INFECTION AND IMMUNITY, Aug. 1975, p. 364-377

Vol. 12, No. 2
Printed in U.S.A.

Evidence for O Antigens as the Antigenic Determinants in "Ribosomal" Vaccines Prepared from *Salmonella*

TOBY K. EISENSTEIN

Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Received for publication 14 April 1975

The nature of the protective substance in ribosomal ribonucleic acid and protein extracts of *Salmonella* has been investigated. The results of experiments in which vaccines were prepared from isogenic strains and strains with defects in lipopolysaccharide synthesis show that O antigens contaminate both ribonucleic acid and protein ribosomal extracts, and are responsible for at least part of their strain-specific protective activity. In addition, it was observed that a ribosomal ribonucleic acid preparation from *S. adelaide* contains a heat-stable immunogen which is not an O antigen or that gives cross-protection across species lines. The contribution of ribosomes to the immunity induced by "ribosomal vaccines" is discussed.

Experiments in several laboratories have shown that vaccination with ribosome-rich extracts of species of *Salmonella* protects mice against subsequent experimental infection with live, virulent organisms of the same species (2, 7, 32). These preparations have been referred to as "ribosomal vaccines." At the present time work from different laboratories has led to conflicting conclusions as to the identity of the protective substance(s), which has yet to be definitively established. Using column chromatography, Venneman (31) purified a highly protective fraction of molecular weight greater than 300,000. By chemical analysis the fraction contained no detectable protein and appeared to be entirely ribonucleic acid (RNA), although he could not completely exclude the possibility of a contaminating polysaccharide. In contrast, Johnson (7, 8) has presented evidence that points to the protein fraction as the protective moiety. Smith and Bigley (28) also found that protein fractions had protective activity, but the protein was not as active as crude ribosomal extracts.

In this laboratory, we have considered the hypothesis that the protection is due to O antigens contaminating the ribosomal extract. This possibility is not unlikely, since it is well known that nucleic acids and lipopolysaccharides (LPS) have a strong affinity (24).

To investigate the contribution of O antigens to the observed immunity, we have used several genetically defined strains of *Salmonella* for preparation of ribosomal vaccines. Protection was assayed by the ability of immunized mice to suppress the multiplication of a challenge inoculum. One approach has been to use two isogenic strains. One is designated GO11, with O antigenic constitution O:9,12, and the other is GO24, with O antigens 4 and 12. Both strains are *his*$^+$ transductants derived from the same *S. enteritidis* parent strain (T. K. Eisenstein, E. P. Ornellas, R. J. Roantree, and J. P. Steward, Bacteriol. Proc., p. 94, 1970). They are known to differ from each other only in the region of the chromosome coding for O antigens 4 and 9. and in the ability to ferment galactose. It is assumed, therefore, that the ribosomal RNA and ribosomal proteins are the same in both strains. Thus, vaccines prepared from either of these strains should cross-protect against the other if the ribosomal components are responsible for the protection. If, however, O antigens are the immunogens, then the protection should be specific for the strain from which the vaccine was prepared.

A second approach to investigating the contribution of O antigens to the immunogenicity of these preparations has been to test the protective capacity of vaccines prepared from mutant strains of *Salmonella* that have known defects in LPS synthesis.

The results presented in this paper point strongly to the conclusion that O antigens are the antigens in RNA-rich extracts of the ribosomes. A preliminary report of some of these experiments was presented at the 71st Meeting of the American Society for Microbiology (T. K. Eisenstein, C. H. O'Keefe, and J. Kremer, Bacteriol. Proc., p. 112, 1971).

Since this work was initiated, Johnson (8) has also shown that RNA fractions of ribosomes contain O antigens and endotoxic activity. He found that purification of the RNA with phenol and guanidine hydrochloride removed the O antigens, and also the protective activity. Berry et al. (2), using gas chromatography, were able to detect O-specific sugars in purified RNA fractions. Thus, our results and those of Johnson and Berry et al. are in agreement that O antigens are present in RNA-rich extracts of ribosomes which have been used as vaccines.

To date, the nature of the protective substance in the protein fraction described by Johnson (8) has not been definitively identified. Results which are presented in this paper point to the conclusion that O antigens also contaminate the protein fraction, and can account for its observed protective capacity. These results raise the question as to whether any of the protection resulting from immunization with "ribosomal vaccines" is due to components other than LPS.

MATERIALS AND METHODS

Mice. Outbred mice from two suppliers were used: Swiss-Webster mice from Huntingdon Farms (Conshohocken, Pa.) and CD-1 mice from Charles River Mouse Farm (Wilmington, Mass.). All animals were females weighing 19 to 21 g. They were housed in plastic cages with Absorb-Dri as bedding. Purina Laboratory Chow and water were available ad libitum.

Bacterial strains. Two genetically defined strains of *Salmonella*, designated GO11 and GO24, were kindly supplied by Eugene Ornellas (Harvard University Medical School). They were prepared as transductants of *S. enteritidis*, strain R147, which is a one-step penicillin-resistant mutant of strain R134. Strain R134 is a genetically marked derivative, *his-1461*, *cys*$^-$, *gal*$^-$, that is smooth but serum sensitive and was obtained by successive mutagen treatments from a wild-type *S. enteritidis*, strain I203 (R. J. Roantree, personal communication). Ornellas used phage P22.L4, grown on an *S. typhimurium* LT2 line (of antigenic character O:4,5,12), to obtain *his*$^+$ transductants from *S. enteritidis* R147 (of antigenic character O:9,12). A minority of the *his*$^+$ transductants lacked O factor 9, as the result of replacement of the recipient *rfb* gene cluster by that of the donor co-transduced with *his*$^+$. These O:4,12 transductants did not have O factor 5—the gene determining this factor, i.e., the addition of an O-acetyl group to the abequose of the O:4 repeating unit, being too far from *his* to be co-transducible with it (29). Strain GO11, which is *S. enteritidis*, of character O:9,12,*his*$^+$,*cys*$^-$,*gal*$^-$, and GO24, which is *S. typhimurium*, of character O:4,12, *his*$^+$,*cys*$^-$,*gal*$^-$, were chosen as representatives of the two classes of *his*$^+$ transductants. These two strains are isogenic except for the *rfb* region of the chromosome. To permit their differentiation on plates, a spontaneous galactose-fermenting mutant was selected from the O9 strain, GO11. In this paper GO24 is designated GO24$^-$ to indicate it does not ferment galactose, and the O:9,12*gal*$^+$ transductant is designated GO11$^+$. Strains GO24$^-$ and GO11$^+$ are of about equal virulence (mean lethal dose [LD_{50}], intraperitoneal [i.p.] route, for CD-1 mice, ca. 5×10^4 bacteria).

The *S. typhimurium* strains used were provided by B. A. D. Stocker (Department of Medical Microbiology, Stanford University School of Medicine). Strain TA1659, isolated by Bruce Ames (Department of Biochemistry, University of California at Berkeley) from an *S. typhimurium* LT2 stock, has a deletion of the *gal* operon (involving also *uvrB*, *chl*, and *bio*). It shows phage sensitivity pattern Epi-1 typical of *Salmonella* making LPS of type Rc (33) due to the inability to manufacture the uridine 5'-diphosphate (UDP)-galactose precursor of the galactose units of the LPS core (Fig. 1). Its phage-sensitivity pattern is unaltered on medium supplemented with galactose, which indicates that it is unable to convert exogenous galactose to UDP-galactose, the expected result of a deletion of the whole of the *gal* gene cluster, including genes *galK* and *galT*, which specify enzymes needed for this conversation. Strain SL1694 is a derivative of the *gal* deletion mutant, TA1659, corrected of its galactose-negative character by acquisition of F'8-*gal*$^+$, which includes the wild-type form of the *gal* gene cluster of *Escherichia coli* K-12. (See references 11 and 12 for restoration of smooth character, virulence, etc., of a *galE* mutant of *S. typhimurium* by introduction of an F'*gal*$^+$ plasmid.) SL1102 is a rough mutant of class *rfaE*, making incomplete core lps of type Re, i.e., deficient in heptose (and sugars distal to it), derived from a genetically marked subline of *S. typhimurium*, strain LT2 (33). Strain SL4507 is a *galU* mutant of a genetically marked derivative, TV253 (11) of an *S. typhimurium* strain of type FIRN (20), which is deficient in UDP-glucose-pyrophosphorylase because of its inability to make the UDP-glucose, a precursor of the glucose of the LPS core. Thus, it is rough and makes LPS of type Rd (Fig. 1). Assay of extracts for UDP-glucose-pyrophosphorylase activity suggests that its enzymic defect is incomplete (X. Kuo, D. G. MacPhee, and B. A. D. Stocker, manuscript in preparation; B. A. D. Stocker, personal communication). Strain SL4522 (19), used for a challenge inoculum in one experiment (Table 1), is a smooth mouse-virulent, genetically marked derivative of an *S. typhimurium* strain of type FIRN. *S. adelaide* was obtained from a collection of clinical isolates kept by Catherine Dietz of Temple University, Department of Microbiology and Immunology.

Isolation of ribosomal fractions. Ribosomal fractions were prepared by a modification of the method of Youmans and Youmans (34, 35) or by the method of Fogel and Sypherd (3). For both extractions, organisms were grown in brain heart infusion broth (BBL) at 37 C, without shaking, and harvested. In the method of Youmons and Youmans, 25 g (wet weight) of cells were washed twice in buffer A (10^{-2} M phosphate. 10^{-4} M $MgCl_2$, pH 7.0), resuspended in 150 ml

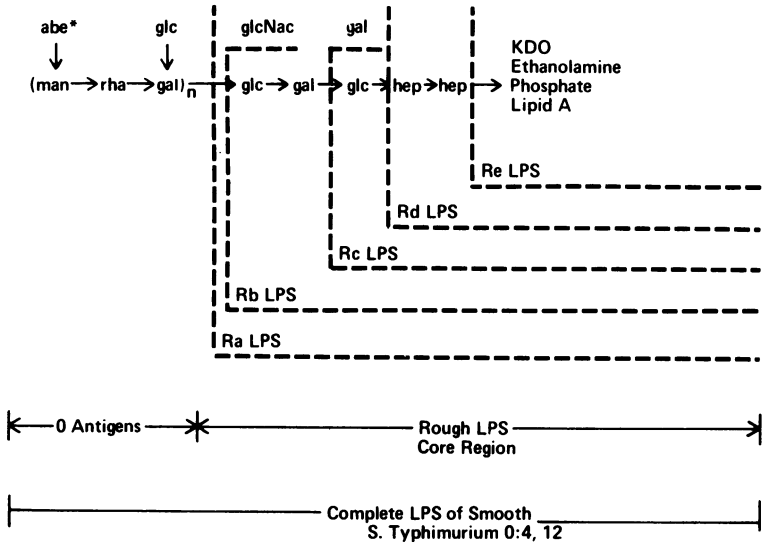

FIG. 1. *LPS structure of A. typhimurium. Abbreviations: Abe, abequose: man, mannose; rha, rhamnose; gal, galactose; glc, glucose; glcNac, N-acetyl glucosamine; KDO, 2-keto-3-deoxyoctonic acid; hep, heptose; n, number of repeating units of O side chain; *, substitute tyvelose for S. enteritidis O:9,12 LPS.*

of buffer B (10^{-4} M phosphate, 2×10^{-4} M $MgCl_2$, 0.44 M sucrose, pH 7.0), and placed in a Roset cooling cell immersed in a salt-ice bath. The cooled suspension was sonically treated using a Branson sonifier (model W185), set at 100 W for 7 min. The sonic extract was centrifuged twice at 27,000 × *g* for 20 min, and the top four-fifths of the supernatant from each spin was decanted. The last supernatant was spun at 37,000 × *g* for 20 min. The supernatant was again decanted and ultracentrifuged at 160,000 × *g* for 2.5 h in a Beckman model L2 centrifuge using a 50 Ti rotor. The pellets were obtained and resuspended in 50 ml of buffer A and an equal volume of 0.5% sodium lauryl sulfate. The solution was stirred slowly for 1 h and stored overnight at 5 C. The solution was spun at 37,000 × *g* for 20 min. The top four-fifths of the supernatant was given a second high-speed centrifugation at 160,000 × *g* for 3 h. The ribosomal pellets were resuspended in 75 ml of buffer A to which were added 2 volumes of 95% ethanol and approximately 70 drops of NaCl. The solution was stored overnight in the freezer and centrifuged at 3,200 × *g* for 15 min. This ethanol-precipitable fraction was used for immunization and was designated Y-RNA. It was stored at −20 C in sterile distilled water.

To prepare ribosomal fractions by the Fogel and Sypherd method (3), their procedure was followed except that cells were broken by sonic treatment as described above under the method of Youmans and Youmans. This extraction procedure yielded a ribosomal pellet, a portion of which was suspended in sterile water and stored at −20 C. Another portion was further extracted with 2-chloroethanol (3) to yield RNA and protein fractions. The RNA precipitate was dissolved in water and stored at −20 C. The protein supernatant was also stored at −20 C.

Immunization. Vaccines were administered in one dose intraperitoneally in a volume of 0.5 ml. Control mice received 0.5 ml of water or buffer. For ribosomes, and RNA extracts of ribosomes, the amount of vaccine was standardized by estimating its RNA content from the optical density at 260 nm (OD_{260}). A conversion factor calculated from *E. coli* base ratios equates one OD_{260} unit to 44 μg of RNA (14). Except where indicated, animals received 1 OD_{260} unit of vaccine. For injection of the protein fraction obtained in the Fogel and Sypherd procedure, the protein concentration was determined by the method of Lowry et al. (15) using bovine serum albumin (Sigma) as the standard.

Vaccines were sterilized by filtration through an 0.45-μm membrane filter (Millex; Millipore Corp.). Before injection they were tested for sterility by inoculating 0.1 ml of the preparation into thioglycolate broth and noting the absence of growth in 48 h. The protein fraction was not filtered, as it was found to be sterile on inoculation into thioglycolate broth, and filtration was found to markedly reduce the protein concentration.

Challenge procedure. Fourteen days after immunization, mice were given an intraperitoneal challenge with live salmonella. A lyophilized vial of the desired strain was rehydrated and inoculated into a 100-ml flask of brain heart infusion broth which was grown overnight. In the morning, 0.1 ml from this starter culture was transferred to a tube containing 10 ml of brain heart infusion broth. The culture was incubated until it reached late log phase, as determined by the OD at 640 nm in a Coleman 20 spectrophotometer (Bausch and Lomb). The organisms were then diluted to the desired concentration in saline. The cultures were kept on ice between standardization and injec-

TABLE 1. *Extent of multiplication of virulent S. typhimurium in mice vaccinated with Y-RNA extracts prepared from an S. typhimurium mutant making LPS of the Rc type, or from its smooth derivative*

Mouse immunized with:[a]	Mouse no.[b]	No. of SL4522 per mouse[c]	Median
Saline	1	5.6×10^5	
	2	5.9×10^5	
	3	2.6×10^6	
	4	4.4×10^6	
	5	6.2×10^7	
	6	8.4×10^7	
	7	1.1×10^8	
	8	7.9×10^8	1.65×10^7
Strain SL1694 (smooth) Y-RNA vaccine	1	$<1.0 \times 10^3$	
	2	1.0×10^3	
	3	1.0×10^3	
	4	1.0×10^3	
	5	3.5×10^3	
	6	6.0×10^3	
	7	1.4×10^4	1.0×10^3
Strain TA1659 (rough) Y-RNA vaccine	1	1.2×10^6	
	2	1.9×10^6	
	3	3.3×10^6	
	4	1.35×10^7	
	5	8.0×10^7	
	6	1.26×10^8	
	7	5.0×10^9	1.35×10^7

[a] Vaccine dose was 3.00 OD_{260} units.

[b] Mice were injected i.p. with 5.2×10^3 cells of strain SL4522 14 days after immunization.

[c] Five days postinfection.

tion. A standard curve was used to estimate the number of bacteria, and the actual number injected was always verified by plate counts.

In many experiments, a "double-challenge" procedure was used (Eisenstein et al., Bacteriol. Proc., p. 94, 1970). Mice received a mixed infection with about equal numbers of GO24$^-$ and GO11$^+$. For this procedure, the organisms were grown and standardized separately. Equal amounts of the two cultures were then mixed and diluted together to the desired final concentration. Animals received a single injection i.p. of 0.5 ml containing both organisms. Dilutions of the inoculum were plated on eosin-methylene blue agar (BBL) with galactose (1% wt/vol) incorporated into the medium. On this agar, colonies of the GO24$^-$ strain were uncolored; those of the GO11$^+$ strain were deep purple or green, due to the metabolism of galactose. Thus, the number of colonies produced by each strain could be determined on a single plate. From plate counts the challenge ratio of *gal*$^-$ to *gal*$^+$ organisms was determined.

Assay for protection. Five days after infection, mice were killed, and the total number of bacteria in each carcass was determined by the method of Berry (1). The skin and digestive tract were removed, and the carcass was homogenized in sterile, distilled water in a Waring Blendor (model 5010) for 3 min. Appropriate dilutions were plated on eosin-methylene blue agar plus 1% galactose as described above. By plating 0.1 ml of the undiluted homogenate, the smallest number of organisms detectable was 1,000. To distinguish the infecting strains from coliform contaminants, slide agglutinations, using typing sera (Lederle Diagnostics), were performed on questionable colonies. A record of the mortality in each group was also kept in most experiments for the 5-day period between infection and sacrifice to provide additional data about protection. Dying mice usually have greater than 1×10^9 organisms in their carcasses (1).

Assessment of specificity of protection. To quantitate the relative growth of the GO24$^-$ (O:4) and GO11$^+$ (O:9) strains in double-challenge experiments, a specificity ratio (SR) was defined as the ratio of *gal*$^-$ (O:4,12)/*gal*$^+$(O:9,12) organisms in the carcass. An average SR for each group of mice was determined.

Passive hemagglutination. To raise antisera, groups of 50 mice were injected i.p. three times at 7-day intervals. Each time, they received either 1 OD_{260} unit of Y-RNA vaccine prepared from GO24$^-$ or GO11$^+$, or 50 μg of LPS of *S. enteritidis* or *S. typhimurium* (Difco). Control serum was collected from animals similarly injected with water. One week after the last injection the animals were anesthetized with chloroform and bled out from the heart. Pooled sera were filtered, first through an 0.45-μm membrane filter (Millex; Millipore Corp.) and then through a 0.22-μm filter and stored at −20 C in small aliquots.

To test the antibody titers to O antigens induced by the vaccine, human group O erythrocytes were coated with LPS (Difco) of *S. enteritidis* (O:9,12) or *S. typhimurium* (O:1,4,5,12) using the method of Neter (22) as described by Nowotny (23). The LPS (2 mg/ml) was boiled for 2 h. One part of packed erythrocytes was added to five parts of LPS and incubated for 1 h at 37 C. The sensitized cells were washed three times with saline and diluted to a final concentration of 1% with saline. Dilutions of the sera were then made in microtiter plates with tapered walls (Microbiological Associates). Typing sera (Lederle Diagnostics) were used as the positive control, and appropriate controls using uncoated cells were always included. Serum (0.025 ml) was diluted with an equal volume of saline containing 1% bovine serum. Erythrocytes were added at a concentration of 1% in a volume of 0.025 ml. Plates were incubated at room temperature for 1 h and then overnight at 4 C. Wells were scored, from 4+ to 1+, and the titer was taken at the highest dilution giving a 3+ reaction. For absorption experiments, serum was mixed with an equal volume of packed erythrocytes coated with the appropriate LPS. The mixture was incubated at 37 C for 15 min and then centrifuged to remove the cells. The procedure was then repeated.

Statistics. A two-way analysis of variance was used to test the significance of differences in bacterial counts between different groups. Comparisons were made among means using Hartley's modification of the Newman-Keul method (5).

RESULTS

Protection by ribosomal vaccines of cell wall mutants. To test the hypothesis that O antigens contaminate the ribosomal vaccines and are responsible for their protective effect, vaccines were prepared from *Salmonella* mutants with known defects in LPS synthesis. It was reasoned that if ribosomal components were responsible for the observed protection, then vaccines prepared from mutant strains should still be protective. If, however, O antigens were responsible for the immunogenicity, then vaccines prepared from strains making LPS without O antigens should not protect. In the first experiment vaccines were prepared by the method of Youmans and Youmans from the *gal* deletion mutant TA1659, making type Rc LPS, and from its restored smooth derivative SL1694. As the LD_{50} of SL1694 was greater than 8×10^6 organisms it could not be used for challenge. Strain SL4522, a virulent *S. typhimurium* with the same O antigenic composition (O:4,5,12) as SL1694, was used instead. The results given in Table 1 show that the Y-RNA vaccine of the rough (*gal* deletion) strain did not protect, but the Y-RNA made from its smooth derivative provided excellent protection against challenge with another strain of the same serotype, as measured by the extent of bacterial multiplication 5 days after challenge. The same experimental design was used in the next experiment in which Y-RNA preparations from two other mutant strains, in addition to TA1659, were used. Strain SL4507 makes LPS of the Rd type, and strain SL1102 makes LPS of the Re type. For challenge, strain GO24$^-$, which has O antigens 4 and 12, was used. Table 2 shows that the growth of the infecting organisms was suppressed over 1,000-fold in animals which had been vaccinated with vaccine prepared from GO24$^-$, the homologous strain, as compared with controls. As in the previous experiment, the vaccine of the Rc strain did not protect to a significant extent. The Y-RNA vaccines of SL4507 and SL1102, the Rd and Re strains, both gave significant protection, although not as great as the GO24$^-$ vaccine. The deaths that occurred in each group were consistent with the degree of protection observed with carcass counts as the measure of immunity. The results do not show a correlation between the degree of roughness and loss of protection.

Protection by ribosomal vaccines of isogenic strains prepared by the method of Youmans and Youmans. A second approach to searching for O antigens in the ribosomal vaccine was to prepare vaccines from the isogenic strains GO11$^+$ and GO24$^-$, which are believed to be identical, except that GO24$^-$ has O antigen 4 and GO11$^+$ has O antigen 9. Since the transductants are haploid, these two antigens are mutually exclusive. Thus, if ribosomal components are responsible for the protection one would expect vaccines prepared from each of the strains to cross-protect against the other. Any specificity of protection would be evidence for the role of O antigens in the protective effect

TABLE 2. *Protection afforded by Y-RNA vaccines prepared from Salmonella strains making complete LPS or LPS with varying degrees of roughness*

Mouse immunized with:[a]	Mouse no.[b]	No. of GO24 per mouse[c]	No. dead 5 days post-challenge
Water	1	2.6×10^7	10/15
	2	8.6×10^8	
	3	$\boxed{1.2 \times 10^9}$[d]	
	4	1.7×10^9	
	5	2.2×10^9	
GO24$^-$ (O:4,12) Y-RNA vaccine	1	$<1.0 \times 10^3$	0/15
	2	1.0×10^4	
	3	$\boxed{1.6 \times 10^4}$	
	4	2.3×10^4	
	5	9.0×10^4	
TA1659 (Rc LPS) Y-RNA vaccine	1	3.2×10^6	5/15
	2	7.1×10^7	
	3	$\boxed{9.7 \times 10^7}$	
	4	$>2.5 \times 10^8$	
	5	9.8×10^8	
SL4507 (Rd LPS) Y-RNA vaccine	1	3.0×10^3	0/15
	2	2.4×10^4	
	3	6.2×10^4	
	4	$\boxed{9.8 \times 10^4}$	
	5	4.1×10^5	
	6	6.0×10^5	
	7	1.4×10^6	
SL1102 (Re LPS) Y-RNA vaccine	1	4.5×10^5	2/15
	2	5.1×10^5	
	3	1.0×10^6	
	4	$\boxed{2.5 \times 10^6}$	
	5	1.4×10^7	
	6	1.7×10^7	
	7	4.2×10^7	

[a] Vaccine dose was 1.00 OD_{260}.

[b] Groups of 15 mice were injected i.p. with 7.5×10^5 cells of GO24$^-$ 14 days after immunization.

[c] Five days postinfection: Rc versus water, not significant; Re versus water, $P < 0.005$; Rc versus Re, $P < 0.025$; Rc versus Rd, $P < 0.005$; Re versus 24$^-$, $P < 0.005$; Rd versus 24$^-$, $P < 0.05$.

[d] Numbers in boxes are median values.

of ribosomal vaccines. Groups of mice were immunized i.p. with Y-RNA vaccine prepared from each of these strains, and also with a similar vaccine prepared from *S. adelaide* (O:35), an organism which has no O antigens in common with GO11$^+$ or GO24$^-$. Fourteen days later all animals were double challenged with GO24$^-$ and GO11$^+$ in a ratio (−/+) of 0.66. As shown in Table 3, the two strains grew to approximately equal numbers in control animals to give an average SR of 0.83. However, mice which received the vaccine prepared from GO24$^-$ (O:4,12) had an SR of 0.5, which represents greater than a 20-fold suppression of multiplication of the strain against which they were vaccinated relative to that of strain

TABLE 3. *Results of a double challenge of mice vaccinated with Y-RNA extracted from isogenic strains GO24$^-$ and GO11$^+$ and from S. adelaide*

Mouse immunized with:[a]	Mouse no.[b]	No. of bacteria cultured from carcass on day 5 postinfection			Sp ratio (GO24$^-$/GO11$^+$)[c]	Avg sp ratio
		GO11$^+$	GO24$^-$	Total no. of bacteria		
Saline	1	1.7×10^{5}[d]	1.8×10^{5}[e]	3.5×10^{5}	1.06	
	2	2.6×10^{5}	1.4×10^{5}	4.0×10^{5}	0.53	
	3	4.3×10^{5}	3.8×10^{5}	8.1×10^{5}	0.88	
	4	$\boxed{1.9 \times 10^{6}}$[f]	$\boxed{9.5 \times 10^{5}}$	$\boxed{2.9 \times 10^{6}}$	0.48	
	5	1.8×10^{6}	1.9×10^{6}	3.7×10^{6}	1.07	
	6	3.2×10^{6}	4.3×10^{6}	7.5×10^{6}	1.34	
	7	4.9×10^{7}	2.3×10^{7}	7.2×10^{7}	.47	0.83
S. adelaide (O:35) Y-RNA vaccine	1	2.0×10^{3}	$<1.0 \times 10^{3}$	$<2.0 \times 10^{3}$	<0.50	
	2	3.2×10^{4}	1.0×10^{3}	3.3×10^{4}	0.03	
	3	7.0×10^{4}	2.4×10^{4}	9.4×10^{4}	0.34	
	4	$\boxed{6.8 \times 10^{4}}$	$\boxed{3.8 \times 10^{4}}$	$\boxed{1.1 \times 10^{5}}$	0.56	
	5	6.7×10^{5}	1.0×10^{4}	6.8×10^{5}	0.01	
	6	5.1×10^{5}	2.7×10^{5}	7.8×10^{5}	0.53	
	7	3.7×10^{8}	5.0×10^{7}	4.2×10^{8}	0.13	0.30
GO24$^-$ (O:4,12) Y-RNA vaccine	1	6.9×10^{4}	$<1.0 \times 10^{3}$	$<6.9 \times 10^{4}$	0.015	
	2	1.0×10^{3}	$<1.0 \times 10^{3}$	$<1.0 \times 10^{3}$	(<1.00)[g]	
	3	9.0×10^{3}	$<1.0 \times 10^{3}$	$<9.0 \times 10^{3}$	<0.11	
	4	$\boxed{1.4 \times 10^{4}}$	$\boxed{1.0 \times 10^{3}}$	$\boxed{1.5 \times 10^{4}}$	0.07	
	5	$<1.0 \times 10^{3}$	2.0×10^{3}	$<2.0 \times 10^{3}$	(>0.50)	
	6	1.8×10^{6}	7.0×10^{3}	1.8×10^{6}	0.004	
	7	3.5×10^{5}	8.0×10^{3}	3.6×10^{5}	0.02	0.05
GO11$^+$ (O:9,12) Y-RNA vaccine	1	$<1.0 \times 10^{3}$	4.0×10^{3}	$<4.0 \times 10^{3}$	>4.00	
	2	$<1.0 \times 10^{3}$	1.0×10^{3}	$<1.0 \times 10^{3}$	(>1.00)	
	3	2.0×10^{3}	1.2×10^{4}	1.4×10^{4}	6.00	
	4	$\boxed{1.0 \times 10^{4}}$	$\boxed{4.0 \times 10^{5}}$	$\boxed{4.1 \times 10^{5}}$	40.00	
	5	5.0×10^{4}	2.1×10^{5}	2.6×10^{5}	4.20	
	6	1.1×10^{5}	1.4×10^{5}	2.5×10^{5}	1.24	
	7	5.7×10^{5}	2.3×10^{5}	8.0×10^{5}	0.40	9.31

[a] Vaccine dose was 1.5 OD_{260} units.

[b] Mice injected i.p. 14 days after immunization with 5.0×10^{5} GO24$^-$ and 7.7×10^{5} GO11$^+$. Challenge ratio GO24$^-$/GO11$^+$ = 0.66.

[c] Compare SR of saline versus *S. adelaide*, $P < 0.05$; saline versus GO24$^-$, $P < 0.01$; saline versus GO11$^+$, $P < 0.01$.

[d] Compare median GO11$^+$ counts, saline versus *S. adelaide*, not significant- saline versus GO24$^-$, $P < 0.05$; saline versus GO11$^+$, $P < 0.05$.

[e] Compare medium GO24$^-$ counts, saline versus *S. adelaide*, $P < 0.05$; saline versus GO24$^-$, $P < 0.05$; saline versus GO11$^+$, $P < 0.05$.

[f] Numbers in boxes are median values.

[g] Numbers in parentheses are not included in the average.

GO11+, which is identical except for the O:9 antigen and the galactose marker. Animals which received the GO11+ (O:9,12) Y-RNA vaccine also showed a significant reduction in total counts and a significant suppression of the GO11+ strain to give an SR of 9.31, an 11-fold suppression of the strain homologous to that of the vaccine preparation.

In addition to the strain-specific effect, mice vaccinated with GO24- Y-RNA or GO11+ Y-RNA suppressed growth of the heterologous organism in the challenge inoculum. Thus, it appears that these two vaccines each contain a protective substance which induces specific protection for the homologous organism and a cross-protective substance. Interestingly, mice receiving the *S. adelaide* vaccine also significantly suppressed the growth of both challenge strains.

Thirty-day survival of vaccinated mice. To show that the bacterial count assay correlates with survival, vaccinated and control mice were challenged with two doses of GO24- and deaths were recorded for 30 days. Table 4 shows that mice which received 1 OD_{260} unit of GO24- Y-RNA vaccine were well protected against both challenge doses. The higher challenge dose is greater than the inoculum used in any of the experiments where protection was assayed by carcass counts.

Antibody to ribosomal vaccines. To further substantiate the presence of O antigens in the Y-RNA vaccines prepared from the isogenic strains, groups of mice were injected with these preparations. Controls received either O:4,5,12 or O:9,12 LPS. Sera were titrated against erythrocytes coated with the 9,12 or 4,5,12 LPS. Mice given either the GO11+ (O:9,12) or GO24- (O:4,12) ribosomal Y-RNA vaccine developed antibodies reactive with the homologous LPS (Table 5). It is interesting that both the 9,12 and 4,5,12 LPS induced substantial specific antibody, and little cross-reactive antibody, in spite of the 12 antigen in common. The sera raised against the 9,12 ribosomal vaccine is also specific for 9,12 LPS as shown by the failure of 4,5,12 LPS-coated cells to absorb out the antibody. In contrast, the anti-4,12 ribosomal serum shows low titers against the homologous LPS and significant cross-reactivity against the heterologous LPS. Furthermore, the titer to the homologous and heterologous LPS is absorbed out by the heterologous 9,12 LPS. Thus the antibody studies confirm that O antigens are present in the ribosomal preparations, but there is no correlation between the pattern of specificity in the antibody response and the specificity of protection induced by the vaccine.

TABLE 4. *Thirty-day survival of control and vaccinated mice*

Mouse immunized with:	GO24- challenge dose[a]	Death/ total[b]
GO24- Y-RNA vaccine[c]	4.3×10^5	0/15
Water	4.3×10^5	13/15
GO24- Y-RNA vaccine	2.6×10^6	0/15
Water	2.6×10^6	14/15

[a] Mice challenged i.p. 14 days after vaccination.
[b] Mortality 30 days postchallenge.
[c] Vaccine dose was 1 OD_{260} unit i.p.

Heat resistance of ribosomal vaccines. The preceding double-challenge experiments indicated that there were two immunogens in the GO24- Y-RNA vaccine, one that was specific, presumably the O:4 antigen, and one that was cross-protective. The cross-protective antigen could be O:12 or it could be some other antigen which the isogenic strains have in common, in their cell walls or in their ribosomes. One of the distinguishing features of LPS is its heat stability (10). Accordingly, the effect of heat on the protective capacity of the GO24- (O:4,12) vaccine was examined. Boiling for 30 min did not alter the homologous or the heterologous protective capacity of the preparation (Table 6). A similar experiment was performed on *S. adelaide* ribosomal vaccine. As shown in Table 7, boiling had no effect on the level of protection it induced.

Protection by ribosomal fractions prepared by the method of Fogel and Sypherd. In the experiment shown in Table 8, mice were immunized with 3.0 OD_{260} units of FS-RNA prepared from GO11+ (O:9,12) or GO24+ (O:4,12). It is evident that the GO24- and GO11+ vaccines induced greater specific protection against the homologous isogenic strain than against the heterologous one, in a manner similar to that seen with Y-RNA vaccines (see Table 3). A comparison of the average SR of saline-injected animals and of mice vaccinated with the GO24- vaccine shows that there was a 15-fold decrease in the ratio in the vaccinated animals. Similarly, mice vaccinated with the GO11+ vaccine had a 39-fold increase in SR as compared with controls. These results show that ribosomal RNA extracts prepared from the isogenic strains by two different methods yield similar data.

Table 9 shows the results of an experiment in which a second batch of ribosomes was prepared from strain GO24+ (O:4,12), by the Fogel and Sypherd method. Groups of mice were immunized with the whole ribosomes, or with the protein or RNA fractions obtained by treatment

TABLE 5. *Titration of anti-O antibody in pooled sera from mice vaccinated with Y-RNA vaccines or with LPS*

Pooled sera from 50 mice immunized with:	Antigenic specificity of LPS on erythrocytes	Hemagglutination titer			
		Unabsorbed serum		Serum absorbed with:	
		Expt 1	Expt 2	*S. typhimurium* LPS (O:1,4,5, 12) (expt 2)	*S. enteriditis* (O:9,12) (expt 2)
4, 12 Y-RNA	1, 4, 5, 12	1:32	1:16	1:8	1:4
4, 12 Y-RNA	9, 12	1:32	1:32	<1:4	<1:4
9, 12 Y-RNA	1, 4, 5, 12	1:32	<1:4	<1:4	<1:4
9, 12 Y-RNA	9, 12	1:128	1:128	1:128	1:4
1, 4, 5, 12 LPS	1, 4, 5, 12	1:128	1:128	ND[a]	ND
1, 4, 5, 12 LPS	9, 12	1:32	1:16	ND	ND
9, 12 LPS	1, 4, 5, 12	1:4	<1:4	ND	ND
9, 12 LPS	9, 12	1:256	1:512	ND	ND
Water	1, 4, 5, 12	<1:4	<1:4	ND	ND
Water	9, 12	<1:4	<1:4	ND	ND
anti-1, 4, 5, 12[b]	1, 4, 5, 12	1:512	1:512	ND	ND
anti-1, 4, 5, 12[b]	9, 12	<1:4	<1:4	ND	ND
anti-9, 12[b]	9, 12	1:512	1:512	ND	ND
anti-9, 12[b]	9, 12	1:4	1:4	ND	ND

[a] ND, not determined.
[b] Typing sera specificity.

of the ribosome fraction with 2-chloroethanol. A comparison of these results with those in Table 8 shows that the FS-RNA fraction, at a dose of 1 OD_{260} unit, gives similar results to those obtained with this vaccine at 3.0 OD_{260} units (Table 8). The overall level of protection is slightly lower, but the specific protection indices are about the same as seen with the higher dose. A new and important observation is that the protein fraction, like the RNA fractions, results in highly significant specificity of protection, in addition to some nonspecific protection.

DISCUSSION

Mice immunized with salmonella ribosomes, or ribosomal subfractions, show high levels of immunity to subsequent challenge with living salmonellae. The present studies have tested the hypothesis that the protective activity of these fractions is due to O antigens which contaminate these preparations.

With regard to the RNA-rich fractions, four lines of evidence support the conclusion that O antigens are present in the vaccine and are responsible for at least part of their observed protective capacity. (i) A vaccine prepared from a deletion mutant which lacks O antigen does not protect, but a vaccine prepared from its smooth derivative gives excellent protection; (ii) the protection afforded by vaccines prepared from isogenic strains shows specificity; (iii) O antibodies are present in antisera raised to RNA-rich vaccines; and (iv) the immunogenicity of the vaccines is not affected by boiling for 30 min, a finding which is consistent with the well-known heat stability of O antigens.

With regard to the protein extract of the ribosomes, the evidence from the experiments using isogenic strains suggests that O antigens are also present in this fraction and account for at least part of its protective capacity.

The use of the isogenic strains in conjunction with the double-challenge technique has been extremely useful in providing insight into the nature of the vaccine immunogen. We have found that RNA and protein "ribosomal" fractions induce a strain-specific protection. Since these strains are isogenic, and are expected to be identical except for their O antigens, at least part of the protective activity of these fractions must be attributed to contamination with O antigens. The only other explanation for the observed specificity would be that these two strains differ also in their ribosomal RNAs or proteins. Although no rigorous proof is available for the assumption that the ribosomal RNA and

TABLE 6. *Effect of heat on the protective activity of GO24⁻ Y-RNA vaccine*

Mouse immunized with:[a]	Mouse no.[b]	No. of bacteria cultured from carcass on day 5 postinfection			Sp ratio[c] (GO24⁻/GO11⁺)	Avg sp ratio
		GO11⁺	GO24⁻	Total no. of bacteria		
Water	1	7.0×10^5 [d]	8.0×10^5 [e]	1.5×10^6	1.1	
	2	9.0×10^5	1.9×10^6	2.8×10^6	2.1	
	3	$\boxed{5.9 \times 10^6}$ [f]	$\boxed{7.3 \times 10^6}$	$\boxed{1.3 \times 10^7}$	1.2	
	4	7.4×10^7	1.0×10^8	1.8×10^8	1.3	
	5	3.9×10^8	9.8×10^8	1.4×10^9	2.5	1.6
GO24⁻ (O:4,12) Y-RNA vaccine held at 0 C for 30 min	1	3.2×10^4	8.0×10^3	4.0×10^4	0.25	
	2	6.0×10^4	2.3×10^4	8.3×10^4	0.38	
	3	$\boxed{1.0 \times 10^5}$	$\boxed{3.0 \times 10^4}$	$\boxed{1.4 \times 10^5}$	0.28	
	4	3.3×10^5	4.0×10^4	3.7×10^5	0.12	
	5	7.4×10^5	2.0×10^4	7.6×10^5	0.02	0.21
GO24⁻ (O:4,12) Y-RNA vaccine held at 25 C for 30 min	1	2.0×10^3	$<1.0 \times 10^3$	$<2.0 \times 10^3$	(<0.50)[g]	
	2	3.0×10^3	$<1.0 \times 10^3$	$<3.0 \times 10^3$	(<0.33)	
	3	2.0×10^4	1.0×10^4	3.0×10^4	0.50	
	4	2.8×10^4	7.0×10^3	3.5×10^4	0.25	
	5	4.0×10^4	1.1×10^4	5.1×10^4	0.27	
	6	2.2×10^5	5.0×10^4	2.7×10^5	0.22	0.31
		$\boxed{2.4 \times 10^4}$	$\boxed{1.0 \times 10^4}$	$\boxed{4.2 \times 10^4}$		
GO24⁻ (O:4,12) Y-RNA vaccine boiled for 30 min	1	1.0×10^4	$<1.0 \times 10^3$	$<1.0 \times 10^4$	(<0.10)	
	2	4.3×10^4	3.0×10^3	4.6×10^4	0.06	
	3	$\boxed{1.4 \times 10^5}$	$\boxed{5.0 \times 10^3}$	$\boxed{1.5 \times 10^5}$	0.03	
	4	1.2×10^5	1.0×10^5	2.2×10^5	0.83	
	5	4.5×10^6	8.0×10^4	4.6×10^6	0.02	0.24

[a] Vaccine dose was 3.00 OD_{260} units.

[b] On day 14 after immunization mice were infected with 5.0×10^5 cells of strain GO24⁻ (O:4,12) and 6.5×10^5 cells of GO11⁺ (O:9,12). Challenge ratio GO24⁻/GO11⁺ = 0.77.

[c] Compare SR of saline versus 0 C Y-RNA, $P < 0.005$; saline versus 25 C, $P < 0.005$; saline versus boil, $P < 0.005$.

[d] Compare median GO11⁺ counts: saline versus O C Y-RNA, $P < 0.05$; saline versus 25 C Y-RNA, $P < 0.05$; saline versus boil Y-RNA, $P < 0.05$; 0 C RNA versus boil RNA, not significant.

[e] Compare median GO24⁻ counts: saline versus 0 C RNA, $P < 0.05$; saline versus 25 C Y-RNA, $P < 0.05$; saline versus boiled RNA, $P < 0.05$.

[f] Numbers in boxes are median values.

[g] Numbers in parentheses are not included in the average.

proteins in the two strains are the same, it is known that no more than 1% of the chromosome is transferred during transduction, which in this case was confined to the loci which code for *his* and the O antigens. These loci map at 65 min on the salmonella chromosome (27). The genes coding for ribosomal RNAs and protein that have been mapped are located far enough from this region to make it unlikely that they were directly affected by the transduction (25, 27).

With regard to the vaccines prepared from strains of *Salmonella* with deficiencies in lipopolysaccharide synthesis, the degree of protection they induce seems to correlate with their degree of "leakiness" rather than with the depth of the lesion in the cell walls, since the vaccine of TA1659, the Rc mutant, clearly did not protect, whereas the vaccines of the rougher Rd and Re mutants were protective. One explanation for these results may be found in the fact that strain TA1659 is a deletion mutant for the whole *gal* operon, which means that it cannot make even small quantities of O-specific material. The distal O sugars are completely absent, which correlates with the observation that extracts of it give no protection. Strain SL4507 has only a partial deficiency of UDP-glucose pyrophosphorylase (Stocker, personal communication), which means it probably can synthesize a small amount of O-antigenic materials, which

TABLE 7. *Effect of heat on the protective activity of S. adelaide Y-RNA vaccine*

Mouse immunized with:[a]	Mouse no.[b]	No. of bacteria cultured from carcass on day 5 postinfection			Sp ratio[c] ($GO24^-/GO11^+$)	Avg sp ratio
		$GO11^+$	$GO24^-$	Total no. of bacteria		
Water	1	3.2×10^{6}[d]	1.1×10^{6}[e]	4.3×10^{6}	0.35	
	2	1.1×10^{7}	7.6×10^{6}	1.8×10^{7}	0.69	
	3	1.8×10^{7}	1.6×10^{7}	3.4×10^{7}	0.86	
	4	2.1×10^{7}	1.3×10^{7}	3.4×10^{7}	0.61	
	5	1.6×10^{9}	9.0×10^{8}	2.5×10^{9}	0.56	
	6	3.4×10^{9}	1.8×10^{9}	5.2×10^{9}	0.52	0.605
		$\boxed{1.9 \times 10^{7}}$[f]	$\boxed{1.4 \times 10^{7}}$	$\boxed{3.4 \times 10^{7}}$		
S. adelaide Y-RNA vaccine held at 0 C for 30 min	1	$<1.0 \times 10^{3}$	$<1.0 \times 10^{3}$	$<1.0 \times 10^{3}$	(—)	
	2	6.0×10^{3}	6.0×10^{3}	1.2×10^{4}	1.00	
	3	8.0×10^{3}	4.0×10^{4}	4.8×10^{4}	5.00	
	4	$\boxed{4.2 \times 10^{4}}$	$\boxed{3.4 \times 10^{4}}$	$\boxed{7.6 \times 10^{4}}$	0.82	
	5	1.3×10^{5}	9.0×10^{4}	2.2×10^{5}	0.69	
	6	1.5×10^{5}	5.6×10^{4}	2.1×10^{5}	0.37	1.57
S. adelaide Y-RNA vaccine held at 25 C for 30 min	1	6.0×10^{3}	3.0×10^{3}	9.0×10^{3}	0.50	
	2	1.0×10^{4}	4.0×10^{4}	5.0×10^{4}	4.00	
	3	4.7×10^{4}	3.2×10^{4}	7.9×10^{4}	0.68	
	4	1.4×10^{5}	1.4×10^{5}	2.8×10^{5}	1.00	
	5	1.1×10^{6}	3.0×10^{4}	1.1×10^{6}	0.03	
	6	9.4×10^{5}	4.1×10^{5}	1.4×10^{6}	0.43	0.681
		$\boxed{8.1 \times 10^{4}}$	$\boxed{3.5 \times 10^{4}}$	$\boxed{1.5 \times 10^{5}}$		
S. adelaide Y-RNA vaccine boiled for 30 min	1	4.5×10^{4}	5.9×10^{4}	9.1×10^{4}	1.31	
	2	1.0×10^{5}	2.0×10^{4}	1.2×10^{5}	0.20	
	3	$\boxed{2.8 \times 10^{5}}$	$\boxed{1.1 \times 10^{5}}$	$\boxed{3.9 \times 10^{5}}$	0.39	
	4	4.0×10^{5}	2.0×10^{5}	6.0×10^{5}	0.50	
	5	3.2×10^{6}	2.0×10^{6}	5.2×10^{6}	0.62	0.604

[a] Vaccine dose was 3.00 OD_{260} units.

[b] On day 14 after immunization mice were infected with 1.0×10^{6} cells of strain $GO11^+$ and 1.0×10^{6} cells of $GO24^-$. Challenge ratio $GO24^-/GO11^+ = 1.0$.

[c] Compare SR of saline versus 0 C Y-RNA, not significant.

[d] Compare median $GO11^+$ counts: water versus Y-RNA at 0 or 25 C or boiled, $P < 0.01$; 0 C Y-RNA versus 25 C RNA or versus boiled RNA, not significant.

[e] Compare median $GO24^-$ counts: water versus Y-RNA at 0 or 25 C or boiled, $P < 0.01$; 0 C Y-RNA versus 25 C RNA versus boiled, not significant.

[f] Numbers in boxes are median values.

could account for the protection induced by its ribosomal extract. Strain SL1102 tends to accumulate smooth revertants (Stocker, personal communication), and may be slightly leaky (29). This might account for the moderate protective capacity of the ribosomal vaccine prepared from it.

The results and conclusions presented here concerning O antigens in the RNA-rich ribosomal fractions are in agreement with those of other investigators (2, 7). However, our interpretation of the species-specific protection of the protein fraction as being due to the presence of O antigens is not the interpretation of other laboratories. Johnson has suggested that unique ribosomal proteins are responsible for the immunogenicity and species specificity of this fraction (8). Smith and Bigley (28) interpreted their results to mean that the active immunogens in their subcellular preparations were proteins absorbed to RNA which they thought was acting as an adjuvant. They searched for O antigens in their preparations by a micro-gel diffusion test with commercial antisera and found reactivity to antigen 4. However, the results were discounted as an artifact, since antisera against other O antigens gave negative reactions. They also found no mitogenically

TABLE 8. *Results of a double challenge of mice vaccinated with FS-RNA extracted from isogenic strains GO24⁻ and GO11⁺*

Mouse immunized with:[a]	Mouse no.[b]	No. of bacteria cultured from carcass on day 5 postinfection			Sp ratio[c] (GO24⁻/GO11⁺)	Avg sp ratio
		GO11⁺	GO24⁻	Total no. of bacteria		
Tris	1	1.6×10^5[d]	2.4×10^5[e]	4.0×10^5	1.5	
	2	4.4×10^5	7.2×10^5	1.2×10^6	1.6	
	3	8.8×10^5	1.4×10^6	2.3×10^6	1.6	
	4	6.3×10^6	1.1×10^7	1.7×10^7	1.7	
	5	7.1×10^6	2.5×10^7	3.2×10^7	3.5	
	6	9.6×10^7	6.8×10^7	1.6×10^8	0.71	1.85
		[2.4×10^6][f]	[3.9×10^6]	[6.3×10^6]		
GO24⁻ (O:4,12) FS-RNA vaccine	1	7.0×10^3	$<1.0 \times 10^3$	$<7.0 \times 10^3$	(<0.14)[g]	
	2	1.7×10^4	$<1.0 \times 10^3$	$<1.7 \times 10^4$	(<0.06)	
	3	4.5×10^4	3.0×10^3	4.8×10^4	0.06	
	4	[5.1×10^4]	8.0×10^3	[5.9×10^4]	0.16	
	5	1.0×10^5	[7.0×10^3]	1.1×10^5	0.07	
	6	1.4×10^5	1.8×10^4	1.6×10^5	0.13	
	7	3.0×10^5	5.3×10^4	3.5×10^5	0.18	0.12
GO11⁺ (O:9,12) FS-RNA vaccine	1	1.0×10^4	7.0×10^3	1.7×10^4	0.7	
	2	$<1.0 \times 10^3$	6.0×10^4	$>6.0 \times 10^4$	(>60.0)	
	3	1.0×10^3	6.6×10^4	6.7×10^4	66.0	
	4	1.0×10^3	[1.1×10^5]	[1.1×10^5]	110.0	
	5	[2.0×10^3]	3.1×10^5	3.1×10^5	155.0	
	6	2.0×10^4	2.0×10^6	2.0×10^6	100.0	
	7	3.8×10^6	1.7×10^7	2.1×10^7	4.5	70.89

[a] Vaccine dose was 3.00 OD_{260}. Tris, Tris(hydroxymethyl)aminomethane.

[b] On day 14 after immunization mice were injected with 0.85×10^4 cells of strain GO24⁻ and 1.55×10^4 cells of strain GO11⁺. Challenge ratio GO24⁻/GO11⁺ = 0.55.

[c] Compare SR of Tris versus GO24⁻ FS-RNA, $P < 0.05$; Tris versus GO11⁺, $P < 0.05$.

[d] Compare median GO11⁺ counts: Tris versus GO24⁻ FS-RNA, $P < 0.05$; Tris versus GO11⁺ FS-RNA, $P < 0.05$.

[e] Compare median GO24⁻ counts: Tris versus GO24⁻ FS-RNA, $P < 0.05$; Tris versus GO24⁻ FS-RNA, $P < 0.05$.

[f] Numbers in boxes are median values.

[g] Numbers in parentheses are not included in the average.

active endotoxin in any of their preparations. These experiments were taken as evidence of the absence of LPS in their fractions. Johnson also looked for LPS contamination of his protein fraction but failed to find any with immunodiffusion and the Limulus-lysate coagulation test. Failure to detect O antigens by immunodiffusion could be due to the relative insensitivity of the immunodiffusion test. It should be noted also that failure to detect endotoxic activity does not exclude the presence of O antigens. The endotoxin activity of LPS is attributable to the lipid (9, 30), whereas the serological specificity is associated with the O sugars (see Fig. 1). It is possible that haptenic O antigenic groups such as those termed "native hapten" (26), which show no endotoxic activity but are still immunogenic, can complex with the ribosomal proteins in the extraction process.

In addition to the specific protection induced by the ribosomal vaccines, double-challenge experiments with the isogenic strains revealed significant cross-protection. The antibody studies indicate that at least part of this cross-protection could be due to the humoral response to the 12 antigen which the two strains have in common. However, the experiments using *S. adelaide* ribosomal vaccines show that in addition to the O antigens, there is another immunogenic determinant in these preparations which induces a protective response. The inhibition of multiplication of both isogenic strains, GO11⁺ and GO24⁻; in mice immunized with *S. adelaide* Y-RNA vaccine indicates that the antigen

TABLE 9. *Results of a double-challenge experiment in which mice were immunized with ribosomes, ribosomal RNA, or ribosomal protein of strain GO24⁻*[a]

Mouse immunized with:	Mouse no.[b]	No. of bacteria cultured from carcass on day 5 postinfection			Sp ratio[c] (GO24⁻/GO11⁺)	Avg sp ratio
		GO11⁺	GO24⁻	Total no. of bacteria		
Water	1	2.0×10^8 [d]	4.8×10^7 [e]	2.4×10^8	0.24	
	2	1.0×10^9	8.3×10^7	1.1×10^9	0.08	
	3	$\boxed{1.5 \times 10^9}$ [f]	$\boxed{1.9 \times 10^9}$	$\boxed{3.4 \times 10^9}$	1.24	
	4	3.8×10^9	1.6×10^9	5.5×10^9	0.42	
	5	4.1×10^9	4.2×10^9	8.4×10^9	1.04	0.60
GO24⁻ ribosome vaccine[g]	1	1.3×10^4	$<1.0 \times 10^3$	1.4×10^4	<0.08	
	2	2.1×10^4	2.0×10^3	2.3×10^4	0.09	
	3	$\boxed{2.4 \times 10^4}$	$\boxed{3.0 \times 10^3}$	$\boxed{2.7 \times 10^4}$	0.13	
	4	2.5×10^4	4.0×10^3	2.9×10^4	0.16	
	5	4.2×10^5	1.1×10^3	4.2×10^5	0.002	0.092
GO24⁻ FS-RNA vaccine[h]	1	6.2×10^5	4.0×10^4	6.6×10^5	0.06	
	2	6.8×10^5	3.8×10^4	7.2×10^5	0.06	
	3	$\boxed{1.1 \times 10^6}$	$\boxed{4.0 \times 10^4}$	$\boxed{1.1 \times 10^6}$	0.04	
	4	2.8×10^6	$<1.0 \times 10^4$	2.8×10^6	(<0.004)[i]	
	5	6.4×10^6	6.0×10^4	6.5×10^6	0.009	0.034
GO24⁻ protein vaccine (50 μg)	1	3.5×10^5	2.0×10^3	3.5×10^5	0.006	
	2	1.1×10^6	9.0×10^3	1.1×10^6	0.008	
	3	$\boxed{1.2 \times 10^6}$	$\boxed{1.0 \times 10^4}$	$\boxed{1.2 \times 10^6}$	0.008	
	4	3.2×10^7	7.0×10^4	3.2×10^7	0.002	
	5	9.8×10^6	1.6×10^6	1.1×10^7	0.163	0.037
GO24⁻ protein vaccine (100 μg)	1	1.0×10^5	3.0×10^4	1.3×10^5	0.300	
	2	8.0×10^5	1.0×10^4	8.1×10^5	0.013	
	3	1.9×10^6	1.0×10^4	1.9×10^6	0.005	
	4	3.6×10^6	1.6×10^5	3.7×10^6	0.044	0.091
		$\boxed{1.2 \times 10^6}$	$\boxed{1.0 \times 10^4}$	$\boxed{1.2 \times 10^6}$		

[a] Prepared by the method of Fogel and Sypherd (3).

[b] On day 14 after immunization mice were injected with 3.9×10^5 GO24⁻ and 1.75×10^6 GO11⁺. Challenge ratio GO24⁻/GO11⁺ = 0.22.

[c] Compare SR of water versus ribosomes, $P < 0.05$; water versus RNA, $P < 0.01$; water versus protein (50 μg), $P < 0.005$; water versus protein (100 μg), $P < 0.025$.

[d] Compare median GO11⁺ counts of water versus each of the other groups, $P < 0.05$ for all comparisons.

[e] Compare median GO24⁻ counts of water versus each of the other groups, $P < 0.05$ for all comparisons.

[f] Numbers in boxes are median values.

[g] Vaccine dose was 1.00 OD_{260} units.

[h] Vaccine dose was 1.00 OD_{260} units.

[i] Numbers in parentheses are not included in the average.

is common to all three strains, and that it is not O antigen 35. The effect is not due to a transient increase in nonspecific resistance induced by endotoxin, as the protection is undiminished 4 weeks after immunization (unpublished observation). The antigen is unaffected by boiling for 30 min. The heat stability of the antigen suggests that it might be part of the LPS.

Several laboratories have described protective effects of antibodies to antigens of the LPS core, particularly to the determinants of Rd and Re8 mutants (17, 18, 39). Anti-lipid A antibodies have been shown to enhance blood clearance of *Salmonella* (16). An as yet unidentified cross-protective antigen present in lipid A extracts of rough strains of *Salmonella* has been described (21). It is possible that in the procedure for extraction of the ribosomal RNA, incomplete, rough LPS chains complex with the ribosome and are rendered immunogenic. Alternatively, the antigen in the *S. adelaide* vaccine might be Kunin's common antigen (13), which

has been shown to induce some cross-protection against salmonella challenge (4). The possibility must also be considered that the cross-protective effects are due to either protein or RNA which is acting as a common antigen. However, the observation that the Y-RNA extracted from the Rc mutant, TA1659, did not protect makes it unlikely that RNA functions as a cross-protective immunogen. Experiments to evaluate the contribution of the protein to cross-protection are in progress.

In light of the evidence presented in this paper, that O antigens can contaminate ribosomal preparations, one might raise the question as to whether the ribosomes make any contribution to the observed protection. In this regard it should be noted that the immunogenicity of whole *Salmonella* ribosomes is not reduced by treatment with RNase, Pronase, or trypsin (32). Furthermore, in the case of another gram-negative organism, *Vibrio cholerae*, Jensen et al. (6) have shown that the protective antigen in ribosomal preparations can be separated from the ribosomes. The antigen is a colloid of cell wall origin which contains protein, lipid, and carbohydrate. It does not need to be complexed with the ribosomes to be protective, although a comparison of protective capacity on a weight basis between complexed and noncomplexed antigen was not reported.

If the ribosomes do serve a function it seems most reasonable that they have an adjuvant role and serve as carriers for O antigens or other wall or membrane antigens. In the case of the *Salmonella*, there is some evidence that ribosomal preparations are more protective on a weight basis than LPS given alone (32). However, a systematic appraisal of the relative efficacy of LPS, ribosome-associated LPS, and LPS complexed with ribosomal fractions has not been reported, and would help to elucidate the contribution of the ribosomal components, if any, to the immunity induced by these preparations.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CC-00624 from the Center for Disease Control, Atlanta, Ga., and AI11860 from the National Institute of Allergy and Infectious Diseases.

I wish to thank Sue O'Donnell for her excellent technical assistance and C. H. O'Keefe and J. Kremer for their help in carrying out the experiments shown in Table 8. I am grateful to Martin Rosenzweig for the statistical analyses, and to Bruce A. D. Stocker and Alois H. Nowotny for critically reading the manuscript.

1. Berry, L. J. 1955. Effect of malonate on growth rate of *Salmonella typhimurium* in mice. Ann. N.Y. Acad. Sci. **62**:327-348.
2. Berry, L. J., G. N. Douglas, P. Hoops, and N. E. Prather. 1975. The background of immunization against salmonellosis, p. 388-398. *In* Proceedings of the Fourth International Convocation of Immunology. Karger, Basel.
3. Fogel, S., and P. S. Sypherd. 1968. Extraction and isolation of individual ribosomal proteins from *Escherichia coli*. J. Bacteriol. **96**:358-364.
4. Gorzynski, E. A., J. J. Ambrus, and E. Neter. 1971. Effect of common enterobacterial antiserum on experimental *Salmonella typhimurium* infection of mice. Proc. Soc. Exp. Biol. Med. **137**:1209-1211.
5. Hartley, H. O. 1955. Some recent developments in analysis of variance. Commun. Pure Appl. Math. **8**:47-72.
6. Jensen, R., B. Gregory, J. Naylor, and P. Actor. 1972. Isolation of protective somatic antigen from *Vibrio cholerae* (Ogawa) ribosomal preparations. Infect. Immun. **6**:156-161.
7. Johnson, W. 1972. Ribosomal vaccines. I. Immunogenicity of ribosomal fractions isolated from *Salmonella typhimurium* and *Yersinia pestis*. Infect. Immun. **5**:947-952.
8. Johnson, W. 1972. Ribosomal vaccines. II. Specificity of the immune response to ribosomal ribonucleic acid and protein isolated from *Salmonella typhimurium*. Infect. Immun. **8**:395-400.
9. Kasai, N., and A. Nowotny. 1967. Endotoxic glycolipid from a heptoseless mutant of *Salmonella minnesota*. J. Bacteriol. **94**:1824-1836.
10. Kauffmann, F. 1947. The serology of the Coli group. J. Immunol. **57**:71-100.
11. Krishnapillai, V. 1971. Uridinediphosphogalactose-4-epimerase deficiency in *Salmonella typhimurium* and its correction by plasmid-borne galactose genes of *Escherichia coli* K-12: effects on mouse virulence, phagocytosis, and serum sensitivity. Infect. Immun. **4**:177-188.
12. Krishnapillai, V., D. G. MacPhee, and B. A. D. Stocker. 1971. Properties of a *Salmonella typhimurium* mutant with an incomplete deficiency of uridinediphosphogalactose-4-epimerase. J. Bacteriol. **107**:155-161.
13. Kunin, C. M. 1963. Separation, characterization and biological significance of a common antigen in Enterobacteriaceae. J. Exp. Med. **118**:565-586.
14. Kurland, C. G. 1966. The requirements of specific sRNA binding by ribosomes. J. Mol. Biol. **18**:90-108.
15. Lowry, O. H., N. J. Rosebrough, A. J. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
16. Luderitz, O., C. Galanos, V. Lehmann, M. Nurminen, E. T. Rietschel, G. Rosenfelder, M. Simon, and O. Westphal. 1973. Lipid A: chemical structure and biological activity. J. Infect. Dis. **128**(Suppl.):17-29.
17. McCabe, W. R. 1972. Immunization with R mutants of *S. minnesota*. I. Protection against challenge with heterologous gram-negative bacilli. J. Immunol. **108**:601-610.
18. McCabe, R. W., B. E. Kreger, and M. Johns. 1972. Type-specific and cross-reactive antibodies in gram-negative bacteremia. N. Engl. J. Med. **287**:261-267.
19. MacPhee, D. G., and B. A. D. Stocker. 1969. Suppression of amber and ochre mutants in *Salmonella typhimurium* by a mutant F'-1-*gal* factor carrying an ochre suppressor gene. J. Bacteriol. **100**:240-246.
20. Morgenroth, A., and J. P. Duguid. 1968. Demonstration of different mutational sites controlling rhamnose fermentation in FIRN and non-FIRN *rha*$^-$ strains of *Salmonella typhimurium*: an essay in bacterial archaeology. Genet. Res. **11**:151-169.
21. Mullan, N. A., P. W. Newsome, P. G. Cunnington, G. H. Palmer, and M. E. Wilson. 1974. Protection against gram-negative infections with antiserum to lipid A from *Salmonella minnesota* R595. Infect. Immun. **10**:1195-1201.
22. Neter, E., L. F. Bertram, D. A. Zola, M. R. Murdock, and

C. E. Arbesman. 1952. Studies on hemmagglutination and hemolysis by *Escherichia coli* antisera. J. Exp. Med. **96**:1-15.
23. Nowotny, A. 1969. Basic exercises in immunochemistry, p. 144. Springer Verlag, New York.
24. O'Neill, G. J., and J. P. Todd. 1961. Extraction of nucleic acid-free lipopolysaccharide from gram-negative bacteria. Nature (London) **190**:344-345.
25. Pace, N. R. 1973. Structure and synthesis of the ribosomal ribonucleic acid of prokaryotes. Bacteriol. Rev. **37**:562-603.
26. Rudback, J. A., R. L. Anacker, W. T. Haskins, and K. C. Milner. 1967. Physical structure of a native protoplasmic polysaccharide from *Escherichia coli*. J. Immunol. **98**:1-7.
27. Sanderson, K. E. 1972. Linkage map of *Salmonella typhimurium*, edition IV. Bacteriol. Rev. **36**:558-586.
28. Smith, R. A., and N. J. Bigley. 1972. Ribonucleic acid-protein fractions of virulent *Salmonella typhimurium* as protective immunogens. Infect. Immun. **6**:377-383.
29. Stocker, B. A. D., and P. H. Makelai. 1971. Genetic aspects of biosynthesis and structure of *Salmonella* lipopolysaccharide, p. 369-438. *In* G. Weingaum et al. (ed.), Bacterial toxins, vol. IV. Academic Press Inc., New York.
30. Tripodi, D., and A. Nowotny. 1966. Relation of structure to function in bacterial O-antigens. V. Nature of active sites in endotoxic lipopolysaccharides of *Serratia marcescens*. Ann. N.Y. Acad. Sci. **133**:604-621.
31. Venneman, M. R. 1972. Purification of immunologically active ribonucleic acid preparations of *Salmonella typhimurium:* molecular-sieve and anion-exchange chromatography. Infect. Immun. **5**:269-282.
32. Venneman, M. R., and N. J. Bigley. 1969. Isolation and partial characterization of an immunogenic moiety obtained from *Salmonella typhimurium*. J. Bacteriol. **100**:140-148.
33. Wilkinson, R. G., P. Gemski, and B. A. D. Stocker. 1972. Non-smooth mutants of *Salmonella typhimurium:* differentiation by phage sensitivity and genetic mapping. J. Gen. Microbiol. **70**:527-554.
34. Youmans, A. S., and G. Youmans. 1965. Immunogenic activity of a ribosomal fraction obtained from *Mycobacterium tuberculosis*. J. Bacteriol. **89**:1291-1298.
35. Youmans, A. S., and G. P. Youmans. 1966. Preparation of highly immunogenic ribosomal fractions of *Mycobacterium tuberculosis* by use of sodium dodecyl sulfate. J. Bacteriol. **91**:2139-2145.
36. Zeigler, E. J., H. Douglas, J. E. Sherman, C. E. Doirs, and A. I. Braude. 1973. Treatment of *E. coli* and klebsiella bacteremia in agranulocytic animals with antiserum to a UDP-gal epimerase deficient mutant. J. Immunol. **111**:433-438.