Induction of Procoagulant Activity in Human Leukocytes with Lipoglycans from Mycoplasmas

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Lipoglycans from mycoplasmas possess the ability to induce the elaboration of procoagulant activity. This represents a third endocytopathic property for these polymers, in addition to the pyrogenicity and clotting of Limulus lysate reported previously. Lipoglycans from three species of Acholeplasma (A. axanthum, A. granularum, and A. oculi) stimulated the elaboration in vitro of procoagulant activity by human mononuclear cells to differing extents. Two serotypes, 3 and 4, of Ureaplasma urealyticum also exhibited this stimulatory activity. The activities of lipoglycans tested were, in decreasing order, A. oculi > U. urealyticum type 4 > A. axanthum > U. urealyticum type 3 > A. granularum.

MATERIALS AND METHODS

Lipoglycans from three species of Acholeplasma (A. axanthum [bovine], A. oculi [caprine], and A. granularum [swine]) and from two serotypes of U. urealyticum (strain P108, serotype 3, and strain 681, serotype 4) were samples from lots of lipoglycans used in previous studies (1, 8–10). All were highly purified and free of detectable nucleic acids and protein. Escherichia coli O111:B4 LPS, purchased from Difco Laboratories (Detroit, Mich.), was used as a reference preparation.

Blood was collected aseptically from apparently healthy donors and treated with trisodium citrate (0.015 M final concentration) to prevent coagulation. Blood samples then were incubated with various concentrations of LPS or lipoglycans for 4 h at 37°C. Procoagulant activity was evaluated either in whole blood-LPS or whole blood-lipoglycan mixtures and in mononuclear cells isolated from the incubation mixtures. When whole blood was used, procoagulant activity was determined by the procedure of Østerud and Bjørkild (6) by recording clotting times after the addition of 0.1 ml of 0.025 M CaC₂ to 0.2 ml of incubation mixture. For examination of isolated mononuclear cells, leukocytes (mixed mononuclear cells) were purified by the Ficoll-Hypaque (Lymphoprep; Nyegaard, Oslo, Norway) gradient technique as described by Büyum (2). Cell preparations were washed four times with citrated Hanks balanced salt solution. The final concentration of cells (5 × 10⁷/ml) was prepared by suspension in Hanks balanced salt solution without citrate. Of these cells, 20% were monocytes, as assessed by cytochemical reactivity for alpha-naphthyl acetate esterase (5), and the remainder were lymphocytes. Granulocyte contamination was less than 5%, and the ratio of platelets to leukocytes was less than 1:1 as determined by light microscopy. The coagulant activity of mononuclear cells was measured by a one-stage recalcification time using the following test system: 0.1 ml of cell suspension, 0.1 ml of plasma substrate, and 0.1 ml of 0.025 M CaC₂ (3). The plasma substrate was normal plasma (pooled from at least three normal subjects) and plasma from individuals congenitally deficient in factor VII or VIII (Dade Division, Pharmaseal, Trieste, Italy). All tests were performed in duplicate in plastic tubes using a manual method. Positive controls consisted of blood exposed to E. coli LPS. Negative controls used blood incubated with sterile pyrogen-free saline.

RESULTS

All lipoglycan preparations (1 to 10 μg/ml of whole blood) were able to induce procoagulant production in the incubation mixtures. Indeed, mononuclear cells isolated from whole blood-lipoglycan mixtures after 4 h of incubation at 37°C shortened the recalcification time of normal plasma, though to differing degrees. Among the lipoglycans from acholeplasmas, the A. oculi and A. axanthum samples were the most effective. Similar activity was exhibited by lipoglycans from both strains of U. urealyticum. This finding parallels that obtained by measuring generation of procoagulant activity in the corresponding whole blood-lipoglycan mixtures. A similar effect was induced by LPS from the typical gram-negative E. coli strain, whereas no effect was found when blood was incubated with sterile, pyrogen-free saline (Table 1). In another series of experiments, each lipoglycan was incubated at various concentrations to investigate the dose dependence of this activity. The lipoglycans from A. oculi, A. axanthum, and both strains of U. urealyticum were the most reactive (Fig. 1). These data demonstrate that production of procoagulant activity is dependent upon the dose of lipoglycan used. To partially characterize the procoagulant activity, mononuclear cells

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TABLE 1. Procoagulant activity in mixtures of whole blood and lipoglycans after 4 h of incubation at 37°C and in suspensions of mononuclear cells (5 × 10^6/ml) isolated from them

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Conc (μg/ml)</th>
<th>No. of expts</th>
<th>Recalcification time (s) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Whole blood-lipoglycans</td>
</tr>
<tr>
<td>A. oculi lipoglycan</td>
<td>1</td>
<td>8</td>
<td>134 ± 11.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8</td>
<td>71 ± 4.5</td>
</tr>
<tr>
<td>A. axanthum lipoglycan</td>
<td>10</td>
<td>5</td>
<td>105 ± 8.2</td>
</tr>
<tr>
<td>A. granularum lipoglycan</td>
<td>10</td>
<td>5</td>
<td>326.5 ± 8.4</td>
</tr>
<tr>
<td>U. urealyticum 681 lipoglycan</td>
<td>30</td>
<td>3</td>
<td>80 ± 8.7</td>
</tr>
<tr>
<td>U. urealyticum P108 lipoglycan</td>
<td>10</td>
<td>3</td>
<td>156.6 ± 3.3</td>
</tr>
<tr>
<td>E. coli LPS</td>
<td>3</td>
<td>3</td>
<td>98.5 ± 3.1</td>
</tr>
<tr>
<td>Saline</td>
<td>8</td>
<td></td>
<td>&gt;360</td>
</tr>
</tbody>
</table>

Isolated from the incubation mixtures were tested in normal plasma and in plasma deficient in factor VII or VIII. When factor VII-deficient plasma was used as a substrate, leukocytes stimulated with lipoglycans were not able to shorten the plasma recalcification time (Table 2).

**DISCUSSION**

The data presented demonstrate that the lipoglycans from three species of Acholeplasma (A. oculi, A. axanthum, and A. granularum) and from U. urealyticum P108 and 681 possess yet another property of the classical gram-negative LPS. They can stimulate the production of procoagulant activity by human mononuclear cells in vitro. Among the acholeplasmal lipoglycans tested, those from A. oculi and A. axanthum appeared to be more reactive than that from A. granularum when compared on a weight basis in their capacity to stimulate human mononuclear cells. Lipoglycans from U. urealyticum displayed a potency similar to that shown by the lipoglycans from A. oculi. While this finding might be of interest for lipoglycans for the human serotypes of U. urealyticum because they are reputed to be pathogenic, the high degree of activity exhibited by lipoglycans from A. oculi and A. axanthum isolated from animal sources merits attention since the role of acholeplasmas in disease remains to be determined. It is noteworthy that the different degrees of potency of acholeplasmal lipoglycans observed in inducing procoagulant production parallel their different abilities to clot Limulus amoebocyte lysate, as shown by Seid et al. (7). These authors speculated that the lipoidal region of mycoplasmal lipoglycans could be responsible for pyrogenicity and Limulus gelation. Therefore, the lipid moiety of the lipoglycan molecule also could be responsible for induction of procoagulant activity. Structural differences in the lipoglycan molecules may account for the different degrees of endotoxic potency. The least active lipoglycan, that from A. granularum, possesses as its lipid moiety a terminal diacylglycerol (9). The more active lipoglycan from A. axanthum, on the other hand, contains its lipid moieties as N-acyl saturated and 3-hydroxy fatty acids distributed along the length of the polysaccharide chain. Phosphorus is present as branched phosphodiesters of glycerol and galactose (10). The third acholeplasmal lipoglycan, that from A. oculi, differs from other acholeplasmal lipoglycans by lacking amino sugars but containing fucose, a neutral sugar not found in the others (1). The structures of the lipoglycans from U. urealyticum are not resolved. However, they contain only neutral sugars and phosphorus (11) in addition to fatty acids, suggesting a similarity to the two active acholeplasmal lipoglycans. Lipoglycans from Ureaplasma spp. may play a role in pathogenesis. This activity of acholeplasmal lipoglycans as a factor in disease processes remains to be established.

**TABLE 2. Characterization of mononuclear cell procoagulant activity induced by lipoglycans**

<table>
<thead>
<tr>
<th>Lipoglycan</th>
<th>Plasma recalcification time (s) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal plasma</td>
</tr>
<tr>
<td>A. oculi</td>
<td>135 ± 12</td>
</tr>
<tr>
<td>A. axanthum</td>
<td>165 ± 3.8</td>
</tr>
<tr>
<td>A. granularum</td>
<td>300 ± 9.1</td>
</tr>
<tr>
<td>U. urealyticum 681</td>
<td>150 ± 7.2</td>
</tr>
<tr>
<td>U. urealyticum P108</td>
<td>163 ± 5.9</td>
</tr>
<tr>
<td>Saline</td>
<td>&gt;360</td>
</tr>
</tbody>
</table>

*Cells (5 × 10^6/ml) were isolated from whole blood after 4 h of incubation at 37°C with lipoglycans (1 μg/ml). Each result shown is the mean of four experiments.*

**FIG. 1.** Procoagulant activity (clotting time) of mononuclear cells (5 × 10^6/ml) isolated from whole blood after 4 h of incubation with lipoglycans at the indicated concentrations. Each point represents the mean ± standard error of three separate experiments. Symbols: ▲, A. granularum; ■, U. urealyticum P108; ★, U. urealyticum 681; ●, A. axanthum; X, A. oculi.

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LITERATURE CITED