Vaccination against *Klebsiella aerogenes*

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SUMMARY

Klebsiella vaccine was prepared from strains of *Klebsiella aerogenes* with capsular types K1, K36, K44 and K Cross (a type which cross-reacts *in vitro* with sera from many klebsiella capsular types). The vaccine was extracted by dialysis and ultrafiltration from capsular material released during growth of the bacteria in a five-day batch culture.

Mice given one dose of vaccine from K1a (1·0 μg/mouse) survived lethal intraperitoneal challenge of 11/11 homologous klebsiella strains four days after vaccination; 14 days after vaccination protection against the same challenge strains had declined to 5/11 strains. Vaccines from K1a, b, c, K36, K44 and K Cross induced homologous protection and protected mice against different ranges of heterologous klebsiella capsular types.

The protective response of the mice was greatly enhanced by administering three doses of the vaccines. Vaccines from K1, K36, K44 and K Cross protected mice against 14/20, 11/20, 10/20 and 9/20 homologous and heterologous klebsiella challenge strains respectively.

None of the klebsiella vaccines was toxic for mice at the immunizing dose (1·0 μg/mouse). Vaccine from K36 was the most lethal, killing mice at 10^3 immunizing doses. The least toxic vaccine was from K44, which killed mice at 10^4 immunizing doses.

INTRODUCTION

Gram-negative bacilli are the leading cause of nosocomial infection (Kreger *et al.* 1980; Bryan, Reynolds & Brenner, 1983). The mortality of patients who develop Gram-negative bacteraemia is between 20 and 50 % (Zinner & Peter, 1983). In England and Wales *Klebsiella aerogenes* was second only to *Escherichia coli* as the most important cause of Gram-negative bacteraemia in hospital-acquired infections (Young, 1982).

Evidence of multiply drug-resistant strains of *K. aerogenes* in patients (Thomas *et al.* 1977); Montgomerie, 1979; Casewell, 1982) suggests a need for an alternative treatment for *K. aerogenes* infections. Immunotherapy offers an alternative form of treatment but has received little attention (Nakashima & Kato, 1977; Jones, 1981; Riottot, Fournier & Jouin, 1981; Cryz, 1983) possibly because of the existence of 77 different capsular types (Palfreyman, 1978) which can be involved in klebsiella infections (Orskov, 1956; Riser & Noone, 1981; Smith, Digori & Eng, 1982).

Currently there is no immunological treatment specifically for klebsiella infections (Cryz, 1983), although immunological treatment against other hospital-acquired Gram-negative bacilli has been successful (Ziegler *et al.* 1982) especially against *Pseudomonas aeruginosa* (Feller, 1966; Alexander *et al.* 1969; Jones, Roe & Gupta, 1980; Roe & Jones, 1983). These clinical studies showed that the surface antigens of Gram-negative bacilli made the most efficacious vaccines especially when these antigens were one of the main virulence factors of the bacteria. In *K. aerogenes* the dominant surface component is the capsule, and it is the capsule which exerts antiphagocytic activity (Robbins *et al.* 1980; Williams *et al.* 1983), interferes with the host raising a protective immune response (Orskov, 1956; Yokochi, Nakashima & Kato, 1979) and is responsible for antigenaemia that acts to adsorb protective antibody (Pollack, 1976; Domenico, Johanson & Strauss, 1982).

In this study we vaccinated mice with capsular material from different capsular types of *K. aerogenes* to test the ability of the capsular material to induce protection against homologous and some heterologous klebsiella strains.

MATERIALS AND METHODS

Bacteria

All strains of *K. aerogenes* used in the study were identified using methods by Davis, Lilly & Lowbury (1968). The bacteria were capsular-typed by counter-immunoelectrophoresis at the Public Health Laboratory Service (PHLS), Coventry using the methods of Palfreyman (1978). Bacteria were stored freeze-dried and during the course of the experiments were maintained at 4 °C by weekly subculture on nutrient agar (Oxoid) slopes.

Vaccine production

The six strains of *K. aerogenes* used for making the vaccines were isolated from patients with burns at Safdarjang Hospital, New Delhi. Three strains were of capsular type 1 – K1 a, K1 b and K1 c – and the other strains were of capsular type K36, K44 and K Cross (a strain of *K. aerogenes* which cross-reacts in Quellung tests with antisera against many other klebsiella capsular types).

Bacterial challenge

The bacteria used for intraperitoneal (i.p.) challenge in the protection tests were obtained from PHLS, Coventry (*K. aerogenes* capsular type 1, strains K1 d – K1 k), from the Birmingham Accident Hospital (strains K2, K16, K41, K48 and K63) and from Safdarjang Hospital, New Delhi (strains K18a, K18b, K29, K23, K25, K62, K79, KNTa and KNTb).

For i.p. challenge strains of *K. aerogenes* were subcultured overnight at 37 °C on nutrient agar (Oxoid). Loopfuls of the bacteria were suspended in saline and shaken on a Whirlimixer (Fisons) to ensure an even suspension of the bacteria. Doubling dilutions of the suspension from 9×10^7 to 7.5×10^5 bacteria/ml were made in 10 ml saline and the number of bacteria was estimated using a turbidity meter (Drott). Bacteria were inoculated (1·0 ml/mouse) into groups of six SPF female, albino mice, BKW2 weighing 22–25 g. The smallest number of bacteria (1·0 MLD, minimum lethal dose) which killed all six mice within 24 h of challenge was recorded, and used for challenging immunized mice.

Vaccine

Vaccines were extracted from culture filtrates of *K. aerogenes* K1a, K1b, K1c, K36, K44 and K Cross. The bacteria were cultured for five days at 37° in 1l batches of a synthetic medium, described by Liu (1964) and used by Carney & Jones (1968) to culture *P. aeruginosa*, prior to vaccine extraction.

The culture medium (1l) was inoculated with 10 ml of a saline suspension of a colony of *K. aerogenes* from an 18 h subculture grown on 4 % blood agar at 37°. The culture was stirred and aerated by bubbling filtered air (0·45 μm Millipore membrane) at 500 ml/min through a sintered glass pad at the bottom of the culture vessel.

After five days aeration at 37° the bacteria were killed by adding formaldehyde to the culture to give a final concentration of formaldehyde of 0·12 %. Bacteria were removed by centrifugation (10000 rev./min, 50 min, 12 °C). As the supernatant was viscous it was pre-filtered through a 25AP Millipore and a 0·65 μm DA filter under positive pressure before filtration through a 15AP Millipore pre-filter and 0·45 μm HA Millipore membrane. The filtrate was ultrafiltered through a No. 1 Visking dialysis membrane (450 mmHg) to reduce the volume to 150 ml, dialysed against running tap water for 24 h and freeze-dried.

Protection tests

Mice were vaccinated i.p. with 1·0 ml of saline containing 1·0 μg or 0·1 μg of klebsiella vaccines from K1a, K1b, K1c, K36, K44 and K Cross. In most experiments groups of six mice were challenged i.p. four days after vaccination with 1·0 MLD of *K. aerogenes*. In one experiment mice were challenged seven days after a single injection of vaccine and in another experiment a group of mice were challenged 14 days after one dose of vaccine.

Mice were also given three doses of (1·0 μg/mouse) klebsiella vaccines (K1a, K36, K44 and K Cross). Groups of 120 mice were vaccinated i.p. on days 0, 3 and 6 with the four vaccines. Four days later mice were challenged i.p. with 1·0 MLD of 20 different strains of *K. aerogenes* (see Table 5 for list of challenge strains).

In all challenge experiments a group of six unvaccinated control mice similar in type, weight, age and sex to the vaccinated mice were subjected to the same bacterial challenge as the vaccinated mice.

RESULTS

Protection against homologous klebsiella strains

Mice were inoculated with a single dose of vaccine (1·0 μg/mouse) from *K. aerogenes* K1a. Groups of six mice were challenged i.p. with 1·0 MLD of 11 different strains of K1 (a–k) 4, 7 and 14 days after vaccination. A control group of six mice was also challenged with the same klebsiella suspensions. By the fourth day after vaccination the mice were protected against a lethal challenge of all 11 strains of K1 (Table 1) which killed unvaccinated control mice.

Seven and 14 days after vaccination mice were protected against fewer challenge strains than four days after vaccination. Mice were not protected against challenge strains, b, e, g and j, and b, e, f, g, h and j on days 7 and 14 respectively.

Table 1. *Protection of mice against homologous strains of* K. aerogenes *K1 a–k by a vaccine extracted from* K. aerogenes *K1, strain a*

Day of challenge after vaccination	Deaths in groups of six vaccinated mice challenged* i.p. with 1·0 MLD of the following *K. aerogenes* K1 strains										
	a	b	c	d	e	f	g	h	i	j	k
4	0	0	0	0	0	0	0	0	0	0	0
7	0	4	0	0	2	0	3	0	0	3	0
14	0	4	0	0	6	6	4	4	0	6	0

* i.p. challenge administered four days after a single dose of vaccine.

Table 2. *Protection of mice against homologous strains of* K. aerogenes *K1 a–k by vaccines extracted from* K. aerogenes *K1, strains a, b and c*

Vaccine used for immunization was extracted from *K. aerogenes* K1 strains	Deaths in groups of six mice challenged* i.p. with 1·0 MLD of the stated *K. aerogenes*, K1 strains										
	a	b	c	d	e	f	g	h	i	j	k
a	0	0	0	0	0	0	0	0	0	0	0
b	0	0	0	0	0	0	0	0	0	0	0
c	0	0	0	0	0	0	0	3	0	4	0
Unvaccinated control mice	6	6	6	6	6	6	6	6	6	6	6

* i.p. challenge administered four days after a single dose of vaccine.

A lower dose of vaccine from K1 a (0·1 µg/mouse) protected mice against all but strains K1 b and j when the mice were challenged four days after vaccination.

Vaccines from two other strains of K1, (strains b and c) were used to vaccinate mice (1·0 µg/mouse). Vaccine from K1 b gave homologous protection similar to the vaccine from K1 a (Table 2), but vaccine from K1 c induced partial protection against two of the challenge strains, K1 h and j; 3/6 and 4/6 of the mice died respectively when challenged four days after vaccination.

Protection against heterologous klebsiella strains

Groups of mice were vaccinated with vaccines from K1 a, b and c. Four days after the single dose of vaccine groups of six mice were challenged with the homologous strain and 13 heterologous klebsiella strains (Table 3).

The three vaccines protected mice against challenge by the three strains of homologous capsular type. There were other similarities in protective response of vaccines from K1 a, b and c. Each vaccine protected mice against strains K16, K18 and K79 and each vaccine failed to protect mice against K20 a and K63. Apart from the three heterologous strains, each vaccine protected mice against two other strains of klebsiella; vaccine from K1 a protected mice against K25 and K Cross, vaccine K1 b protected against K2 and KNT and K1 c protected against K2 and K25.

Table 3. *Protection of mice against homologous and heterologous klebsiella capsular types with three vaccines from different strains of* K. aerogenes *K1*

Vaccine used for immunization was extracted from *K. aerogenes* K1 strains	Deaths in groups of six mice challenged* i.p. with 1·0 MLD of *K. aerogenes* of capsular types													
	K1a	K1b	K1c	K2	K16	K18	K20a	K20b	K25	K44	K63	K79	K Cross	KNTa
a	0	0	0	3	0	0	6	3	0	4	6	0	0	3
b	0	0	0	0	0	0	6	3	6	6	6	0	2	0
c	0	0	0	0	0	0	6	3	0	3	6	0	3	4
Unvaccinated control mice	6	6	6	6	6	6	6	6	6	6	6	6	6	6

* i.p. challenge administered four days after a single dose of vaccine.

12-2

Table 4. *Protection of mice against heterologous klebsiella capsular types with vaccines extracted from K. aerogenes K36, K44 and K Cross*

Vaccine used for immunization was extracted from K. aerogenes capsular types	Deaths in groups of six mice challenged* i.p. with 1·0 MLD of K. aerogenes of capsular types											
	K2	K16	K18	K25	K36	K41	K44	K48	K62	K63	K79	K Cross
K36	6	3	4	6	0	0	6	6	6	6	6	5
K44	6	0	0	6	0	6	0	6	6	6	0	0
K Cross	0	0	0	3	2	6	0	4	0	6	6	0
Unvaccinated control mice	6	6	6	6	6	6	6	6	6	6	6	0

* i.p. challenge administered four days after a single dose of vaccine.

Table 5. *Protection of mice against homologous and heterologous klebsiella capsular types with three inoculations of klebsiella vaccines*

Vaccine used for immunization was extracted from K. aerogenes capsular types	Deaths in groups of six mice challenged* i.p. with 1·0 MLD of K. aerogenes of capsular types and strains																		
	K1a	K1b	K1c	K2	K16	K18a	K20a	K23	K25	K36	K41	K44	K48	K62	K63	K79	K Cross	KNTa	KNTb
K1a	0	0	0	0	0	0	0	0	0	0	3	0	0	0	6	3	3	3	3
K36	0	4	3	0	0	3	3	0	0	0	0	0	0	0	3	4	6	4	4
K44	3	4	3	3	3	6	0	0	0	0	0	0	2	0	3	6	0	3	4
K Cross	6	6	6	0	0	2	0	0	0	3	1	0	0	3	6	6	0	2	2
Unvaccinated control mice	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6

* i.p. challenge administered four days after three doses of vaccine.

Table 6. *Toxicity of klebsiella vaccines for mice*

Vaccine extracted from *K. aerogenes* capsular type	Deaths in groups of five mice inoculated i.p. with the stated doses of vaccine (μg/mouse)						
	1.0×10^4	5×10^3	2.5×10^3	1.25×10^3	10^2	10	1.0*
K1a	5	5	0	0	0	0	0
K36	5	5	5	2	0	0	0
K44	5	0	0	0	0	0	0
K Cross	5	4	2	0	0	0	0

* Immunizing dose of vaccine.

Vaccines from K36, K44 and K Cross induced homologous protection and, like vaccine from K1a, b and c, protected mice against different selections of heterologous challenge strains (Table 4).

Vaccine from K36 was a poor immunogen, it only protected mice against 1/11 of the heterologous strains, but vaccines from K44 and K Cross protected mice against 5/11 and 4/11 of the same challenge strains respectively.

The range of heterologous klebsiella strains which vaccines from K1a, K36, K44 and K Cross protected against was enhanced by increasing the frequency of injection of the vaccines from one to three (Table 5). After three injections, vaccine from K36 protected mice against K2, K16, K25, K44, K48 and K62, bacteria which it failed to protect against after one dose.

Vaccine from K1a failed to give protection against only one challenge strain, K63. It gave complete protection to mice against 14/20 heterologous strains after three injections and protected 50% of the mice against the other five challenge strains.

The least protective vaccine, after three injections, was K Cross, which failed to given any protection at all to mice challenged with the three K1 strains, K63 and K79.

Toxicity of klebsiella vaccines

Vaccines from K1a, K44 and K Cross varied considerably in their lethality for mice on i.p. inoculation (Table 6). The least toxic vaccine extracted from K44 killed mice at 10000 immunizing doses (1.0 μg/mouse). The most toxic vaccine was extracted from K36. It killed 2/5 mice at 1250 immunizing doses.

DISCUSSION

The immunogenic properties of capsular material from *K. aerogenes* were studied as a first step towards making an anti-klebsiella vaccine. The klebsiella vaccines were similar to other vaccines from opportunistic Gram-negative bacilli (Zinner & Peter, 1983; Jones, 1983) in that they were simple to extract with mild physical techniques, centrifugation, dialysis and ultrafiltration. The toxicity for mice of vaccines from K1a, K30, K44 and K Cross was low, between 10^3 and 10^5 immunizing doses per mouse. An immunizing dose (1.0 μg/mouse) was based on a single effective protective dose of vaccine given to mice four days before challenge.

Protection induced by the vaccines was not type-specific as is sometimes claimed (Cryz, 1983). All six vaccines induced both homologous and heterologous protection as early as four days after one dose of vaccine. The range of capsular types each vaccine protected against was however unpredictable. For future preparations of vaccine it would be worth searching for bacteria that yield vaccines giving a broad spectrum of protective cover, especially as epidemiological studies show a wide range of klebsiella capsular types appearing in clinical isolates (Casewell & Talsania, 1979; Riser & Noon, 1981).

The number of heterologous capsular types each vaccine protected against was increased by increasing the frequency of vaccination. There are limits to this approach; for example, after three injections of vaccine from K Cross, mice were still not protected against K1a, b, c, K63 or K79. It seems that, to achieve protection with these vaccines against any desired range of klebsiella capsular types, a multivalent vaccine will be required. Currently work is in progress to study the protective properties of multivalent klebsiella vaccines.

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