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Nature of the Cross-Protective Antigen in Subcellular Vaccines of *Streptococcus pneumoniae*

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Studies have been carried out to investigate the nature of the antigen present in subcellular extracts of a rough strain of *Streptococcus pneumoniae* $A66_{2b}$ which has been shown to confer protection in mice against challenge with smooth, virulent organisms of the homologous and heterologous serotypes. The finding that whole, heat-killed cells were also capable of immunizing mice against challenge with organisms of heterologous serotypes suggests that the immunogen is present on the surface of the rough pneumococcal cell. Ribosomes purified by sucrose gradient centrifugation were not protective, but material recovered in the pellet retained activity. Subcellular extracts prepared from spheroplasts with a partial absence of cell wall showed decreased protective capacity, and extracts prepared from wall-deficient protoplasts were not protective. Crude cell walls evidenced cross-serotype protection, but purified walls did not protect. These results are interpreted as suggesting that the active moiety in the subcellular vaccine is present on the surface of rough pneumococci and is either a wall antigen that must be part of a larger macromolecular complex to be immunogenic, or a substance associated with the cell wall that is present in crude, but not purified, cell wall fractions.

Thompson and Snyder (20) in 1971 were the first to report that a subcellular ribosome-rich fraction prepared from a non-encapsulated type 3 pneumococcus could be used as a vaccine and could confer cross-serotype immunity in mice against encapsulated pneumococci of homologous and heterologous serotypes. The properties of this cross-protective preparation were interesting in that immunogenicity was destroyed by prior treatment of the vaccine with ribonuclease, was reduced over 80% by protease, and was reduced 40% by deoxyribonuclease (20). Swendsen and Johnson, in studies on partial purification of the cross-protective moiety, found that it was retained in protein extracts prepared by 2-chloroethanol extraction of ribosomal fractions (18). The identity of the substance which gives cross-serotype protection is presently still unknown. However, studies carried out by Thompson and Eisenstein (19) showed that immune antiribosomal serum could passively transfer protection and that the protection could be absorbed out with smooth or rough type 3 cells. Swendsen and Johnson confirmed the observation that whole organisms could absorb out the protective capacity of antiribosomal serum (18). To explain these results, Thompson and Eisenstein proposed that the protection afforded by the ribosomes is due to the presence of one or more pneumococcal surface antigens (other than the capsular polysaccharide) which contaminate the ribosomes during the extraction process, and that the ribosomes may act as adjuvants for the contaminating antigens (19).

In the literature two pneumococcal antigens have been shown to be immunogenic on the cell surface and to be species specific, i.e., common to all serotypes. These are the C-carbohydrate (6, 9, 15), which is a cell wall antigen, and the F-polysaccharide (pneumococcal Forssman antigen) (5, 7-10), which is membrane associated. The experiments described in this paper were designed to examine more closely the hypothesis that the ribosomal vaccine protects because it is contaminated with cell wall antigens or cell membrane antigens.

MATERIALS AND METHODS

Bacterial strains. The following strains of *Streptococcus pneumoniae* were used in this study. All vaccines were prepared from $A66R_{2b}$, a type 3 rough strain. For challenge, smooth strains of types 3 (A66), 4, and 6A were used. All strains were kindly supplied by Robert Austrian of the University of Pennsylvania and maintained either in the lyophilized state or on blood agar plates containing 5% sheep blood. The 50% lethal dose for type 3 and type 6A was approximately 1 colony-forming unit (CFU) per mouse when injected intraperitoneally (i.p.). For type 4, the 50% lethal dose was about 10 CFU per mouse when injected i.p.

Experimental animals. Outbred CD-1 mice from Charles River Breeding Laboratories (Wilmington,

Mass.) were used. All animals were female, weighing 19 to 21 g. They were housed in plastic cages with Absorb-Dri as bedding. Purina Mouse Chow and water were available ad libitum.

Preparation of crude subcellular ribosomal fraction. Bacteria were grown in infusion broth (Difco), and crude ribosomes were extracted according to the procedure described by Thompson and Eisenstein (19). Three liters of strain $A66R_{2b}$ cells were collected at late log phase and washed three times in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-magnesium acetate buffer (pH 7). The cells were then suspended in 50 ml of 0.01 M magnesium acetate-potassium phosphate buffer (pH 7), and sodium deoxycholate was added to a final concentration of 1%. The suspension was incubated at 37°C for 30 min with occasional mixing. The lysed cells were then centrifuged at 41,000 × *g* for 15 min at 4°C. Crude ribosomes were pelleted from the supernatant by centrifugation at 152,000 × *g* for 3 h at 4°C. The pellet was suspended in cold magnesium acetate-potassium phosphate buffer.

Preparation of heat-killed rough cells. Heat-killed rough pneumococci were prepared as described by Goebel and Adam (9). Cells were collected at late log phase, suspended in phosphate-buffered saline, and heated in a 100°C water bath for 5 min. Formalin was then added at room temperature to a final concentration of 0.1%, and the cell concentration was determined by counting in a Petroff-Hausser chamber.

Preparation of spheroplasts and cell fractionation. Wall-deficient forms (spheroplasts) of $A66R_{2b}$ cells were prepared as described by Lacks and Neuberger (14). As a positive control, pneumococcal strain R6 was obtained from Lacks at Brookhaven National Laboratory, Uptown, N.Y. Cells were grown at 37°C in a chemically defined medium with casein hydrolysate base (13) to an optical density reading at 650 nm (OD_{650}) of about 0.2. The culture was then transferred to a 30°C water bath and allowed to grow to an OD_{650} of about 0.4. The cells were finally harvested by centrifugation and suspended to one-fifth the original volume in a 0.05 M Tris-malate buffer containing 1 M sucrose at pH 7.6. The cells were incubated at 30°C, and at various time periods samples were tested for sensitivity to osmotic shock. This was done by pipetting 1-ml samples of the culture either into 3 ml of the Tris-malate buffer containing 1 M sucrose or into 3 ml of distilled water. The OD_{650} of these dilutions was measured. A decrease in OD upon dilution into water indicated that spheroplasts had been formed.

After the cells had been converted into spheroplasts, they were pelleted by centrifugation at 8,000 × *g* for 15 min. The supernatant fluid was saved for retrieval of cell wall fragments, and the spheroplasts were lysed by suspending in a 0.01 M Tris-hydrochloride buffer containing 0.0001 M $MgCl_2$ at pH 7.6 (buffer A). The lysate was centrifuged at 41,000 × *g*, and the supernatant was then spun at 152,000 × *g*, as described above, to pellet the ribosomes. The supernatant fluid obtained after the spheroplasts had been pelleted was dialyzed against buffer A. This fraction was assumed to contain partially digested cell walls as a result of the spheroplast formation. After dialysis, this fraction was pelleted by centrifugation at 20,000 × *g* for 20 min and then washed six times with distilled water. The amount of cell wall material was quantitated in terms of hexosamine content by the Elson-Morgan assay (17).

Preparation of protoplasts and cell fractionation. Pneumococcal protoplasts were prepared using mutanolysin M-1, a bacteriolytic enzyme produced by *Streptomyces globisporus* 1829 (23). Cells were harvested at late log phase and suspended in one-fifth the original volume in a buffer composed of 0.005 M Tris-hydrochloride (pH 7), 0.0025 M $MgSO_4$, and 12.5% (wt/vol) polyethylene glycol no. 6000 (Baker). Mutanolysin M-1 was then added to a final concentration of 25 μg/ml. The culture was incubated at 37°C and tested for osmotic sensitivity as described above.

After the cells had been converted to protoplasts, they were pelleted by centrifugation at 8,000 × *g* for 20 min and washed once in the above buffer containing polyethylene glycol. The washed protoplasts were then lysed in a buffer composed of 0.01 M Tris-hydrochloride, 0.01 M $MgCl_2$, and 0.05 M KCl (pH 7.8) (buffer B). The lysate was centrifuged at 41,000 × *g* for 20 min, and the supernatant was spun at 152,000 × *g* for 3 h to pellet the ribosomes.

Purification of ribosomes by sucrose gradient centrifugation. About 40 OD_{260} units of the crude subcellular ribosomal preparation in a volume of 0.45 ml was layered on top of 13 ml of a linear 15 to 30% sucrose gradient (wt/vol) in buffer B. A total of about 160 OD_{260} units of crude ribosomes in a volume of 1.8 ml was run in four tubes. As a positive control, *Escherichia coli* 70S ribosomes, provided by F. Chang, Department of Biology, Temple University, Philadelphia, Pa., were included in different tubes and run at the same time. They were centrifuged at 30,000 × *g* for 18 h in an SW40 rotor. After centrifugation, 10-drop fractions were collected, and the OD_{260} of each fraction was monitored by continuous flow in a spectrophotometer. The fractions under the peaks, representing the 70S, 50S, and 30S ribosomal subunits, were pooled and dialyzed against buffer B. After dialysis, the volume was concentrated in a Minicon filter to the original volume of 1.8 ml.

Preparation of cell walls. Crude pneumococcal cell walls were prepared according to the procedure described by Mosser and Tomasz (15). A portion of the crude cell walls was purified by adding deoxyribonuclease (10 μg/ml), ribonuclease (50 μg/ml), and a few drops of chloroform, followed by incubation at 37°C for 12 h with constant mixing with a magnetic stirrer. Trypsin (50 μg/ml) and $CaCl_2$ (0.1 mM) were then added, and incubation was continued for another 12 h. This was followed by the addition of a second portion of trypsin (50 μg/ml) and incubation for an additional 12 h. The purified cell walls were pelleted by centrifugation and washed six times with phosphate-buffered saline and six times with distilled water. The amount of cell wall material in the crude and purified cell wall preparations was quantitated in terms of hexosamine content by the Elson-Morgan assay (17).

Immunization and challenge procedures. All vaccines, with the exception of the heat-killed cells, were emulsified in an equal volume of Freund incomplete adjuvant (Difco). Before injection into mice,

sterility of all vaccines was established by inoculation of 0.1 ml into both brain heart infusion and thioglycolate broths (Difco). Vaccines were injected i.p. into mice in a volume of 0.5 ml.

Mice were challenged 3 weeks post-immunization with virulent, encapsulated pneumococci of the desired serotypes. Immunity was assessed by survival at 7 days after challenge. For challenge, the organisms were grown to late log phase in infusion broth supplemented with 10% fetal calf serum, 2% glucose, and 0.15% cysteine-hydrochloride. The culture was chilled on ice, and the number of bacteria per milliliter was determined by counting in a Petroff-Hausser counter. The culture was then diluted in infusion broth to obtain the desired number of cells for challenge. The actual number of organisms in the challenge inoculum was calculated from colony counts obtained by making spread plates on 5% sheep blood agar.

Statistics. The levels of significance for the observed frequencies (P_i) were determined by Fisher's exact test using 2 × 2 tables (3).

RESULTS

Cross-serotype protection by crude subcellular ribosomal preparation. Previous studies by different investigators (19, 20) have shown that crude ribosomes extracted from different type 3 rough strains of *S. pneumoniae* afforded cross-serotype protection against pneumococcal infection in mice. To test whether the crude ribosomal preparation extracted from this strain would protect across serotype lines, groups of mice (14 per group) were injected i.p. with crude subcellular ribosomal extract (1.5 OD_{260} units per mouse) mixed with an equal volume of Freund incomplete adjuvant. Control mice were injected with buffer plus adjuvant alone. Three weeks later, these mice were challenged with virulent pneumococci of serotypes 3, 4, or 6A. As shown in Table 1, excellent protection was found when the immunized animals were challenged with the homologous serotype at a dose of 30 CFU. In addition, there was significant protection against challenge with heterologous serotypes 4 and 6A in the immunized mice. The results suggest the presence of a species-specific antigen in the crude ribosomal extract prepared from this bacterial strain.

Cross-serotype protection by heat-killed $A66R_{2b}$ cells. Groups of mice were immunized with heat-killed $A66R_{2b}$ cells to look for cross-serotype protection. This was done to test the hypothesis proposed by Thompson and Eisenstein (19) that the protective immunogen in the "ribosomal" vaccine is a species-specific antigen exposed on the cell surface. Three groups of 10 mice each were injected i.p. with 5×10^8 heat-killed bacteria suspended in 0.5 ml of phosphate-buffered saline. Control mice received 0.5 ml of phosphate-buffered saline alone. Three weeks after immunization, all mice were challenged with virulent *S. pneumoniae* of serotypes 3, 4, or 6A (Table 2). There was 90% protection against a challenge dose of 180 CFU of the homologous serotype and significant but lesser protection against challenge with lower doses of heterologous serotypes 4 and 6A. These results, therefore, indicate the presence of a cross-protective antigen on the surface of this rough bacterial strain. This experiment was not intended to compare the degree of homologous protection with heterologous protection, although the results do suggest that better protection was achieved when challenge was with the homologous serotype.

Purification of ribosomes by sucrose gradient centrifugation and protection studies. To test the hypothesis that the protection af-

TABLE 1. *Protection by crude subcellular ribosomal preparation against challenge with homologous and heterologous serotypes of S. pneumoniae*

Challenge serotype	Challenge dose[a] (CFU per mouse)	No. of survivors[b] (%)		P_i[e]
		Immune[c]	Control[d]	
3	30	13 (93)	0 (0)	0.005
4	50	11 (79)	2 (14)	0.005
6A	130	12 (86)	2 (14)	0.005

[a] Challenge given i.p. 3 weeks post-immunization.
[b] $N = 14$.
[c] Mice immunized i.p. with 1.5 OD_{260} units of crude subcellular ribosomal preparation in Freund incomplete adjuvant.
[d] Mice received buffer in Freund incomplete adjuvant i.p.
[e] P_i value compares immune with control for each group.

TABLE 2. *Protection by heat-killed S. pneumoniae $A66R_{2b}$ against challenge with homologous and heterologous serotypes*

Challenge serotype	Challenge dose[a] (CFU per mouse)	No. of survivors[b] (%)		P_i[e]
		Immune[c]	Control[d]	
3	180	9 (90)	0 (0)	0.005
4	20	5 (50)	0 (0)	0.025
6A	30	7 (70)	1 (10)	0.01
6A	100	6 (60)	0 (0)	0.01

[a] Challenge give i.p. 3 weeks post-immunization.
[b] $N = 10$.
[c] Mice immunized with 5×10^8 cells suspended in phosphate-buffered saline i.p.
[d] Mice sham-immunized with phosphate-buffered saline i.p.
[e] P_i value compares immune with control for each group.

forded by the crude ribosomal extract might be due to contamination of the ribosomes with a pneumococcal surface antigen, crude ribosomes were purified by sucrose gradient centrifugation, and the purified fractions were used to test for protection. A volume of 1.8 ml of the crude ribosomal preparation (92 OD_{260} units per ml) was layered on top of a linear 15 to 30% sucrose gradient and centrifuged at 30,000 × *g* for 18 h in an SW40 rotor. To see whether the pneumococcal ribosomal subunits were located at the regions where intact 70S, 50S, and 30S subunits would be expected to band, a positive control of 70S *E. coli* ribosomes was included in different tubes and run at the same time. After centrifugation, fractions were collected, and a membranous pellet was left behind. The profiles of the gradients are shown in Fig. 1. From the gradient profiles, it can be seen that the pneumococcal ribosomes seem to be degraded by the extraction process, since a majority of the subunits banded near the region of the *E. coli* 30S subunits (peak III). Nevertheless, the fractions below peaks I, II, and III were pooled together and dialyzed against buffer B to remove the sucrose. The volume of this pooled fraction was then concentrated to the original 1.8 ml in a Minicon filter, and this purified ribosomal fraction was used to immunize mice. The membranous pellet obtained after sucrose gradient centrifugation was rinsed with buffer and also suspended in a volume of 1.8 ml of buffer B for subsequent protection studies.

To test for the ability to confer protection against pneumococcal infection with the homologous serotype, groups of mice were immunized with either the crude ribosomal extract, the purified pooled ribosomal fraction, the membranous pellet, or buffer alone. Since the gradient-purified ribosomal fraction had been dialyzed to remove the sucrose, an additional group of mice were immunized with dialyzed crude ribosomes. For each fraction, three doses were used for immunization. For the crude subcellular ribosomal fraction, doses of 1.0, 1.5, and 2.0 OD_{260} units per mouse were obtained by diluting the concentrated material. Comparable doses of the purified pooled ribosomal fraction, the membranous pellet, and the dialyzed crude ribosomes were obtained by making similar dilutions of the material which had been collected or which had been dialyzed and previously standardized to the crude ribosomal preparation on an equal volume basis. All vaccines were mixed with an equal volume of Freund incomplete adjuvant before injection into mice. Three weeks after immunization, all mice were challenged with 80 CFU of smooth, virulent type 3 *S. pneumoniae*. It can be seen from the data presented in Table 3 that the crude ribosomal preparation and the dialyzed crude ribosomes conferred good protection in the immunized mice against this challenge dose. Significant protection was also achieved in mice immunized with the membranous pellet. However, the pooled ribosomal frac-

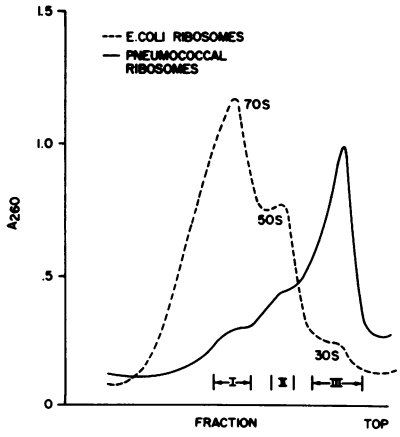

FIG. 1. *Profiles of ribosomal fractions collected from the sucrose gradient. The crude subcellular ribosomal fraction from S. pneumoniae A66R2b was purified by sucrose gradient centrifugation. E. coli 70S ribosomes were run at the same time as a standard. The pneumococcal ribosomal fractions below peaks I, II, and III were pooled together for subsequent protection studies.*

TABLE 3. *Protection afforded by crude subcellular ribosomal preparation and sucrose gradient-purified ribosomal fractions against challenge with type 3 S. pneumoniae*

Mice immunized with:	Immunizing dose[a]	No. of survivors[b]	Pooled % survival	P_i[c]
Crude subcellular ribosomal preparation	Low	8		
	Medium	5	67	0.005
	High	7		
Dialyzed crude ribosomes	Low	6		
	Medium	6	67	0.005
	High	8		
Gradient-purified ribosomes	Low	4		
	Medium	0	23	NS
	High	3		
Pellet from gradient	Low	6		
	Medium	3	47	0.025
	High	5		
Buffer	—	0	0	—

[a] Mice immunized with crude subcellular ribosomal preparation received a dose standardized by the OD_{260} as follows: low, 1 OD_{260}; medium, 1.5 OD_{260}; high, 2 OD_{260}. Doses for the other fractions were standardized against the crude ribosomes on a comparable volume basis as described in the text.

[b] Challenge dose: 80 CFU of type 3 *S. pneumoniae* given 3 weeks post-immunization. $N = 10$.

[c] Significance calculated by comparing pooled survival values for each group versus buffer. NS, Not significant: $P_i > 0.05$.

tions from the sucrose gradient did not afford significant protection. Since no dose response was evident for any of the vaccines, the percent survival was calculated on composite values for the three dose groups for each vaccine. One important conclusion from this experiment is that the protective immunogen is not dialyzable.

In further experiments, mice were immunized with the same fractions described above and tested for protection against heterologous serotypes. Groups of mice were immunized with 2.0 OD_{260} units or a comparable dose on a volume basis of the above fractions, and challenged with virulent *S. pneumoniae* of either serotype 4 or 6A (Table 4). When challenge was with 45 CFU of type 6A, significant protection was observed in mice immunized with the crude ribosomal extract, dialyzed crude ribosomes, and the membranous pellet. Mice immunized with the gradient-purified ribosomal fraction did not show significant protection as compared with control mice which received buffer alone. The results from this experiment on heterologous protection are similar to those obtained for homologous protection. Both experiments identify the protective immunogen as being present in the membranous pellet, but not in the purified ribosomal fraction. None of the fractions afforded any protection in the immunized mice when they were challenged with type 4 pneumococcus at a dose of 90 CFU per mouse. In this case, the challenge dose might have been sufficiently high that it overcame the protective capacity of the vaccines.

TABLE 4. *Evaluation of protection afforded by crude subcellular ribosomal preparation and sucrose gradient-purified ribosomal fractions against challenge with S. pneumoniae types 4 and 6A*

Mice immunized with[a]:	Protection against challenge with:			
	Type 4[b]		Type 6A[c]	
	No. of survivors[d] (%)	P_i[e]	No. of survivors[d] (%)	P_i[e]
Crude subcellular ribosomal preparation	2 (14)	NS	11 (79)	0.025
Dialyzed crude ribosomes	2 (14)	NS	12 (86)	0.025
Gradient-purified ribosomes	0 (0)	NS	7 (50)	NS
Pellet from gradient	0 (0)	NS	14 (100)	0.005
Buffer	0 (0)	—	4 (29)	—

[a] Mice immunized with crude subcellular ribosomal preparation received a dose of 2 OD_{260}. Doses for the other fractions were standardized against the crude ribosomes on a comparable volume basis as described in the text.

[b] Challenge dose: 90 CFU of type 4 *S. pneumoniae* given 3 weeks post-immunization.

[c] Challenge dose: 45 CFU of type 6A *S. pneumoniae* given 3 weeks post-immunization.

[d] $N = 14$.

[e] P_i values were calculated for immunized groups versus buffer group. NS, Not significant: $P_i > 0.05$.

Studies on immunogenicity of ribosomes extracted from pneumococcal spheroplasts. The observation that the protective immunogen residues in the membranous pellet but not in the purified ribosomal fraction tends to support the hypothesis that the immunogen is a contaminant. To investigate whether or not the immunogen in the crude ribosomal extract is a cell wall contaminant, spheroplasts and protoplasts were prepared from strain $A66R_{2b}$ cells. Ribosomes were then extracted from these wall-deficient forms to immunize mice for protection against pneumococcal infection.

Spheroplasts were prepared by suspending exponentially growing cells in Tris-malate buffer containing 1 M sucrose, and their formation was monitored spectrophotometrically by a decrease in optical density due to osmotic shock after dilution into water (Fig. 2). Lacks and Neuberger (14) reported that autolytic enzymes are involved in the formation of spheroplasts in a concentrated sugar solution and that genetically different strains of *S. pneumoniae* form spheroplasts at different rates. It can be seen from Fig. 2 that the bacterial strain used in this study, $A66R_{2b}$, appeared to form spheroplasts at a slow rate as compared with strain R6 used by Lacks and Neuberger (14). As a result, the $A66R_{2b}$ cells were allowed to incubate overnight for 18 h before a significant decrease in optical density was detected when the culture was diluted into water. After the spheroplasts were formed, a

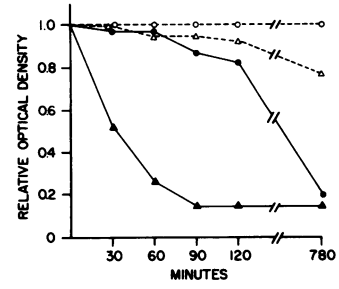

FIG. 2. *Rate of spheroplast formation for S. pneumoniae strains $A66R_{2b}$ and R6. Cells from the two strains were incubated in 1 M sucrose in buffer and tested for osmotic sensitivity as described in the text. Dilution into 1 M sucrose: △, R6; ○, $A66R_{2b}$. Dilution into water: ▲, R6; ●, $A66R_{2b}$. Relative optical density = (OD_{650} at time X)/(OD_{650} at time 0).*

subcellular ribosomal fraction was extracted according to the procedures described in Materials and Methods.

To compare the immunogenicity of the ribosomes extracted from the spheroplasts with the immunogenicity of ribosomes extracted from whole cells after lysis with deoxycholate, two groups of mice were immunized with 1 OD_{260} unit of the two different ribosomal preparations emulsified in Freund incomplete adjuvant. Another group of mice was injected with buffer in adjuvant alone. Three weeks later, all mice were challenged with virulent pneumococci of serotype 3, 4, or 6A. The results are given in Table 5 for mice challenged with 100 CFU of smooth type 3 pneumococci. Both ribosomal extracts gave highly significant protection as compared with the controls. Protection against challenge with heterologous serotypes was also tested (Table 6). Significant protection was observed in the immunized group challenged with the low dose of serotype 6A. The ribosomes extracted from the spheroplasts did not protect against challenge with the type 4 pneumococcus or type 6A at the higher challenge dose. Comparing the degree of heterologous protection afforded by the ribosomes extracted from the spheroplasts (Table 6) with the crude ribosomal preparation extracted from whole cells (Table 1), it appears that partial removal of the cell walls before the extraction of ribosomes resulted in a reduction in the protective capacity of the vaccine.

TABLE 5. *Comparison of the protective capacity afforded against challenge with type 3 S. pneumoniae by a ribosomal fraction extracted from spheroplasts with the crude subcellular ribosomal preparation*

Mice immunized with[a]:	No. of survivors (%) after challenge[b]	P_i
Crude subcellular ribosomal preparation	15 (100)	0.005
Spheroplast ribosomes	13 (87)	0.005
Buffer	0 (0)	—

[a] Immunizing dose: 1 OD_{260} in Fruend incomplete adjuvant i.p.

[b] Challenge dose: 100 CFU of type 3 *S. pneumoniae* given 3 weeks post-immunization. $N = 15$.

TABLE 6. *Evaluation of protection afforded by ribosomes extracted from spheroplasts against challenge with homologous and heterologous serotypes of S. pneumoniae*

Challenge serotype	Challenge dose[a] (CFU per mouse)	No. of survivors[b] (%)		P_i[e]
		Immune[c]	Control[d]	
3	100	10 (100)	3 (30)	0.005
4	30	3 (30)	2 (20)	NS
6A	30	10 (100)	2 (20)	0.005
6A	100	5 (50)	1 (10)	NS

[a] Challenge given i.p. 3 weeks post-immunization.

[b] $N = 10$.

[c] Mice immunized i.p. with 1 OD_{260} of ribosomes extracted from spheroplasts suspended in Freund incomplete adjuvant.

[d] Mice received buffer in Freund incomplete adjuvant i.p.

[e] P_i value compares immune with control for each group. NS, Not significant: $P_i > 0.05$.

Studies with protoplasts. Protoplasts are, by definition, bacterial forms completely devoid of cell walls, as compared to spheroplasts, which may still have small pieces of cell wall fragments attached to the cell surface. Lacks and Neuberger reported that pneumococcal spheroplasts, even though they are osmotically sensitive, still retain some cell wall material (14). Since ribosomes prepared from the spheroplasts still conferred significant protection against challenge with serotypes 3 and 6A, the role of the cell walls in inducing immunity in the ribosomal vaccine was further investigated by preparing pneumococcal protoplasts. Ribosomes were then extracted from these wall-less forms and subsequently tested in protection studies.

Pneumococcal protoplasts were prepared by adding the bacteriolytic enzyme mutanolysin M-1 to *S. pneumoniae* $A66R_{2b}$, with polyethylene glycol as an osmotic stabilizer. Initial experiments were carried out to see whether this enzyme would digest pneumococcal cell walls, and to determine the necessary enzyme concentration. From the curve shown in Fig. 3, it can be seen that the enzyme was active at various concentrations in lysing the cells. Figure 4 shows the suitability of using polyethylene glycol as an

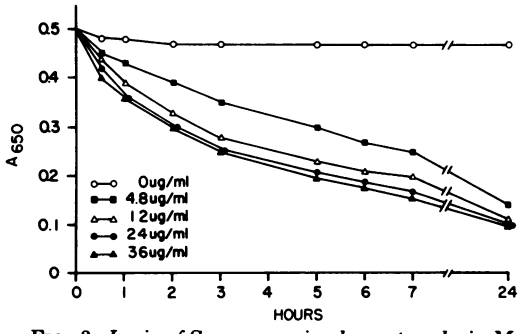

FIG. 3. *Lysis of S. pneumoniae by mutanolysin M-1. Cells were heated at 70°C for 20 min to inactivate the autolytic enzymes before suspending to OD_{650} of 0.5 in 0.005 M Tris-hydrochloride buffer (pH 7) containing 0.0025 M $MgSO_4$. Mutanolysin M-1 was then added in various concentrations, the cultures were incubated at 37°C, and changes in OD were observed.*

osmotic stabilizer. The protoplasts obtained in this way were washed once in the same buffer containing 12.5% polyethylene glycol to remove the cell walls from the supernatant. They were then lysed by suspension in buffer B. A crude ribosomal preparation was extracted from the lysate by ultracentrifugation as previously described. This ribosomal preparation was used to immunize mice to test for its ability to confer protection against pneumococcal infection. Groups of mice were immunized with 1.5 OD_{260} units of this ribosomal preparation emulsified in Freund incomplete adjuvant. Control mice were given buffer in adjuvant alone. Three weeks later, they were challenged with virulent pneumococci of serotype 3, 4, or 6A. It was observed that the ribosomes extracted from the protoplasts did not afford any significant protection in the immunized mice against challenge with either homologous or heterologous serotypes of the pneumococcus (Table 7). Since the challenge doses employed were moderate and in the range used in previous experiments where protection was demonstrated, it is unlikely that the observed lack of protection is attributable to an overwhelming of the protective capacity of the vaccine. It appears, therefore, that removal of the cell walls before the extraction of ribosomes resulted in a loss of the protective capacity of the vaccine.

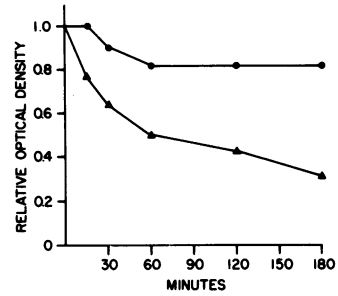

FIG. 4. *Formation of pneumococcal protoplasts. Mutanolysin M-1 at 25 mg/ml was used with 12.5% polyethylene glycol as an osmotic stabilizer. Protoplast formation was determined by osmotic shock monitored at OD_{260}. Dilution into water, ▲; dilution into buffer containing polyethylene glycol, ●.*

TABLE 7. *Evaluation of protection afforded by ribosomes extracted from protoplasts against challenge with homologous and heterologous serotypes of S. pneumoniae*

Challenge serotype	Challenge dose[a] (CFU per mouse)	No. of survivors[b] (%)		P_i[e]
		Immune[c]	Control[d]	
3	60	4 (26)	2 (13)	NS
4	60	1 (7)	0 (0)	NS
6A	45	1 (7)	0 (0)	NS

[a] Challenge given i.p. 3 weeks post-immunization.
[b] $N = 15$.
[c] Mice received 1.5 OD_{260} of ribosomes extracted from protoplasts in Freund incomplete adjuvant i.p.
[d] Mice received buffer in Freund incomplete adjuvant i.p.
[e] P_i value compares immune with control for each group. NS, Not significant: $P_i > 0.05$.

Evaluation of protection by cell walls. Crude and purified cell walls were extracted from *S. pneumoniae* $A66R_{2b}$ to test for their ability to immunize mice against pneumococcal infection. The wall fragments shed from the cells in the preparation of spheroplasts were also retrieved from the sucrose buffer for the same purpose. The amount of hexosamine in each fraction was determined by the Elson-Morgan assay (17). Assuming that pneumococcal cell walls contain 14.7% (by weight) hexosamine (15), the immunizing doses for each fraction were standardized on a dry-weight basis.

Groups of mice (10 per group) were immunized with several doses of the different cell wall fractions in Freund incomplete adjuvant. Control mice were given buffer plus adjuvant. Three weeks after immunization, all mice were challenged with 40 CFU of *S. pneumoniae* type 6A. None of the fractions gave significant protection against this low challenge dose except the crude cell wall fraction at the higher immunizing dose of 25 μg per mouse (Table 8). The ability of the crude, but not the purified, preparation to afford protection seems to indicate that the immunogenicity of the crude cell wall preparation is lost upon purification by enzymatic (deoxyribonuclease, ribonuclease, and protease) treatment.

TABLE 8. *Evaluation of the protection afforded by cell wall fractions and the F-polysaccharide of S. pneumoniae $A66R_{2b}$ against challenge with S. pneumoniae type 6A*

Mice immunized with[a]:	Dose[b] (μg/ml)	No. of survivors (%) upon challenge[c]	P_i
Crude cell walls	10	2 (20)	NS[d]
Crude cell walls	25	6 (60)	0.01
Purified cell walls	10	1 (10)	NS
Purified cell walls	25	0 (0)	NS
Spheroplast cell walls	10	0 (0)	NS
Saline	—	0 (0)	—

[a] All vaccines were mixed with an equal volume of Freund incomplete adjuvant before i.p. injection into mice.
[b] Determined from the hexosamine content of the preparations.
[c] Challenge dose: 40 CFU of type 6A *S. pneumoniae* injected i.p. $N = 10$.
[d] NS, Not significant: $P_i > 0.05$.

DISCUSSION

These experiments show that a crude subcellular ribosomal extract from *S. pneumoniae* $A66R_{2b}$, a type 3 rough strain, gives excellent protection in mice against challenge with virulent pneumococci of the homologous serotype, as well as with heterologous serotypes 4 and 6A. This observation is in agreement with previous reports in which similar ribosomal preparations were used and shown to afford protection across serotype lines (19, 20). The observation that whole, heat-killed $A66R_{2b}$ cells give cross-serotype protection in mice suggests the presence of a cross-protective antigen on the surface of this strain. Similar observations were reported by Tillett in the later 1920s (21, 22): rabbits immunized with heat-killed rough pneumococci were found to have an increase in resistance against challenge with smooth pneumococci of serotype 1, 2, or 3. The presence of a species-specific antigen on the surface of the pneumococcus would explain previous observations that pneumococci of the homologous (19) or heterologous serotype (18) could adsorb out the protective capacity of antiribosomal serum, and thus would support the hypothesis proposed by Thompson and Eisenstein (19) that the protective immunogen in the ribosomal vaccine is a contaminating surface antigen. The results from protection studies using gradient-purified ribosomes tend to support the contaminant theory since these ribosomes were not protective. Instead, the protective immunogen was found in the pellet, which presumably was rich in cell membranes and cell wall fragments. Protection studies using ribosomes extracted from wall-deficient spheroplasts showed that they afforded less protection against challenge with homologous or heterologous serotypes than did similar ribosomal preparations from whole cells. Ribosomes extracted from protoplasts gave no protection. A crude cell wall preparation was found to give significant levels of protection at a dose of 25 μg per mouse. However, purified cell walls, which were derived from the crude wall preparation by extensive enzymatic treatment with deoxyribonuclease, ribonuclease, and protease, did not protect.

There are two interpretations of the results obtained using the crude and purified cell walls. The first hypothesis proposes that the reason crude walls protect, but purified walls do not, is because the crude wall fraction contains a contaminating nonwall antigen, which is removed upon further purification, thus rendering the purified walls nonprotective. The second hypothesis proposes that the protective antigen is a cell wall constituent, but that this wall antigen is not immunogenic unless it is complexed with other bacterial components. Previous studies on the pneumococcal ribosomal vaccine, showing that the immunogenicity of the vaccine is markedly reduced by ribonuclease treatment (19, 23) or other enzyme treatments (20), support the model of an antigen complex as the active moiety in these extracts. It could be envisaged that ribosomal ribonucleic acid or protein or both act as adjuvants or as carriers for a cell wall antigen in both the crude ribosomal and the crude cell wall preparations. In the literature, both double-stranded synthetic ribonucleic acid (polyadenylic-polyuridylic acid) and oligonucleotides have been shown to act as adjuvants in mice (4, 12). There are also reports in the literature in which highly purified pneumococcal carbohydrate antigens were found to be non-immunogenic in rabbits, but the same antigen presented as part of a complex did induce antibody production. It was established by Avery and Morgan in 1925 that purified capsular polysaccharide from the type 3 pneumococcus is not immunogenic in rabbits (2). However, if the animals were injected with whole, smooth, killed microorganisms, they made a good immune response to the pneumococcal polysaccharide (2). A similar situation is found in rabbits presented with purified C-carbohydrate. They do not make antibody to the purified molecule, but make an excellent immune response when immunized with the F-polysaccharide, a lipid-rich extract of the pneumococcus which contains an antigen that cross-reacts with the C-carbohydrate (9). No similar studies using purified C-carbohydrate have been carried out in mice.

The studies described in this paper support the concept that the active moiety in the subcellular vaccine is not a ribosomal component, but they do not definitively identify the protective antigen. It can be postulated that the C-carbohydrate might be the cross-protective antigen present in the ribosomes, as this is one of the already documented species-specific antigens of the pneumococcus. The other well-characterized species-specific surface antigen in the pneumococcus is the F-polysaccharide (pneumococcal Forssman antigen). Results presented in a companion study show that, even though the F-polysaccharide is a contaminant in the crude ribosomal extract, it is not the protective immunogen (1). Studies carried out by Schalla and Johnson (16) and by Green and Johnson (11), investigating a cross-protective antigen in a subcellular vaccine of *Streptococcus pyogenes*, point to a protein antigen as the immunogen in this closely related organism. Our studies do not exclude the possibility that an as yet unidentified species-specific protein antigen could be the protective antigen in the pneumococcal system.

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