Interactions of Radio-Detoxified *Escherichia coli* Endotoxin Preparations with the Complement System

GYÖRGY FÜST, LÓRÁND BERTÓK,* AND SÁNDOR JUHÁSZ-NAGY

National Institute of Haematology and Blood Transfusion, "Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene,* and Semmelweis Medical University, Department of Vascular Surgery, Budapest, Hungary

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*Escherichia coli* O89 lipopolysaccharide (LPS) was treated with different doses of gamma irradiation (5, 10, 15, and 20 Mrad). Various biological activities such as lethal effect, decrease in arterial blood pressure in dogs, and interaction with the complement system were determined for the parent and irradiated preparations. Irradiation of LPS significantly and in a dose-dependent manner decreased its lethal and blood pressure-depressing effects along with its ability to activate the complement system. In contrast, radio-detoxified LPS fixed more strongly the isolated human C1 than did the parent LPS. The possible connection between the toxicity of endotoxin and endotoxin-induced complement activation is discussed.

Various chemical and physical procedures that lead to a decrease in toxicity of bacterial lipopolysaccharide (LPS), including acidic or alkaline hydrolysis, treatment with detergents, formalin, and enzymes, irradiation, etc., proved to be useful in studying the connection between the structure and biological properties of endotoxins (28). Gewurz et al. (16) and Galanos et al. (14) reported that detoxified LPS preparations lost their anticomplementary activity.

It is known that LPS can activate the complement system through both the classical and the alternative pathways (11, 22). Recently, it was demonstrated that the abilities of different LPS preparations to initiate the two complement pathways strongly differ (6). Lipid A isolated from LPS activates both the alternative and classical pathways. Furthermore, Loos et al. (21) found that LPS and lipid A interact with isolated human C1.

In earlier experiments, we studied some biological parameters (toxicity, endotoxin tolerance-inducing capacity, shock-preventing effect) of radio-detoxified LPS preparations (3 to 5). In the present experiments, the interaction between endotoxin that has been detoxified by gamma irradiation and the complement system was studied. An *Escherichia coli* O89 LPS preparation was treated with different doses of gamma irradiation, and its lethality, ability to decrease blood pressure, and activation of complement were determined for the parent and detoxified preparations. The extent of the endotoxin-induced activation of the classical and alternative pathways as well as the interaction of the untreated and irradiated LPS preparations with the isolated human C1 were also studied.

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MATERIALS AND METHODS

LPS preparation. LPS was isolated by the warm phenol-water method (32) from a fermentor-grown culture of *E. coli* O89 and purified by repeated ultracentrifugation in a Beckman L2 65B ultracentrifuge at 100,000 \(*\) g.

Detoxification of the LPS preparation. For detoxification, the LPS was dissolved in distilled water and irradiated at a concentration of 10 mg/ml in a \(^{60}\)Co source (NORATOM-Gamma 350C) by the method of Previte et al. (26) as modified by us.

Hypotension in dogs. To determine the hypotensive effect of various LPS preparations, female and male mongrel dogs, weighing 15 ± 6 kg, were used. A 2-mg/kg amount of LPS dissolved in isotonic saline was injected intravenously, and the blood pressure was measured in the carotid artery under chloralose anesthesia.

Determination of the LD\(_{50}\) of LPS in rats. To determine 50% lethal dose (LD\(_{50}\)) values of various LPS preparations, female Wistar-R/AxLong Evans/Hooded F\(_{1}\), hybrid rats (Laboratory Animal Institute, Gödöllő, Hungary) weighing 110 ± 5 g were injected intravenously. The LD\(_{50}\) values were calculated by the method of Reed and Muench (27).

Preparation of purified complement components and intermediate of immune hemolysis. Human C1 and guinea pig C1 were isolated in functionally pur-
ified form according to Nelson et al. (25). EAC1 was made by the method of Nelson et al. (25); EAC4 was made according to Borsos and Rapp (7).

Measurement of the anticomplementary activity of LPS preparations. A 0.1-ml amount of various dilutions of LPS was incubated with 0.1 ml of normal human serum at 37°C for 60 min. After incubation, the residual hemolytic complement activity was determined by the standard method (23). Anticomplementary activity of various doses of LPS was measured by the percent consumption of available complement (number of 50% hemolytic complement [CH50] units measured in control of samples incubated with the diluent). Veronal-buffered saline, pH 7.3, was used as diluent.

Measurement of the complement consumption via the alternative pathway. This measurement was performed as described by Fine et al. (9), using magnesium - ethyleneglycol - bis(2-aminoethyl ether)-N,N'-tetraacetic acid (Mg-EGTA) instead of EGTA for chelation of the Ca2+ in the serum (10). Normal human serum was mixed with 100 mM EGTA (Fluka AG, Buchs, Switzerland) in serum at a final concentration of 10 mM and with MgCl2 at a final concentration of 8 mM. One-tenth milliliter of the Mg-EGTA serum was incubated with 0.1 ml of the various dilutions of LPS for 60 min at 37°C. After incubation, the serum was recalcified and the residual complement activity was determined.

Measurement of C4 consumption in normal human serum. One-tenth milliliter of normal human serum was incubated with 0.1 ml of LPS at 37°C for 60 min, and the residual C4 activity in serum was measured by the effective titration method of Nelson et al. (25).

Measurement of consumption of isolated human C1. The ability of LPS preparations to consume human C1 was studied as outlined by Augener et al. (2). The extent of C1 consumption was expressed as the dose of LPS consuming 50% of the available C1 activity (measured in control samples incubated with diluent).

Detection of conversion of C3 in normal human serum by two-dimensional electrophoresis. The conversion of C3 was studied according to the method of Laurell (20), using monospecific goat antihuman C3 serum (Hyland Laboratories, Elkhart, Ind.). Electrophoresis was first performed in 1% agarose in Veronal-lactate buffer, pH 8.6 (μ = 0.25; 15 V/cm; 10 A for 2 h). The running buffer was Veronal-lactate, pH 8.6 (μ = 0.065). The agarose layer was put on a new glass slide. The remaining part of the slide was filled with 1% agarose in Veronal-lactate buffer, pH 8.6 (μ = 0.025), containing 1% antiserum. The second electrophoresis was run on a cooled plate for 16 h (2.5 V/cm; 4.5 A). The slides were washed, dried, and stained with Coomassie brilliant blue.

RESULTS

Effect of 60Co gamma irradiation on the various biological activities of LPS. E. coli O89 LPS was irradiated with different doses (5, 10, 15, and 20 Mrad) of 60Co, and the LD50 of the original and treated preparations was determined in rats (Fig. 1). Irradiation significantly increased the LD50 of the LPS, and detoxification was dose dependent. The LD50 of the parent preparation was 20 mg/kg; but after irradiation with 10 Mrad it increased to 70 mg/kg, and after irradiation with 20 Mrad it was more than 100 mg/kg.

In another experiment, five dogs were injected intravenously with 2 mg of the parent endotoxin per kg and nine animals were given the same dose of LPS irradiated with 20 Mrad. Arterial blood pressure was recorded continuously. Between 4 and 6 min after administration of the original preparation, blood pressure decreased by 61 ± 24 mm of Hg. In contrast, the detoxified preparation induced only a minimal decrease, 9.8 ± 8.8 mm.

Effect of 60Co gamma irradiation on the anticomplementary activity of LPS. Figure 2 shows the anticomplementary activity of the parent LPS and that of preparations treated with different doses of gamma irradiation. The activity of the irradiated LPS was significantly less than that of the control, and the effect was dependent on the irradiation dose. For consumption of 50% of the available complement activity, 32 μg of the parent LPS, 290 μg of the LPS irradiated with 10 Mrad, and more than 1,000 μg of the LPS irradiated with 20 Mrad were required.

Conversion of C3 in human serum treated with LPS. Figure 3 shows the results obtained with Laurell's two-dimensional electrophoresis technique (20). On the right is the arc of the intact C3 in normal human serum (Fig. 3D). In the center are shown the electrophoresis pat-

![Fig. 1. LD50 values of untreated and irradiated E. coli O89 LPS preparations in rats.](http://iai.asm.org/)
terns of C3 in normal serum treated at 37°C for 15 min with either 150 (Fig. 3C) or 50 (Fig. 3B) μg of parent LPS. Most of the C3 converted to faster-moving conversion products. On the left (Fig. 3A) is shown the electrophoresis pattern of human serum treated with 150 μg of LPS irradiated with 20 Mrad. C3 conversion was minimal since most of it remained intact.

Activation of the classical and alternative complement pathways by LPS preparations. Complement consumption by different doses of the parent and irradiated LPS in normal human serum treated with Mg-EGTA was measured. In the treated serum, C1 was nonfunctional due to the absence of Ca²⁺ ions. Activation could occur, therefore, only through the alternative pathway. Untreated LPS consumed much less complement in Mg-EGTA serum than in normal serum (Table 1). This indicates that the preparation activated the complement system primarily via the classical pathway. Irradiation decreased significantly the anticomplementary activity of parent LPS in normal serum. The activation of the alternative pathway decreased, but to a lesser degree than activation via the classical pathway. Preparations irradiated with 20 Mrad activated complement almost exclusively through the alternative pathway.

Similar results were obtained when C4 consumption in normal human serum by LPS was measured. A 500-μg amount of parent LPS consumed 4,700 U (57%) of the available C4 activity (8,100 U). The same dose of LPS that had been irradiated with 10 Mrad consumed 600 U (7%), whereas no C4 consumption was observed in serum incubated with 500 μg of LPS irradiated with 20 Mrad.

Comparison of complement consumption and C1 consumption of untreated and radio-detoxified LPS. Consumption of complement in normal human serum and of the isolated C1 by the same dose (22 μg) of parent and irradiated LPS was measured (Table 2).

As in the previous experiments, irradiated endotoxin consumed fewer CH₅₀ units in normal human serum than did the original preparation. However, gamma irradiation increased significantly the ability of LPS to consume isolated human C1 in a dose-dependent manner. In further experiments, the dose of different LPS preparations required for consumption of 50% of the available C1 activity was determined. For this, 64 μg of the untreated, 7.2 μg of the 10-Mrad-treated, and 3.0 μg of the 20-Mrad-treated preparations were necessary. The ratio of dose needed for 50% consumption of complement activity to 50% reduction in C1 activity was 1:2 for the parent LPS, 39:1 for LPS irradiated with 10 Mrad, and more than 1,000:1 for LPS irradiated with 20 Mrad.

DISCUSSION

Gewurz et al. (16) reported that the detoxification procedure also resulted in a loss in anticomplementary activity of LPS. Similar results were described by Galanos et al. (14), who found that different treatments of LPS had similar effects on its toxicity and anticomplementary properties.

Findings in this paper indicate that gamma irradiation decreases the lethal and complement-activating effects of endotoxin. The diminution of these two biological properties was dependent on the dose of irradiation and occurred in parallel. It seems, therefore, that different chemical and physical procedures affect...
equally the sites responsible for the toxicity and complement-activating ability on the LPS molecule. These results do not prove that the two sites are the same. Recently Galanos (13) succeeded in preparing a toxic preparation that was not anticomplementary in vitro. This material was electrodialyzed and neutralized with triethylamine and had a molecular weight of 50,000. It was free of aggregates. In contrast, preparations that activated the complement system were at least partly in aggregated form. It is possible, however, that under in vivo conditions the triethylamine preparation reaggregates and regains its complement-activating ability.

More studies are needed, therefore, to clarify the role that the complement system plays in the complex mechanisms involved in the death of endotoxin-treated animals. There are, however, certain effects of endotoxin that are known to be complement dependent (17). One of these is the initial decrease in arterial blood pressure in dogs elicited by an intravenous injection of LPS (12, 15). We demonstrated in this study that radio-detoxification abolished almost completely this effect in LPS. This finding suggests that the hypotensive effect of endotoxin is mediated via the complement system not only in the dogs but in rats as well. In contrast, LPS-induced hypotension was demonstrated to be complement independent in rabbits (29) and in felines (19). It is difficult to explain this difference between various species; presumably it is connected with the absence or presence of the anaphylatoxin inactivator (30).

It is known that bacterial LPS can activate both the classical and alternative pathways of complement (11, 22). The proportion of activation in the two pathways is said to be different when LPS is isolated from different strains of the same species of gram-negative bacteria (6). The endotoxin used in this study was isolated from E. coli 089 by the phenol-water method (28). The ability of this preparation to activate the alternative pathway was measured in human serum chelated with EGTA and supplemented with Mg2+ ions. We have demonstrated (G. Füst and M. Ádám, Acta Microbiol. Acad. Sci. Hung., in press) that in Mg-EGTA the "detoxification" of soluble LPS observed by Fine (8) in serum chelated with EGTA without Mg2+ does not occur. This method can be used, therefore, to estimate the activation of the alternative pathway induced not only by cell-bound but also by soluble LPS.

On the basis of the consumption of complement measured in normal human serum and in Mg-EGTA serum, respectively, the original LPS preparation was shown to be capable of activating both complement pathways. Irradiation of the LPS decreased its ability to activate both pathways in a dose-dependent manner. The diminution of the alternative pathway-activating capacity of the LPS was, however, less marked. This finding seems to confirm our earlier observation (Füst and Ádám, Acta Microbiol. Acad. Sci. Hung., in press) that different parts of the LPS molecule are responsible for activation of the two pathways. Very recently, Morrison et al. (D. C. Morrison, P. M. Henson, and L. F. Kline, Fed. Proc. 35:655, 1976) suggested that the lipid A part of the LPS macro-molecule initiated the classical pathway activation, whereas complement activation through the alternative pathway was triggered by the polysaccharide part of the molecule.

In studies of the interaction of parent and radio-detoxified endotoxin with isolated human C1, it was found that consumption of C1 by detoxified preparations did not diminish in parallel with its loss in complement-activating capacity. In contrast, a sharp increase in C1 consumption occurred. The amount necessary for the consumption of 50% of the available C1 activity by LPS irradiated with 20 Mrad was

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**Table 1. Complement consumption by different doses of untreated and 56Co-irradiated E. coli 089 LPS preparations in normal and Mg2+-EGTA sera**

<table>
<thead>
<tr>
<th>LPS dose (μg)</th>
<th>Normal serum</th>
<th>Mg2+-EGTA serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0°</td>
<td>10</td>
</tr>
<tr>
<td>7.4</td>
<td>22.6</td>
<td>8.0</td>
</tr>
<tr>
<td>22.2</td>
<td>46.8</td>
<td>20.0</td>
</tr>
<tr>
<td>66.6</td>
<td>62.2</td>
<td>35.8</td>
</tr>
<tr>
<td>200.0</td>
<td>84.2</td>
<td>53.2</td>
</tr>
</tbody>
</table>

*CH50 units available: 5.0.
*Mg2+-EGTA serum.

**Table 2. Complement and Cl consumption induced by 22.2 μg of untreated and irradiated E. coli 089 LPS preparations**

<table>
<thead>
<tr>
<th>Irradiation dose (Mrad)</th>
<th>Complement activity consumed (% of the CH50 units available)</th>
<th>Cl activity consumed (% of the Cl activity available)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>41</td>
<td>36</td>
</tr>
<tr>
<td>5</td>
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<td>83</td>
</tr>
<tr>
<td>15</td>
<td>11</td>
<td>34</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>84</td>
</tr>
</tbody>
</table>

*CH50 units available: 5.0.
*Cl activity available: 1.0 (α).
about 1/30 the dose of parent LPS required. Loos et al. (21) first described the ability of LPS to interact with isolated C1, but no comparison was made with the complement-activating capacity of different preparations. In studies not reported here (G. Füst, and M. Ádám, unpublished observations), we have observed no correlation between the extent of activation of the classical pathway and the consumption of isolated C1 induced by preparations isolated from 12 strains of Pseudomonas aeruginosa. Similar results were obtained with rat myeloma immunoglobulin G proteins (24). These observations demonstrate that the consumption of isolated C1 cannot necessarily be taken as a measure of the activation of the classical pathway. This suggests that the interaction of C1 with LPS is not necessarily followed by the activation of other components of complement. Similar results were reported recently by Allen and Isliker (1) and Hurst et al. (18). Further studies designed to explain why irradiated LPS shows increased consumption of C1 are in progress.

LITERATURE CITED
