Lipopolysaccharide Alteration is Associated with Induced Resistance of Neisseria gonorrhoeae to Killing by Human Serum

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(Received 11 November 1985)

On SDS-PAGE, solubilized and proteinase K treated preparations of Neisseria gonorrhoeae strain BS4 (agar) showed differences in silver stained lipopolysaccharide (LPS) patterns, before and after induction to resistance to serum killing by incubation for 3 h at 37°C with low M₉ fractions from lysates of guinea pig red blood cells. Preparations from the original serum susceptible gonococci and LPS purified from such bacteria showed two components, but the preparations from the serum resistant gonococci were deficient in the higher M₉ component. Furthermore, on immunoblotting with fresh human serum (FHS), the two LPS components of the susceptible gonococci reacted strongly with IgM. With preparations from the serum resistant gonococci there was no reaction in the area corresponding to the higher M₉ component and a weaker reaction with the component of low M₉. Purified LPS from the susceptible gonococci neutralized the bactericidal activity of FHS against N. gonorrhoeae strain BS4 (agar) probably by reacting with the relevant antibody, since heated FHS was no longer bactericidal when mixed with a source of complement (human placental serum) after prior reaction with the LPS. These neutralization tests coupled with the results of immunoblotting strongly suggest that increased serum resistance is due to the lack of the high M₉ LPS moiety.

INTRODUCTION

When examined directly from patients, gonococci are resistant to complement mediated killing by normal human serum. This resistance is important in the pathogenesis of gonorrhoea (Brooks et al., 1978) and is of two types, stable and unstable. Strains from disseminated infection retain their resistance on subculture (Brooks et al., 1978) but most of those from urogenital infections become susceptible (Ward et al., 1970). We have investigated the latter, unstable type of resistance using first a guinea pig model.

A strain of Neisseria gonorrhoeae (BS4) selected from a small colony forming, piliated laboratory strain (BS) by four passages through plastic chambers implanted subcutaneously in guinea pigs was resistant to killing by human serum when examined without subculture, but it became susceptible [BS4 (agar)] when cultured on laboratory media (Penn et al., 1976, 1977). The change in serum resistance of BS4 (agar) was phenotypic: serum resistance could be restored by incubation at 37°C for 3 h with low M₉ fractions of either guinea pig or some human sera in a defined medium (DM) containing 0.1% (w/v) bovine serum albumin (BSA) (Veale et al., 1981; Martin et al., 1981). The low M₉ inducer(s) from guinea pig serum: (1) was similar to that from human serum (Martin et al., 1981); (2) converted all of 30 recent isolates of N.

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Abbreviations: FHS, fresh human serum; HHS, heated human serum; KDO, 2-keto-3-deoxyoctonate; RBC, red blood cells.
gonorrhoeae back to serum resistance (Martin et al., 1983); and (3) was present in glucopeptide containing fractions that are possibly derived from red blood cell (RBC) membranes (Patel et al., 1984b). Recently the inducer(s) was obtained in greater quantity from lysates of guinea pig RBC (Patel et al., 1984a) and this allowed an investigation of the mechanism of resistance induction and the gonococcal determinants involved.

Induction of BS4 (agar) to serum resistance is a two step process; adsorption of the inducing factor which per se does not confer resistance, and then probably a metabolic event that occurs within 3 h at 37°C (Goldner et al., 1984). A change in pyocin sensitivity pattern during induction, which indicated either an alteration in lipopolysaccharide (LPS) structure or a masking of some determinants on the LPS, was associated with conversion to resistance (Winstanley et al., 1984). A possible change in LPS structure seemed relevant because LPS appears to be the target for the 'natural antibody' responsible for bactericidal activity of normal human serum (Brooks et al., 1978; Schneider et al., 1982). Also, the degree of serum sensitivity of gonococcal strains from disseminated infection has been related to differences in LPS (Sadoff et al., 1984). Recently the inducer(s) was obtained in greater quantity from lysates of guinea pig RBC (Roantree, 1967). It should be remembered, however, that differences in serum sensitivity of N. gonorrhoeae have also been related to differences in outer membrane proteins (Brooks et al., 1978; James et al., 1982) and to serum blocking factors (McCutchan et al., 1978).

This paper describes a comparison of the LPS of N. gonorrhoeae strain BS4 (agar) before and after induction to resistance, and its interaction with serum factors involved in bactericidal action.

METHODS

Neisseria gonorrhoeae. Strain BS4 (agar) was derived, stored, cultured and counted as described previously (Veale et al., 1975; Penn et al., 1976, 1977; Goldner et al., 1979; Patel et al., 1984b).

Fresh human serum (FHS). This was a pooled sample from at least 10 individuals, collected as described by Veale et al. (1981).

Test for gonococcal resistance to killing by FHS. The method used was that described by Veale et al. (1981) as modified by Patel et al. (1984a).

Semipurified, low M, inducer of gonococcal resistance. A water lysate of guinea pig RBC (60 ml) was ultrafiltered as described by Patel et al. (1984a). The ultrafiltrate from the YM-5 Diaflo membrane (nominal cut-off 5000 Da) was freeze dried, dissolved in 5 ml low conductivity water (Milli-Q; Millipore) and filter sterilized (0.22 μm Millipore membrane); 1.0–1.2 ml converted 5 × 10^3 BS4 (agar) to serum resistance. For control samples (see below), the inducer in the ultrafiltrate was inactivated by adjusting the pH to 2 with 1 M-HCl, incubating at 37°C for 1 h, then readjusting the pH to 7.0 (Patel et al., 1984a).

Induction of strain BS4 (agar) to serum resistance. Eight Erlenmeyer flasks (50 ml) each containing 20 ml of the defined medium (DM) described by Veale et al. (1981) were inoculated with about 10^6 viable gonococci in 1.0 ml of a diluted 16 h culture (37°C) of BS4 (agar) in DM. The initial culture of BS4 (agar) in DM was achieved without adding BSA (cf. Veale et al., 1981) by inoculating sufficient agar-grown bacteria to give 10^7–10^8 c.f.u. ml⁻¹ (Veale et al., 1975); after 16 h about 4 × 10^6 c.f.u. ml⁻¹ were present. To each of four flasks, 1.0–1.2 ml of the RBC ultrafiltrate (see above) was added and to each of the remaining flasks 1.0–1.2 ml of acid inactivated inducer (see above). The flasks were incubated (37°C, 3 h) on a rotary shaker (50 cycles min⁻¹) and samples taken for the test for resistance to killing by FHS. The gonococci from each flask were centrifuged (3000 g, 4°C, 15 min) in separate tubes and the supernatants removed. When the results of the test for resistance to killing by FHS were known, the gonococci from experimental and control flasks were separately pooled. Except on rare occasions (less than 10% of flasks in more than 20 tests) the gonococci from the experimental flasks were > 95% resistant to serum killing and those from the control flasks < 5% resistant.

Solubilization of gonococci and digestion with proteinase K. The pooled resistant and control gonococci (about 5 × 10^7 c.f.u.) were treated separately with 100 μl solubilizing buffer (Hitchcock & Brown, 1983) containing 2% (w/v) SDS, 4% (w/v) 2-mercaptoethanol and 10% (v/v) glycerol in 1 M-Tris buffer (pH 6.8) with bromophenol blue. After the heating at 100°C for 10 min, a solution of 50 μg proteinase K (Boehringer-Mannheim) in 20 μl solubilizing buffer was added and the mixture incubated at 60°C for 60 min.

SDS-PAGE. This was done as described by Tsai & Frasch (1982), using an 18% (w/v) polyacrylamide gel with 4% (w/v) urea and a constant current of 20 mA. The amounts of proteinase K treated material applied corresponded to about 5 × 10^7 gonococci. The gels were silver stained by the following modification of the method of Hitchcock & Brown (1983). After fixing overnight at room temperature in a 25% (v/v) 2-propanol solution containing 7% (v/v) acetic acid, the gels were reacted with a solution of periodic acid (1.05 g) and fixative (4 ml) in Milli-Q water. After removal of the oxidizing solution, the gels were washed eight times (30 min each) in 200 ml
Milli-Q water and stained for 15 min in a freshly prepared solution containing 0.1 M-NaOH (28 ml), ammonia solution 0.88 sp. gr. (2 ml), silver nitrate (5 ml of a 20% w/v, solution) and Milli-Q water (115 ml), then washed three times for 10 min with 200 ml Milli-Q water. Components reacting with the silver strain were developed at 25 °C in a solution (200 ml) of formalin (37%, w/v, formaldehyde; 0.5 ml) and citric acid (50 mg) in water (1 l). When the darkly stained components reached the desired intensity (2–10 min) the developer was removed and the gel immersed in 0.35% acetic acid (200 ml) for 1 h at room temperature. The gel was then washed with water (200 ml) and photographed within 48 h.

**Transfer of LPS to nitrocellulose membranes and immunoblotting with FHS.** The method of Towbin et al. (1979) was modified. Immediately after SDS-PAGE (see above), the LPS components were transferred from the gels electrophoretically (negative to positive) to nitrocellulose membranes (0.45 μm pore size; Sartorius) in an EC Transphoelectrophoresis transfer cassette and electrophoresis unit (Applied Corporation) in 25 mM-Tris/192 mM-methylene glycol buffer pH 8.2 containing 25% (v/v) methanol at a current of 0.4 A for 16 h. The nitrocellulose membranes were treated for 90 min at room temperature with 50 ml phosphate buffered saline (PBS) (Penn et al., 1976), containing 3% (w/v) BSA to saturate all remaining binding sites, washed quickly twice with PBS (50 ml), and reacted with FHS (2 ml in 50 ml PBS containing 0.1% BSA) for 45 min at room temperature. Tween-20 was omitted from all buffers because it caused elution of the gonococcal LPS from the membranes (cf. Caldwell & Hitchcock, 1984). After treatment with FHS, the membranes were washed three times for 5 min with PBS (50 ml), incubated with goat anti-human IgG (Sigma) diluted 1:100 in PBS (50 ml) containing 0.1% BSA for 1 h at room temperature and the washings repeated. The membranes were then incubated for 1 h at room temperature with rabbit anti-goat IgG conjugated to alkaline phosphatase (Sigma) diluted 1:100 in PBS (50 ml) containing 0.1% BSA, washed twice with PBS (50 ml), then placed in 0.1 M-Tris buffer pH 8.2 (50 ml). To visualize the immune complexes, membranes were placed in a mixture of (a) 10 ml 0.2 M-Tris buffer pH 8.2 containing 60 mg Fast Red TR salt (Sigma) and (b) 10 ml water containing 4 mg sodium naphthol AS-MX phosphate (Sigma) until the desired intensity was achieved. Development was stopped by placing membranes in 0.1 M-Tris buffer pH 8.2 (100 ml).

**Preparation of LPS from serum susceptible BS4 (agar).** About 10³ c.f.u. of BS4 (agar) were obtained from 40 AG agar plates (Veale et al., 1975; Penn et al., 1976) as a suspension in 50 ml 50 mM-sodium phosphate buffer pH 7.0 containing 5 mM-EDTA. LPS was extracted by the hot phenol/water procedure of Westphal & Jann (1965) as modified by Johnson & Perry (1976) to include pretreatment of the cells with lysozyme. The yield of LPS was about 50 mg. The preparation contained 11.8% 2-keto-3-deoxyoctonoic acid (KDO: assayed by the method of Karkhanis et al., 1978, with sodium KDO as standard), <1% protein (by the method of Lowry with BSA as standard), and glucosamine 2-1% and galactosamine 3-6% (by the method described by Patel et al., 1984b). Apicella et al. (1978) detected both glucosamine and galactosamine in the LPS of N. gonorrhoeae.

**Neutralization of FHS with LPS from serum susceptible BS4 (agar).** A suspension of LPS in DM (2 mg ml⁻¹) was sonicated at 4°C (Rapidis Ultrasonics; 50 kW, 20 kHz) until the solution became clear. The LPS solution was filter sterilized (0.22 μm Millipore membrane); KDO analysis indicated that about 75–80% of the LPS passed the membrane.

In one series of experiments, LPS suspension (2.5–100 μl) was added to 100 μl FHS and the volume adjusted to 200 μl with DM containing 0.1% BSA. The mixture was incubated at 37°C for 30 min, a suspension (0.1 ml) of BS4 (agar) (about 10⁶ c.f.u. ml⁻¹) added and incubation continued for a further 45 min. Viable gonococci were then counted in experimental and control (LPS not added) samples.

In a second series of experiments, the suspension of LPS (25–100 μl) was added to 100 μl heated human serum (56°C, 30 min; HHS) and the volume adjusted to 200 μl with DM containing 0.1% BSA. The mixture was incubated at 37°C for 30 min. To 100 μl of this mixture, fresh human placental serum (50 μl, a source of human complement, lacking natural antibodies, kindly donated by Birmingham Maternity Hospital) and a suspension of BS4 (agar) (0.1 ml, about 10⁵ c.f.u. ml⁻¹) were added. The mixture was incubated for 45 min at 37°C and viable gonococci were counted in experimental samples and three control samples [as experimental sample but with LPS omitted; placental serum alone with BS4 (agar); and HHS alone with BS4 (agar)].

**RESULTS**

During the induction of BS4 (agar) to serum resistance, the approximate concentration of gonococci was 5 × 10⁶ c.f.u. ml⁻¹ at the beginning and 1 × 10³ c.f.u. ml⁻¹ after 3 h at 37°C, i.e. about 1 generation occurred. A similar increase in count occurred in control flasks. Any slight differences in numbers of gonococci in control and experimental flasks were adjusted to obtain standard numbers of organisms for the analytical procedures.

**LPS patterns on SDS-PAGE of susceptible and resistant BS4 (agar)**

Fig. 1 shows the SDS-PAGE patterns of proteinase K treated preparations from susceptible and resistant BS4 (agar) (lanes 1 and 2). The pattern for the LPS isolated from susceptible BS4...
Fig. 1. LPS patterns of susceptible and resistant BS4 (agar) on SDS-polyacrylamide gels (18%) containing 4 M-urea. Lanes 1 and 2, proteinase K digested preparation from susceptible and resistant gonococci (amounts $= 5 \times 10^7$ gonococci) respectively; lane 3, LPS isolated from susceptible cells (amount $= 5 \times 10^7$ gonococci). These patterns are typical of at least three separate preparations of each of the materials.

Fig. 2. Immunoblotting with FHS of LPS moieties separated by SDS-PAGE (see Fig. 1). Lanes 1 and 2, proteinase K digested preparation from susceptible and resistant gonococci (amounts $= 5 \times 10^7$ gonococci) respectively; lane 3, LPS isolated from susceptible cells (amount $= 5 \times 10^7$ gonococci).

(agar) is shown in lane 3. The isolated LPS and the preparation from the susceptible BS4 (agar) contain a higher $M_r$ component (cf. Tsai & Frasch, 1982) not seen in the preparation from the same number ($5 \times 10^7$) of resistant organisms. The fact that both high and lower $M_r$ components in all preparations were carbohydrate in nature was confirmed by staining duplicate gels by the method of Dubray & Bezard (1982). The absence of other silver staining bands on the gels of the LPS from susceptible organisms (lane 3) supported the chemical
**LPS alteration in serum-resistant gonococci**

Table 1. *Neutralization of bactericidal activity of FHS and of HHS plus human placental serum (HPS) by LPS isolated from serum susceptible BS4 (agar)*

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<th>Serum</th>
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<td>&gt;95</td>
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* Two separate batches of LPS were used, one in Expt 1 and the other in Expt 2.

† The results quoted are the means of two similar tests with each batch of LPS.

Evidence (see Methods) for little protein contamination. The nature of the high *M*<sub>r</sub> material found near the origin of the gels from both susceptible and resistant gonococci (Fig. 1; top of lanes 1 and 2) is unknown. It may be undigested protein since it did not stain with the carbohydrate stain.

**Immunoblotting with FHS**

After immunoblotting with FHS, both the high and low *M*<sub>r</sub> components of the proteinase K treated susceptible BS4 (agar) and the corresponding components of the LPS from these organisms reacted strongly with the IgM (Fig. 2, lanes 1 and 3). As expected from the SDS-PAGE gels of the proteinase K treated preparation from the resistant organisms, reaction with IgM was not detectable in the area corresponding to the high *M*<sub>r</sub> component (Fig. 2, lane 2). There was, however, a reaction with the component of lower *M*<sub>r</sub>, but it was weaker than for the preparations from the susceptible organisms (Fig. 2, lanes 1 and 3).

**Neutralization of the bactericidal activity of FHS and of HHS plus human placental serum, by LPS isolated from serum susceptible BS4 (agar)**

The top part of Table 1 shows that the bactericidal activity of FHS against BS4 (agar) is neutralized by pretreatment with differing amounts of LPS (two separate batches) from serum susceptible BS4 (agar). The bottom part of Table 1 shows the results of first incubating the LPS with HHS, to adsorb any antibody that might be contributing to bactericidal activity, and then testing for the latter with the addition of an excess of complement but not antibody, i.e. human placental serum. Again, the bactericidal activity was neutralized, indicating that the specific interaction of LPS with antibody is the cause of the neutralization, not an anticomplementary effect.
DISCUSSION

Most studies on the LPS of *N. gonorrhoeae* and *N. meningitidis* (Apicella et al., 1981; Connelly & Allen, 1983) have indicated a 'rough' structure, i.e. the molecule contains core carbohydrate and short O antigenic chains. One study, however, reported some high M, 'smooth' type LPS molecules in both *Neisseria* spp. (Thomas et al., 1984). Our findings indicate that the serum susceptible gonococcal strain BS4 (agar) contains a 'rough' type LPS with two discernible, relatively low M, components (Fig. 1).

Strikingly, when BS4 (agar) was induced to serum resistance, production of the higher M, component was inhibited. Thus, the alteration in LPS structure indicated by the change in pyocin sensitivity pattern (Winstanley et al., 1984) has been confirmed. Furthermore, immunoblotting suggested that the higher M, component, present in the susceptible organisms and not detected in the same number (5 × 10^7) of resistant ones, contained a binding site for IgM antibody (Glynn & Ward, 1970) present in bactericidal FHS. The relevance of the lack of the high M, moiety in the resistant BS4 (agar) was thus strongly indicated. Two types of tests showed that LPS isolated from susceptible BS4 (agar) neutralized the bactericidal agent in FHS. First there was a simple neutralization of bactericidal activity which was probably due to a combination of the LPS with the relevant antibody but could be ascribed to an anticomplementatory effect as shown by some LPS (Shafer et al., 1984). The second test ruled out the latter by allowing the reaction of the LPS with HHS in which complement had been destroyed, and then adding back for the bactericidal test excess complement in the form of human placental serum. It is reasonable to conclude therefore that the LPS from the susceptible form of BS4 (agar) neutralizes the bactericidal activity of FHS by interacting with the relevant antibody. Coupled with the results of the immunoblotting experiments these neutralization tests strongly suggest that induced serum resistance is due to the absence of the higher M, LPS moiety. When sufficient low M, inducer is available to allow the separation of LPS in quantities from resistant organisms, the results of similar neutralization tests and immunoblotting experiments with it and FHS will be interesting.

There are several possible explanations for the change in LPS which occurs during induction to serum resistance. The inducer could change the course of carbohydrate metabolism by affecting hexose transferases. The factor might induce an enzyme(s) capable of degrading LPS. Apicella et al. (1978) found such an enzyme in cell extracts of *N. gonorrhoeae* strain 1291. It is unlikely that the change of LPS is due to an induced change in growth rate (Morse et al., 1983) because marked differences in growth rate were not indicated in experimental and control flasks during the 3 h inducing period.

Finally it should be mentioned that although the change in LPS structure appears significant, the inducing factor may produce biologically relevant changes in other surface components, for example outer membrane proteins, which have yet to be investigated.

Our thanks are due to Dr M. Bailey for helping with the immunoblotting technique.

REFERENCES


