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Humoral Immunity to *Streptococcus pneumoniae* Induced by a Pneumococcal Ribosomal Protein Fraction

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Isolation of a protective subfraction of ribosomes from *Streptococcus pneumoniae* has been achieved, and the immune response it induces has been investigated. Mice immunized with pneumococcal ribosomes or purified protein extracted from the ribosomal preparation (2-CE protein) exhibit similar survival rates upon challenge by virulent *S. pneumoniae*. In contrast, recipients of purified ribosomal ribonucleic acid were never protected against pneumococcal challenge. Serum from mice immunized with pneumococcal ribosomes or 2-CE protein passively immunized syngeneic recipients against pneumococcal challenge, whereas spleen cells from the same donors were unable to transfer immunity. Passive immunization with antiribosome serum could be abrogated by absorption with whole ribosomes, 2-CE protein, or various serotypes of *S. pneumoniae* (capsular types 2, 3, 6, and 14). Antiribosome serum significantly enhanced clearance of *S. pneumoniae* from mouse blood in vivo and in vitro. This required phagocytic cells, since antiribosome serum alone, with or without complement, supported growth of *S. pneumoniae* to an extent comparable to normal serum. The data suggest that the primary immunogen of pneumococcal ribosomes resides in the protein fraction. Further, the immunity induced by the protein fraction is mediated by antibody that appears to function as an opsonin for *S. pneumoniae*.

Since 1965, ribosomes and ribosome-derived preparations have repeatedly been shown to be effective immunogens against a virulent challenge by the homologous organism (3, 10, 24, 28, 29, 31, 34, 37, 39). Induction of protective immunity appears to be a common property among ribosomes from a broad spectrum of eukaryotic and prokaryotic organisms, but extensive investigation has failed to define conclusively the immunogenic component of ribosomal preparations. Evidence exists supporting ribosomal ribonucleic acid (RNA) (32, 40, 41), ribosomal protein (15, 24; C. W. Feit, R. P. Tewari, and M. DeMaria, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, Mm5, p. 136), or some combination of the two (1, 10, 26, 38) as the primary immunogen in subcellular vaccines. Others (7, 14) have suggested that protection is not induced by ribosomes themselves but rather from the presence of some contaminant that is regularly purified with the ribosomal preparation.

Although the mechanism of action of ribosomal vaccines remains to be worked out, reports suggest cell-mediated immunity may play a significant role in the system developed for *Mycobacterium tuberculosis* (17, 22) and *Salmonella typhimurium* (27, 33).

In contrast to the type-specific immunity classically associated with conventional streptococcal vaccine preparations (2, 5), ribosomal preparations from both *Streptococcus pneumoniae* (31) and *Streptococcus pyogenes* (24) have been shown to produce significant protection against heterologous challenge by various serotypes of pneumococci or streptococci, respectively. Recently, Schalla and Johnson (24) demonstrated purified ribosomal protein from *S. pyogenes* to be the primary immunogen in their streptococcal ribosomal vaccine, but the ability of streptococcal ribosomal protein to induce cross-immunity to other streptococcal serotypes was not investigated.

The results presented in this paper suggest that the immunogenic component of ribosomes from *S. pneumoniae* is also protein in nature. In addition, data are presented suggesting that the immunity induced by pneumococcal ribosomes or the protein extracted from the ribosomes is due to antibody that is able to opsonize numerous serotypes of *S. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains. A heavily encapsulated type 3 strain of *S. pneumoniae*, obtained as a fresh clinical isolate from the Iowa State Hygienic Laboratory,

Iowa City, Iowa, was used as the standard challenge organism throughout the study. An unencapsulated avirulent strain (3R) was isolated by serial culture of the type 3 strain in Todd-Hewitt broth (Difco). *S. pneumoniae* types 2 (ATCC 6302), 6 (ATCC 6306), 7 (ATCC 6307), and 14 (ATCC 6314) were purchased from the American Type Culture Collection. Virulence of all encapsulated strains was maintained by serial passage in mice. The mouse median lethal dose (LD_{50}) of types 2, 3, 6, and 7 was determined by the method of Reed and Muench (23) to be approximately 1 colony-forming unit (CFU) when administered intraperitoneally and 10^2 CFU when injected subcutaneously. Type 14 was relatively avirulent despite repeated mouse passage. The mouse LD_{50} for this strain was usually 10^3 CFU when injected intraperitoneally and 10^5 CFU via the subcutaneous route.

Growth of bacteria. Slants and plates were composed of meat infusion agar containing 5% defibrinated sheep blood. Large-scale broth cultures used Todd-Hewitt broth (Difco) supplemented with 2% neutralized yeast extract and 0.05% sodium thioglycolate. Carboys containing 15 liters of medium received a 20% inoculum of a log-phase culture and were incubated, with occasional agitation, at 37°C for 6 h. The pH was monitored throughout the growth period and was maintained at pH 7.8 with 1 N NaOH.

Preparation of ribosomes. Bacteria (strain 3R) from 40 to 45 liters of culture were collected at 4°C by means of continuous-flow centrifugation at 31,000 × *g*. The cell pellet (60 to 70 g of cells) was washed three times with sterile tris(hydroxymethyl)aminomethane(Tris)-buffered saline composed of 0.01 M Tris and 0.15 M NaCl, pH 7.5. The cells were then suspended in 80 ml of Tris buffer, containing 0.01 M $MgCl_2$, 0.35 M NH_4Cl, and 0.25% sodium dodecyl sulfate and 50 μg of deoxyribonuclease, pH 7.5. Sodium deoxycholate was added to a final concentration of 0.25%, and the mixture was incubated at 37°C for 15 min or until a Gram stain of the mixture failed to demonstrate intact bacteria. After cell lysis, all further steps were carried out at 4°C. Cellular debris was removed by centrifugation at 30,000 × *g* for 15 min, and the supernatant fluid was placed in a beaker. The fluid was brought to a volume of 200 ml by the addition of cold 0.01 M Tris containing 0.01 M $MgCl_2$ (TM buffer), pH 7.5. The ammonium sulfate precipitation procedure described by Fogel and Sypherd (11) was followed for the isolation of ribosomes. The precipitated ribosomes were suspended in 100 ml of TM buffer and dialyzed for 24 h against three changes of TM buffer. After dialysis, the contents of the sac were centrifuged at 35,000 × *g* for 15 min to remove any insoluble material and sterilized by positive-pressure filtration through a chilled 0.45-μm membrane filter (Millipore Corp.). The filtrate was then centrifuged at 105,000 × *g* for 2.5 h. The pelleted ribosomes were gently resuspended in a minimal amount of TM buffer with the aid of a glass tissue homogenizer and stored in small volumes at −70°C.

Preparation of ribosomal subfractions. Protein (2-CE protein) was prepared from 1 g of ribosomes (wet weight) by 2-chloroethanol extraction as described by Fogel and Sypherd (11). After 2 h of stirring at 4°C, the RNA precipitate was removed by centrifugation at 30,000 × *g* for 15 min, and the supernatant fluid containing the protein was dialyzed at 4°C against 200 volumes of sterile distilled water changed three times over 24 h. Due to its insolubility in physiological buffers after lyophilization, the protein was stored as a distilled-water solution. The protein solution was concentrated by placing the dialysis sac in polyvinylpyrrolidone (1 kg/liter of water) for 8 h. Protein prepared in this manner was free of detectable RNA, possessed an absorbancy at 260 nm/absorbancy at 280 nm ratio of 1, and showed an absorbance maximum at 230 nm. Preparations were stored at −70°C.

RNA was extracted with 100% sodium perchlorate as described by Wilcockson (36). One gram (wet weight) of ribosomes was suspended in 25 ml of Tris buffer containing 1% sodium dodecyl sulfate, pH 7.5. To this suspension was added 75 ml of 100% $NaClO_4$. The mixture was stirred for 15 min and then centrifuged at 5,000 × *g* for 30 min. The bottoms of the tubes were punctured, and the liquid below the sodium dodecyl sulfate-protein layer was collected into 2 volumes of ice-cold 95% ethanol, which was then allowed to stand at −20°C overnight. The precipitate was collected by centrifugation at 10,000 × *g* for 15 min and washed three times with cold 95% ethanol. The RNA was dissolved in a minimal amount of phosphate-buffered saline (0.01 M PO_4, 0.15 M NaCl), pH 7.5, and stored at −20°C. RNA preparations contained from 5 to 7% protein. Pneumococcal type 3 capsular polysaccharide was prepared according to Kabat and Meyer (16). Pneumococcal C carbohydrate was prepared as described by Liu and Gotschlich (20).

Experimental animals. Swiss-Webster mice were obtained from a colony maintained by the University of Iowa, Iowa City, Iowa. Inbred C3H/HeJ mice were purchased from Jackson Laboratories, Bar Harbor, Me. Experiments were conducted using either male or female mice but never together in the same experiment. All mice were used at 5 weeks of age.

Immunization and challenge procedures. Where possible, all immunizing doses were calculated on the basis of protein content; the optimal immunizing dose of ribosomes for Swiss-Webster mice was 30 μg of protein, whereas 150 μg was required to immunize the C3H/HeJ strain. The immunization protocol involved emulsifying the ribosomal preparations in an equal volume of incomplete Freund adjuvant (Difco) and injecting 0.2 ml of the mixture intraperitoneally. Animals were bled for serum or challenged 3 weeks after immunization. Rabbit antiserum to pneumococcal ribosomes was prepared by an initial footpad injection of antigen, emulsified in Freund incomplete adjuvant as described, followed 2 weeks later by a subcutaneous booster injection. Serum was collected 1 week later. In all experiments, animals injected with TM buffer in Freund incomplete adjuvant were used as controls.

Mice were challenged subcutaneously with 10 to 20 LD_{50} suspended in Hanks balanced salt solution

(HBSS). Bacterial count at time of challenge was confirmed by the spread plate technique. The results of each experiment were recorded as percentage of survival at 7 days postchallenge.

Passive transfer of serum and lymphocytes. Three weeks postimmunization, five mice from each experimental group and a control group were exsanguinated by cardiac puncture. Their spleens were removed, and single cell suspensions were prepared at room temperature in HBSS containing 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.3. Erythrocytes were lysed in hypotonic ammonium chloride solution, and the remaining lymphocytes were adjusted to a concentration of 10^8 viable cells/ml in HBSS-HEPES buffer. Serum and lymphocytes from each experimental group were pooled and injected into normal C3H/HeJ recipients. Recipients received either 0.1 ml of serum or 2×10^7 viable lymphocytes via the lateral tail vein. Twenty-four hours posttransfer, all recipients plus representative samples from each donor group were challenged with 10 LD_{50} of virulent pneumococci.

Immunological procedures. Double immunodiffusion was done in 1% agarose in Tris-buffered saline, pH 8.6. Reactions were stained with amido black after 24 h and then photographed. Passive hemagglutination was done in a microtiter system (Cooke Engineering Co.) using sheep erythrocytes as antigen carriers. Antigens were coupled to sheep erythrocytes using chromium chloride (9). Assays for macrophage migration inhibition factor were done as described by Falk and Zabriski (8) using 0.01-ml capillary pipettes (Drummond Scientific). Footpad testing for delayed hypersensitivity in mice was done according to Smith and Bigley (27).

Biochemical analysis. Protein concentrations were determined using the method of Lowry et al. (21). RNA and deoxyribonucleic acid were measured using the orcinol and diphenylamine reactions, respectively (6). Total carbohydrates were detected by the anthrone reaction (12), and the method of Randall and Morgan (12) was used to determine hexosamines.

In vivo clearance of bacteria. Mice were challenged with 10 LD_{50} of virulent type 3, and the number of viable pneumococci per milliliter of blood was determined by spread plate count at 4-h intervals for 24 h and, thereafter, once every 24 h for 27 days or until all experimental animals were dead. Counts of viable bacteria in the spleens of infected animals were also obtained by aseptically removing the organs at the time of bleeding and emulsifying them in 2 ml of sterile HBSS with a glass tissue homogenizer.

Growth of bacteria in serum. For each experiment, fresh serum was obtained, and one-half was heated at 56°C for 30 min while the other half was kept on ice. One-milliliter volumes of the various serum specimens were inoculated with 10^2 to 2×10^2 CFU of virulent pneumococci in 0.1 ml of phosphate-buffered saline, and 0.01-ml volumes (25) were removed for a colony count at time zero. The tubes were incubated at 37°C, and 0.01-ml samples were removed at designated intervals over 24 h to monitor the bacterial growth.

In vitro clearance of bacteria. In vitro clearance of pneumococci was done using the indirect bactericidal assay of Lancefield (19). Three-tenths milliliter of heparinized normal mouse blood was placed in a siliconized culture tube along with 0.05 ml of test serum, and an inoculum of 50 to 2×10^2 CFU in 0.1 ml of HBSS was added. Such samples were prepared in triplicate and all were incubated at 37°C on a slowly revolving, end-over-end rotary shaker. The number of bacteria in 0.1 ml was determined initially (T_0) and after 3 h of incubation (T_3). The percentage of change was expressed as $[(T_0 - T_3)/T_0] \times 100\%$.

Absorption of antiribosome serum. Rabbit antiserum to pneumococcal ribosomes was absorbed with *S. pneumoniae* by suspending approximately 10^9 CFU of a washed logarithmic-phase culture in each 1-ml volume of serum. Absorption of serum with pneumococcal ribosomes or 2-CE protein was done by adding the antigens to the serum specimens at a final concentration of 1 mg of protein per ml of serum. After absorption for 18 h at 4°C, all mixtures were diluted 1:2 in phosphate-buffered saline and sterilized by filtration (2.2-μm pore size, Millipore Corp.).

Statistical methods. Comparison of two sample means for significant differences was done using the Student's *t* test. The significance of survival in an immunized group of animals over that of a control group was determined using Fischer's exact probability test (42).

RESULTS

Determination of the ribosomal immunogen. Four groups of mice, 40 mice/group, were immunized with pneumococcal ribosomes, 2-CE protein, or ribosomal RNA or were sham immunized with buffer. The ribosomal subfractions were injected in amounts corresponding to those in the optimum immunizing dose for the intact ribosome, i.e., 30 μg of protein or 60 μg of RNA per mouse. At 7-day intervals after immunization, 10 mice from each group were challenged and the onset of immunity to *S. pneumoniae* induced by the ribosomal immunogens was recorded.

Mice immunized with ribosomes or 2-CE protein exhibited similar survival rates at each of the challenge periods postimmunization (Table 1). In contrast, the mice injected with ribosomal RNA were never significantly protected against pneumococcal challenge. Biochemical analysis of the 2-CE protein failed to detect RNA or polysaccharide contamination. In addition, no antibody to pneumococcal cell wall or capsular polysaccharide antigens was detectable by passive hemagglutination in the serum of mice immunized with the 2-CE protein.

Subsequent experiments (Fig. 1) demon-

TABLE 1. *Comparative immunogenicity of pneumococcal ribosomes and purified ribosomal subfractions assessed at various intervals postimmunization*[a]

Days postimmunization	Immunogenicity of antigens[b]			
	Buffer control	Ribosomes[c]	2-CE protein	Ribosomal RNA
7	0	0	40[d]	0
14	0	60	80	0
21	0	80	60	0
28	0	60	60	20[d]

[a] All mice were challenged subcutaneously with 10 LD_{50} of *S. pneumoniae* type 3.

[b] Expressed as percentage of survival 7 days postchallenge.

[c] Mice were injected with 30 μg of ribosomes or 2-CE proteins (based on protein content).

[d] $P > 0.05$, not significantly different from control values. All other values were significantly different from controls.

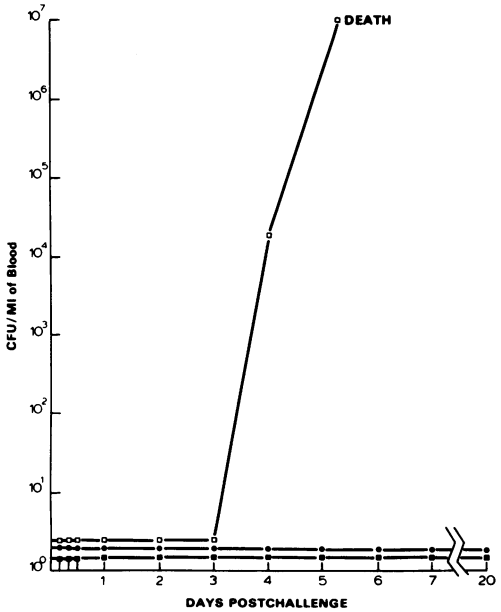

FIG. 1. *Clearance of a pneumococcal challenge by mice optimally immunized with pneumococcal ribosomes or 2-CE protein. All mice were challenged subcutaneously with 10 LD_{50} of S. pneumoniae type 3. Mice immunized with: ■, pneumococcal ribosomes; ●, 2-CE protein. □, Sham immunized.*

strated that both ribosomes and 2-CE protein induced immunity, which inhibited in vivo multiplication of a virulent pneumococcal challenge. Pneumococci could not be detected in the blood or spleens of immunized mice up to 20 days postchallenge, whereas sham-immunized mice consistently exhibited a significant bacteremia 3 to 4 days postchallenge and succumbed 5 to 6 days after pneumococcal infection.

Passive transfer of immunity. Passive transfer experiments were designed to assess the importance of humoral and cellular mechanisms in the antipneumococcal response of mice immunized with ribosomal preparations. Two groups of inbred C3H/HeJ mice were optimally immunized with pneumococcal ribosomes or 2-CE protein (150 μg of protein per mouse). A third group was sham immunized with TM buffer in Freund incomplete adjuvant. After 3 weeks, five donor mice from each group were sacrificed, and their serum and spleen cells were prepared as described. Unimmunized syngeneic recipients, of the same population as the donors, were divided into six groups of 10 mice/group. These animals were given either 0.1 ml of serum or 2×10^7 viable spleen cells from the donor mice. Twenty-four hours after transfer, all recipients plus 10 remaining animals from each donor group were challenged with 10 LD_{50} of *S. pneumoniae* type 3 and deaths were recorded for 7 days.

Pneumococcal ribosomal preparations appear to induce a strong humoral response against *S. pneumoniae* (Table 2). Recipients of 0.1 ml of serum from donors immunized with pneumococcal ribosomes or 2-CE protein exhibited survival rates that paralleled those of their respective donor groups. In contrast to these results, the transfer of one spleen equivalent from any of the donor groups failed to protect recipients against pneumococcal challenge. Attempts to

TABLE 2. *Passive transfer of pneumococcal immunity by serum or lymphocytes from donors immunized with pneumococcal ribosomes or ribosomal protein*

Donors immunized with:[a]	% Survival of donors	% Survival[b] of recipients of:	
		Serum[c]	Spleen cells[d]
Ribosomes	100[e]	90[e]	0
2-CE protein	60[e]	60[e]	0
TM buffer control	0	0	0

[a] Mice were immunized with 150 μg of pneumococcal ribosomes or 2-CE protein (based on total protein content).

[b] All mice were challenged with 10 LD_{50} of *S. pneumoniae* type 3 24 h after transfer. Survival was calculated 7 days postchallenge.

[c] 0.1 ml of serum intravenously.

[d] 2×10^7 viable spleen cells intravenously.

[e] $P < 0.05$, statistically significant as compared to control values.

detect a delayed hypersensitivity response to ribosomes were also negative. Mice immunized with pneumococcal ribosomes were skin test negative, as determined by footpad swelling (26), and assays for macrophage migration inhibition factor in vitro using spleen or peritoneal exudate cells from mice immunized with ribosomal preparations were equivocal.

Analysis by Ouchterloney immunodiffusion (Fig. 2) of serum used in the passive transfer experiments demonstrated precipitating antibody to pneumococcal ribosomes in the serum from mice or rabbits immunized with whole ribosomes but not in the serum of mice immunized with 2-CE protein. In our system, repeated experiments have failed to detect a precipitin reaction between ribosomal antigens and the serum of mice immunized with 2-CE protein.

Specificity of antibody to ribosomal antigens. Specificity of antibody to ribosomal antigens was assessed by studying the effect absorption with various pneumococcal antigens would have on the ability of antiribosome serum to passively protect mice against pneumococcal challenge. Serum samples were absorbed with pneumococcal capsular type 2, 3, 6, or 14 as well as with whole pneumococcal ribosomes or 2-CE protein. After absorption, the filter-sterilized preparations (0.2 ml) were injected intravenously into Swiss-Webster mice, 10 mice/group, and the recipients were challenged 24 h later with 10 LD_{50} of type 3 pneumococci.

Unabsorbed rabbit antisera to pneumococcal ribosomes protected 100% of adoptively immunized recipients (Table 3). Absorption of this serum preparation with intact *S. pneumoniae*, regardless of serotype, abolished its ability to passively transfer immunity. Similar results were observed in mice given serum absorbed with pneumococcal ribosomes or 2-CE protein. Ouchterloney immunodiffusion analysis (Fig. 3) of the absorbed serum samples showed that precipitating antibody to pneumococcal ribosomes was removed from antiribosome serum by absorption with all the pneumococcal serotypes tested, as well as with the pneumococcal ribosomes and 2-CE protein.

Growth of *S. pneumoniae* in serum. Serum specimens were inoculated with very small numbers of *S. pneumoniae* type 3 and allowed to incubate at 37°C for 24 h. Pooled data from three experiments are recorded in Fig. 4. Virulent *S. pneumoniae* grew in rabbit antiribosome serum at a rate identical to normal rabbit serum. At the conclusion of the experiment,

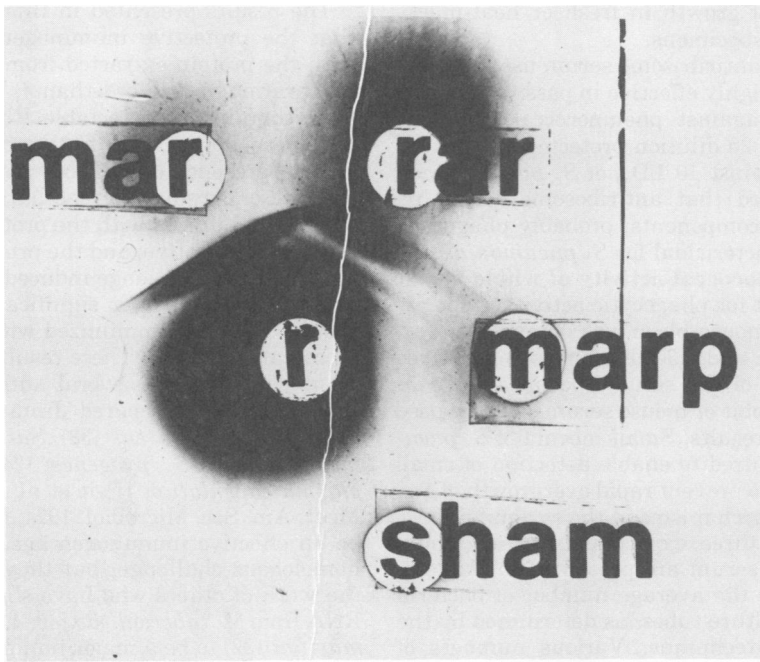

FIG. 2. *Immunodiffusion analysis of serum used to passively transfer antipneumococcal immunity. Center well contains pneumococcal ribosomes. Peripheral wells contain: mar, mouse antipneumococcal ribosomes; rar, rabbit antipneumococcal ribosomes; marp, mouse antipneumococcal 2-CE protein; sham, serum from mouse injected with TM buffer.*

TABLE 3. *Effect of absorption with pneumococcal ribosomal antigens or intact S. pneumoniae on the ability of rabbit antiribosome serum to passively transfer immunity against a pneumococcal challenge*[a]

Absorbant	% Survival in recipients of:	
	Normal rabbit	Rabbit antiribosome
None	0	100[b]
S. pneumoniae		
Type 2[c]	0	0
Type 3	0	10[d]
Type 6	0	10[d]
Type 14	0	10[d]
Pneumococcal ribosomes[e]	0	0
Ribosomal protein[e]	0	20[d]

[a] All mice were challenged with 10 LD_{50} of *S. pneumoniae* type 3.

[b] $P < 0.05$, statistically significant as compared to normal rabbit serum.

[c] Adsorbed with 10^9 CFU/ml of serum.

[d] $P > 0.05$, not significantly different from normal rabbit serum.

[e] Absorbed with 1 mg of protein per ml of serum.

each serum specimen contained approximately 10^9 CFU/ml. Bacteriolysis due to complement activity was not observed, as evidenced by the similarities of growth in fresh or heat-inactivated serum specimens.

The rabbit antiribosome serum used in these studies was highly effective in passively immunizing mice against pneumococcal challenge (0.1 ml of a 1:20 dilution protected 100% of all recipients against 10 LD_{50} of *S. pneumoniae*). This suggested that antiribosome serum required other components, probably phagocytic cells, to be bactericidal for *S. pneumoniae*.

Antipneumococcal activity of whole mouse blood. To test for phagocytic activity, heparinized normal mouse blood was obtained by cardiac puncture and 0.3-ml volumes were mixed with 0.05 ml of the serum specimens. It was found that rabbit or mouse serum could be used with similar results. Small inocula of *S. pneumoniae* were used to enable detection of small changes and to prevent rapid overgrowth of any bactericidal mechanisms in the system used.

Results of three experiments using mouse antiribosome serum are presented in Table 4. Each value is the average number of bacteria from three culture tubes, as determined by the spread plate technique. Various numbers of pneumococci were used as an initial inoculum, as indicated.

An opsonic function by antiribosome serum is clearly suggested by the results presented in Table 4. Depending on the initial inoculum, blood mixtures containing antiribosome serum showed a 4% increase or a 53 to 83% decrease in viable pneumococci over the 3-h test period. In contrast, viable pneumococci in blood mixed with serum from sham-immunized mice did not decrease but, in fact, significantly increased during the test period, demonstrating a 17 to 91% rise over the initial inoculum.

DISCUSSION

Enzyme degradation of pneumococcal ribosomes has suggested that both RNA and protein are necessary for optimum immunogenicity of pneumococcal ribosomes (31). We have extended these observations by investigating the immunogenicity of purified ribosomal RNA and protein fractions. Our data have shown that purified RNA from pneumococcal ribosomes is not a protective immunogen in mice. In addition, RNA did not appear to function as an adjuvant, since the protective immunity induced by pneumococcal ribosomes and 2-CE protein was not significantly different after 4 days postimmunization. Further, the immunity induced by 2-CE protein appeared 7 days before that induced by pneumococcal ribosomes. This would not be expected if the RNA in the whole ribosomes possessed adjuvant activity.

The results presented in this paper suggest that the protective immunogen is associated with the protein extracted from our ribosomal preparation by 2-chloroethanol. The protein extract contains no detectable RNA, deoxyribonucleic acid, or carbohydrate; serological assays for the presence of antibody to pneumococcal cell wall or capsular polysaccharides in the sera of mice immunized with the protein extract are repeatedly negative; and the protection against pneumococcal challenge induced by the protein extract does not differ significantly from that observed in mice immunized with whole pneumococcal ribosomes. These results are in agreement with those of several authors who have shown protein prepared from ribosomes of *Staphylococcus aureus* (38), *Salmonella typhimurium* (15), *S. pyogenes* (24), and *Histoplasma capsulatum* (Feit et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, Mm5, p. 136) to be an effective immunogen against a virulent homologous challenge, but they contrast with the work of others who have shown ribosomal RNA from *M. tuberculosis* (40, 41) and *S. typhimurium* (32) to be a major immunogen in their ribosomal vaccines. The reason for these discrepancies has not yet been elucidated.

At present we are unable to determine if the protective antigen in 2-CE protein is ribosomal protein(s) or a protein contaminant that is puri-

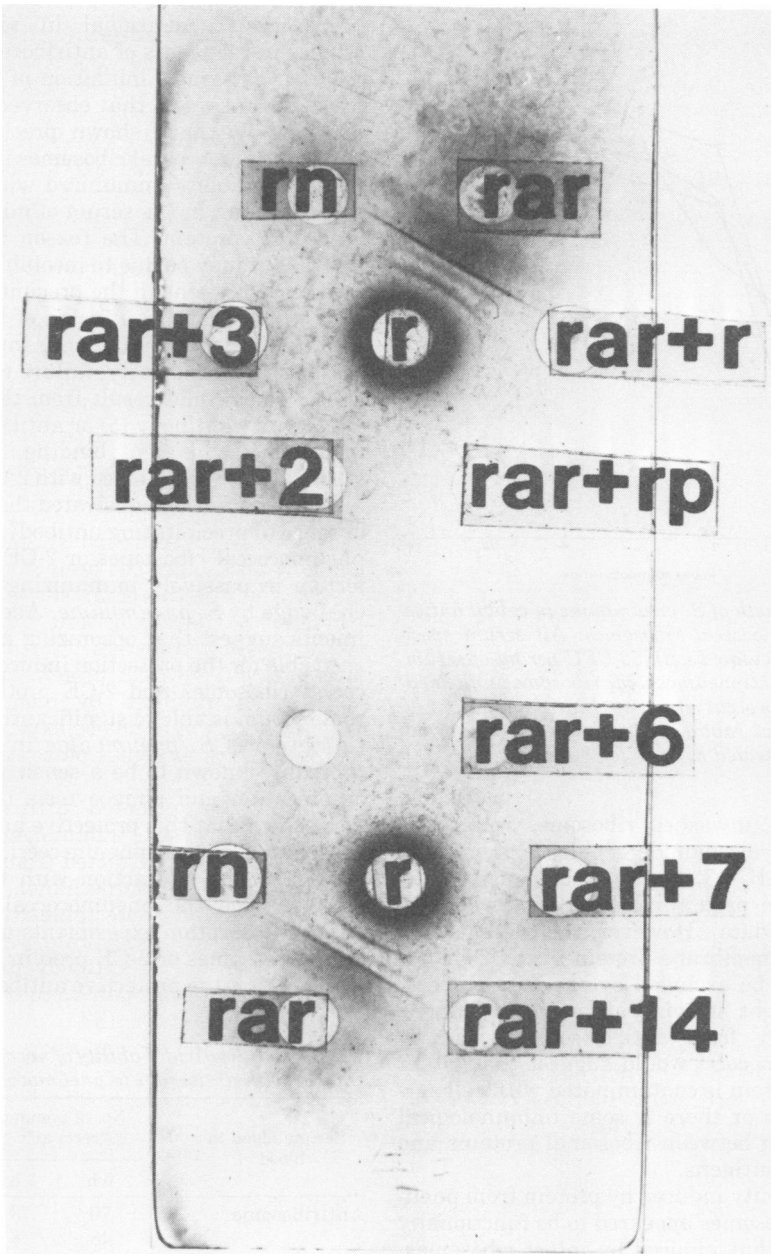

FIG. 3. *Immunodiffusion analysis of rabbit serum specimens adsorbed with intact S. pneumoniae or pneumococcal ribosomal antigens. r, Center well contains pneumococcal ribosomes. rar, Rabbit antipneumococcal ribosome unabsorbed; rar + r, rar absorbed with pneumococcal ribosomes; rar + rp, rar absorbed with 2-CE protein; rar + 2, 3, 6, 7, 14, rar absorbed with the designated pneumococcal serotype; rn, normal rabbit serum.*

fied with the ribosome. Precipitation by $(NH_4)_2SO_4$ is considered a standard method for the preparation of ribosomes relatively free of contaminating cytoplasmic proteins (18), and we have further examined our preparation for such contaminants by washing the ribosomes with 1 M NH_4Cl, a procedure that has been shown to remove acidic cytoplasmic proteins from ribosomes of *Escherichia coli* (35). Preliminary experiments in which the proteins of

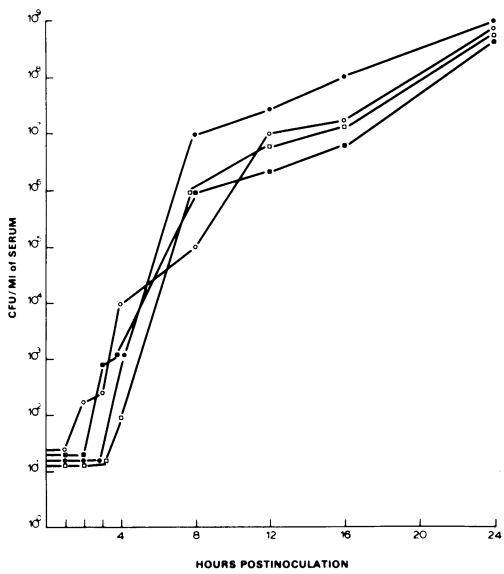

FIG. 4. *Growth of S. pneumoniae in rabbit antiserum to pneumococcal ribosomes. All serum specimens were inoculated with 50 CFU per ml of serum. Symbols: ○, antipneumococcal ribosomes unheated; ●, antipneumococcal ribosomes heated at 56°C for 30 min; □, normal rabbit serum unheated; ■, normal rabbit serum heated at 56°C for 30 min.*

washed and unwashed ribosomes were compared by polyacrylamide gel electrophoresis in 6 M urea, pH 4.3, have failed to detect any differences in protein composition (Swendsen, unpublished data). However, the presence of a cell wall or membrane protein in the 2-CE extract cannot be excluded by our data. Indeed, the experiment showing absorption of protective antibody from antiribosome serum by whole pneumococci would suggest that either the 2-CE protein is contaminated with cell surface antigens or there is some immunological cross-reaction between ribosomal proteins and cell surface antigens.

The immunity induced by protein from pneumococcal ribosomes appeared to be functionally identical to that induced by intact ribosomes. Mice immunized with either of these preparations were able to inhibit multiplication of virulent pneumococci and did not exhibit a detectable bacteremia. By comparison, control animals regularly succumbed to an extensive bacteremia within 3 to 4 days of challenge. Our data suggest that this immunity is dependent upon humoral mechanisms. The protection achieved by immunization with pneumococcal ribosomes or 2-CE protein was reflected only in recipients of serum from donors immunized with these preparations. Additional in vivo clearance studies in recipients of antiribosome serum detected a pattern of inhibition of pneumococcal growth identical to that observed in the donor animals. We have shown precipitating antibody to pneumococcal ribosomes in the serum of mice and rabbits immunized with intact ribosomes but not in the serum of mice immunized with 2-CE protein. The reason for this is not known but may be due to insolubility of certain ribosomal proteins in the precipitating system. This would prevent diffusion of these proteins into the agar and subsequent interaction with antibody. Alternatively, failure to observe precipitin lines could result from the presence of low-affinity antibody (5) or antibody capable of monogomous bivalent binding (5, 13) in the serum of mice immunized with 2-CE protein.

Our work has demonstrated that, even in the absence of precipitating antibody, antiserum to pneumococcal ribosomes or 2-CE protein is effective in passively immunizing mice against challenge by *S. pneumoniae*. Additional experiments suggest that opsonizing antibody is responsible for the protection induced by pneumococcal ribosomes and 2-CE protein. Antiribosome serum is able to significantly inhibit multiplication of *S. pneumoniae* in vitro using a procedure known to be a sensitive measure of opsonization and phagocytosis (19). We have also shown that this protective antibody can be absorbed by intact pneumococci, thus further suggesting an interaction with the protective antibody and the pneumococcal cell surface. Similar absorption experiments using pneumococcal ribosomes or 2-CE protein as absorbants indicate that the protective antibody is directed

TABLE 4. *Bactericidal ability of normal mouse blood mixed with antiserum to pneumococcal ribosomes*

Serum added to blood[a]	No. of pneumococci at[b]:		% Change
	0 h	3 h	
Antiribosome	70	73	+4[c]
	48	8	−83[c]
	30	14	−53[c]
Sham immunized	70	134	+91
	48	53	+10
	30	35	+17

[a] 0.05 ml of serum was added to 0.3 ml of fresh heparinized blood.

[b] Blood mixtures were inoculated with dilutions of a pneumococcal culture. Numbers are CFU/0.1 ml of mixture.

[c] Significantly different as compared to mixtures containing serum from sham-immunized mice; $P < 0.05$.

towards an antigen that is associated with the 2-CE protein fraction.

Our data are in close agreement with recent work presented by Thompson and Eisenstein (30), who have demonstrated that passive immunization with antiribosome serum protects against a homologous challenge by pneumococci. However, we have expanded these observations and shown that adsorption of antiribosome serum with not only type 3, the homologous organism, but also with types 2, 6, and 14 removes the protective antibody from the serum. The absorption of the protective activity by heterologous serotypes is consistent with earlier work by Thompson and Snyder (31), who demonstrated induction of immunity to heterologous serotypes of pneumococci by immunization with ribosomes from *S. pneumoniae* type 3.

No manifestations of delayed hypersensitive or cell-mediated immunity in animals immunized with pneumococcal ribosomes or 2-CE protein have been observed. This is in keeping with the classical concept (5) that only humoral immunity is effective against the pneumococcus, but the complete absence of delayed hypersensitivity to ribosomal antigens, as determined by skin testing of guinea pigs or footpad testing in mice, was unexpected. In assays for macrophage migration inhibition factor, pneumococcal ribosomes were observed to inhibit the migration of spleen cells from mice immunized with ribosomes or ribosomal protein, but statistical analysis demonstrated a similar inhibition of migration in cultures of spleen cells from normal mice. This could be a result of nonspecific stimulation of lymphocytes by the ribosomal RNA as suggested by Casavant and Youmans (4), who observed such nonspecific inhibition of macrophage migration by mycobacterial RNA in a system very similar to ours. Elicitation of macrophage migration inhibition factor by 2-CE protein in the absence of RNA could not be investigated due to the insolubility of 2-CE protein under physiological conditions.

We have shown that immunity induced by pneumococcal ribosomes is due to humoral antibody specific for the 2-CE fraction of the ribosome. It appears that the major function of this antibody is opsonic, suggesting a coincidental antigenic similarity between ribosomal proteins and the proteins of the pneumococcal cell wall or contamination of the ribosomal protein with cell wall proteins during the purification procedure. We are currently attempting to resolve this question by isolating the immunogenic protein or proteins from 2-CE protein and identifying their antigenic specificity and cellular location.

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