Lipid A as the Biologically Active Moiety in Bacterial Endotoxin (LPS)-initiated Generation of Procoagulant Activity by Peripheral Blood Leukocytes

By Julian Niemetz and David C. Morrison

Preparations of rabbit or human leukocytes, when incubated with bacterial endotoxins (lipopolysaccharides, LPS) are stimulated to generate a procoagulant-tissue factor activity (TFa). As LPS has been shown to consist of specific repeating oligosaccharide side chains (O-antigen) linked to a central polysaccharide core region that is, in turn, linked to the lipid region of the molecule (lipid A), we have examined the biochemical requirement of the LPS necessary for generation of TFa. Using preparations of LPS from mutant strains of bacteria, which contain varying amounts of polysaccharide in relation to lipid A, we have demonstrated that activity is associated with the lipid A region of the LPS molecule. These observations have been confirmed using isolated lipid A, which is a potent stimulator of TFa, as well as a native protoplasmic polysaccharide that is both devoid of lipid A and without detectable TFa stimulatory activity. Modification of LPS by treatment with mild alkali abrogated its capacity to stimulate TFa generation. In addition, such altered preparations of LPS partially inhibit the stimulatory effect of native LPS. Similarly, treatment of LPS (or lipid A) with the antibiotic polymyxin B substantially inhibited the stimulatory effect of LPS.

BACTERIAL ENDOTOXINS, or lipopolysaccharides (LPS), a major constituent of the outer cell wall of most gram-negative bacteria, can initiate a number of potent pathophysiologic changes when administered to experimental animals. Many of these changes parallel those seen in patients with gram-negative sepsis, including hypotension, peripheral blood cell changes, and disseminated intravascular coagulation (DIC). Although the precise mechanism(s) for many of these changes is not understood at present, it is clear that LPS has the capacity to interact with a number of mediation systems in vitro, in particular, with mediators of the clotting system. Thus LPS has been shown to activate purified Hageman factor and initiate intrinsic clotting, and to induce release of vasoactive and coagulation-promoting substances from rabbit platelets. In addition, preparations of leukocytes are known to exhibit a procoagulant activity related to the extrinsic coagulation pathway which is greatly enhanced by LPS.

The basic biochemical structure of LPS may be defined in three major re-
regions: (1) the repeating 0-antigenic oligosaccharide units, which are linked to (2) the core polysaccharide region, which is linked via a trisaccharide of 2-keto-3-deoxyoctulosonate (KDO) to (3) the lipid portion of the molecule (lipid A). The structure of the lipid A region of the molecule has recently been elucidated and basically consists of two N-acetylglucosamines, glycosidically linked, containing pyrophosphates and both ester- and amide-linked long-chain fatty acids. The isolation and characterization of a rough mutant from Salmonella minnesota (R-595) by Westphal and co-workers has provided the first clear evidence that the lipid A region of the LPS molecule is responsible for the endotoxic and pyrogenic properties of LPS. Since that time, lipid A has been demonstrated to be critical for a number of the biologic properties of LPS, and recent experiments by Ulevitch et al. have established that preparations of R-595-LPS can initiate almost all of the pathophysiologic changes produced by smooth preparations of LPS, including hypotension, thrombocytopenia, leukopenia, and DIC.

If the enhancement of procoagulant activity of peripheral blood leukocytes by LPS in vitro is to play a potentially significant role in the in vivo generation of DIC initiated by the lipid A region of LPS, it becomes imperative to demonstrate that the leukocyte procoagulant activity is also associated with lipid A. The experiments described in this report have been designed to examine this question in detail. We conclude that the generation of procoagulant activity is a property of the lipid A part of the LPS molecule.

MATERIALS AND METHODS

Preparation of Leukocytes

Rabbit leukocyte preparations were obtained from arterial blood anticoagulated with 0.1 volume 4% trisodium citrate dihydrate. The whole blood was sedimented with dextran 250 (Pharmacia Fine Chemicals, Rahway, N.J.) and the leukocyte suspension washed four times in citrated saline (5 parts 0.15 M saline, 1 part 4% trisodium citrate dihydrate at 4°C as previously described). The leukocytes were then suspended at the desired concentration in Hank’s balanced salt solution (Difco Laboratories, Detroit, Mich.) that contained 100 U/ml each of penicillin and streptomycin. The final concentration of leukocytes in the incubation mixture was 2 x 10^6/ml.

Human leukocytes were obtained from venous blood and processed in a similar manner. Final concentration of human leukocytes in the incubation mixture was 2.0 x 10^7/ml. Leukocytes were suspended in medium 199 (Difco) containing 20% serum obtained from the same donor. Prior to use, serum was first absorbed three times with 2.0 mg/ml tricalcium phosphate (Baker, Philipsburg, N.J.) for 10 min at room temperature, followed by centrifugation at 2200 g for 10 min to remove tricalcium phosphate.

Preparation of Lipopolysaccharides

LPS was extracted from Escherichia coli 0111:B4 by the phenol-water procedure of Westphal and Jann, digested with RNAase, and fractionated into two fractions, containing a high percentage (LPS II) and low percentage (LPS I) by weight of lipid A, by gel filtration chromatography. LPS from S. minnesota R-595 was extracted by the phenol-chloroform-petroleum ether procedure described by Galanos et al. Lipid A was prepared by mild acid hydrolysis (1%, glacial acetic acid, 100°C, 2 hr) of a phenol-water extract of S. minnesota R-60 and was solubilized by the addition of 1% triethyl amine followed by sonication prior to use.*

*The R-595 LPS and lipid A were prepared while one of the authors (D.C.M.) was a guest in the laboratory of Prof. Otto Luderitz, Max Planck Institut für Immunbiologie, Freiburg im Br., West Germany.
LPS from *E. coli* 026:B6 was obtained from Difco Laboratories. LPS from the galactose-epimeraseless (J-S) mutant of *E. coli* 0111:B4 was the generous gift of Dr. Abraham Braude, Division of Infectious Diseases, University of California, San Diego, Calif. Native protoplasmic polysaccharide (NPP) from *E. coli* 0113 was the generous gift of Dr. Tony Rudbach, Department of Microbiology, U. of Montana, Missoula, Montana. These various preparations of LPS are diagrammed schematically in Table 1. All preparations of LPS were made into distilled water.

Base-hydrolyzed LPS (BHLPS) was prepared by treating LPS with 0.5 N NaOH at 56°C as described previously. Preparations of LPS or lipid A were treated with polymyxin B (PB-LPS, Sigma Chemical Co., St. Louis, Mo.) as described earlier. Three- to fivefold excesses of PB were added to LPS preparations, incubated for 60 min at room temperature, and either used immediately or dialyzed extensively at 2°C to remove unbound PB. We, and others, have previously demonstrated that PB formed a tight noncovalent complex with the lipid A region of the LPS molecule. As it was established that PB also inhibited coagulant activity, experiments were done to insure that unbound PB was removed. After dialysis of comparable amounts of PB, the content of the bag was added to leukocytes and lipid A and the mixture allowed to generate TFα.

Meticulous care was taken in all experiments to avoid contamination with extraneous endotoxins and to preserve sterility.

**Assay of Coagulant Activity**

The coagulant activity was tested in two ways, by the one-stage test, as described previously, and by the two-stage assay by the method of Nemerson with minor modifications. The activities reported by the two-stage assay are given in units derived from comparison with dilutions of a saline extract of commercial rabbit brain thromboplastin (Difco) prepared as previously described. One milliliter of this extract contained 1000 units of TFα.

The results presented here are the average of at least three separate experiments.

**RESULTS**

**Generation of Tissue Factor Activity**

We initially examined the capacity of a number of preparations of LPS with varying amounts of polysaccharide in relation to lipid A (Table 1) to generate TFα. For these experiments, increasing amounts of LPS were added to rabbit leukocytes and the amount of TFα generated after 18 hr of incubation was quantitated. Results of these experiments (Fig. 1) indicated that all preparations of LPS tested had the capacity to generate significant amounts of TFα. In general, however, those preparations of LPS with a higher percentage by weight of lipid A were significantly more active than those of lower lipid A content. When the concentration of LPS required to generate 15 units of TFα was determined, LPS from the rough mutants J-S and R-595 were the most potent (Table 2), with 2–4 ng/ml sufficient to give significant activity. This observation was further supported by the use of two preparations of LPS (from *E. coli* 0111:B4) that differed only in their relative amount of lipid A to polysaccharide. Thus LPS II was approximately tenfold more active than LPS I. Finally, isolated lipid A itself was a potent stimulator of TFα although somewhat less

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Table 1. Structure of Preparations of LPS Used in This Study

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> 026:B6 LPS</td>
<td>lipid A-(KDO)₃-core PS-(0-Ag) -(0-Ag)</td>
</tr>
<tr>
<td><em>E. coli</em> 0111:B4 LPS I</td>
<td>lipid A-(KDO)₃-core PS-(0'-Ag)-(0'-Ag)</td>
</tr>
<tr>
<td><em>E. coli</em> 0111:B4 LPS II</td>
<td>lipid A-(KDO)₃-core PS-(0'-Ag)-(0'-Ag)</td>
</tr>
<tr>
<td><em>E. coli</em> 0111:B4 J-5-LPS</td>
<td>lipid A-(KDO)₃-core PS</td>
</tr>
<tr>
<td><em>S. minnesota</em> R-595-LPS</td>
<td>lipid A-(KDO)₃</td>
</tr>
<tr>
<td>Isolated lipid A</td>
<td>lipid A</td>
</tr>
</tbody>
</table>
Fig. 1. TFa generated from rabbit peripheral blood leukocytes by various preparations of LPS. Approximately 2 × 10⁶ leukocytes/ml were incubated in Hanks' balanced salt solution with the indicated amounts of LPS; the amount of TFa generated after 18 hr was assayed. Schematic representation of the structures of the LPS preparations used are given in Table 1.

active than the rough mutant. Taken together, these observations provided strong evidence that the lipid A region of the LPS molecule played a major role in LPS-initiated generation of TFa from leukocytes. It is also apparent from Fig. 1 and Tables 1 and 2, however, that the percentage by weight of lipid A in preparations of LPS was not the sole determining factor for TFa generation. For example, the LPS from *E. coli* 026:B6, while less active than the LPS from J-5 or R-595, was more active than either isolated lipid A itself or LPS II from *E. coli* 0111:B4, both of which contained more lipid A than 026:B6-LPS (Table 1).

Several preparations of LPS were also examined for their ability to generate TFa from human peripheral blood leukocytes. As shown in Fig. 2, all three preparations tested were able to generate TFa, although, in contrast to rabbit leukocytes, significantly higher cell concentrations were required. Since both the R-595-LPS and the isolated lipid A had activity, a prominent role for lipid A was again suggested.

The generation of TFa by rabbit leukocytes initiated by the various preparations of LPS was also examined by their effect on the one-stage clotting times. As shown in Fig. 3, parallel results were obtained to those with the two-stage

| Table 2. Relative Quantities of LPS Required for the Generation of TFa* |
|------------------------|----------------------|
| LPS J-5                | 1†                   |
| LPS R-595              | 1.7                  |
| LPS 026:B6             | 8                    |
| Lipid A                | 20                   |
| LPS II                 | 50                   |
| LPS I                  | 500                  |

*Data obtained from experiment described in Fig. 1.
†Base number 1 represents 2 ng/ml required for 15 units of TFa.
assay (Fig. 1), with the LPS from the J-5 mutant being the most efficient at reducing clotting times, and LPS I from *E. coli* 0111:B4 being the least efficient. It was also apparent from these experiments that LPS from *E. coli* 026:B6 appeared to have higher activity than either isolated lipid A or LPS II from *E. coli* 0111:B4, both of which contained a greater percentage by weight of lipid A.

**Effect of Chemical Modification of Lipid A on Generation of TFa**

The experiments described above suggested that, as with many of the other biologic properties of LPS, activity was associated with the lipid A region of
the molecule. A number of investigators used mild alkali treatment of LPS to hydrolyze selectively the ester-linked fatty acids on the lipid A and thus altered the biologic properties, e.g., toxicity, mitogenicity, or immunogenicity of the LPS molecule. We therefore examined the effect of base hydrolysis on the ability of LPS to generate TFa from rabbit leukocytes.

We initially examined the effect of alkali treatment on $E. coli$ 0111:B4 LPS I since an LPS with a low content of lipid A should be most sensitive to minor alterations in the lipid A region. As a control for these experiments we have utilized a high molecular weight polysaccharide from the related strain $E. coli$ 0113. This NPP, as has been shown by Rudbach and his colleagues, has a carbohydrate composition like that of LPS but is free of lipid A. As expected, such a preparation (Fig. 4) is without TFa activity, even when concentrations as high as 20 μg/ml are tested, in contrast to LPS I, with which 0.5–1.0 μg generates substantial activity. Treatment of LPS I with alkali for 20 min significantly reduces its capacity to stimulate TFa. After 40 min, activity is reduced almost to the level of NPP.

Experiments were also performed to examine the effect of alkali treatment on the capacity of the high-lipid A-containing LPS II from $E. coli$ 0111:B4 to promote generation of TFa. As shown in Table 3, treatment of LPS II under conditions which almost completely abrogated the generation of TFa by LPS I had a smaller, although still significant, effect upon LPS II. Most surprising,
Table 4. Inhibitory Effect of Base-hydrolyzed LPS (BHLPS II) on LPS-initiated Generation of TFα

<table>
<thead>
<tr>
<th>Time of Addition to Leukocytes*</th>
<th>TFα Generated (units)</th>
</tr>
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<tbody>
<tr>
<td>0 hr</td>
<td>1 hr</td>
</tr>
<tr>
<td>LPS</td>
<td></td>
</tr>
<tr>
<td>BHLPs II</td>
<td></td>
</tr>
<tr>
<td>LPS + BHLPs II</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>BHLPs II</td>
</tr>
<tr>
<td>LPS</td>
<td>Sonicate</td>
</tr>
<tr>
<td>LPS</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td></td>
</tr>
</tbody>
</table>

*Experiment performed essentially as described in the legend of Fig. 1. IPS from E. coli 026:B6 was used at a concentration of 5 μg/ml, and BHLPs from E. coli 0111:B4 (LPS II) was used at a concentration of 20 μg/ml.

However, was the pronounced inhibition of TFα generation at high concentrations of BHLPs II. Although inhibition was only moderate at concentrations of BHLPs in the range 0.1–1.0 μg/ml, virtually complete abrogation of the generation of TFα was observed at 10 μg/ml.

We next examined the capacity of preparations of BHLPs II to inhibit the activity of untreated preparations of LPS. For these experiments, rabbit peripheral blood leukocytes were incubated with E. coli 026:B6 LPS for various times, after which an inhibitory concentration of BHLPs II from E. coli 0111:B4 (20 μg/ml) was added. Control cells were incubated with either preparation alone. As shown in Table 4, the positive preparation of LPS (026:B6) generated high TFα activity, whereas the BHLPs II preparation was without activity. Significantly, the simultaneous addition of the BHLPs II to 026:B6 LPS caused a drastic reduction (83%) in the ability of the latter to generate TFα. The temporal importance of the addition of the inhibitory BHLPs II is also shown in Table 4. Addition of BHLPs 1 hr after stimulation with positive LPS caused only a 50% inhibition in activity, and a 2-hr delay before addition of BHLPs II was without detectable effect. Thus the time of interaction of LPS with the leukocyte which committed the cell to the generation of TFα appeared to be within the first 2 hr. Control cells incubated with positive LPS (026:B6) in which further generation of TFα was arrested by sonic disruption showed that very little TFα was generated after 1 hr of incubation, suggesting that the BHLPs II inhibitory effect was not due to cell death (Table 4).

As an alternative method of demonstrating a role for lipid A in the generation of TFα by peripheral blood leukocytes, we have utilized the observation that PB forms a molecular complex with the lipid A region of LPS. The

Table 5. Inhibitory Effect of Polymyxin B (PB) on Lipid A*

<table>
<thead>
<tr>
<th>TFα Generated (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC + lipid A</td>
</tr>
<tr>
<td>WBC + lipid A + PB</td>
</tr>
<tr>
<td>WBC + (lipid A + PB)</td>
</tr>
<tr>
<td>WBC + PB dialyzed</td>
</tr>
</tbody>
</table>

*PB (15 μg) added to 5 μg lipid A and either tested directly for effect on generation of TFα (as described in the legend of Fig. 1) or after dialysis to remove unbound PB. Control indicates that unbound PB is substantially dialyzable.
formation of such complexes has been demonstrated to abrogate lipid A-dependent activities of LPS such as toxicity, murine lymphocyte mitogenicity, and activation of the classical pathway of complement. When such complexes are formed and either examined directly or after dialysis to remove unbound PB, significant inhibition of TFa generation is observed (Table 5). The reason for a lack of complete inhibition is unclear, although recent experiments have shown that, under some circumstances, PB does not bind to LPS, which may in part account for the lack of complete inhibition. Nevertheless, the demonstration of significant inhibition by PB further supports the conclusion that lipid A has a role in LPS-initiated TFa generation by leukocytes.

DISCUSSION

In the experiments reported here, rabbit and human peripheral blood leukocytes were incubated with various preparations of LPS and the generated procoagulant activity assayed. In general, LPS preparations having the highest amount of lipid A by weight, e.g., S. minnesota R-595 and E. coli 0111:B4 J-5, were the most active. In addition, preparations of LPS in which the lipid A was modified either by base hydrolysis or by treatment with PB showed decreased stimulatory activity when compared to control preparations. These findings strongly supported the concept that lipid A was the part of LPS responsible for generation of the procoagulant activity. However, preparations of lipid A, while very active, were not the most active, which raised the question whether a very short polysaccharide chain in the R-595 LPS (such as KDO) or in the J-5 preparations (such as KDO and core polysaccharide) was required for full stimulatory activity. The presence of such a polysaccharide would be expected to increase partially the solubility of such preparations. Thus, the limited activity of lipid A might result from its very poor solubility; as a consequence, in these experiments less lipid A was available to the cells for the stimulatory effect to occur. Another explanation for the lower stimulatory activity of lipid A is that activity may have been lost during the separation procedure used in the isolation of lipid A. Such possibilities do not fully account for the increased activity of 026:B6 LPS as compared to 0111:B4 LPS II. A complete explanation will await further investigation.

A significant observation in these experiments was that BHLPS II, when used at higher concentrations, had a profound inhibitory effect on the TFa stimulatory effect of positive preparations of LPS. Its mode of action is as yet unclear, but it may act by occupying a “site” for LPS on the leukocyte. The fact that BHLPS II can inhibit the response of an LPS with a completely unrelated O-antigenic polysaccharide provides additional support for lipid A being the functionally active portion of the LPS. Finally, PB shows a partial but significant inhibition of lipid A stimulatory activity (care was taken to exclude direct effect on the procoagulant activity). As PB complexes with the lipid A directly, these experiments would suggest that PB blocks the available lipid A-binding sites necessary for interaction with the leukocyte membrane.

Some of the experiments described in this manuscript have been presented earlier and are essentially in agreement with the preliminary studies recently reported by Rickles et al.
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