THE PATHOGENICITY OF *KLEBSIELLA PNEUMONIAE* FOR MICE: THE RELATIONSHIP TO THE QUANTITY AND RATE OF PRODUCTION OF TYPE-SPECIFIC CAPSULAR POLYSACCHARIDE[1]

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It is well established that among certain groups of microorganisms the presence of a capsule is essential for virulence; these studies are largely limited to all-or-none effects, i. e. the presence or absence of capsule. Among a few species, e. g. *Diplococcus pneumoniae*, some quantitative studies have been made which indicate that there is a relationship between size of capsule and degree of pathogenicity (Shaffer *et al.*, 1936; Rich and McKee, 1936; MacLeod and Kraus, 1950). In an attempt to extend to organisms other than the pneumococcus the quantitative studies of the relationship of capsule size to pathogenicity, 4 strains of *Klebsiella pneumoniae* type 2 were investigated. While it was found that to some extent the pathogenicity for mice was influenced by capsule size, the rate of capsule production also proved to be a determining factor.

MATERIALS AND METHODS

The parent strain, P, was from the stock collection of the Department of Microbiology. Strain 4 and 13 were mutants of P obtained by ultraviolet irradiation followed by penicillin treatment (Lederberg and Zinder, 1948; Davis, 1948) and had additional growth requirements for leucine and methionine, respectively. Strain 15 was obtained accidentally from P when on one occasion the stock solution used for the preparation of the agar medium was inadvertently not diluted and hence nutrient salts were 10 times the usual final strength; it possessed no special growth requirements.

The media employed for growth were heart infusion agar or broth (Difco) or a synthetic medium (Witkin *et al.*, 1952).

The mice, originally of the CF1 strain, had been inbred and maintained at Tulane University; they usually weighed 20–25 g at the time of infection. Mouse infectivity was determined as follows: broth cultures grown overnight were centrifuged, washed 3 times with saline and so diluted that the inoculating dose was contained in 0.3 ml. The approximate number of viable organisms in the inoculum was estimated by measuring the turbidity of the whole broth culture; the actual number of viable cells was determined by spread plate counts. All surviving mice were observed for 7–9 days before an experiment was terminated. In those groups in which the majority succumbed, a number of animals either recently dead or moribund were necropsied and a heart blood culture prepared. In every instance it was found that the blood contained large numbers of Klebsiella, which were shown to be of the original type by capsular swelling; in addition, the nutritional requirements were tested to insure that during reproduction in the mouse no back mutation had occurred in those strains that had special growth requirements.

Phagocytosis experiments were performed by the method of Davies (1951), using whole fresh mouse blood extracted by cardiac puncture or fresh human blood from a venepuncture.

The production of type-specific polysaccharide was studied by determining (a) the volume of packed cells as a measure of relative capsule size and (b) the quantity of type-specific polysaccharide present in soluble form in a centrifuged supernatant. The volume of packed cells was measured by placing an aliquot of broth culture into a Hopkins tube (Kimble Glass Co. #45225) and centrifuging for 30 min at 2600 rpm in an International type 1 SB centrifuge using a #277 head. This determination assumes that there is no large variation in the size of the soma among the different strains. To estimate the quantity of type-specific polysaccharide in solution, broth cultures were centrifuged at 10,000 rpm in a Sorvall SS1 angle centrifuge to remove bacterial cells and debris. The clear supernatants, heated at 60–65 C for 30 min to insure sterility, were

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recentrifuged to eliminate the last traces of sediment, then were analyzed by the quantitative precipitin method (Heidelberger and McPherson, 1943) using an antiserum that had been calibrated with purified type-specific substance as previously described (Baer *et al.*, 1954). All estimations were made in the zone of antibody excess. No appreciable quantity of antigen other than type-specific polysaccharide was measured as evidenced by the fact that antiserum adsorbed with purified type-specific substance yielded no precipitate when mixed with broth supernatants. In addition no precipitates were obtained when broth cultures of *Klebsiella* type 1 were used.

RESULTS

The results of testing the various strains of *K. pneumoniae* type 2 for their ability to produce fatal infection on intraperitoneal injection in mice are listed in table 1. The parent strain usually produced a fatal infection when about 100 viable organisms were injected; this number is sufficiently small so that there can be no doubt that death resulted from true infection involving extensive multiplication of the organism in the mouse. Mutant strains 4 and 13, requiring leucine and methionine respectively, had about the same degree of virulence as the parent strain. Strain 15, however, was markedly different from the other 3 strains since it did not produce fatal infection even when the dose was increased a hundredfold. With an inoculum of 1,500,000 organisms death usually ensued, but with such a large dose it is difficult to know whether the fatal outcome was the result of infection, toxicity, or more likely, a combination of these and other factors. It is well known that gram negative rods possess toxic somatic antigens; the *Klebsiella* type 2 polysaccharide is also known to be toxic (Baer *et al.*, 1954), although only in relatively high concentration. Thus even the small quantity of toxic antigen present in the inoculum may have added to the effect of other factors.

TABLE 1

Virulence of various strains of Klebsiella pneumoniae type 2 for mice when injected intraperitoneally

Strain	Challenge		Outcome	
	No. mice	No. organisms	No. died	No. survived
Parent	35	70–150	33	2
4	7	70–150	6	1
13	7	70–150	7	0
15	35*	70–150	0	35
15	28	10–15,000	0	28
15	7	1,500,000	6	1
15M†	7	150	0	7
15M	7	15,000	0	7
15M	7	1,500,000	7	0

* Seven of these animals were injected with organisms suspended in 0.3 ml of heart infusion broth, rather than in saline as usual.

† Strain 15M was isolated from the blood of a mouse that had succumbed to an inoculum of 1,500,000 organisms of strain 15.

Animals dying of strain 15 infection showed a positive blood culture. To determine whether the large inoculum enabled more virulent back mutants of the parent type to appear, a culture re-isolated from heart's blood, called strain 15M (table 1), was injected into previously unexposed mice. The results clearly showed that no change had occurred and that these organisms displayed the same relationship of dose to infectivity as the original strain 15.

Since strain 15 was relatively non-virulent for mice even though it showed capsular swelling, further experiments were carried out to determine what differences other than infectivity existed among the various strains. It was considered possible that this organism was more readily susceptible to phagocytosis but qualitative tests using human and mouse blood revealed that, with 24-hr cultures, none of the *Klebsiella* strains was ingested to an appreciable extent by mouse or human phagocytes while in control mixtures with *Micrococcus pyogenes* var. *albus* the cocci were found intracellularly in large numbers.

Since the type-specific polysaccharide is largely involved in anti-Klebsiella immunity it was decided to study the rate and quantity of this substance produced by the various strains. The growth intervals selected were 3, 6, 24 and 96 hr, and the amount of type-specific polysaccharide produced at the various times was measured in two ways. The relative size of the capsule on the various strains was estimated by sedimenting the whole culture and measuring the volume of packed cells. The quantitative precipitin test was used to measure the amount of soluble polysaccharide in the supernatant. It was thus possible to determine not only how much was produced but whether or not it remained on the cell

as capsule or was released into the medium in soluble form. When comparing different strains these results can have meaning only in terms of the total number of cells involved. Since it is relatively easy to determine the number of viable organisms, a comparison was made between viable and total microscopic counts at 3 and 24 hr of growth; when it was found that these did not differ significantly, viable counts were thereafter employed and are given in table 2. In addition, the ratio of turbidity to viable count was constant from 3 to 24 hr, confirming the fact that most of the organisms in the culture were viable during this period. No calculations were made using the viable counts obtained at 96 hr since the ratio of turbidity to viable count was found to rise indicating that a considerable number of organisms were no longer viable.

From table 2 it will be seen that the ratio of viable count to volume of packed cells is smallest for the parent strain at all times, indicating that this strain has the largest capsule and that it is well retained on the surface of the cell for at least 24 hr. Strain 4 and 13 show ratios about 3 times greater than the parent strain at 3 and 6 hr and about double the ratio of the parent strain at 24 hr; these organisms therefore produce a capsule considerably smaller than that of the parent strain. The actual values for the ratios for the parent and for mutant strains 4 and 13 show very little change with time, from which it would appear that the capsule size remains constant throughout the time interval studied.

Strain 15, the relatively non-infectious strain, presented a somewhat different picture. The ratio of viable count to volume was more than 6 times larger than the parent strain at 3 hr and about 2 times greater than strains 4 and 13. Unlike the other 3 strains, the ratio gradually diminished until at 24 hr it was only slightly greater than strains 4 and 13 and about 3 times larger than the parent strain. The cells of strain 15, therefore, were able to increase the size of their capsule with continued incubation. These data are further confirmed by the studies showing the quantity of capsular polysaccharide produced (table 2). After the 3 hr of growth the parent strain produced the largest amount of capsular material, strains 4 and 13 less, and strain 15 only about ¼ as much as the parent strain. At 24 hr all strains had produced increased amounts of capsular polysaccharide but strain 15 exhibited the largest increase since at this time interval it produced as much as strains 4 and 13.

TABLE 2

*Relationship between the number of bacteria, size of capsule, and quantity of soluble type-specific polysaccharide produced during various periods of growth of certain strains of Klebsiella pneumoniae type 2**

1	2	3	4	5	6
Strain	Incubation Time	Vol. Bacteria† per Ml	Viable Count × 10^7 per Ml	μg SSS‡ per Ml	(Col. 4)/(Col. 3)
	hr				
p	3	0.0112	46	6.5	4,100
4		0.0041	54	2.8	13,000
13		0.0044	56	2.7	13,000
15		0.0015	40	1.9	27,000
p	6	0.0103	53		5,100
4		0.0044	66		15,000
13		0.0050	61		12,000
15		0.0017	35		21,000
p	24	0.0122	65	14.3	5,300
4		0.0072	68	9.9	9,500
13		0.0071	74	9.6	11,000
15		0.0055	81	10.4	15,000
p	96	0.0700	46	87.5	§
4		0.0385	73	67.5	
13		0.0370	42	46.0	
15		0.0245	42	42.5	

* The figures in the tables represent the average values for 2 or 3 experiments for the 3- and 24-hr growth periods and 1 experiment for the 6- and 96-hr intervals.

† Volume of packed bacterial cells; ml in all instances refers to whole broth culture.

‡ SSS refers to the type-specific capsular polysaccharide.

§ Calculations not made because turbidity to viable count studies revealed that there were large numbers of dead organisms at this time interval.

Another aspect of the problem examined was whether the capsular polysaccharide produced by strain 14 was antigenically different from that produced by the other 3 strains. All strains showed capsular swelling and the supernatant of broth cultures gave positive interfacial ring tests with the same antiserum. It thus appeared that if any differences existed between the polysaccharides of the 4 strains it was not very great. A more critical test is one which would determine

TABLE 3

Immunity against Klebsiella pneumoniae type 2 infection produced in mice by the injection of type-specific polysaccharides from various strains

Klebsiella Antigen Injected	Quantity Type 2 SSS Injected	No. of Mice		
		Inoc.*	Died	Survived
	μg			
Type 2				
Parent strain supernatant	2†	8	0	8
Mutant 15 supernatant	2†	7	0	7
Purified type 2 SSS	2	7	0	7
Type 1				
Supernatant	0	7	7	0
None	0	7	7	0

* Approximately 200 viable organisms were contained in the challenge dose.

† Estimated by quantitative precipitin test.

whether the polysaccharide of strain 15 would protect mice from infection by the parent strain. To accomplish this broth culture supernatants that had been analyzed for their content of type-specific polysaccharide were diluted in such a manner that when they were injected into mice, each animal received 1 μg of type-specific polysaccharide in each of 2 injections. As controls, purified polysaccharide isolated from the parent strain and a broth culture supernatant of *K. pneumoniae* type 1 were employed. Table 3 shows that the culture supernatant of strain 15 was as effective as that of the parent strain or the purified polysaccharide in protecting mice against challenge with about 200 organisms of the parent strain. The various tests employed thus indicated that the type-specific polysaccharide of strain 15 is qualitatively the same as that produced by the other 3 strains.

DISCUSSION

Variation in pathogenicity with variation in capsule size has been the subject for quantitative study mainly among the pneumococci. For example, MacLeod and Krauss (1950) showed that non-virulent, or slightly virulent strains of pneumococci produced much less type-specific capsular substance than the fully virulent strains. Shaffer *et al.* (1936) and Rich and McKee (1936) found that a strain of Type III pneumococcus non-virulent for rabbits produced a somewhat smaller capsule than a virulent strain and that in broth cultures the avirulent strain lost its capsule much sooner than the virulent one.

The present study extends this concept to the genus *Klebsiella*. When the capsule diminishes sufficiently in size, as in strain 15, the organism largely loses its virulence for mice. Strain 15, however, shows an additional interesting property; in contrast to the pneumococci studied by Shaffer *et al.* (also Rich and McKee) it is capable of increasing the size of its capsule when grown in broth culture so that after 24 hr its capsule is almost as large as that of the pathogenic strains 4 and 13. This phenomenon probably cannot take place in the mouse because the new cells formed by division have small capsules and very likely are unable to survive long enough to grow larger capsules. With a sufficiently large inoculum of a 24-hr culture—1,500,000 organisms—the cells have had time to grow large capsules *in vitro* and may themselves be capable of causing death or inhibiting the defense mechanism of the animal.

Since strains 4 and 13 have special nutritional requirements for leucine and methionine it is noteworthy that their pathogenicity does not differ significantly from the parent strain. This confirms the work of Garber *et al.* (1952) using *Klebsiella* and Bacon *et al.* (1950) using *Bacterium typhosum*.

SUMMARY

Four strains of *Klebsiella pneumoniae* type 2 were examined with respect to mouse virulence following intraperitoneal inoculation.

A study of relative capsule size revealed that the parent strain had the largest capsule while 2 pathogenic mutants had capsules about half as large. The non-pathogenic strain had the smallest capsule but in broth culture the size of its capsule increased with time so that at 24 hr it was almost as large as that of the pathogenic mutants.

REFERENCES

BACON, G. A., BURROWS, T. W., AND YATES, M. 1950 The effects of biochemical mutation on the virulence of Bacterium typhosum. Brit. J. Exptl. Pathol., **31**, 714.

BAER, H., BRINGAZE, J. K., AND MCNAMEE, M. 1954 The effect of the dose of bacterial polysaccharide antigen on antibody production in mice. J. Bacteriol., **67**, 123.

DAVIS, B. D. 1948 The isolation of biochemically deficient mutants of bacteria by means of penicillin. J. Am. Chem. Soc., **70,** 4267.

DAVIES, G. E. 1951 A method for obtaining accurate and reproducible results in experiments on phagocytosis. J. Pathol. and Bacteriol., **68,** 149.

GARBER, E. D., HACKETT, A. J., AND FRANKLIN, R. 1952 The virulence of biochemical mutants of *Klebsiella pneumoniae*. Proc. Natl. Acad. Sci. U. S., **38,** 693.

HEIDELBERGER, M. AND MCPHERSON, C. F. C. 1943 Quantitative micro-estimation of antibodies in the sera of man and other animals. Science, **97,** 405.

LEDERBERG, J. and ZINDER, N. 1948 Concentration of biochemical mutants of bacteria with penicillin. J. Am. Chem. Soc., **70,** 4267.

MACLEOD, C. M. AND KRAUSS, M. R. 1950 Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharide formed in vitro. J. Exptl. Med., **92,** 1.

RICH, A. R. AND MCKEE, C. M. 1936 The mechanism of a hitherto unexplained form of native immunity to the type III pneumococcus. Bull. Johns Hopkins Hosp., **59,** 171.

SHAFFER, M. F., ENDERS, J. F., AND WU, C. J. 1936 Studies on natural immunity to Pneumococcus type III. II. Certain distinguishing properties of two strains of Pneumococcus type III varying in their virulence for rabbits, and the reappearance of these properties following R→S reconversion of their respective rough derivatives. J. Exptl. Med., **64,** 281.

WITKIN, E. M., BRYSON, V., DEMEREC, M., LABRUN, E. L., GALINSKY, I., AND SZYBALSKI, W. 1952 *Laboratory outline for a course on selected methods in bacterial genetics*. Biological Laboratory, Cold Spring Harbor, N. Y.