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Ribosomal Vaccines

I. Immunogenicity of Ribosomal Fractions Isolated from *Salmonella typhimurium* and *Yersinia pestis*

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The immunogenicity of ribosomes and ribosomal subfractions isolated from *Yersina pestis* and *Salmonella typhimurium* has been studied. Ribosomes and ribosomal protein isolated from *S. typhimurium* protected mice against lethal challenge. Ribosomal ribonucleic acid isolated by phenol extraction failed to induce any significant level of protection in mice. None of the ribosomes or ribosomal subfractions isolated from *Y. pestis* were effective in inducing immunity to lethal challenge. These results suggest that the immunogen of the ribosomal vaccine is protein.

The demonstration by Youmans and Youmans (20) that ribosomal antigens prepared from *Mycobacterium tuberculosis* are effective in inducing in mice immunity to challenge with a live homologous organism has led to the investigation of the potential protective ability of ribosomal antigens from several species of bacteria (14, 16, 18, 19). Recent reports have shown ribosomal vaccines from *Salmonella typhimurium* to be as effective in inducing immunity in mice as the live attenuated vaccine, and they have suggested that ribonucleic acid (RNA) may be the immunogenic moiety of the ribosomal vaccine preparations (16, 17, 21).

In this study we have compared the immunogenic properties of ribosomal antigens isolated from fully virulent cultures of *Yersinia pestis* and *S. typhimurium* to identify the immunogenic moiety of the ribosomal vaccines and to determine whether the immunizing potential of ribosomal antigens could be extended to another gram-negative pathogen, *Y. pestis*.

MATERIALS AND METHODS

Organisms. *S. typhimurium* strain SR-11 (kindly supplied by L. Joe Berry, University of Texas, Austin) was maintained on Brain Heart Infusion (BHI) agar. *Y. pestis* strain Kim 10, obtained from our stock culture collection, was maintained as a glycerol suspension at −23 C to avoid loss of virulence. To prepare inocula for mass cultivation of cells, several drops of the glycerol suspension were placed on blood agar base (BBL) slants containing 2.5 mmoles of $CaCl_2$ and 10 mmoles of glucose (calcium agar). Cultures of *Y. pestis* were routinely plated on the magnesium oxalate medium of Higuchi and Smith (8) to determine the number of avirulent cells. Challenge doses of *Y. pestis* are reported as the number of fully virulent cells.

Preparation of cultures. Two-liter flasks containing 250 ml of BHI broth were inoculated with 2.5 ml of an exponential-phase broth culture of *S. typhimurium*. The flasks were incubated at 37 C for 6 to 8 hr on a reciprocal shaker. Cultures of *Y. pestis* were grown at 26 C for 10 to 12 hr in a medium containing 3% N-Z Amine (Sheffield Chemical, Norwich, N.Y.), 2.5×10^{-3} M sodium thiosulfate, 1% xylose, and basal salts. Cells were harvested by centrifugation at 27,000 × *g* and washed four times in cold (4 C) 0.02 M phosphate buffer, *p*H 7.0.

Animals. Albino, Swiss-Webster male mice weighing 18 to 21 g were obtained from the Fort Detrick Animal Farm. The median lethal dose (LD_{50}) values, as determined by the method of Reed and Muench (12), were 8×10^2 for *S. typhimurium* and 1.5×10 for *Y. pestis*.

Isolation of ribosomes. Ribosomes were isolated by a modification of the procedure described by Venneman and Bigley (16). All procedures were performed at 4 C unless otherwise indicated. Washed, packed cells were suspended in four volumes of 0.02 M phosphate buffer, *p*H 7.0, containing 10^{-2} M $MgCl_2$, 0.25% sodium dodecyl sulfate (SDS), and 2 μg of deoxyribonuclease per ml and were broken in a French pressure cell at 10,000 psi. The crude extract was centrifuged at 27,000 × *g* for 15 min to remove intact cells and cellular debris. The top four-fifths of the supernatant fluid was removed by suction and centrifuged at 40,000 × *g*. The top three-fourths of the supernatant fluid was removed, and samples were taken for plate counts to insure the removal of viable cells.

The 40,000 × *g* supernatant fluid was centrifuged at 105,000 × *g* for 2 hr. The pellet, designated as crude ribosomes, was suspended with several strokes of a Dounce homogenizer in 0.02 M phosphate buffer, *p*H 7.0, containing 10^{-2} M $MgCl_2$. An equal volume of 1% SDS in phosphate buffer with 10^{-2} M $MgCl_2$ was added

to the suspension and stirred for 1 hr at room temperature. After refrigeration overnight at 4 C, the precipitated SDS was removed by centrifugation at 35,000 × *g* for 15 min. The upper three-fourths of the supernatant fluid was removed by suction and centrifuged at 105,000 × *g* for 2 hr. To eliminate trace contamination with nucleases, final purification of the ribosomes was carried out according to a modification of the procedure described by Takanami (13). The ribosomal pellet was washed seven times by suspending it in HS buffer containing 0.01 M $MgCl_2$, 0.35 M NH_4Cl, 0.005 M β-mercaptoethanol in 0.01 M tris(hydroxymethyl)aminomethane (Tris) (*p*H 7.6) and concentrating the ribosomes as a pellet by centrifugation at 105,000 × *g* for 3 hr. The final pellet was suspended in HS buffer and applied to a Sephadex G-200 column (3 by 50 cm). After elution with HS buffer, the fractions containing ribosomes were pooled and dialyzed against Tris buffer (*p*H 7.6) containing 0.01 M $MgCl_2$ 0.05 M NH_4Cl, and 0.005 M β-mercaptoethanol (LS buffer). The dialyzed material was then centrifuged at 105,000 × *g* for 2.5 hr to sediment the ribosomes. Ribosomes were suspended in LS buffer with several strokes of a hand homogenizer and passed through a Nalge sterile, disposable filter containing a 0.45-μm grid membrane. To insure sterility, samples of the *S. typhimurium* and *Y. pestis* ribosomes were plated on blood agar base and calcium agar, respectively. Ribosomes were then pelleted by centrifugation at 105,000 × *g* for 3 hr and resuspended in sterile 0.01 M phosphate buffer (*p*H 7.2) containing 10^{-2} M $MgCl_2$.

Isolation of ribosomal RNA and protein. Proteins were extracted with 2-chloroethanol (Eastman Organic Chemicals, Rochester, N.Y.) according to the procedure described by Fogel and Sypherd (7). Three milliliters of a suspension containing 300 μg (wet weight) of purified ribosomes per ml was added to five volumes of cold 2-chloroethanol containing 0.06 N HCl. The resulting suspension was homogenized with a mortar and pestle and placed at 0 C for 3 hr with periodical homogenization. The suspension was then centrifuged for 10 min at 8,000 × *g* to remove the precipitated RNA. The top four-fifths of the supernatant fluid was removed and dialyzed against water. The dialyzed protein was then lyophilized and stored at −20 C.

Two methods were used for the extraction of RNA. The first employed 80% phenol equilibrated with 10^{-2} M phosphate buffer (*p*H 7.1) containing 0.5% (w/v) SDS and 10^{-3} M ethylenediaminetetraacetate (EDTA) according to the procedure described by Venneman et al. (17). Briefly, an equal volume of buffer-saturated phenol was added to ribosomes. The mixture was shaken vigorously and placed at 65 C for 10 min. The aqueous phase was separated by centrifugation at 4 C for 10 min at 10,000 × *g*. The upper three-fourths of the aqueous phase was removed and extracted twice more at 65 C with buffer-saturated phenol. The final aqueous phase was removed, and an equal volume of anhydrous ether was added to extract any residual phenol. The ether was removed by bubbling nitrogen through the mixture. The RNA was then precipitated overnight by the addition of two volumes of cold (−20 C) 95% ethanol.

The second method of RNA extraction employed water-saturated phenol by a modification of the procedure described by Moldave (10). Ribosomes were suspended in 10^{-2} M (2-amino-2-hydroxymethylpropane-1,3-diol) Tris-hydrochloride buffer, *p*H 7.4. An equal volume of water-saturated phenol was added and the mixture was stirred vigorously for 1 hr at 4 C. The aqueous phase was separated by centrifugation at 25,000 × *g* for 20 min. The upper three-fourths of the aqueous phase was removed and extracted once more with water-saturated phenol. The aqueous phase was separated by centrifugation at 25,000 × *g* for 20 min and the upper three-fourths was removed. RNA was precipitated overnight by the addition of a 0.1 volume of cold (4 C) 20% potassium acetate, *p*H 5.0, and two volumes of cold (−20 C) 95% ethanol. The RNA was pelleted by centrifugation at 4,000 × *g* for 15 min and dissolved in 2% potassium acetate (*p*H 5.0). Insoluble material was removed by centrifugation at 4,000 × *g* for 10 min, and the RNA was precipitated by the addition of two volumes of cold (−20 C) 95% ethanol. Precipitated RNA was collected by centrifugation and dissolved in 10^{-2} M phosphate buffer, *p*H 7.2.

RNA isolated by both the water and buffer-saturated phenol extraction procedures was further purified by the method of Cox (4). Briefly, one volume of ribosomes suspended in 10^{-2} M phosphate buffer (*p*H 7.2) containing 10^{-2} M magnesium acetate was added to two volumes of 6 M guanidinium chloride. The mixture was placed at 4 C for 30 min. A one-half volume of cold (−20 C) 95% ethanol was added. The mixture was kept at 4 C for 2 hr. The precipitated RNA was pelleted by centrifugation (4 C) at 4,000 × *g* for 15 min. The RNA was dissolved in 4 M guanidinium chloride containing 10^{-3} M EDTA (*p*H 7.0). The temperature of the mixture was brought to 20 C and a one-half volume of cold (0 C) 95% ethanol was added to precipitate the RNA. Precipitated RNA was pelleted by centrifugation at 4,000 × *g* for 15 min and dissolved in 10^{-2} M phosphate buffer.

Separation of high-molecular-weight RNA from transfer RNA was carried out by molecular-sieve chromatography on a Sephadex G-200 column. The optical density at 260 nm (OD_{260}) of each fraction was determined in a Beckman DB spectrophotometer. The major RNA peak risided in the fractions representing 20 to 50 ml of the column void volume. These fractions were pooled, and the RNA was precipitated overnight by the addition of cold (−20 C) 95% ethanol. The RNA was pelleted by centrifugation at 10,000 × *g* for 10 min and dissolved in 10^{-2} M phosphate buffer (*p*H 7.4). RNA concentration was determined by the orcinol procedure (1). In view of the known lability of the immunogenic RNA isolated by others (16), the RNA was injected into animals as quickly as possible.

Sucrose density gradient ultracentrifugation. Samples of RNA were placed on 5 to 20% linear sucrose gradients containing 10^{-2} M magnesium acetate in 0.02 M phosphate buffer. After centrifugation at 25,000 rev/min for 18 hr in a Beckman model L ultracentrifuge (SW25.1 rotor), 1-ml fractions were

collected from the bottom of the tubes and the OD_{260} was recorded.

Immunization. In initial experiments the vaccine preparations were mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously in 0.2-ml amounts. Subsequent experiments indicated that adjuvant did not enhance the immune response of the ribosomal preparations or subfractions. Consequently, as standard procedure each animal was immunized subcutaneously with 0.2 ml of material. Animals were challenged by the intraperitoneal route 15 days after immunization. Deaths were recorded 30 days after challenge. Statistical analyses were done by the chi-square test.

Treatment of ribosomal proteins with pronase, trypsin, and ribonuclease. Pronase (Calbiochem), trypsin, and ribonuclease (Sigma Chemical Co.) were added to the ribosomal proteins at a concentration of 100 μg of enzyme to 2 mg of ribosomal protein. The mixtures were then incubated at 37 C for 30 min.

Biochemical assay. Protein was determined by the method of Lowry et al. (9). RNA and deoxyribonucleic acid (DNA) were measured by the orcinol procedure and by diphenylamine, respectively (1, 5). Bovine serum albumin, yeast RNA, and pancreatic DNA (Nutritional Biochemicals Corp.) served as standards. Carbohydrates were assayed by the indole procedure (6).

RESULTS

Immunogenicity of whole ribosomes. The procedure for washing the ribosomes in HS buffer was found to be a critical step in the preparation of pure ribosomes from *Y. pestis*. Ribosome preparations washed fewer than seven times had significant pesticin activity when assayed by the procedure of Brubaker and Surgalla (3). These same preparations were also lethal to mice in quantities of more than 25 μg (wet weight). The injection of unwashed ribosomes into mice uniformly produced death within 24 hr. Since no viable *Y. pestis* could be recovered from moribund animals, the lethality of these ribosome preparations was ascribed to the presence of small amounts of plague murine toxin. Ribosomes washed seven times in LS buffer and further purified on a Sephadex G-200 column had no pesticin activity and were not lethal to mice.

Table 1 shows the survival of mice immunized with whole ribosomes from *S. typhimurium* and *Y. pestis*. The animal received 25, 50, 100, or 200 μg (wet weight) of ribosomes. Immunization of mice with as little as 25 μg of *S. typhimurium* ribosomes induced significant protection against challenge with the homologous organism. However, mice immunized with as much as 200 μg of *Y. pestis* ribosomes failed to show any significant degree of protection against challenge with the homologous organism.

Immunogenicity of purified ribosomal proteins. The ribosomal protein preparation from *Y. pestis* was free from fraction I (FI) and VW antigens, as shown in Fig. 1. The ribosomal proteins gave no bands of precipitation against antiserum to VW antigens. Likewise, no bands were observed against a commercial preparation of plague antiserum (Lederle Laboratories, Pearl River, N.Y.) or against II antiserum prepared by immunization or rabbits with the Alexander strain of *Y. pestis* containing the FI antigen. Precipitin bands were observed only between the ribosomal protein preparation and the homologous antiserum.

TABLE 1. *Immunogenicity of whole ribosomes isolated from Yersinia pestis and Salmonella typhimurium*

Amt injected[a]	Animals immunized with ribosomes (no. dead/total)	
	Y. pestis[b]	*S. typhimurium*[c]
200	40/40	0/40
100	39/40	0/40
50	40/40	0/40
25	40/40	6/40

[a] Expressed as micrograms (wet weight) of ribosomes. In the unimmunized controls, the number of animals dead per total was: *Y. pestis*, 40/40; *S. typhimurium*, 38/40.

[b] Challenge dose: 10 LD_{50} (Kim 10).

[c] Challenge dose: 100 LD_{50} (SR-11).

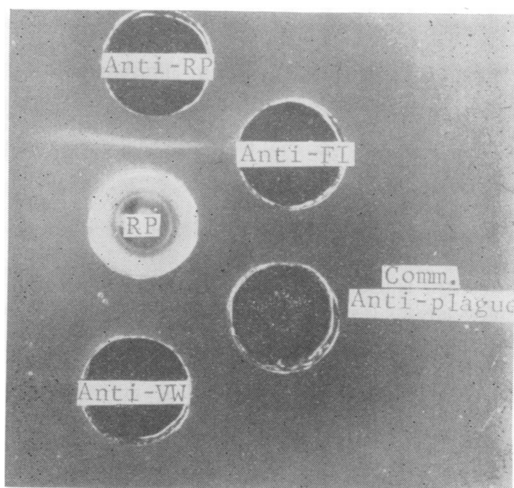

FIG. 1. *Immunodiffusion plate showing the reactions of ribosomal proteins from Yersinia pestis with various antisera. Anti-RP, antiserum to ribosomal protein; Anti-FI, antiserum to fraction I antigen; Comm. Anti-plague, commercial antiserum to Y. pestis; Anti-VW, antiserum to VW antigen; RP, ribosomal proteins.*

TABLE 2. *Immunogenicity of ribosomal proteins isolated from Yersinia pestis and Salmonella typhimurium*[a]

Amt injected (μg)	Mice immunized with ribosomal proteins (no. dead/total)	
	Y. pestis[b]	*S. typhimurium*[c]
200	39/40	0/40
100	40/40	0/40
50	38/40	1/40
25	40/40	14/40

[a] In the unimmunized controls, the number of animals dead per total was 40/40.
[b] Challenge dose: 10 LD_{50} (Kim 10).
[c] Challenge dose: 100 LD_{50} (SR-11).

The results of immunization of mice with ribosomal proteins is given in Table 2. The ribosomal proteins extracted from *S. typhimurium* induced significant protection at doses as low as 25 μg and complete protection at doses of 100 μg or more. In contrast, the ribosomal protein preparation from *Y. pestis* failed to show any significant protection at any of the dose levels tested.

Effects of Pronase, trypsin, and ribonuclease. Table 3 shows the effects of Pronase, trypsin, and ribonuclease treatment on the immunogenicity of the ribosomal proteins. Treatment with either Pronase or trypsin caused a marked reduction in the protective effects of the protein preparations. Ribonuclease treatment had no effect on the immunity induced by the ribosomal protein.

Immunogenicity of purified ribosomal RNA. The initial ribosomal RNA isolated from *Y. pestis* and *S. typhimurium* had been extracted from ribosomes that had not undergone a final purification step on a Sephadex G-200 column. These RNA preparations were not immunogenic, and subsequent analysis by analytical ultracentrifugation and sucrose-gradient density ultracentrifugation indicated that the high-molecular-weight ribosomal RNA species had been rapidly degraded to material corresponding to approximately 5 to 20*S*. Because of this degradation, ribosomes were purified on a Sephadex G-200 column before RNA extraction to eliminate any traces of nuclease activity.

The RNA isolated by extraction of ribosomes with SDS-phosphate-buffered phenol failed to elicit any immune response in animals injected with as much as 200 μg of RNA. Because this preparation of RNA contained approximately 0.1% protein contamination (wet weight), a further purification using guanidinium chloride was employed. Biochemical analysis of this RNA preparation failed to detect any DNA or protein contamination in the preparations used for immunization. Although no markers were employed, almost all of the RNA was found in peaks generally corresponding to 16 and 23*S* when analyzed in sucrose gradients. Some low-molecular-weight material of approximately 3 to 8*S* was observed, but it never represented more than 3% of the total material (wet weight).

The results of attempts to immunize animals with guanidinium chloride-purified ribosomal RNA are given in Table 4. The first line shows the normal immunization procedure. Because the RNA preparations failed to induce any significant level of protection, the immunization schedule was changed to two injections given 7 days apart as shown in the second line. Again no protection was observed for either preparation.

TABLE 3. *Effects of enzymatic degradation on the immunogenicity of ribosomal proteins from Salmonella typhimurium*[a]

Treatment	No. dead/total[b]
Untreated	1/40
Ribonuclease	0/40
Pronase	36/40
Trypsin	34/40
Control[c]	29/30

[a] Animals were immunized with 200 μg of ribosomal protein.
[b] Challenge dose: 100 LD_{50} of *S. typhimurium*.
[c] Control animals were divided into three groups of 10 animals each. One group received trypsin, one Pronase, and one ribonuclease.

TABLE 4. *Effects of injection schedule on the immunogenicity of guanidinium chloride-purified ribosomal RNA isolated from Yersinia pestis and Salmonella typhimurium*

Injection schedule[a]	Mice injected with RNA (No. dead/total)	
	Y. pestis[b]	*S. typhimurium*[c]
One injection (challenged on day 14)	40/40	39/40
Controls	30/30	29/30
Two injections 7 days apart (challenged 14 days after second injection)	40/40	38/40
Controls	29/30	28/30

[a] Each injection contained 200 μg of purified RNA.
[b] Challenge dose: 10 LD_{50} (Kim 10).
[c] Challenge dose: 100 LD_{50} (SR-11).

Because Youmans and Youmans (22) have shown that the use of SDS in the phenol extraction of RNA from ribosomes of *M. tuberculosis* may greatly reduce the immunogenic activity of the RNA preparations, ribosomal RNA was isolated from *S. typhimurium* by using water-saturated phenol. RNA isolated by this procedure failed to elicit any immune response in animals receiving doses as high as 200 μg of RNA. Further purification of this RNA by precipitation with guanidinium chloride failed to enhance the immunogenic activity.

DISCUSSION

Although immunogenic ribosomal material has been isolated from several species of bacteria, there are conflicting results as to the exact nature of the immunogenic moiety. The immunogenic material isolated from *M. tuberculosis* by Youmans and Youmans (20, 21, 23) is reported to be a heat-labile, ribonuclease-sensitive, double-stranded RNA which is effective only when administered in adjuvant. The ribosomal immunogen isolated from *S. typhimurium* has been described as a heat-stable ribonuclease-resistant RNA or RNA-protein complex which can be administered without adjuvant (16, 17). In contrast to the mycobacterium and salmonella preparations, the ribosomal immunogen isolated from *Staphylococcus aureus* and *Psudomonas aeruginosa* by Winston and Berry (18, 19) may be protein, possibly attached to RNA which may serve as an adjuvant. The *S. aureus* immunogen is insensitive to ribonuclease and is only effective when injected subcutaneously. The ribosomal immunogen isolated from *Diplococcus pneumoniae* (14) is sensitive to degradation by both ribonuclease and Pronase and is effective when injected without adjuvant.

The results of our experiments support, in part, the work of other investigators (16, 17) who have shown that ribosomes and ribosomal subfractions isolated from *S. typhimurium* can induce significant levels of immunity in mice. However, the experiments presented in this paper show that ribosomal RNA extracted with either water or SDS buffer-saturated phenol is not immunogenic in mice. Further purification of phenol-extracted RNA by precipitation with guanidinium chloride resulted in a RNA preparation which contained no detectable protein and was not immunogenic in animals. In contrast, a significant degree of protection was observed in mice immunized with ribosomal proteins isolated from *S. typhimurium*. Further evidence which supports the importance of the protein as the immunogen is that treatment of the ribosomal protein with trypsin or Pronase resulted in a large reduction in the immunogenicity of the preparation. Ribonuclease treatment had no effect on the immunizing potential of the preparations. These results support the finding of Winston and Berry (19), who obtained an immunogenic protein fraction by extraction of *S. aureus* ribosomes with 2-chloroethanol, but are in contrast to the reports of Venneman and Bigley (16) and Venneman et al. (17) who have suggested that the immunogenic ribosomal material of *S. typhimurium* is an RNA or RNA-protein complex which does not lose its immunogenic activity when treated with ribonuclease, Pronase, or trypsin. It is possible that the presence of large amounts of ribonuclease-insensitive double-stranded RNA in their preparations serves to coat an immunogenic protein moiety and protect this protein from degradation by proteolytic enzymes.

One of the unanswered questions regarding ribosomal vaccines is the mechanism by which they induce immunity. Venneman et al. (17) and Winston and Berry (19) have suggested that the ribosomal RNA may act as an adjuvant for an as yet unidentified ribosomal immunogen. In view of the evidence that cellular immunity plays an important role in infection with *S. typhimurium* (2), Venneman et al. (17) have suggested that the ribosomal RNA or RNA-protein complex may stimulate the production of an immune effector in mononuclear phagocytes. In support of this, Patterson and Youmans (11) have shown that splenic lymphocytes from mice immunized with ribosomal vaccines prepared from *M. tuberculosis* produce a filterable substance which inhibits the multiplication of tubercle bacilli in normal mouse peritoneal macrophages. Venneman and Berry (15) have shown that immunity induced by their ribosomal RNA vaccines can be passively transferred to normal mice with peritoneal cells from immunized donors but not with serum. In contrast, immunity induced by whole ribosomes was shown to be passively transferred by either peritoneal cells or serum. The failure of the ribosomal preparations from *Y. pestis* to induce any protection in mice may indicate that cellular immunity to plague plays only a minor role in the total defense mechanisms of the host. Our laboratory is currently investigating the mechanism by which this ribosomal subfraction from *S. typhimurium* induces immunity.

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