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Immunoprotective Activity of Capsular Polysaccharide in *Klebsiella pneumoniae* Ribosomal Preparations Does Not Involve Ribonucleic Acid

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

Unité des Antigènes Bactériens[2] and Unité d' Immunologie Microbienne[1], Institut Pasteur, 75724 Paris Cédex 15, France

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Two peaks were obtained by cesium chloride density gradient ultracentrifugation of *Klebsiella pneumoniae* ribosomal preparations. Peak I contained capsular polysaccharide, lipopolysaccharide, protein, and less than 0.5% ribonucleic acid. Peak II consisted mainly of ribonucleic acid, with low amounts of protein and capsular polysaccharide. Expressed as capsular polysaccharide content, the 50% protective dose of peak I and of nonfractionated ribosomal preparations was nearly constant (2.6 and 1.2 ng, respectively). Since peak I contained less than 0.5% ribonucleic acid, these results provide evidence that ribosomal ribonucleic acid is not required for protection of mice by *K. pneumoniae* capsular polysaccharide which contaminates ribosomal preparations.

Youmans and Youmans (28) reported in 1964 that ribosomal preparations of *Mycobacterium tuberculosis* could be highly immunoprotective. Since that time, immunogens of similar origin have been prepared from many bacterial species (4), including *Klebsiella pneumoniae* (5, 6, 17, 22, 23). The use of *K. pneumoniae* K2 bacteriophage-associated glycanase which specifically hydrolyzes *K. pneumoniae* K2 capsular polysaccharide (CP) allowed us to demonstrate the involvement of an extraribosomal antigen in the immunoprotective activity of *K. pneumoniae* ribosomal preparations (22). Nevertheless, purified CP was poorly protective to mice (6). Two hypotheses could be put forward to explain the immunoprotective activity of ribosome-associated CP: (i) the immunoprotective activity of CP may be due to its association with ribosomal ribonucleic acid (RNA), which may act as an adjuvant or as a carrier according to the hypothesis of Youmans and Youmans (29, 30), or (ii) CP contaminating ribosomal preparations may be associated with outer membranous vesicles which copurify with ribosomes. These membranous vesicles may contain immunoprotective CP in high-molecular-weight form, native form, or both. Alternatively, other antigens present in outer membranous vesicles (lipopolysaccharide [LPS] or proteins) may render CP immunoprotective.

By comparative study of the immunoprotective activity of *K. pneumoniae* cell surface and ribosomal preparations, we determined that CP present in cell surface preparations was as immunoprotective as CP contaminating ribosomal preparations (6). The low level of nucleotide components detected in purified cell surface preparations led us to infer that the immunoprotective activity of CP may be independent of the adjuvant activity of RNA. Nevertheless, this work gave only indirect proof of this contention.

This report presents results obtained with *K. pneumoniae* ribosomal preparations fractionated on cesium chloride density gradient and provides direct evidence that ribosomal RNA is not involved in the immunoprotective activity of CP which contaminates *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 *K. pneumoniae* 7825 (O1:K1), *K. pneumoniae* 7823 (O1:K2), and *K. pneumoniae* 52-145 (O1:K2), obtained from the Pasteur Institute collection. The intraperitoneal 50% lethal dose (LD_{50}), calculated by the Reed-Muench formula (21), was less than 5 colony-forming units per mouse for these strains.

Bacteriophage. The bacteriophage used was *K. pneumoniae* phage K2, which was isolated originally from Freiburg sewage and was kindly supplied by H. Geyer, Zentrum für Biochimie am klinikum der Justus-Liebig-Universität, Lahn-Giessen, West Germany. This bacteriophage carries an enzymatic activity which cleaves the *K. pneumoniae* K2 CP at the β-

glucose-1,4-mannose linkage, giving rise to a series of mono-, di-, and oligomeric repeating-unit split products (8). Bacteriophage stock was prepared and titrated as previously described (22).

Bacterial growth. Large-scale growth of *K. pneumoniae* was performed as previously described (22).

CRP extraction. Crude ribosomal preparations (CRP) were prepared from the *K. pneumoniae* 7823 (O1:K2) cell suspension as previously described (22).

CsCl density gradient ultracentrifugation of CRP. CRP was dialyzed for 48 h against several changes of cold 10 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer containing 30 mM NH_4Cl, 0.3 mM $MgCl_2$, and 6 mM β-mercaptoethanol (dissociation buffer), pH 7.5. Four milliliters of CRP solution (containing 780 μg of protein per ml of dissociation buffer) was added to 31 ml of CsCl solution (928 mg of CsCl per ml of dissociation buffer). The mixture was centrifuged at 100,000 $\times$ *g* for 48 h. The gradient was fractionated from the top of the centrifuge tube. Twenty-seven fractions of 1.3 ml each were collected, and their absorbance at 260 and 280 nm was determined. Two peaks were obtained. The fractions of each peak were pooled, dialyzed for 48 h against phosphate-buffered saline, and stored in small volumes at −20°C.

Bacteriophage degradation of preparations. A 1-ml amount of a solution containing bacteriophage and ribosomal preparation (1.4×10^8 plaque-forming units of bacteriophage per μg of preparation expressed as CP content) and 2 mg of sodium azide were incubated for 72 h at 37°C. Then the mixture was diluted in sterile pyrogen-free saline for immunization. Preparations 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. (16). Bovine serum albumin (Sigma Chemical Co.) served as the standard. RNA was evaluated by the orcinol method (1). Yeast RNA (Sigma) served as the standard. *K. pneumoniae* K2 CP was determined by measuring the amount of uronic acid by the method of Blumenkrantz and Asboe-Hansen (2), assuming that K2 CP contains 26% glucuronic acid (7). Glucuronic acid (Sigma) served as the standard. LPS was determined either in vitro by the carbocyanin dye method of Janda and Work (11) or in vivo in mice sensitized with actinomycin D (Sigma) by the method of Pieroni et al. (18). *Salmonella enteritidis* LPS (Difco Laboratories no. 3126) served as the standard.

Immunization and challenge. Mice were immunized subcutaneously with 0.5 ml of the vaccine preparation diluted in sterile pyrogen-free saline without adjuvant. 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* 52-145 (O1:K2) 14 days after immunization. The bacterial count at the time of the challenge was confirmed by the spread plaque technique. Deaths were recorded 14 days after challenge. The 50% protective dose (PD_{50}) was calculated by the Reed-Muench formula (21). By using data from biochemical assays, PD_{50} was expressed either on a dry weight basis or as protein, CP, LPS, and RNA content of these different preparations. The total amount of the PD_{50} recovered before and after ultracentrifugation was calculated for each preparation.

RESULTS

CsCl density gradient fractionation of CRP. When CRP was fractionated on the CsCl gradient, a typical two-peak profile was obtained (Fig. 1). Peak I banded at the top of the gradient (density of <1.513) and peak II banded at the bottom of the gradient (density of 1.760).

Chemical composition of CRP, peak I, and peak II. Table 1 gives the chemical composition of CRP, peak I, and peak II. CRP contained 7% CP and 8% LPS as contaminating antigens. Peak I contained less than 0.5% RNA, whereas peak II contained 84% RNA. CP was present in both peaks (22% in peak I and 10% in peak II). Ribosomal proteins were recovered mainly in peak I.

Protection induced by CRP, peak I, and peak II. Table 2 presents the PD_{50} expressed as protein, CP, LPS, and RNA content of CRP, peak I, and peak II and the total amount of the PD_{50} recovered in these preparations. Although peak I contained less than 0.5% RNA, its protective activity expressed as CP content was quite as effective as the protective activity of initial CRP (PD_{50}, 2.6 and 1.2 ng of CP, respectively). On the other hand, peak II, which contained both CP and RNA, was less protective (PD_{50}, 28 ng of CP). After CsCl density gradient ultracen-

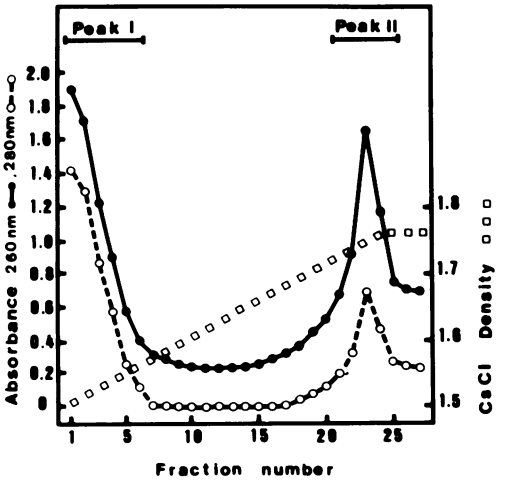

FIG. 1. *CsCl density gradient ultracentrifugation of CRP. Four milliliters of CRP solution containing 780 μg of protein per ml was added to 31 ml of CsCl solution (928 mg of CsCl per ml of dissociation buffer). Centrifugation was carried out for 48 h at 100,000 $\times$ g at 4°C. Fractions of 1.3 ml were collected, and their absorbance at 260 and 280 nm was determined.*

TABLE 1. *Chemical composition of peak I and peak II obtained by CsCl density gradient ultracentrifugation of CRP from K. pneumoniae*[a]

Prepn	% Protein	% CP	% LPS	% RNA
CRP	33	7	8	52
Peak I	65	22	13	<0.5
Peak II	5	10	0.5	84

[a] Theoretical values assuming that protein, CP, LPS, and RNA were the main constituents of the preparations as previously established (6).

TABLE 2. *Immunoprotective activity of CRP, peak I, and peak II*[a]

Vaccine	PD_{50} (ng) in:				Total amt of PD_{50} recovered in each prepn ($\times 10^3$)
	Protein	CP	LPS	RNA	
CRP	6	1.2	1.5	9.6	1,040
Peak I	8	2.6	1.6	<0.05	398
Peak II	14	28	1.4	233	1.3

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

trifugation, the recovered immunoprotective activity was almost integrally localized in peak I (398×10^3 PD_{50} recovered in peak I versus 1.3×10^3 PD_{50} recovered in peak II).

Specificity of the protection induced by CRP, peak I, and peak II. Groups of mice were inoculated with either CRP, peak I, or peak II at different dilutions. Control groups received saline. After 14 days, each group was divided in two subgroups that were challenged intraperitoneally with 100 LD_{50} of either *K. pneumoniae* 7825 (O1:K1) or *K. pneumoniae* 52-145 (O1:K2). The results of this challenge (Table 3) showed the capsular serotypic specificity of the protection induced by these preparations.

The involvement of the CP in the immunoprotective activity of these preparations was demonstrated by using bacteriophage K2-associated glycanase, which dramatically diminished the immunoprotective activity of these preparations (Table 4).

DISCUSSION

Arguments alternatively disproving or proving the role of RNA in immunoprotective activity of ribosomal preparations have been proposed. Since Youmans and Youmans (29) reported that the isolated active fraction of *M. tuberculosis* is a double-stranded RNA, no one has reported similar results on mycobacteria. Portelance et al. (19, 20) suggested that polysaccharide or RNA-polysaccharide complexes may play an important role in the induction of immunity against tuberculosis. However, these investigators did not identify the hypothetic polysaccharide involved. In studies on immunization against *Salmonella typhimurium*, contrasting evidence has been presented by Johnson (12, 13) and Venneman et al. (25–27). Johnson found that a ribosomal protein is active as a protective antigen, whereas Venneman et al. indicated the immunogen to be either a stable RNA molecule or an undefined polysaccharide. Smith and Bigley (24) suggested that both RNA and protein may be required to obtain optimal immunogenicity. In contrast, Lin and Berry (15), Eisenstein (3), and Hoops et al. (10) have strong evidence that cell wall components are needed in ribosomal vaccines to protect against *S. typhimurium* infections. Controversial results have been also found in the case of *Pseudomonas aeruginosa*. Lieberman (14) demonstrated that protection induced by the purified ribosomal preparation was RNA independent, whereas Gonggrijp et al. (9) con-

TABLE 3. *Specificity of the protection conferred on mice by CRP, peak I, and peak II from K. pneumoniae strain 7823 (O1:K2)*[a]

Prepn	*K. pneumoniae* challenge strain	PD_{50}[b] (ng)
CRP	7825 (O1:K1)	>50,000
	52-145 (O1:K2)	18
Peak I	7825 (O1:K1)	>11,000
	52-145 (O1:K2)	12
Peak II	7825 (O1:K1)	>1,500
	52-145 (O1:K2)	276

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

[b] Expressed on a dry weight basis.

TABLE 4. *Immunoprotective activity of untreated and phage-treated CRP, peak I, and peak II*[a]

Antigen type	PD_{50} (ng)[b]
Untreated CRP	18
Phage-treated CRP	>50,000
Untreated peak I	12
Phage-treated peak I	>11,000
Untreated peak II	276
Phage-treated peak II	>1,500

[a] All animals were immunized subcutaneously at zero time and 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 per mouse) or injected with saline and then challenged with *K. pneumoniae* 52-145 (O1:K2); all control mice died.

[b] Expressed on a dry weight basis.

cluded that RNA was required for protection by purified ribosomal preparations from *P. aeruginosa*.

To explain the immunoprotective activity of *K. pneumoniae* ribosomal preparations, Michel et al. (17) and Fontanges et al. (5) proposed that RNA-polysaccharide complexes were involved. However, these investigators did not specify which polysaccharide was complexed with RNA: CP or LPS. In a previous work, we provided direct evidence that CP was involved in the immunoprotective activity of *K. pneumoniae* ribosomal preparations (22). As CP is undoubtedly an extraribosomal antigen, the question remained whether the immunoprotective potency of this CP was either dependent on or independent of its association with ribosomal RNA. We compared the immunoprotective activity of ribosomal preparations and bacterial cell surface preparations from *K. pneumoniae* (6). The PD_{50} of both preparations was practically the same on the basis of CP content. Thus, CP was as immunogenic in association with cell surface preparations as in association with ribosomal preparations. These results suggested that the immunoprotective activity of CP was not due to its association with ribosomal RNA, but they did not provide direct evidence for this contention. The experiment did not eliminate the possibility of two distinct mechanisms to explain the immunoprotective activity of *K. pneumoniae* CP: (i) a role of RNA-CP complexes in the case of immunoprotective ribosomal preparations or (ii) a role either of high-molecular weight or native-form CP or of surface antigens-associated CP in the case of immunoprotective cell surface preparations.

Our results provide evidence that the immunoprotective activity of CP present in CRP does not depend on its association with ribosomal RNA. However, more experimentation is required to explain the high immunoprotective activity of *K. pneumoniae* cell surface-associated CP.

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LITERATURE CITED

1. **Ashwell, G.** 1957. Colorimetric analysis of sugars. Methods Enzymol. **3**:87–89.
2. **Blumenkrantz, N., and G. Asboe-Hansen.** 1973. New method for quantitative determination of uronic acids. Anal. Biochem. **54**:484–489.
3. **Eisenstein, T. K.** 1975. Evidence for O antigens as the antigenic determinants in "ribosomal" vaccines prepared from *Salmonella*. Infect. Immun. **12**:364–377.
4. **Eisenstein, T. K.** 1978. Ribosomal vaccines: a review, p. 211–222. *In* A. Voller and H. Friedman (ed.), New trends and developments in vaccines. MTP Press Limited, St. Leonard Gate.
5. **Fontanges, R., D. Robert, Y. Content, and G. Nis.** 1980. Study of the immunogenicity of ribosomes and ribosomal RNA extracted from *K. pneumoniae* and *S. pneumoniae*. Arzneim. Forsch. **30**:142–172.
6. **Fournier, J. M., C. Jolivet-Reynaud, M. M. Riottot, and H. Jouin.** 1981. Immunoprotective activity in mice of cell surface preparations from *Klebsiella pneumoniae*: comparative study with ribosomal preparations. Infect. Immun. **32**:420–426.
7. **Grahan, 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.
8. **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.
9. **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.
10. **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.
11. **Janda, J., and E. Work.** 1971. A colorimetric estimation of lipopolysaccharides. FEBS Lett. **16**:343–345.
12. **Johnson, W.** 1972. Ribosomal vaccines. I. Immunogenicity of ribosomal fractions isolated from *Salmonella typhimurium* and *Yersinia pestis*. Infect. Immun. **5**: 947–952.
13. **Johnson, W.** 1973. Ribosomal vaccines. II. Specificity of the immune response to ribosomal ribonucleic acid and protein isolated from *Salmonella typhimurium*. Infect. Immun. **8**:395–399.
14. **Lieberman, M. M.** 1978. *Pseudomonas* ribosomal vaccines: preparation, properties, and immunogenicity. Infect. Immun. **21**:76–86.
15. **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 vaccines. RES J. Reticuloendothel. Soc. **8**:135–143.
16. **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.
17. **Michel, F. B., L. Dussourd d'Hinterland, J. Bousquet, A. M. Pinel, and G. Normier.** 1978. Immunostimulation by a ribosomal vaccine associated with a bacterial cell wall adjuvant in humans. Infect. Immun. **20**:760–769.
18. **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.
19. **Portelance, V., R. P. Boulanger, and R. Brasseur.** 1977. Induction of antituberculous immunity by polysaccharidic contaminants of crude ribosomal vaccines isolated from *Mycobacterium bovis*, strain BCG. Rev. Can. Biol. **36**:265–275.
20. **Portelance, V., R. Brasseur, and R. P. Boulanger.** 1975. Factors affecting the immunizing activity of ribosomal fractions isolated from *Mycobacterium tuberculosis* var. *bovis* strain BCG. Can. J. Microbiol. **21**:1492–1499.
21. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent end points. Am. J. Hyg. **27**: 493–497.
22. **Riottot, M. M., J. M. Fournier, and H. Jouin.** 1981. Direct evidence for the involvement of capsular polysaccharide in the immunoprotective activity of the *Klebsiella pneumoniae* ribosomal preparations. Infect.

Immun. **31**:71-77.

23. **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.
24. **Smith, R. A., and N. J. Bigley.** 1972. Ribonucleic acid-protein fractions of virulent *Salmonella typhimurium* as protective immunogens. Infect. Immun. **6**:377-383.
25. **Venneman, M. R.** 1972. Purification of immunologically active ribonucleic acid preparations of *Salmonella typhimurium*: molecular-sieve and anion exchange chromatography. Infect. Immun. **5**:269-282.
26. **Venneman, M. R., and N. J. Bigley.** 1969. Isolation and partial characterization of an immunogenic moiety obtained from *Salmonella typhimurium*. J. Bacteriol. **100**: 140-148.
27. **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.
28. **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.
29. **Youmans, A. S., and G. P. Youmans.** 1970. Immunogenic mycobacterial ribosomal and ribonucleic acid preparations: chemical and physical characteristics. Infect. Immun. **2**:659-668.
30. **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.