The Role of Surface Polysaccharide in Determining the Resistance of *Serratia marcescens* to Serum Killing

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Two O14:H12 strains of *Serratia marcescens* with different sensitivities to killing by normal pooled human serum were investigated. Complement binding, studied by measuring hydrophobicity and using rocket immunoelectrophoresis with anti-human C3, showed the sensitive cells (S1220) rapidly bound and fixed complement whereas the resistant cells (4444-60) bound less C3b. The strains had identical membrane protein composition. Crossed immunoelectrophoresis suggested that in S1220 cells the polysaccharide material including LPS was less antigenic and present in smaller amounts than in 4444-60 cells. This was confirmed by examining extracted polysaccharide material chemically and by SDS-PAGE. The resistant strain had 33% more phenol-extractable polysaccharide material than the sensitive strain, possibly comprising LPS with longer O antigen chain lengths, or a microcapsule of O antigen polysaccharide. Extra polysaccharide material on the surface of the resistant strain prevents complement components binding and reaching the hydrophobic membrane where lytic lesions occur.

INTRODUCTION

Serratia marcescens is becoming an important opportunistic pathogen that can cause outbreaks of sepsis in special care baby units (Lewis *et al.*, 1983) and urinary and respiratory tract infections in immunocompromised patients (Yu, 1979; Bullock *et al.*, 1982). Serotyping is based on the O (lipopolysaccharide, LPS) and H (flagella) antigens, the most prevalent combination isolated clinically being O14:H12 (Pitt, 1982). K (capsular) antigens have been noted (Ewing *et al.*, 1959; Traub *et al.*, 1985), but await further elucidation.

The complement-mediated bactericidal effect of normal human serum plays an important role in host defence against bacterial infection. A vital step on activation of either the classical or the alternative pathway is covalent fixation of C3b to the cell surface. The early acting components of both pathways through C3 combine to form a cleaving enzyme which starts the sequence of reactions leading to membrane attack complex (MAC) formation, the latter being responsible for lesions in the lipid bilayers (Joiner *et al.*, 1984). The virulence of many Gram-negative bacteria has been linked to the O and K antigens, their presence often enabling the cells to resist direct complement killing (Taylor, 1983). Outer membrane proteins (OMPs), for example the TraT protein of *Escherichia coli*, have also been associated with resistance (Moll *et al.*, 1980).

In previous work we suggested that in *S. marcescens* New CDC O14:H12 the O antigen masks other antigenic sites on the cell surface and is the dominant surface exposed antigen (Jessop & Lambert, 1985). In this paper we investigate the influence of variation in the amount of surface polysaccharide material upon the sensitivity of two O14:H12 strains to complement.

Abbreviations: MAC, membrane attack complex; NPHS, normal pooled human serum; OMP, outer membrane protein.

METHODS

Organisms and growth conditions. The two O14:H12 serotyped strains of *S. marcescens* used were reference strain 4444-60 and a urinary tract infection isolate, S1220 (both kindly donated by Dr T. L. Pitt, PHLS, Colindale, London). Cells were grown in an orbital shaker at 37 °C to mid-exponential phase in tryptone soy broth (TSB; Lab M) to an OD₄₇₀ of 0.3 and a doubling time of 20 min.

Serum bactericidal assays. A suspension of bacteria in saline (0.6 ml; OD₄₇₀ 0.2) was added to 0.6 ml normal pooled human serum (NPHS) obtained from six healthy volunteers and the mixture was incubated at 37 °C in a reciprocal shaking water bath. Samples (100 µl) were taken at intervals, diluted with sterile saline and viable counts made according to the method of Miles & Misra (1938).

Opsonization of bacteria. NPHS was added to cells resuspended in saline (OD₄₇₀ 1.0) to give a serum concentration of 10% (v/v). After incubation, with shaking, for 15 min at 37 °C the bacteria were harvested, the supernate was retained, and the cells were washed twice in saline. NPHS, heat-inactivated at 56 °C for 30 min, was used in a control experiment.

Bacterial adherence to hydrocarbon. The technique developed by Rosenberg *et al.* (1980) was used where 100 µl octane was added to 1.2 ml normal or opsonized bacteria resuspended to OD₄₇₀ 0.3 in PUM buffer (K₂HPO₄·3H₂O, 22.2 g; KH₂PO₄, 7.26 g; urea, 1.8 g; MgSO₄·7H₂O, 0.2 g; distilled water to 1000 ml, pH 7.1). After 10 min preincubation at 25 °C, the mixture was vortex mixed for 120 s. When separation of the layers was complete, the OD₄₇₀ of the aqueous phase was measured.

Solubilization of bacteria. Opsonized or normal cells were solubilized in 4% (v/v) Triton X-100 in 50 mM-Tris/HCl, pH 8.6, containing 5 mM-EDTA (Triton/EDTA) for 2 h at 25 °C to give a protein concentration of 2 mg ml⁻¹ for rocket or 10 mg ml⁻¹ for crossed immunoelectrophoresis. Unsolubilized cells were removed by centrifugation and the remaining soluble antigen extract was analysed.

Rocket immunoelectrophoresis. The method of Laurell (1966) was used where 0.1% (v/v) goat anti-human complement C3 (Sigma) was absorbed with *S. marcescens* cells and added to 1% (w/v) agarose in Tris/barbiturate buffer, pH 8.6 (LKB), which was then cast onto Gel bond film (LKB).

Crossed immunoelectrophoresis (CIE). This was done as described by Weeke (1973) using 1% (w/v) agarose in Tris/barbiturate buffer cast onto Gel bond film. Antiserum was raised in rabbits to UV-killed whole cells of strain 4444-60 or strain S1220 as described by Driver & Lambert (1984). Electrophoresis was at 10 V cm⁻¹ for 2 h in the first dimension and at 2 V cm⁻¹ for 18 h in the second dimension, both at 10 °C. Gels were washed, pressed and stained with Coomassie Blue. A blank intermediate gel was interposed between the first and second dimension gels to increase resolution.

In one experiment, samples of solubilized whole cells (30–40 µl) were treated in various ways before CIE. They were (1) autoclaved (121 °C) for 15 min or (2) digested with proteinase K (Sigma; 10 µg in 5 mM-Tris/HCl, pH 6.8) at 60 °C for 1 h, or (3) oxidized with 0.1 M-sodium periodate in sodium acetate buffer (pH 4.5) at room temperature for 16 h, excess periodate then being consumed by adding butane-2,3-diol.

Tandem CIE of solubilized whole cells with phenol-extractable polysaccharide material, 10 mg ml⁻¹ (see below), was done according to Kroll (1973). Briefly, samples of solubilized whole cells and phenol-extractable material were placed in adjacent wells in the agarose, with the polysaccharide material in the well nearer the anode. After allowing the material to diffuse for 1 h at 20 °C the wells were filled with cooled molten agarose and subjected to CIE.

Iodination of cells. Cells were iodinated using [¹²⁵I]lactoperoxidase (Lambert & Booth, 1982) and the activity of the labelled cells was measured with a γ-counter. After solubilization and CIE, autoradiographs of the CIE plates of the labelled cells were developed at room temperature for 7 d using Singul-XRP X-ray film (Ceaverken AB, Strängnäs, Sweden).

Extraction and chemical analysis of LPS. LPS and associated acidic exopolysaccharides produced by the strains (Brigden & Wilkinson, 1985) were extracted from 2-litre cultures of cells by the phenol/water method of Westphal & Jann (1965). After dialysis of the material for 24 h against running tap water, DNAase and RNAase (10 µg of each per ml) were added to digest any contaminating nucleic acid (incubated for 16 h at 37 °C). The polysaccharide material was then re-extracted, dialysed, freeze-dried and the total weight determined. The following chemical assays were done on a 10 mg ml⁻¹ solution of purified material. (1) Total carbohydrate (hexose) by the phenol/sulphuric acid method (Dubois *et al.*, 1956). (2) Total KDO (3-deoxy-D-manno-2-octulosonic acid) by a method based on that of Osborn (1963). Briefly, material was hydrolysed in sealed tubes at 100 °C for 30 min and oxidized with periodate (12 mM) at 55 °C for 20 min. Excess periodate was consumed with sodium arsenite and the colours generated by heating with thiobarbituric acid at 100 °C for 20 min. (3) Total fatty acids by gas-liquid chromatography after acid hydrolysis (6 M-HCl) at 100 °C for 4 h (Lambert & Moss, 1983) with an internal standard of tridecanoic acid (Sigma).

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The phenol-extractable material was analysed by SDS-PAGE (Lugtenberg *et al.*, 1975) using 12% (w/v) acrylamide gels containing 4 M-urea, and detected by silver-staining (Tsai & Frasch, 1982).

Fig. 1

Fig. 2

Fig. 1. Kinetics of killing of *S. marcescens* by normal pooled human serum (NPHS) (filled symbols) or by heat-inactivated NPHS (open symbols): ○, ●, strain 4444-60; △, ▲, strain S1220. The survival values indicate viable counts to time zero. Similar results were obtained in three separate experiments.

Fig. 2. Affinity of bacterial whole cells to octane. Cell suspensions (1.2 ml) were vortex mixed with 0.1 ml octane, the phases allowed to separate and the optical density of the aqueous phase determined. Results are expressed as a percentage of the initial optical density of the aqueous cell suspension. A, strain 4444-60; B, strain 4444-60 after contact with 10% (v/v) NPHS for 15 min; C, strain S1220; D, strain S1220 after contact with 10% (v/v) NPHS for 15 min; E, as D, but heat-inactivated NPHS was used.

RESULTS

Serum bactericidal assays

The assays showed that strain S1220 is sensitive to rapid killing by complement whereas strain 4444-60, by comparison, is resistant (Fig. 1). Both strains grew in heat-inactivated NPHS. Using SDS-PAGE and the immunoblotting techniques described by Jessop & Lambert (1985), we confirmed that the NPHS contained a low titre of antibodies to *S. marcescens* antigens, particularly the OmpA protein (data not shown).

Bacterial adherence to hydrocarbon

The sensitive cells became hydrophobic after 15 min incubation with 10% (v/v) NPHS, remaining hydrophilic when heat-inactivated serum was used (Fig. 2, D and E). This suggested that fixation and opsonization of complement components to the S1220 cells was occurring. With 4444-60 cells, it appeared that there was virtually no binding (Fig. 2, A and B).

Rocket immunoelectrophoresis

Rocket immunoelectrophoresis, using goat-anti-human C3 antisera of whole cells solubilized after being in contact with 10% NPHS for 15 min, showed that strain S1220 rapidly bound C3b (Fig. 3, B) whereas strain 4444-60 bound small amounts only (Fig. 3, A). Controls with bacteria solubilized after contact with heat-inactivated NPHS gave no rockets (data not shown). Analysis of the 10% (v/v) NPHS after removal of the cells, showed that the quantity of C3 in the supernate after contact with strain S1220 increased, indicating activation of the complement pathway (Fig. 3, D), involving splitting of C3 into C3a and C3b, all of which are presumably detected by the anti-C3 serum.

Fig. 3. Rocket immunoelectrophoresis using anti-human C3 antiserum of: A, B, solubilized whole cells of *S. marcescens* reacted with 10% (v/v) NPHS for 15 min; C, D, the supernate (10%, v/v) NPHS remaining after removal of the cells. The strains used were 4444-60 (A, C) and S1220 (B, D). E to H are dilutions (10%, 7.5%, 5%, 2.5%) of NPHS in saline.

Fig. 4. CIE patterns of: (a) solubilized whole cells of strain 4444-60 (20 μ l = 200 μ g protein) with homologous antiserum (200 μ l); (b) solubilized whole cells of strain S1220 (30 μ l = 300 μ g protein) with homologous antiserum (250 μ l). Tandem CIE reactions of: (c) as in (a) with phenol-extractable material from strain 4444-60 (20 μ l = 200 μ g dry weight) in the second well; (d) as in (b) with phenol-extractable material from strain S1220 (20 μ l = 200 μ g dry weight) in the second well.

Fig. 5. CIE patterns of strain 4444-60 antiserum (200 μ l). (a) Solubilized whole cells of strain 4444-60 (20 μ l = 200 μ g protein); (b) as (a) but extract treated first with heat (121 $^{\circ}$ C for 15 min); (c) as (a) but extract treated first with proteinase K (10 μ g in 5 mM-Tris/HCl pH 6.8, 60 $^{\circ}$ C for 1 h); (d) as (c) but without proteinase K; (e) as (a) but extract treated first with 0.1 M-sodium periodate in sodium acetate buffer (pH 4.5) overnight; (f) as (e) but without sodium periodate.

SDS-PAGE of OMPs

OMP profiles of the two strains revealed by SDS-PAGE of Sarkosyl-insoluble membranes failed to show any differences (results not published). There was no evidence, on the basis of M_r analysis, of a TraT-like protein known in *Escherichia coli* to be associated with resistance to serum killing (Moll *et al.*, 1980).

Iodination of cells

[125 I]Lactoperoxidase specifically labels proteins on the surface of bacteria (Lambert & Booth, 1982); strain 4444-60 bound 50% less radioactive label than strain S1220. This suggested that a non-protein surface component present on the resistant cells was preventing the [125 I]lactoperoxidase reaching its binding sites.

Fig. 6. CIE patterns of (a) solubilized [^{125}I]lactoperoxidase-labelled whole cells of strain 4444-60 ($20\ \mu\text{l} = 200\ \mu\text{g}$ protein) with homologous antiserum ($200\ \mu\text{l}$). (b) Autoradiograph of (a) developed after 7 d.

CIE

Reaction of solubilized whole cells of strain 4444-60 with homologous antisera showed the presence of one dominant precipitin arc and several minor arcs (Fig. 4a). The major arc was not observed in the profile of strain S1220 with its homologous antisera (Fig. 4b).

Tandem CIE is a technique that allows two antigen samples to be applied in the same run in such a way that related precipitin peaks form as fused double peaks in the final pattern (Kroll, 1973). Running solubilized cells and isolated phenol-extractable material from strain 4444-60 suggested that the dominant arc was polysaccharide (Fig. 4c). In comparison, the polysaccharide peak in strain S1220 was a small weakly formed precipitin line (Fig. 4d).

Heating the solubilized cells of strain 4444-60 ($121\ ^\circ\text{C}$ for 15 min) destroyed all antigens apart from one that appeared to correspond with the dominant peak (Fig. 5b). The polysaccharide arc remained intact after digestion with proteinase K, and the fainter wide arc disappeared (Fig. 5c). The two precipitin lines that formed in the intermediate gel became very pronounced, suggesting they were not protein material, but their appearance on the CIE plates was irregular (Fig. 5d, the control). Periodate treatment led to the disappearance of the major antigen arc, confirming it was polysaccharide in nature (Fig. 5e). Taken together these results indicate that the major precipitin line is polysaccharide.

CIE of solubilized iodinated whole cells of strain 4444-60 (Fig. 6a) followed by autoradiography (Fig. 6b) showed that the slower migrating portion of the polysaccharide peak was labelled and that the fainter wider arc was not. This suggested that part of the major peak was a surface protein polysaccharide complex although its antigenicity was due to the polysaccharide.

Cross-reactions by CIE of strain 4444-60 antigen with strain S1220 antiserum showed that the polysaccharide peak was indistinct (Fig. 7a) but could be located by running authentic phenol-extractable material in tandem (Fig. 7c). With strain S1220 antigen and strain 4444-60 antiserum the polysaccharide peak became more defined than with homologous antiserum (Fig. 7b, d).

CIE is a quantitative technique, the strength of the reaction and the area subtended by an immunoprecipitate being proportional to the antigen/antibody ratio (Weeke, 1973). All the results therefore suggest that in strain 4444-60 the polysaccharide material and LPS is more antigenic and is present in greater amounts than in strain S1220.

Chemical analysis

Analysis of the phenol-extractable material gave the following results (expressed as μg of each component in 1 mg dry weight of cells for strains 4444-60 and S1220 respectively $\pm$ SD): total weight extracted, 83 ± 4.9 and 56 ± 3.6 ; total hexose, 36 ± 2.8 and 20 ± 1.9 ; total fatty acids,

Fig. 7. CIE patterns of: (a) solubilized whole cells of strain 4444-60 (20 μ l = 200 μ g protein) with strain S1220 antiserum (250 μ l); (b) solubilized whole cells of strain S1220 (30 μ l = 300 μ g protein) with strain 4444-60 antiserum (200 μ l). Tandem CIE reactions of: (c) as (a) with phenol-extractable material from strain 4444-60 (20 μ l = 200 μ g dry weight) in the second well; (d) as (b) with phenol-extractable material from strain S1220 (20 μ l = 200 μ g dry weight) in the second well.

1.8 $\pm$ 0.1 and 1.7 $\pm$ 0.1; total KDO, 6.7 $\pm$ 0.35 and 0.84 $\pm$ 0.04; ratio hexose : fatty acids, 20 : 1 and 12 : 1. The serum resistant strain had 33% more phenol-extractable material than the sensitive one. The KDO measurements for strain 4444-60 are probably influenced by the presence of a related sugar which gives a positive response with the periodate/thiobarbituric assay (Brigden & Wilkinson, 1983). We assume that this component is not present in LPS from strain S1220. We therefore used fatty acid analysis (after acid hydrolysis to release amide-linked fatty acids) as an independent measure of LPS. The nature and relative proportions of fatty acids in each LPS were identical, viz.: laurate, 5%, myristate, 27%; 3-hydroxymyristate, 60%; unknown, 8%. These values are close to those reported by Alaupovic *et al.* (1966), although their proportion of β -hydroxymyristic acid was lower, due to milder conditions of acid hydrolysis. The ratio of hexose to fatty acids suggested that, in phenol-extractable material from strain 4444-60, the average sugar chain length of the LPS is longer than in strain S1220, or a polysaccharide microcapsule is present.

SDS-PAGE of polysaccharides

Identical amounts of phenol-extractable material were loaded to each track in Fig. 8. It appears that strain 4444-60 contained a proportion of material which migrated slowly, was not resolved into the familiar LPS ladder pattern and is thought to be molecules with long O antigen chains (Peterson & McGroarty, 1985). Strain S1220 contained far less of this material but the ladder pattern was more pronounced near the fast migrating lipid A region (probably representing rougher LPS). Attempts to resolve the slow migrating material into discrete bands

Fig. 8. Silver-stained phenol-extractable material of *S. marcescens* after SDS-PAGE from (A) strain 4444-60 (50 µg) and (B) strain S1220 (50 µg). Similar patterns were produced on three separate occasions.

by increasing the SDS concentration in the gel were unsuccessful (Peterson & McGroarty, 1985). Usually, capsular polysaccharides can be separated from LPS by ultracentrifugation (Sutherland & Wilkinson, 1971). On subjecting samples of phenol-extractable material to ultracentrifugation, a characteristic ladder pattern was obtained from both the supernate (monomeric LPS) and pellet fractions on SDS-PAGE (results not shown). We were therefore unable to distinguish capsular material in the supernate.

DISCUSSION

Analysis of two O14:H12 strains of *S. marcescens* with different sensitivities to serum killing showed that the sensitive strain rapidly bound large quantities of C3 to its cells, a trend found by other workers in different bacteria (Taylor & Kroll, 1985; Engels *et al.*, 1985). C3b bound to early acting complement components initiates the cascade responsible for MAC formation and direct cell death (Joiner *et al.*, 1984). The attached molecules also increase the hydrophobicity of the cells, making them more sensitive to phagocytosis (Ofek & Beachey, 1980), and C3b bound on its own opsonizes the cells, C3b receptors being found on phagocytic cells (Joiner *et al.*, 1984).

The reasons for the differences in sensitivity could not be attributed to alterations in the OMPs. No evidence for a TraT protein was found, confirming the results of other workers (Montenegro *et al.*, 1985).

Goldman *et al.* (1984) showed that the quantity of LPS covering the cells is important in determining serum sensitivity of *E. coli* O111B4. A mutant with 50% more LPS and an increased coverage of lipid A core with O antigen was more resistant to complement killing than its parent,

due to restricted access of the MAC to hydrophobic membrane sites. CIE and chemical analysis showed that the serum-resistant *S. marcescens* 4444-60 had 33% more polysaccharide material than the serum-sensitive strain S1220. SDS-PAGE suggested that strain 4444-60 contains more high M_r LPS, presumably as longer O antigen chains. The increased LPS content and O antigen chain length might direct complement components to sites further from the cell surface, preventing them reaching the hydrophobic domains and making the cells resistant to killing. By contrast, in strain S1220 the C3b and MAC can reach the outer membrane.

Little is known about the capsular antigens of *S. marcescens*, although Traub *et al.* (1985) isolated K antigen extracts from four strains. *S. marcescens* produces a complex range of surface carbohydrates and Brigden & Wilkinson (1985) suggested that strain 4444-60 may have a microcapsule associated with its LPS. The increase in phenol-extractable material and the hexose:fatty acid ratio in strain 4444-60 compared to strain S1220 could reflect the presence of such an acidic glucomannan. The polymer might run on SDS-PAGE as slowly migrating material and would be immunologically indistinguishable from the LPS on CIE. However, we could not separate any such microcapsular material from the phenol-extractable material on ultracentrifugation, even in the presence of magnesium chloride. Goldman *et al.* (1984) found the possession of an O antigen capsule in *E. coli* O111B4 did not affect serum sensitivity, the quantity of LPS being more important. Strain 4444-60 contains additional surface polysaccharide material compared to strain S1220, which restricts access of complement components to the hydrophobic membrane. We cannot conclude whether this material is in the form of more LPS with longer O antigen chains, a microcapsule, or a combination of both.

Traub *et al.* (1985) showed that capsular polysaccharides and LPS from a range of *S. marcescens* serotypes separately afford protection against homologous strains in mice. Therefore we need to have further information both on their antigenicity and on their role in protecting the cells from serum killing.

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