Relationships among Capsular Structure, Phagocytosis, and Mouse Virulence in *Klebsiella pneumoniae*

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Klebsiella pneumoniae strains of the K2 capsular serotype are usually highly virulent in mice, which is in contrast to the low virulence of most other serotypes. Here we used a genetic approach to examine the relative contribution of capsule type to the virulence of *K. pneumoniae* in mice. We used wild-type strains expressing capsular polysaccharide (CPS) serotypes K2 (strain KPA1) and K21a (strains KPB1 and KPC1), which were then used to construct capsule-switched derivatives. The close proximity of the *cps* gene cluster to selectable *his* markers made it possible to mobilize the *cps* genes by conjugation from one serotype (donor) to another (recipient) and to obtain recombinants in which interserotype switching had occurred by reciprocal recombination. Each capsule-switched derivative examined of the KPA and KPC strain backgrounds produced a CPS that was immunologically and structurally identical to that of the donor. Strain background was confirmed by demonstrating restriction fragment length polymorphism patterns identical to those of the respective recipients. The parent strains were then compared with capsule-switched recombinants for phenotypic properties associated with virulence. Clearance from the bloodstreams of mice was rapid in serotype K21a strains of either wild-type or recombinant origin, whereas K2 strains remained viable in the blood during the period examined. These differences appeared to be dependent upon the CPS type but independent of strain background. Binding to macrophages was higher in K21a strains than in those with the K2 capsule and was also independent of the strain background. Both blood clearance and macrophage-binding activities were completely inhibited by yeast mannan, suggesting that they were mediated via the macrophage mannose receptor. The K2 parent strain was highly virulent to mice (50% lethal dose [LD_{50}], 3×10^3), while the K21a parent strains demonstrated low virulence (LD_{50} , $>2 \times 10^8$). Interestingly, the virulence of recombinant KPC10(*cps*K2), originally of the KPC1(*cps*K21a) background, was intermediate (LD_{50} , 4×10^5). In contrast, both *cps*K21a recombinants of the originally virulent KPA1(*cps*K2) background became nearly avirulent (LD_{50} , $>2 \times 10^8$). Six additional serotypes (K12, K24, K32, K55, K62, and K67) were examined, and all showed a positive correlation between the ability of the *Klebsiella* serotype to interact with a human mannose receptor, as expressed by Cos I cell recombinants, and the LD_{50} of the serotype. These results suggest that expression of a capsule which is recognized by the mannose receptor markedly affects the interaction with macrophages and blood clearance. The virulence of the *cps*K2 recombinant of the KPC background may have been enhanced because it was expressing a heterologous capsule not recognized by the mannose receptor. Thus, this study shows that the capsule type plays an important role in the rate of blood clearance and phagocytosis but contributes only partially to the virulence of *K. pneumoniae* in mice.

Klebsiella pneumoniae causes suppurative infections, bacteremia, and septicemia and also accounts for a substantial percentage of nosocomial infections in neonates (27). Mortality rates reported for *K. pneumoniae* bacteremia range from 25 to 50%, indicating the relative ineffectiveness of current therapy for such infections. Research on the pathogenesis of experimental infections with *K. pneumoniae* has shown the capsular polysaccharide (CPS) to be a prime factor in virulence (26). The majority of clinically isolated *K. pneumoniae* strains have a well-defined capsule. The production of a hydrophilic CPS (K antigen) is characteristic of the genus *Klebsiella*, and the antiphagocytic property of CPS is believed to be an important

virulence mechanism. Almost all clinical isolates of *K. pneumoniae* are encapsulated, and at least 77 chemically and antigenically distinct CPS types have been recognized (18). In a large study of bacteremic *K. pneumoniae* isolates, and in a recent study of clinical isolates of *K. pneumoniae*, it has been shown that some capsular serotypes (e.g., K2) are isolated at significantly higher frequency than other capsular serotypes (5, 23). However, there is no apparent correlation between CPS type and any particular isolation site or disease state.

It has been shown that certain CPS types can actually promote the binding of *K. pneumoniae* to guinea pig alveolar macrophages in serum-free media and that this binding is followed by ingestion and killing of the bacteria (3). The molecular basis for this type of phagocytosis, termed lectinophagocytosis (17), involves a macrophage mannose receptor. This macrophage receptor recognizes sequences such as Man- α 2/3-Man and Rha- α 2/3-Rha found in the CPS of a number of

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capsular serotypes. The rapid clearance of certain *K. pneumoniae* serotypes in serum-poor environments, such as the respiratory tract, may be mediated in large part via direct recognition of the organisms by such mannose receptor-dependent phagocytosis. To better understand the role of capsule in virulence, we recently employed genetic techniques to exchange the *cps* gene clusters of a strain (KPA1) of the virulent K2 serotype with a strain (KPB1) of the low-virulence K21a serotype (16). In these studies, we found that the recombinant strains which express heterologous *cps* genes display the macrophage binding phenotype of the donor, thus confirming that nonopsonic recognition by macrophages is determined by the capsule type expressed.

In the present study, genetically manipulated strains expressing heterologous *cps* gene clusters and their parental strains were compared for interaction with macrophages, blood clearance, organ distribution, and virulence in mice. In addition, we employed other wild-type strains of *K. pneumoniae* to compare their mouse virulence with their ability to interact with macrophage mannose receptors, as expressed on the surface of cells transfected with cDNA encoding the human mannose receptor. The results suggest that the capsule type expressed by *K. pneumoniae* plays an important role in blood clearance and phagocytosis and contributes partially to the level of virulence.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. We employed the previously described *K. pneumoniae* parent strains KPA1(*cps*K2) and KPB1(*cps*K21a) (16). Another strain of serotype K21a, designated KPC1 and expressing the K21a1 CPS, was kindly provided by S. J. Cryz, Swiss Serum and Vaccine Institute, Bern, Switzerland. Other strains used were capsule-switched derivatives of KPA1 and KPC1 called KPA5(*cps*K21a), KPA6(*cps*K21a1), and KPC10(*cps*K2) (reference 15 or constructed in the present study). *K. pneumoniae* wild-type strains of serotypes K12, K24, K32, K55, K62, and K67 were kindly provided by Ida Ørskov, International *Escherichia* and *Klebsiella* Center, Statens Serum Institut, Copenhagen, Denmark, and are described elsewhere (3). L broth and M-9 media were solidified with 1% Bacto Agar and supplemented with amino acids or antibiotics as needed for growth of the bacterial strains and their auxotrophic, antibiotic-resistant, and capsule-switched derivatives, as described previously (16). For assays of biological activity, bacteria were grown overnight on agar plates at 37°C, harvested in phosphate-buffered saline (PBS; 0.1 M NaCl, 0.02 M PO₄ [pH 7.2]), and washed twice by centrifugation. Stock suspensions of the bacteria (10⁹ cells per ml) were stored at -70°C.

Construction of capsule-switched strains. We took advantage of the close proximity of selectable *his* auxotrophic markers to the capsular polysaccharide gene cluster (*cps*) to construct strains with a donor strain's heterologous *cps* cluster that was often coinherited following selection for the *his*⁺ marker. Donor strains used were spontaneous nalidixic acid-resistant (Nal^r) mutants and also carried the conjugative plasmid R68.45, which has generalized chromosome mobilization ability as described previously (16). In addition to the strains and auxotrophs described in a previous study (16), we constructed a His⁻ auxotroph (KPC5) of strain KPC1 for strain construction in this background. *K. pneumoniae* chromosomal DNA was mobilized from Nal^r donor strains to His⁻ recipients in plate matings as described previously (16). His⁺ Nal^r recombinant colonies were then selected for possible coinheritance of the adjacent *cps* genes of the donor. As a control, a recombinant strain (KPC7) was constructed which expressed the homologous K21a1 capsule type.

Manipulation of DNA. Procedures for small-scale isolations of *K. pneumoniae* genomic DNA were performed as described previously (8). Detection of restriction fragment length polymorphism in genomic DNA was performed as described previously (16, 20). Briefly, genomic DNA samples (2 to 3 mg) were completely digested with *Eco*RI, subjected to agarose (0.8%) gel electrophoresis, and blotted onto nitrocellulose as described previously (25). A probe was prepared by amplifying a segment of *Escherichia coli* DNA encoding rRNA by a PCR technique as described before (15). The amplified fragments were purified from the agarose gel and labeled by nick translation with [³²P]dCTP (11). Hybridization and autoradiography were performed as described previously (11).

Analysis of CPSs. Rabbit antisera to formalin-treated suspensions of *K. pneumoniae* cells were raised by intravenous (i.v.) injection as described previously (18) and used in a test for capsule serotype as described previously (16). CPSs were prepared from *Klebsiella* strains as described previously (6). For analysis of

polymers by nuclear magnetic resonance spectroscopy, samples were deuterium exchanged by freeze-drying solutions in D₂O and then dissolved in 99.99% D₂O (0.45 ml) containing a trace of acetone for use as an internal reference (d 2.23 for ¹H and 31.07 ppm for ¹³C). Spectra were recorded at 40, 80, and 95°C on a Bruker WH-400 or AM-400 spectrometer, equipped with an Aspect 3000 computer and array processor, by using standard Bruker software. The molecular weights of polysaccharides were approximated by sieve chromatography as described previously (6).

Macrophage adherence assay. Association of the bacteria with the phagocytes was determined as described previously (12). Briefly, bacteria (10¹⁰) in 9 ml of carbonate buffer (0.5 M; pH 9.5) containing 0.006% fluorescein isothiocyanate were stirred at room temperature for 20 h, washed three times (5 min at 8,000 × g), and suspended in 3.5 ml of Hanks' balanced salt solution plus 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HSS-HEPES) as described previously (12). Rat alveolar macrophages were isolated by lung lavage from Wistar rats (250 to 300 g [body weight]) as described previously (12) and suspended in HSS-HEPES. Macrophages (2 × 10⁵) in 90 ml of HSS-HEPES were mixed with 2 × 10⁶ bacteria in 10 ml of buffer, incubated in a shaking incubator with 5% CO₂ for 30 min at 37°C, and washed three times (10 min at 400 × g). The percentage of macrophages containing bound bacteria was determined by fluorescence light microscopy as described previously (3). The Mann-Whitney U test was used to estimate the significance of the differences observed.

Blood clearance, organ distribution, and survival of bacteria in blood. Blood clearance of bacteria in mice after i.v. injection and its inhibition by mannan (1 mg/ml) were determined as described previously (22). Organ distribution of bacteria was studied in mice injected i.v. with 2 × 10⁷ bacteria. Mice were killed 20 min after injection, and then the livers, spleens, kidneys, and lungs were excised, washed with saline, and homogenized in distilled water. Samples were diluted in PBS and used to determine CFU in aliquots spread on nutrient agar plates. The bactericidal effect of whole mouse blood was assayed as described previously (22). Briefly, fresh mouse blood (500 µl) supplemented with 20 U of heparin was mixed with 50 µl of bacterial suspension at a concentration of 10⁷ to 10⁸ bacteria per ml and then incubated at 37°C. At various times, samples of 10 ml were diluted in distilled water or PBS. The number of CFU was determined after incubating 0.1 ml from the dilutions on nutrient agar plates overnight at 37°C.

Mouse virulence and passive immunization tests. Each mouse virulence test was performed with six ICR white male mice (18 to 20 g). Each mouse was injected in the peritoneum with 0.5 ml of a bacterial dilution, and survivors were counted on day 7 (16). Fifty percent lethal dose (LD₅₀) values were determined as described previously (10). In some experiments, five mice were each passively immunized by injecting intraperitoneally 0.5 ml of undiluted anti-K2 or anti-K21a serum 24 h prior to challenging the mice with a lethal dose of bacteria.

Interaction with Cos I cells. Cos I cells were transfected with a cDNA clone encoding the human macrophage receptor as described previously (1, 7). Cos I cells (2 × 10⁵) in 90 µl of HSS-HEPES were mixed with fluorescein-labeled bacteria (2 × 10⁶) in 10 µl of buffer. The reaction mixture was treated as described above. The percentage of Cos I cells containing bound bacteria was determined by fluorescent light microscopy as described above. In each experiment, the results were compared with those of mock-transfected Cos cells. The Mann-Whitney U test was used to estimate the significance of the differences observed.

RESULTS

Genetic and phenotypic analysis of capsule-switched *K. pneumoniae* strains. We have shown that the close proximity of the *cps* gene cluster to selectable *his* markers in *K. pneumoniae* allows for the conjugal exchange of *cps-his* chromosomal DNA, thus permitting the construction of strains expressing new capsule types (e.g., type K2 or K21a). In previous studies (16), strain KPA1(*cps*K2) was converted to KPA5(*cps*K21a), and KPB1(*cps*K21a) was converted to KPB20(*cps*K2). In previous studies, it has been shown that the parental KPA1 and KPB1 strains exhibited identical properties in various phenotypic assays, and a restriction fragment length polymorphism analysis, using an *E. coli* rRNA probe, proved to be effective in demonstrating the strain background of capsule-switched recombinants (16). To confirm and extend these findings, we employed the same techniques here to construct new capsule-switched strains from another K21a wild-type strain, called KPC1, which produced a capsule that is distinguished here as K21a1 (Table 1).

In tests to establish the strain background, restriction fragment length polymorphism DNA fingerprint patterns of parent strain KPA1(*cps*K2) and recombinant KPA6(*cps*K21a1) were

TABLE 1. Properties and biological activities of *K. pneumoniae* strains and their capsule-switched derivatives

Property or activity	Parent strains			Capsule-switched recombinants ^a		
	KPA1	KPB1	KPC1	KPA5	KPA6	KPC10
Strain background	Wild type	Wild type	Wild type	KPA1	KPA1	KPC1
CPS by NMR ^b	K2	K21a	K21a1	K21a	K21a1	K2
Agglutination titer:						
Anti-K21a	1:<2	1:40	1:80	1:20	1:40	1:<2
Anti-K2	1:40	1:<2	1:<2	1:<2	1:10	1:40
CPS molecular weight	2.5×10^6	4×10^5	3×10^6	6.4×10^4	2.8×10^5	3.2×10^4
Blood clearance ^c	—	+	+	+	++	—
Binding to macrophages ^d						
In buffer alone	2 ± 0.5	13 ± 2	10 ± 1	8 ± 1	8 ± 2	3 ± 1
In buffer mannan	NT	4 ± 1	5 ± 2	5 ± 1	4 ± 0.5	NT
Mouse virulence ^e	3×10^3	$\geq 6 \times 10^8$	2.5×10^8	4×10^8	2.5×10^8	4×10^5

^a See text for source of strains.^b NMR, nuclear magnetic resonance.^c See Fig. 2 for rate of blood clearance. Symbols: —, +, and ++, negligible, intermediate, and high rates of blood clearance, respectively.^d Percentage of macrophage associated with bacteria by counting 100 macrophages. In representative experiments, we found that strains exhibiting 8 to 13% phagocytosis bound in the range of six to eight bacteria per macrophage, while strains exhibiting 2 to 3% phagocytosis bound in the range of one to four bacteria per macrophage. Compared with that of the parental strain KPB1, only the percent binding of KPA1 and KPC10 was significantly decreased ($P < 0.001$).^e Values represent LD₅₀s.

shown to be the same (Fig. 1). Likewise, the patterns for the parent strain KPC1(*cpsK21a1*) were the same as those for its His⁺ auxotroph KPC5, recombinant KPC10(*cpsK2*), and control recombinant KPC7(*cpsK21a1*), which expressed the homologous capsule (Fig. 1). Thus, recombinants KPA6 and KPC10 (Table 1) appear to contain the genomic backgrounds of their respective recipient strains, except for the *his-cap* region of the chromosome, which was replaced with donor DNA. As determined by nuclear magnetic resonance, the CPS structures of recombinants KPC10(*cpsK2*), KPA5(*cpsK21a*), and KPA6(*cpsK21a1*) were indistinguishable from those of their donor parent strains KPA1(*cpsK2*), KPB1(*cpsK21a*), and KPC1(*cpsK21a1*), respectively (data not shown). However, the CPS molecular weights were generally reduced in the recombinant strains when compared with those of the respective donor strains, especially for KPC10(*cpsK2*) (Table 1). CPS-specific antisera (16) were also employed to verify capsule

type of the recombinant strains. Agglutinating titers ranging between 1:80 and 1:40 were obtained with anti-K21a against the parent K21a strains KPB1 and KPC1 (Table 1). The agglutination titers of the same serum against recombinant strains KPA6(*cpsK21a1*) prepared in the present study and KPA5(*cpsK21a*) from a previous study (16) were 1:40 and 1:20, respectively. None of these strains reacted with anti-K2 sera, except strain KPA6(*cpsK21a1*), which gave a low titer of 1:10. Titers of 1:40 were obtained with anti-K2 sera against the KPA1(*cpsK2*) parent and the KPC10(*cpsK2*) recombinant strain; these strains did not react with anti-K21a serum (Table 1).

Similar to our previous studies of capsule-switched strains to generate KPA5, the results of these genetic, chemical, and immunological analyses suggest that the recombinant KPA6(*cpsK21a1*) is of the KPA1 background that has acquired the donor *cpsK21a1* gene cluster. Likewise, strain KPC10(*cpsK2*) is a recombinant of the KPC1 background that has acquired the donor *cpsK2* gene cluster from KPA1. However, the molecular weight of K2 CPS of this recombinant strain is about 1/100 lower than that of the KPA1 parent strain. The altered molecular weight of CPS in strain KPC10 may be due to regulatory genes that were not co-inherited from the donor strain and required for determining K2 capsule size.

Binding of *K. pneumoniae* and recombinant strains to alveolar macrophages. Our previous studies showed that *K. pneumoniae* strains expressing a K21a capsule, which contains Man- α 2/3-Man, are recognized by a macrophage mannose receptor which promotes lectinophagocytosis (3). Binding to rat alveolar macrophages by K21a strains KPB1 and KPC1 was approximately fivefold higher than that by K2 strain KPA1 (Table 1). Percent binding of recombinant strains KPA5(*cpsK21a*) and KPA6(*cpsK21a1*) was about threefold higher than that of K2 parent strain KPA1. The alveolar macrophages bound approximately threefold-fewer bacteria of recombinant KPC10(*cpsK2*) than they did of the parent strain KPC1(*cpsK21a1*). Mannan (i.e., rich in Man- α 2/3-Man sequences) inhibited the binding of all strains expressing K21a or K21a1 CPS (Table 1), suggesting that the binding to the macrophage is mediated via the mannose receptor. Thus, high-level binding (8 to 13%) to macrophages correlated with the K21a capsule type, whereas low-level binding (2 to 3%) correlated with the K2 type.

FIG. 1. Restriction fragment length polymorphism analysis of *K. pneumoniae* KPA1, KPC1, and their derivatives. An autoradiograph of hybridization of a ³²P-labeled *E. coli* rRNA probe to a blot of *Eco*RI restriction fragments of *Klebsiella* genomic DNA is shown. Lanes: 1, KPA1; 2, KPA4; 3, KPA11; 4, KPA6; 5, KPC1; 6, KPC5; 7, KPC7; 8, KPC10. Note that the hybridization patterns of lanes 1 to 4 are the same in all KPA strains. The patterns in lanes 5 to 8 are the same in all KPC strains and distinguishable from the KPA strain patterns. A detailed description of the strains not described in the present study was given elsewhere (16).

FIG. 2. Blood clearance of the parent strains of *K. pneumoniae* and their capsule-switched derivatives in mice and their inhibition by mannan. Mice were injected in the vein with bacteria, and blood samples were withdrawn at the times indicated to determine the CFU per milliliter of blood as described in Materials and Methods. The shaded area represents the range of blood clearance of all strains injected with mannan (1 mg/ml). The types of capsule expressed by the parent strains (KPC1, KPA1, and KPB1) and their capsule-switched derivatives (KPC10, KPA5, and KPA6) are shown (see Table 1 for description of strains).

Blood clearance of *K. pneumoniae* and its recombinants. It has long been accepted that in nonimmune animals, the liver and spleen clear the blood of invading bacteria (4, 13). Previous studies have shown that a mannose receptor is responsible for the blood clearance of *E. coli* (22). To examine the blood clearance of *K. pneumoniae* strains, bacteria were injected i.v. into mice, and blood samples were taken at 5-min intervals for quantitative bacteriology. The rates of blood clearance of K21a strains KPB1, KPC1, KPA6, and KPA5 were rapid compared with those of K2 strains KPA1 and KPC10 (Fig. 2). The enhanced blood clearance of the K21a CPS-expressing strains was completely inhibited by mannan (Fig. 2, shaded area), suggesting that it is mediated by the macrophage mannose receptor.

Examination of the organ distribution of the parent KPC1(*cps*K21a1) strain revealed that most of the bacteria were recovered in the liver and spleen more than 20 min after i.v. injection (Fig. 3). There was no detectable killing of the bacteria when they were incubated with fresh whole mouse blood *in vitro*; survival, relative to time zero counts, was in the range of 95 to 109% (data not shown). This suggests that blood clearance of the bacteria was not due to bactericidal activity of either blood phagocytes or serum components but rather to the sequestration of the bacteria by the liver and spleen.

Mouse virulence of *K. pneumoniae* and recombinant strains. We established previously that wild-type strain KPA1(*cps*K2) is highly virulent in mice, whereas wild-type strain KPB1(*cps*K21a) is not (16). Here, we determined the LD₅₀s of these parent strains and their capsule-switched derivatives. The LD₅₀ values for the recombinants KPA5(*cps*K21a) and KPA6(*cps*K21a1) were high, i.e., 4×10^8 and 2.5×10^8 , respectively, whereas that of the highly virulent KPA1(*cps*K2) parent strain was 5 orders of magnitude lower (Table 1). The LD₅₀ of recombinant KPC10(*cps*K2) was intermediate at 4×10^5 , while that of the less-virulent parent strain KPC1(*cps*K21a1) was 3 orders of magnitude higher (Table 1). The LD₅₀ values of the parental recipient K2 His⁻ and K21a His⁻ auxotrophs were 4×10^3 and

FIG. 3. Organ distribution of the parent KPC1 strain in mice. Mice were injected i.v. with 2×10^7 bacteria and sacrificed 20 min later. The various organs were excised and homogenized to determine the number of CFU per organ.

5×10^8 , respectively, suggesting that the mutagenesis of the *his* locus did not affect the virulence of *Klebsiella* strains.

To show specificity, groups of five mice were each passively immunized with anti-K2 or anti-K21a rabbit serum and then challenged intraperitoneally after 24 h with a lethal dose of the parent KPA1 strain (i.e., 5×10^4 bacteria per mouse) or the recombinant KPC10(*cps*K2) strain (5×10^5 bacteria per mouse). All mice that received anti-K21a serum or saline died, whereas none of the mice which received anti-K2 serum died, suggesting a serotype-specific protection.

Correlation between mannose receptor binding and virulence. The relationship between capsule type, interaction with the mannose receptor, and mouse virulence was examined further by employing an additional six wild-type strains expressing different capsule serotypes. For these studies, isogenic Cos I cells that contained a plasmid expressing the human mannose receptor or only the relative vector were used. In addition, we employed six different serotypes, three of which (K12, K32, and K55) were found in a previous study to bind poorly to macrophages compared with the other three (K24, K62, and K67), and mannan inhibited the binding of the latter (3). The LD₅₀s of strains of serotypes K24, K62, and K67, which bound to the mannose receptor expressed by Cos I cells, were considerably higher (i.e., 3 to 4 orders of magnitude) than those of serotypes K12, K32, and K55, which showed poor binding to the receptor (Table 2). The results were clear even though there was a relatively low percentage of Cos I cells expressing receptors for *K. pneumoniae*. This may have been due to either a low transfection efficiency or a low percentage of cells expressing the appropriate threshold number of receptors per cell required to bind the bacteria, and these possibilities are currently under investigation. However, the results taken together support a positive correlation between receptor-binding activity and mouse virulence.

DISCUSSION

Lectinophagocytosis of *K. pneumoniae* was found to be mediated by CPSs that contain sequences recognized by the macrophage mannose receptor (3). To better understand the relative contribution to virulence of capsules that may be

TABLE 2. Relationship between interaction with mannose receptor expressed by Cos I cells and mouse virulence

Serotype	Binding to Cos I (% cells) ^a		Mouse virulence (LD ₅₀)
	Mock transfected	Transfected	
K12	4 ± 0.32	5 ± 0.25	0.8 × 10 ⁴
K32	3 ± 0.57	2 ± 0.45	4.0 × 10 ⁴
K55	5 ± 0.22	4 ± 0.32	2.0 × 10 ⁴
K24	5 ± 0.8	11 ± 0.35 ^b	3.0 × 10 ⁸
K62	4 ± 0.47	8 ± 0.61 ^b	0.8 × 10 ⁸
K67	6 ± 0.45	9 ± 0.35 ^b	2.0 × 10 ⁸

^a Percentage of cells associated with bacteria.^b These values are significantly higher compared with those obtained with nontransfected cells.

recognized or not recognized by this macrophage receptor, we developed a genetic technique to construct strains of *K. pneumoniae* that possessed a heterologous capsule gene cluster. This technique enabled us to construct strains that maintained the overall genetic background of the parent strain but expressed a heterologous capsule type (16). In the present study, we employed two strains that express the K21a CPS (KPB1 and KPC1), a capsule that contains the Man- α 2-Man sequence which is recognized by the macrophage lectin. These were compared with strain KPA1, which expresses K2 CPS, a polymer that lacks such sequences. The parent KPA1(*cps*K2) strain is highly virulent in mice (14), while parent strains KPB1 and KPC1 expressing K21a CPS have low virulence in mice (16). The differences in levels of mouse virulence and interactions with macrophages enabled us to evaluate the importance of lectinophagocytosis mediated by mannose receptor in the overall virulence of *Klebsiella* spp. in mice.

Blood clearance rates of the *K. pneumoniae* strains and their capsule-switched derivatives appeared to reflect the magnitude of binding to alveolar macrophages. Parent and recombinant strains expressing K21a CPS (recognized by the macrophage lectins) were cleared from the blood at a considerably higher rate than strains expressing K2 capsule. This proposed mechanism is supported by the demonstration that blood clearance was inhibited by mannan and mainly due to sequestration of the bacteria into the liver and spleen, which contain macrophages (Kupffer cells) that express the mannose receptor (2). Other studies have shown the involvement of the mannose receptor in the blood clearance of bacteria (21, 22) and yeast cells (24).

Mouse virulence probably reflects the ability of the bacteria to survive, proliferate, and secrete tissue-damaging constituents for a relatively long period of time. The determination of the LD₅₀s of the parent strains and their capsule-switched derivatives revealed that the mouse virulence of the K2 strain is multifactorial, and one factor appears to be related to the capsule structure. We hypothesize that the parent KPA1 strain was not recognized by the macrophage lectin, and therefore its clearance by the macrophages was reduced, thus allowing the proliferation of the bacteria and expression of virulence factors. In contrast, the capsule-switched derivatives expressing K21a (i.e., KPA5 and KPA6) were cleared rapidly by the macrophages. Their inability to proliferate and express other virulence factors encoded by their genomic background resulted in high LD₅₀ values and reduced virulence. The K21a and K21a1 parent strains (KPB1 and KPC1) were not virulent, probably because they were cleared rapidly by the macrophages and failed to express other virulence factors required for pathogenesis in the mouse. In contrast, the capsule-

switched derivative KPC10(*cps*K2) showed reduced clearance, thus allowing proliferation of the bacteria. The reduced molecular weight of the K2 CPS of this recombinant strain did not affect the magnitude of binding to macrophages nor its blood clearance as compared with that of the parental KPA1 strain. We believe, therefore, that the diminished virulence is due to the genetic background of the KPC10 recombinant strain, which does not express other virulence factors of the K2 strain background, and, therefore, its mouse virulence in terms of LD₅₀ was intermediate. Thus, the capsule appears to play a more important role in virulence if it is not recognized by the macrophage lectin. This enables the bacteria to escape lectinophagocytosis, proliferate, and express other virulence factors. Conversely, it appears that strains which express genes for other virulence factors, but express a CPS recognized by the macrophage lectin, demonstrate low virulence because they cannot escape lectinophagocytosis.

The above hypothesis is strengthened by the results showing a positive correlation between the ability of *Klebsiella* serotypes to interact with the macrophage lectin, as expressed by Cos I cells, and the LD₅₀ of these serotypes in the mouse. The virulence of strains expressing CPS structures not recognized by the macrophage lectin (e.g., serotypes K12, K32, and K55) was intermediate (compared with that of the K2 strain KPA1), whereas that of strains expressing CPS structures recognized by the macrophage lectin (e.g., serotypes K3, K24, K62, and K67) was low. The data on binding to macrophages (Table 1) and to mannose receptor (Table 2) taken together suggest that *Klebsiella* serotypes recognized by the macrophage lectin are expected to exhibit low virulence while those not recognized by the lectin may exhibit either low or high virulence depending on the genetic background of the strain. Recently, mouse virulence assays of genetic switching of capsular serotype in *Streptococcus pneumoniae* also confirmed the partial contribution to virulence of the type of capsule expressed by the capsule-switched derivatives of this species (9). However, their strains were not examined for the ability to interact with macrophages expressing the mannose receptor.

Overall, our study highlights the role of capsule structure in virulence, and it provides support for the hypothesis that the degree of virulence is enhanced when strains express CPS structures that are not recognized by the macrophage lectin. Compelling evidence, both from this study and our previous work (16), comes from our observations that virulence is reduced considerably when an otherwise virulent strain (e.g., expressing *cps*K2) is genetically altered to express a capsule recognized by the macrophage lectin (e.g., expressing *cps*K21a). Future studies will examine the relationships of capsule type and other virulence factors.

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REFERENCES

1. Aruffo, A., and B. Seed. 1987. Molecular cloning of a CD28 cDNA by a high efficiency COS cell expression system. *Proc. Natl. Acad. Sci. USA* **84**:8573-8576.
2. Ashwell, G., and J. Harford. 1982. Carbohydrate-specific receptors of the liver. *Annu. Rev. Biochem.* **51**:531-554.
3. Athamna, A., I. Ofek, Y. Keisari, S. Markowitz, G. G. S. Dutton, and N. Sharon. 1991. Lectinophagocytosis of encapsulated *Klebsiella pneumoniae* mediated by macrophage surface lectins of guinea pig alveolar macrophages and human monocyte-derived macrophages. *Infect. Immun.* **59**:1673-1682.
4. Benacerraf, B., M. M. Sebestyen, and S. Schlossman. 1959. A quantitative

- study of the kinetics of blood clearance of p32 labelled *Escherichia coli* and *Staphylococci* by the reticuloendothelial system. *J. Exp. Med.* **110**:24–48.
5. Cryz, S. J., Jr., P. M. Mortimer, V. Mansfield, and R. Germanier. 1986. Seroepidemiology of *Klebsiella* bacteremic isolates and implications for vaccine development. *J. Clin. Microbiol.* **23**:687–690.
 6. Dutton, G. G. S., S. K. Ng, L. A. S. Parolis, and H. L. Parolis. 1989. A re-investigation of the structure of the capsular polysaccharide of *Klebsiella* K10. *Carbohydr. Res.* 147–155.
 7. Ezekowitz, R. A. B., K. Sastry, P. Baily, and A. Warner. 1990. Molecular characterisation of the human macrophage mannose receptor: demonstration of multiple carbohydrate recognition-like domains and phagocytosis of yeast in Cos-1 cells. *J. Exp. Med.* **172**:1785–1794.
 8. Goldberg, J. B., and D. E. Ohman. 1984. Cloning and expression in *Pseudomonas aeruginosa* of a gene involved in the production of alginate. *J. Bacteriol.* **158**:1115–1121.
 9. Kelly, T., J. P. Dillard, and J. Yother. 1994. Effect of genetic switching of capsular type on virulence of *Streptococcus pneumoniae*. *Infect. Immun.* **62**:1813–1819.
 10. Kuehne, R. W. 1983. Rapid determination of log₁₀ 50% lethal doses or 50% infective doses. *J. Clin. Microbiol.* **17**:702–703.
 11. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 12. Manz-Keike, H., H. Plattner, and J. Schlepper-Schafer. 1992. Lung surfactant protein A (SP-A) enhances serum-independent phagocytosis of bacteria by alveolar macrophages. *Eur. J. Cell Biol.* **57**:95–100.
 13. Mims, C. A. 1976. The pathogenesis of infectious disease, p. 80–84. Academic Press Ltd., London.
 14. Mizuta, K., M. Ohta, M. Mori, T. Hasegawa, I. Nakashima, and N. Kato. 1983. Virulence for mice of *Klebsiella* strains belonging to the O1 group: relationship to their capsular (K) types. *Infect. Immun.* **40**:56–61.
 15. Mullis, K. B., and F. A. Faloon. 1987. Specific synthesis of DNA in vitro via a polymerase catalysed chain reaction. *Methods Enzymol.* **155**:335–350.
 16. Ofek, I., K. Kabha, A. Athamna, G. Frankel, D. J. Wozniak, D. L. Hasty, and D. E. Ohman. 1993. Genetic exchange of determinants for capsular polysaccharide biosynthesis between *Klebsiella pneumoniae* strains expressing serotypes K2 and K21a. *Infect. Immun.* **61**:4208–4216.
 17. Ofek, I., and N. Sharon. 1988. Lectinophagocytosis: a molecular mechanism of recognition between cell surface sugars and lectins in the phagocytosis of bacteria. *Infect. Immun.* **56**:539–547.
 18. Ørskov, F., and I. Ørskov. 1978. Serotyping of Enterobacteriaceae with special emphasis on K antigen determination, p. 37–38. In J. R. Norris and T. Bergen (ed.), *Methods in Microbiology*. Academic Press Ltd., London.
 19. Ørskov, I., and F. Ørskov. 1984. Serotyping of *Klebsiella pneumoniae*. *Methods Microbiol.* **14**:143–164.
 20. Pennington, T. H., C. Harker, and F. Thomson-Carter. 1991. Identification of coagulase-negative staphylococci by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and rRNA restriction patterns. *J. Clin. Microbiol.* **29**:390–392.
 21. Perry, A., Y. Keisari, and I. Ofek. 1985. Liver cell and macrophage surface lectins as determinants of recognition in blood clearance and cellular attachment of bacteria. *FEMS Microbiol. Lett.* **27**:345–350.
 22. Perry, A., and I. Ofek. 1984. Inhibition of blood clearance and hepatic tissue binding of *Escherichia coli* by liver lectin-specific sugars and glycoproteins. *Infect. Immun.* **40**:257–262.
 23. Podschun, R. 1990. Phenotypic properties of *Klebsiella pneumoniae* and *K. oxytoca* isolated from different sources. *Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A.* **189**:527–535.
 24. Sawyer, R. T., M. N. Horst, and A. L. Richardson. 1990. Altered hepatic clearance and killing of *Candida albicans* in the isolated perfused mouse liver model. *Infect. Immun.* **58**:2869–2874.
 25. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503–517.
 26. Williams, P., and J. M. Tomas. 1990. The pathogenicity of *Klebsiella pneumoniae*. *Rev. Med. Microbiol.* **1**:196–202.
 27. Youmans, G. P. 1975. *Haemophilus influenzae* and *Klebsiella pneumoniae*, p. 300–314. In G. P. Youmans, P. Y. Paterson, and H. M. Sommers (ed.), *In The biological and clinical basis of infectious diseases*. The W. B. Saunders Co., Philadelphia.