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The role of capsular polysaccharide K21*b* of *Klebsiella* and of the structurally related colanic-acid polysaccharide of *Escherichia coli* in resistance to phagocytosis and serum killing

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Summary. The behaviour of strains of *Klebsiella aerogenes* of capsular serotype K21 and strains of *Escherichia coli* producing a structurally related polysaccharide (colanic acid) was analysed by phagocytic and serum-killing assays. The cell-surface characteristics of these strains and of non-capsulate strains derived from them were also investigated by partitioning experiments in aqueous two-polymer phase systems. The possession of K21-type capsule by *K. aerogenes* or colanic-acid polysaccharide by *E. coli* conferred a strong negative charge on capsulate bacteria. Negatively charged bacteria of *E. coli* producing colanic-acid capsules, however, like non-capsulate *K. aerogenes*, were susceptible to uptake by polymorphonuclear leukocytes. In contrast, K21 polysaccharide conferred on klebsiellae considerable resistance to phagocytic uptake. The finding that ingested non-capsulate derivative strains of *K. aerogenes* were less rapidly degraded by phagocytes than *E. coli* strains suggested that other components of the cell surface of *Klebsiella*, notably lipopolysaccharide, may be involved in protection against phagocytic killing. The presence of colanic-acid capsules on *E. coli* conferred little resistance to the bactericidal activity of human serum or phagocytic uptake and did not protect against intracellular killing by polymorphonuclear leukocytes.

Introduction

Klebsiellae of capsular serotypes K2 and K21 are much more frequently involved in outbreaks of nosocomial infection than strains of other serotypes (Casewell and Talsania, 1979). The K21 serotype of *Klebsiella* is not homogeneous but consists of strains producing one or other of two chemically distinct types of capsular polysaccharide. Type K21*a* refers to those capsules which have the structure reported by Choy and Dutton (1973). Type K21*b* is a novel type more commonly encountered among clinical isolates than type K21*a* (Allen *et al.*, 1987*b*). To analyse the contribution of K21*b* capsular polysaccharide to pathogenicity, we have compared the resistance to phagocytic and serum killing of a capsulate strain of *K. aerogenes* with that of a non-capsulate strain spontaneously derived from it.

Though the colanic-acid capsular polysaccharide of *Escherichia coli* is antigenically similar to K21 polysaccharide of *Klebsiella* and reacts strongly with antiserum against K21 polysaccharide, it is chemically distinct. We have, therefore, investigated the behaviour of capsulate colanic-acid producing strains of *E. coli* obtained spontaneously and after genetic manipulation (Allen *et al.*, 1987*a*). It was of particular interest to analyse the role of colanic acid as a virulence factor because capsulate strains of *E. coli* producing that polysaccharide have been isolated at higher frequencies from patients with cystic fibrosis than from healthy controls (Macone *et al.*, 1981).

In this paper we have compared the behaviour of capsulate and non-capsulate strains of *Klebsiella* and *E. coli* by observing the degree of their phagocytosis by polymorphonuclear leukocytes (PMNL) and their susceptibility to killing by serum. Because physico-chemical properties are important in determining interactions between bacteria and phagocytes (Stendåhl, 1983), cell-surface characteristics were also investigated by

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electronmicroscopy and by partitioning in aqueous two-polymer phase systems.

Materials and methods

Bacterial strains

K. aerogenes strain 1L918 of capsular type K21 and the non-capsulate strain 1L919 were obtained from Dr J. G. Barr, Royal Victoria Hospital, Belfast (Barr, 1981). *E. coli* strain DH1 (Hanahăn, 1983) was a non-capsulate laboratory strain of *E. coli*. The capsulate strain 2L223, derived spontaneously from *E. coli* strain DH1, produced large amounts of colanic acid. The capsulate strain 2L245 was *E. coli* strain DH1 carrying the plasmid pLV213, which carries a fragment of the genome of *K. aerogenes* bearing the *rcsA* gene conferring on that strain the ability to produce colanic-acid capsule at <30°C (Allen *et al.*, 1987*a*).

Growth of bacteria

The different strains were grown overnight at 37°C in nutrient broth and harvested by centrifugation; *E. coli* strain 2L245 (pLV213), however, was grown at 28°C. For partitioning in two-polymer phase systems, bacteria were washed and resuspended in 0·03M Tris buffer (*p*H 7·2) to 2×10^9 cfu/ml. For serum bactericidal and phagocytic assays, bacteria were washed in Hanks's Balanced Salts Solution (HBSS) and resuspended in HBSS to 2×10^8 cfu/ml.

Partitioning in aqueous two-polymer phase systems

A two-phase system containing polyethylene glycol (PEG) 6000 4·4% w/w and Dextran T500 6·2% w/w in 0·03M Tris buffer (*p*H 7·2) was prepared from stock solutions of PEG 6000 (BDH Ltd) 20% w/w, Dextran T500 (Pharmacia) 20% w/w, 0·1M Tris buffer (*p*H 7·2) and distilled water and allowed to equilibrate overnight at 4°C. The charged system was prepared by including trimethyl-ammonium PEG (TMA-PEG) 0·55% w/w in the phase system. After separation of phases, 1 ml of the dextran-rich lower phase and 1 ml of the PEG-rich upper phase were pipetted into test tubes before addition of 0·1 ml of bacterial suspension. Tubes were inverted 20 times and allowed to stand for 30 min at 4°C. After separation of the phase, 0·5-ml samples from the upper phase were withdrawn and the number of bacteria therein determined. The number of bacteria present in the upper phase was expressed as a percentage of the total number added. Bacterial viability in the phase systems was routinely monitored and no effect on viability was observed.

Serum bactericidal assay

Sera from eight normal adults not receiving antimicrobial chemotherapy were collected, pooled and stored at -70°C until required. To determine resistance to the bactericidal activity of serum, 0·1 ml of bacterial suspension was added to increasing concentrations of pooled serum (concentration range 5–50% v/v in HBSS) in a final test volume of 1 ml. Mixtures were incubated at 37°C and the number of viable bacteria present after 90 min was determined.

Preparation of phagocytes and serum for phagocytosis assays

PMNL were obtained from the heparinised blood of normal donors. Blood (10 ml) was added to 3 ml of Dextran T500 6% w/v in saline. The blood was then drawn into a syringe and allowed to stand for 1 h. The leukocyte-rich supernate was decanted and centrifuged at 160 *g* for 5 min. Any erythrocytes remaining in the pellet of PMNL were lysed by shaking for 30 s in 9 ml of distilled water. The addition of 1 ml of NaCl 9% w/v restored isotonicity; PMNL were centrifuged at 160 *g* for 3 min, resuspended in 5 ml of HBSS, layered on 5 ml of Ficoll-Paque (Pharmacia) and centrifuged at 160 *g* for 25 min. PMNL were resuspended in 1 ml of HBSS containing bovine serum albumin (BSA) 0·1 v/v. Normal human serum was obtained from blood clotted at room temperature and diluted 1 in 5. When required, serum was heat-inactivated at 56°C for 30 min.

Uptake and killing by PMNL

A mixture of 0·5 ml of PMNL suspension (5×10^6/ml), 0·4 ml of diluted serum and 0·1 ml of bacterial suspension in polypropylene vials was rotated on a mixer (Matburn) at 37°C. For the uptake assay, samples (20 μl) taken at 0, 5, 15, 30 and 90 min were suspended in 2 ml cold HBSS. PMNL were removed from the samples by centrifugation at 110 *g* for 3 min and the number of viable bacteria in the supernate was determined by plating appropriate serial dilutions in triplicate on nutrient agar. The viable counts were expressed as the percentages of viable bacteria from the initial inocula remaining in the mixtures at each sampling time. For the phagocytic-killing assay, samples (20 μl) taken at 0, 5, 15, 30 and 90 min were suspended in 2 ml of distilled water for 5 min to disrupt the leukocytes. The number of viable bacteria in each sample was calculated as before. Phagocytosis was assessed visually by taking samples (20 μl) at 5, 15, 30 and 60 min and suspending in 1 ml of cold phosphate-buffered saline (*p*H 7·2) containing BSA 4% v/v. Slide preparations, made by centrifuging 0·5-ml samples in a centrifuge (Cytospin) at 150 rpm for 10 min, were stained with Giemsa's stain and examined by light microscopy. The numbers of bacteria associated with 50 randomly chosen PMNL were determined. All experiments were performed at least three times.

Electronmicroscopy

Bacteria were grown on plates of MacConkey agar for 2 days at room temperature and resuspended in saline to

1×10^9 cfu/ml. Bacteria were fixed and capsules stained in cacodylate-buffered glutaraldehyde 2·5% v/v containing ruthenium red 1% w/v. After 2 h, bacteria were pelleted, embedded in araldite, sectioned and further stained with lead citrate and uranyl acetate 2% v/v. Stained bacteria were examined with a Phillips 301 Electronmicroscope. Bacteria and PMNL were mixed and incubated as described for the phagocytic-uptake and killing assays. After 20 min, the mixture was fixed by the addition of 5 ml of cold glutaraldehyde 0·5% v/v. The mixture was embedded in araldite, sectioned, stained and examined.

Statistical analysis

For each experiment the mean of replicate samples was calculated together with the standard error of the mean (SEM) and levels of significance were determined by Student's *t* test.

Results

Surface properties of bacteria

K. aerogenes strain 1L918 (fig. 1a), *E. coli* strain 2L245 (pLV213) (fig. 1b) and strain 2L223 were shown by electronmicroscopy to possess capsular material that was stained by ruthenium red. *E. coli* strain DH1 (fig. 1c) and *K. aerogenes* strain 1L919 were non-capsulate.

The results from partitioning experiments with strains of *Klebsiella* and *E. coli* in aqueous two-polymer phase systems are shown in table I. In the normal (i.e., uncharged) phase system *c.* 20% of the capsulate bacteria of *K. aerogenes* strain 1L918 were found in the PEG-rich upper phase. Non-capsulate bacteria of *K. aerogenes* strain 1L919 had a stronger affinity for the dextran-rich lower phase with few (< 1%) of the bacteria present in the upper phase. But, similar experiments showed that both the non-capsulate bacteria of *E. coli* strain DH1 and the capsulate bacteria of *E. coli* strains derived from strain DH1 had a strong affinity for the lower phase (table I). When positively charged TMA-PEG was incorporated to the upper phase, however, capsulate bacteria of *K. aerogenes* strain 1L918 were found in significantly larger numbers in the upper phase; non-capsulate bacteria of *K. aerogenes* strain 1L919, on the other hand, remained in the lower phase. Although incorporation of TMA-PEG to the upper phase resulted in more bacteria of *E. coli* strain DH1 being found in the upper phase, significantly larger numbers of capsulate bacteria of *E. coli* strain 2L245 (pLV213) were found in that phase ($p < 0.01$). Furthermore, in the presence of TMA-PEG, the capsulate mutant strain 2L223 of *E. coli* had a significantly stronger affinity for the top phase than the capsulate *E. coli* strain 2L245 carrying the *Klebsiella rcsA* gene ($p < 0.01$).

Table I. Partitioning of strains of *Klebsiella* and *E. coli* in aqueous two-polymer phase systems

Species and strain no.	Capsule	Percentage of bacteria in the upper phase* of system that was	
		Normal†	Charged†
K. aerogenes 1L918	+	19·1 (1·8)	88·6 (5·2)
K. aerogenes 1L919	−	0·9 (0·1)	4·3 (0·4)
E. coli DH1	−	3·0 (0·3)	18·7 (0·8)
E. coli 2L245 (pLV213)	+	1·9 (0·2)	66·3 (3·0)
E. coli 2L223	+	2·5 (0·4)	82·5 (4·3)

* Each result represents the mean of four replicate samples and is shown together with the standard error of the mean (SEM) in parentheses.
† See *Materials and methods.*

Susceptibility of bacteria to complement-mediated serum killing

At serum concentrations $\geq 20\%$, capsulate bacteria of *K. aerogenes* strain 1L918 appeared significantly more sensitive ($p < 0.001$) to complement-mediated serum killing than bacteria of the related non-capsulate *Klebsiella* strain 1L919 (fig. 2). Moreover, growth of the non-capsulate *K. aerogenes* strain 1L919 was apparently stimulated at serum concentrations $< 10\%$. In contrast, both the non-capsulate *E. coli* strain DH1 and capsulate strains derived from it were extremely sensitive to the bactericidal activity of serum (fig. 3). Capsulate strains of *E. coli*, however, showed a small, but significant ($p < 0.001$), increase in serum resistance at serum concentrations $< 5\%$ compared with the non-capsulate *E coli* strain DH1.

Uptake and killing of bacterial strains by PMNL

Phagocytic uptake and killing by PMNL of *K. aerogenes* strain 1L918 of capsular type K21*b* and the non-capsulate strain 1L919 derived from it are shown in fig. 4. Capsulate bacteria of *K. aerogenes* strain 1L918 were resistant to phagocytosis and there was little, or no, killing by PMNL; the non-capsulate bacteria of *K. aerogenes* strain 1L919, on the other hand, were rapidly phagocytosed and after 90 min, only 1–2% of the latter bacteria were viable. Phagocytic assays with the serum-sensitive *E. coli* strain DH1 and with the capsulate strains of *E. coli* were performed with heat-inactivated (i.e., complement-deficient) serum. Both the non-capsu-

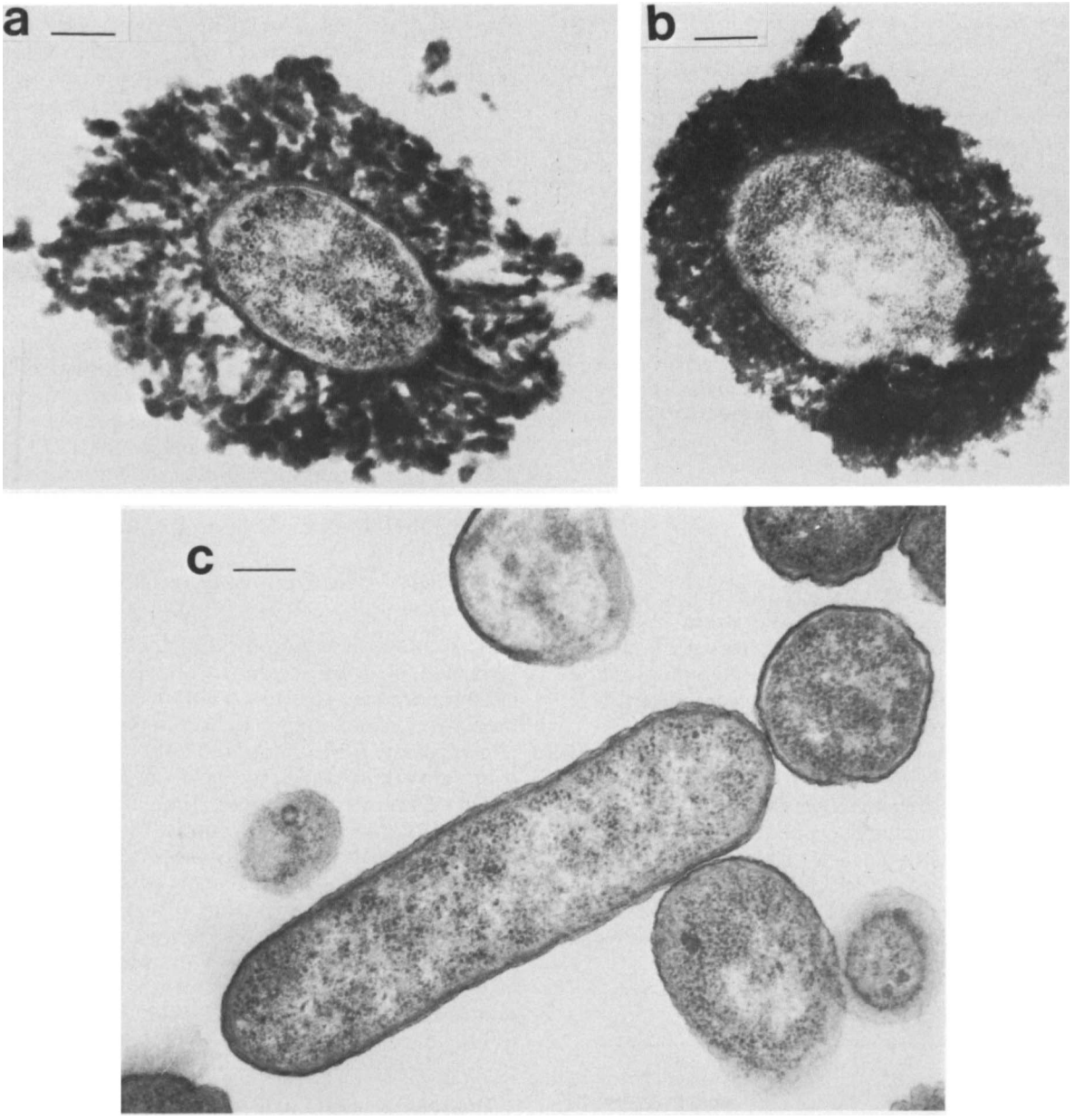

Fig. 1. Electronmicrographs of thin sections of: (a) *K. aerogenes* strain 1L918; (b) *E. coli* strain 2L245 (pLV213); and (c) *E. coli* strain DH1. Capsular material was stained with ruthenium red. Bar = 0·2 μm.

late strain DH1 and the capsulate strains of *E. coli* were extremely sensitive to phagocytosis by PMNL, although capsulate bacteria of *E. coli* strain 2L245 and the spontaneously derived capsulate mutant strain 2L223 of *E. coli* showed slight resistance to phagocytic uptake (fig. 5). The capsulate strains of *E. coli*, however, were not resistant to intracellular killing by PMNL.

Visualisation of endocytosed bacteria

Capsulate bacteria of *K. aerogenes* strain 1L918 were extremely resistant to uptake by PMNL, most bacterial cells remaining extracellular even after incubation for 60 min. Non-capsulate bacteria of *K. aerogenes* strain 1L919, on the other hand, were

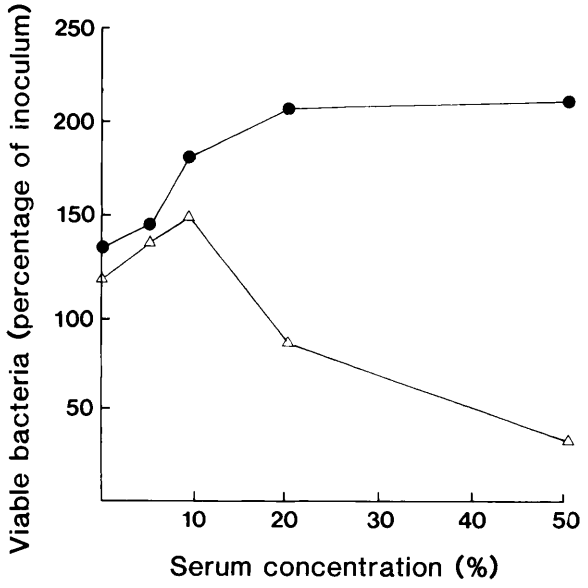

Fig. 2. Susceptibility to the bactericidal activity of pooled normal human serum of *K. aerogenes* strain 1L918 of capsular type K21*b* (△) and a non-capsulate mutant strain 1L919 (●) derived spontaneously from it. Each point represents the mean of four replicate samples.

readily phagocytosed and many of the bacteria of strain 1L919 were seen in the phagocytes after incubation for only 15 min. At each sampling interval, the mean number of bacteria of the capsulate strain 1L918 of *K. aerogenes* associated

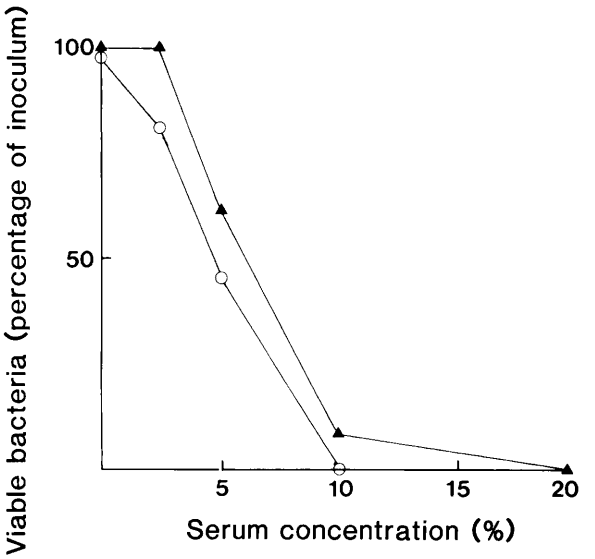

Fig. 3. Susceptibility to the bactericidal activity of pooled normal human serum of the non-capsulate *E. coli* strain DH1 (○) and a capsulate mutant strain 2L223 (▲) derived spontaneously from it. Each point represents the mean of three replicate samples.

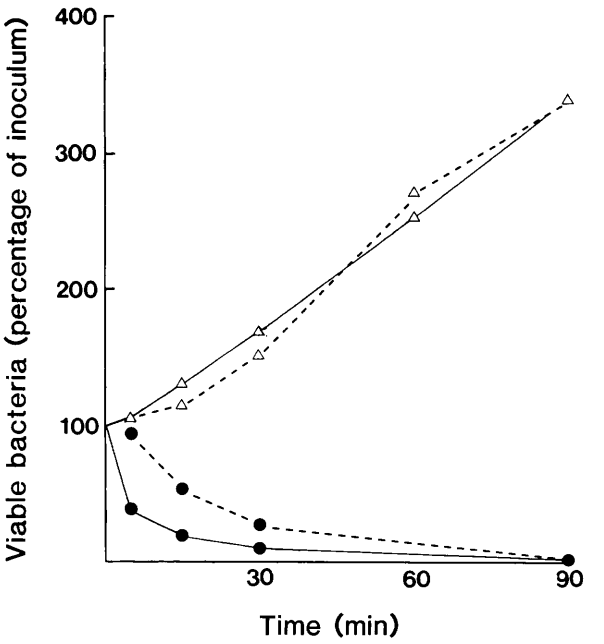

Fig. 4. Uptake (——) and killing (----) by PMNL in the presence of unheated serum of *K. aerogenes* strain 1L918 of capsular type K21*b* (△) and a non-capsulate strain 1L919 (●) derived spontaneously from it. Each point represents the mean of three replicate samples.

with PMNL was significantly lower ($p<0{\cdot}001$) than the mean number of non-capsulate bacteria of strain 1L919 (table II). *K. aerogenes* strain 1L918 exhibits instability of the K21*b* capsular phenotype at a frequency of about 10^{-2}/cell; thus, the small numbers of bacteria associated with phagocytes may have resulted from the phagocytosis of spontaneously occurring non-capsulate mutant bacteria rather than from the phagocytic uptake of capsulate klebsiellae.

After incubation for 5 min, phagocytic uptake of capsulate bacteria of *E. coli* strains 2L223 and 2L245 was significantly lower ($p<0{\cdot}001$) than that of the non-capsulate bacteria of strain DH1 (table II). After incubation for 15 min, the majority of cells of *E. coli* strain DH1 were intracellularly located. It was not possible, however, to count the number of phagocyte-associated bacteria, most of which were in an advanced stage of destruction (fig. 6a). Again, few extracellular bacteria of *E. coli* strains 2L223 and 2L245 were visible after incubation for 15 min and most intracellular bacteria had disintegrated (fig. 6b). In contrast, electronmicrographs of ingested non-capsulate bacteria of *K. aerogenes* strain 1L919 revealed that intracellular bacteria showed little, if any, structural change after incubation for 20 min (fig. 6c).

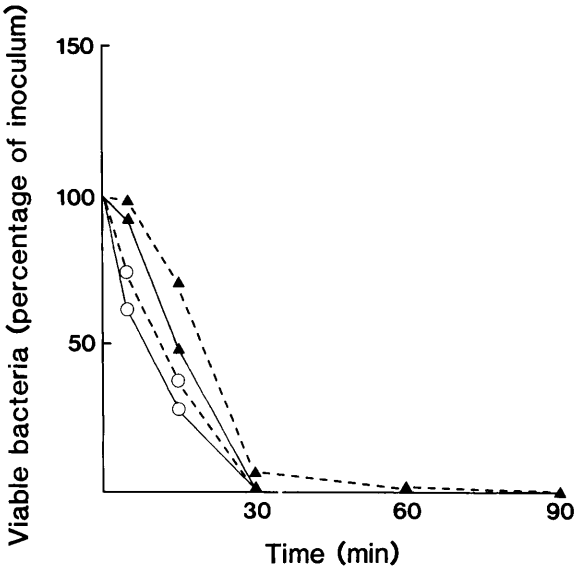

Fig. 5. Uptake (——) and killing (----) by PMNL in the presence of heat-inactivated serum of capsulate *E. coli* strain 2L223 (▲) and the non-capsulated strain DH1 (○) derived spontaneously from it. Each point represents the mean of three replicate samples.

Table II. Endocytosis of capsulate and non-capsulate bacteria by PMNL

Species and strain no.	Capsule	Time of incubation (min)	Mean number of bacteria/ phagocyte*
K. aerogenes 1L918	+	5	0·3 (0·1)
		15	0·8 (0·2)
		30	0·7 (0·2)
		60	1·2 (0·3)
K. aerogenes 1L919	−	5	10·8 (1·3)
		15	15·4 (2·2)
		30	17·2(2·5)
		60	13·4 (2·0)
E. coli DH1	−	5	4·0 (0·6)
E. coli 2L245 (pLV213)	+	5	1·4 (0·1)
E. coli 2L223	+	5	0·6 (0·1)

* Each result represents the mean of four replicate samples and is shown together with the standard error of the mean (SEM) in parentheses.

Discussion

The importance of K-antigens other than K21 as virulence determinants of *Klebsiella* has been reported by Williams *et al.* (1983) and Simoons-Smit *et al.* (1986). We have compared the resistance to phagocytosis and serum killing of a capsulate strain of *K. aerogenes* (1L918) with that of an otherwise isogenic non-capsulate strain (1L919); the former reacted strongly with anti-K21 antiserum by "Quellung" reaction, immunofluorescence and counter-immunoelectrophoresis whereas the latter was non-typable. *K. aerogenes* strain 1L918 possessed a thick capsule that imparted a strong negative surface charge to the bacteria whereas strain 1L919 was non-capsulate and its bacteria were less negatively charged. The presence of the negatively charged capsule on bacteria of strain 1L918 conferred considerable resistance to

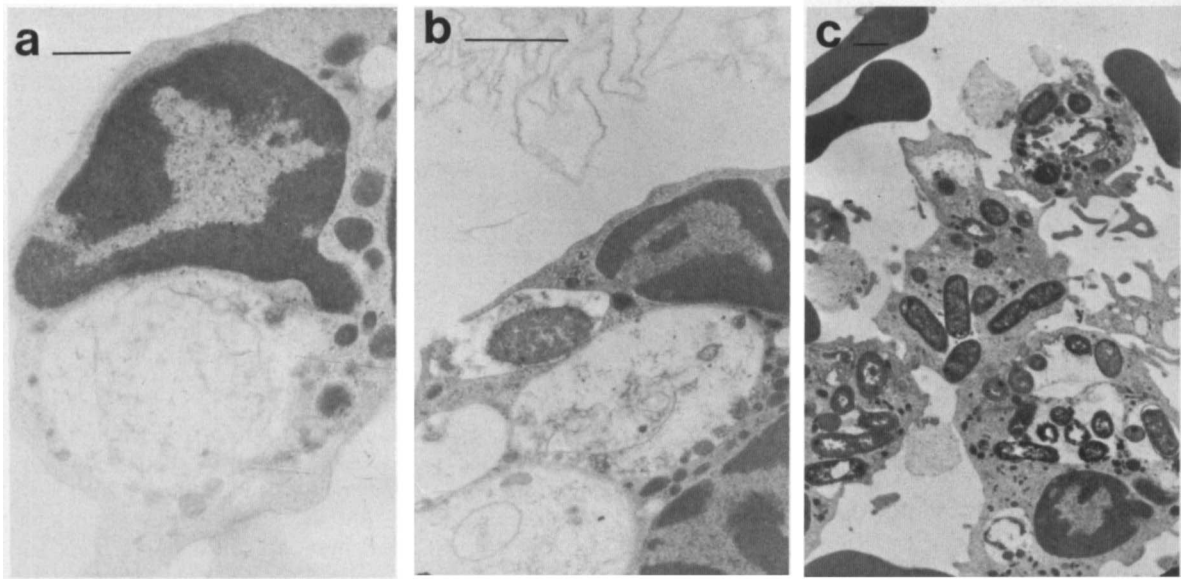

Fig. 6. Electronmicrographs of thin sections of human PMNL after incubation for 15 min with: (a) non-capsulate *E. coli* strain DH1; (b) capsulate *E. coli* strain 2L245 (pLV213); and (c) non-capsulate *K. aerogenes* strain 1L919. Bar = 1 μm.

phagocytic uptake and killing compared with strain 1L919. The capsulate bacteria of *Klebsiella* strain 1L918, however, were significantly more susceptible to serum killing than the non-capsulate strain derived from it but at concentrations of serum greater than 20% v/v only. It is probable that at these serum concentrations there is sufficient naturally occurring anti-K21 capsular antibody to permit activation and appropriate deposition of complement proteins on the bacterial surface. At serum concentrations of *c.* 10% v/v, however, phagocytic uptake of the capsulate strain was poor suggesting that at these concentrations insufficient antibody was present for effective opsonisation. The resistance of strain 1L919 to serum killing emphasises the importance of other bacterial envelope components such as lipopolysaccharide in that kind of resistance. Although the non-capsulate strain 1L919 was not resistant to phagocytic uptake, it was less susceptible to phagocytic killing than *E. coli* strain DH1 because, after incubation for 30 min, intact bacteria of *Klebsiella* strain 1L919 present within the phagocytes could be seen by electronmicroscopy, whereas all of the bacteria of *E. coli* strain DH1 were disrupted.

Capsules were clearly visible by electronmicroscopy on the variant strains 2L223 and 2L245 of *E. coli* that expressed a mucoid phenotype but were not present on bacteria of *E. coli* strain DH1. Each of the capsulate strains of *E. coli* was antigenically similar to capsulate bacteria of *K. aerogenes* strain 1L918 because they reacted with anti-K21 antiserum as determined by immunofluorescence and "Quellung" reaction (Allen *et al.*, 1987*a*). In aqueous two-polymer phase systems the presence of a capsule conferred a strong negative charge on the bacteria of *E. coli* strains 2L223 and 2L245, and it has been suggested that the presence of a capsule and a strong negative surface charge inhibits contact between bacteria and phagocytes (Stendahl, 1983). In marked contrast, however, to the capsulate strains of *Klebsiella*, the negatively-charged capsules of bacteria of *E. coli* strains 2L223 and 2L245, which produced colanic acid as the capsular polysaccharide, did not confer great resistance to phagocytic uptake or killing. Colanic acid has a side chain that is antigentically similar to the side chain of K21 polysaccharide but the two polysaccharides are otherwise chemically distinct (Henriksen, 1954; Garegg *et al.*, 1971; Choy and Dutton, 1973). Although the capsules of bacteria of *Klebsiella* strain 1L918 and of *E. coli* strains 2L223 and 2L245 appeared identical on electronmicroscopy, the capsule of strains of *Klebsiella* is closely attached to the underlying cell surface whereas colanic-acid capsular polysaccharide is often present as loosely attached slime (Sutherland, 1977). It has also been observed that different capsules may possess different anti-phagocytic properties (Glynn and Howard, 1970).

Mucoid strains of *E. coli* producing colanic-acid capsules have been associated with lung infections in patients with cystic fibrosis (Macone *et al.*, 1981). In these cases, however, the viscid bronchial mucus provides a favourable environment for the production of capsular polysaccharide because colonisation of the respiratory tract first occurs with non-mucoid *Pseudomonas aeruginosa* followed thereafter by the emergence of mucoid strains (Hoiby, 1974). It has been suggested that the mucoid coat of *P. aeruginosa* may provide a barrier against phagocytosis and limit access of opsonic antibodies (Hoiby, 1974; Lam *et al.*, 1980). It is clear, however, that the colanic-acid capsule of bacteria of *E. coli* strains 2L223 and 2L245 neither protected against serum killing nor against phagocytic uptake and killing.

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