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Mechanisms of *Klebsiella pneumoniae* Resistance to Complement-Mediated Killing

SUSANA MERINO,[1] SILVIA CAMPRUBÍ,[1] SEBASTIAN ALBERTÍ,[2] VICENTE-JAVIER BENEDÍ,[2] AND JUAN M. TOMÁS[1]*

Departamento de Microbiología, Universidad de Barcelona, Diagonal 645, 08071 Barcelona,[1] and Area de Microbiología, Departament de Biología Ambiental, Universitat de les Illes Balears, Palma de Mallorca,[2] Spain

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The different mechanisms of *Klebsiella pneumoniae* resistance to complement-mediated killing were investigated by using different strains and isogenic mutants previously characterized for their surface components. We found that strains from serotypes whose K antigen masks the lipopolysaccharide (LPS) molecules (such as serotypes K1, K10, and K16) fail to activate complement, while strains with smooth LPS exposed at the cell surface (with or without K antigen) activate complement but are resistant to complement-mediated killing. The reasons for this resistance are that C3b binds far from the cell membrane and that the lytic final complex C5b-9 (membrane attack complex) is not formed. Isogenic rough mutants (K^+ or K^-) are serum sensitive because they bind C3b close to the cell membrane and the lytic complex (C5b-9) is formed.

The complement system plays a crucial role in humoral defense against microbial pathogens and has recently been reviewed (25, 26). This series of serum proteins which are sequentially activated produces two major effects in terms of host defense: (i) deposition onto the microbial surface of an opsonic protein (C3b or iC3b), which activates the C5 cascade, and (ii) the result of the C5 cascade, the formation and assembly of a membrane attack complex (C5b-9) capable of lysing susceptible bacteria. The latter effect of direct bacterial killing is known as the serum bactericidal reaction.

Complement activation may take place by either of two pathways (classical or alternative) resulting in activation of the vital third component of complement, C3. Bacterial resistance to complement-mediated killing may result from the failure or limitation of complement activation by either of the two pathways or by the failure of activated complement to exert its effect. Various surface antigens which render bacterial cells resistant to complement-mediated killing, such as lipopolysaccharide (LPS), outer membrane proteins, and capsules (8, 17, 24), have been identified.

Klebsiella pneumoniae biotypes are widely recognized as opportunistic pathogens acting as agents of bacteremias and respiratory and genitourinary infections, particularly in patients under stress (13). Unlike other enterobacteria, *K. pneumoniae* contains a large capsular polysaccharide involved in colonization and proliferation in its host (3). We previously obtained isogenic unencapsulated mutants (serum resistant) (2), as well as mutants lacking the O-antigen chains of the LPS (serum sensitive) (28). Also, a previous study of the surface accessibility of the O-antigen LPS in different capsular serotypes of *K. pneumoniae* (29) allowed us in this study to examine the molecular mechanisms of resistance to complement-mediated killing used by different *K. pneumoniae* strains by defining at a molecular level the roles of capsular type and LPS in complement evasion.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and media. The *K. pneumoniae* strains used in this study are listed in Table 1. Luria broth (15) was routinely used for bacterial growth and solidified with 1.5% agar (Luria broth agar).

Bacterial survival in fresh nonimmune serum. The survival of exponential-phase bacteria in fresh nonimmune human serum (NHS) was measured at 37°C as previously described (28). Control measurements with bacteria in phosphate-buffered saline (PBS) (containing 0.15 M sodium chloride and 0.15 M sodium phosphate, pH 7.2) or heat-inactivated NHS (56°C for 30 min) were performed.

Strains and survival in NHS. *K. pneumoniae* strains from capsular serotypes K1, K10, and K16 showed the O1-antigen LPS masked by the K antigen, while strains from serotypes K2, K7, K19, K21, K22, and K66 exposed at the bacterial surface both antigens (the O1-antigen LPS and the corresponding K antigen) (29). All of the *K. pneumoniae* strains with O1-antigen (smooth) LPS were resistant to serum bactericidal activity; they showed over 100% survival after 3 h of incubation at 37°C in NHS, regardless of whether they were K^+ or K^-. Only strains lacking the O1-antigen LPS (strains KT707, KT701, and KT859), regardless of whether they were K^+ (KT707 and KT701) or K^- (KT859), showed a great decrease in viable cells on NHS (i.e., were serum sensitive) (27). If we depleted NHS of complement by heating it at 56°C for 30 min or by treating it with 20 mM EDTA for 1 h at 37°C, serum-sensitive strains, such as KT707, showed over 100% survival after 3 h of incubation at 37°C in treated serum (28).

Cell surface isolation and analyses. Cell envelopes were prepared by lysis of whole cells in a French press at 16,000 lb/in^2. Unbroken cells were removed by centrifugation at 10,000 × *g* for 10 min, and the envelope fraction was collected by centrifugation at 100,000 × *g* for 2 h. Cytoplasmic membranes were solubilized twice with sodium *N*-lauryl sarcosinate (7), and the outer membrane fraction was collected as described above. Outer membrane proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by a modification (1) of the Laem-

TABLE 1. *K. pneumoniae* strains used in this study

Strain	Relevant characteristics[a]	Reference or source[b]
KP1LC	O1:K1, S^r	6
7825	O1:K1, S^r	18
DL1	O1:K1, S^r	30
KT836	Isogenic K^- mutant derived from DL1, S^r	30
52145	O1:K2, S^r	18
B5055	O1:K2, S^r	11
KT739	O1:K7, S^r	I. Ørskov
KT759	O1:K10, S^r	I. Ørskov
KT112	O1:K10, S^r	I. Ørskov
KT762	O1:K16, S^r	I. Ørskov
KT1119	O1:K16, S^r	I. Ørskov
KT763	O1:K19, S^r	I. Ørskov
KT765	O1:K21, S^r	I. Ørskov
KT741	O1:K22, S^r	16
C3	O1:K66, S^r	28
KT791	Isogenic K^- mutant derived from C3, S^r	2
KT707	Isogenic O^- mutant derived from C3, S^s	28
KT701	Isogenic O^- mutant derived from C3, S^s	28
KT859	Isogenic O^- mutant derived from 52145, S^s	Our laboratory

[a] S^r, serum resistant; S^s, serum sensitive.
[b] Strains from I. Ørskov are from the International Klebsiella Center, Copenhagen, Denmark.

mli procedure (12). Protein gels were fixed and stained with Coomassie blue. LPS was isolated and purified by the method of Westphal and Jann (33) as modified by Osborn (20). LPS was analyzed by SDS-PAGE and silver stained by the method of Tsai and Frasch (32).

Capsular polysaccharide was isolated and purified as supernatant fluid by the method of Wilkinson and Sutherland (34) and was proved to be essentially free of LPS by SDS-PAGE (32) and by an enzyme-linked immunosorbent assay (ELISA) using specific antiserum against purified LPS (28).

Inhibition of serum bactericidal activity. The effect of treating the serum with bacterial cells or purified cell components in the serum bactericidal reaction was studied as follows.

(i) Whole cells. NHS (1.5 ml) was incubated at 37°C for 1 h with 10^8 cells and centrifuged for 5 min in an Eppendorf microcentrifuge. The supernatant was filtered through a 0.45-μm-pore-size filter to remove the cells, and the treated serum (0.9 ml) was added to 0.1 ml of an exponential-phase culture (5×10^7 CFU) of *K. pneumoniae* KT707 (serum sensitive [28]) or a similar strain and incubated at 37°C for 3 h. Samples were taken hourly, and bacterial concentrations were determined by dilution and plating on Luria broth agar.

(ii) Purified LPS. LPS was suspended in PBS at a final concentration of 1 to 5 mg/ml and briefly sonicated at 4°C until the solution cleared. LPS solution in a final concentration range of 0.01 to 0.2 mg/ml was added to 1 ml of NHS and incubated for 30 min at 37°C. After this treatment, the bactericidal activity of the serum was determined with strain KT707 or a similar strain as described above.

(iii) Capsular polysaccharide. Purified capsular polysaccharide was suspended in PBS at a final concentration of 5 mg/ml and briefly sonicated at 4°C until the solution cleared. The solution, in a final concentration range of 0.01 to 0.4 mg/ml, was added to 1 ml of NHS and incubated for 30 min at 37°C. After this treatment, the bactericidal activity of the serum was determined as described above.

Controls consisting of NHS incubated for 1 h at 37°C in PBS without cells, LPS, or capsular polysaccharide showed no inhibition of serum bactericidal activity.

Measurement of the anticomplement activity of whole cells or purified molecules. The anticomplement activity of whole cells, purified LPS, or purified capsular polysaccharide was measured according to the method of Shafer et al. (23). The positive control consisted of sensitized sheep erythrocytes plus NHS alone, and the negative control consisted of cells or purified molecules without added NHS.

Concentrations of C1q and C3 complement components were measured with specific antibodies as described by Theofilopoulos et al. (27). Briefly, specific anti-C1q or anti-C3 antisera (Sigma) were coated onto a microtiter plate, incubated overnight at 4°C, washed, and incubated for 1 h at 37°C with 1% bovine serum albumin. Meanwhile, NHS was treated with *K. pneumoniae* whole cells, purified LPS, or purified capsular polysaccharide for 30 min at 37°C. Untreated NHS was used as a standard with the same incubation period. After the plates were washed, the treated or untreated NHS was added and incubated for 90 min at 37°C. The plates were then washed again and incubated for 1 h at 37°C with protein A-alkaline phosphatase conjugate (Boehringer). After the plates were washed, the color reaction was developed with 4-nitrophenyl phosphate (1 mg/ml) and the A_{405} was recorded.

Binding of C3b and C5b-9 to whole cells. The interaction between whole *K. pneumoniae* cells and complement components C3b and C5b-9 was quantified by an enzyme immunoassay and observed by immunogold electron microscopy. Bacteria that were preincubated for 5 to 20 min with 90% NHS at 37°C were washed twice with cold PBS by microcentrifugation, incubated for 45 min at 37°C in suspension with anti-C3b or anti-C5b-9 (Calbiochem) (1:100 dilution in PBS plus 1% bovine serum albumin), and washed again by microcentrifugation. Next, in the case of the immunoassay, the bacteria were incubated with protein A-alkaline phosphatase (1:100 dilution in PBS) at 37°C for 45 min; in the case of immunogold electron microscopy, they were incubated with protein A conjugated to 20-nm gold particles (1:20 dilution in PBS). After the washing, the color reaction in the immunoassay was developed as described above and the A_{405} was recorded. In the case of immunogold electron microscopy, after the washing, the bacterial suspensions were placed on Formvar-coated copper grids, air dried, and examined in a Hitachi H600 transmission electron microscope.

Controls consisted of cells treated with protein A-alkaline phosphatase for the immunoassay or protein A conjugated to 20-nm gold particles for immunogold electron microscopy in the absence of specific antibodies.

RESULTS

Inhibition of serum bactericidal activity by whole cells or purified surface molecules. Preincubation of NHS with whole cells of *K. pneumoniae* strains from serotypes O1:K2, O1:K7, O1:K19, O1:K21, O1:K22, and O1:K66 inhibited serum bactericidal activity when tested against serum-sensitive strains such as KT707. This inhibitory effect was also observed with unencapsulated (K^-) isogenic mutants, such as KT791 or KT847 (Table 1), irrespective of the K serotype of their wild-type strain. However, whole cells from sero-

TABLE 2. Inhibition of bactericidal activity of NHS against serum-sensitive strain KT707 by whole cells of *K. pneumoniae*

Strain used for NHS treatment (serotype)	% Survival of KT707 after incubation[a] for indicated time with:			
	Untreated NHS (3 h)	Treated NHS		
		1 h	2 h	3 h
KP1LC (O1:K1)	<0.1	0.6	<0.1	<0.1
7825 (O1:K1)	<0.1	0.5	<0.1	<0.1
DL1 (O1:K1)	<0.1	0.6	<0.1	<0.1
KT836 (O1:K$^-$)	<0.1	98	110	139
52145 (O1:K2)	<0.1	95	108	128
B5055 (O1:K2)	<0.1	96	112	145
KT739 (O1:K7)	<0.1	98	111	133
KT759 (O1:K10)	<0.1	0.5	<0.1	<0.1
KT112 (O1:K10)	<0.1	0.6	<0.1	<0.1
KT762 (O1:K16)	<0.1	0.7	<0.1	<0.1
KT1119 (O1:K16)	<0.1	0.6	<0.1	<0.1
KT763 (O1:K19)	<0.1	99	121	146
KT765 (O1:K21)	<0.1	96	107	131
KT741 (O1:K22)	<0.1	95	104	123
C3 (O1:K66)	<0.1	97	115	138
KT791 (O1:K$^-$)	<0.1	100	129	156

[a] The bacteria were incubated at 37°C.

types O1:K1, O1:K10, and O1:K16 were completely unable to inhibit the bactericidal activity of NHS (Table 2). Similar results were obtained with two other serum-sensitive strains (KT701 and KT859) of *K. pneumoniae* (data not shown), both of which lack O1-antigen LPS.

Various concentrations of purified whole LPS (a mixture of O-antigen-containing and O-antigen-deficient LPS molecules) from *K. pneumoniae* DL1 (O1:K1), 52145 (O1:K2), or C3 (O1:K66) inhibited the bactericidal activity of NHS in a dose-dependent manner when tested against strain KT707. For instance, these purified LPSs at 0.1 mg/ml were able to completely inhibit the bactericidal activity of NHS against strain KT707 (Table 3). It is important to point out that whole cells of strains DL1 (O1:K1), KT759 (O1:K10), and KT762 (O1:K16) were unable to inhibit the bactericidal activity of NHS, while their purified LPSs were able to do so.

This fact prompted us to examine the interaction between purified capsular polysaccharide and NHS. Purified capsular polysaccharides (with LPS contamination of <1%, as determined with specific antibodies and gels) were obtained from strains DL1 (O1:K1), 52145 (O1:K2), KT739 (O1:K7), KT759 (O1:K10), KT762 (O1:K16), KT763 (O1:K19), KT765 (O1:K21), KT741 (O1:K22), and C3 (O1:K66). None of these capsular polysaccharides were able, even at very high concentrations (0.2 or 0.4 mg/ml), to inhibit the bactericidal activity of NHS when tested against the serum-sensitive strains of *K. pneumoniae* (data shown in Table 3 for strain KT707). Similar results were obtained with other serum-sensitive strains of *K. pneumoniae* lacking the O-antigen LPS (KT701 and KT859 [data not shown]).

Anticomplement activity of whole cells and purified surface molecules. The complement-absorbing activity of whole cells and purified surface molecules was measured to determine whether inhibition of serum bactericidal activity was due to the depletion of serum complement. Whole cells of *K. pneumoniae* strains of serotypes O1:K2, O1:K7, O1:K19, O1:K21, O1:K22, and O1:K66, as well as isogenic K$^-$ mutants (regardless of the K serotype of their wild-type strains), inhibited complement-mediated hemolysis of sensitized sheep erythrocytes. However, whole cells of serotypes O1:K1, O1:K10, and O1:K16 were completely unable to inhibit complement-mediated hemolysis of sensitized erythrocytes. Furthermore, complement components C1q and C3 were depleted when NHS was treated with whole cells of serotypes that exposed both antigens at the cell surface (such as O1:K2 or O1:K66) or isogenic K$^-$ mutants, while whole cells of K serotypes that masked the O1-antigen LPS were unable to deplete these complement components in treated NHS (Table 4).

TABLE 3. Inhibition of bactericidal activity of NHS against serum-sensitive strain KT707 by different purified surface molecules of *K. pneumoniae* strains

Surface molecules used for NHS treatment	% Survival of KT707 after incubation[a] for indicated time with:			
	Untreated NHS (3 h)	Treated NHS		
		1 h	2 h	3 h
LPS from strain[b]:				
DL1 (O1:K1)	<0.1	98	104	125
KT836 (O1:K$^-$)	<0.1	97	105	128
52145 (O1:K2)	<0.1	96	104	126
KT759 (O1:K10)	<0.1	97	107	130
KT762 (O1:K16)	<0.1	96	102	125
C3 (O1:K66)	<0.1	98	109	131
KT791 (O1:K$^-$)	<0.1	95	104	124
K antigen from strain[c]:				
DL1 (O1:K1)	<0.1	0.7	<0.1	<0.1
52145 (O1:K2)	<0.1	0.6	<0.1	<0.1
KT739 (O1:K7)	<0.1	0.6	<0.1	<0.1
KT759 (O1:K10)	<0.1	0.6	<0.1	<0.1
KT762 (O1:K16)	<0.1	0.8	<0.1	<0.1
KT763 (O1:K19)	<0.1	0.5	<0.1	<0.1
KT765 (O1:K21)	<0.1	0.7	<0.1	<0.1
KT741 (O1:K22)	<0.1	0.5	<0.1	<0.1
C3 (O1:K66)	<0.1	0.6	<0.1	<0.1

[a] The bacteria were incubated at 37°C.
[b] Purified LPS at a concentration of 0.1 mg/ml.
[c] Purified K antigen (capsular polysaccharide) at a concentration of 0.2 mg/ml.

The complement-absorbing activity of LPS from strains DL1 (O1:K1) and C3 (O1:K66) was dose dependent (Fig. 1). Also, the C3 concentration was depleted when NHS was treated with these purified LPSs (Table 5). Again, it is important to point out that whole cells from strains masking

TABLE 4. Concentrations of complement components C1q and C3 in untreated NHS and NHS treated with whole *K. pneumoniae* cells

Strain used for treatment	Concn of component[a]:	
	C1q	C3
None	1.32	1.98
DL1	1.29	1.87
KT836	0.35	0.45
52145	0.39	0.48
KT759	1.30	1.89
KT762	1.27	1.90
C3	0.36	0.44
KT791	0.32	0.41

[a] Concentrations were determined by ELISA and are given in arbitrary A_{405} units. Results are means from experiments done in triplicate at least twice. Standard deviations were all <0.07.

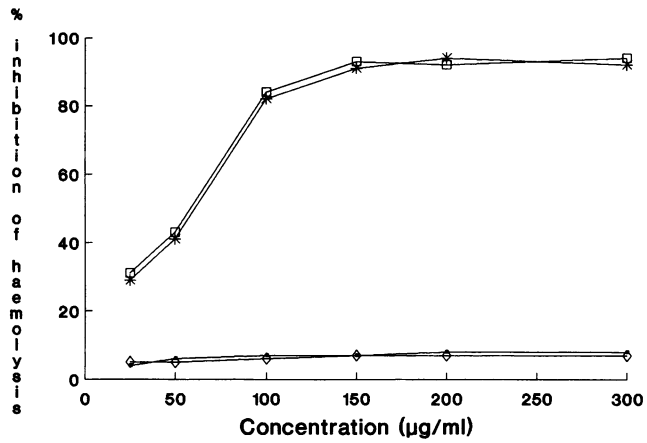

FIG. 1. Inhibition of complement-mediated hemolysis of sensitized sheep erythrocytes. Erythrocytes were incubated for 30 min with NHS as a control, with NHS containing LPS from *K. pneumoniae* DL1 (O1:K1) (✱) or C3 (O1:K66) (□), or with NHS containing purified capsular polysaccharide from strain DL1 (◇) or C3 (■).

the O1-antigen LPS are unable to deplete complement in treated NHS, while their purified LPSs are able to do so.

When we tested purified capsular polysaccharides from different *K. pneumoniae* strains (Table 5), we found that none of them were able to inhibit the complement-mediated hemolysis of sensitized sheep erythrocytes (Fig. 1, data for K1 and K66) or to deplete C3 from capsular polysaccharide-treated NHS (Table 5).

Binding of C3b and C5b-9 to whole cells. As shown in Table 6, whole cells of *K. pneumoniae* strains of serotypes K2, K7, K19, K21, K22, and K66 and isogenic K^- mutants (serum resistant) bound less C3b than serum-sensitive strains (O^- mutants). Also, serum-resistant strains did not bind C5b-9,

TABLE 5. Concentrations of complement component C3 in untreated NHS and NHS treated with purified *K. pneumoniae* surface molecules

Treatment and strain	Concn of C3[a]
None	1.98
LPS[b]	
DL1	0.47
KT836	0.50
52145	0.43
KT759	0.45
KT762	0.45
C3	0.47
K antigen[c]	
DL1	1.88
52145	1.86
KT759	1.91
KT762	1.89
KT765	1.85
C3	1.89

[a] Concentrations were determined by ELISA and are given in arbitrary A_{405} units. Results are means from experiments done in triplicate at least twice. Standard deviations were all <0.09.

[b] Purified LPS at a concentration of 0.1 mg/ml.

[c] Purified K antigen (capsular polysaccharide) at a concentration of 0.2 mg/ml.

TABLE 6. Interaction of complement components C3b and C5b-9 with *K. pneumoniae* whole cells

Strain (serotype)	Relative concn (mean ± SD)[a] of:	
	C3b	C5b-9
KT836 (O1:K^-)	0.82 ± 0.12	0.09 ± 0.03
52145 (O1:K2)	0.62 ± 0.09	0.10 ± 0.03
B5055 (O1:K2)	0.67 ± 0.10	0.09 ± 0.02
KT739 (O1:K7)	0.61 ± 0.09	0.08 ± 0.03
KT763 (O1:K19)	0.66 ± 0.11	0.10 ± 0.02
KT765 (O1:K21)	0.62 ± 0.09	0.09 ± 0.03
KT741 (O1:K22)	0.66 ± 0.07	0.10 ± 0.02
C3 (O1:K66)	0.63 ± 0.08	0.08 ± 0.03
KT791 (O1:K^-)	0.84 ± 0.12	0.11 ± 0.03
KT707 (O^-:K66)	1.69 ± 0.23	1.45 ± 0.12
KT701 (O^-:K66)	1.72 ± 0.19	1.45 ± 0.15
KT859 (O^-:K2)	1.71 ± 0.19	1.47 ± 0.13

[a] Results are given in arbitrary A_{405} units from ELISAs done in triplicate at least twice. When control cells were incubated in the absence of specific antibodies, the concentrations of C3b and C5b-9 were always <0.1 A_{405} units.

while a high level of binding of this final complement component was observed for the serum-sensitive strains (Table 6). Identical results were obtained by immunogold electron microscopy (Fig. 2 and 3). It is interesting to notice that strain C3 bound C3b farther away from the cell membrane than strain KT707 (serum sensitive) (Fig. 2). This was also true for the isogenic K^- mutants of these two strains. Similar immunogold electron microscopy results were found with other serum-resistant and -sensitive *K. pneumoniae* strains. No specific gold particles were found on control cells incubated in the absence of specific antibodies (data not shown).

DISCUSSION

The bactericidal effects of immune or nonimmune sera are mediated by activated components of the classical and alternative complement pathways (25). Activation of either pathway can lead to membrane damage, usually resulting in cell death. Some gram-negative bacteria, such as *Neisseria gonorrhoeae* (23), *Pseudomonas aeruginosa* (22), *Haemophilus ducreyi* (19), and *Aeromonas hydrophila* (14), activate only the classical complement pathway; however, other gram-negative bacteria, such as enterobacteria, activate both complement pathways (31). *Klebsiella pneumoniae* C3 (O1:K66) was previously shown to activate both complement pathways (4).

Bacterial resistance to complement-mediated killing may be due to either of two main factors: (i) a complete or nearly complete inability to activate complement or (ii) a failure of activated complement to exert its effect (26). We clearly demonstrated that *K. pneumoniae* strains of serotypes O1:K1, O1:K10, and O1:K16, which have only the K antigen exposed at the cell surface (29), resist complement-mediated killing by impeding complement activation. It is also clear that purified capsular polysaccharides (K antigen) from nine different serotypes (able or unable to mask the LPS) were unable to activate complement. This situation is similar to that observed with the K1 capsule of *Escherichia coli* and the capsule of group B meningococci (5, 21).

However, for *K. pneumoniae* strains of K serotypes (such as K2, K7, K19, K21, K22, and K66), which have the capsule and LPS exposed together on the cell surface (29) or

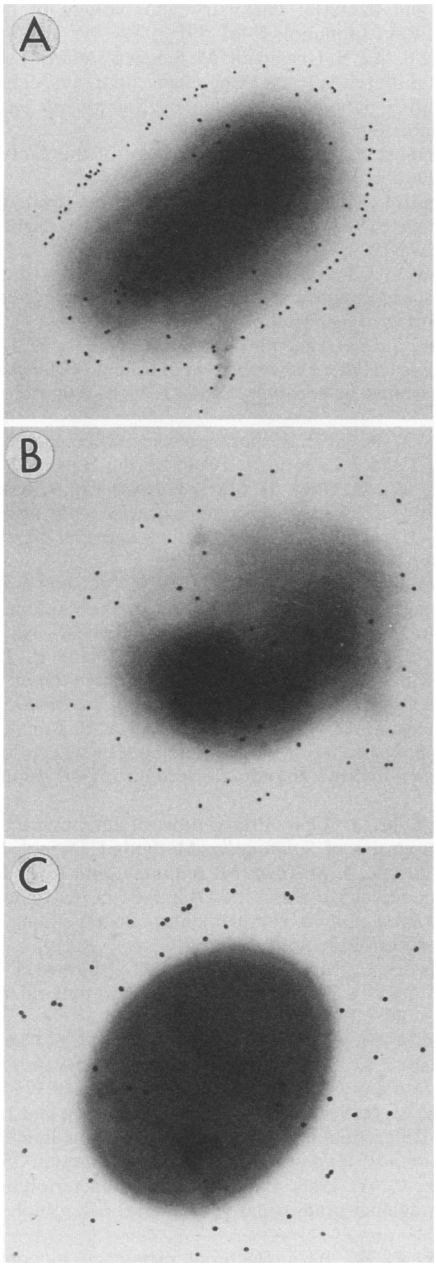

FIG. 2. Immunogold electron microscopy of *K. pneumoniae* strains preopsonized with NHS, incubated with anti-C3b, and labeled with protein A–20-nm gold spheres. (A) Strain KT707 (O^-:K66) (serum sensitive); (B) strain KT791 (O1:K^-) (serum resistant); (C) strain C3 (O1:K66) (serum resistant).

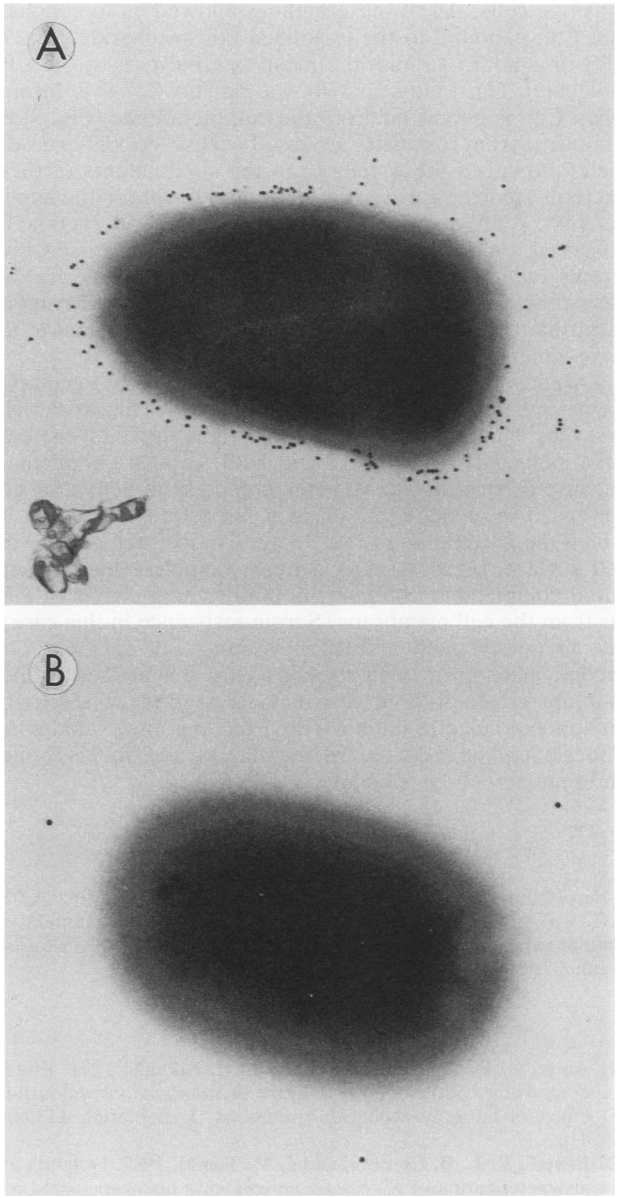

FIG. 3. Immunogold electron microscopy of *K. pneumoniae* strains preopsonized with NHS, incubated with anti-C5b-9, and labeled with protein A–20-nm gold spheres. (A) Strain KT707 (O^-:K66) (serum sensitive); (B) strain KT791 (O1:K^-) (serum resistant).

are unencapsulated mutants, the resistance to complement-mediated killing should be explained by the second reason (failure of activated complement to exert its effect). We clearly showed that these strains were able to activate complement by measuring the inhibition of complement-mediated hemolysis of sensitized sheep erythrocytes or directly measuring C1q or C3 complement component depletion, which occurs with their purified LPSs. LPSs from strains in which this molecule is masked by the K antigen are also able to activate complement.

It has been postulated that smooth strains of *Salmonella*, which are serum-resistant organisms, fix C3b to the longest O-polysaccharide side chains of the LPS (9, 10), preventing the formation of C5b-9 or the insertion of these side chains into the critical sites of the bacterial cell membranes, which causes membrane damage and cell death (9, 10).

Our study clearly shows that cells of strains with exposed smooth LPS at the cell surface (with or without K antigen) are able to bind C3b but are unable to form C5b-9. These cells (which have smooth LPS and are serum resistant) bind less C3b than cells of serum-sensitive strains (rough LPS devoid of O antigen). Also, immunogold electron microscopy reveals that C3b deposition on serum-resistant cells is farther away from the cell membrane than it is in serum-

sensitive cells. All of these findings allowed us to conclude that C3b is bound to the O-antigen polysaccharide units of LPS on smooth strains (the most external part of the LPS molecule). Our study also shows that no C5b-9 is formed when C3b is bound far from the cell membrane (Fig. 3) on smooth (serum-resistant) strains, because no C5b-9 is detected bound to these cells or in the supernatants of these bacteria incubated in serum (data not shown for the supernatants). A large amount of this compound (C5b-9) could be observed on the cell membrane of the serum-sensitive strains (which have rough LPS devoid of O antigen). We have thus clearly explained the defect of activated complement that renders the cells with smooth LPS resistant to the complement-mediated killing of *K. pneumoniae*.

Two mechanisms of *K. pneumoniae* resistance to complement-mediated killing have been defined by this work. The first mechanism, characteristic of strains in which the capsular polysaccharide masks the LPS, evades complement because these strains have a non-complement-activating cell surface. The second mechanism is characteristic of strains in which the capsule and the LPS are exposed together at the cell surface. These strains activate complement, but activated complement components bind to long chains of LPS far from the cell membrane. Serum resistance in this case is due to complement activation without cell damage. This second mechanism also explains why the unencapsulated mutants remained serum resistant and confirms, as shown by immunogold electron microscopy, the hypothesis that when C3b is bound far from the cell membrane, no C5b-9 is formed and thus no cell lysis occurs.

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