Role of Lipopolysaccharide and Complement in Susceptibility of
Escherichia coli and Salmonella typhimurium to Non-immune Serum

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The role of lipopolysaccharide (LPS) in the susceptibility of Escherichia coli and Salmonella typhimurium to non-immune human serum was investigated using serum-sensitive strains of both enterobacteria. LPS from serum-resistant strains of E. coli and S. typhimurium could activate and completely remove the serum bactericidal activity, and also showed dose-dependent anti-complement activity. These properties were mainly due to the high-molecular-mass LPS: the low-molecular-mass LPS from serum-resistant strains of E. coli and S. typhimurium had only a slight effect on the serum bactericidal activity, and showed only low anti-complement activity, even at high concentration. The results suggest that LPS composition, especially the O-antigen polysaccharide chains, contributes to the susceptibility of E. coli and S. typhimurium strains to complement-mediated serum bactericidal activity.

INTRODUCTION

The bactericidal effect of non-immune serum plays an important role in host defence against bacterial infection. This phenomenon has been widely noted and studied since the late 1800s (Buchner, 1889) and has been shown to be complement mediated (Roantree & Pappas, 1960). Although serum resistance of Gram-negative bacteria probably has a multifactorial basis in vim, the outer membrane clearly is involved as the most peripheral component of the bacterial cell envelope. Several studies have implicated outer membrane components such as lipopolysaccharide (LPS) (Glynn & Ward, 1970, Munn et al., 1982; Nelson & Roantree, 1967), capsular polysaccharide (Glynn & Howard, 1970; Sutton et al., 1982) and outer membrane proteins (Hildebrant et al., 1978; Moll et al., 1980; Taylor & Parton, 1977) in resistance of bacterial strains to the bactericidal activity of serum.

Complement activation by Gram-negative bacteria can occur via the classical or the alternative pathway. The classical pathway can be activated directly by the interaction of antibody with bacterial surface antigens such as the lipid A moiety of LPS (Morrison & Kline, 1977). The alternative pathway may be activated by bacterial surface polysaccharides independent of antibody (Morrison & Kline, 1977).

In the present study we have investigated the mechanism of complement activation by the LPS of Escherichia coli and Salmonella typhimurium. We have also examined the role of high-molecular-mass LPS (HMM-LPS) (O-antigen enriched) and low-molecular-mass (LMM-LPS) (core and lipid A enriched) in serum susceptibility.

METHODS

Bacterial strains and media. The E. coli and S. typhimurium strains used are listed in Table 1. S. typhimurium strains SA2380 and SA1377 are isogenic mutants of strain Su453. The basal medium for bacterial growth was Luria broth (LB) (Miller, 1972) or LB with 1.5% (w/v) agar (LB-agar).

Abbreviations: HMM-LPS, high-molecular-mass lipopolysaccharide; LMM-LPS, low-molecular-mass lipopolysaccharide; KDO, 2-keto-3-deoxyoctulosonic acid; NHS, non-immune human serum.

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serum (NHS) was measured as previously described (Tomás et al., 1986). Controls (bacteria in phosphate-buffered saline: PBS, containing 0.15 M-sodium chloride and 0.15 M-sodium phosphate, pH 7.2) showed no significant changes in viable counts over the incubation period. We used heat-inactivated NHS (56 °C for 30 min) as an additional control. Serum was usually used on the day of collection or stored at −80 °C.

LPS isolation and subfractionation. LPS from E. coli O55:B5 or S. typhimurium Sa453 was purified by the method of Westphal & Jann (1965) or purchased from Sigma. LPS from serum-sensitive strains of E. coli and S. typhimurium was purified according to Westphal & Jann (1965) with the modification of Osborn (1966).

Lyophilized LPS was solubilized at a final concentration of 7.5 mg ml⁻¹ in buffer containing 3% (w/v) sodium deoxycholate, 0.2 M-NaCl, 5 mM-EDTA, 20 mM-Tris/HCl (pH 8.3), and was applied at room temperature to a column of Sephacryl S-300 (Pharmacia) equilibrated in buffer containing 0.25% sodium deoxycholate, 0.2 M-NaCl, 5 mM-EDTA, 10 mM-Tris/HCl (pH 8.0). Fractions (2.5 ml) were collected at a flow rate of 12 ml h⁻¹ and were analysed directly by SDS-PAGE. Before chemical analyses and serum inhibition studies, fractions were dialysed extensively against distilled water, first at room temperature and then at 4 °C.

Electrophoretic techniques. SDS-PAGE was done according to the procedure of Laemmli (1970) as modified by Ames et al. (1974). Samples were mixed 1:1 with sample buffer (containing 4%, w/v, SDS) and boiled for 5 min, and 10 μl portions were applied to the gel. LPS bands were detected by the silver stain method of Tsai et al. (1982).

Analytical procedures. Organic phosphate was measured by the method of Ames & Dubin (1960) using NaH₂PO₄, 2H₂O as standard, and total carbohydrates by the phenol reaction procedure (Hanson & Phillips, 1981) with glucose as standard. 2-Keto-3-deoxyoctulosonic acid (KDO) was measured by the thiobarbituric acid method after hydrolysis of samples in 0.05 M-sulphuric acid for 30 min (Karkhanis et al., 1978).

Inhibition of serum bactericidal activity by complete and fractionated LPS. LPS was suspended in PBS (pH 7.2) to a final concentration of 1–5 mg ml⁻¹ and briefly sonicated at 4 °C until the solution cleared. LPS solution in the concentration range 0.01–0.2 mg ml⁻¹ was added to 0.5 ml serum in a tube. The volume was adjusted to 0.9 ml with PBS, and the solution was incubated at 37 °C with shaking (200 r.p.m.) for 30 min. Then, 0.1 ml of bacterial suspension (5 × 10⁶ c.f.u.) in the exponential phase was added to the tube and incubated at 37 °C for an additional 1 h before dilution plating. Similar experiments were done with LPS fractions lyophilized and suspended in PBS and added at a final concentration of 0.05 mg ml⁻¹ to 0.5 ml serum. Controls without LPS or LPS fractions showed no inhibition of serum bactericidal activity.

Measurement of the anti-complement activity of LPS from E. coli and S. typhimurium. The anti-complement activity of LPS or LPS fractions was measured according to Shafer et al. (1984) with slight modifications. Serum (0-1 ml) was mixed with LPS (0.01–0.2 mg ml⁻¹) or LPS fractions (0-05 mg ml⁻¹) suspended in PBS, or with PBS alone, to a final volume of 0.2 ml, and incubated with shaking at 37 °C for 30 min. Antibody-sensitized sheep erythrocytes in 0.2 ml PBS were added to a fourfold dilution of treated NHS and incubated for an additional 30 min at 37 °C. Ice-cold saline (3 ml) was added to the mixture, the cells were pelleted by centrifugation, and the absorbance of the supernatant was measured at 412 nm. The positive control was sensitized sheep erythrocytes plus serum without added LPS or LPS fraction, and the negative control was LPS or LPS fraction without added serum.

Radiolabelled LPS. Bacteria were grown with the addition of D-[2-³H]glucose (5 mCi l⁻¹, 185 MBq l⁻¹; Amersham) and LPS was extracted and fractionated as described above.

Liposome preparation. Liposomes were prepared according to Porat et al. (1987) and mixed with ³H-labelled LPS or ³H-labelled LPS fractions (0.5 mg) in a total volume of 0.2 ml. The liposome suspension was agitated vigorously for 5 min, washed twice with PBS and resuspended in 1 ml PBS. The suspension (0.2 ml) was then incubated at 37 °C for 30 min with one of the following: 20% (v/v) NHS, 20% heat-inactivated NHS, or PBS alone, in a final volume of 1 ml. Each mixture was chromatographed on Sephadex G-25 and 1 ml fractions were collected and assayed for radioactivity.
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Table 2. Inhibition of serum bactericidal activity against E. coli and S. typhimurium strains by homologous and heterologous LPS

The LPS concentration used was 0.1 mg ml⁻¹. The results are the means of three independent experiments. NHS pretreated with LPS from S. typhimurium Su453 (serum-resistant) was unable to kill E. coli strain PL-2 (serum-sensitive), and NHS pretreated with LPS from E. coli O55: B5 (serum-resistant) was unable to kill S. typhimurium strain SA2380 (serum-sensitive).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control NHS</th>
<th>Serum-sensitive</th>
<th>Serum-resistant</th>
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<tr>
<td>E. coli</td>
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<tr>
<td>PL-2</td>
<td>&lt;1</td>
<td>5</td>
<td>108</td>
</tr>
<tr>
<td>Y10</td>
<td>&lt;1</td>
<td>4</td>
<td>107</td>
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<tr>
<td>S. typhimurium</td>
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</tr>
<tr>
<td>SA2380</td>
<td>&lt;1</td>
<td>4</td>
<td>109</td>
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<tr>
<td>SA1377</td>
<td>&lt;1</td>
<td>7</td>
<td>111</td>
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RESULTS

Inhibition of serum bactericidal activity by E. coli and S. typhimurium LPS

Purified LPS from E. coli O55: B5 at various concentrations was able to inhibit the bactericidal activity of NHS against E. coli serum-sensitive strain PL-2. The survival of PL-2 cells in NHS after 60 min incubation at 37 °C was 0, 39, 86, 108, 109, 112% at LPS concentrations of 0, 0.025, 0.05, 0.1, 0.2, 0.4 mg ml⁻¹, respectively. Purified LPS from S. typhimurium Su453 at various concentrations was also able to inhibit the bactericidal activity of NHS against S. typhimurium serum-sensitive strain SA2380. The survival of SA2380 cells in NHS after 60 min incubation at 37 °C was 0, 41, 87, 109, 111, 115% at LPS concentrations of 0, 0.025, 0.05, 0.1, 0.2, 0.4 mg ml⁻¹, respectively.

Table 2 shows the percentage survival of serum-sensitive strains of E. coli and S. typhimurium in NHS treated with LPS (0.1 mg ml⁻¹) from serum-sensitive and serum-resistant strains of both enterobacteria. Serum-sensitive strains of E. coli (PL-2 and Y10) and S. typhimurium (SA2380 and SA1377) showed a survival in control NHS of less than 1% after 60 min incubation, whereas these strains survived well in NHS pre-incubated with LPS obtained from serum-resistant strains of E. coli and S. typhimurium. LPS from serum-sensitive strains only slightly inhibited the bactericidal activity of NHS (Table 2).

Subfractionation of LPS

LPS from the serum-resistant strains E. coli O55: B5 and S. typhimurium Su453 were fractionated as described in Methods. The presence of LPS in column fractions was monitored by determination of organic phosphate, KDO and total hexose, as well as by SDS-PAGE (Fig. 1 for E. coli O55: B5 LPS; Fig. 2 for S. typhimurium Su453 LPS).

Purified LPS from E. coli O55: B5 showed a main peak of total hexose centred in fraction 30. This fraction also coincided with a peak of KDO (Fig. 1a) and was HMM-LPS (Fig. 1b). In these peak fractions (28–32) the apparent ratio of hexose to KDO was approximately 25–30:1. This value decreased in the following fractions until the main peak of KDO (Fig. 1a), centred in fraction 42, where the apparent ratio of hexose to KDO was approximately 1–2:1, a feature consistent with LPS fractionation on the basis of a decreasing number of repeating units in the O-antigen side chains. Fractions 38–48 contained LMM-LPS (Fig. 1b).

A similar profile was obtained by fractionation of the S. typhimurium Su453 LPS. The main peak of total hexose was centred in fraction 28 (Fig. 2a) and coincided with a peak of KDO and HMM-LPS (Fig. 2b). In these peak fractions (26–30) the apparent ratio of hexose to KDO was approximately 25–35:1. As in the E. coli LPS subfractionation, the hexose:KDO ratio
Fig. 1. (a) Subfractionation of LPS from *E. coli* O55:B5 (serum-resistant) by column chromatography. LPS was applied to a column of Sephacryl S-300 and eluted with 0.25% buffered deoxycholate as described in Methods. Eluted fractions were analysed for KDO (●●●) and total hexose (○○○) after extensive dialysis, and by SDS-PAGE in conjunction with silver staining for carbohydrate (see b). V₀, void volume. (b) Analysis of subfractionated LPS from *E. coli* O55:B5 by SDS-PAGE. LPS fractions from the gel filtration (a) were analysed and stained with silver. The leftmost lane contains purified LPS from *E. coli* O55:B5; the other lanes are labelled by fraction number (see a).

decreased in the following fractions until the main peak of KDO (Fig. 2a) centred in fraction 42, where the apparent ratio was approximately 1:2. Fractions 38–50 were LMM-LPS (Fig. 2b).

*Inhibition of serum bactericidal activity by *E. coli* and *S. typhimurium* LPS fractions*

Serum-sensitive strains of *E. coli* (PL-2) and *S. typhimurium* (SA2380) showed a high percentage survival (>50%) in NHS treated with LPS fractions having a high hexose:KDO ratio (HMM-LPS), such as fractions 28–32 from the *E. coli* O55:B5 subfractionation, or
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Fig. 2. (a) Subfractionation of LPS from *S. typhimurium* Su453 (serum-resistant) by column chromatography. Details as for Fig. 1(a). (b) Analysis of subfractionated LPS from *S. typhimurium* Su453 by SDS-PAGE. The leftmost lane contains purified LPS from *S. typhimurium* Su453. Other details as for Fig. 1(b).

Fractions 26–30 from the *S. typhimurium* Su453 LPS subfractionation. When the hexose : KDO ratio decreased in the LPS fractions, there was also a decrease in the survival of serum-sensitive strains in the NHS pretreated with these LPS fractions.

When NHS was treated with LPS fractions of low hexose : KDO ratio (LMM-LPS) (fractions 38–50 in *E. coli* O55:B5 LPS or *S. typhimurium* Su453 LPS), the survival of serum-sensitive strains of both bacteria was always below 10%.

**Measurement of the anti-complement activity of *E. coli* and *S. typhimurium* LPS**

The anti-complement activity of LPS from *E. coli* O55:B5 and *S. typhimurium* Su453 was measured to determine whether the inhibitory effect of the LPS on serum bactericidal activity was due to its ability to activate and deplete complement. The anti-complement activity of the unfractionated LPS from both strains was dose-dependent, as was the anti-complement activity of the HMM-LPS fractions (Fig. 3). The LMM-LPS fractions of both bacteria had low anti-complement activity even at high concentration (0.2 mg ml⁻¹) (Fig. 3).
Fig. 3. Inhibition of complement-mediated haemolysis of sensitized sheep erythrocytes after 30 min incubation in non-immune rabbit serum with LPS fractions from E. coli O55:B5 and S. typhimurium Su453. ○, HMM-LPS from E. coli O55:B5; ●, LMM-LPS from E. coli O55:B5; □, HMM-LPS from S. typhimurium Su453; ■, LMM-LPS from S. typhimurium Su453.

Fig. 4. Distribution of $^3$H after incubation of liposomes containing $^3$H-labelled HMM-LPS (a) or LMM-LPS (b) (both from S. typhimurium Su453) with NHS (○—○) or with heat-inactivated NHS (●—●) for 30 min at 37 °C. Only a single peak, representing unlysed liposomes, is seen with heat-inactivated NHS (a, b) and with liposomes containing $^3$H-labelled HMM-LPS incubated with NHS (a). Two peaks are seen with liposomes containing $^3$H-labelled LMM-LPS (b) after incubation with NHS, the second peak representing $^3$H released from lysed liposomes.

**Lysis of liposomes containing LPS fractions**

Pooled fractions 26–30 (HMM-LPS) and 40–46 (LMM-LPS) from S. typhimurium Su453 $^3$H-labelled LPS were incorporated separately into liposomes, and treated with NHS or heat-inactivated NHS. HMM-LPS protected liposomes from lysis by NHS (Fig. 4a): there was no release of $^3$H from liposomes containing this LPS fraction. In contrast, liposomes containing similar concentrations of LMM-LPS were lysed and $^3$H was released (Fig. 4b). Similar experiments were done with pooled HMM-LPS and LMM-LPS fractions of E. coli O55:B5 $^3$H-labelled LPS incorporated into liposomes. As for the S. typhimurium LPS, HMM-LPS, but not LMM-LPS, protected the liposomes from lysis by NHS (data not shown).

**DISCUSSION**

The bactericidal effects of immune or non-immune serum are mediated by activated components of the classical or alternative complement pathway (Pangburn, 1983; Taylor, 1983). Activation of either can lead to membrane damage culminating in cell death (Taylor, 1983). E. coli (Taylor, 1975) or Salmonella spp. (Joinier et al., 1982) are known to activate both complement pathways.

Sensitivity of a number of Gram-negative bacteria to the bactericidal activity of non-immune or immune sera has been attributed to their LPS composition (Munn et al., 1982; Rice & Kasper, 1977; Schiller et al., 1984; Shafer et al., 1984). We found that LPS from serum-resistant strains of E. coli and S. typhimurium inhibited serum bactericidal activity against serum-sensitive strains
from both enterobacteria, whereas LPS from serum-sensitive strains of *E. coli* and *S. typhimurium* was only slightly inhibitory even at high concentrations. Furthermore, we found a correlation between the anti-complement activity of LPS, measured as the percentage inhibition of haemolysis with sensitized erythrocytes, and its inhibitory effect on serum bactericidal activity.

As described previously (MacIntyre et al., 1986; Peterson & McGroarty, 1985), the LPS fractions with HMM-LPS contained a high number of O-antigen side-chain repeating units. This feature was consistent with the apparent ratio of hexose to KDO in the subfractionation of LPS from *E. coli* O55:B5 and *S. typhimurium* Su453. In the *E. coli* O55:B5 LPS subfractionation we also found, as described by Peterson & McGroarty (1985), LPS bands of very high molecular mass, which we also assume represented very long polymers of O-antigen not covalently attached to lipid A (capsular polysaccharide) as described by some authors (Goldmann et al., 1984). These carbohydrate bands of very high molecular mass were not observed in *S. typhimurium* LPS subfractionation, as was also described by Peterson & McGroarty (1985) for *S. typhimurium* and *S. minnesota*.

The *E. coli* and *S. typhimurium* LPS fractions containing HMM-LPS could activate and remove the serum bactericidal reaction, and could also reduce the percentage of haemolysis in a mixture with sensitized sheep erythrocytes, indicating a correlation between the effect on serum bactericidal activity and the anti-complement activity of these LPS fractions. Also, 3H-labelled HMM-LPS from *E. coli* or *S. typhimurium* prevented lysis of liposomes containing these LPS fractions when mixed with NHS.

*E. coli* and *S. typhimurium* LPS fractions containing LMM-LPS with an apparent hexose:KDO ratio of 1:2:1 (i.e. a small number of O-antigen side chains per lipid A-KDO molecule) were unable to significantly activate and remove the serum bactericidal reaction, or to inhibit the haemolysis of sensitized sheep erythrocytes. Furthermore, 3H-labelled LMM-LPS fractions from *E. coli* or *S. typhimurium* were unable to prevent lysis of liposomes containing these LPS fractions when treated with NHS.

All these facts suggest that the O-antigen side-chains are the main part of the LPS molecules from *E. coli* and *S. typhimurium* involved in serum resistance by virtue of their anti-complement activity. This point seems to correlate with the results of other laboratories indicating that C3b (a component of the complement system) preferentially attaches to the longest O-antigen chains in LPS molecules (Goldmann et al., 1984; Liang-Takasaki et al., 1983).

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