omitted, but first and second antibodies were present (Fig. 1*B*, *C*). We also found intense staining of the axenically grown free-living green algae, *Chlorella* (strain NC64A) (Fig. 1*D*) and the related species *Prototheca* (strain 289, an achlorophyli, white algae that is an obligate heterotroph and an opportunistic pathogen of livestock and humans; data not shown). The intracellular symbiotic green algae of the ciliate protozoan, *Paramecium bursaria* (Fig. 1*E*), and of the green coelenterate, *Chlorohydra viridissima* (Fig. 1*F*), also stained. Chloroplasts isolated from pea seedlings (Fig. 1*G*) and chloroplasts of paraffin-sectioned pea seedling leaves (Fig. 1*I*) stained with both

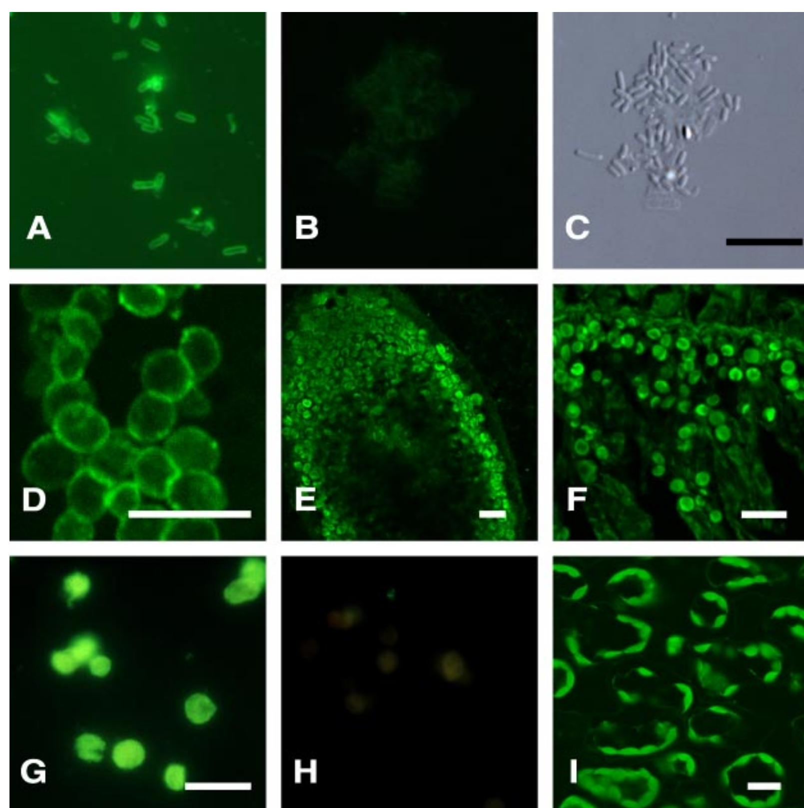

Figure 1. Staining of lipid A, the core component of LPS, utilized the lipid A binding moieties, LALF (*A*, *D*, *G*) and biodipy-conjugated polymyxin B (*E*, *F*, *I*). For all micrographs, the specimens were fixed in freshly prepared 4% paraformaldehyde in PBS (10 min, room T), then blocked successively with 0.1 M glycine in TBS and 5 mg/ml BSA in the same buffer. Specimens were examined with Zeiss Axioplan and Leica DMIRBE confocal microscopes. To reduce the likelihood of contamination with exogenous bacterial LPS, all reagents were prepared in pyrogen-free water (Sigma cat W3500), and glassware and microscope slides (Fisher Superfrost/Plus cat 12–550-15) were treated at 180°C for 4 h to remove LPS. Staining is seen with our positive control, the Gram-negative bacterium, *E. coli* (*A*), the green alga *Chlorella* sp., strain NC64A (*D*), the endosymbiotic algae of the cilliate protozoan, *Paramecium busaria* (*E*), the intracellular algae of the green hydra, *Chlorohydra viridissima* (*F*), isolated chloroplasts from the garden pea (*G*), and chloroplasts in paraffin sections of pea leaf tissue (*I*). The intracellular algae (*E*, *F*) and chloroplasts *in situ* (*I*) stained more intensely than the organelles and other membrane systems of the host cells. When LALF was omitted from the staining regimen (*B, C*, fluorescence and phase contrast micrographs of a clump of *E. coli* exposed to all reagents for LALF immunostaining but with the omission of LALF; *H*, fluorescence micrograph of isolated pea seedling chloroplasts stained identically to the bacteria of panel *B*) or when polymyxin B was preincubated with bacterial LPS (data not shown), staining failed to occur. The pairs of photomicrograpys (*A, B* and *G, H*) were photographed and processed identically. The brighter background fluorescence in panel *A* is due to staining of LPS that has adsorbed onto the glass microscope slide from the buffer in which the bacteria were suspended and presumably is material that had been shed into the buffer by the bacteria. The autofluorescence of chlorophyl did not contribute to the fluorescent signal from algae (not shown) or chloroplasts (*H*). Bar, 10 μm.

lipid A binding agents. Isolated membranes from pea seedling chloroplasts (a gift from Prof. Dr. Soll) stained with LALF and biodipy-polymyxin B (data not shown). Chlorophyl, an abundant molecule of green algae and chloroplasts, is strongly fluorescent at longer wavelengths than those used for the fluorescein and biodipy reporter dyes used in these assays (fluorescence emission for chlorophyl, 640–685 nm) and did not contribute to the fluorescent signal for confocal (not shown) or epifluorescence (Fig. 1*H*) images.

Bacterial LPS is a frequent contaminant of fluids and surfaces. Contamination of biological samples with bacterial LPS has been the cause of a number of false attributions of novel biological activities to particular agents where the responses were actually due, not to the agents themselves, but to contaminating LPS (23–25). To minimize the possibility that contaminating LPS of bacterial origin was bound to the surfaces of the cells and organelles, we processed axenically grown algae and all specimens with fixatives and other reagents prepared in LPS-free water (Sigma). Presumably the natural intracellular location of the symbiotic algae of *Paramecium* (Fig. 1*E*) and *Chlorohydra* (Fig. 1*F*) and of chloroplasts (Fig. 1*I*) would protect them from exposure to bacterial LPS from the environment prior to paraffin sectioning. Our results suggest that the lipid A detected on these cells and organelles is endogenously synthesized.

DISCUSSION

It is generally believed that LPS and its membrane anchor, lipid A, are found uniquely in select prokaryotes. Recently this view has been challenged by reports that glycolipids with the chemical signature moieties of lipid A can be purified from green algae and by the observation that genes encoding the proteins of the enzymatic pathway for biosynthesis of lipid A are present in vascular plants. Genetic studies do not show that lipid A is actually synthesized by vascular plants, nor do they indicate its localization in the cell. Our histochemical observations indicate that lipid A is synthesized by vascular plants and is localized to the chloroplasts. Chloroplasts are thought to be evolved endosymbiotic relatives of present-day Gram-negative cyanobacteria (26). It is possible that the genes encoding the biosynthetic pathway responsible for lipid A biosynthesis were introduced into the primitive collectives that represented the ancestral eukaryote cells of vascular plants via these endosymbiotic cyanobacteria. The adaptive significance of lipid A in the chloroplast is uncertain but it can be presumed to be important because lipid A is still present even after hundreds of millions of years of evolutionary integration of the chloroplast into the economy of the plant cell. FJ

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SPECIFIC AIM

The lipopolysaccharides (LPS) are a set of glycolipids that constitute major constituents of the outer leaflet of the outer membrane of the Gram-negative bacteria and are thought be restricted to this class of prokaryotes (**Fig. 1**). We investigated the presence and localization of lipid A, the lipid core of LPS, in eukaryotic cells using specific lipid A binding reagents for histochemical staining and light microscopy.

PRINCIPAL FINDINGS

1. The lipid A binding agents, polymyxin B and LALF, can be used to localize lipid A in cells

Biodipy-conjugated polymyxin B, an antibiotic of fungal origin, and the lipid A binding protein, LALF (Limulus antilipopolysaccharide factor), a 12 kDa protein from the secretory granules of the blood cells of the horseshoe crab, can be used to localize lipid A in prokaryotic cells. Biodipy-conjugated polymyxin B can be visualized directly; indirect immunohistochemistry using an anti-LALF antibody (Ab) was used to visualize the localization LALF. Both probes bound to the surface of the positive control, the Gram-negative bacterium, *Escherichia coli* (**Fig 2*A–C***).

2. Both lipid A binding agents stain green algae

The monocellular algae, *Chlorella* (strain NC64A) (Fig. 2*D*) and *Prototheca* (strain 289), bound polymyxin B and LALF. Both agents also stained cells of endosymbiotic green algae in the ciliate protozoan, *Paramecium bursaria* (Fig. 2*E*), and the coelenterate, *Chlorohydra viridissima* (Fig. 2*F*). Care was taken to ensure that exogenous bacterium-derived LPS was not present in these systems. Algae were cultured under axenic conditions, then fixed and processed with reagents prepared with LPS-free water. Algal cells and *Paramecium bursaria* were processed as intact cells. Presumably intracellular elements such as intracellular algae would be protected from contact with exogenous LPS in the external environment. Paraffin sections of *Chlorohydra* were attached to LPS-free glass slides in LPS-free water. This significantly reduces the chance that staining is of LPS from bacterial sources that might have bound to the surfaces of the algal cells and suggests that lipid A staining is of endogenously produced glycolipid.

3. Both probes stained the chloroplasts of the garden pea

The vascular plant, *Arabidopsis thalii*, possesses the entire complement of genes to encode the enzymes of the lipid A biosynthetic pathway, suggesting that this glycolipid may be present in vascular plants. Isolated chloroplasts of the pea seedling (Fig 2*G*) and the chloroplasts in paraffin sections of pea seedling leaf tissue (Fig. 2*I*) stained with both agents, indicating that this proposition is valid and that lipid A is found in the chloroplast. The staining is not a general membrane staining because organelles other than the endosymbiotic algae of *Paramecium* and *Chlorohydra* and chloroplasts of the garden pea did not stain with either lipid A probe. The autofluorescence of chlorophyll did not contribute to the staining signal of the chloroplasts (Fig. 2*H*)

CONCLUSIONS AND SIGNIFICANCE

LPS constitutes the principal lipid of the outer leaflet of the outer membrane of Gram-negative bacteria and the cyanobacteria; lipid A, its lipid core, is the causative agent of Gram-negative sepsis, a potentially lethal condition claiming >100,000 lives annually in the U.S. Lipid A is a potent activator of the innate immune systems of metazoans. It is thought that lipid A is

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unique to Gram-negative bacteria and that this moiety is diagnostic for this class of prokaryotes. Thus, the discovery of a lipid A-like molecule in unicellular algae, members of the eukaryotes, was unanticipated. Biochemical characterization of the algal molecule has documented the presence of several chemical moieties found in bacterial lipid A. The vascular plant *Arabidopsis thaliana* (Angiospermae, Dicotyledonae) has been found to contain genes that encode all of the enzymes of the lipid A biosynthetic pathway, although it has not been shown that vascular plants synthesize lipid A or where lipid A might be located in the tissues. This study indicates that lipid A is indeed present and in chloroplasts. The observation that the lipid A binding probes, polymyxin B and LALF, bind green algae and the chloroplasts of vascular plants supports the hypothesis that lipid A is not restricted to the Gram-negative bacteria, but is found, as well, in select eukaryotes.

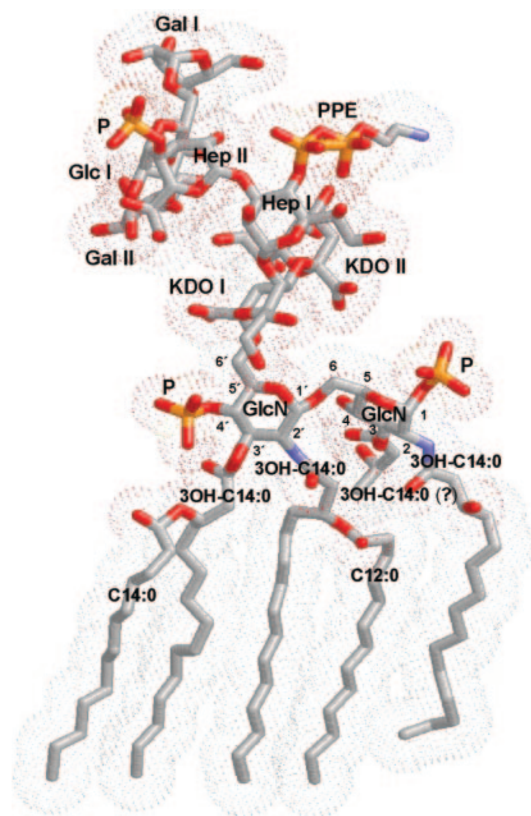

Figure 1. Lipid A is the major lipid of the outer leaflet of the outer membrane of Gram-negative bacteria and an important agent of morbidity and mortality in humans. Lipid A activates the innate immune systems of a variety of higher metazoans, including arthropods and mammals. This illustration depicts LPS from *E. coli* K-12, with its proximate lipid A moiety, which is conjugated with two molecules of 3-deoxy-D-manno-oct-2-ulosonic acid (KDO I and KDO II) sugar, and the sugar moieties of the inner core oligosaccharide chain (Hep, heptose; Gal, galactose; Glc, glucose and the phospho-amino compound pyrophosphate-ethanolamine, PPE. Lipid A has multiple, fully saturated acyl chains (bottom) that attach by amide and ester bonds to two residues of the phosphorylated glucosamine backbone (GlcN). Biochemical studies provide evidence for lipids from the green alga, *Chlorella*, with several diagnostic characters of bacterial lipid A. The present study uses lipid A binding agents from fungi and arthropods to demonstrate the presence of this lipid in chloroplasts of algae and vascular plants. Adapted from Fig. 1 of Ferguson *et al.* (1998) *Science* **282,** 2215–2220, kindly supplied by Professor Ulrich Zahringer and used with his permission.

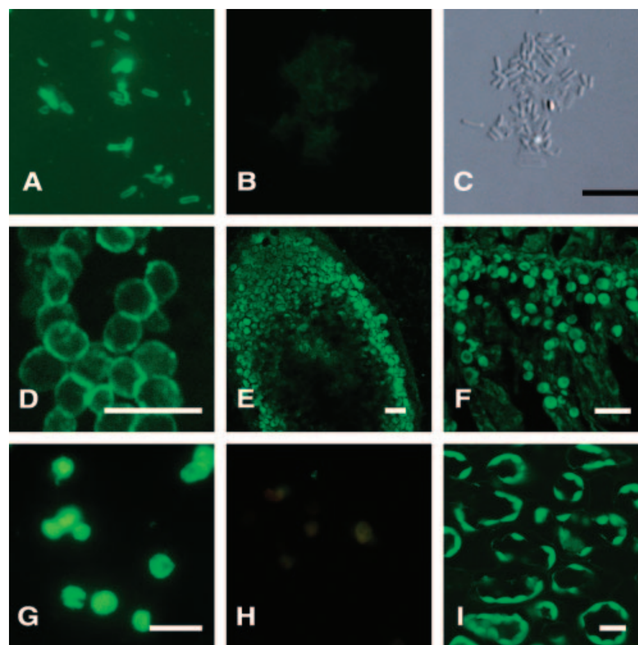

Figure 2. The staining of lipid A, the core component of LPS, utilized the lipid A binding moieties LALF (*A, D, G*) and biodipy-conjugated polymyxin B (*E, F, I*). For all micrographs, the specimens were fixed in freshly prepared 4% paraformaldehyde in PBS (10 min, room T), then blocked successively with 0.1 M glycine in Tris-buffered saline (TBS) and with 5 mg/ml BSA in the same buffer. Specimens were examined with Zeiss Axioplan and Leica DMIRBE confocal microscopes. To reduce the likelihood of contamination with exogenous bacterial LPS, all reagents were prepared in pyrogen-free water (Sigma cat W3500); glassware and microscope slides (Fisher Superfrost/Plus cat 12–550-15) were treated at 180°C for 4 h to remove LPS. Staining is seen with our positive control, the Gram-negative bacterium, *E. coli* (*A*), the green alga *Chlorella* sp., strain NC64A (*D*), the endosymbiotic algae of the cilliate protozoan, *Paramecium busaria* (*E*), the intracellular algae of the green hydra, *Chlorohydra viridissima* (*F*), isolated chloroplasts from the garden pea (*G*), and chloroplasts in paraffin sections of pea leaf tissue (*I*). The intracellular algae (*E, F*) and chloroplasts *in situ* (*I*) stained more intensely than the organelles and other membrane systems of the host cells. When LALF was omitted from the staining regimen (*B, C*-fluorescence and phase contrast micrographs of a clump of *E. coli* exposed to all reagents for LALF immunostaining but with the omission of LALF; *H:* fluorescence micrograph of isolated pea seedling chloroplasts stained in a manner identical to the bacteria of panel *B*) or when polymyxin B was preincubated with bacterial LPS (data not shown), staining failed to occur. The pairs of photomicrographs (panels *A, B* and *G, H*) were photographed and processed identically. The brighter background fluorescence in panel *A* is due to staining of LPS that has adsorbed onto the glass microscope slide from the buffer in which the bacteria were suspended and presumably is material that had been shed into the buffer by the bacteria. The autofluorescence of chlorophyl did not contribute to the fluorescent signal from algae (not shown) or chloroplasts (*H*). Bar, 10 μm.

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