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Ribonucleic Acid-Protein Fractions of Virulent *Salmonella typhimurium* as Protective Immunogens

RANDALL A. SMITH AND NANCY J. BIGLEY
Department of Microbiology, University of Health Sciences, The Chicago Medical School, Chicago, Illinois 60612

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Mice were injected with virulent *Salmonella typhimurium* SR-11 subfractions containing varied amounts of ribonucleic acid (RNA) and protein or with living attenuated *S. typhimurium* RIA. In these mice, maximal resistance to lethal infection by 1,000 or 5,000 median lethal doses of *S. typhimurium* SR-11 was seen 2 to 3 weeks after immunization. The *S. typhimurium* RIA vaccine and a crude ethanol-precipitated RNA fraction (E-RNA) prepared from lysates of *S. typhimurium* SR-11 were the most efficient immunogens inducing protection against salmonellosis. The contribution of the components present in the E-RNA fractions to host protection against lethal salmonella infection was also examined. RNA-rich fractions (P-RNA) prepared from lysates of the virulent salmonellae contained several bands of protein when examined by disc electrophoresis. P-RNA fractions stimulated protective immunity in mice to infection with *S. typhimurium* SR-11 but to a much lesser degree than did the E-RNA fractions or strain RIA vaccine. Protein-rich fractions (NP), separated from E-RNA by salt precipitation, exhibited the same number and distribution of protein bands by disc electrophoresis as did the parent E-RNA fractions. Mixtures of either bovine liver soluble RNA or various synthetic polynucleotides and NP were examined, as was NP fraction alone, for the ability to confer protection in mice to challenge infections by the virulent strain of salmonella. Polyadenylic-uridylic acid plus NP conferred significant protective immunity to challenge infections in mice immunized with this mixture, being nearly as effective an immunogen as were the E-RNA fractions of *S. typhimurium* SR-11 or the attenuated *S. typhimurium* RIA.

The precise antigenic moiety responsible for the protective immunity induced by ribosomal "vaccines" or ribonucleic acid (RNA) subfractions of bacterial pathogens is not clearly defined. Winston and Berry (21) found that a protein fraction obtained from *Staphylococcus aureus* ribosomes afforded protection in mice against multiplication of staphylococci, whereas the RNA fraction of the ribosomes containing 10% protein stimulated even better protection. Thompson and Snyder (14) were able to protect mice against lethal pneumococcal infection after immunization with ribosomal extracts of *Diplococcus pneumoniae*. Both RNA and protein moieties appeared to be necessary components of the effective immunogen.

Venneman and Bigley (18) and Venneman et al. (19) have shown that both a ribosomal subfraction from *Salmonella typhimurium* SR-11 and its subunits are effective immunogens in stimulating immunity to lethal salmonellosis in mice. They also observed that an ethanol-precipitated, crude RNA fraction of the ribosomal preparations induced protective immunity comparable to that developed after immunization with the whole ribosomal fraction. Moreover, RNA fractions prepared by phenol extractions of whole-cell lysates or ribosomal preparations from *S. typhimurium* SR-11 also induced immunity to lethal salmonella infection in mice. The objective of this present study was to examine the contribution of the protein components contained in the RNA extracts of *S. typhimurium* SR-11 to the induction of resistance to lethal salmonella infection in mice.

The immunogenic efficacy of RNA-protein fractions from lysates of *S. typhimurium* SR-11 and the corresponding RNA-rich and protein-rich subfractions were compared for the capacity to stimulate protective immunity to challenge in-

fection in mice. The RNA-protein fractions (E-RNA) were prepared by ethanol precipitation of whole-cell lysates of *S. typhimurium* SR-11 and were rich in both RNA and protein. Other RNA-rich fractions were prepared by phenol extractions of the RNA present in whole-cell lysates. The protein-rich fractions (NP) were prepared by salt precipitation of the proteins present in E-RNA fractions. Host resistance in mice protected against lethal salmonellosis after injections of RNA-rich subfractions (E-RNA and P-RNA) of virulent *S. typhimurium* SR-11 was examined first. The protein fractions (NP) isolated from the E-RNA preparations were then used as immunogens in mice either alone or in combination with bovine liver soluble RNA (sRNA) or with several synthetic polynucleotides. The effectiveness of these various immunizations in mice was compared with that immunity imparted after immunization with living attenuated *S. typhimurium* RIA. Evaluation of the immunogenic efficacy of each preparation was based on survival of the mice for 35 days after either 1,000 or 5,000 median lethal doses (LD_{50}) of the virulent *S. typhimurium* SR-11.

MATERIALS AND METHODS

Bacterial cultures. *S. typhimurium* strains SR-11 and RIA were used throughout this study.

Preparation of bacterial cultures for nucleic acid extractions. The techniques for culturing, harvesting, and rupturing the virulent *S. typhimurium* were those of Venneman and Bigley (18) with the following exceptions. A 0.01 M phosphate buffer, *p*H 7.1, containing 4×10^{-4}% polyvinyl sulfate (20) was used in the washing and harvesting of the bacteria. Also, the bacterial suspensions were passed through a cold (4 C) French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) three times at 14,000 psi instead of once.

Preparation of whole-cell RNA fractions by ethanol precipitation. One-half of the cell-free supernatant fluid was brought to 0.1 M sodium concentration with the addition of NaCl. After the addition and solubilization of the NaCl, two volumes of cold (−20 C) 95% ethanol were added to the cell-free supernatant fraction. The mixture was left at −20 C for 24 hr to allow precipitation of the nucleic acids and protein. The mixture was then centrifuged at 10,000 × *g* for 10 min in a Sorvall (model RC-2) refrigerated centrifuge with an SS34 rotor head. The supernatant fluid was discarded, and the precipitate was left at 4 C for 6 to 8 hr to dry. The smallest volume of 0.16 M NaCl necessary to dissolve the precipitate was added to the dried precipitate. The nucleic acid-protein solution was adjusted to 10^{-3} M Mg^{2+} concentration by the addition of $MgCl_2$. Deoxyribonuclease (I, Sigma Chemical Co., St. Louis, Mo.) at a concentration of 5 μg/ml was added to the nucleic acid-protein solution, and the mixture was incubated at 25 C for 1 hr to destroy deoxyribonucleic acid (DNA) (20). Again, two volumes of cold ethanol were added to the nucleic acid-protein mixture. After standing for 24 hr at −20 C, the mixture was centrifuged, and the precipitate was collected and allowed to dry. The nucleic acid-protein pellet was resuspended as described, lyophilized in 5-ml quantities, and stored at −80 C.

Preparation of whole-cell RNA fraction by phenol extraction. From the remaining half of the cell-free supernatant fluid, RNA was prepared by phenol extraction by the method of Venneman et al. (19), with the exception that a 90% phenol solution was used instead of an 80% solution.

Preparation of bacterial protein fraction (NP). To lyophilized E-RNA fraction suspended in 0.16 M NaCl were added two volumes of saturated $(NH_4)_2SO_4$ solution. The solution was mixed on a magnetic stirrer for 4 hr at 4 C, after which it was centrifuged at 10,000 × *g* for 10 min in a Sorvall (model RC-2) refrigerated centrifuge. The pellet was solubilized in 0.16 M NaCl and dialyzed against 0.16 M NaCl for 2 days at 4 C with frequent changes of saline. The material was examined for protein, DNA, and RNA as described and for free carbohydrate by the method of Margolis and Bigley (9). Absorbancy ratios (A_{280}/A_{260}) were determined in a Beckman DB spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.).

Chemical determinations. Estimation of the protein content of the various preparations was determined by the method of Lowry et al. (8). Standard curves for protein determinations were made with bovine serum albumin (fraction V, powder, Pentex Biochem. Corp., Kankakee, Ill). RNA content of the various salmonella fractions was determined by using the Bial reaction for pentoses (20). RNA standards were made with bovine liver RNA (Nutritional Biochem. Corp., Cleveland, Ohio). Determination of DNA content was performed by the Dische method (5). Standards for the DNA determinations were made with DNA, sodium salt (Nutritional Biochem. Corp., Cleveland, Ohio).

Disc electrophoresis of RNA and protein fractions. The method described by Williams and Chase (20) for disc electrophoresis was used with the exception that Coomassie brilliant blue was used to develop the protein bands in the ethanol-precipitated RNA (E-RNA), the phenol-extracted RNA (P-RNA), and in the protein-rich NP fractions.

Estimation of virulence. Non-inbred, albino, female mice SCH:ARS Ha (ICR) were purchased from the A. R. Schmidt Co., Madison, Wis., and were used throughout this study. The method for the LD_{50} determinations of the *S. typhimurium* strains SR-11 and RIA was that of Dragstead and Lang (6).

Immunizations of mice. Mice were injected subcutaneously (sc) with 0.1-ml quantities of specified amounts of E-RNA and P-RNA fractions incorporated in 0.2 ml of incomplete Freund's adjuvant (Difco Labs., Detroit, Mich.). Mice were immunized intraperitoneally (ip) with 10^6 cells of *S. typhimurium* RIA. For protection studies, mice were challenged 2 to 3 weeks after immunization with either 1,000 or 5,000 LD_{50} doses of *S. typhimurium* SR-11 ip in 0.2 ml of sterile saline. Mice also were injected with 200-μg

amounts of the following nucleotide materials mixed with 100-μg amounts of NP fraction: polyadenylic-cytidylic-uridylic acid, potassium salt (poly ACU); polyinosinic-cytidylic acid, potassium salt (poly IC); polyadenylic-uridylic acid, potassium salt (poly AU); and bovine liver sRNA. The synthetic polynucleotides and sRNA were purchased from Nutritional Biochem. Corp., Cleveland, Ohio. Also, 10-μg amounts of the NP fraction were mixed with 200-μg quantities of poly AU. The nucleotide or sRNA and NP mixtures were incubated for 1 hr at 37 C prior to being mixed with two parts of incomplete Freund's ajduvant and subsequently injected sc into mice. Mice were also injected sc at two different sites, one site on each mouse injected with 100-μg amounts of NP fraction in incomplete Freund's adjuvant and the other site with 200-μg of poly AU in incomplete Freund's adjuvant.

Collection of mouse lymphoid cells. Spleens and lymph nodes (inguinal and axillary) from groups of immunized and unimmunized mice were aseptically removed, and single cell suspensions were prepared by the method of Adler et al. (1). Peritoneal exudate cells were removed from mice and prepared for use by the method of Saito and Mitsuhashi (11, 12).

Passive transfer of lymphoid cells and sera. Normal mice were injected intravenously (iv) in the tail vein with spleen cells, lymph node cells, and peritoneal exudate cells from immunized and unimmunized mice. All suspensions of lymphoid cells were adjusted to a concentration of 10^7/ml, and each mouse received 10^6 cells. All sera were concentrated twofold in polyethylene glycol (Fisher Scientific Co., Pittsburgh, Pa.) and injected in 0.1-ml quantities iv into normal recipient mice. All mice receiving lymphoid cells were challenged 5 days later with 1,000 LD_{50} doses of *S. typhimurium* SR-11, whereas those receiving sera were injected with the same dose of virulent salmonellae 1 day after injection of the sera. All mice were observed for a 35-day period after injection of the salmonellae.

RESULTS

Stimulation of protection to challenge infection by RNA-rich subfractions of S. typhimurium SR-11. Three weeks after immunization with doses of 250 μg of either E-RNA or P-RNA subfractions of *S. typhimurium* SR-11 per mouse, the immunized mice as well as the unimmunized mice were injected with 5,000 LD_{50} doses of virulent *S. typhimurium* SR-11. As shown in Table 1, mice immunized with three preparations of E-RNA exhibited marked survival to this challenge infection. As the ratio of RNA to protein in the three E-RNA preparations used as immunizing materials increased from 99:1 (four phenol extractions) to 90:10 (one phenol extraction), survival increased from 35.7 to 52.7%. This difference in survival is statistically significant ($P < 4 \times 10^{-3} > 2 \times 10^{-3}$). All unimmunized mice were dead by 7 days after injection of the same dose of *S. typhimurium* SR-11.

TABLE 1. *Comparative immunogenicity in mice of RNA-rich subfractions of S. typhimurium SR-11*

Immunizing preparation	Ratio (RNA: protein)	% Survival at 35 days[a]
E-RNA.......	65:35	80.0 (12/15)
E-RNA.......	65:35	81.3 (13/16)
E-RNA.......	55:45	89.5 (17/19)
P-RNA.......	99:1	35.7 (5/14)[b]
P-RNA.......	98:2	40.0 (8/20)[c]
P-RNA.......	90:10	52.7 (10/19)[d]
None.........		0 (0/45)[e]

[a] All mice injected with 5,000 LD_{50} doses of *S. typhimurium* SR-11.
[b] $P < 0.01 > 0.0008$ based on 15 control mice.
[c] $P < 0.004 > 0.003$ based on 15 control mice.
[d] $P < 0.004 > 0.003$ based on 15 control mice.
[e] In 3 groups of 15/group.

Stimulation of protection to challenge infections by protein-rich subfractions of S. typhimurium SR-11. The subfractions (E-RNA and NP) of *S. typhimurium* SR-11 which contained appreciable amounts of protein were compared for the capacity to induce resistance to lethal salmonella infections. Mice were immunized with doses of 200 to 250 μg of protein with 3 E-RNA preparations per mouse and with doses of 100 to 200 μg of protein of the NP fractions from *S. typhimurium* SR-11 per mouse. At the same time, groups of mice were injected with 10^6 cells of the RIA vaccine strain of *S. typhimurium*. Fifteen days after immunization (Table 2), one group of mice injected with the RIA vaccine (II), one group of mice injected with E-RNA (III), and one group of mice injected with NP fraction (III) were injected with 1,000 LD_{50} doses of *S. typhimurium* SR-11. All other groups of mice listed in Table 2 received the same dose of virulent salmonellae 21 days after immunization. All mice either vaccinated with the RIA vaccine or immunized with at least 200 μg of protein/mouse exhibited marked survival rates (66.6–89.5%) to 1,000 LD_{50} doses of virulent *S. typhimurium* SR-11. All unimmunized mice were dead by 11 to 13 days after injection of this same dose of virulent salmonellae.

Effect of protein (NP) isolated from E-RNA fraction on protective host immunity. The induction of protective host immunity in mice by the protein-rich subfractions prepared from *S. typhimurium* SR-11 was examined further. Groups of mice were immunized with the purest NP fraction (NP I < 0.1 part RNA: >99.9 parts protein) alone or mixed with bovine liver sRNA or synthetic polynucleotides. As sham controls, mice were injected with either sRNA or synthetic

TABLE 2. *Comparative immunogenicity of S. typhimurium RIA vaccine and protein-rich subfractions from S. typhimurium SR-11*

Immunizing material	Ratio (RNA:protein)	Dose (μg protein)	% Survival at 35 days
RIA vaccine I[a]			83.9 (26/31)
RIA vaccine II[b]			89.5 (17/19)
E-RNA I[a]	65:35	250	85.0 (17/20)
E-RNA II[a]	50:50	200	80.0 (16/20)
E-RNA III[b]	50:50	200	77.7 (7/9)[c]
NP I[a]	<0.1:>99.9	100	30.0 (3/10)[d]
NP II[a]	6:94	100	50.0 (9/18)[e]
NP III[b]	10:90	200	66.6 (6/9)[f]
None			0 (0/100)[g]

[a] Mice injected with 1,000 LD_{50} doses of *S. typhimurium* SR-11 21 days after immunization.
[b] Mice injected with 1,000 LD_{50} doses of *S. typhimurium* SR-11 15 days after immunization.
[c] $P < 0.006 > 0.0005$ based on 10 control mice.
[d] $P < 0.07 > 0.05$ based on 10 control mice.
[e] $P < 0.008 > 0.006$ based on 20 control mice.
[f] $P < 0.003 > 0.002$ based on 10 control mice.
[g] In four groups of 10/group and in three groups of 20/group.

TABLE 3. *Immunogenicity of NP fraction in the presence and absence of synthetic polynucleotides and bovine liver sRNA*

Immunizing preparation	% Survival at 35 days[a]	
Poly IC + NP	40	(4/10)[b]
Poly IC	0	(0/5)
Poly ACU + NP	55.5	(5/9)[c]
Poly ACU	0	(0/5)
Poly AU + 10 μg of NP	47	(14/30)[d]
Poly AU + 100 μg of NP	70	(28/40)[e]
Poly AU + 100 μg of NP[f]	60	(12/20)[g]
Poly AU	0	(0/5)
Bovine liver sRNA + NP	50	(5/10)[h]
Bovine liver sRNA	0	(0/5)
NP	30	(3/10)[i]
S. typhimurium RIA	83.3	(25/30)
None	0	(0/40)[j]

[a] All mice injected with 1,000 LD_{50} doses of *S. typhimurium* SR-11 challenge infection.
[b] $P < 0.03 > 0.02$ based on 10 control mice.
[c] $P < 0.0007 > 0.006$ based on 10 control mice.
[d] $P < 0.003 > 0.0002$ based on 20 control mice.
[e] $P < 4 \times 10^{-6} > 3 \times 10^{-6}$ based on 20 control mice.
[f] Injected at separate sites in each mouse.
[g] $P < 10^{-4} > 5 \times 10^{-5}$ based on 10 control mice.
[h] $P < 0.02 > 0.01$ based on 10 control mice.
[i] $P < 0.07 > 0.05$ based on 10 control mice.
[j] In two groups of 10/group and in one group of 20.

polynucleotides incorporated in incomplete Freund's adjuvant. Three weeks later all mice, in addition to mice injected with 10^6 vaccine cells (*S. typhimurium* RIA) 3 weeks previously and groups of normal control mice, received ip 1,000 LD_{50} doses of virulent *S. typhimurium* SR-11. None of the unimmunized or sham control mice survived the dose of salmonellae used in the challenge infections, whereas 83.3% of the RIA vaccinated mice were alive and healthy 35 days after challenge (Table 3). When the NP fraction was mixed with poly IC or with poly ACU or with bovine liver sRNA and used as immunizing agent or when the NP fraction was injected alone into mice, varying degrees of survival to challenge infection were observed. The NP fraction alone stimulated the poorest immunity. NP in 100-μg amounts mixed with poly AU stimulated a more effective immunity to challenge infection in mice than did 10-μg amounts of NP mixed with poly AU. When mice were injected with 100-μg amounts of NP at a different site from that into which 200-μg amounts of poly AU were injected, significant (60%) survival to 1,000 LD_{50} doses of virulent salmonellae was observed.

Chemical nature of immunogenic subfractions of S. typhimurium SR-11. All subfractions (E-RNA, P-RNA, and NP) of *S. typhimurium* SR-11 were routinely assayed for the relative content of RNA, DNA, and protein.

To minimize the degradation of the membrane-cell wall units, the E-RNA fractions were purposefully prepared in the absence of sodium dodecyl sulfate. Initially, this was considered an effective procedure in that Margolis and Bigley (9) found no serological evidence of O antigen carbohydrate with Lederle (Pearl River, N.Y.) antiserum nor chemically detectable carbohydrate moieties *free* in the E-RNA, P-RNA, and NP samples examined. The test employed was sensitive to the detection of carbohydrate materials above a concentration of 10 μg. Similar to the observations of Johnson (W. Johnson, *personal communication*), these samples were found to be reactive with Difco (Detroit, Mich.) antiserum to factor 4 (nonacetylated abequose portion of the O antigen complex) in micro-gel diffusion tests. Curiously, none of the preparations were reactive with Difco antisera: anti-factors 4 and 5 (acetylated abequose): anti-factor 5; anti-factor 12; nor anti-factors 4, 5, and 12. The specificity of anti-factor 4 (Difco) needs to be examined further and verified. However, mitogenically active endotoxin activity was not present in the E-RNA, P-RNA, or NP fractions as determined by ^3H-thymidine uptake and inhibition of migration of spleen cells from normal mice (R. A. Smith, M.S. thesis, Univ. of Health Sciences/The Chicago Medical School, 1971).

The NP preparations generally contained 5% or less of orcinol-positive material and usually less than 4% diphenylamine-positive material. Of eight NP preparations made, only one was found to be free from detectable orcinol- and diphenylamine-positive materials and exhibited an absorbancy ratio (A_{280}/A_{260}) of 1.4. NP preparations were subjected to examination by disc electrophoresis, as were several samples of E-RNA. As shown in Fig. 1, the two E-RNA preparations (1 and 2) exhibited comparable numbers and distribution of protein bands. The NP sample shown here was prepared from the E-RNA sample 2. Note that both contain the same distribution of protein bands. These three samples were solubilized in distilled water prior to addition to the acrylamide gels. The protein bands were detected in concentrations as low as 35 μg of protein. Better resolution of the protein materials was obtained when a 100-μg amount of protein was contained in each sample, as in samples 1, 2, and 3 of Fig. 1. With the P-RNA fraction (sample 4) this concentration of protein was not possible. When the P-RNA was dissolved in phenol-acetic acid-water (2:1:0.5; w/v/v) (10) and then subjected to electrophoresis in the typical acrylamide gels (20), several bands (2 or 3) of protein-staining material as seen in sample 4 of Fig. 1 were regularly observed.

Passive transfer of immune resistance. Passive transfer of host protection was attempted in a limited number of animals with the sera and lymphoid cells from mice immunized with the E-RNA fraction of *S. typhimurium* SR-11. Only lymphoid cell populations from immune mice transferred host protection to infection by 1,000 LD_{50} doses of virulent salmonellae (Table 4). Sera from immune mice did not transfer resistance to lethal salmonellosis nor did sera or lymphoid cells from normal mice. Sera from immune mice did afford transient protection to normal recipients briefly in that the time interval before death was prolonged by 4 days as compared to that of normal mice receiving the same dose of virulent salmonellae.

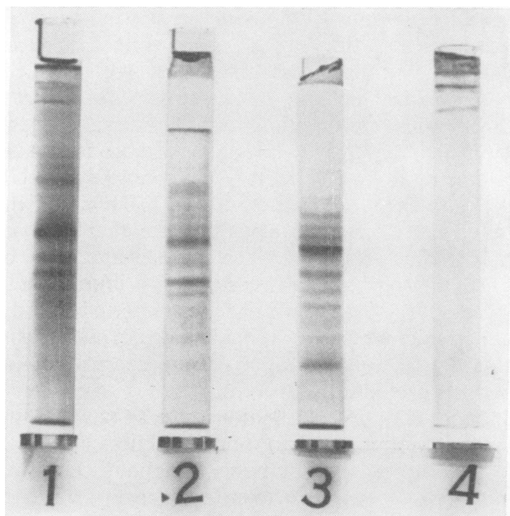

FIG. 1. *Disc electrophoresis of E-RNA, P-RNA, and NP fractions of S. typhimurium SR-11. Samples of ethanol-precipitated RNA (E-RNA), 1 and 2; $(NH_4)SO_4$-precipitated proteins (NP) from E-RNA sample 2, 3; phenol-extracted RNA (P-RNA), 4.*

DISCUSSION

An evaluation of the efficacy of the RNA and protein components in subfractions of virulent *S. typhimurium* SR-11 was performed. Two RNA-rich fractions (ethanol-precipitated, E-RNA and phenol-extracted, P-RNA) were prepared from whole-cell lysates of *S. typhimurium* SR-11 and used as immunizing agents in mice. By 3 weeks after immunization, the E-RNA fractions stimulated a significantly higher degree of resistance to lethal salmonellosis (80–89.5% survival) than did the P-RNA fractions (35.7–52.7% survival). With both types of RNA-rich fractions, greater protection to 5,000 LD_{50} doses of virulent salmonellae was conferred by those fractions containing higher concentrations of protein. This was more readily apparent with the P-RNA preparations. Those P-RNA fractions prepared from whole-cell lysates by only one phenol extraction were more effective immunogens and contained 100 times more protein than P-RNA fractions prepared by three to four phenol extractions. It is of interest to note that Venneman

TABLE 4. *Passive transfer of protective immunity to normal mice from donors immunized with E-RNA fraction*

Material transferred	% Survival at 35 days[a]
Immune serum	0 (0/10)
Immune peritoneal exudate cells	40 (2/5)[b]
Immune lymph node cells	20 (1/5)[c]
Immune spleen cells	40 (2/5)[d]
Normal serum	0 (0/5)
Normal peritoneal exudate cells	0 (0/5)
Normal spleen cells	0 (0/5)
None	0 (0/10)

[a] All mice injected with 1,000 LD_{50} doses of *S. typhimurium* SR-11 in challenge infection.
[b] $P < 0.15 > 0.10$.
[c] $P > 0.20$.
[d] $P < 0.15 > 0.10$.

(15) finds the protein portion isolated chromatographically from phenol-extracted RNA subfractions of *S. typhimurium* SR-11 to be nonimmunogenic. The nonimmunogenicity of such material may reflect denaturation of the protein by phenol. In this present study, similar phenol-extracted RNA preparations from *S. typhimurium* SR-11 contained several bands of protein upon examination by disc electrophoresis, and two of these bands did not migrate in the same positions as any of the bands of protein in either the E-RNA samples or the NP fractions isolated from the E-RNA samples. This difference may reflect denaturation of the proteins in the P-RNA fraction.

Since the E-RNA preparations consisted of 30 to 50 parts protein, protein-rich NP fractions were prepared from them by salt precipitation. Apparently the same proteins were present in both the NP and E-RNA fractions since the same number and distribution of protein bands were detected in the isolated NP as were in the parent E-RNA fraction upon examination by disc electrophoresis. The potency of NP fractions as protective immunogen was evaluated against resistance induced in mice 2 to 3 weeks after immunizations with several samples of E-RNA or vaccination with *S. typhimurium* RIA. With 100-μg doses of protein, the NP sample containing less than 0.1% RNA was the poorest immunogen in that mice receiving it and subsequently challenged with virulent salmonellae exhibited a lesser survival rate (30%) than did mice receiving an NP preparation at the same dosage level of protein but with a 6% RNA content. This latter group of animals exhibited a 50% survival rate 35 days after injection of virulent salmonellae. The immunogenic efficacy of the NP as a protective immunogen was enhanced when 200-μg doses of protein were injected. This sample (NP III) contained approximately 22 μg of RNA and stimulated a 66.6% survival rate in mice to lethal infection by the virulent strain of salmonella. Even though all materials used in this study to immunize mice were contained in incomplete Freund's adjuvant, except the RIA vaccine, the presence of the RNA in the NP samples appeared to enhance their potency as protective immunogens. No NP fractions were able to stimulate resistance to challenge infections as well as did the E-RNA fractions (77.7–85% survival rates) or the living attenuated *S. typhimurium* RIA (83.9–89.5% survival rates) to the 1,000 LD_{50} doses of virulent salmonellae.

In an attempt to determine whether the nucleotides in the salmonella subfractions were unique in their combination with the protein components as immunogens, the effects of synthetic polynucleotides and a eukaryotic source of RNA (bovine liver sRNA) on the immunogenicity of the purest salmonella protein subfraction were examined. The most effective polynucleotide in enhancing the immunogenicity of NP was poly AU.* Mice immunized with a mixture of poly AU and NP (100 μg of protein) exhibited a 70% survival rate in contrast to mice immunized with the attenuated salmonella strain (RIA) which demonstrated an 83.3% survival rate to 1,000 LD_{50} doses of virulent salmonellae. The average amount of protein received by each mouse immunized with the most effective E-RNA preparation studied was 200 to 250 μg. Other combinations of NP fraction and synthetic polynucleotides or bovine liver sRNA imparted varying degrees of protection to challenge infection, whereas the NP fraction alone stimulated the poorest protection (30% survival to virulent salmonellae). These results are in contrast to those of Youmans and Youmans (22) who did not effect protective immunity in mice to challenge infection with virulent *Mycobacterium tuberculosis* after injection of synthetic polynucleotides mixed with varying amounts of ribosomal protein isolated from an attenuated strain of *M. tuberculosis*.

The nucleotide sequence AU has been shown by Braun et al. (2, 3) to function as an immunological adjuvant. Schmidtke and Johnson (13) have shown that the polynucleotide AU did not have to be injected by the same route or site as the antigen to exert the adjuvant effect on the host. Consequently, in this study mice were injected with poly AU at a different site from that used for injection of NP proteins. Three weeks later these mice were challenged with virulent salmonellae, and 60% were alive 35 days later. From these observations it is apparent that the polynucleotide AU does exert a considerable adjuvant effect on the mouse response to the NP fraction of *S. typhimurium* SR-11. Thus, it is of interest to raise the question: are the bacterial ribosomal and RNA-protein subfractions of certain bacteria effective protective immunogens because of the adjuvant effect exerted by the inherent complement of nucleotides in addition to containing antigens(s) of either inherent or adsorbed protein?

Since Collins (4) demonstrated that effective protective immunity to murine salmonellosis was transferrable with immune lymphoid cells and not sera, we were curious to determine whether this was also demonstrable with the highly immunogenic E-RNA subfractions of *S. typhimurium* SR-11. Peritoneal exudate and spleen cells from mice immunized with E-RNA fraction transferred host protection to normal mice slightly more effectively than did lymph node cells

* column 2, line 6: Insert the following statement: "In contrast to double-stranded polynucleotides used for their adjuvant effect by others, the polynucleotides in this study were single-stranded random copolymers of molecular weights in excess of 100,000."

in the limited number of animals studied. The ICR mice used are a non-inbred strain. However, if an allograft response on the part of the recipients contributed to survival of challenge infection, the mice receiving normal lymphoid cell populations should have exhibited signs of increased or transient protection. They did not. Sera from the same immune mice did not confer protection to normal mice but did impart transient protection for a short period of time in that the time interval before the mice succumbed to lethal salmonellosis was prolonged by 4 days as has previously been described by Collins (4) in passive transfer studies with sera from mice immunized with salmonella vaccines. Similar observations have also been made by Venneman and Berry (16, 17) in a non-inbred mouse host.

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