Selective Induction of Metabolic Activation Programs in Peritoneal Macrophages by Lipopolysaccharide Substructures

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The structural elements of Salmonella typhimurium lipopolysaccharides (LPS) that are able to stimulate peritoneal macrophages to produce increased amounts of prostaglandin E₂, ornithine, and citrulline, agents known to modulate immune responses, are described. Two different incomplete lipid A structures which lack the carbohydrate portion, the nonhydroxylated fatty acids lauric acid and myristic acid (lipid A precursor IB), and additional palmitic acid (lipid A precursor IA) stimulated increased prostaglandin E₂ synthesis but were unable to augment ornithine and citrulline production at concentrations of up to 0.5 μg/ml. Acyl-deficient smooth LPS containing lipid A precursors IA and IB substituted by the complete carbohydrate region were able to augment prostaglandin E₂ and ornithine production but failed, even at a high concentration (0.5 μg/ml), to stimulate citrulline production. Moreover, Re glycolipids and smooth intact LPS containing the lipid A region with 3-acyloxyacyl residues possessed all of the structural requirements to induce increased prostaglandin E₂, ornithine, and citrulline synthesis. Finally, all of the LPS structures, including lipid A precursors IA and IB substituted, in combination with gamma interferon, production of citrulline with similar efficiencies. These results demonstrate that LPS contains various substructures including regions of the carbohydrate and lipid A structure that can deliver signals for the activation of peritoneal macrophages. Signals for partial activation of macrophages to produce prostaglandins and ornithine can be delivered by acyl-deficient LPS structures. In contrast, full activation of macrophages to produce citrulline requires an additional signal that is delivered by 3-acyloxyacyl residues of the lipid A region or gamma interferon.

Macrophages play a crucial role in the defense against tumors and microbial agents. When appropriately activated, they produce increased amounts of prostaglandins, ornithine, or citrulline, agents that are known to modulate immune responses. Thus, prostaglandins are able to down-regulate cytotoxic responses of peritoneal macrophages (16) or are involved in lipopolysaccharide (LPS)-induced activation of arginase within macrophages, which converts arginine into ornithine (1). Ornithine, on the other hand, is thought to be involved in T-cell activation (5), whereas L-arginine-dependent mechanisms mediate Kupffer cell inhibition of hepatocyte protein synthesis (2). This study analyzed the abilities of various LPS substructures, including acyl-deficient LPS, to influence the production of these compounds. The studies were facilitated by the availability of mutants of Salmonella typhimurium that are conditionally defective in the synthesis of the complete lipid A structure. One of these mutants (Ts7) is conditionally defective in the transfer of lauric and myristic fatty acid residues to lipid A precursor structures (12). Under appropriate conditions, this mutant accumulates acyl-deficient LPS possessing a polysaccharide structure of the normal polymer but a lipid A structure that lacks lauric and myristic fatty acids. Another mutant (Ts5) is conditionally defective in 3-deoxy-D-manno-octulosonic acid (KDO)-8-phosphate synthetase (8, 9) and, under nonpermissive conditions, produces lipid A precursors IA that lack lauric, myristic, and palmitic fatty acids, as well as precursor IB, which is devoid of the lauric and myristic acids of the complete lipid A structure. By using these substructures, it was possible to define some structural features of LPS required to stimulate peritoneal macrophages to produce increased amounts of prostaglandin E₂, ornithine, and citrulline.

MATERIALS AND METHODS

Animals. C3H mice were obtained from a stock of the German Cancer Research Center. Eight- to twelve-week-old mice were used in most experiments.

Preparation of peritoneal exudate cells and macrophages. Peritoneal exudate cells were prepared as described previously (1, 5). Briefly, mice were treated with 1 ml of phosphate-buffered saline intraperitoneally and sacrificed 18 to 24 h later. The peritoneal cells were collected by intraperitoneal injection of 8 to 10 ml of RPMI 1640 medium (GIBCO) and subsequent aspiration of the cell suspension with a syringe. The cells were washed several times and adjusted to 1 × 10⁶ to 2 × 10⁹/ml in Dulbecco’s modified Eagle medium containing glucose (4.5 g/liter), 10% fetal calf serum, and streptomycin-penicillin (each at 100 U/ml; GIBCO). This cell suspension contained about 50 to 58% macrophages as determined in staining cytocentrifuge preparations. Peritoneal macrophages were obtained by transferring 1 ml of this cell suspension to 16-mm-diameter flat-bottom Costar wells and allowing the cells to adhere for 2 h at 37°C. Nonadherent cells were removed by washing with medium four times. The homogeneity of these macrophages was determined by microscopic inspection.

Consumption of [¹⁴C]arginine and production of ornithine and citrulline. Cultures with 5 × 10⁹ macrophages in 0.5 ml of Dulbecco’s modified Eagle medium were mixed with 1 μCi of L-¹⁴C]arginine (New England Nuclear) and incubated at 37°C. Small aliquots (4 μl) of the supernatant were analyzed after different intervals and analyzed by thin-layer chromatography (TLC) on Si-DC plates (no. 31298921;
TABLE 1. Properties of the Salmonella mutants used

<table>
<thead>
<tr>
<th>S. typhimurium strain</th>
<th>Phenotype*</th>
<th>Growth conditions*</th>
<th>Structure of LPS (chemotype or precursor synthesized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ts5</td>
<td>UDP galactose epimerase deficient KDO&lt;sup&gt;+&lt;/sup&gt;</td>
<td>30°C</td>
<td>Re</td>
</tr>
<tr>
<td></td>
<td></td>
<td>42°C</td>
<td>Lipid A precursors IA and IB</td>
</tr>
<tr>
<td>Ts7</td>
<td>UDP galactose epimerase deficient HT&lt;sup&gt;+&lt;/sup&gt; C&lt;sub&gt;12&lt;/sub&gt;T&lt;sup&gt;+&lt;/sup&gt; C&lt;sub&gt;14&lt;/sub&gt;T&lt;sup&gt;+&lt;/sup&gt;</td>
<td>30°C</td>
<td>Re</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30°C + galactose</td>
<td>Smooth</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35°C</td>
<td>Re</td>
</tr>
<tr>
<td></td>
<td></td>
<td>42°C</td>
<td>Acyl-deficient, Re</td>
</tr>
<tr>
<td></td>
<td></td>
<td>42°C → 30°C + galactose</td>
<td>Acyl-deficient, smooth</td>
</tr>
</tbody>
</table>

* KDO<sup>+</sup>, temperature-sensitive KDO synthesis; HT<sup>+</sup>, temperature-sensitive heptosyl I transferase; C<sub>12</sub>T<sup>+</sup> and C<sub>14</sub>T<sup>+</sup>, temperature-sensitive transfer of lauric and myristic acids, respectively.

<sup>a</sup> → shift down of the culture.

Smooth LPS with a complete lipid A portion was isolated from Ts7 cells grown at 30°C in the presence of galactose. LPS was extracted by the phenol-water method as described by Westphal et al. (20) and subsequently with phenol-chloroform-petroleum ether (7).

To isolate acyl-deficient smooth LPS, cultures of mutant Ts7 (1 liter) kept at 35°C were used. Such cells were diluted 10-fold with Loeb medium and allowed to grow to a density of 6 × 10<sup>8</sup>/ml. Cultures were then shifted to 42°C and permitted to incubate for 1 h at this temperature. At 42°C, transfer of lauric and myristic acids to LPS precursor structures was irreversibly blocked in mutant Ts7 (unpublished data). Cultures were then shifted down to 30°C. At the same time, D-galactose was added. The incubation was then continued for 45 min. During this time, transfer of lauric and myristic acid residues was still blocked but mutant Ts7 regained its ability to add the carbohydrate chain to the acyl-deficient lipid A structure. Accordingly, mutant Ts7 produced underacylated smooth LPS lacking lauric and myristic acids. Cells were then extracted with phenol-water (20), followed by the PCP method (7). The yield of decylated LPS was 50 mg.

**Chemicals.** All of the reagents used were commercial products.

**Analytical procedures.** 3-Deoxy-D-manno-octulosonic acid was measured by the thio-barbituric acid method (19), glucosamine was measured as described by Strominger et al. (18), and organic phosphate was measured as described by Lowry et al. (14). Fatty acids were determined by gas-liquid chromatography after acid hydrolysis in 4 M HCl for 4 h at 100°C, followed by treatment with 2.0 M methanolic HCl for 10 h at 85°C. The analysis was carried out with a temperature gradient of 120 to 175°C (3°C/min) in a Varian 3700 gas chromatograph using a capillary glass column (12 cm long) packed with SE54 (Weeke, Duisburg, Germany). D-Galactose was determined by gas-liquid chromatography (3) with a Varian 1400 aerograph equipped with a flame ionization detector and connected to an automatic integrator (Hewlett Packard 3380A). The sugar was determined as alditol acetate on an ECNSS-M column (3% on Gaschrom Y, 100/200 mesh, 200°C, 0.32 by 200 cm). Prostaglandin E<sub>2</sub> concentrations were determined by radioimmunoassay, NEN Research Products catalog no. NEK-020 and 4 (1).

**Radioactive material and counting procedure.** All radiochemicals were obtained from Amersham Buchler (Frankfurt, Germany). Radioactivity was measured in a Mark III 6880 liquid scintillation system from Searle Nuclear, Chicago Division, with a commercially available scintillation...
FIG. 1. Structures of the LPS of S. typhimurium (A) and its lipid A region (B). (A) The point of termination of the incomplete LPS of UDP galactose epimerase-deficient (chemotype Re) and Re mutants is shown. Hep, 1-glycerod-mannoheptose. (B) Structure of lipid A, smooth LPS, and various acyl-deficient (a.d.) compounds from S. typhimurium. The numbers in circles indicate the numbers of C atoms in the acyl chains. The C12,0,0 fatty acid; C14,0,0 myristic acid; C16,0,0 palmitic acid. The phosphates at positions 1 and 4 may be substituted by phosphoethanolamine and aminoarabinose, respectively (6, 15). Precursors IA and IB were isolated from S. typhimurium mutant Ts5, which possesses a conditional lethal defect in the biosynthesis of KDO. Acyl-deficient Re glycolipid and acyl-deficient smooth LPS were isolated from S. typhimurium mutant Ts7, which is conditionally defective in the transfer of the first heptose residue and in the transfer of lauric and myristic acids. Acyl-deficient Re glycolipid, acyl-deficient smooth LPS, and smooth LPS are a mixture of analogs containing and devoid of C16 fatty acids. Polys., polysaccharide.

FIG. 2. Effects of LPS structures on prostaglandin E2 (PGE_2) production by peritoneal macrophages. Resident peritoneal macrophages (10^6) from C3H/1if mice were incubated with graded concentrations of LPS structures in 0.2 ml cultures. Samples of supernatants were removed after 4 h and analyzed for prostaglandin E2 by radioimmunoassay (NEN): a.d., acyl-deficient; prec, precursor.

RESULTS

LPS structures used to activate peritoneal macrophages to produce prostaglandin E_2, ornithine, and citrulline. Various LPS structures (Fig. 1) were used to stimulate peritoneal macrophages to produce prostaglandin E_2, ornithine, and citrulline (Table 2). These included two acyl-deficient lipid A structures, lipid A precursors IA and IB, which contain glucosamine, phosphate, 3-hydroxyimyrstic acid, and palmitic acid in molar ratios of 1.0:1.1:2:0:0.005 and 1.0:1.2:2:1:0.48, respectively. As previously shown (9), lipid A precursors are made up by a diphosphorylated glucosamine disaccharide substituted by four 3-hydroxyimyrstic acids. They lack the KDO residues, the nonhydroxylated fatty acids lauric acid and myristic acid (lipid A precursor IB), and additional palmitic acid (lipid A precursor IA). Another acyl-deficient LPS structure (acyl-deficient Re glycolipid) is composed of glucosamine, KDO, phosphate, 3-hydroxyimyrstic acid, and palmitic acid in a molar ratio of 1.0:1.0:1.2:2.2:0.23, consistent with an underacylated glycolipid of the Re chemotype composed of lipid A precursors IA and IB substituted by two KDO residues. Finally, a fourth acyl-deficient LPS product (acyl-deficient smooth LPS) contains glucosamine, 3-hydroxyimyrstic acid, palmitic acid, and galactose in a molar ratio of 1.5:2.2:0:18:18 and only trace amounts of lauric and myristic acids, consistent with an LPS product that is made up of the smooth-type polysaccharide attached to lipid A precursor IA and IB structures.

Effects of LPS and acyl-deficient LPS structures on the ability of peritoneal macrophages to produce PGE_2. Lipid A precursors IA and IB, acyl-deficient LPS of the smooth and Re chemotypes, and intact LPS were equally able to stimulate peritoneal macrophages to produce prostaglandin. The effects of three of these structures on prostaglandin E_2 production are shown in Fig. 2. Within 4 h, 10^6 macrophages generated 100 pg of prostaglandin E_2 per ml of medium (i.e., 1.6 x 10^{-9} M). This concentration was increased approximately three- to fourfold by all of the LPS structures. These findings demonstrated that the lipid A precursor structures are sufficient to stimulate macrophages to augment prostaglandin E_2 synthesis.

Effects of LPS structures on the ability of peritoneal macrophages to consume arginine. We next investigated the abilities of the various LPS substructures to activate peritoneal macrophages to consume [3H]arginine. As shown previously (1) and in Fig. 3, lipid A precursors IA and IB failed to augment consumption of [3H]arginine. Weak stimulation fluid from Roth (Karlsruhe, Germany) designated Rotisint 22.
was obtained with the acyl-deficient Re glycolipid, whereas Re glycolipids with the complete lipid A structure and acyl-deficient LPS containing the smooth carbohydrate chain linked to precursors IA and IB were potent stimulators and almost equaled the stimulatory capacity of fully acylated smooth LPS.

Abilities of LPS substructures to stimulate peritoneal macrophages to produce ornithine and citrulline. As expected from the previous experiment, lipid A precursors IA and IB at concentrations of up to 0.5 µg/ml were unable to stimulate peritoneal macrophages to produce ornithine and citrulline (Fig. 4). Acyl-deficient Re glycolipid weakly stimulated ornithine production but did not stimulate citrulline formation. In contrast, acyl-deficient smooth LPS was a potent stimulator of ornithine production but was unable (even at high concentrations such as 0.5 µg/ml) to stimulate peritoneal macrophages to convert arginine into citrulline. Finally, Re glycolipids or complete LPS containing 3-acyloxyacyl residues predominantly augmented ornithine production at low concentrations (10 and 100 ng/ml) but at a higher concentration (500 ng/ml) converted arginine mostly into citrulline. These results demonstrated that partial activation of peritoneal macrophages to produce increased amounts of ornithine can be achieved by acyl-deficient smooth LPS structures, while increased citrulline synthesis requires 3-acyloxyacyl residues of the lipid A region.

Acyl-deficient LPS structures, in conjunction with gamma interferon, stimulate macrophages to produce citrulline. Figure 5 shows that the failure of acyl-deficient LPS structures to stimulate citrulline production can be circumvented by gamma interferon. Lipid A precursors IA and IB and acyl-deficient smooth LPS and LPS at a concentration of 100 ng/ml, in conjunction with gamma interferon, were fully able to convert arginine into citrulline.

![FIG. 3. Effects of various LPS substructures on [1H]arginine consumption by peritoneal macrophages. Peritoneal macrophages were obtained from C3H mice 24 h after injection of 1 ml of phosphate-buffered saline intraperitoneally and allowed to adhere for 2 h to flat-bottom wells of Costar microtiter plates. The adherent-cell preparation (5 × 10^5/0.5 ml) was then incubated in Dulbecco's modified Eagle medium with various LPS substructures. After 18 h, the culture medium was changed, 1 µCi of L-[U-14C]arginine was added, and the radioactive material in the supernatant was analyzed after an additional 24 h by TLC. Prec, precursor; a.d., acyl deficient.](image1)

![FIG. 4. Effects of various LPS structures on ornithine and citrulline production by peritoneal macrophages. Peritoneal macrophages were allowed to adhere to flat-bottom wells of Costar microtiter plates. The adherent-cell preparation (5 × 10^5/0.5 ml) was then incubated in culture medium with graded concentrations of LPS structures. After 18 h, the culture medium was changed, 1 µCi of [U-14C]arginine was added, and the radioactive material in the supernatant was analyzed by TLC. The data are mean values of triplicate cultures ± standard deviations. Prec, precursor; a.d., acyl deficient.](image2)
FIG. 5. Effects of various LPS structures on ornithine and citruline production in the presence and absence of gamma interferon (IFN-γ) by peritoneal macrophages. Peritoneal macrophages were prepared as described in the legend to Fig. 4. Cells (5 × 10^7/0.5 ml) were then incubated with 100 ng of LPS structures in the absence or presence of 100 U of gamma interferon. l-[U-14C]larginine (1 μCi) was added 22 h later, and the radioactive material in the supernatant was analyzed by TLC after a further 12 h. The results are expressed as mean values of triplicate cultures ± standard deviations. Prec, precursor; a.d., acyl deficient.

DISCUSSION

The present report defines the structural regions of LPS that are able to stimulate peritoneal macrophages to produce increased amounts of prostaglandin E₂, ornithine, and citruline, substances that are known to modulate immune responses (1, 2, 5, 16).

It was shown that incomplete lipopolysaccharide structures are composed of a glucosamine disaccharide substituted by two phosphate residues, four 3-hydroxyxymyrstic acids (lipid A precursor IA), and palmitic acid (lipid A precursor IB) were able to augment prostaglandin E₂ synthesis but failed to influence ornithine and citruline synthesis.

To increase ornithine production, additional features are required which include the 3-acyl-oxyacyl residues of the complete lipid A structure and the carbohydrate region of smooth LPS. This conclusion is based on the finding that Re glycolipids with a complete lipid A structure and smooth LPS, devoid of lauric and myristic acids (acyl-deficient smooth LPS), were able to augment ornithine production, while acyl-deficient Re glycolipids were poor stimulators.

On the other hand, acyl-deficient smooth LPS was a poor stimulator of citruline production. To stimulate macrophages to produce citruline, LPS structures which contain a lipid A region with 3-acyl-oxyacyl residues are required.

Moreover, all of the LPS structures, including the most amputated lipid A structures, such as precursors IA and IB, in combination with gamma interferon, increased conversion of arginine into citruline with similar efficiencies. These results confirm previous observations that LPS, together with gamma interferon, stimulates macrophages to convert arginine into citruline (4, 10). Moreover, they demonstrated that conversion of arginine into citruline occurs at the expense of ornithine production (Fig. 5). This suggests that one of the physiological functions of citruline production is downregulation of ornithine and putrescine by competition for their common precursor, arginine.

Finally, the results also show that both lipid A substruc-

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REFERENCES


