Penetration of immunoglobulins through the *Klebsiella* capsule and their effect on cell-surface hydrophobicity

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Summary. The ability of antibodies to cell-surface components of *Klebsiella* to increase surface hydrophobicity and to gain access to antigens potentially masked by the capsule was investigated. Treatment of capsulate or non-capsulate strains with the respective autologous antiserum resulted in a marked increase in surface hydrophobicity. Antisera raised against a rough non-capsulate ($K^{-}O^{-}$) strain had little effect on the surface hydrophobicity of either of the capsulate strains $K1^{+}O1^{+}$ and $K2^{+}O1^{+}$, or of the non-capsulate $K^{-}O1^{+}$ strain. Whereas anti- $K^{-}O1^{+}$ sera or anti- $K2^{+}$ sera increased the surface hydrophobicity of the $K2^{+}O1^{+}$ strain, only antisera containing anti- $K1^{+}$ antibodies increased the hydrophobicity of the $K1^{+}O1^{+}$ strain. Immunoabsorption of anti- $K^{-}O1^{+}$ serum by whole capsulate cells revealed that neither the K1 nor the K2 capsular polysaccharide acted as a barrier to anti-O antibodies but that the K1 capsular polysaccharide masked the presence of the immunoglobulin at the cell surface. The *Klebsiella* capsular polysaccharide does not appear to present a permeability barrier to immunoglobulins although failure to detect outer-membrane proteins in the immune complexes of either of the capsulate strains or of the $K^{-}O1^{+}$ strain suggests that the O antigen may prevent access of antibodies to these antigens.

Introduction

Phagocytic cells such as polymorphonuclear leucocytes appear to recognise foreign particles by at least two mechanisms (Stendahl, 1983; van Oss, 1978). The first is mediated via specific ligand-receptor interactions. Such ligands include the Fc portion of IgG and the C3b component of complement. However, general physico-chemical properties such as surface hydrophobicity and surface charge also play a role, and IgG and C3b are known to increase surface hydrophobicity of opsonised micro-organisms (Stendahl, 1983; van Oss, 1978).

Capsular polysaccharides (K antigens) and the complete O antigen of lipopolysaccharides (LPS) confer hydrophilic characteristics upon bacterial cell surfaces. These polysaccharides are important virulence determinants (Robbins *et al.*, 1980; Wicken and Knox, 1980). The ability of capsulate micro-organisms to resist phagocytosis in the

absence of specific anti-capsular antisera is well documented (Wolberg and DeWitt, 1969; Welch *et al.*, 1979; Robbins *et al.*, 1980; Van Dijk *et al.*, 1981; Williams *et al.*, 1983). However, the ability of antibodies to other cell-surface components of capsulate bacteria (e.g., LPS and outer-membrane proteins) to promote phagocytosis is less clear (Wolberg and DeWitt, 1969; Van Dijk *et al.*, 1981; Williams *et al.*, 1983).

Klebsiella species are important pathogens of man and are frequently isolated from hospital-acquired infections (Rennie and Duncan, 1974; Bryan *et al.*, 1983; Cryz, 1983). Most clinical *Klebsiella* isolates possess a well defined capsule (Cryz, 1983) that confers serotype specificity (Kaufmann, 1949). In experimental infections, those strains expressing K antigen types K1 or K2 generally appear to be the most virulent (Mizuta *et al.*, 1983; Simoons-Smit *et al.*, 1986). Both the K and O antigens of *Klebsiella* play an important role in protecting the organism from complement-mediated serum killing and phagocytosis (Williams *et al.*, 1983, 1986; Simoons-Smit *et al.*, 1986). In some strains, anti-O antibodies as well as anti-K antibodies can opsonise the organism, whereas

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antisera to other outer-membrane (OM) components have little effect on phagocytosis (Williams *et al.*, 1983). Loss of the K antigen by mutation renders an organism (now $K^{-}O^{+}$) susceptible to phagocytosis following opsonisation by complement in the absence of specific antisera (Williams *et al.*, 1983, 1986; Simoons-Smit *et al.*, 1986). Opsonisation of a $K^{-}O^{+}$ strain by complement alone markedly increases its surface hydrophobicity (Williams *et al.*, 1986). In the present study, we investigated the effect of antisera to specific cell-envelope components on surface hydrophobicity. We also studied the role of the capsule as a barrier to the penetration of antibodies to cell-envelope components masked by the exopolysaccharide matrix.

Materials and methods

Bacterial strains

Klebsiella aerogenes (also called *K. pneumoniae*) strain 5055 (K2, O1) was obtained from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT. Mutants M10 ($K^{-}O1^{+}$) and M10B ($K^{-}O^{-}$), both derived from strain 5055, were kindly donated by Drs I. R. Poxton and I. W. Sutherland. Strain DL1 ($K1^{+}O1^{+}$) was a clinical isolate (Williams *et al.*, 1983). Bacteria were grown for 18 h on Nutrient Agar (Lab M, Ford Lane, Salford, M6 6PB) at 37°C and resuspended in the appropriate buffer (see below). Growth on solid media reduces the loss of capsular material associated with growth in shaking incubators.

Preparation of antisera

Antisera to whole formalin-treated cells were raised in New Zealand White rabbits as described before (Williams *et al.*, 1983). Absorption of antiserum raised against the K2O1 strain with whole M10 ($K^{-}O1^{+}$) cells was also performed as previously described (Williams *et al.*, 1983). All antisera were heat-inactivated (30 min at 56°C) before use, because complement alone increases the surface hydrophobicity of the non-capsulate *Klebsiella* strains (Williams *et al.*, 1986).

Opsonisation of bacteria

To investigate the influence of specific antisera on cell-surface hydrophobicity and the ability of immunoglobulins to penetrate through the *Klebsiella* capsule, bacteria were resuspended in 10 mM phosphate-buffered saline pH 7.2 (PBS) to an absorbance at 470 nm (E_{470}) of 1.0 and incubated for 30 min at 37°C with 1.0 ml of the appropriate heat-inactivated antiserum or heat-inactivated non-immune rabbit serum. Bacteria were harvested

by centrifugation and washed twice in PBS before use in hydrophobicity or immunoadsorption assays.

Cell-surface hydrophobicity

Surface hydrophobicity of opsonised and non-opsonised bacterial cells was determined by the two-phase partition method described by Rosenberg (1984), as modified by Williams *et al.* (1986); octane was used as the hydrocarbon phase.

Isolation of outer membranes and LPS

Outer membranes were prepared by a modification of the method of Filip *et al.* (1973) with sodium N-lauryl sarcosinate (Sarkosyl) 2% w/v, as described by Williams *et al.* (1984). LPS was extracted by digestion of OM's with proteinase K (Sigma) according to the method of Hitchcock and Brown (1983). After digestion, the preparation was incubated in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) denaturing buffer for 10 min before electrophoresis.

Electrophoresis and immunoblotting

Outer membranes, LPS and antigen-antibody complexes were subjected to SDS-PAGE by the method of Lugtenberg *et al.* (1975). For separation of LPS, 4M urea was added to the gels. After separation by SDS-PAGE, the antigens were transferred to nitrocellulose paper in a Bio-Rad Transblot Cell as described by Towbin *et al.* (1979). After transfer, antigens were detected by the method of Jessop and Lambert (1985), modified by the use of staphylococcal protein A-horse radish peroxidase conjugate (Sigma) diluted 1 in 2000 for visualisation of the immunoreactive components.

Permeability of the *Klebsiella* capsule to immunoglobulins

The permeability of the *Klebsiella* capsule to immunoglobulins was investigated by a modification of the method of Swanson (1981) as adapted by Jessop and Lambert (1985). Briefly, cells were treated with antiserum to *K. aerogenes* strain M10 ($K^{-}O^{+}$), or with PBS for controls, as described above. Bacterial cells were resuspended in 10 ml of Empigen 2% w/v and shaken for 90 min at 37°C to lyse the cells without dissociating antigen-antibody complexes. Unlysed cells were removed by centrifugation and the supernate passed through a 0.5 ml bed volume of protein A-Sepharose CL4B (Pharmacia) packed in a Pasteur pipette. The column was eluted with Empigen 2% w/v to remove material remaining within the Sepharose gel but not bound to protein A via IgG. The protein A-Sepharose column containing the bound antigen-antibody complexes was then washed three times in 10-ml volumes of distilled water and then resuspended in 0.5 ml of ethanol for storage overnight at -20°C. The ethanol was removed by aspiration and the

Sepharose was suspended in 0.5 ml of water. After incubation in SDS-PAGE denaturing buffer at 100°C for 10 min the Sepharose was subjected to SDS-PAGE and immunoblotting. Immunoblots were developed with anti M10 (K^-O1^+) serum. By use of this method, the visualisation of heavy and light immunoglobulin chains on the immunoblots was avoided. As a further control, antiserum was passed over the protein A-sepharose column. SDS-PAGE of the resulting immune-complexes revealed the presence of immunoglobulin heavy and light chains on the immunoblots only by amido black staining; they were not visualised by immunodetection (data not shown).

Results

Influence of specific antisera on cell-surface hydrophobicity

Treatment of either capsulate or non-capsulate *Klebsiella* strains with heat-inactivated non-immune rabbit serum had little effect on cell-surface hydrophobicity (fig. 1A). $K1^+O1^+$, $K2^+O1^+$ and K^-O1^+ strains are all hydrophilic and failed to partition into the hydrocarbon layer. With the K^-O^- strain, there was a small but significant increase in hydrophobicity compared with the $K1^+O1^+$, $K2^+O1^+$ and K^-O1^+ strains in the presence (fig. 1A) or absence (Williams *et al.*, 1986) of non-

immune heat-inactivated serum. In contrast, exposure of the capsulate and non-capsulate strains to the corresponding autologous antisera resulted in marked increases in hydrophobicity of all strains (fig. 1B). Therefore sera containing anti-capsular antibodies profoundly affect the cell-surface hydrophobicity of the capsulate strains. Treatment of the $K2^+O1^+$ and $K1^+O1^+$ strains with anti-M10 (anti- K^-O1^+) serum increased the surface hydrophobicity of the *Klebsiella* strain expressing the type 2 capsular polysaccharide but had no effect upon strain DL1 ($K1^+O1^+$) (fig. 2A). Absorption of the anti- $K2^+O1^+$ serum with whole K^-O1^+ cells to remove all antibodies except the anti-K2 antibodies confirmed that either anti-capsular antibodies alone or antibodies to other OM components increased the cell-surface hydrophobicity of *K. aerogenes* NCTC 5055 ($K2^+O1^+$) (fig. 2A). Exposure of this strain to antiserum raised against the K^-O^- strain had no effect on cell-surface hydrophobicity (fig. 2B) which suggests that the anti-O antigen component of the anti- K^-O1^+ serum was responsible for increasing the cell-surface hydrophobicity of the $K2^+O1^+$ *Klebsiella* strain. Fig. 2B also shows that anti- K^-O^- serum had little effect on the cell-surface hydrophobicity of either $K1^+O1^+$ or K^-O1^+ strains.

Fig. 1. Effect of antibodies on cell-surface hydrophobicity measured by adherence to octane: **A**, heat-inactivated non-immune serum on $K1^+O1^+$ (■), $K2^+O1^+$ (○), K^-O1^+ (▲) and K^-O^- (●) strains; **B**, heat-inactivated autologous antiserum on each of the four strains.

Fig. 2. Effect of antibodies on cell-surface hydrophobicity measured by adherence to octane: A, anti-K⁻O₁⁺ serum on K₁⁺O₁⁺ (■) and K₂⁺O₁⁺ (○) strains, and of anti-K₂⁺O₁⁺ serum absorbed by whole K⁻O₁⁺ cells on the K₂⁺O₁⁺ strain (●); B, anti-K⁻O⁻ serum on K₁⁺O₁⁺ (■), K₂⁺O₁⁺ (○), K⁻O₁⁺ (▲) and K⁻O⁻ (●) strains.

Penetration of immunoglobulins through the *Klebsiella* capsule

Surface hydrophobicity experiments revealed that antibodies to the O₁ antigen of LPS could alter the surface properties of one capsulate *Klebsiella* strain possessing the type 2 capsular polysaccharide but not of a strain sharing the same O₁ antigen but with a type 1 capsular polysaccharide. To investigate whether the K₁ capsular polysaccharide presented a barrier to these anti-O₁ antibodies, a procedure was devised in which immunoabsorption of anti-K⁻O₁⁺ antiserum by whole cells (K₁⁺O₁⁺, K₂⁺O₁⁺ and K⁻O₁⁺) was followed by recovery and analysis of the immune complexes. In fig. 3 the OM protein profiles (A) of the three strains and immunoblots (B and C) developed with antiserum raised against the K⁻O₁⁺ strain are compared. The immunoblots clearly show that this antiserum contained antibodies to the OM proteins (B) and to the LPS (C). Immunoblots of the immune complexes recovered after immunoabsorption of the anti-K⁻O₁⁺ serum by capsulate or non-capsulate *Klebsiella* strains are shown in fig. 4. The major antigenic component of the immune complexes was

LPS. However, because this immunoblot was obtained from an SDS-polyacrylamide gel run without urea so that the presence of OM proteins would be clearly apparent, the LPS from these *Klebsiella* strains has run predominantly as a smear. Therefore the presence of LPS as the major antigenic component of the immune complexes reveals that the capsular polysaccharides do not form a barrier to the penetration of anti-O antibodies. Comparison of lanes 1, 2 and 3 in fig. 4 with an immunoblot of the OM of the K₂⁺O₁⁺ strain (lane 4) also shows that, even in the non-capsulate strain, the OM proteins appear to be poorly accessible to antibodies. This suggests that the O antigen may act as a barrier to their penetration.

Discussion

Autologous heat-inactivated antiserum exerted a profound effect on the cell-surface hydrophobicity of capsulate and non-capsulate *Klebsiella* strains. These increases in surface hydrophobicity, which should favour phagocytic recognition, correlate well with the pattern of susceptibility to phago-

Fig. 3. A, SDS-PAGE of the OM proteins of *Klebsiella* strains $K1^+O1^+$ (lane 1), $K2^+O1^+$ (lane 2) and K^-O1^+ (lane 3); lane 4 shows mol. wt (10^3) marker proteins. B and C, immunoblots of the OM proteins (B) and the LPSs (C) of the strains shown in A developed with antiserum raised against the K^-O1^+ strain. A and B were run on 15% polyacrylamide gels. To enhance LPS ladder patterns, the immunoblot in C was obtained from a 10% polyacrylamide gel containing 4M urea.

cytosis in the presence of specific antisera previously reported (Williams *et al.*, 1983). Such opsonising antibodies must, therefore, enhance the specific ligand-receptor interaction as well as rendering the bacterial surface more liable to hydrophobic interaction.

Antiserum to the O antigen as well as to the K antigen have been found to increase surface hydrophobicity and promote phagocytosis (Williams *et al.*, 1983) of the $K2^+O1^+$ *Klebsiella* strain. The portion of the immunoglobulin molecule responsible for increasing surface hydrophobicity is the Fc fragment (van Oss, 1978; Stendahl, 1983). This fragment must, therefore, be exposed outside the capsule and on the bacterial surface. However, anti-O1 antiserum failed to increase cell-surface hydrophobicity or promote phagocytosis (Williams *et al.*, 1983) of a strain possessing the same O1 antigen but with a K1 capsular serotype. This K antigen must either mask, or prevent access of antibodies to, the O antigen. Immunoabsorption of the anti- K^-O1^+ serum by whole cells revealed that both K1 and K2 capsules were permeable to anti-

O antibodies, indicating that, in the K1 serotype, anti-O antibodies were bound but not exposed at the cell surface. Therefore, surface hydrophobicity and phagocytosis (Williams *et al.*, 1983) were not enhanced.

By exploiting the presence of protein A in the cell wall, King and Wilkinson (1981) showed that the capsule of *Staphylococcus aureus* did not act as a diffusion or permeability barrier to IgG. They suggested that the resistance of capsulate staphylococci to phagocytosis was the result of masking of immunoglobulins and complement components bound to the cell wall. In gram-negative bacteria, several investigators have reported that only anti-K antibodies were opsonic and protective in experimental animal infections (Welch *et al.*, 1979; van Dijk *et al.*, 1981). Anti-KO but not anti-O serum alone enhanced clearance of capsulate *Escherichia coli* injected intravenously (Howard and Glynn, 1971). However, others have shown that antibodies to somatic antigens react with capsulate *E. coli* and can promote binding of complement to the bacterial cell surface (Horwitz and Silverstein,

Fig. 4. Immunoblot of the immune complexes obtained after immunoadsorption of anti-K⁻O1⁺ serum by whole cells of the K1⁺O1⁺ (lane 1), K2⁺O1⁺ (lane 2) and K⁻O1⁺ (lane 3) strains. Lane 4 is an immunoblot of the OM proteins of the K2⁺O1⁺ strain also developed with anti K⁻O1⁺ serum. These immunoblots were obtained from 15% polyacrylamide gels. (Immunoblots of controls with whole cells absorbed with buffer instead of antiserum showed no immunoreactive components.)

1980) and protect experimental animals against lethal infections (Kaijser *et al.*, 1972).

In *Klebsiella*, we have shown that the K antigen does not prevent access of anti-O antibodies but may mask their presence. Further investigation is required to determine whether the size of the capsule, the specific K antigen, or both, are important. Several investigators have explored the relationship between capsular size and pathogenicity in *Klebsiella* (Ehrenworth and Baer, 1956;

Domenico *et al.*, 1982; Cryz *et al.*, 1984). Colonial variants of *Klebsiella* possessing small capsules were reported to be much less virulent than the parent strains in burn-traumatized mice (Cryz *et al.*, 1984) and in a rat lobar pneumonia model (Domenico *et al.*, 1982). We have also shown that physical reduction in the amount of capsule promoted phagocytosis of the K1⁺O1⁺ *Klebsiella* strain after opsonisation with anti-O1 sera (Williams *et al.*, 1983). Thus, although the capsule does not present a permeability barrier, its size appears to determine whether antibodies to somatic antigens are exposed on the cell surface and are accessible to phagocytic cells.

The failure of antibodies to gain access to OM proteins in both capsule and non-capsule strains indicates that the O antigen acts as a barrier to their penetration. In this respect, *Klebsiella* resembles *Serratia marcescens*, with which immunoadsorption of antiserum with whole cells revealed that only the O antigen chains of LPS and the flagellar (H) antigen were accessible to antibodies (Jessop and Lambert, 1985). Furthermore, van der Ley *et al.* (1986) reported that the presence of a complete O antigenic side chain prevented access of antibodies to the OM pore protein Pho E at the surface of intact enterobacteria.

The growth environment, including the presence of antibiotics, exerts a profound effect on bacterial cell-envelope components in general (Brown and Williams, 1985), including bacterial exopolysaccharides (Sutherland, 1977). The nutritional conditions *in vivo* during infections may not only influence capsule size but also the size of the pores and channels within the exopolysaccharide matrix. Growth in the presence of sub-inhibitory concentrations of antibiotics, including cephalosporins (Kadurugamuwa *et al.*, 1985a; Williams, 1987) and ciprofloxacin (Williams, 1987) influenced the *Klebsiella* capsule such that a greater number of protein antigenic sites became exposed at the cell surface and accessible to antibodies (Kadurugamuwa *et al.*, 1985b) and complement (Williams, 1987). Further work is required, therefore, to determine whether growth conditions can influence penetration of antibodies to subcapsular envelope components and the subsequent opsonisation and phagocytosis of capsule *Klebsiellae*.

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