Evidence for an Extrinsic Immunogen in Effective Ribosomal Vaccines from *Salmonella typhimurium*

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Previous work has indicated that ribosomes isolated from *Salmonella typhimurium* were highly immunogenic and afforded excellent protection against homologous challenge. Effective protection was obtained also when ribonucleic acid (RNA) extracted from these ribosomes was used as a vaccine. In this investigation ribosomes prepared by another method and washed repeatedly in 1 M NH$_4$Cl lost much of their prophylactic potency and yielded poorly protective RNA. The high-salt wash of the ribosomes was immunogenic. The RNA and the protein isolated from the salt wash of the ribosomes were effective vaccines. No intrinsic component of the ribosomes was removed by the NH$_4$Cl wash, since the ability of both "crude" and "clean" ribosomes to function equally well in an in vitro protein synthesizing system was demonstrated. The presence of a component with toxic properties similar to those of endotoxin was found in active vaccines but not in weak ones. This was shown by the ability of effective vaccines to kill lead acetate-sensitized mice and to induce tolerance to endotoxin.

Youmans and Youmans were the first to isolate a highly protective ribosomal vaccine (44–47). Their work was with *Mycobacterium tuberculosis,* and since that time immunogens of similar origin have been derived from *Salmonella typhimurium* (9, 11, 27, 38, 42); T. K. Eisenstein, Ph.D. thesis, Bryn Mawr College, Bryn Mawr, Pa., 1969), *Staphylococcus aureus* (43), *Pseudomonas aeruginosa* (28, 42), *Pneumococcus pneumonia* (33), *Neisseria meningitidis* (32), *Vibrio cholerae* (8, 10), *Listeria monocytogenes* (31), *Streptococcus pyogenes* (24), and *Histoplasma capsulatum* (7). In no case has the antigenic component been chemically identified and confirmed, even though the Youmans (48) have good reason to believe that double-stranded ribonucleic acid (RNA) is responsible for the immunity they observe.

There is considerable disagreement between laboratories that have attempted to identify the nature of the effective moiety present in ribosomal vaccines isolated from *S. typhimurium* (1, 5, 9, 11, 12, 17, 26, 27, 34–37, 39). All agree, with the exception of Vas and his associates (Medina et al. [18]), at least with one strain of inbred mice they have used, that the highest possible level of immunity can be produced by these vaccines. There is inadequate information as to how strains of mice differ in their

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response to ribosomal vaccines, so some of the uncertainty about the identity of antigenic components may relate to the animals used in the experiments. Keeping this in mind, Johnson believes that the protective component is a ribosomal protein (11, 12). Venneman and Berry found high activity in an RNA fraction, immunogenic at doses of 10 μg or less (1, 35–37, 39). No one else has reported a preparation equally effective at a dose level as small. Smith and Bigley believe that the active antigen is an RNA-protein complex (17, 26, 27). A glycoprotein or mucopolysaccharide was credited by Houchens and Wright for the antigenicity they observed in a ribosomal vaccine (9). Medina et al. (18) attributed protection, in the strain of mouse that responded, to a "Venneman-type" RNA (34). Most recently, Eisenstein (5) has concluded that it is the O antigens contaminating either the crude ribosomes or the RNA and protein derived from them that are responsible for immunity. This agrees with our observations reported more than a year ago at the Convocation of Immunology in Buffalo (1).

The work reported in this paper compares the immunizing efficacy of several fractions prepared from *S. typhimurium* ribosomes. The results suggest that the protective moiety becomes adherent to the ribosomes during the extraction procedure or is normally associated with them. Because of this conclusion, a detailed description of the steps involved in isolat-

ing the fractions used in the experiments is given below.

MATERIALS AND METHODS

Animals. CF-1 female albino mice were obtained from the Carworth Farms Division of Charles River Laboratories, Portage, Mich. Young adults weighing 18 to 25 g were used in all experiments. They were housed 10/cage and given Purina Mouse chow and water ad libitum.

Organism. *S. typhimurium* strain SR-11 was the source of vaccines and was used also as the challenge organism. The 50% lethal dose (LD_{50}), estimated by the method of Reed and Muench (22), was 20 organisms/mouse. Survival was measured for 30 days after intraperitoneal challenge.

Immunizations. Mice were immunized subcutaneously with a single injection. The desired amount was suspended in 0.1 ml of sterile, nonpyrogenic isotonic saline. Control animals received saline alone.

Immune assay. Mice were challenged intraperitoneally 15 days postimmunization with 1,000 LD_{50} (10,000 colony-forming units) of the virulent strain. Immunity is reported as the number of animals surviving 30 days postchallenge over the total number infected. The significance of the data was determined by the chi-square test (29).

Preparation of crude ribosomes. Crude ribosomes were prepared by a modification of the procedure described by Remold-O'Donnell and Thach (23). A 10-liter broth culture of *S. typhimurium* SR-11, in midlog phase of growth, was harvested by continuous-flow centrifugation at 40,000 × g at 40 C. The bacteria were washed in 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.2, and sedimented at 27,000 × g for 20 min at 4 C. The cell mass was drained and frozen at −80 C until used. The frozen cells were ruptured by alumina grinding and suspended in 0.02 M Tris-hydrochloride buffer, pH 7.5, containing 0.01 M $MgCl_2$ and 0.005 M 2-mercaptoethanol (BME) (buffer A) such that 1.5 ml of buffer was added per g(wet weight) of cells. The alumina was removed by centrifugation at 10,000 × g for 15 min. Deoxyribonuclease was added to a final concentration of 3 μg/ml, and after approximately 15 min of incubation at 0 C the mixture was centrifuged twice for 30 min, the first at 20,000 × g and the second at 30,000 × g, to remove cell debris. Crude ribosomes were isolated from the resulting supernatant solution by centrifugation for 3 h at 270,000 × g. The pellet was resuspended in 0.05 M Tris-hydrochloride buffer, pH 7.7, containing 0.05 M NH_4Cl, 0.02 M $MgCl_2$, and 0.005 M BME (buffer B) at approximately 50 mg of ribosomes per ml. The ribosome concentration was estimated from the absorbance of the solution at 260 nm (as described below). The crude ribosomes were stored at −80 C until used.

Preparation of high-salt wash. To a solution of crude ribosomes solid reagent grade NH_4Cl was added, with gentle stirring, to bring the final concentration to 1 M. The solution was held at 4 C overnight and then centrifuged at 270,000 × g for 3

h. The resulting supernatant solution (high-salt wash) was retained and stored at −80 C until used.

Preparation of washed ribosomes. The pellet obtained from the salt wash was resuspended in a 0.05 M Tris-hydrochloride buffer, pH 7.7, containing 1 M NH_4Cl, 0.02 M $MgCl_2$, and 0.005 M BME (buffer C) at a concentration of approximately 50 mg/ml. The ribosomes were resedimented by centrifugation at 270,000 × g for 3 h, and the pellet was again suspended in buffer C and allowed to stand overnight at 4 C. The mixture was once again centrifuged at 270,000 × g for 3 h, and the ribosomal pellet was resuspended at a concentration of about 50 mg/ml in buffer D (0.01 M Tris-hydrochloride, pH 7.5, 0.05 M NH_4Cl, 0.01 M $MgCl_2$, and 0.005 M BME). The solution was clarified by centrifugation at 30,000 × g for 20 min. The washed ribosomes were stored at −80 C.

Preparation of protein fractions. Protein was extracted from the high-salt wash by the method of Kurland et al. (14). An aliquot of the high-salt wash was stirred vigorously in an ice bath. To the mixture, one-tenth volume of 1 M aqueous $MgCl_2$ was added, and as quickly as possible 2 volumes of glacial acetic acid were added. The mixture was kept in the ice bath and stirred for 45 min and then centrifuged at 30,000 × g for 20 min. The supernatant solution was retained and stored at 4 C. The precipitate was suspended in a small amount of 67% acetic acid containing 0.032 M $MgCl_2$, stirred on ice for 20 min, and centrifuged as above. The two supernatant solutions were combined and dialyzed overnight against 0.05 M Tris-hydrochloride buffer, pH 7.5, containing 0.05 M NaCl and 0.005 M BME. A precipitate formed during dialysis, but no attempt was made to remove it. The dialyzed mixture was adjusted to approximately pH 7 with 1 M Tris base and stored at −80 C.

Extraction of RNA. RNA from the crude and washed ribosomes was isolated by the method of Venneman (34). To the crude or washed ribosomes, 1 volume of hot (65 C) phenol saturated with buffer E (0.05 M Tris-hydrochloride buffer, pH 7.2, containing 0.5% sodium dodecyl sulfate [SDS]) was added, and the mixture was agitated vigorously for 10 min in a 65 C water bath. The aqueous phase was separated from the phenol phase by centrifugation at 1,000 × g. The aqueous phase was extracted twice again with hot buffer-saturated phenol. Subsequent to the final centrifugation, the upper two-thirds of the aqueous phase was carefully removed and brought to 0.1 M NaCl concentration by the addition of 1 M NaCl in 0.05 M Tris-hydrochloride buffer, pH 7.2 (buffer F). To this solution, 2 volumes of cold (−20 C) absolute ethanol were added, and the mixture was permitted to stand overnight at −20 C.

The following day, the nucleic acid precipitate was collected by centrifugation at 27,000 × g for 5 min. After resuspension in buffer F, 2 volumes of cold (−20 C) absolute ethanol were added, and the mixture was stored at −20 C for 4 h. The precipitate was again collected and resuspended in 50 ml of buffer F and 1 volume of 1% SDS. The mixture was stirred for 30 min at 25 C. Two volumes of cold absolute ethanol were added to precipitate the nucleic acid. The mixture was incubated at −20 C for 4

h. After collection by centrifugation, the nucleic acid was suspended and reprecipitated four more times as just described. The final pellet was suspended in buffer F and stored either frozen at −80 C or after lyophilization.

RNA was extracted from the high-salt wash of crude ribosomes with SDS-phenol according to a modification of the method described by Moldave (19). To an aliquot of the high-salt wash, solid SDS was added, with gentle stirring, to give a final SDS concentration of 1.5%. An equal volume of phenol that had been saturated with 0.01 M potassium phosphate buffer, pH 7.2, containing 0.1 M NaCl and 4% SDS was added. The mixture was stirred vigorously, by intermittent use of a Vortex mixer, for 30 min at room temperature. The mixture was cooled on ice and then centrifuged at 30,000 × g for 30 min. The resulting layers were separated by aspiration and retained for further treatment. To the aqueous phase, an equal volume of buffer-saturated phenol was added. The mixture was stirred at room temperature, cooled, and centrifuged as before. The two phenol phases were combined, and a small amount of water (about one-fifth the volume of the original high-salt wash aliquot) was added. The mixture was stirred and centrifuged as above. The phenol was discarded and the water layer was combined with the aqueous phase from the previous centrifugation. The solution was reduced to approximately one-half its original volume in a rotary evaporator at room temperature. If necessary, just enough water was added to dissolve any precipitate formed during condensation. To the clear solution, 2 volumes of cold absolute ethanol were added and the mixture was stored at −20 C overnight. The precipitated RNA was collected by centrifugation at 30,000 × g at −10 C for 30 min. The RNA was washed twice by dissolving it in a minimum volume of 0.2 M sodium acetate buffer, pH 6. The RNA was reprecipitated with ethanol, allowed to stand at −20 C for at least 2 h, and recovered by centrifugation as above. After the final washing operation, the precipitated RNA was dissolved in the sodium acetate solution and stored at −80 C.

The RNA preparations were separated from contaminating small-molecular-weight carbohydrates, nucleotides, SDS, and phenol by chromatography on Bio-Gel P-6 (molecular exclusion limit, 4,600). An RNA preparation in 5 ml of 0.01 M phosphate buffer, pH 7.2, containing 0.1 M NaCl was placed on a column of Bio-Gel P-6 (50 by 1.5 cm) equilibrated in the same buffer. Fractions (5 ml) were collected at a flow rate of 60 ml/h. The fractions were monitored with an Isco model UA-2 recording ultraviolet analyzer at an absorbancy at 254 nm (A_{254}).

Assay for ribosomal integrity. Crude and washed ribosomes prepared as described above were assayed for the ability to bind N-acetylphenylalanyl-transfer RNA (tRNA) and to catalyze poly(U)-directed incorporation of phenylalanine into polypeptide. The N-acetylphenylalanyl-tRNA binding assay was conducted as previously described (31) using highly purified $E. coli$ initiation factors and rate-limiting amounts of $S. typhimurium$ ribosomes. Polyphenylalanine synthesis was measured by a modification of the procedure described by Ravel and Shorey (21)

using highly purified $E. coli$ elongation factors. The reaction mixture was supplemented with 2.5 µg of EF-TS, 8 µg of EF-TU, 16 µg of EF-G, 0.15 mg of $E. coli$ tRNA charged with 100 pmol of [^{14}C]phenylalanine, and rate-limiting amounts of $S. typhimurium$ ribosomes.

Tests for endotoxin. An intrvenous injection of 2 mg of lead acetate sensitizes mice at least 100-fold to the lethal effect of intraperitoneally administered endotoxin (25). Graded amounts of the different vaccines were tested for their ability to kill lead-sensitized mice.

The $Limulus$ lysate coagulation test of Levin and Bang (15) is probably the most sensitive measure for endotoxin known. It suffers from lack of specificity (6) but was used to test the vaccines.

Vaccines were injected in graded doses to see if recipient mice were able to survive 48 h after challenge with 2 LD_{50} of $S. typhimurium$ endotoxin prepared by the phenol-water extraction method (20).

Analyses. The protein concentration of solutions was estimated by the method of Warburg and Christian (40) and also by the procedure described by Lowry et al. (16). Carbohydrate concentrations were assayed by the phenol-sulfuric acid method of Dubois et al. (4) using D-glucose as the standard. RNA concentration was estimated by the orcinol test (3) using $E. coli$ B tRNA as the standard and also by the absorbance of solutions at 260 nm assuming 24 A_{260} units correspond to 1 mg of RNA per ml (41). Deoxyribonucleic acid was determined by the diphenylamine test of Burton (2). The concentration of ribosomes was estimated spectrophotometrically assuming that 14.4 A_{260} units corresponds to 1 mg of ribosomes per ml (21).

RESULTS

Immunizations with crude and washed ribosomes. To determine whether the active moiety of a ribosomal vaccine was an integral component or a contaminant of the preparation, mice were injected subcutaneously with 100, 50, or 5 µg of either crude or washed ribosomes. Animals injected with saline served as controls. The mice were challenged intraperitoneally 15 days later with 1,000 LD_{50} of salmonellae. Deaths were recorded daily for 30 days. The results are presented in Table 1. One hundred, 50, and 5 µg of crude ribosomes were all highly immunogenic. Only 100 µg of washed ribosomes, however, provided a similar level of protection. This suggests that the active antigenic component was removed by washing the crude ribosomes with NH_4Cl.

Ability of crude and washed ribosomes to bind N-acetylphenylalanyl-tRNA. Table 2 summarizes the evidence that establishes the ability of crude and washed $S. typhimurium$ ribosomes in combination with $E. coli$ initiation factors to bind N-acetyl-[^{14}C]phenylalanyl-tRNA. Also shown are results obtained in an identical system in which homologous $E. coli$

ribosomes were used. It is evident that, even though the latter were more active, washed *S. typhimurium* ribosomes were no less active than the crude ribosomes. No component of the ribosomes needed to initiate protein synthesis was lost, therefore, in the washing procedure. In separate experiments crude and washed *S. typhimurium* ribosomes were found to be equally active in catalyzing poly(U)-direct polyphenylalanine synthesis.

Immunization with high-salt wash from crude ribosomes. Crude ribosomes lose between 20 and 30% of their weight as a result of the high-salt wash. The approximate amount of material derived from 50, 15 μg, and 5 μg of crude ribosomes was used as a vaccine. Its efficacy is shown in Table 3 and is compared with that of both crude and washed ribosomes.

The materials removed from the crude ribosomes were highly immunogenic and protected mice against lethal challenge in approximate proportion to the quantity orginally present on crude ribosomes. Even 1.5 μg of the high-salt wash afforded significant resistance to infectious challenge. When the two smaller amounts of high-salt wash (5 and 1.5 μg) were added back to 50 and 5 μg of washed ribosomes, the resulting immunity was about equivalent to that obtained with the high-salt wash alone. It was also similar to the protection obtained with crude ribosomes. Quite clearly, therefore, protective antigens can be removed from immunogenic crude ribosomes, and when these are added back to poorly immunogenic washed ribosomes no synergistic effect appears. It is important to note that washed ribosomes afford only about one-tenth the protection obtained with the same weight of crude ribosomes.

Immunization with high-salt wash protein and RNA. Mice were immunized with 18, 9, 3, or 1 μg of protein and with 3.0, 1.0, or 0.1 μg of RNA, isolated from the high-salt wash. The approximate weight of crude ribosomes that had to be subjected to the high-salt wash to

TABLE 1. *Comparative immunogenicity of crude and washed ribosomes*

Vaccine	Dose (μg)	Living/ total	% Survival[a]
Crude ribosomes	100	19/20	95
	50	19/20	95
	5	16/20	80
Washed ribosomes	100	19/20	95
	50	11/20	55
	5	4/20	20
Saline		0/20	0

[a] Thirty-day survival after intraperitoneal challenge with 1,000 LD_{50} of *S. typhimurium* SR-11.

TABLE 2. *Ability of crude and washed ribosomes to bind N-acetylphenylalanyl-tRNA*

Prepn	Concn (μg)	N-acetyl-[^{14}C]-phenylalanine bound (pmol)
Crude *S. typhimurium* ribosomes	100	4.0
	200	5.8
Washed *S. typhimurium* ribosomes	100	3.8
	200	7.8
Washed *E. coli* ribosomes	100	12.4
	200	21.9

TABLE 3. *Immunogenicity of crude and washed ribosomes and of high-salt wash*

Vaccine	Dose (μg)	Living/total	% Survival[a]
Crude ribosomes	100	19/20	95
	50	19/20	95
	5	16/20	80
Washed ribosomes	100	19/20	95
	50	11/20	55
	5	4/20	20
High-salt wash	15[b]	35/40	88
	5	33/40	83
	1.5	24/40	60
Washed ribosomes + high-salt wash	50 + 5	8/10	80
	5 + 1.5	7/10	70
Saline		1/20	5

[a] Thirty-day survivial after intraperitoneal challenge with 1,000 LD $_{50}$ of *S. typhimurium* S-11.

[b] Amounts derived from 50 15, and 5 μg of crude ribosomes, respectively.

yield the antigenic dose administered to the mice is indicated in column 1 of Table 4. The percentage of survival 30 days after challenge is also shown in the table. The closest equivalence in protection provided by the materials derived from crude ribosomes was provided by both the high-salt wash and the protein. In order for the RNA to be protective, a much larger quantitiy of crude ribosomes had to be processed. Even though the two larger amounts of RNA afforded significant resistance to the challenge infection, it is not likely to be the antigenic component in the high-salt wash. Both preparations were contaminated with approximately 2% carbohydrate as measured by the phenol-sulfuric acid test of Dubois et al. (4). Its contribution to the immunity these substances elicited is not likely to have been significant because of the quantity involved.

Immunization with RNA extracted from crude and washed ribosomes. To determine whether RNA acts as an adjuvant for a contaminating immunogen in the crude ribosomal vaccines, RNA was extracted by the method of Venneman (34) from crude and washed ribosomes and injected into mice. The mice received 100, 10, 1, or 0.1 μg of RNA from crude ribosomes and 100, 50, or 5 μg of RNA extracted from washed ribosomes. The percentage of survival after challenge is summarized in Table 5. RNA extracted from washed ribosomes protected 20% of mice given 50 μg, but all animals died when the immunizing dose was only 5 μg.

By comparison, 10 and 0.1 μg of RNA from crude ribosomes permitted, respectively, 80 and 40% of the mice to live. This provides additional evidence for the fact that an active moiety associates with the RNA during its isolation from crude ribosomes. Presumably this substance is removed during the washing process used to purify ribosomes.

Assays for the presence of endotoxin in vaccines. Because of the implied need for the presence of "contaminating" substances for the prophylactic activity of crude ribosomes and the RNA derived from them, several of the vaccines were assayed for the presence of cell wall fragments, specifically for endotoxin. The first as-

TABLE 5. *Immunogenicity of ribosomal RNA*

Vaccine	Dose (μg)	Living/total	% Survival[a]
RNA from crude ribosomes	100	10/10	100
	10	8/10	80
	1	4/10	40
	0.1	4/10	40
RNA from washed ribosomes	100	5/10	50
	50	2/10	20
	5	0/10	0
Saline		0/10	0

[a] Thirty-day survival after intraperitoneal challenge with 1,000 LD_{50} of *S. typhimurium* SR-11.

TABLE 4. *Immunogenicity of protein and RNA extracted from high-salt wash (HSW)*

Vaccine	Initial wt of crude ribosomes[a] (μg)	Antigenic dose (μg)	Living/total	% Survival[b]
Crude ribosomes		100	19/20	95
		50	19/20	95
		5	16/20	80
HSW	50	15	34/40	88
	17	5	33/40	83
	5	1.5	24/40	60
HSW protein	100	18.0[c]	10/10	100
	50	9.0	9/10	90
	17	3.0	6/10	60
	8	1.0	5/10	50
HSW RNA	1,500	3.0[d]	9/10	90
	500	1.0	7/10	70
	50	0.1	0/10	0
Saline			0/20	0

[a] Estimated weight of crude ribosomes from which the antigenic dose was derived.
[b] Thirty-day survival after intraperitonial challenge with 1,000 LD_{50} of *S. typhimurium* SR-11.
[c] Micrograms of protein.
[d] Micrograms of RNA.

say made use of mice sensitized to endotoxin by an intravenous injection of 2 mg of lead acetate. Immediately thereafter the animals were given an injection of one of a series of graded doses of a vaccine. When purified lipopolysaccharide from *S. typhimurium* was injected into lead-treated mice, 5 µg was 100% lethal (25). The results with vaccines are summarized in Table 6. It is clear that smaller amounts of protective vaccines were required to kill lead-sensitized mice than of poorly protective vaccines. On this basis, endotoxin seems to be associated with immunogenic preparations.

The second test used is specific for endotoxin. A 500-µg amount of each of the vaccines was given to groups of seven mice. Two days later, 2

LD_{50} of endotoxin were administered. The results are given in Table 7. Sharp differences emerge. Only the protective vaccines were able to induce tolerance. Poorly protective vaccines did not. There can be no question, therefore, but that the effective vaccines contained endotoxin in an amount estimated to be about 1% by weight. This is based on the amount of endotoxin required to induce tolerance. It is not surprising that such small amounts are difficult to detect by chemical means (20).

The *Limulus* lysate assay was used also as a test for endotoxin, but it proved to be too sensitive. All vaccines clotted the lysate, but since RNA alone has this effect (6) no meaningful information emerged from this experiment.

TABLE 6. *Lethality of ribosomally derived vaccines in lead-sensitized mice*

Vaccine	Dose (µg)	Living/total	% Survival
Crude ribosomes (immunogenic)	500	0/5	0
	300	0/5	0
	150	1/5	20
Washed ribosomes (poorly immunogenic)	500	1/5	20
RNA extracted from crude ribosomes by Venneman method (immunogenic)	500	0/5	0
	300	0/5	0
	150	1/5	20
RNA extracted from washed ribosomes by Venneman method (poorly immunogenic)	500	4/5	80
Endotoxin	10	0/5	0
Lead control		5/5	100

TABLE 7. *Induction of tolerance to endotoxin by ribosomally derived vaccines*

Vaccine	Dose (µg)	Living/total	% Survival
Crude ribosomes (immunogenic)	500	7/7	100
Washed ribosomes (poorly immunogenic)	500	0/7	0
RNA extracted from crude ribosomes by Venneman method (immunogenic)	500	7/7	100
RNA extracted from washed ribosomes by Venneman method (poorly immunogenic)	500	0/7	0
Salt wash from ribosomes (immunogenic)	500	7/7	100
Endotoxin (induces tolerance)	10	7/7	100
Saline control		0/7	—

DISCUSSION

The results presented in this paper clearly establish that some substance(s) separable from ribosomes is responsible for the protection against infection provided by ribosomal vaccines. The removal of the antigenic contaminant by washing with NH_4Cl solution is made all the more evident by the efficacy of the wash itself. The highly protective RNA extracted consistently from crude ribosomes that are themselves excellent vaccines cannot be derived from the poorly immunogenic washed ribosomes. This fact supports the argument that surface adherents and not internal constituents of ribosomes are responsible for the excellent level of immunity these substances provide.

The chemical nature of the antigenic determinant has not been resolved. Conflicting reports from different laboratories attempting to resolve the matter are not easily explained. Even though there is no reason to doubt the accuracy of observations that are in disagreement, there are sufficient differences in experimental details to make identical results unlikely. This conclusion is best evidenced by the details summarized in Table 8.

No laboratory has attempted to duplicate precisely experiments done by another, and yet there is a tendency for one group to assume that lack of agreement is attributable to technical inadequacies. Such variables as the strain of mouse used, route of immunization and challenge, use of adjuvants, dose level of vaccine administered, the number of LD_{50}'s used for the challenge, the assessment of level of immunity, and procedures for preparing vaccines and for challenge are evident. A diversity of approaches is desirable as long as some effort is made to explain the basis for nonconformity of results. This has not been done.

Bacteria have been ruptured by at least three methods. These include use of the French pressure cell, the sonicator, and alumina grinding. Each is capable of producing some undesirable alteration in the chemistry of the final product, especially sonication and, to a lesser extent, the French press.

TABLE 8. *Summary of results with S. typhimurium ribosomal vaccines*

Type of immunogen	Dose of vaccine (μg)	Challenge dose (LD_{50})	Immune assay	Effectiveness of vaccine (%)	Mouse strain	Reference
RNA	250	5,000	35-day survival	37–52	Ha (ICR)	Bigley[a] & others
RNA-protein	250	5,000	35-day survival	80–90	Outbred	(17, 26, 27)
Protein	100–200	1,000	35-day survival	30–67	Outbred	
Ribosomes	25–200	100	30-day survival	85–100	Swiss Webster	Johnson (11)
Protein	25–200	100	30-day survival	65–100	Outbred	
RNA	200	100	30-day survival	2–5	Outbred	
Ribosomes	25–400	1,000	30-day survival	15–100	Swiss Webster	Johnson[b] (12)
Protein	25–400	1,000	30-day survival	20–100	Outbred	
Protein	25–400	1,000	30-day survival	10–100	Outbred	
Ribosomes	100	25	30-day survival	46	NIH/NMRI	Houchens &
Protein	100	25	30-day survival	25	Outbred	Wright[c] (19)
RNA	100	25	30-day survival	17	Outbred	
RNA (crude)	6	10,000	30-day survival	94	Albino Swiss	Venneman et al.
RNA ("purified")	10	100+	30-day survival	100	Outbred	(34–37, 39)
RNA (crude)	44	2.6×10^6 cells	30-day survival	100	Swiss Webster or CD-1	Eisenstein[d] (5)
RNA	50	100	20-day survival	100	A/J inbred	Medina et al.[e]
RNA	50	100,000	20-day survival	0	C57Bl/6J	(18)
RNA (crude ribosomes)	10	1,000	30-day survival	80	CF-1	This paper[f]
RNA (washed ribosomes)	50	1,000	30-day survival	20	Outbred	
Protein	9	1,000	30-day survival	90	Outbred	

[a] Vaccines incorporated in Freund incomplete adjuvant; not used by others.
[b] First protein listed extracted with 2-chloroethanol, the second with acetic acid.
[c] Route of infection not specified; all others except Vas were intraperitoneally.
[d] Route of immunization, intraperitoneal; all others were subcutaneous.
[e] Route of infection, intravenous.
[f] Protein obtained from salt wash.

Our results (1) agree essentially with those of Eisenstein (5). She made use of well-characterized mutants of *S. typhimurium, S. adelaide,* and *S. enteritidis* to conclude that O antigens must be present for a vaccine to be effective. We used a cell wall-deficient mutant of *S. typhimurium* and were unable to prepare an immunogenic "Venneman type" of RNA (34). Eisenstein, while demonstrating a high degree of protection with the RNA she prepared from smooth strains, used an estimated minimum of 44 μg as the immunizing dose. The challenge was with 20 to 40 LD_{50}.

One of the difficulties in dealing with the identification of the active fraction of a crude ribosomal vaccine is the "stickiness" of these particles. Kurland (13) has reported that about 30% of the weight of freshly isolated ribosomes can be removed by (diethylaminoethyl) column chromatography without altering their ability to function in an in vitro system. We estimate that our crude ribosomes lost between 20 and 30% of their initial weight as a result of the high-salt wash. The immunogenic activity they retained could be attributed to remaining contaminants. Because lipopolysaccharides seem to be present in amounts too small for detection by conventional analytical means, it has not been possible to exclude them as the active component of the vaccines. Venneman (34) recognized this and stated it in his last paper. Johnson (12) identified O antigens in an immunodiffusion assay of preparations that were inactive as vaccines. Why his preparation was inactive is not clear.

Since biological tests for endotoxin may be more sensitive than chemical determinations of small amounts of lipopolysaccharides, the proof of their presence in active, but not in inactive, vaccines is significant (cf. Tables 6 and 7), especially when combined with the other types of evidence that point to the same conclusion. Other investigators might profit from the use of these assays. When polynucleotides are known to be present, the *Limulus* lysate clotting test has dubious value, since Elin and Wolff have shown that substances other than endotoxin can produce coagluation of the lysate (6).

It is important to recognize that the type of active antigen present in ribosomally derived vaccines from *S. typhimurium* is not necessarily the same as that found in vaccines similarly prepared from other pathogenic microorganisms. The Youmans (48) may be correct in attributing the activity they have so convincingly demonstrated for their *M. tuberculosis* vaccine to double-stranded RNA. It is active only when protected by incorporating it into Freund incomplete adjuvant, and it is far more labile to heat and ribonuclease than the salmonellae preparations. It would be premature to assume, therefore, an identity of antigenic types in all ribosomal vaccines. Even though unanswered questions remain in work so far reported, there is no better justification for continued experimentation than the remarkably high degree of protection they confer. No one can deny that O antigens, either in combination with isolated cell walls or when incorporated into adjuvants, fail to provide the infection immunity elicited by ribosomal vaccines. Not only do the latter protect as well as any observed, but they may also provide cross-immunity against strains (5). These are the considerations that make further work imperative.

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