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Capsular Serotypic Specificity of the Protection Conferred on Mice by *Klebsiella pneumoniae* Ribosomal Preparations

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Klebsiella pneumoniae ribosomal preparations protect mice immunized by the subcutaneous route against an intraperitoneal challenge of 100 50% lethal doses. The minimal protective doses are 5 and 0.4 μg of proteins for preparations extracted from strains of capsular serotypes 1 and 2, respectively. This difference in protective activity is also found in bacteria killed by Formalin. The protective activity of these preparations is not diminished by their purification on sucrose gradient, which eliminates most of the membrane vesicles which are visible by electron microscopy. The use of four strains of *K. pneumoniae* belonging to capsular serotypes 1 and 2 allowed us to show that the immunoprotective capacity of the ribosomal preparations was specific to the capsular serotype of the origin strain. This was confirmed by experiments in which the serum of immunized mice was transferred passively. The experimental data favor the presence in the ribosomal preparation of antigens belonging to the bacterial surface and resisting elimination by ultracentrifugation on sucrose gradient. These surface antigens (possibly capsular polysaccharide) at least play a role in the orientation of the specificity of the protection induced by the ribosomal preparations.

The immunoprotective capacity of bacterial ribosomal preparations has been studied by Youmans and Youmans (28, 29) in mice with *Mycobacterium tuberculosis*. Since that time, immunogens of similar origins have been derived from *Salmonella typhimurium* (4, 8, 10, 14, 25, 26), *Staphylococcus aureus* (27), *Pseudomonas aeruginosa* (11), *Streptococcus pneumoniae* (6, 19, 23, 24), *S. pyogenes* (17), *Neisseria meningitidis* (22), *Vibrio cholerae* (7, 9), *Pasteurella multocida* (2), *Klebsiella pneumoniae* (6), *Histoplasma capsulatum* (5, 20), *Brucella abortus* (3), and *Haemophilus influenzae* (13, 21). However, considerable controversy exists in the literature concerning the immunogenic principle of these subcellular extracts. For example, Johnson (10) found that a ribosomal protein is active as a protective antigen against *S. typhimurium*, but Venneman et al. (26) favor the ribosomal RNA as a protective antigen, and Eisenstein (4) presents evidence that O antigens contaminate both ribonucleic acid and protein ribosomal extracts and are responsible for at least part of their strain-specific protective activity.

In fact, the ribosomal origin of immunoprotective antigen is doubtful if we consider that humoral or cellular immunoprotective mechanisms are obliged to act on intact viable bacterial cells and should imply only surface bacterial antigen (18). We can ask if the immunoprotective antigen is effectively a ribosomal constituent, or if it is a contaminant (for example, issued from the cell surface) that is carried by ribosomes during their extraction. In the present investigation, ribosomal preparations were extracted from four strains of *K. pneumoniae*, two capsular serotype 1 strains and two capsular serotype 2 strains. We have studied the specificity of the protection conferred in mice with these different preparations to determine whether the immunizing potential of ribosomal preparations has the same specificity as the strain capsular serotype.

The results presented in this paper clearly show the serotypic specificity of ribosomal immunoprotective capacity and suggest that capsular polysaccharides are responsible for at least part of the ribosomal immunoprotective activity.

MATERIALS AND METHODS

Mice. Outbred Swiss OF_1 from IFFA-CREDO (F 69210 L'Arbresle) were used. Experiments were carried out, using males at 4 or 5 weeks of age.

Bacterial strains. Strains used in this study included the following: *K. pneumoniae* 7825, capsular serotype 1, biotype b (Richard 15); *K. pneumoniae* A-215, capsular serotype 1, biotype b; *K. pneumoniae* 52-145, capsular serotype 2, biotype e, obtained from the Pasteur Institute Collection; and *K. pneumoniae* 7823, capsular serotype 2, biotype d, kindly supplied by L. Chedid (Pasteur Institute).

The intraperitoneal 50% lethal dose (LD_{50}), calculated by the Reed-Muench formula (15), was less than 5 colony-forming units (CFU) per mouse for 7825, 52-

145, and 7823 strains, and 10^3 CFU/mouse for strain A-215.

Growth of bacteria. The lyophilized bacterial strain was inoculated in a 10-ml, then a 500-ml, and then a 5,000-ml flask of nutrient broth (Institut Pasteur Production no. 64-067). The flasks were incubated at 37°C for 12 to 15 h without agitation, and the bacterial cells were harvested by continuous-flow centrifugation at 40,000 × *g* at 4°C.

Extraction of CRP. Crude ribosomal preparations (CRP) were prepared by a slight modification of the method described by Schalla and Johnson (17). All procedures were conducted at 4°C. Bacterial cells were washed twice in cold Tris-hydrochloride 0.01 M buffer containing 0.01 M $MgCH_3CO_2$, 0.06 M NH_4Cl, (TMN; pH 7.4) and suspended in the same buffer (100 mg/ml, wet wt/vol). Lysozyme (ICN Pharmaceutical Co.) was added at a concentration of 15 mg/g of packed cells. After incubation for 1 h at 37°C, 2.0 μg of deoxyribonuclease (Sigma Chemical Co.) per ml was added, and the cells were broken in a Ribi press at 40,000 lb/in^2. Sodium dodecyl sulfate (0.5% wt/vol) was added, and NH_4Cl was added to a final concentration of 0.35 M. The extract was centrifuged at 25,000 × *g* for 20 min to remove cellular debris and intact cells. A 21-g amount of dry $(NH_4)_2SO_4$ was added to 100 ml of the supernatant fluid with gentle stirring for 3 min. The slurry was centrifuged at 25,000 × *g* for 20 min, and the pellet was discarded. An additional 21 g of dry $(NH_4)_2SO_4$ was added to the supernatant fluid with gentle stirring for 3 min. The slurry was centrifuged at 25,000 × *g* for 20 min, and the supernatant fluid (SF-1) was saved. The precipitate was suspended in TMN and centrifuged for 20 min at 25,000 × *g*.

The supernatant fluid (SF_2) from this last centrifugation was combined with SF_1 and centrifuged at 150,000 × *g* for 3 h. The pellet was suspended in TMN, dialyzed 48 h against several changes of TMN, and centrifuged at 150,000 × *g* for 2 h. The final CRP was suspended in TMN and stored in small volumes at −20°C.

Sucrose density gradient. A 0.5-ml sample of CRP (1.5 mg of protein/ml) was layered on a 20 to 50% (wt/vol) linear sucrose gradient on TMN and centrifuged at 100,000 × *g* for 18 h. Fractions, 0.5 ml each, were collected and read at 260 and 280 nm. The purified ribosomal peak (PRP) was pooled and concentrated on an XM 50 Amicon membrane.

Biochemicals assays. Protein was determined by the method of Lowry (12), ribonucleic acid was measured by the orcinol method (1). Bovine serum albumin and yeast ribonucleic acid (Sigma Chemical Co.) served as standards.

Electron microscopy. Ribosomal preparations were negatively stained with 3% phosphotungstic acid (pH 5). The negative stains were viewed in a Siemens 101 electron microscope at 80 kV.

Whole cell vaccines. A bacterial suspension of 10^8 CFU/ml of 0.15 M NaCl was prepared from a 15-h nutrient agar culture. Formalin was added to a final concentration of 0.5%. After 12 h at 4°C, no viable *K. pneumoniae* were noticeable by plate culture technique.

Immunization and challenge. Mice were immunized subcutaneously with 0.5 ml of the vaccine preparation diluted in sterile apyrogenic saline without adjuvant. Immunizing doses of ribosomal preparations were calculated on the basis of protein content. Controls were inoculated subcutaneously with 0.5 ml of sterile pyrogen-free saline.

All mice were challenged intraperitoneally with 100 LD_{50}s of *K. pneumoniae*. The bacterial count at the time of the challenge was confirmed by the spread plate technique. Deaths, which occurred principally between days 2 to 8, were nevertheless recorded 14 days after challenge.

Passive transfer of serum. Mice immunized with 15 or 50 μg of CRP were exsanguinated 14 days later, and their serum was pooled. Normal recipients received 0.2 to 0.5 ml of serum via lateral tail vein. All recipients, plus control group injected with saline, were challenged intraperitoneally with 100 LD_{50}s of *K. pneumoniae* 24 h after transfer.

RESULTS

Chemical studies. Chemical assays indicated that the ratio of RNA to protein varies from about 0.8 to 2. The ratio between absorbances at 260 and 280 nm varies from 1.5 to 1.8.

Immunoprotective capacity of Formalin-treated cells and CRP: dose response. Seven groups of mice were inoculated with from 10^5 to 10^8 Formalin-treated cells of *K. pneumoniae* 7825 or 52-145, and 11 other groups were immunized with from 0.4 to 100 μg (expressed as protein content of preparation) of CRP. Two control groups received saline. All animals were challenged 14 days later with 100 LD_{50}s of homologous strain.

A dose response relationship was observed (Table 1). 1×10^8 *K. pneumoniae* 7825 whole cells, 1×10^6 *K. pneumoniae* 52-145 whole cells, 5 μg of *K. pneumoniae* 7825, or 0.4 μg of *K. pneumoniae* 52 145 CRP protected at least 60% of the mice. The better activity of *K. pneumoniae* 52-145 whole cells compared with *K. pneumoniae* 7825 was corroborated with CRP extracted from these strains.

Effect of rest period upon survival. Three groups of mice were immunized subcutaneously at day zero with 5 μg of *K. pneumoniae* 7825, and three other control groups received saline. At days 7, 14, and 28 after immunization, one immune and one control group were challenged intraperitoneally with 100 LD_{50}s. The survival at days 3, 5, 7, and 14 after challenge is reported in Table 2. When animals were challenged at day 7, the only result observed was a mortality delay of the immune group compared with the control group from day 3 to 5 after challenge. In contrast, animals challenged 14 and 28 days after immunization were definitively protected.

Immunogenic capacity of purified ribosomal preparations. Ultracentrifugation on sucrose gradient of CRP eliminated most of the

TABLE 1. *Effect of antigen concentration on immunity induced by whole cells or crude ribosomal vaccine*[a]

Immunization and challenge strain	Antigen type	Dose[b]	No. of survivors/total	Survival (%)	P[c]
K. pneumoniae 7825, capsular serotype 1	Formalin-treated cells	1×10^8	10/10	100	<0.01
		1×10^7	2/10	20	NS
		1×10^6	1/10	10	NS
	CRP	100 μg	10/10	100	<0.01
		50 μg	25/30	83	<0.01
		15 μg	17/20	85	<0.01
		5 μg	13/20	65	<0.01
		2 μg	3/10	30	NS
	Saline		0/10	0	
K. pneumoniae 52-145, capsular serotype 2	Formalin-treated cells	1×10^8	10/10	100	<0.01
		1×10^7	8/10	80	<0.05
		1×10^6	6/10	60	NS
		1×10^5	0/10	10	NS
	CRP	50 μg	64/66	97	<0.01
		10 μg	35/38	92	<0.01
		2 μg	17/20	85	<0.01
		0.4 μg	20/30	67	<0.01
		0.08 μg	11/30	37	<0.01
		0.016 μg	1/20	5	NS
	Saline		0/10	0	

[a] All animals were immunized subcutaneously on day zero and challenged intraperitoneally with 100 LD_{50}s 14 days after immunization.
[b] Values indicate number of Formalin-treated cells, unless indicated otherwise. CRP doses are expressed as protein content of ribosomes (1 μg of ribosomal vaccine was extracted from 5×10^7 cells).
[c] NS, Not significant.

TABLE 2. *Effect of delay between immunization and challenge on immunoprotective activity of crude ribosomal preparations*[a]

Delay between immunization and challenge (days)	Experimental lot[b]	No. of survivors (day after challenge)				
		0	3	5	7	14
7	R	10	9	8	6	1
	C	10	1	0	0	0
14	R	10	10	10	10	9
	C	10	3	2	1	1
28	R	15	14	12	12	12
	C	15	1	0	0	0

[a] All animals where immunized subcutaneously with 5 μg (expressed as protein content) of CRP, and challenged intraperitoneally with 100 LD_{50}.
[b] R, Animals immunized with CRP; C, controls, sham immunization with saline.

visible membrane vesicle in this preparation (Fig. 1A and B). However, the immunoprotective capability of PRP did not diminish (Table 3).

Specificity of active immunization. CRP were extracted from four strains of *K. pneumoniae*, including two strains of capsular serotype 1 (strains 7825 and A.215) and two strains of capsular serotype 2 (strains 52-145 and 7823). Four groups of mice were immunized subcutaneously with each of the CRP; one control group received saline. After 14 days, each group (immune and control) was divided into four subgroups that were challenged intraperitoneally with 100 LD_{50}s of each *K. pneumoniae* strain. The results of this challenge (Table 4) show the serotypic specificity of the protection induced by ribosomal preparations.

Specificity of passive immunization. Passive transfer experiments were designed to confirm specificity of the protection demonstrated in the last paragraph for active immunization. Four groups of mice received, intravenously, serum of the CRP-immunized donor, and after 24 h, were divided in four subgroups that were challenged intraperitoneally with 100 LD_{50}s of each *K. pneumoniae* strain. The control group received saline intravenously 24 h before challenge. As with active immunization, the results (Table 5) argued in favor of the serotypic specificity of passive protection. Serum of mice immunized with type 1 CRP are less effective in immunity transfer than serum of type 2 CRP-immunized mice. These results confirmed data obtained in active immunization with type 1 or type 2 CRP or Formalin-treated whole cells.

DISCUSSION

Ribosomal preparations isolated from *K. pneumoniae* are good immunoprotective anti-

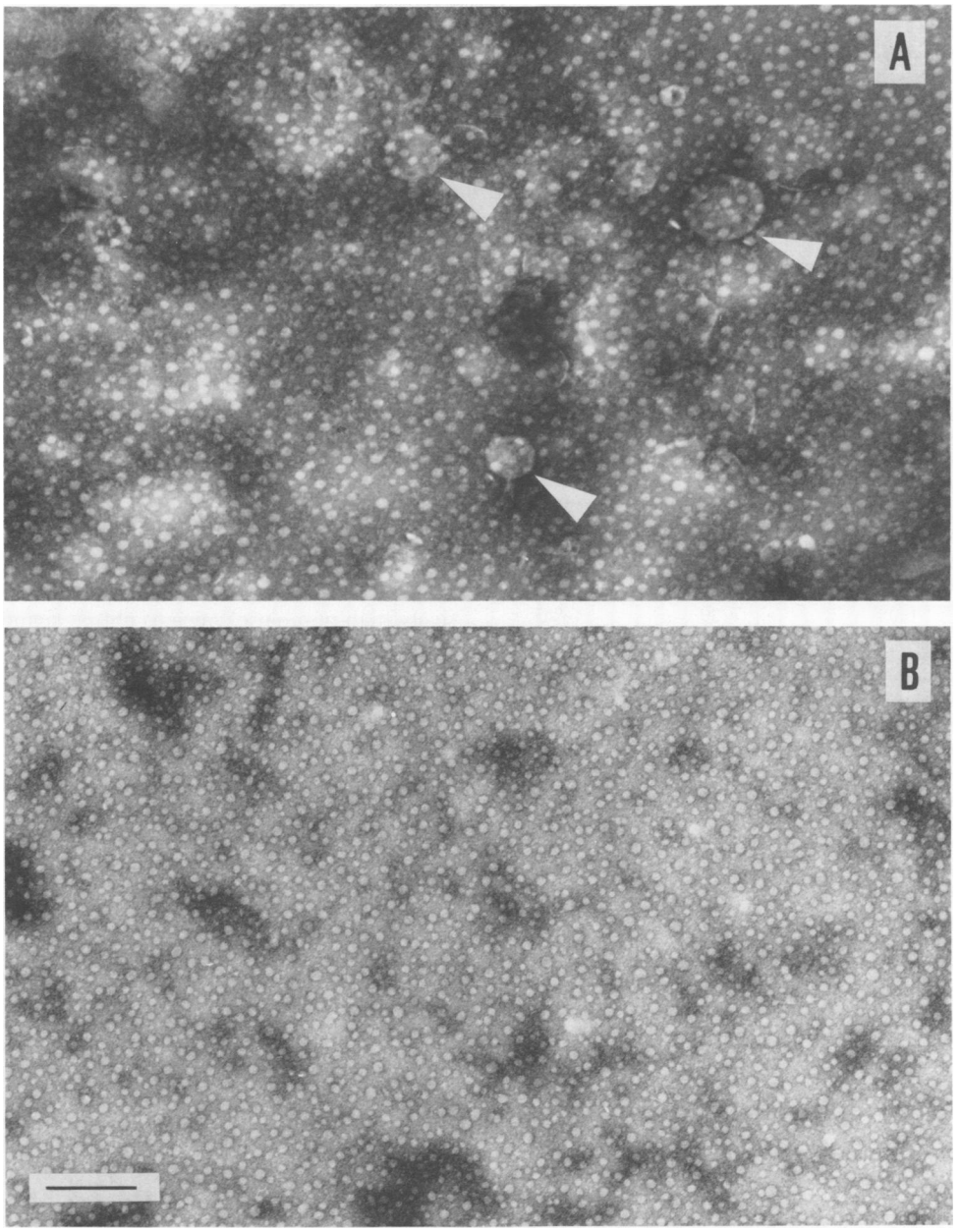

FIG. 1. *Electron photomicrograph of ribosomal preparations. (A) Crude ribosomal preparations. Vesicles of different size are visible (arrows). (B) Ribosomal preparation purified on sucrose gradient. Bar, 200 nm.*

gens in mice. This agrees with data presented in the literature. However, we find protective capacity at lower doses (0.4 and 5 μg of proteins for types 2 and 1, respectively) than the doses utilized by Schalla and Johnson (17) with *S. pyogenes* (100 to 1,500 μg) and Thompson and Snyder (23) and Thompson and Eisenstein (24) with *S. pneumoniae* (100 to 10,000 μg). Our doses are, in contrast, similar to those utilized by Fontanges et al. (6) with *K. pneumoniae* (5 μg), Venneman and Bigley (25, 26) with *S. typhimurium* (0.1 to 10 μg), and Lieberman (11) with *P. aeruginosa* (1 μg).

Protection was conferred 14 days after immunization with one subcutaneous injection of ribosomal preparations. This result agrees with

the literature, although Misfeldt and Johnson (14) with *S. typhimurium* and Fontanges et al. (6) with *K. pneumoniae* had to use two injections to improve protection.

Protection is specific for the capsular serotype of the strain from which that ribosome was extracted. Eisenstein (4) has demonstrated O antigen-dependent specificity of *S. typhimurium* ribosomal preparation immunoprotective capacity.

However, ribosomal preparations isolated from *S. pyogenes* (17), *S. pneumoniae* (19, 23, 24), and *Neisseria meningitidis* (22) protect mice against heterologous challenge. Our results suggest, as do Eisenstein's results, that surface antigens are copurified with ribosomes during their preparation and are responsible for at least part of their strain-specific protective activity. These contaminants were not eliminated during purification of ribosomes by sucrose gradient centrifugation. In fact, the presence of surface components, for example lipopolysaccharide, in ribosomal preparations has been demonstrated by Hoops et al. (8) and Misfeldt and Johnson (14) on *S. typhimurium*, and by Lieberman (11) on *P. aeruginosa*.

The passive transfer protection with immune serum demonstrated that anti-*K. pneumoniae* immunity is humoral, in keeping with the results of Thompson and Eisenstein (24), Swendsen and Johnson (19), and Fontanges et al. (6) with *S. pneumoniae*. In contrast, our results differ from those of Fontanges et al. (6) who did not observe transfer protection with serum of mice immunized with serotype 1 *K. pneumoniae* 7825. In fact, the protection reported in our work with this strain is slight and we must utilize a relatively high number of mice to obtain significant protection. In active immunization experiments, this lesser immunoprotective activity of serotype 1 preparation (Formalin-treated whole cells and

TABLE 3. *Comparison of the protective ability of CRP and PRP of K. pneumoniae 52-145*[a]

Dose (μg of protein)	No. of survivors/total	
	CRP	PRP
10	9/10	10/10
2	10/10	9/10
0.4	17/20	11/20
0.08	7/20	5/20
0.016	1/20	1/20

[a] All animals were challenged intraperitoneally with 100 LD_{50}s 14 days after subcutaneous immunization.

TABLE 4. *Serotypic specificity of the immunoprotective capacity of ribosomal preparation extracts from 4 different strains of K. pneumoniae*[a]

Origin of ribosomal vaccine		Challenge		No. of survivors/total	Survival (%)	P^b
Strain	Capsular serotype	Strain	Capsular serotype			
7825	1	7825	1	25/30	83	<0.01
		A.215	1	8/8	100	<0.01
		52-145	2	0/10	0	NS
		7823	2	2/8	25	NS
A.215	1	7825	1	20/30	67	<0.01
		A.215	1	9/10	90	<0.01
		52-145	2	0/10	0	NS
		7823	2	1/10	10	NS
52-145	2	7825	1	1/10	10	NS
		A.215	1	1/10	10	NS
		52-145	2	64/66	97	<0.01
		7823	2	10/10	100	<0.01
7823	2	7825	1	0/10	0	NS
		A.215	1	3/10	30	NS
		52-145	2	19/20	95	<0.01
		7823	2	19/20	95	<0.01
Saline		7825	1	0/10	0	
		A.215	1	0/10	0	
		52-145	2	0/10	0	
		7823	2	0/10	0	

[a] All animals were immunized subcutaneously on day zero with 50 μg (protein) of ribosomal preparations, and challenged at day 14 with 100 LD_{50}s intraperitoneally.

[b] NS, Not significant.

TABLE 5. *Serotypic specificity of transferred protection by serum of immunized mice with ribosomal preparations*[a]

Origin of ribosomal prepn		Challenge		Mice injected with immune serum		Control mice injected with saline		P^b
Strain	Capsular serotype	Strain	Capsular serotype	No. of survivors/total	%	No. of survivors/total	%	
7825	1	7825	1	24/62	39	0/35	0	<0.01
		A.215	1	18/20	90	4/20	20	<0.01
		52-145	2	1/8	12	1/10	10	NS
		7823	2	2/10	20	1/10	10	NS
A.215	1	7825	1	28/89	32	0/70	0	<0.01
		A.215	1	24/30	80	0/20	0	<0.01
		52-145	2	1/30	3	0/30	0	NS
		7823	2	0/10	0	1/10	10	NS
52-145	2	7825	1	1/10	10	2/10	20	NS
		A.215	1	0/10	0	0/10	0	NS
		52-145	2	12/12	100	0/12	0	<0.01
		7823	2	10/10	100	1/10	10	<0.01
7823	2	7825	1	1/28	4	0/30	0	NS
		A.215	1	4/20	20	0/20	0	NS
		52-145	2	36/38	95	0/40	0	<0.01
		7823	2	20/20	100	3/20	15	<0.01

[a] Animals received intravenously 0.2 to 0.5 ml of serum of donors immunized subcutaneously 14 days before with 15 to 50 μg of ribosomal preparations. They were then challenged 24 h later with 100 LD_{50}s of *K. pneumoniae* intraperitoneally.

[b] NS, Not significant.

ribosomal preparations) is also observed.

We can formulate two hypotheses to explain this fact: (i) the presence of smaller quantities of immunoprotective antigens in serotype 1 strain; and (ii) the presence in serotype 1 strain of an immunodepressive substance that facilitates in vivo multiplication of bacteria. In favor of this second hypothesis is the observation that *K. pneumoniae* strain 7825 (serotype 1) is much more virulent than strain 52-145 (serotype 2) when the intravenous route is utilized for challenge (unpublished data), whereas intraperitoneal lethal doses are similar for the two strains.

We have shown that active and passive immunity induced by *K. pneumoniae* ribosomal preparations is specific for the capsular serotype of the origin strain, results that suggest the contamination of ribosomal preparations by capsular polysaccharide. We have also shown that, in spite of the elimination of a major part of ribosomal preparations containing visible membrane vesicles, their immunoprotective ability is conserved. We propose two hypotheses to explain this apparent contradiction.

(i) The same polysaccharidic antigen exists in ribosomal preparations and on the *K. pneumoniae* cell surface. This hypothesis seems unlikely in the light of current knowledge of ribosomal constitution.

(ii) Ribosomes are contaminated during their purification procedure by *K. pneumoniae* surface components that cosediment with ribosomes in sucrose gradient centrifugation either by adsorption or because they have the same sedimentation coefficient.

This second hypothesis, the more likely in our opinion, implies high immunogenicity of the protective contaminating antigen. Indeed, this antigen would represent only a slight proportion of ribosomal preparations of which immunoprotective activity persists down to 0.4 μg of proteins for strains of *K. pneumoniae* 52-145. We are currently attempting to resolve this question by isolating such an immunogen on the *K. pneumoniae* bacterial surface.

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