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Ribosomal Vaccines

II. Specificity of the Immune Response to Ribosomal Ribonucleic Acid and Protein Isolated from *Salmonella typhimurium*

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Ribosomal proteins isolated from *Salmonella typhimurium* were effective in inducing immunity in mice. This immunity was specific since animals immunized with ribosomal proteins from *S. typhimurium* were not protected against challenge with *S. cholerae-suis* or *S. enteritidis*. Immunity was evident as early as 5 days after immunization. Ribosomal ribonucleic acid (RNA) failed to provide any effective immunity in mice. Polyinosinic acid:polycytidylic acid (poly I:C) induced a rapid, short-lived immunity to all three *Salmonella* species. In contrast, ribosomal RNA failed to elicit any rapid nonspecific response to infection.

Venneman and Bigley (21) were the first investigators to show that ribosomes isolated from *Salmonella typhimurium* could provide effective immunity in mice to challenge with the live homologous organism. Since ribosomal immunogens are not derived from cell surface material, which usually provides protection against only one or several closely related serotypes, ribosomal vaccines could provide protection against several serologically unrelated members of a genus. This type of vaccine would be ideal for immunization against salmonellae which represent a serologically heterogeneous group of bacteria. However, little effort has been devoted to determining the specificity of the immune response induced by ribosomal vaccines. Eisenstein et al. (Bacteriol. Proc., p. 112, 1971), working with *S. typhimurium* serotype 0:9,12 and *S. typhimurium* serotype 0:4,12, showed that immunization with ribonucleic acid (RNA) extracted from these two strains provided significantly better protection against challenge with the homologous serotype and suggested that part of the immunity was due to O antigen contamination of the RNA preparations. In contrast, Thompson and Snyder (17) have shown that ribosomes isolated from *Diplococcus pneumoniae* type 3 provide significant protection against challenge with *D. pneumoniae* types 1, 2, 3, and 7.

The investigations reported here were initiated to elucidate further the specificty of the immune response to immunogenic ribosomal fractions isolated from *S. typhimurium*.

MATERIALS AND METHODS

Organisms. *S. typhimurium* strain SR-11 (kindly supplied by L. Joe Berry, University of Texas, Austin) and *S. cholerae-suis* and *S. enteriditis* (kindly supplied by F. Koontz, State Hygenic Laboratory, University of Iowa, Iowa City) were maintained as stock cultures on brain heart infusion agar (Difco).

Preparation of cultures. Fernback flasks containing 1.8 liters of brain heart infusion broth were inoculated with 250 ml of exponential-phase broth cultures of *S. typhimurium*. The flasks were incubated at 37 C for 10 to 12 h on a New Brunswick Scientific shaker. Cells were harvested by centrifugation at 18,000 × *g* in a Sorvall RC2-B centrifuge equipped with an SZ-14 continuous-flow rotor and were washed four times in cold (4 C) 0.02 M phosphate buffer, pH 7.0.

Animals. Albino, Swiss-Webster male mice weighing 18 to 21 g were obtained from a colony maintained by the Department of Microbiology, University of Iowa.

Isolation of ribosomes. Washed, packed cells were suspended in 0.02 M phosphate buffer containing 10^{-2} M $MgCl_2$ (POM buffer) and 2 μg of deoxyribonuclease per ml and were broken in a French pressure cell at 10,000 lb/in^2. The crude extract was centrifuged at 25,000 × *g* for 20 min to remove intact cells and cellular debris. Ribosomes were isolated by $(NH_4)_2SO_4$ fractionation as described by Kurtland (6) and modified by Fogel and Sypherd (3). The final ribosome pellet was resuspended in POM buffer and filtered through a Nalge sterile, disposable filter containing a 0.45-μm grid membrane. Ribosomes were pelleted by centrifugation at 350,000 × *g* for 90 min and resuspended in POM buffer.

Isolation of ribosomal RNA and protein. Ribosomal RNA was isolated by phenol POM buffer extrac-

tion (P-RNA) and guanidinium chloride precipitation (G-RNA) as previously described (5).

Ribosomal proteins were extracted with 2-chloroethanol (Eastman Kodak Organic Chemicals, Rochester, N.Y.) by the procedure of Fogel and Sypherd (3). A second method of isolation of proteins was the acetic acid procedure of Hardy et al. (4). Briefly, ribosomes were suspended in 10^{-2}M (2-amino-2-hydroxy-methyl propane-1, 3-diol)tris -hydrochloride buffer, pH 8.0, containing 3×10^{-3} M succinic acid and 10^{-2}M $MgCl_2$ (TSM buffer). One volume of 0.1 M $MgCl_2$ and 2 volumes of glacial acetic acid were added in rapid succession. The mixture was stirred in an ice-water bath for 45 min, and the precipitated RNA was removed and dialyzed in the cold (4 C) against TSM buffer. The dialyzed protein was then lyophilized. Proteins were dissolved in TSM buffer before injection into mice.

Endotoxin detection. A limulus extract was prepared from the horseshoe crab *Limulus polyphemus* as described by Levin and Band (7). The extract was stored at −20 C and was stable for at least 6 months. The assay consisted of mixing 0.5 ml of limulus extract with 0.5 ml of varying dilutions of test material. The end point was read as the last dilution to show gelation. A positive control consisting of varying dilutions of *S. typhimurium* endotoxin (Difco, control 248105) was included in all tests. This assay detected as little as 1 ng of *S. typhimurium* endotoxin.

The passive hemagglutination technique (PHA) described by Rudbach (12) was used to detect antibodies to endotoxin in mice immunized with the various ribosomal fractions. Two controls sera were used in each test. The first was pooled serum obtained from unimmunized mice. The second control serum was obtained from mice given two injections each containing 1 μg of endotoxin 7 days apart. Fourteen days after the second injection, the mice were bled, and the serum was used as a positive control in the PHA test.

Immunization with ribosomal fractions. The ribosomal RNA was emulsified in an equal volume of Freund incomplete adjuvant before injection. Proteins were injected without adjuvant. Animals were immunized subcutaneously with 0.2 ml of material and challenged by the intraperitoneal route 15 days after immunization unless otherwise indicated. Deaths were recorded 30 days after challenge.

Biochemical assay. Protein was determined by the method of Lowry et al. (8). RNA and deoxyribonucleic acid (DNA) were measured by the orcinol procedure and by diphenylamine, respectively (1, 2). Bovine serum albumin, yeast RNA, and pancreatic DNA (Nutritional Biochemicals Corp.) were used as standards.

Chemicals and antisera. Antisera to the *S. typhimurium* O antigens 1, 4, 5, 12 and H phase 1 and 2 antigens were obtained from Difco. Polyinosinic acid:polycytidylic acid (poly I:C) was purchased from Sigma Chemical Co.

RESULTS

Purity of ribosomal RNA and ribosomal protein. The ribosomal RNA obtained by phenol extraction contained 0.1% protein. Further purification of the RNA by guanidinium chloride precipitation resulted in an RNA preparation which contained no detectable protein. Immunodiffusion studies showed that the phenol-extracted RNA contained the factor 1, 4, and 12 antigens even after six to eight phenol extractions. Figure 1 shows the immunodiffusion pattern of the RNA preparations when reacted against antiserum to factor 4. No O antigens were found in the RNA preparation obtained after guanidinium chloride precipitation (Fig. 1).

The results of PHA tests for antibodies to endotoxin are shown in Table 1. Pooled sera from unimmunized mice showed a titer of 1:2, whereas the sera obtained from mice receiving two injections each containing 1 μg of endotoxin

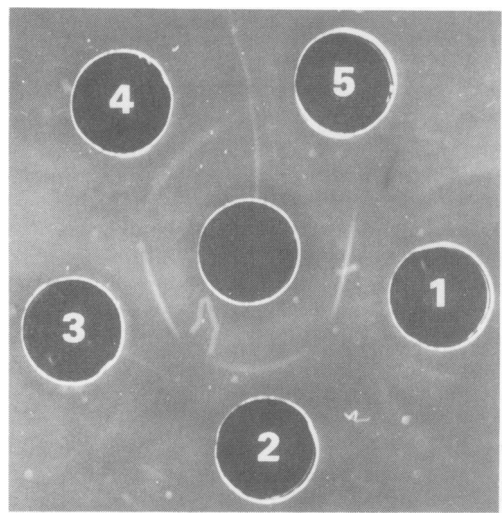

FIG. 1. *Immunodiffusion plate showing the reactions of P-RNA and G-RNA with antiserum to factor 4 antigen. The center well contains antiserum to factor 4 antigen. 1, P-RNA extracted two times with phenol; 2, P-RNA extracted six times with phenol; 3, lysate of S. typhimurium (positive control); 4, P-RNA extracted eight times with phenol; 5, G-RNA.*

TABLE 1. *Passive hemagglutination titers to endotoxin in mice immunized with ribosomal RNA*

Mice immunized with	Titer[a]
Unimmunized control	1:2
Endotoxin	1:512
P-RNA[b]	1:64
G-RNA[b]	1:4

[a] Titers were obtained from sera pooled from five mice.

[b] Mice were immunized with 1 mg of RNA and bled 14 days after immunization.

given 7 days apart had a titer of 1:512. Mice immunized with 1 mg of P-RNA had a PHA titer of 1:128. No significant antibody to endotoxin was observed in mice immunized with 1 mg of G-RNA. The P-RNA contained 1 μg of endotoxin per 600 μg of RNA, and the G-RNA contained 1 μg per 2.4 mg of RNA when assayed by the limulus coagulation test.

Proteins isolated by the acetic acid procedure contained 1% contamination with RNA, but proteins extracted with 2-chloroethanol contained no detectable RNA. Both of these protein preparations gave equivalent degrees of protection when equal amounts of protein were injected (Table 2). No endotoxin was detected in either preparation of protein when assayed by the limulus coagulation test, and immunodiffusion studies failed to detect any O or H antigens. The protein obtained by extraction with 2-chloroethanol was used in all subsequent experiments.

Specificity of the immune response. Mice immunized with ribosomal proteins from *S. typhimurium* had significant levels of protection against a challenge of 100 mean lethal doses (LD_{50}) of the homologous organism (Table 3). No protection was observed in animals immunized with such ribosomal proteins and challenged with *S. enteriditis* or *S. cholerae-suis*. The ribosomal RNA preparations failed to elicit any significant degree of protection against homologous or heterologous challenge. Since mice immunized with the RNA fractions failed to show any immune response, an experiment was done to determine whether the ribosomal RNA fractions would induce a rapid short-lived, nonspecific response to infection as has been reported for synthetic RNA (23). Mice immunized with various ribosomal fractions were challenged at varying intervals after immunization (Table 4). Mice immunized with poly I:C showed protection against all three organisms when the mice were challenged 1 or 2 days after injection of poly I:C. Very little protection was observed in mice challenged 3 days after immunization with poly I:C. No rapid, nonspecific protection was observed in animals immunized with either *S. typhimurium* RNA preparation. Mice immunized with ribosomal proteins showed evidence of protection when challenged 5 days after immunization. The immunity induced by the ribosomal proteins was specific since no immunity was observed in animals immunized with *S. typhimurium* ribosomal proteins and challenged with heterologous organisms.

TABLE 2. *Comparison of the immunogenicity of ribosomal proteins extracted with acetic acid and 2-chloroethanol with whole ribosomes*

Amount injected (μg of protein)	No. dead/total[a]		
	2-chloroethanol-extracted proteins[b]	Acetic acid-extracted proteins	Whole ribosomes
400	0/20	0/20	0/20
200	0/20	0/20	0/20
100	0/20	0/20	1/20
50	2/20	3/20	2/20
25	16/20	18/20	17/20
Controls[c]	20/20	20/20	20/20

[a] Animals were challenged with 1,000 LD_{50} of *S. typhimurium* 14 days after immunization.
[b] Compound with which animals were immunized.
[c] Controls received 0.2 ml of POM buffer.

TABLE 3. *Specificity of the immune response induced by ribosomal RNA and ribosomal protein*

Material injected	Challenge organism[a]	No. dead/total
S. typhimurium protein[b]	*S. typhimurium*	0/20
	S. enteriditis	19/20
	S. cholerae-suis	20/20
S. typhimurium P-RNA[c]	*S. typhimurium*	20/20
	S. enteriditis	20/20
	S. cholerae-suis	20/20
S. typhimurium G-RNA[c]	*S. typhimurium*	18/20
	S. enteriditis	20/20
	S. cholerae-suis	20/20
Controls[d]	*S. typhimurium*	20/20
	S. enteriditis	20/20
	S. cholerae-suis	20/20

[a] Mice were challenged with 100 LD_{50} of the appropriate organism 14 days after immunization.
[b] Mice were immunized with 200 μg of ribosomal protein.
[c] Mice were immunized with 250 μg of ribosomal RNA.
[d] Controls were injected with 0.1 ml of POM buffer.

DISCUSSION

Since Youmans and Youmans (24) first demonstrated the immunogenicity of ribosomes isolated from *Mycobacterium tuberculosis*, attempts to identify the immunogenic moiety of these vaccines have produced conflicting results. Youmans and Youmans (25–27) have reported that the ribosomal immunogen isolated from *M. tuberculosis* is a heat-labile, ribonuclease-sensitive, double-stranded RNA. In support of this, the ribosomal immunogen

TABLE 4. *Comparison of the immune response induced by poly I:C with ribosomal RNA and protein*

Material injected	Challenge organism[a]	No. dead/total					
		1[b]	2	3	5	7	14
Poly I:C[c]	*S. typhimurium*	1/10	2/10	8/10	10/10	10/10	10/10
	S. enteriditis	1/10	3/10	9/10	10/10	10/10	10/10
	S. cholerae-suis	2/10	2/10	10/10	10/10	10/10	10/10
S. typhimurium P-RNA[d]	*S. typhimurium*	10/10	10/10	9/10	10/10	10/10	10/10
	S. enteriditis	9/10	10/10	10/10	10/10	10/10	10/10
	S. cholerae-suis	10/10	10/10	10/10	9/10	10/10	10/10
S. typhimurium G-RNA[d]	*S. typhimurium*	10/10	10/10	10/10	10/10	10/10	10/10
	S. enteriditis	9/10	10/10	10/10	9/10	10/10	10/10
	S. cholerae-suis	10/10	10/10	9/10	10/10	10/10	10/10
S. typhimurium ribosomal proteins[e]	*S. typhimurium*	10/10	9/10	9/10	6/10	4/10	0/10
	S. enteriditis	9/10	10/10	10/10	10/10	10/10	10/10
	S. cholerae-suis	10/10	9/10	9/10	10/10	10/10	10/10
Controls[f]	*S. typhimurium*	10/10	10/10	10/10	10/10	10/10	10/10
	S. enteriditis	9/10	10/10	10/10	9/10	10/10	10/10
	S. cholerae-suis	10/10	10/10	9/10	10/10	10/10	10/10

[a] Mice were challenged with 1,000 LD_{50} of the appropriate organism.
[b] Days of challenge after immunization.
[c] Mice received 1 μg of poly I:C.
[d] Mice were immunized with 250 μg of RNA.
[e] Mice were immunized with 200 μg of ribosomal protein.
[f] Controls were injected with 0.1 ml of POM buffer.

isolated from *S. typhimurium* has been reported to be RNA or an RNA-protein complex (18, 21, 22). In contrast, Johnson (5) has reported that the immunogenicity of ribosomal preparations from *S. typhimurium* resides in the protein fraction and not the RNA fraction of the ribosomes. More recently, Smith and Bigley (14) have shown that protein-rich ribosomal fractions provide better immunity than RNA-rich fractions isolated from *S. typhimurium* ribosomes. These same investigators found that the immunogenicity of a relatively pure protein fraction could be enhanced if the protein was mixed with polyadenylate:polyuridylic acid before injection. This would suggest that RNA may serve as an adjuvant for some as yet unidentified immunogen. Thomas and Weiss (16) have shown that the immunogenic moiety of ribosomes isolated from *Neisseria meningitidis* is sensitive to treatment with Pronase and suggest that the immunogen is protein or a carbohydrate liberated by the action of Pronase. The results of our current experiments support the contention that the immunogenic moiety of the ribosomes is a protein or group of proteins. A comparison of the immunogenicity of ribosomal proteins with whole ribosomes (Table 2) has shown that, based on protein content, the ribosomal proteins are as effective as whole ribosomes in inducing immunity. RNA fractions failed to elicit any immune response corresponding to that observed with the ribosomal proteins. In addition, P-RNA and G-RNA fractions failed to elicit any rapid nonspecific response to challenge with the homologous or heterologous organism. This is in direct contrast to the rapid, nonspecific response to infection observed in animals injected with poly I:C. It is difficult to reconcile our results which indicate that ribosomal protein is the immunogen with those of previous investigators (18, 21, 22) who have suggested that ribosomal RNA is the immunogen. However, it has been suggested (14) that phenol extraction of RNA may result in an RNA preparation contaminated with small amounts of immunogenic protein which has been partially denatured. The RNA then may serve as an adjuvant for the protein.

The immune response induced by the ribosomal proteins is specific since mice immunized with proteins from *S. typhimurium* showed no protection against challenge with *S. cholerae-suis* or *S. enteriditis*. This supports the observations of Eisenstein et al. (Bacteriol. Proc., p. 112, 1971). However, the mechanism for the specificity of the immune response elicited by ribosomal protein is unknown. The protein preparations contained no O or H antigens, so

the specificity is not due to contamination with cell surface antigens. It has been shown that the ribosomal proteins isolated from eukarytotic and prokaryotic cells are quite different (13) and, in *Escherichia coli*, certain 30S ribosomal proteins are unique to certain strains (10). It is possible therefore that a single 30S protein or group of proteins is responsible for the specificity observed with the ribosomal immunogens. The specificity of the immune response induced by ribosomes isolated from bacteria other than *S. typhimurium* does not appear to be specific. Thompson and Snyder (17) have shown that ribosomes isolated from *Diplococcus pneumoniae* type 3 will protect mice against challenge with *D. pneumoniae* types 1, 2, 3, and 7. Also, Thomas and Weiss (16) have shown that mice immunized with ribosomes isolated from group B *N. meningitidis* are protected against infections with *N. meningitidis* groups A, B, and C. Further studies are necessary to determine why some ribosomal vaccines induce specific protection whereas others seem to induce a broader degree of protection.

Cellular immunity appears to play an important role in the immunity induced by ribosomal vaccines. Venneman and Berry (19, 20) have shown that immunity induced by ribosomal RNA vaccines can be passively transfered with peritoneal cells but not with serum. Patterson and Youmans (11) have reported that splenic lymphocytes from mice immunized with ribosomal RNA from *M. tuberculosis* produce a filterable substance which inhibits the multiplication of tubercle bacilli in normal mouse peritoneal macrophages. Margolis and Bigley (9) demonstrated the presence of cytophilic macroglobulin in animals immunized with ribosomal proteins, and Smith and Bigley (15) have shown that mice immunized with ribosomal fractions develop manifestations of delayed hypersensitivity as early as 4 days after immunization, with peak responses occuring in 2 to 3 weeks. Our results are in agreement with these observations. Animals immunized with ribosomal proteins showed significant levels of protection 5 to 7 days after immunization, and maximal protection was observed in animals challenged 14 days after immunization. Preliminary experiments have shown that this immunity can be passively transfered with peritoneal cells but not with serum (unpublished observations). Further investigations are necessary to isolate a single immunogenic ribosomal protein or group of proteins so that we might better explain the basis for the specificity of the immune response and more clearly define the mechanism by which this protein or proteins induce immunity.

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