VACCINATION AGAINST 77 CAPSULAR TYPES OF *KLEBSIELLA AEROGENES* WITH POLYVALENT *KLEBSIELLA* VACCINES

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SUMMARY. A method is described for producing monovalent and polyvalent vaccines from culture filtrates of *Klebsiella aerogenes*. With a single injection, each monovalent vaccine protected mice against lethal intraperitoneal challenge by more than 30 capsular types; and polyvalent vaccines containing 2–12 monovalent components protected against 46–61 of the 77 capsular types of *K. aerogenes*. One vaccine with 12 components, administered in two doses, induced full protection against 71 types and protected half of the mice challenged with the other six types.

INTRODUCTION

The importance of *Klebsiella aerogenes* as a major opportunistic pathogen is well documented (Steinhauer *et al.*, 1966; Rennie and Duncan, 1974; Young, 1982; Bryan, Reynolds and Brenner, 1983). Specific associations include debilitated patients (Foster and Bragg, 1962; McHenry and Hawk, 1974; McCabe *et al.*, 1977; Young *et al.*, 1977; Wardle, 1979; Ziegler *et al.*, 1982), cancer (Umsawasdi *et al.*, 1973), renal transplants (Montgomerie *et al.*, 1970), neurosurgery (Price and Sleight, 1970), bone marrow disease (Young, 1983), urinary tract complications (Casewell *et al.*, 1977), ankylosing spondylitis (Ebringer *et al.*, 1978), burns (Jones, Roe and Gupta, 1980; Jones, 1981) and bronchopneumonia (Darrell and Hurdle, 1964; Pennington and Menkes, 1981). The emergence of strains of *K. aerogenes* with multiple drug resistance (Thomas *et al.*, 1977; Montgomerie, 1979; Casewell, 1982), together with its high ranking as a main cause of gram-negative bacteraemia (Montgomerie and Ota, 1980), suggests a need for an alternative approach.

Immunisation is a relatively untried method of combatting klebsiella infections. Recent research has been mainly on the protective properties of lipopolysaccharides or core-glycolipids that are common to all members of the Enterobacteriaceae (Young, Stevens and Ingram, 1975; Braude *et al.*, 1977; Young, 1980). There have been few studies on the immunogenicity of type-specific capsular polysaccharides (Jones, 1971; Nakashima, Nagase and Kato, 1977; Riottot, Fournier and Jouin, 1981). Experimental evidence suggests that type-specific vaccines from virulent strains of gram-negative

bacilli afford a higher degree of protection than the common protective antigens (McCabe *et al.*, 1977; Roe and Jones, 1983; Zinner and Peter, 1983).

In this study we have immunised mice with type-specific vaccines from virulent strains, and tested the cross-protection of single and polyvalent vaccines against the 77 capsular types of *K. aerogenes*.

MATERIALS AND METHODS

Sources of bacteria. Strains of *K. aerogenes* were isolated from blood cultures of patients with burns at Birmingham Accident Hospital, and at Safdarjang Hospital, New Delhi, by methods described by Davis, Lilly and Lowbury (1969). Other hospital strains of *K. aerogenes* were kindly supplied by Dr R. Warren, Addenbrookes Hospital, Cambridge, and Dr Reeves, University Hospital, Nottingham. All strains of *K. aerogenes* were serotyped by their capsular antigens, using counter-current immunoelectrophoresis and the Quellung reaction (Palfreyman, 1978), at the Public Health Laboratory, Coventry. A representative strain of each of the 77 capsular types of *K. aerogenes* was kindly provided also from that laboratory, having been isolated originally from hospital patients in the UK. The bacteria were freeze-dried, and also maintained in subculture on nutrient agar (Oxoid) at 4°C.

K. aerogenes was grown for 18 h at 37°C on 1.2% Blood Agar Base No. 2 (Oxoid), and resuspended in 10 ml of saline to a concentration of 10^{10} bacteria/ml, estimated by turbidity. The bacterial suspension was added to 1.5 L of synthetic medium containing glucose 25 g, ammonium lactate (60% syrup) 15 ml, NaCl 4.5 g, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 20 g, KH_2PO_4 2.25 g, Supplement A 1.5 ml, Supplement B 1.5 ml; and KH_2PO_4 crystals were added to adjust the pH to 7.2. Supplement A: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25 g, $\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.25 g, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.25 g, 0.1 N H_2SO_4 5 ml, H_2O 95 ml. Supplement B: $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1 g, KCl 10 g, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 7 g, made up to 100 ml with H_2O .

The medium (1.5 L) was pumped into a Biolab Fermenter (F.T. Biotechnology, Station Industrial Estate, Bredon, Tewkesbury, Gloucester GL20 7HH) through a 0.2- μm membrane filter (Millipore). The fermenter had automatic monitor and control facilities for pH, temperature, antifoam, dissolved oxygen and stirring. The pH was set to a lower limit of 7.15 and upper limit of 7.25, control was by automatic addition of 1.0 N H_2SO_4 or 1.0 N KOH as required by pH sensor; the temperature of the culture was kept at 37°C; antifoaming agent was 1.0% polypropylene glycol 2025, and the control was set to add antifoam agent as needed; air was initially pumped into the medium at a rate of 2500 ml/min through a 0.2- μm Acro 50 hydrophobic filter (Gelman Sciences Ltd, 10 Harrowden Road, Brackmills, Northampton NN4 0EB) until a stable state of maximum dissolved oxygen was reached; the monitor was then set to 100% dissolved oxygen, and the air supply was switched to 'control supply' which attempted to maintain this level of oxygen in the medium as conditions altered during culture (the minimum air flow during culture was 600 ml/min); the culture was stirred constantly at 500 rpm with four stainless steel paddles, each 2.5 cm².

Strains of *K. aerogenes* were grown for 48 h in controlled conditions; then 4.5 ml of concentrated formaldehyde (40% w/v) was added to the culture and the bacteria were removed from the medium by centrifugation (16 000 g, 1 h, 12°C), followed by positive pressure filtration through a 0.2- μm membrane on a 142-mm plate filter holder (Sartorius Instruments Ltd, 18 Avenue Road, Belmont, Surrey). The culture filtrate was filtered through a Nuclepore Ultrafiltration Unit with MFS membranes (90 mm diam.) type M0220A090E (V.A. Howe and Co. Ltd, 12-14 St Ann's Crescent, London SW18 2LS) which retained molecules with a mol. wt > 20 000, and reduced the volume of the retained fraction to 100 ml. Traces of medium and remaining small molecules were removed from this concentrate by diafiltration by passing 2 $\times$ 2.5 L of de-ionised water through the Ultrafiltration Unit. When the retained fraction had been reduced to 400 ml it was shell-frozen and freeze-dried. The yield of each vaccine was a minimum of 1 g/L.

For polyvalent vaccine, stock solutions of monovalent vaccines (1.0 ml of saline containing 1 mg of klebsiella vaccine) were mixed and made up to 1 L with saline. Thus a 3-part polyvalent vaccine contained 3 μg of combined klebsiella vaccines per ml.

Animal experiments. Female albino BKW2 mice, 22–25 g, were vaccinated and given challenge doses of live bacteria by intraperitoneal injection. They were kept at 24°C, with food and water *ad libitum*; deaths were recorded up to 24 h after challenge or, for tests of vaccine toxicity, up to 48 h after vaccination.

For virulence tests, strains of *K. aerogenes* were grown on nutrient agar, and colonies of bacteria were suspended in physiological saline by shaking on a mixer. After estimation of the bacterial count by turbidity, each suspension was diluted to the required concentration. Groups of three or six mice were given 1.0-ml inocula of serial dilutions from a maximum dose of 3×10^9 bacteria/ml. The highest dilution that killed a group of six mice was considered to contain a minimum lethal dose (MLD) (Jones, 1970).

Vaccines were prepared initially from strains with an MLD of 10^8 bacteria or less, such as type 1 and type 20 (table I). Subsequently, however, in order to protect against all 77 capsular types, we had to include vaccines prepared from other capsular types, even though the most virulent strains that we possessed had an MLD greater than 10^8 bacteria, such as types 50 and 70.

For protection tests, groups of mice were given 1.0 ml of single or polyvalent vaccine, and challenged 4 days later with 1 MLD of live bacteria. For those that received two doses of vaccine, the second dose was given 3 days after the first and the mice were challenged 4 days after the second dose.

For toxicity tests, groups of mice received graded doses of vaccine (table V). The six-part polyvalent vaccine was made up by adding 16 mg of each of the six component vaccines to 10 ml of saline to give a final concentration of combined vaccine of approximately 10 mg/ml. The nine-part polyvalent vaccine was made by adding 11 mg of each of the nine vaccines to 10 ml of saline, giving a final concentration of combined vaccine of approximately 10 mg/ml.

RESULTS

Vaccine strains of K. aerogenes

Of the 324 strains of *K. aerogenes* in our collection, 20 strains were classed as highly virulent, killing groups of six mice at doses of 1.0×10^8 bacteria/mouse or less; 24 strains were classed as avirulent, not killing mice at doses of 2.0×10^9 bacteria/mouse.

TABLE I
Virulence of vaccine strains of K. aerogenes for mice

Strains of <i>K. aerogenes</i>		Minimum lethal dose (MLD)*
Source	Capsular type	
SJH	1	1.0×10^7
BAH	2	1.0×10^7
PHLS	3	6.0×10^8
PHLS	15	6.0×10^8
SJH	20	1.0×10^8
PHLS	35	8.0×10^8
SJH	36	2.0×10^8
SJH	44	1.0×10^8
PHLS	50	1.4×10^9
BAH	63	1.0×10^8
PHLS	70	1.8×10^9
SJH	74	2.8×10^8

SJH = Safdarjang Hospital, New Delhi.

BAH = Accident Hospital, Birmingham.

PHLS = Coventry area laboratory of Public Health Laboratory Service.

* Intraperitoneal injection in BKW2 mice.

TABLE II
Cross-protection of mice after one dose of monovalent klebsiella vaccine

Capsular types of <i>K. aerogenes</i> present in monovalent vaccines	Number of capsular types (n=77) against which vaccine gave			Capsular types against which vaccine gave no protection
	complete* protection	partial protection	no† protection	
1	43	13	21	2 15 20 24 25 32 35 44 46 48 49 52 55 57 58 59 60 62 63 64 69
2	42	17	18	3 5 6 15 20 23 24 27 35 40 42 44 47 49 50 63 65 74
3	34	19	24	1 2 5 10 11 14 17 20 24 29 31 35 36 45 46 49 55 63 68 69 71 72 73 74
15	30	19	28	1 2 3 4 5 8 12 13 16 18 19 20 22 24 26 32 35 36 40 44 46 57 58 69 71 73 74 75
20	38	18	21	2 3 4 6 24 26 31 35 40 41 44 45 49 50 63 68 69 70 71 73 74
35	42	17	18	1 2 10 14 17 20 22 24 25 30 59 64 67 68 69 71 73 74
44	46	21	10	1 2 20 35 50 58 59 63 64 65

* 6/6 mice protected.

† 0/6 protected.

The 12 vaccine strains (table I) were derived mainly from highly virulent bacteria, e.g., types 1, 2, 20, 44, 63, and only three (types 35, 50 and 70) were from relatively avirulent strains.

Protection by single dose of monovalent vaccine

Seven monovalent vaccines each protected mice against different ranges of 30–46 types of *K. aerogenes* (table II). The groups of vaccinated mice were challenged with 77 types, at a time when the maximum cross-protective response occurs, i.e., 4 days after a single dose of vaccine (Roe and Jones, 1984). Vaccine 44 induced protection against the largest number of types (46), and failed to protect all the mice in a group against only 10 types. Each monovalent vaccine protected against the type from which it was made and also against a random set of other types, and there seemed to be no particular strain or set of strains (table II) against which the vaccines were incapable of inducing protection.

Protection by single dose of polyvalent vaccine

Ten polyvalent vaccines, containing 2–12 components, all protected mice against more types than did the individual components (table III). The 7–12-part polyvalent vaccines protected against more types than the two-part vaccines. Also, more groups of mice showed partial protection (2–5 survivors out of 6 challenged), with a consequent reduction in the number of groups that received no protection. One polyvalent vaccine (1/2/3/15/20/35/44) induced full or partial protection against all but five of the 77 types.

Single injections of two-part vaccines resulted in only small gains in protection compared with monovalent vaccine. Both of the two-part vaccines (1/44 and 2/20)

TABLE III
Cross-protection of mice after one dose of polyvalent *klebsiella* vaccine

Capsular types of <i>K. aerogenes</i> present in polyvalent vaccines	Number capsular types (n=77) against which vaccine gave			Capsular types against which vaccine gave no protection
	complete*	partial	no†	
1 44	48	16	13	2 15 20 23 32 35 46 48 50 59 63 69 73
2 20	46	23	8	15 35 50 52 65 70 71 74
1 2 20 44	51	15	11	11 15 25 32 35 41 45 50 60 73 74
1 2 20 35 44	49	18	10	2 4 6 15 16 17 36 60 63 74
2 3 20 35 44	58	7	12	1 2 11 20 23 25 32 46 69 71 72 74
1 2 3 20 35 44	55	12	10	12 15 27 29 55 63 67 69 73 74
1 2 3 15 20 35 44	49	23	5	35 56 73 74 75
1 2 15 20 35 36 44	53	18	6	4 36 63 70 74 76
1 2 3 15 20 35 36 44 50 63	53	17	7	1 4 36 63 70 74 76
1 2 3 15 20 35 36 44 50 63 70 74	61	10	6	2 35 50 52 69 70

* 6/6 mice protected.

† 0/6 protected.

failed to protect mice against some types of *K. aerogenes* for which the component monovalent vaccines had given protection; but, conversely, they induced protection against types for which neither of the individual component vaccines had protected.

Protection by two doses of vaccine

Two injections of monovalent vaccines 1 and 2 (table IV) gave protection against an almost identical range of types to that protected by one injection (table II); also the protection induced by one or two injections of two-part vaccines (1/44, 2/20) was nearly identical.

However, with vaccines containing more than seven components, two injections induced protection against more types than one injection. For example, vaccine 1/2/3/15/20/35/36/44/50/63/70/74 protected completely against 61 types after one dose (table III) and against 71 types after two doses (table IV). Indeed, two doses of this vaccine gave some degree of protection against all 77 types.

When each of the two doses of polyvalent vaccine 1/2/3/15/20/35/36/44/63 was increased 10-fold there was a substantial broadening of the protective response (table IV): the higher dose (90 µg/mouse), gave complete protection against 73 types, whereas the lower dose (9 µg) protected against only 46 types.

Toxicity of vaccine

Monovalent vaccines 2, 3 and 44 were the least toxic, failing to kill mice with 5000 immunising doses (table V). The two most toxic vaccines were 36 and 50, which killed 50% of the mice with 1250 immunising doses. The polyvalent vaccines were found to be less toxic than the most toxic of the individual components: a 6-part polyvalent vaccine killed only 50% of mice with 5000 immunising doses, and a 9-part vaccine killed no mice with 1250 immunising doses.

TABLE IV

*Cross-protection of mice after two doses of monovalent or polyvalent klebsiella vaccine**

Capsular types of <i>K. aerogenes</i> present in monovalent or polyvalent vaccine	Number of capsular types (n = 77) against which vaccine gave			Capsular types against which vaccine gave no protection
	complete† protection	partial protection	no‡ protection	
1	43	13	21	2 15 20 24 25 32 35 44 46 48 49 52 55 57 58 59 60 62 63 64 69
2	41	18	18	3 5 6 15 20 23 24 27 35 40 42 44 47 49 50 63 65 74
20	38	18	21	2 3 4 6 24 26 31 35 40 41 44 45 49 50 63 68 69 70 71 73 74
44	44	24	9	1 2 35 50 58 59 63 64 65
1 44	46	17	14	2 12 20 23 32 35 46 48 51 57 59 63 69 74
2 20	46	23	8	15 35 50 52 65 70 71 74
1 2 20 44	51	13	13	11 15 20 25 32 35 41 45 50 60 70 73 74
1 2 3 15 20 35 44 50 63 74	54	20	3	57 71 74
1 2 15 20 35 44 63 70 74	56	13	8	2 3 49 50 68 70 73 74
1 2 15 20 35 36 44 63 70 74	64	9	4	5 15 29 35
1 2 3 15 20 35 36 44 50 63 70 74	71	6	0	
1 2 3 15 20 { 90µg	73	2	2	58 65
35 36 44 63 { 9µg	46	19	12	1 3 16 17 18 19 27 35 58 69 71 73

* Mice received a second dose of the same vaccine, 3 days after the first, and were challenged 4 days later.

† 6/6 mice protected.

‡ 0/6 protected.

TABLE V

Toxicity of monovalent and polyvalent klebsiella vaccines

Capsular types of <i>K. aerogenes</i> present in vaccines	Number of deaths out of 6 mice given the following doses of vaccine						
	10 mg	5 mg	2.5 mg	1.25 mg	100 µg	10 µg	1 µg*
1	6	6	0	0	0	0	0
2	6	0	0	0	0	0	0
3	6	0	0	0	0	0	0
15	6	6	6	0	0	0	0
20	6	6	6	0	0	0	0
35	6	6	0	0	0	0	0
36	6	6	6	3	0	0	0
44	6	0	0	0	0	0	0
50	6	6	6	3	0	0	0
63	6	3	0	0	0	0	0
1 2 3 20 35 44	6	3	0	0	0	0	0
1 2 3 15 20 35 36 44 63	6	6	6	0	0	0	0

* 1 µg was the dose used for immunisation (table II).

DISCUSSION

Most *K. aerogenes* infections occur in patients who are already weakened by disease (Young, 1982) and in whom contemporary chemotherapy seems ineffective (Thomas *et al.*, 1977). The experiments with polyvalent klebsiella vaccines are a promising step towards an immunological approach to such infections. In previous experiments (Roe and Jones, in press) mice were found to be protected against lethal infection as early as 4 days after a single injection of a monovalent vaccine. The present experiments have shown that polyvalent vaccines can induce a similar early protection against almost all 77 capsular types. Broad spectrum early protective responses are not unique to klebsiella vaccines; they were found in mice immunised with pseudomonas vaccines (Jones, 1971) and in severely burned patients whose lives were saved by early protective responses induced by a polyvalent pseudomonas vaccine (Roe and Jones, 1983).

Each klebsiella vaccine induced protection against 30 or more capsular types of *K. aerogenes* and the vaccines that induced the widest protective cover were usually derived from mouse-virulent strains. Thus if culture filtrates were to be used for making a polyvalent vaccine against all 77 types, it would be advantageous to seek virulent strains for vaccine preparation.

The pooling of monovalent vaccines produced some confusing protective responses in the mice, as protection against a challenge strain could be either lost or gained as a result of pooling the vaccines. One explanation for this observation is that each monovalent vaccine contains several immunogenic constituents, and when combined with other vaccines the amounts of the minor constituents in the polyvalent vaccine are above or below a level capable of initiating an immune response. The practical outcome was that there seemed no way of predicting what protective response might be produced after pooling. Thus the polyvalent vaccines were made on a 'trial and error' basis, adding extra monovalent vaccines that gave protection against capsular types for which the initial polyvalent vaccine failed to protect, in the hope that protection against the remaining types might be achieved.

The protective response was enhanced, and became more predictable and stable, by increase in the number of doses and in the size of each dose; some of the protection lost on combining monovalent vaccines re-appeared. We are investigating this further.

The medium used for vaccine production was intended to enhance the yield of capsular polysaccharide; it had a high sugar:nitrogen ratio (Duguid and Wilkinson, 1953; Wilkinson, Duguid and Edmunds, 1954). After 24 h, growth was limited by nitrogen deficiency, and the excess sugar became available during the next 24 h for synthesis of capsular polysaccharide. We attempted to extract the vaccines from the culture filtrates, undenatured and free from medium and toxic contaminants; this was achieved by culturing in a synthetic dialysable medium and using low stress physical methods for extraction (diafiltration and centrifugation). The resulting vaccines were water-soluble and caused no harmful effects in mice at the immunising doses: the level of lethal toxicity was > 1000 protective doses of monovalent vaccine.

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