Effect of Polymyxin B and Colimycin on Induction of Plasminogen Antiactivator by Lipopolysaccharide in Human Endothelial Cell Culture

FRÉDÉRIC DUBOR, ANNE M. DOSNE,* AND LOUIS A. CHEDID
Centre National de la Recherche Scientifique 04-0579, Immunothérapie Expérimentale, Institut Pasteur, 75015 Paris, France

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The effect of lipopolysaccharide (LPS) on the production of fibrinolytic inhibitor by human endothelial cells was determined because results of previous experiments have shown us that it is possible to stimulate this synthesis with muramyl dipeptide. Treatment of these cells with LPS resulted in a marked enhancement of fibrinolytic inhibitor, as estimated in a urokinase-induced fibrinolysis assay. A dose-response curve was obtained for LPS concentrations ranging from 10 to 1,000 ng/ml, thus demonstrating the great sensitivity of these cells. This inhibitor did not reduce plasmin activity and formed complexes with high- and low-molecular-weight urokinase as visualized by fibrin enzymography on sodium dodecyl sulfate-polyacrylamide electrophoretic gels. The molecular weight of this inhibitor was estimated to be 54 to 58 kilodaltons. These findings led us to conclude that LPS stimulates formation of a plasminogen antiactivator. This LPS effect could be suppressed by polymyxin B and colimycin. The stimulatory effect of muramyl dipeptide required doses which were at least 1,000 times greater than those of LPS and was not decreased by polymyxin B. These results show the possibility of independent modulation of plasminogen antiactivator production at the endothelial level, which could be important in endotoxemia. Under these conditions colimycin might have an additional advantage for clinical use because of its ability to prevent fibrinolytic inhibition.

Microvascular alterations in response to in vivo administration of endotoxin have been recognized for a long time and have been shown to involve leukocyte adherence, platelet aggregation, and activation of several steps of coagulation that lead to vascular obstruction (25). Direct effects of endotoxin on vascular endothelium also have been described, such as injury of bovine endothelial cells (16, 31), induction of thromboplastin (13), and colony-stimulating factor synthesis (29) in human cells. However, neither cytotoxicity nor modifications in synthesis of factor VIII antigen, angiotensin-converting enzyme, and prostacyclin release were found when human cells were used (16). In this study we examine the effects of lipopolysaccharide (LPS) on the production of fibrinolytic inhibitor by cultured human endothelial cells. The ability of these cells to synthesize inhibitor(s) of plasminogen activators has been demonstrated previously (7, 10, 12, 22, 28). Recently, we have observed that muramyl dipeptide (MDP), the immunoadjuvant properties of which have been well documented (3, 20), could stimulate this fibrinolytic inhibitor synthesis (6). This led us to compare the sensitivity of the endothelial response toward LPS and MDP. It has been described previously that LPS reduces the production of plasminogen activator by macrophages in vitro (1, 9). The involvement of an inhibitor was not demonstrated in vitro (9), although fibrinolytic inhibitory activity was found in vivo in the ascite liquid following endotoxin injection (2). This led us to determine whether LPS might increase fibrinolytic inhibitor production by human endothelial cells. The data obtained from this study provide evidence that LPS induces the generation of a plasminogen antiactivator.

MATERIALS AND METHODS

Endothelial cell culture. Endothelial cells were obtained from human umbilical vein and cultured in medium 199 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 20% fetal calf serum (Hy-Clone; Sterile Systems Inc., Logan, Utah) (19). These cells were characterized by the presence of Weibel-Palade bodies and the synthesis of factor VIII-related antigen (7) and by their ability to degrade ADP (8). At confluency, i.e., 6 to 8 days after seeding, the cell cultures (3 × 10^6 to 4 × 10^6 cells per 4-cm^2 dish) were washed 4 times with medium 199 containing 3.5 g of human nonpyrogenic albumin (Centre National de Transfusion, Paris, France) per liter and then incubated in medium 199 with LPS (from Salmonella enteritidis; Difco Laboratories, Detroit, Mich.) or MDP (Colo Chime Réactifs, Paris, France). In some experiments polymyxin B (8,000 U/mg; Sigma Chemical Co., St. Louis, Mo.) or colimycin (12,500 U/mg; Roger Bellon, Paris, France) were also added to the incubation medium. Control incubations were done with a 0.9% nonpyrogenic NaCl solution instead of the tested compounds. After a 24-h incubation, culture supernatants were collected, centrifuged, and used for fibrinolytic inhibition assays.

Inhibition of urokinase-induced fibrinolysis. Supernatants were mixed (1:1, vol/vol) with 6 to 25 U of urokinase solution (Institut Choay, Paris, France) per ml and 30 μl of the mixture was deposited on a standard fibrin plate prepared with rich plasminogen bovine fibrinogen (Koch and Light Laboratories, Colnbrook, England) as described previously (6). The lysis area was measured after 18 h at 37°C. Results were expressed as the inhibition percentage of the lysis area induced by urokinase mixed with unconditioned medium. Because this assay could not discriminate plasminogen
antiactivator from antiplasmin, another test specific for antiplasmin was also used.

**Inhibition of plasmin-induced fibrinolysis.** Fibrin plates were heated at 80°C for 45 min to denature the plasminogen. This treatment rendered fibrin unsusceptible to urokinase lysis and allowed us to test specifically for plasmin activity. Different concentrations (0.3 to 2.5 U/ml) of porcine plasmin (Sigma) were mixed (1:1, vol/vol) with unconditioned medium or cell supernatants from untreated or LPS-treated cells. A total of 30 μl was deposited on a heated fibrin plate, and the lysis area was measured after 18 h at 37°C.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fibrin enzymography.** Sodium dodecyl sulfate-(SDS) polyacrylamide gel electrophoresis (PAGE) and fibrin enzymography were used to visualize complexes between plasminogen antiactivator and urokinase, as described previously (15) with some modifications. A total of 50 μl of cell supernatant or unconditioned incubation medium was mixed (1:1, vol/vol) with 50 μl of 0 U of urokinase per ml for 20 min and then supplemented with 100 μl of SDS sample buffer, giving a final SDS concentration of 2.5%. A total of 100 μl of this mixture was applied to a 10% acrylamide gel for electrophoresis at a constant current of 80 mA for 2.40 h. The gel was washed by the original method and placed on a thin agarose-fibrin gel which was prepared as follows. A total of 1.8 ml of 2% indubiose (kept at 44°C) was mixed with 1.1 ml of 0.2 U of thrombin (kept at 44°C) per ml and 1.7 ml of 0.6 mg of bovine fibrinogen per ml that was rich in plasminogen (kept at 37°C). The solution was spread on plastic covers (9 by 14 cm) of Falcon dishes. The electrophoretic gel was incubated for 5 h at 37°C on this revelator gel. The fibrin gel was washed, dried, and stained with 0.25% Coomassie brilliant blue in 50% methanol-7% acetic acid. The clear area in the fibrin gel corresponded to urokinase molecular species or complexes that were transferred from the electrophoretic gel.

**Viability of the endothelial cell cultures.** Incorporation of [14C]leucine in cell protein was used to verify the integrity of cell metabolism during incubation with different compounds. [U-14C]Leucine (300 mCi/mmol; Centre d’Energie Atomique, Paris, France) was added to the incubation medium (0.25 μCi/ml) for 24 h. Cultures were then washed twice with medium 199 and twice with 10% ice-cold trichloroacetic acid. Cell protein radioactivity was counted after hydrolysis with 25 N formic acid and expressed as a percentage of the total radioactivity of [14C]leucine that was added to the culture.

**RESULTS**

**LPS induction of an inhibitor of urokinase-induced fibrinolysis.** Whatever the variability in the basal activity of different cell supernatants, LPS addition always induced an increase in urokinase inhibition (Fig. 1). This increase appeared to be related to LPS doses, but its detection depended on the urokinase concentration used in the assay. When supernatants were mixed with 6 U of urokinase per ml, a 100% inhibition was reached after treatment with 0.05 μg of LPS per ml. A more progressive dose-response curve was observed when 12 U of urokinase per ml was used. 50% was obtained with 0.05 μg of LPS per ml, with the maximum being induced by 1 μg/ml. When 25 U of urokinase per ml was used, the effect of LPS was generally less evident, with 10 μg/ml generating only a 30% inhibition. It was verified that LPS alone did not directly modify urokinase lysis of fibrin.

**Lack of antiplasmin activity in the supernatant from untreated or LPS-treated cells.** The results presented in Table 1 correspond to the lysis area obtained when the same supernatants were mixed either with urokinase or plasmin and tested on standard fibrin plates or heated plates, respectively. Supernatants from untreated or treated cells did not reduce plasmin activity, whereas they strongly decreased urokinase-induced fibrinolysis. This suggests that LPS does not induce formation of an antiplasmin and that a plasminogen antiactivator is more probably implicated.

**Revelation of antiactivator-urokinase complexes by fibrin enzymography.** Under our experimental conditions, endogenous plasminogen activator could not be detected, and only lysis bands corresponding to urokinase or its complexes appeared on the fibrin revelator gel (Fig. 2). The two molecular species of the urokinase solution used are shown (Fig. 2, lane A) and corresponded to apparent molecular masses of 35 and 57 kilodaltons (kDa). The addition of cell supernatant to urokinase induced a modification in the area and the distribution of urokinase lysis bands. After urokinase was mixed with untreated cell supernatant (Fig. 2, lane B), these lysis bands were reduced, and two supplementary bands appeared that corresponded to 93 and 107 kDa. After incubation with the supernatant from LPS-treated cells (Fig. 2, lane C), the 53-kDa band was barely visible, the 53-kDa band was further reduced, and the two bands which were present at 93 and 107 kDa became visible.

**TABLE 1. Comparative effect of endothelial cell supernatant on urokinase and plasmin activity as estimated by lysis area on fibrin plates**

<table>
<thead>
<tr>
<th>Treatment (U/ml)</th>
<th>Unconditioned medium</th>
<th>Supernatant from untreated cells</th>
<th>Supernatant from LPS-treated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urokinase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>144</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>196</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>361</td>
<td>144</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>484</td>
<td>484</td>
<td>324</td>
</tr>
<tr>
<td>Plasmin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>56</td>
<td>49</td>
<td>49</td>
</tr>
<tr>
<td>0.6</td>
<td>81</td>
<td>81</td>
<td>90</td>
</tr>
<tr>
<td>1.2</td>
<td>126</td>
<td>121</td>
<td>130</td>
</tr>
<tr>
<td>2.5</td>
<td>143</td>
<td>169</td>
<td>169</td>
</tr>
</tbody>
</table>

*Unconditioned medium and cell supernatants were mixed (1:1, vol/vol) with either urokinase, which was tested on standard fibrin plates (rich in plasminogen), or plasmin, which was tested on heated fibrin plates (devoid of plasminogen).*
higher molecular weight bands were also present. This provides evidence that the cell supernatant contains a component that is bound to the two molecular species of urokinase, leading to the formation of higher molecular weight complexes. These modifications were more important after treatment of the cells with LPS.

**Effect of polymyxin B and E (colimycin) on LPS-induced fibrinolytic inhibition.** Polymyxin B has been shown to block several LPS effects (5, 18, 23, 27, 30, 33), probably by binding to the lipid A moiety (24). Therefore, we tested the ability of this antibiotic at various concentrations (8 to 400 U/ml, i.e., 1 to 50 µg/ml) to suppress the urokinase fibrinolytic inhibition induced by 1 µg of LPS per ml (Fig. 3A). At 8 U/ml the effect of LPS was greatly reduced, and maximum suppression occurred with 80 U of polymyxin B per ml. In the following experiments 160 U/ml was used because polymyxin B has no direct effect on the cells or on the fibrinolytic reaction, even at 400 U/ml. Complementary experiments were performed, with a constant concentration of polymyxin B (160 U/ml) being added to increasing amounts of LPS. This polymyxin B concentration was able to neutralize entirely the effect of 0.5 µg of LPS per ml but not of 5 or 10 µg/ml.

Polymyxin E or colimycin was also tested in this model (Fig. 3B). Colimycin also neutralized the effect of LPS, although less efficiently than did polymyxin B. LPS (1 µg/ml) incubated alone with endothelial cells produced a 100% inhibition of 6 U of urokinase per ml. Simultaneous treatment with colimycin led to a dose-dependent suppression of the LPS-induced inhibition, and total blockage was observed with 400 U of colimycin per ml. Incubation with colimycin alone did not change the basal inhibitory activity.

**Effect of polymyxin B on MDP-induced fibrinolytic inhibition.** As described previously (6), MDP also stimulates the synthesis of fibrinolytic inhibitor in cultured endothelial cells. The susceptibility of this MDP effect to polymyxin B therefore was determined (Table 2). Incubation with MDP (10 to 100 µg/ml) produced a dose-dependent inhibition of urokinase fibrinolytic inhibition. However, it was necessary to use at least 1,000 times more MDP than LPS to induce a similar inhibition. A 100% inhibition of 6 U of urokinase per ml generally required treatment of 50 µg of MDP per ml instead of 0.05 µg of LPS per ml. This MDP response was not reduced by polymyxin B (160 U/ml).

Protein synthesis in the cells exposed to the compounds used in this study was verified by the cell incorporation of [3H]leucine for 24 h. A 5% uptake was obtained in control cultures, as was also obtained in those treated with 50 µg of LPS, 50 µg of MDP, or 20 µg of polymyxin B per ml. Moreover, no cell detachment was noticed during incubation with any of these compounds.

**DISCUSSION**

This study was undertaken to determine whether LPS can stimulate production of fibrinolytic inhibitor in human endothelial cell cultures and to characterize the fibrinolytic

| Table 2. Absence of effect of polymyxin B on the fibrinolytic inhibition induced by MDP* |
|---------------------------------|-----------------|------------------|
| Cell treatment (µg/ml) | % Fibrinolytic inhibition by: | |
| Control | No polymyxin B | 160 U of polymyxin B/ml |
| | 11 ± 1 | 26 ± 14 |
| 10 | 60 ± 17 | 75 ± 25 |
| 50 | 87 ± 12 | 92 ± 7 |
| 100 | 89 ± 11 | 95 ± 5 |

* Cells were treated with MDP in the absence or presence of polymyxin B (160 U/ml = 20 µg/ml), and the supernatant was tested against 6 U of urokinase per ml. Values are means ± standard deviations of two different experiments.

![FIG. 2. Fibrin enzymographic revelation of urokinase complexes formed by the addition of cell supernatants. Urokinase (6 U/ml) was mixed with unconditioned medium (lane A), supernatant from untreated cells (lane B), or LPS-treated (10 µg/ml) cells (lane C) before SDS-PAGE. This gel was applied for 5 h on a fibrin agarose revelator gel. Clear bands correspond to fibrinolysis due to the transfer of enzymatic activity from the electrophoretic gel.](http://iai.asm.org/)

![FIG. 3. Neutralization by polymyxin B and colimycin of the LPS-induced inhibition of urokinase fibrinolysis. Supernatants of the cells treated with increasing concentrations of polymyxin B (A) or colimycin (B) in the presence or absence of LPS (1 µg/ml) were tested against urokinase solution (6 U/ml). Means ± standard deviations of three different experiments are shown.](http://iai.asm.org/)
inhibitor. Results of recent study indicated the generation by LPS of a fast-acting plasminogen antiactivator by human endothelial cells which could not be detected when cells were incubated in human albumin (4). Our previous results have shown that it is possible to stimulate, with MDP, production of inhibitor in the presence of 0.35% bovine albumin (6). Under these conditions LPS had no visible effect, but substitution of clinically injectable nonpyrogenic human albumin allowed detection of a clear stimulating activity of LPS. This led us to think that contamination of bovine albumin by an endotoxin might explain the lack of cell response to additional LPS. This was further supported by the fact that polymyxin B addition to untreated endothelial cells incubated in bovine albumin decreases the basal level of fibrinolytic inhibitor (unpublished data).

Results of this study, performed with an incubation medium containing nonpyrogenic human albumin, demonstrate that LPS (10 ng to 10 μg per ml) strongly stimulates production of a factor that inhibits urokinase-induced fibrinolysis. Because plasmin activity was not modified, this argues against the presence of an antiplasmin. Results of SDS-PAGE and fibrin enzymography provide evidence for the implication of a supernatant component which decreases the activity of 35- and 53-kDa urokinase and forms complexes of 93 and 107 kDa. From these results it can be calculated that the urokinase-binding factor has a molecular mass of 54 to 58 kDa. This is in agreement with data reported by others (28, 32). LPS treatment stimulated the formation of such a urokinase-binding factor, resulting in a further reduction of the two lysis bands of urokinase.

The complexes of high molecular weight retained their catalytic activity after SDS-PAGE. Similar observations on protease-antiprotease complex activity separated in the presence of SDS have been carried out previously (15, 28). It has been proposed that SDS induces changes in the structure of the protease-antiprotease complex so that its catalytic site is exposed (15, 21).

It has been shown that endothelial cell supernatant also forms a complex with melanoma plasminogen activator (28, 32) and inhibits fibrinolytic activity from venoestasis human plasma (6). This suggests that the plasminogen antiactivator formed by endothelial cells might interfere with tumoral cell plasminogen activator, thrombolytic therapy, and physiological hyperfibrinolytic reactions.

The inductivity of plasminogen antiactivator by LPS could be suppressed by polymyxin B. This cationic antibiotic was shown to form a complex with the lipid A part of the LPS molecule (24), and it could be proposed that this lipid moiety is involved in the LPS-endothelial interaction. It was found that 160 U of polymyxin B per ml (20 μg/ml) neutralized 0.5 to 1 μg of LPS per ml, but this was not sufficient to abolish the effect of higher LPS concentrations. When expressed on a weight basis, the requirement of polymyxin B for neutralizing LPS in our model appeared greater than was reported by other investigators, who used a different experimental system (9, 33). This might be due to the fact that polymyxin B also has a great affinity for phospholipids (5) and interacts with enzymes that contain a phospholipid-binding domain (26).

The finding that colimycin had the same effect as polymyxin B might be expected from their structural similarities. This is of interest with regard to clinical use of colimycin. The amounts of colimycin used in this study are compatible with the concentration reached in blood after intravenous administration. Therefore, colimycin, in addition to its bactericidal activity, might have a potential advantage during endotoxemia for preventing endotoxin-induced reduction of the fibrinolytic capacity.

Plasminogen antiactivator was detected in blood after experimental endotoxin injection or in patients with septicemia (4). These findings underline the clinical relevance of such LPS-induced generation of antifibrinolytic component(s) which might originate from endothelium but also, perhaps, from monocytes (14) and platelets (11).

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LITERATURE CITED


LPS INDUCTION OF FIBRINOLYTIC INHIBITOR