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Direct Evidence for the Involvement of Capsular Polysaccharide in the Immunoprotective Activity of *Klebsiella pneumoniae* Ribosomal Preparations

MARIE-MADELEINE RIOTTOT,[2] JEAN-MICHEL FOURNIER,[1]* AND HÉLÈNE JOUIN[1]

Unité des Antigènes Bactériens (ERA Centre National de la Recherche Scientifique 794)[1] and Unité d'Immunologie Microbienne,[2] Institut Pasteur, 75724 Paris Cedex 15, France

Previous work has demonstrated the capsular serotypic specificity of the protection conferred on mice by *Klebsiella pneumoniae* ribosomal preparations. The data in these studies support the hypothesis that capsular polysaccharide plays at least some role in the specificity of the protection conferred by ribosomal preparations. In this investigation, the presence of capsular polysaccharide and lipopolysaccharide in *K. pneumoniae* ribosomal preparations was demonstrated by using immunodiffusion tests. Lipopolysaccharide content was determined for mice treated with actinomycin D. The serotype of O antigen did not play a role in the orientation of the specificity of the protection. The possibility that lipopolysaccharide might act as an adjuvant was not unlikely since the ribosomal preparations which contained the greatest amounts of lipopolysaccharide appeared to be the most immunoprotective preparations. Ribosomal preparations extracted from a noncapsulated mutant of *K. pneumoniae* did not protect mice. This finding suggested that capsular polysaccharide might play a role in the immunoprotective activity of ribosomes. This hypothesis was tested by using *K. pneumoniae* K2 bacteriophage-associated-glycanase, which specifically hydrolyzed *K. pneumoniae* K2 capsular polysaccharide and thereby suppressed the immunoprotective activity of *K. pneumoniae* K2 ribosomal preparations. In contrast, the K2 bacteriophage-associated glycanase did not interfere with the immunoprotective activity of *K. pneumoniae* K1 ribosomal preparations. These results clearly demonstrate that capsular polysaccharide, which is an extraribosomal antigen, is involved in the immunoprotective activity of *K. pneumoniae* ribosomal preparations.

Youmans and Youmans (45, 46) first reported that vaccination with ribosomes extracted from mycobacteria protected mice against infection with virulent *Mycobacterium tuberculosis*. Since then, immunogens of similar origins have been derived from various microorganisms (3-5, 8-10, 13-17, 19-24, 26-29, 33, 34, 36-42, 44). However, although the immunoprotective activity of ribosomal preparations has been well established, there is considerable controversy in the literature concerning the immunogenic principle of these ribosomal preparations. Workers have presented evidence suggesting that ribosomal ribonucleic acid (13, 42) or proteins (17, 27, 36) may be responsible for the protection associated with ribosomal vaccines. Other experiments have shown that ribosomal preparations have been contaminated with cell surface material (4, 22, 28). It has been reported that O antigen contaminates ribosomal preparations and can contribute to the protection afforded by these vaccines (8, 9, 24). In a previous article (33), we presented data demonstrating the capsular serotypic specificity of the protection conferred on mice by *K. pneumoniae* ribosomal preparations. The results of this study supported the hypothesis that capsular polysaccharide (CP) plays at least some role in the orientation of the specificity induced by ribosomal preparations. However, these results did not provide direct evidence for the involvement of CP in the immunoprotective activity of *K. pneumoniae* ribosomal preparations.

In the present paper we report the results of a study on the immunoprotective activity of *K. pneumoniae* K1 and K2 ribosomal preparations before and after degradation with K2 bacteriophage-associated glycanase, which specifically hydrolyzes *K. pneumoniae* K2 capsular polysaccharide. The specific suppression of the immunoprotective activity of *K. pneumoniae* K2 ribosomal preparations was demonstrated, whereas the immunoprotective activity of *K. pneumoniae* K1 ribosomal preparations remained unaffected. This finding provides direct evidence that CP is involved in the immunopro-

tective activity of *K. pneumoniae* ribosomal preparations.

MATERIALS AND METHODS

Mice. Outbred Swiss white mice were obtained from the Pasteur Institute Experimental Farm, Rennemoulin, France. Experiments were performed with males 4 or 5 weeks old.

Bacterial strains. The bacterial strains used in this study included the following: *K. pneumoniae* 7825 (O1:K1) biotype b (32); *K. pneumoniae* A-215 (O1:K1) biotype b; and *K. pneumoniae* 52-145 (NCTC B 5055) (O1:K2) biotype e, obtained from the Pasteur Institute collection.

The intraperitoneal 50% lethal doses (LD_{50}), as calculated by the Reed-Muench formula (31), were less than 5 colony-forming-units per mouse for strains 7825 and 52-145 and 10^3 colony-forming units per mouse for strain A-215.

A noncapsulated mutant of *K. pneumoniae* 52-145 (O1:K-) was selected by culture in the presence of *K. pneumoniae* K2 bacteriophage. This mutant was avirulent for mice at a dose of 5×10^6 colony-forming units per mouse.

Bacteriophage strain. The bacteriophage used was *K. pneumoniae* K2 bacteriophage, which was isolated originally from Freiburg sewage and was kindly supplied by H. Geyer, Zentrum für Biochemie am Klinikum der Justus-Liebig-Universität, Lahn-Giessen, West Germany. This *K. pneumoniae* K2 bacteriophage carries an enzymatic activity which cleaves the K2 CP at the β-glucose-(1-4)-mannose linkages (Fig. 1), giving rise to a series of mono-, di-, and oligomeric repeating unit split products (12).

Growth of bacteria. Each lyophilized bacterial strain was inoculated into a 10-ml tube, transferred to a 500-ml flask, and finally transferred to a 5,000-ml flask containing 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 $40,000 \times g$ at 4°C.

Extraction of CRP. Crude ribosomal preparation (CRP) were prepared by a slight modification of the method of Schalla and Johnson (34). All procedures were conducted at 4°C. Bacterial cells were washed twice in cold 0.01 M tris(hydroxymethyl)aminomethane hydrochloride buffer containing 0.01 M $MgCH_3CO_2$ and 0.06 M NH_4Cl (TMN) (pH 7.4) and then suspended in the same buffer (100 mg [wet weight] per ml). 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 then NH_4Cl was added to a final concentration of 0.35 M. The extract was centrifuged at $25,000 \times g$ 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 $\times g$ 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 $25,000 \times g$ for 20 min, and the supernatant fluid (SF_1) was saved. The precipitate was suspended in TMN and centrifuged at $25,000 \times g$ for 20 min.

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

Extraction of LPS. Lipopolysaccharide (LPS) was prepared from the noncapsulated mutant of *K. pneumoniae* 52-145 (O1:K-) by the phenol-water procedure of Westphal and Jann (43).

Isolation of CP. CPs were prepared by the method of Joseleau et al. (18); 7 g of packed cells and mucus was harvested and diluted with 100 ml of water containing 1% phenol. After 24 h at 4°C, the suspension was centrifuged for 1 h at $100,000 \times g$. The clear supernatant solution was concentrated in a rotary evaporator to 30 ml, poured into 150 ml of ethanol, and centrifuged for 30 min at $30,000 \times g$. The crude polysaccharide pellet was dissolved, precipitated with 3% Cetavlon, dissolved in 50 ml of 2 M sodium chloride, and precipitated with 300 ml of ethanol. The purified polysaccharide was then dissolved in 100 ml of distilled water, deionized on Amberlite IR-120 (H^+) resin, dialyzed, and freeze-dried.

Bacteriophage stocks. High-titer bacteriophage stocks were prepared by the agar layer method of Swanstrom and Adams (35), as modified by Adams and Park (1). Briefly, 2.5 ml of melted 0.7% nutrient agar was inoculated with 0.2 ml of broth containing 10^8 bacteria and 10^4 phage particles and then was poured onto plates of hardened 1.5% nutrient agar. After agar solidification, the plates were incubated overnight at 37°C. Then, 3 ml of broth and a few drops of chloroform were added to each plate, and the agar layer was scraped off with a sterile glass rod into a centrifuge tube.

The broth and agar were shaken vigorously and centrifuged at $2,000 \times g$. The supernatant containing the phage was preserved and stored over chloroform at 4°C.

Phage titration. Phage titration was performed by

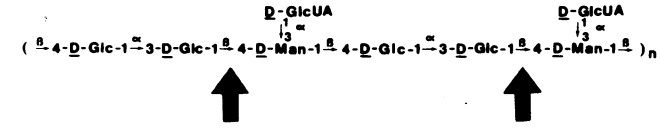

FIG. 1. *Structure of the K. pneumoniae CP. From reference 11. The arrows indicate the linkages which are cleaved by K. pneumoniae K2 bacteriophage enzyme (12). Glc, Glucose; Man, mannose; GlcUA, Glucuronic acid.*

the agar layer method; nutrient agar plates were incubated with serial dilutions of phage mixed with 10^8 host bacteria in melted 0.7% agar. After incubation at 37°C overnight, plates were examined for plaques.

Bacteriophage degradation of CRP. A 0.2-ml amount of nutrient broth containing 1.4×10^{10} (or fewer in certain experiments [see below]) *K. pneumoniae* K2 bacteriophage was added to 0.5 ml of TMN containing 0.5 mg of *K. pneumoniae* 52-145 (O1:K2) or A-215 (O1:K1) CRP (expressed as protein content). Sodium azide was added to a final concentration of 0.2% (wt/vol). After incubation for 72 h at 37°C, the mixture was diluted in sterile pyrogen-free saline for immunization.

CRP incubated under the same conditions with bacteriophage-free nutrient broth served as positive controls.

Biochemical assays. Protein was determined by the method of Lowry et al. (25), ribonucleic acid was measured by the orcinol method (2), and neutral sugars were detected by the method of Dubois et al. (7). Bovine serum albumin, yeast ribonucleic acid (Sigma Chemical Co.), and glucose served as standards.

LPS was measured by intraperitoneally inoculating Swiss mice with 12.5 μg of actinomycin D (0.25 ml; Sigma Chemical Co.) and 0.25 ml of different dilutions of CRP (30). Deaths were scored at 48 h, and the LD_{50} were calculated by the method of Reed and Muench (31). *Salmonella enteritidis* LPS (Difco Laboratories, no. 3126) served as the standard.

Antisera. Rabbits were immunized with 500-μg amounts (proteins) of ribosomal preparations emulsified in Freund complete adjuvant; the first dose was injected intradermally in the back. The rabbits were boosted by four intramuscular injections (100 μg of proteins) at 14-day intervals with antigens emulsified in Freund complete adjuvant. Rabbits were bled at 10 days after the last injection. The sera were stored at −20°C.

Immunodiffusion. CRP were analyzed for the presence of CP and LPS by double diffusion; 0.6% agarose in saline was used. The antigen concentrations used were 1 to 3 mg/ml for CRP, 1 mg/ml for LPS, and 50 μg/ml for CP.

Whole-cell vaccines. A bacterial suspension containing 10^8 colony-forming units per 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* cells were detected by the plate culture technique.

Immunization and challenge. Mice were immunized subcutaneously with 0.5 ml of the vaccine preparation diluted in sterile pyrogen-free 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} of *K. pneumoniae*. The bacterial count at the time of challenge was confirmed by the spread plaque technique. Deaths, which occurred principally within 8 days after challenge, were recorded 14 days after challenge; 50% protective doses (PD_{50}) were calculated by the Reed-Muench formula (31).

RESULTS

Chemical studies. Chemical assays of the CRP indicated that the ratio of ribonucleic acid to protein ranged from about 0.8 to 2. The ratio of absorbance at 260 nm to absorbance at 280 nm was between 1.5 and 1.8. The protein content of the CRP varied from about 35 to 48% of the dry weight. *K. pneumoniae* 52-145 CRP contained 10% LPS, and *K. pneumoniae* A-215 and 7825 CRP contained 2% LPS. CP contained 6% protein and 1% LPS. LPS contained less than 0.5% protein.

Identification of CP in CRP. Immunochemical testing with rabbit antiserum against strain A-215 (O1:K1) CRP showed partial cross-reactivity between K1 CP and strain A-215 (O1:K2) CRP (Fig. 2A). Similarly, K2 CP and strain 52-145 (O1:K2) CRP showed partial cross-reactivity with antiserum against strain 52-145 (O1:K2) CRP (Fig. 2B). The patterns obtained indicated that the CRP contained contaminant CP.

Identification of LPS in CRP. The immunodiffusion pattern in Fig. 2C shows a continuous precipitation line between (O1) LPS and strain 52-145 (O1:K2) CRP reacting with antiserum against strain 52-145 (O1:K2) CRP. This pattern was direct evidence for the presence of contaminating LPS in strain 52-145 (O1:K1) CRP. The fact that antiserum to strain A-215 (O1:K1) CRP precipitated with (O1) LPS provided indirect evidence for the presence of LPS in strain A-215 (O1:K1) CRP. (The same pattern [data not shown] was observed with strain 7825 [O1:K1] CRP.) However, strain A-215 (O1:K1) CRP did not show a continuous precipitation line with (O1) LPS, indicating that the level of contamination of strain A-215 (O1:K1) CRP by

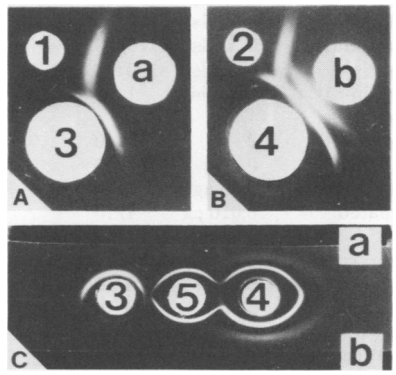

FIG. 2. *Identification of CP and LPS in K. pneumoniae CRP. Well 1, K1 CP; well 2, K2 CP; well 3, strain A-215 (O1:K1) CRP; well 4, strain 52-145 (O1:K2) CRP; well 5, O1 LPS; well a, antiserum to strain A-215 (O1:K1) CRP; well b, antiserum to strain 52-145 (O1:K2) CRP.*

LPS was low. These immunodiffusion patterns were corroborated by biological assays of the LPS in CRP; strain A-215 CRP contained 20 μg of LPS per mg (dry weight) of CRP, and strain 52-145 CRP contained 100 μg of LPS per mg (dry weight) of CRP.

Immunoprotective activity of capsulated and noncapsulated *K. pneumoniae* Formalin-treated cells. Four groups containing 10 mice each were inoculated with 10^5 to 10^8 Formalin-treated cells of *K. pneumoniae* 52-145 (O1:K2), and four other groups were immunized with 10^5 to 10^8 Formalin-treated cells of the *K. pneumoniae* 52-145 (O1:K-) noncapsulated mutant. One control group received saline. All animals were challenged 14 days later with 100 LD_{50} of virulent *K. pneumoniae* 52-145 (O1:K2). Table 1 shows the survival on day 14 after challenge and the PD_{50}. Whereas the PD_{50} of capsulated cells was 10^6 cells, the PD_{50} of the noncapsulated mutant was greater than 10^8 cells. These results indicated that the noncapsulated mutant had lost the immunoprotective activity of the original strain.

Immunoprotective capacity of CPR extracted from capsulated and noncapsulated *K. pneumoniae*. Five groups containing 10 mice each were inoculated with 0.032 to 2 μg (expressed as protein content of ribosomes) of CRP extracted from *K. pneumoniae* 52-145 (O1:K2), and three other groups were immunized with 2 to 50 μg of CRP extracted from the *K. pneumoniae* 52-145 (O1:K-) noncapsulated mutant. One control group received saline. All animals were challenged 14 days later with 100 LD_{50} of *K. pneumoniae* 52-145 (O1:K2). Table 1 shows the survival on day 14 after challenge and the PD_{50}. As with Formalin-treated cells, the immunoprotective activity of the CRP extracted from the noncapsulated mutant (PD_{50}, 25 μg) was lower than the activity of the CRP extracted from the capsulated original strain (PD_{50}, 0.016 μg).

TABLE 1. *Immunoprotective activities of whole cells and CRP from capsulated and noncapsulated K. pneumoniae*[a]

Antigen type	Dose[b]	No. of survivors/ total no.	PD_{50}[b]
(O1:K2) 52-145 Formalin-treated cells	10^8 cells	10/10	10^6 cells
	10^7 cells	8/10	
	10^6 cells	6/10	
	10^5 cells	0/10	
(O1:K-) 52-145 noncapsulated mutant Formalin-treated cells	10^8 cells	4/10	$>10^8$ cells
	10^7 cells	0/10	
	10^6 cells	0/10	
	10^5 cells	1/10	
CRP extracted from (O1:K2) 52-145 capsulated cells	2 μg	9/10	0.016 μg
	0.4 μg	9/10	
	0.08 μg	8/10	
	0.016 μg	4/10	
	0.032 μg	3/10	
CRP extracted from (O1:K-) 52-145 noncapsulated mutant cells	50 μg	7/10	25 μg
	10 μg	2/10	
	2 μg	0/10	
Saline		0/10	

[a] All animals were immunized subcutaneously at zero time and were challenged intraperitoneally with 100 LD_{50} 14 days after immunization.

[b] Number of Formalin-treated cells or protein content of ribosomes (in micrograms).

Immunoprotective activity of phage-degraded CRP. *K. pneumoniae* 52-145 (O1:K2) CRP were treated with *K. pneumoniae* K2 bacteriophage at doses of 1.4×10^8 to 1.4×10^{10} plaque-forming units. *K. pneumoniae* A-215 (O1:K1) CRP were treated with 1.4×10^{10} plaque-forming units of K2 bacteriophage. Groups containing 10 mice each were inoculated with either positive control sham-treated or phage-treated CRP at different dilutions. Control groups received saline or a phage suspension at a dose of 1.4×10^{10} plaque-forming units per mouse. All animals were challenged 14 days later with 100 LD_{50} of the homologous strain. The results of these challenges (Table 2) showed a dose relationship between the concentration of K2 bacteriophage used for the treatment of CRP and the decrease in the immunoprotective activity of phage-treated *K. pneumoniae* 52-145 (O1:K2) CRP. The treatment of *K. pneumoniae* 52-145 (O1:K2) CRP with 1.4×10^{10} plaque-forming units increased the PD_{50} from 0.030 μg (untreated CRP) to >50 μg (phage-treated CRP). In contrast, K2 bacteriophage-treated *K. pneumoniae* A-215 (O1:K1) CRP had essentially the same PD_{50} as untreated CRP (1.8 and 1.6 μg, respectively). These results indicate the serotypic specificity of the phage-associated glycanase activity. In controls, mice injected with 1.4×10^{10} plaque-forming units of K2 phage per mouse were not protected against challenge with either *K. pneumoniae* 52-145 (O1:K2) or *K. pneumoniae* 7825 (O1:K1) cells.

DISCUSSION

We showed in a previous paper (33) that the active immunity and the passive immunity induced in mice by *K. pneumoniae* ribosomal preparations were specific for the capsular serotype of the original strain. These results suggested that the ribosomal preparations were contaminated by CP and prompted us to investigate the possible presence of CP in our ribosomal preparations. In this paper we present direct

TABLE 2. *Immunoprotective activities of untreated and phage-treated CRP*[a]

Origin of CRP	Challenge strain	Antigen		PD_{50} (μg)[b]
		Type	Dose of phage K2 treatment (PFU)	
K. pneumoniae 52-145 (O1:K2)	*K. pneumoniae* 52-145 (O1:K2)	Untreated CRP		0.016
		Treated CRP	1.4×10^8	0.030
			1.4×10^9	10
			1.4×10^{10}	>50
K. pneumoniae A-215 (O1:K1)	*K. pneumoniae* 7825 (O1:K1)	Untreated CRP		1.6
		Treated CRP	1.4×10^{10}	1.8

[a] All animals were immunized subcutaneously at zero time and were challenged intraperitoneally with 100 LD_{50} 14 days after immunization. Control mice were sham-immunized with K2 bacteriophage (1.4×10^{10} plaque-forming units [PFU] per mouse) or were injected with saline and then challenged with either *K. pneumoniae* 52-145 (O1:K2) or *K. pneumoniae* 7825 (O1:K2); all control mice died.

[b] PD_{50} values are expressed as protein contents of ribosomes.

evidence for the presence of CP in *K. pneumoniae* ribosomal preparations. These results differ from those of Tewari et al. (37), who did not find CP in *Haemophilus influenzae* ribosomal preparations.

On the other hand, contaminating LPS has been observed frequently in ribosomal preparations extracted from *Escherichia coli* (6), *Pseudomonas aeruginosa* (13, 22), and *Salmonella typhimurium* (8, 9, 15, 26). Therefore, the presence of LPS in our *K. pneumoniae* ribosomal preparations was not surprising. However, the presence of an extraribosomal antigen (for example, CP or LPS) in ribosomal preparations is not, by itself, direct evidence that the contaminating antigen contributes to the immunoprotective activity of the ribosomal preparations. Eisenstein (8) suggested that the protective capacity of *S. typhimurium* ribosomes might be due to contaminating O antigen; in this study Eisenstein used genetically defined strains of *S. typhimurium* which were unable to make O antigens. Lin and Berry (24) reported similar results; they used mutant *S. typhimurium* LT2-M1 strains, which lack uridine-diphosphate-D-galactose epimerase. All of these results suggested that LPS participates in the immunoprotective activities of ribosomal vaccines, but they did not furnish direct evidence for this contention because the experiments did not eliminate the possibility that there was variation in characters other than LPS structure in the different mutant strains used. We were faced with the same problem, namely, the interpretation of our results, which were obtained with either whole cells or CRP extracted from the noncapsulated mutant of *K. pneumoniae*. The inability of these preparations to protect mice supported a role for CP but was not direct evidence for it. Indeed, these experiments did not eliminate the possibility of variation in characters other than the presence or absence of CP.

The lack of cross-protection between *K. pneumoniae* (O1:K1) 7825 or A-215 CRP and *K. pneumoniae* (O1:K2) 52-145 CRP (33) indicated that the specificity of the protection did not depend upon the O-antigen specificity. However, these results did not rule out a possible contribution of the LPS as an adjuvant for the immunoprotective activity of the ribosomal preparations. The greater activity of *K. pneumoniae* 52-145 CRP compared with the activity of *K. pneumoniae* A-215 CRP, as well as the highest content of contaminating LPS in strain 52-145 CRP, supported this hypothesis, which was in keeping with the results of Hoops et al. (15) with *S. typhimurium* ribosomes.

The identification of cell surface proteins in *S. typhimurium* ribosomal preparations by Misfeldt and Johnson (28) did confirm the presence of extraribosomal antigens in ribosomal preparations, but it did not prove that the immunoprotective antigens are these extraribosomal antigens.

The specificity of the protection, the identification of CP in ribosomal preparations, and the inability of ribosomal preparations extracted from a noncapsulated mutant to protect mice support the hypothesis that an extraribosomal antigen (CP) is involved in the immunoprotective activity of *K. pneumoniae* ribosomal preparations, but these findings do not prove this hypothesis.

Only the experiments with K2 bacteriophage demonstrated beyond doubt that CP participates in the immunoprotective activity of ribosomal preparations. To our knowledge, this is the first time that anyone has provided direct evidence concerning the involvement of an extraribosomal antigen in the immunoprotective

activity of a ribosomal vaccine.

In preliminary experiments we found that purified CP did not protect mice (unpublished data). We propose the following two hypotheses to explain this apparent contradiction of the above-described demonstration of involvement of CP in the immunoprotective activity of *K. pneumoniae* ribosomes.

(i) The immunogenicity of CP is due to its association with ribosomal ribonucleic acid, which may act as an adjuvant or a carrier for this polysaccharide in the same way that Youmans and Youmans (47) showed ribonucleic acid to be an adjuvant for bovine γ-globulin. However, an in vitro mixture of CP with ribosomal preparations extracted from the noncapsulated (K-) *K. pneumoniae* mutant did not protect mice in preliminary experiments (unpublished data).

(ii) The CP present in ribosomal preparations is associated with outer membranous vesicles which copurify with ribosomes. These membranous vesicles contain LPS, protein, and lipids, which may render CP immunoprotective. This hypothesis is in agreement with the results of Jensen et al. (16), who obtained from *Vibrio cholerae* ribosomal preparations an immunoprotective fractions containing protein, lipid, and carbohydrate but not ribonucleic acid.

We are presently studying this second hypothesis by isolating such an immunoprotective antigen from *K. pneumoniae* cell surfaces and comparing the immunoprotective activity of this antigen with that of ribosomal preparations.

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LITERATURE CITED

1. **Adams, M. H., and B. H. Park.** 1956. An enzyme produced by a phage-host cell system. II. The properties of the polysaccharide depolymerase. Virology **2**:719–736.
2. **Ashwell, G.** 1957. Colorimetric analysis of sugars. Methods Enzymol. **3**:87.
3. **Baba, T.** 1977. Immunogenic activity of a ribosomal fraction obtained from *Pasteurella multocida*. Infect. Immun. **15**:1–6.
4. **Cofré, G., I. Calderón, and G. C. Mora.** 1978. Immunogenic capacity of ribosomes of *Salmonella typhi* interfered with by a flagellin-like material contaminant. Infect. Immun. **20**:161–166.
5. **Corbel, M. J.** 1976. The immunogenic activity of ribosomal fractions derived from *Brucella abortus*. J. Hyg. **76**:65–74.
6. **Dandeu, J. P., G. Quash, and E. Barbu.** 1964. Presence d'endotoxine dans les préparations de ribosomes et antigénicité des acides nucléiques. C. R. Acad. Sci. **258**: 3932–3935.
7. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Dosage des sucres neutres par la méthode au phénol H_2SO_4. Anal. Chem. **28**:350–356.
8. **Eisenstein, T. K.** 1975. Evidence for O antigens as the antigenic determinants in "ribosomal" vaccines prepared from *Salmonella*. Infect. Immun. **12**:364–377.
9. **Eisenstein, T. K., and C. R. Angerman.** 1978. Immunity to experimental *Salmonella* infection: studies on the protective capacity and immunogenicity of lipopolysaccharide, acetone-killed cells, and ribosome-rich extracts of *Salmonella typhimurium* in C3H/HeJ and CD-1 mice. J. Immunol. **121**:1010–1014.
10. **Fontanges, R., D. Robert, Y. Content, and G. Nis.** 1977. Pouvoir vaccinant des ribosomes extraits de *K. pneumoniae, S. pneumoniae, S. pyogenes, H. influenzae* et d'une fraction membranaire de *K. pneumoniae*. Rev. Fr. Allergol. **17**:35–81.
11. **Gahan, L. C., P. A. Sandford, and H. E. Conrad.** 1967. The structure of the serotype 2 capsular polysaccharide of *Aerobacter aerogenes*. Biochemistry **6**:2755–2767.
12. **Geyer, H., S. Stirm, and K. Himmelspach.** 1979. Immunochemical properties of oligosaccharide-protein conjugates with *Klebsiella* K2 specificity. I. Specificity and cross-reactivity of anti-conjugate versus anti-bacterial antibodies. Med. Microbiol. Immunol. **165**:271–288.
13. **Gonggrijp, R., W. J. H. A. Mullers, P. J. M. R. Lemmens, and C. P. A. van Boven.** 1980. Ribonuclease-sensitive ribosomal vaccine of *Pseudomonas aeruginosa*. Infect. Immun. **27**:204–210.
14. **Guentzel, M. N., and L. J. Berry.** 1974. Protection of suckling mice from experimental cholera by maternal immunization: comparison of the efficacy of whole-cell, ribosomal-derived, and enterotoxin immunogens. Infect. Immun. **10**:167–172.
15. **Hoops, P., N. E. Prather, L. J. Berry, and J. M. Ravel.** 1976. Evidence for an extrinsic immunogen in effective ribosomal vaccines from *Salmonella typhimurium*. Infect. Immun. **13**:1184–1192.
16. **Jensen, R., B. Gregory, J. Naylor, and P. Actor.** 1972. Isolation of protective somatic antigen from *Vibrio cholerae* (Ogawa) ribosomal preparations. Infect. Immun. **6**:156–161.
17. **Johnson, W.** 1972. Ribosomal vaccines. I. Immunogenicity of ribosomal fractions isolated from *Salmonella typhimurium* and *Yersinia pestis*. Infect. Immun. **5**: 947–952.
18. **Joseleau, J. P., M. Lapeyre, M. Vignon, and G. G. S. Dutton.** 1978. Chemical and N.M.R.-spectroscopic investigation of the capsular polysaccharide of *Klebsiella* serotype K 41. Carbohydr. Res. **67**:197–212.
19. **Leon, L. L., W. Leon, L. Chaves, S. C. G. Costa, M. Q. Cruz, H. M. Brascher, and A. O. Lima.** 1980. Immunization of mice with *Trypanosoma cruzi* polyribosomes. Infect. Immun. **27**:38–43.
20. **Levenson, V. I., L. L. Subbotina, I. L. Gofman, and L. M. Yarovaya.** 1978. Ribosomal dysentery vaccine. Report 1. Preparative technique and physico-chemical characteristics. Zh. Mikrobiol. Epidemiol. Immunobiol. **3**:60–65.
21. **Levenson, V. I., and Y. L. Subbotina.** 1978. Ribosomal vaccine against dysentery. Report 2. Biological assay in experiments of guinea pigs and mice active protection. Zh. Mikrobiol. Epidemiol. Immunobiol. **7**:81–86.
22. **Lieberman, M. M.** 1977. Direct evidence for the presence of lipopolysaccharide components in a *Pseudomonas* ribosomal vaccine. Infect. Immun. **17**:471–473.
23. **Lieberman, M. M.** 1978. *Pseudomonas* ribosomal vaccines: Preparation, properties, and immunogenicity. Infect. Immun. **21**:76–86.
24. **Lin, J. H., and L. J. Berry.** 1978. The use of strain LT2-MI in identifying the protective antigens in a *Salmonella typhimurium*-derived ribosomal vaccine. RES J. Reticuloendothel. Soc. **8**:135–143.
25. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin

phenol reagent. J. Biol. Chem. **193**:265–275.
26. **Misfeldt, M. L., and W. Johnson.** 1977. Role of endotoxin contamination in ribosomal vaccines prepared from *Salmonella typhimurium*. Infect. Immun. **17**:98–104.
27. **Misfeldt, M. L., and W. Johnson.** 1978. Protective ability of *Salmonella* ribosomal protein and RNA in inbred mice. Infect. Immun. **21**:286–291.
28. **Misfeldt, M. L., and W. Johnson.** 1979. Identification of protective cell surface proteins in ribosomal fractions from *Salmonella typhimurium*. Infect. Immun. **24**:808–816.
29. **Morris, J. A., and H. S. Orr.** 1979. Immunity induced in hamsters vaccinated with a ribosome extract of *Leptospira interrogans* serogroup *Icterohaemorrhagiae*. J. Biol. Stand. **7**:81–87.
30. **Pieroni, R. E., E. J. Broderick, A., Bundeally, and L. Levine.** 1970. A simple method for the quantitation of submicrogram amounts of bacterial endotoxin. Proc. Soc. Exp. Biol. Med. **133**:790–794.
31. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent end points. Am. J. Hyg. **27**: 493–497.
32. **Richard, C.** 1973. Etude antigenique et biochimique de 500 souches de *Klebsiella*. Ann. Biol. Clin. **31**:295–303.
33. **Riottot, M. M., J. M. Fournier, and J. Pillot.** 1979. Capsular serotypic specificity of the protection conferred on mice by *Klebsiella pneumoniae* ribosomal preparations. Infect. Immun. **24**:476–482.
34. **Schalla, W. O., and W. Johnson.** 1975. Immunogenicity of ribosomal vaccines isolated from group A, type 14 *Streptococcus pyogenes*. Infect. Immun. **11**:1195–1202.
35. **Swanstrom, M., and M. H. Adams.** 1951. Agar layer method for production of high titer phages stocks. Proc. Soc. Exp. Biol. Med. **78**:372–375.
36. **Swendsen, C. L., and W. Johnson.** 1976. Humoral immunity to *Streptococcus pneumoniae* induced by a pneumococcal ribosomal protein fraction. Infect. Immun. **14**:345–354.
37. **Tewari, R. P., M. Lynn, A. J. Birnbaum, and M. Solotorovsky.** 1978. Characterization of the immunoprotective antigen of ribosomal preparations from *Haemophilus influenzae*. Infect. Immun. **19**:58–65.
38. **Tewari, R. P., D. Sharma, M. Solotorovsky, R. Lafemina, and J. Balint.** 1977. Adoptive transfer of immunity from mice immunized with ribosomes or live yeast cells of *Histoplasma capsulatum*. Infect. Immun. **15**:789–795.
39. **Thomas, D. W., and E. Weiss.** 1972. Response of mice to injection of ribosomal fraction from group B *Neisseria meningitidis*. Infect. Immun. **6**:355–363.
40. **Thompson, H. C. W., and T. K. Eisenstein.** 1976. Biological properties of an immunogenic pneumococcal subcellular preparation. Infect. Immun. **13**:750–757.
41. **Thompson, H. C. W., and I. S. Snyder.** 1971. Protection against pneumococcal infection by a ribosomal preparation. Infect. Immun. **3**:16–23.
42. **Venneman, M. R., N. J. Bigley, and L. J. Berry.** 1970. Immunogenicity of ribonucleic acid preparations obtained from *Salmonella typhimurium*. Infect. Immun. **1**:574–582.
43. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further application of the procedure. Methods Carbohydr. Chem. **5**:83–91.
44. **Winston, S., and L. J. Berry.** 1970. Immunity induced by ribosomal extracts from *Staphylococcus aureus*. RES J. Reticuloendothel. Soc. **8**:66–73.
45. **Youmans, A. S., and G. P. Youmans.** 1964. Nature of the labile immunogenic substance in the particulate fractions isolated from *Mycobacterium tuberculosis*. J. Bacteriol. **88**:1030–1037.
46. **Youmans, A. S., and G. P. Youmans.** 1970. Immunogenic mycobacterial ribosomal and ribonucleic acid preparations: chemical and physical characteristics. Infect. Immun. **2**:659–668.
47. **Youmans, G. P., and A. S. Youmans.** 1972. The effect of mycobacterial RNA on the primary antibody response of mice to bovine γ-globulin. J. Immunol. **109**: 217–221.