Klebsiella capsular type K7 in relation to toxicity, susceptibility to phagocytosis and resistance to serum

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Summary. *Klebsiella* strains possessing capsule type K7 are found predominantly in respiratory secretions. To investigate the importance of this K antigen in virulence, 13 K7 strains were compared with K2 capsulate isolates which are generally regarded as highly virulent. The toxicity of the strains was determined in a mouse peritonitis model. Generally, K7 isolates were significantly less toxic for mice than K2 strains. In the absence of serum, neither capsule type showed much stimulation of leucocytes, measured as the chemiluminescence (CL) response of human polymorphonuclear leucocytes (PMNL). However, in the presence of normal human serum, CL values with K7 strains increased considerably, whereas the CL response to K2 isolates was unaffected. Correspondingly, intracellular killing by PMNL was observed with K7 strains only, whereas K2 isolates proved to be relatively resistant to phagocytic destruction. No correlation was found between capsule type K7 and serum resistance. These data suggest that, in contrast to K2, capsule type K7 may not be a critical factor in the virulence of K7-capsulate *Klebsiella* strains nor does it seem to act as an antiphagocytic barrier.

Introduction

Nosocomial infections due to *Klebsiella* spp. are an important cause of morbidity and mortality.^{1,2} Most clinical isolates of *Klebsiella* possess a well-developed polysaccharide capsule, which appears to be a critical factor in the virulence of these bacteria.^{3–6} The significance of capsules in pathogenicity has been explained by an interference of the capsular K antigen with phagocytosis by polymorphonuclear leucocytes (PMNL) and with killing of the bacteria by serum, as shown for *Klebsiella* spp. and *Escherichia coli*.^{4,7–11}

Klebsiella spp. are classified serologically into 77 different capsular types.¹² Differences in virulence have been observed between particular serotypes: Kauffmann¹³ found that most *Klebsiella* strains belonging to types K1 and K2 were virulent for mice in a peritonitis model, and that strains belonging to other K types were not virulent. In an intraperitoneal model in mice it could be demonstrated that type K2 was more lethal than K1 in strains with O-antigen type 1, whereas serotypes other than K1 and K2 showed little or no virulence.¹⁴ In experimentally induced skin lesions in mice, K1, K2, K4 and K5 strains proved to be more virulent than those of K6 and serotypes higher than K6.¹⁵ Correspondingly, strains expressing K1, K2, K4 or K5 stimulated human neutrophils much less than other capsular types investigated.⁹

Previously, we reported that capsule type K7 was

predominant in *Klebsiella* strains isolated from the respiratory tract of diseased individuals.¹⁶ Furthermore, this serotype was found more often in human isolates than in strains from the environment.¹⁷ However, little is known about the role of the type K7 capsule in host-parasite interactions. The present study was to investigate the behaviour of 13 K7 clinical isolates. The toxicity of the strains for mice, their ability to stimulate the chemiluminescence (CL) response of human PMNL, and their susceptibility to killing by PMNL and to the bactericidal effect of human serum were examined. Because serotype K2 is well-characterised in these respects, K2 clinical isolates were included for comparison with the K7 strains.

Materials and methods

Bacterial strains and growth conditions

Human clinical *Klebsiella* isolates of capsular types K7 (13 strains) and K2 (four strains) were obtained from diseased individuals. Nine of the K7 strains were isolated from the respiratory tract, two were wound isolates, and two were from the genito-urinary tract. Of the K2 strains, one isolate each was from the respiratory tract, a wound infection, significant bacteriuria, and the genito-urinary tract. Strains were biotyped and serotyped as described previously,¹⁶ and stored in brain heart infusion (BHI) broth containing

glycerol 30% v/v at -80°C until use. Bacteria were grown to log-phase in BHI broth at 37°C , harvested by centrifugation, washed twice in phosphate-buffered saline (PBS, pH 7.2), and adjusted to 1×10^9 bacteria/ml.

Serum

Human blood was obtained from healthy individuals. After clotting at room temperature, serum was separated by centrifugation. Serum from c. 100 donors was pooled and stored in small volumes at -80°C until use.

Toxicity assay

The toxicity of the isolates for mice was determined by the method of Hughes *et al.*¹⁸ Briefly, 0.2 ml of bacterial suspensions (2×10^8 bacteria) were injected intraperitoneally (i.p.) into each of 10 NMRI mice (female, c. 16 g, Savo-Ivanovas, Gesellschaft für medizinische Versuchstierzucht, Kislegg, Germany), and the number of mice dead after 24 h was recorded. Lethality was expressed as percentage of mice killed.

Preparation of PMNL

Human PMNL were obtained from heparinised blood of normal donors. Leucocytes were separated by a Ficoll gradient (Pharmacia), followed by dextran sedimentation as described previously.¹⁹ Residual erythrocytes were removed by hypotonic lysis. PMNL were washed twice in Hanks's Balanced Salts solution (HBSS) and adjusted to 1×10^7 cells/ml.

Chemiluminescence (CL) response

Luminol-dependent CL was assayed at 37°C in a luminometer (Biolumat LB 9505, Berthold, Wildbad, Germany), as described by Duncker and Ullmann.¹⁹ Ten μl of PMNL suspension (1×10^7 /ml), 10 μl of luminol (2 mg/ml), and 500 μl of HBSS with or without normal human serum (NHS, final concentration 10% v/v) were mixed in Lumac® polystyrene tubes (Bio-Vials, USA), and the background CL was measured over a 5-min period. CL was induced by adding 10 μl of a bacterial suspension (1×10^9 /ml) or zymosan (50 mg/ml) pre-opsonised with NHS. The bacteria:leucocyte ratio was c. 200:1. The CL response was measured every 20 s over a period of 60 min. After curve-area integration, the results were expressed as percentage of CL obtained with zymosan. All tests were run in duplicate, and each strain was tested at least three times, with PMNL from different donors.

Serum bactericidal assay

The susceptibility of bacteria to human serum was determined by the method of Hughes *et al.*²⁰ Bacteria

were diluted to 2×10^6 cells/ml in physiological saline. Bacterial suspensions (25 μl) and NHS (75 μl) were dispensed into microtitration trays, mixed, and incubated at 37°C . Viability was determined immediately and after incubation for 1, 2 and 3 h. After mixing, samples were taken and serial dilutions were plated on BHI agar for colony counts. Responses were graded from 1 to 6 as follows (each grade is shown by an example strain in fig. 3a): grade 1, viable counts (VC) after 1 and 2 h were $<10\%$ of the inoculum, after 3 h $<0.1\%$ (strain 92, serotype K2); grade 2, VC after 1 h 10–100%, after 3 h $<10\%$ (strain 54, K7); grade 3, VC after 1 h $>100\%$, after 2 and 3 h $<100\%$ (strain 48, K7); grade 4, VC after 1 and 2 h $>100\%$, after 3 h $<100\%$ (strain 66, K2); grade 5, VC after 1, 2 and 3 h $>100\%$, but VC fell at some time during the 3-h period (strain 23, K2); grade 6, VC after 1, 2 and 3 h $>100\%$ of the inoculum and rising throughout the 3-h period (strain 57, K7). Each strain was tested three times.

Killing by PMNL

Killing of bacterial strains by PMNL was determined by mixing 0.1 ml of bacterial suspension (4×10^7 /ml), 0.4 ml of HBSS, 0.4 ml of PMNL (1×10^7 cells/ml), and 0.1 ml of serum (final concentration 10% v/v) in siliconised glass tubes. HBSS was substituted for PMNL in the controls. Vials were incubated in a shaking water-bath at 37°C . Samples (0.1 ml) were taken after 0, 30 and 60 min. After adding 9.9 ml of ice-cold distilled water to lyse PMNL, serial dilutions were plated on BHI agar for viable counts. Each test was run in duplicate, and each strain was tested three times.

Statistical analysis

The significance of differences between results for the two K types was evaluated by the U test of Mann and Whitney.

Results

Toxicity

The toxicity of bacterial strains for mice was determined by a mouse peritonitis test. Results are given in fig. 1. Toxicity of K7 isolates varied over a great range, showing lethality rates of 0–100%. Of the 13 K7 strains examined, four isolates killed $>50\%$ of mice within 24 h. Capsule type 2 strains, which are considered to be highly virulent, were assayed for comparison and, without exception, the four isolates examined killed $\geq 80\%$ of the mice. Toxicity of K7 and K2 strains differed significantly (<0.05).

Chemiluminescence response

The CL response of PMNL after stimulation by bacterial strains was determined in the presence and

Fig. 1. Toxicity of K7 and K2 isolates for mice. Lethality is expressed as the percentage of mice dead 24 h after i.p. injection of 2×10^8 bacteria.

in the absence of NHS. In the absence of NHS, K7 isolates induced only low CL responses (4–40% of CL values obtained with zymosan). NHS significantly enhanced the stimulation of PMNL by K7 strains ($p < 0.001$) and CL values of up to 100% of the corresponding zymosan response were obtained (fig. 2). Unopsonised K2 isolates showed low stimulation of PMNL, the CL values being similar to those with unopsonised K7 strains, but NHS did not increase the CL responses significantly ($p > 0.1$). Responses of PMNL with K7 isolates in the presence of NHS differed significantly from those with K2 strains ($p < 0.01$).

Serum resistance

Bacterial survival in undiluted NHS was determined and graded from 1 to 6 (fig. 3a). Results are shown in fig. 3b. The majority of K7 strains (eight isolates) proved to be serum resistant (grade 5 or 6). Two strains only were found to be highly sensitive (grade 2). Similarly, three of the four K2 isolates investigated showed moderate to high serum resistance.

Killing by PMNL

Killing of bacteria by PMNL was assayed in the presence of NHS 10% v/v. To avoid serum bactericidal effects, only those strains were tested that had proved to be serum resistant (eight K7 and two K2 strains).

Fig. 2. Chemiluminescence (CL) of human PMNL after stimulation with *Klebsiella* isolates in the presence and in the absence of NHS. Values are expressed as the percentage of CL obtained with opsonised zymosan.

Survival of bacteria was determined after incubation for 30 and 60 min. No loss of viability was observed in controls consisting of NHS 10% v/v without PMNL. In all K7 strains with PMNL, 50–90% of the bacteria were killed within 60 min (fig. 4). For six of the eight isolates, cell counts declined to <50% after 30 min, and to <25% after 60 min. In comparison, both K2 strains tested seemed to be resistant to the action of PMNL. After a brief reduction, cell counts increased to c. 200% after 60 min (fig. 4).

Discussion

Little is known about the significance of the *Klebsiella* type K7 capsular polysaccharide in host-parasite interactions, whereas the importance of type K2 capsule as a determinant of virulence is well documented. As expected, all K2 strains investigated were highly toxic for mice, killing >80% of the animals within 24 h. In contrast, K7 isolates were generally significantly less toxic. However, considerable differences in lethality were observed between the K7 strains, suggesting that the K7 capsule may not be the critical factor in the toxicity of *Klebsiella* strains possessing this K antigen.

Stimulation of PMNL by bacterial isolates was investigated in the presence and in the absence of fresh human non-immune serum. This serum did not contain specific antibodies to K2 or K7 as determined

Fig. 3. Sensitivity of *Klebsiella* isolates to normal human serum: (a) grades of response as described in *Materials and methods*; (b) responses of the K7 (■) and K2 (□) strains.

by the capsular swelling reaction and counter immunoelectrophoresis (data not shown). In the absence of opsonins, both K7 and K2 strains produced only low-level CL responses. However, in the presence of normal serum the CL responses induced by K7 isolates increased markedly, whereas no enhancement was observed with K2 strains. For effective phagocytosis of *Klebsiella* serotype K2 strains, specific antibody is necessary, as has been shown by others.^{5,21,22} In contrast, strains possessing capsular polysaccharide K7 may be phagocytosed in the absence of specific antibodies.

Uptake of bacteria by phagocytes does not necessarily lead to intracellular killing. Therefore, phagocytic killing was evaluated by a PMNL bactericidal assay. K2 isolates showed considerable resistance to the action of human PMNL, whereas with all K7 strains, 50–93% of the bacteria were killed within 1 h. These results are consistent with the CL data, indicating that the K7 antigen is not antiphagocytic.

K antigens are considered to be involved in the serum resistance of *Klebsiella* strains.²¹ However, it is unclear whether there is an association between particular serotypes and serum resistance. Bengt²³ found that K21 strains were significantly more often serum resistant than other strains he examined. We did not observe any relationship between capsule type and killing by human serum; similar proportions of serum-sensitive and serum-resistant strains were found in both serotypes. However, serum resistance may depend on the amount of capsular material produced and may require a threshold level of capsule expression.^{24,25} The differences in serum resistance observed in this study may be due to different levels of capsule production. On the other hand, lipopolysaccharides have been reported to confer resistance to serum killing in *Klebsiella* strains,^{26,27} and serum-resistance properties may depend on the composition of O antigens. The exact nature of resistance of *Klebsiella* strains to killing by serum is unknown and probably both O and K antigens are responsible, as has been suggested by Williams *et al.*^{21,22}

Generally, K antigens of *Klebsiella* are considered to be virulence factors *per se*. However, Simoons-Smit *et al.*¹⁵ demonstrated that K1, K2, K4 and K5 capsule strains are significantly more virulent than K3 and K6 strains. Correspondingly, these strains were phagocytosed to a much less extent than K3 and

Fig. 4. Killing of K7 and K2 isolates by human PMNL. Values are expressed on the percentage of the inoculum surviving after 60 min.

K6.⁹ Apparently, particular capsule types contribute differently to virulence properties, dividing *Klebsiella* strains into high and low virulence groups. The results of the present study suggest that, in contrast to K2,

capsule type K7 does not seem to determine the virulence of K7-capsulate strains.

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