VIRULENCE OF *KLEBSIELLA* STRAINS IN EXPERIMENTALLY INDUCED SKIN LESIONS IN THE MOUSE

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SUMMARY. The virulence of 93 clinical isolates of *Klebsiella* was compared in a mouse model by subcutaneous injection. Skin pathogenicity was measured by estimating the number of viable bacteria in the lesions 24 h after infection with a dose of 10^7 bacteria. Strains of serotypes K1-6 were compared with strains of serotypes higher than K6. All K1 and K5, and some K2 and K4 strains were more virulent for mice than strains with a serotype higher than K6. The K3 strains were significantly less virulent than the strains with a serotype higher than K6. The bacteriological findings were confirmed by histological examination with some strains. No differences in virulence were observed between strains of the same serotype isolated from patients with cystitis or from those with pyelonephritis, nor between strains of the same serotype isolated from the blood of patients with septicaemia or from other sites. The mouse model has been found satisfactory for observing differences in virulence between *Klebsiella* isolates.

INTRODUCTION

Klebsiella strains have long been known to cause several different infections in man. Since Friedländer (1882) discovered that this bacillus was the micro-organism responsible for a severe form of lobar pneumonia, several authors have described cases of acute pneumonia with a high mortality caused by it (Solomon, 1937; Julianelle, 1941; Perlman and Bullowa, 1941; Hyde and Hyde, 1943).

Julianelle (1926) classified Friedländer's bacillus into three groups by agglutination: groups A, B and C. Group A appeared to be the most virulent and caused pneumonia with the highest mortality. In later years, Friedländer's bacillus was shown to be an uncommon cause of pneumonia and *Klebsiella* spp. were generally considered to be secondary invaders that could give rise to a variety of infections as opportunistic pathogens.

Since the introduction of chemotherapy, gram-negative bacilli, especially klebsiellae, have become increasingly important as causes of serious hospital infections

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(Montgomerie, 1979). On intensive care wards, epidemics of respiratory and urinary tract infections have been frequently reported. However, not all patients with *Klebsiella* spp. isolated from, for example, their sputum, are severely ill. One possible explanation for this phenomenon is a difference in virulence between *Klebsiella* strains.

In the literature, virulence is associated with capsular serotype. Capsular serotypes K1-6 were, historically, thought to be involved only in respiratory tract infections (Henriksen, 1954; Wilson and Miles, 1975). Orskov (1955), however, found that types K1-6 occurred in only 15.6% of upper respiratory tract infections caused by *Klebsiella* spp. Later studies (Eickhoff, Steinhauer and Finland, 1966; Steinhauer *et al.*, 1966; Tillotson and Finland, 1969; Riser and Noone, 1981; Smith, Digori and Eng, 1982) showed that although there is a prevalence of the lower numbered serotypes in respiratory tract infections, these serotypes are also recovered from infections at other sites; conversely higher numbered serotypes may produce infections of the respiratory tract. In view of the clinical course of *klebsiella* infections in epidemics it seems likely that differences in virulence between capsular serotypes exist. Serotypes K1 and K2 are reported to be highly virulent for mice when injected intraperitoneally, whereas other types are either non-virulent or of low virulence (Orskov, 1974; Wilson and Miles, 1975). We describe the use of a subcutaneous infection model in mice, originally described by Miles, Miles and Burke (1957), to compare the pathogenicity of *Klebsiella* isolates from different human sites, with special reference to capsular serotypes K1-6.

MATERIALS AND METHODS

Bacterial strains. The micro-organisms used in this study, their capsular serotype, source and isolation site are listed in table I. All strains were identified by API 20E (API system S.A., Montalieu Vercien, France). Serotyping was performed by the capsule swelling technique with antisera raised in rabbits (Edmondson and Cooke, 1979). Strains were maintained on agar slants at room temperature and in a mixture of 1 ml of trypticase soy broth and 1 ml of glycerol 60% (v/v) at -70°C and at -20°C . In each experiment strains were checked for purity and a single colony was taken.

Bacterial suspensions. Log phase cultures of strains in nutrient broth were harvested by centrifugation, resuspended in phosphate buffered saline (PBS) and stored overnight at 4°C . Viable counts were determined by plating serial dilutions of the suspensions on nutrient agar plates. The bacterial suspensions were diluted to a concentration of 10^8 bacteria/ml. After infecting mice, viable counts of the suspensions were made again to confirm the dose.

Animals. Eight-week-old female mice (Swiss strain, TNO, Zeist, the Netherlands) were used. The hair was clipped over the dorsal skin and flanks before subcutaneous injection.

Virulence test. The bacterial suspension (0.1 ml; 10^8 bacteria/ml) was injected subcutaneously in the backs of mice about 1 cm lateral to the midline; duplicate injections were made in each animal and at least two animals per strain were used. The viable bacterial content of the skin lesions was estimated 24 h after infection by the method of Miles, Pillow and Khimji (1976) and of Maskell (1981). At that time the animals were killed by chloroform inhalation; the skin was cleaned with 70% (v/v) alcohol and allowed to dry. Skin fragments including the site of injection were excised from dorsal to ventral and stretched. After macroscopic examination, 5-mm parallel cuts at 4 mm intervals were made starting from the periphery and working to the centre of each injection site. Each lesion was sampled by rubbing a loop along both walls of each cut and plating on nutrient agar. After incubation at 37°C overnight the growth was graded as: 8 (confluent growth), 4 (semiconfluent growth), 2 (≥ 20 colonies), 1 (< 20 colonies) or 0 (no growth); we refer to this figure as the growth index. In each animal at least the central cut and the cuts 4 mm from the centre of the lesion were sampled. For each strain mean values of the growth indices in the separate cuts of the skin lesions were calculated. The sum of the mean growth indices in the centre of the lesion and 4 mm from the centre was called the total growth index.

TABLE I
Klebsiella strains

Serotype	Species*	Number of strains	Number isolated from	
			Respiratory sites	Other sites
K 1	<i>K. pneumoniae</i>	9	1	8
K 2	<i>K. pneumoniae</i>	10	2	8
K 3	<i>K. pneumoniae</i>	13	...	13
K 3	<i>K. rhinoscleromatis</i>	1	...	1
K 4	<i>K. ozaenae</i>	8	5	3
K 4	<i>K. pneumoniae</i>	1	...	1
K 5	<i>K. pneumoniae</i>	3	...	3
K 5	<i>K. pneumoniae</i> (NCTC9960)	1	...	1
K 6	<i>K. pneumoniae</i>	6	4	2
K 7	<i>K. pneumoniae</i>	1	...	1
K 8	<i>K. pneumoniae</i>	1	1	...
K 10	<i>K. pneumoniae</i>	1	...	1
K 18	<i>K. pneumoniae</i>	3	...	3
K 21	<i>K. pneumoniae</i>	4	...	4
K 24	<i>K. pneumoniae</i>	1	1	...
K 26	<i>K. pneumoniae</i>	1	...	1
K 27	<i>K. pneumoniae</i>	4	1	3
K 29	<i>K. pneumoniae</i>	1	...	1
K 33	<i>K. pneumoniae</i>	5	1	4
K 35	<i>K. pneumoniae</i>	2	...	2
K 43	<i>K. pneumoniae</i>	2	...	2
K 55	<i>K. pneumoniae</i>	2	...	2
K 61/63/10†	<i>K. pneumoniae</i>	2	...	2
K 64	<i>K. pneumoniae</i>	2	...	2
K 69	<i>K. pneumoniae</i>	4	2	2
NT	<i>K. pneumoniae</i>	5	1	4
Total		93	19	74

NT = non-typable.

* Identified by API 20E.

† Cross reactions not separated.

Histological procedures. The excised skin fragments were stretched to avoid deformation and fixed in 4% buffered formalin for at least 48 h at room temperature. After fixation, sections in dorso-ventral direction of the whole length of the excised skin fragment were taken through the centre of the lesions and embedded in paraffin. Sections of 6 μ m were stained with haematoxylin and eosin.

Determination of LD50. Log-phase cells resuspended in PBS were injected i.v. in serial dilutions into groups of 8-week-old female Swiss mice; each group contained six mice. After 14 days the LD50 values were calculated by the method of Spearman and Kärber (Finney, 1964).

RESULTS

Evaluation of the virulence model

Macroscopic findings. Macroscopic examination of the lesions produced revealed variations between different strains of *Klebsiella*. Some strains produced lesions with only mild induration whereas other strains produced lesions with swelling or small abscesses. Most of the K1 strains, some K2 and K4 strains and all K5 strains produced abscesses. No necrosis was seen. Injection of sterile PBS alone did not give rise to any effect on the skin of the animals.

We did not find it easy to measure the size of the lesions, and so this technique was abandoned.

Dose-response experiments. Serial 10-fold dilutions (10^{10} – 10^6 bacteria/ml) of three *Klebsiella* strains (K33, K18 and a non-typable strain) were injected subcutaneously in 0.1-ml volumes in mice (four injection sites/dose). After 24 h, the mean of the growth indices in the centre of the lesions was determined and plotted graphically against the $\log_{10}$ dose of micro-organisms injected. The mean growth index in the centre of the lesions gave a roughly linear plot against the $\log_{10}$ dose within the range of concentrations used (fig. 1). The virulence experiments were performed subsequently with a dose of 10^7 bacteria.

FIG. 1.—Dose-response curves of three *Klebsiella* strains (serotypes K33, K18 and NT).

Length of time after infection. The optimal length of time after the injections before killing the animals was determined by killing animals at five different time intervals after inoculation (18, 24, 42, 48 and 66 h). There was no further increase of the growth indices in the samples after 24 h (data not shown). In subsequent experiments animals were killed 24 h after injection.

Reproducibility. Reproducibility of the gradation of growth in the centre and 4 mm from the centre of the lesions was tested in at least four lesions per strain. In each lesion the growth index in the centre of the lesion was higher than or, in some lesions, the same as at 4 mm from the centre. In fig. 2, reproducibility of the bacterial concentration profiles of two different *Klebsiella* K2 strains in four lesions per strain is shown. We have chosen one of the best and one of the poorest examples. Although variations between the individual experiments with the same strain occurred, differences in virulence between different strains were quite reproducible. We, therefore, considered this method adequate for comparing the virulence of *Klebsiella* strains.

FIG. 2.—Reproducibility of the growth index in eight lesions induced by two *Klebsiella* K2 strains. Results obtained in duplicate tests in four mice are indicated by the symbols ▲, ▼, ◆ and ■; mean of 4 experiments is shown by ●—●.

Virulence testing of Klebsiella isolates

Virulence tests were performed with the 93 isolates listed in table I. For every strain the mean of the growth indices in the centre and on both sides 4 mm from the centre of the lesions was determined. Bacterial concentration profiles of the lesions induced by all *Klebsiella* K2 strains are shown in fig. 3.

FIG. 3.—Bacterial concentration profiles of *Klebsiella* K2 strains in skin lesions in mice. Each point is the mean of the growth index values in at least four lesions. For each strain the inoculum is shown on the left.

Relation of virulence to serotype. Nearly all the K1 and K5 strains gave a central growth index of 8, whereas the central growth index of K2 and K4 strains was 2–8. K3 strains gave a central growth index of ≤ 3 and strains of serotype K6 or a serotype higher numbered than 6 gave central growth indices that ranged from 1–5. The sum of the mean growth indices in the centre and at 4 mm from the centre (total growth index value) was plotted graphically for each strain (fig. 4), by serotype (K1, K2, K3, K4, K5, K6 and K > 6, including non-typable strains).

FIG. 4.—Total growth index values of strains, arranged according to serotype groups. The total growth index values are the sum of the mean growth index values in the centre and 4 mm from the centres of the lesions.

Statistical analysis by the Wilcoxon test at a significance level of $\alpha=0.01$, showed that the hypothesis that the total growth index value of the strains in serogroups K1, K2, K3, K4, K5 and K6 separately was equal to the total growth index value of the strains with a serotype higher than 6, was rejected for the groups of strains of serotypes K1–5, but not for the K6 strains ($\alpha > 0.05$).

Relation of virulence to origin of isolates. Within the group of strains with a serotype number higher than 6, a group of strains could be distinguished which had been isolated from urines of patients with pyelonephritis (seven strains) and a group of

FIG. 5.—Total growth index values of strains, arranged according to the origin of the isolates. The total growth index values are the sum of the mean growth index values in the centre and 4 mm from the centres of the lesions.

strains with the same serotype which had been isolated from urines of patients with cystitis (five strains). The diagnosis of cystitis or pyelonephritis was based on clinical data only. In the same way a "septicaemia" group of strains (eight strains isolated from blood cultures) and a non-septicaemia group of strains (six strains of the same serotype from sites other than blood) could be compared. The total growth index values of the strains in these groups are plotted graphically in fig. 5. Statistical analysis of the results obtained with these groups of strains by the Wilcoxon test revealed neither a significant difference between the cystitis and pyelonephritis groups of strains, nor between the septicaemia and non-septicaemia groups of strains ($\alpha > 0.05$).

Histological findings. Histological examination was performed on the lesion induced by single strains of serotypes K1, K3 and K5 and by six strains with a serotype higher than 6 (including a nontypable strain). The lesions induced by the K1 and K5 strains showed an extended infiltrate with many intact polymorphonuclear leukocytes (PMNs) and spreading of the inflammatory cells between the subcutaneous muscles, whereas the lesions induced by the other strains showed a small infiltrate with many accumulated PMNs, with partly pyknotic nuclei, localised at the site of infection (figs 6 and 7). Bacteria were only seen in the preparations of the lesions produced by the K1 and K5 strains. The microscopical measurements of the lesions induced by the strains are given in table II.

LD50 determination. We studied the LD50 values of a strain of serotype K1 and of a strain of serotype K18. The LD50 values calculated by the method of Spearman and Kärber were 7.2×10^8 for the K18 strain and $< 3.5 \times 10^6$ for the K1 strain.

FIG. 6.—Photomicrograph of a part of the skin of a mouse given an injection of *Klebsiella* K1 showing an extended layer of inflammatory cells spreading between and above the subcutaneous muscles. ($\times 48$).

FIG. 7.—Photomicrograph of a part of the skin of a mouse given an injection of *Klebsiella* K21 showing a small, localised infiltrate with no spreading of inflammatory cells. ($\times 48$).

TABLE II
Microscopical examinations of lesions induced by nine Klebsiella strains

Serotype	Length of infiltrate (mm)	Width of infiltrate (mm)	Presence of bacteria
K 1	5.8	0.9	++
K 5	9.2	2.1	++
K 10	4.4	0.2	—
K 24	7.2	0.2	—
NT	7.3	0.1	—
K 7	4.9	0.1	—
K 21	4.0	0.1	—
K 29	2.4	0.1	—
K 3	2.9	0.4	—

DISCUSSION

We have described a mouse model for studying the virulence of clinical isolates of *Klebsiella*. This model, in which skin infectivity is measured by subcutaneous injection appeared to be a simple and reproducible technique for testing the virulence of strains. Measurement of the size of the skin lesions, which has been used in virulence tests in guinea-pigs for several micro-organisms, including *Klebsiella* spp. (Miles *et al.*, 1957, 1976; Maskell, 1981) was not satisfactory in our hands in mice. For our test, we needed to estimate the number of viable bacteria in the skin lesions to discriminate between strains. Although this model does not simulate human infections by *Klebsiella* spp., it has the advantage of permitting us to test a larger number of strains in a smaller number of animals and in a shorter period than in the LD50 tests. How far skin virulence in mice can be extrapolated to human infections has not been determined.

With a given inoculum, the *Klebsiella* isolates tested in our model showed differences in virulence. All K1 and K5 strains, and some K2 and K4 strains were more virulent for mice than strains with a serotype higher than 6. The differences in the mean growth index values for the K2 strains (fig. 3) and the K4 strains (data not shown) were greater than could be explained by variation in inoculum size. All K3 isolates, including a *K. rhinoscleromatis* strain, showed a significantly lower virulence for mice than strains with a serotype higher than 6. None of our K1, K2 or K3 strains showed the biochemical characteristics of *K. edwardsi* var. *edwardsi*, *K. edwardsi* var. *atlantae* or *K. pneumoniae* (*sensu stricto*) in the classification of Cowan *et al.* (1960) and referred to as Friedländer's bacillus.

Although much attention has been paid to the relationship between serotype or biotype or both and the site of isolation (Orskov, 1955; Eickhoff *et al.*, 1966; Steinhauer *et al.*, 1966; Riser and Noone, 1981; Smith *et al.*, 1982), few studies are known in which the virulence of *Klebsiella* strains is compared. The use of an intraperitoneal mouse virulence test by Foster and Bragg (1962) to distinguish Friedländer's bacillus from the non pathogenic *K. aerogenes* showed, although exceptions occurred, a higher virulence of *K. edwardsi* var. *edwardsi*.

Our findings agree with those reported in the literature which, on the basis of isolation site, have suggested that the lower-numbered serotypes of *Klebsiella* are more

virulent. In virulence tests in mice, however, only K1 and K2 strains have been reported to be highly virulent (Orskov, 1974; Wilson and Miles, 1975). However, it is not clear from these reports how many strains were tested. A larger number of strains in our experiments could be an explanation of the heterogeneity of the K2 strains in virulence tests. Significantly lower virulence of K3 strains in virulence tests has not been reported previously as far as we know. Because we have examined a selected group of *Klebsiella* strains, no conclusion can be drawn about predominance of different serotypes at different infection sites. The reports in the literature that the lower-numbered serotypes are more virulent, especially in respiratory tract infections, could not be confirmed in this study. In our group of strains of serotypes K1-6, no prevalence of sputum isolates was noted.

Confirmation of differences in virulence between our *Klebsiella* isolates, based upon bacterial counts, was afforded by the histological examination of the skin lesions of a selected group of strains. Differences were seen in the size of the infiltrate and the presence of bacteria between the virulent K1 and K5 strains and the less virulent K3 strain, and strains with a serotype higher than 6. The >200-fold difference between LD50 values of the K1 and the K18 strains tested, also supports the differences in virulence tests.

No differences in virulence could be detected between cystitis strains and pyelonephritis strains, or between septicaemia and non-septicaemia strains of the same serotype. These data are not surprising because, especially in hospitalised patients, apart from the virulence of the bacterial strain many host factors such as age, underlying disease and antibiotic treatment also determine whether a micro-organism becomes invasive.

With the mouse model described in this paper it is possible to detect differences in virulence of *Klebsiella* strains and possibly of other gram-negative micro-organisms. Although there is a correlation between serotype and virulence, we conclude from these results that capsular antigens are not the only virulence factors.

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