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Role of Endotoxin Contamination in Ribosomal Vaccines Prepared from *Salmonella typhimurium*

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Ribosomal vaccines prepared from *Salmonella typhimurium* SR-11 and 6707, an Re mutant bacterium of strain LT2, were effective immunogens in A/J and C_3H/He_{Dub} inbred mice. Only SR-11 ribosomes were able to induce significant protection in C_3H/HeJ mice. $C_{57}BL/6J$ mice were not protected by either ribosomal preparation. A/J mice were protected against salmonella infection by purified SR-11 endotoxin preparations. Neither the C_3H/He_{Dub}, the C_3H/HeJ, nor the $C_{57}BL/6J$ mice were protected by the endotoxin preparation. Passive hemagglutination studies showed that C_3H/HeJ mice had no antibodies to O antigen but were significantly protected by SR-11 ribosomes. In contrast, $C_{57}BL/6J$ mice, which had the highest titers of O antibodies of the four inbred mouse strains, were not protected by SR-11 ribosomes. Endotoxin cannot totally account for the effectiveness of ribosomal vaccines prepared from *S. typhimurium*.

Ribosomal fractions prepared from *Salmonella typhimurium* have been shown to be effective immunogens against experimental salmonellosis. However, the immunogenic moiety of ribosomal fractions remains the subject of controversy. The immunogenic moiety was described by Venneman and co-workers (40–42) to be a heat-stable, ribonuclease-resistant ribonucleic acid (RNA) or RNA protein complex, which was effective even when administered without adjuvant. In contrast to these results, Johnson (14, 15) reported the protective immunogen to be ribosomal protein. Smith and Bigley (33, 34) showed that an ethanol-precipitated RNA preparation induced significant protection when increasing amounts of ribosomal protein were present. Houchens and Wright (12) also showed protective immunity by ribosomal subfractions rich in proteins. More recently, Medina et al. (18) and Eisenstein (8) reported that fractions rich in RNA were protective against salmonellosis.

These conflicting results may be due to the differences in the amount of contamination with nonribosomal material during isolation of the ribosomal subfractions. Berry et al. (3) have proposed that, when a small amount of contaminating carbohydrate is associated with the RNA, the RNA-O antigen complex may act as a superantigen. Their investigations also showed that ribosomes, but not RNA, prepared from an Rd mutant of *S. typhimurium* were protective. Eisenstein (8) has also suggested that the protection associated with the ribosomal fraction is due in part to O antigen contamination. Eisenstein's investigations utilizing isogenic strains of *S. enteritidis* and mutant strains of *S. typhimurium* with defects in lipopolysaccharide (LPS) synthesis showed that O antigens may play some role in the protective ability of "ribosomal" vaccines. Other investigators examining the protective role of salmonella ribosomes have also acknowledged that endotoxin or cell wall contaminants may be responsible for the protective nature of ribosomes (11, 21, 37). Therefore, this investigation was undertaken to examine the role O antigen contamination may play in the protective ability of salmonella ribosomes.

MATERIALS AND METHODS

Animals. Male C_3H/He mice were obtained from Flow Laboratories, Dublin, Va., or Jackson Laboratory, Bar Harbor, Me. Male A/J and $C_{57}BL/6J$ mice were also obtained from Jackson Laboratory. Adult male mice weighing 16 to 24 g were used in all experiments. Mice were housed 10 per cage and given mouse chow and water ad libitum.

Organisms. *S. typhimurium* SR-11 was obtained from L. J. Berry, University of Texas, Austin. *S. typhimurium* 6707 was obtained from N. Bigley, Wright State University, Dayton, Ohio. Strain 6707 was first described by B. Ames et al. (1) as TA1538, an Re mutant of *S. typhimurium* LT2. Cultures were maintained on brain heart infusion agar (Difco). The mean lethal dose was determined by the method of Reed and Muench (25). Survival was measured over 30 days.

Preparation of cultures. Eight-hour cultures of *S. typhimurium* were centrifuged in a Sorvall RC-2B

centrifuge equipped with an SZ-14 continuous-flow rotor, and the cells were washed as described previously (20).

Isolation of ribosomes. Ribosomes were prepared from the bacterial cell suspension as previously described (20).

Endotoxin. LPS was prepared from *S. typhimurium* SR-11 by the method of Westphal and Jann (45). A 25-g amount of bacteria pretreated with diluted formaldehyde (0.1%) was suspended in 350 ml of water at 65 to 68°C. An equal amount of 90% phenol, preheated to 65 to 68°C, was added, and the mixture was shaken for 15 min in a 65°C water bath and then centrifuged at 3,000 × *g* for 40 min. The upper water layer was aspirated off. Another 350 ml of 65°C water was added to the phenol layer, and the mixture was treated as described above. The second water layer aspirated from the centrifuged mixture was combined with the first water layer, and the mixture was dialyzed for 3 to 4 days against water to remove the phenol. The dialyzed material was centrifuged at 6,000 × *g* to remove any insoluble material, and the supernatant fluid containing the LPS and bacterial RNA was lyophilized. To remove the RNA, the lyophilized material was dissolved in sufficient 0.5 M sodium chloride to give a 1% solution. A 2% solution of cetyltrimethyl ammonium bromide (Cetavlon) was added until the proportion of Cetavlon to crude material was about 1.5:1. This solution was then diluted with water, and precipitates were collected by centrifugation as they appeared. The fractions that precipitated at greater than 0.35 M NaCl and the last solution below 0.25 M contained the LPS. The fraction of 0.35 M and above was dissolved in 0.1 M NaCl, and 10 volumes of ethanol was added. The 0.35 M fraction was then centrifuged, and the sediment was dissolved in water and dialyzed. Both the >0.35 M fraction and the <0.25 M fraction were lyophilized.

Immunizations and challenge. Groups of 10 mice were immunized intraperitoneally with 0.2 ml of the appropriate vaccine preparation. Mice immunized with LPS preparations were only given 0.1 ml of the appropriate preparation. Each mouse received two injections of equal concentrations of antigen 14 days apart. Ribosome doses were calculated in micrograms of protein. Mice were challenged with 100 mean lethal doses 14 days after immunization.

Endotoxin detection. An intravenous injection of 2 mg of lead acetate has been shown to sensitize mice to the lethal effects of an intraperitoneal injection of endotoxin (29). Vaccine preparations were tested for their ability to kill lead acetate-sensitized mice.

The passive hemagglutination technique described by Rudbach (27) and also by Nowotny (23) was used to detect antibodies to endotoxin. Sheep erythrocytes were coated with SR-11 endotoxin, and the mixture was incubated at 37°C for 60 min. The suspension was placed in the cold (4°C) and allowed to incubate overnight. The final LPS-erythrocyte mixture was diluted 1:5 before use in the assay. Fifty microliters of antigen was added to serial dilutions of antiserum, and the reciprocal of the highest dilution giving agglutination was considered the end point. Serum was obtained from groups of five mice immunized with various preparations 14 days after the second injection and was examined for antibodies to endotoxin. Results obtained from the passive hemagglutination assay with SR-11 endotoxin were compared with those obtained using sheep erythrocytes coated with *S. typhimurium* LT2 endotoxin. The LT2 endotoxin was kindly provided by Otto Lüderitz, Max Planck Institute, Freiburg, West Germany. Serum titers were identical and did not differ when either preparation was used.

Polyacrylamide gel electrophoresis. Vertical sodium dodecyl sulfate-slab gel electrophoresis was performed by the method of Laemmli (16) with the following modifications. The electrode buffer was composed of: 0.025 M tris(hydroxymethyl)aminomethane, 0.384 M glycine, and 0.1% sodium dodecyl sulfate, pH 8.3. Gel concentrations were 7% as described by Russell and Johnson (28). Samples were diluted 1:1 with a solution composed of: 0.13 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 6.8), 5% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue. Samples were heated for 2 min at 100°C, and 100 μl was applied to the gels. After electrophoresis at 35 mA/slab, the gels were fixed in 20% sulfosalicylic acid for 2 h and stained overnight with the appropriate stain. Gels were stained with Coomassie brilliant blue for protein, periodic acid-Schiff stain for carbohydrates and glycoproteins, and Sudan black B for lipids and lipoproteins (12).

Electron microscopy. Samples containing either 100 μg of endotoxin or 50 μg of ribosomes were negatively stained with 1% uranyl acetate on carbon-stabilized, Formvar-coated 400-mesh grids. The negative stains were viewed in a JEOL 100B electron microscope at 60 kV.

Biochemical assay. Protein was determined by the method of Lowry et al. (17), with bovine serum albumin fraction V as the standard. RNA was measured by the orcinol method (7), and deoxyribonucleic acid was measured by the method of Ashwell (2). Yeast RNA and pancreatic deoxyribonucleic acid served as standards. LPS was estimated by the carbocyanine method of Janda and Work (13). 2-Keto-3-deoxy sugars were assayed by the method of Waravdekar and Saslaw (44). LPS from *S. typhimurium* LT2 and 2-keto-3-deoxy sugar, which served as standards, were the gift of Otto Lüderitz, Max Planck Institute, Freiburg, West Germany.

Statistical evaluation. Significance levels for protection were determined by the Fisher exact probability test by the method of Siegel (30).

RESULTS

Comparative immunogenicity of ribosomal vaccines. To compare the immunogenicities of ribosomal preparations of *S. typhimurium* SR-11 with those of an Re mutant, mice were immunized with the appropriate preparation and given a booster immunization 2 weeks later (Table 1). A/J and C_3H/He_{Dub} mice were completely protected by SR-11 ribosomes at the dos-

TABLE 1. *Comparative immunogenicity of ribosomes prepared from S. typhimurium SR-11 or 6707*

Vaccine	Immunizing dose (μg of protein)	Survival[a] (%)			
		A/J	$C_{57}BL/6J$	C_3H/HeJ	C_3H/He_{Dub}
SR-11 ribosomes	200	100[b]	0	50[b]	100[b]
SR-11 ribosomes	100	100[b]	0	30	100[b]
6707 ribosomes	200	100[b]	0	10	90[b]
6707 ribosomes	100	100[b]	0	0	70[b]
Control		0	0	0	0

[a] Mice were challenged with 100 mean lethal doses of SR-11 14 days after immunization.
[b] $P \leq 0.025$.

age levels of 200 and 100 μg. C_3H/HeJ mice were significantly protected at a dosage level of 200 μg of SR-11 ribosomes. However, at 100 μg, C_3H/HeJ mice were not significantly protected. The difference in protection at the 200-μg level of SR-11 ribosomes between C_3H/HeJ and C_3H/He_{Dub} mice was significant. $C_{57}BL/6J$ mice were not protected at either dose of SR-11 ribosomes. A/J and C_3H/He_{Dub} mice also showed significant levels of protection when immunized with either 200 or 100 μg of 6707 ribosomes. C_3H/HeJ mice were not protected at either dose by the 6707 ribosomes. $C_{57}BL/6J$ mice were not protected by 6707 ribosomes.

Immunogenicity of *S. typhimurium* SR-11 endotoxin. Phenol-water-extracted endotoxin was tested in the four inbred mouse strains for its ability to protect against lethal challenge. Mice were immunized intraperitoneally with varying amounts of endotoxin and given a booster immunization 2 weeks later (Table 2). The $C_{57}BL/6J$ and the C_3H/HeJ mice were not protected by any dose of endotoxin used. Although the C_3H/He_{Dub} mice showed some survival at the higher dosages, the levels of protection observed were not significant. A/J mice were the only strain of mice that were significantly protected by endotoxin at all levels tested.

Analysis of preparations for endotoxin. Carbocyanine analysis of ribosome preparations showed approximately a 0.1% contamination of both the 6707 and SR-11 ribosomes with endotoxin. Another assay used for detecting the presence of cell wall fragments in vaccine preparations was the lead-sensitized mouse assay. Mice were given an intravenous injection of 2 mg of lead acetate and immediately afterward were given an intraperitoneal injection of an appropriate vaccine preparation. Mean lethal doses of purified endotoxin were determined in A/J mice that had been sensitized with lead acetate (Table 3). The mean lethal dose of LPS in lead acetate-sensitized A/J mice was 0.1 μg, a 500- to 1,000-fold increase in toxicity compared with that of untreated mice. The lethality of ribosome-derived vaccines in lead acetate-sensitized mice is presented in Table 4. A/J mice, which were found to be the most sensitive to endotoxin, were killed by all vaccines tested. Purified endotoxin preparations and SR-11 ribosomes were toxic to lead acetate-sensitized C_3H/He_{Dub} mice, but 6707 ribosomes were not significantly toxic. C_3H/HeJ, which are nonresponders to endotoxin, were not killed by any of the preparations.

TABLE 2. *Immunogenicity of SR-11 endotoxin*

Dose (μg)	Survival[a] (%)			
	$C_{57}BL/6J$	A/J	C_3H/HeJ	C_3H/He_{Dub}
10	0	90[b]	0	40
1	0	80[b]	0	11
0.1	0	100[b]	0	0
0.01	ND[c]	70[b]	0	ND
Control	0	20	0	0

[a] Mice were challenged with 100 mean lethal doses of SR-11 14 days after immunization.
[b] $P \leq 0.05$.
[c] ND, Not determined.

TABLE 3. *Lethality of S. typhimurium SR-11 endotoxin in lead-sensitized A/J mice*

Dose (μg)	Survival (%)
50	0[a]
10	0[a]
1	20[a]
0.1	50[a]
0.01	100
0.001	100
Control	100

[a] $P \leq 0.025$.

The third test used to assay for the presence of endotoxin was the passive hemagglutination assay. Mice that had been immunized with the various preparations were bled for their sera. The sera was titered for the presence of O antibodies by using sheep erythrocytes coated with *S. typhimurium* endotoxin. The results (Table 5) indicate that C_3H/HeJ mice were totally devoid of any antibody to O antigen. C_3H/He_{Dub} mice and A/J mice showed identical patterns of

O antibody response. Mice immunized with SR-11 ribosomes had a 1:16 titer, whereas mice immunized with 6707 ribosomes had no titer. C_3H/He_{Dub} and A/J mice immunized with endotoxin had a 1:8 titer. $C_{57}BL/6J$ mice had the highest titers of O antibody. SR-11 ribosome-immunized $C_{57}BL/6J$ mice had a 1:32 titer, and endotoxin-immunized $C_{57}BL/6J$ mice had a 1:6 titer.

Electron microscopy studies showed the presence of membranous material both in the SR-11 ribosome preparation and in the 6707 ribosome preparation.

Electrophoresis (Fig. 1) showed the presence of lipids or lipoproteins in both ribosomal preparations.

DISCUSSION

Previous investigations by our laboratory (20), along with those of Medina et al. (18), Robson and Vas (26), Plant and Glynn (24), and VonJeney et al. (43), have shown that protection against salmonella infection may depend on the strain of mouse used. In addition, Vas et al. (39) and VonJeney et al. (43) have shown that inbred mouse strains vary in their sensitivity to endotoxin. Since resistance to salmonella infection may not correlate with the degree of sensitivity to endotoxin, both of these facts should be taken into consideration when investigating the immunogenic moiety of salmonella ribosomes.

In this study the four strains of inbred mice were selected because of their different sensitivities to salmonella infection and endotoxin. A/J mice are considered resistant to salmonella infection but are quite sensitive to endotoxin (26, 39). $C_{57}BL/6J$ mice are quite sensitive to salmonella infection and sensitive to endotoxin, (26, 39, 43) and cannot be immunized against salmonella infection (20). C_3H/He_{Dub} mice are sensitive to salmonella infection and sensitive to endotoxin but can be protected against infection by several vaccines. In contrast, C_3H/HeJ mice are sensitive to salmonella infection but are totally nonresponsive to the toxic and immunogenic activity of endotoxin (5, 9, 31, 32, 35).

In this study both the A/J mice and the C_3H/He_{Dub} mice were significantly protected by the SR-11 ribosomes and the ribosomes isolated from 6707, an Re mutant of *S. typhimurium* LT2. The 6707 ribosomes induced slightly higher levels of protection in the A/J mice than in the C_3H/He_{Dub} mice. C_3H/HeJ mice were significantly protected by ribosomes isolated from the SR-11 strain but not by the ribosomes isolated from the 6707 strain. However, the protec-

TABLE 4. *Lethality of preparations in lead acetate-sensitized mice*

Vaccine	Dose (μg)	Survival (%)		
		A/J	C_3H/HeJ	C_3H/He_{Dub}
SR-11 ribosomes	200[a]	0	60[b]	0
6707 ribosomes	200[a]	40	100[b]	80[c]
SR-11 endotoxin	50	0	90[b]	0
SR-11 endotoxin	1	20	100[b]	60
Lipid A	10	20	ND[d]	40
Pneumococcal ribosomes	200[a]	20	ND[d]	40
Pneumococcal ribosomal RNA	200	60[d]	ND[d]	60
Control		100	100	100

[a] Micrograms of protein.
[b] $P \leq 0.01$.
[c] $P \leq 0.05$.
[d] ND, Not determined.

TABLE 5. *Passive hemagglutination titers to endotoxin in mice immunized with various preparations*

Vaccine	Immunizing dose (μg)	Titer			
		A/J	C_3H/HeJ	C_3H/He_{Dub}	$C_{57}BL/6J$
Controls		1:2	1:2	1:2	1:2
SR-11 ribosomes	200[a]	1:16	1:2	1:16	1:32
6707 ribosomes	200[a]	1:2	1:2	1:2	ND[b]
SR-11 endotoxin	50	1:8	1:2	1:8	1:16
SR-11 ribosomes[c]	200	1:64	ND	1:64	ND
Difco group B antiserum		1:64			

[a] Micrograms of protein.
[b] ND, Not determined.
[c] Survived salmonella challenge.

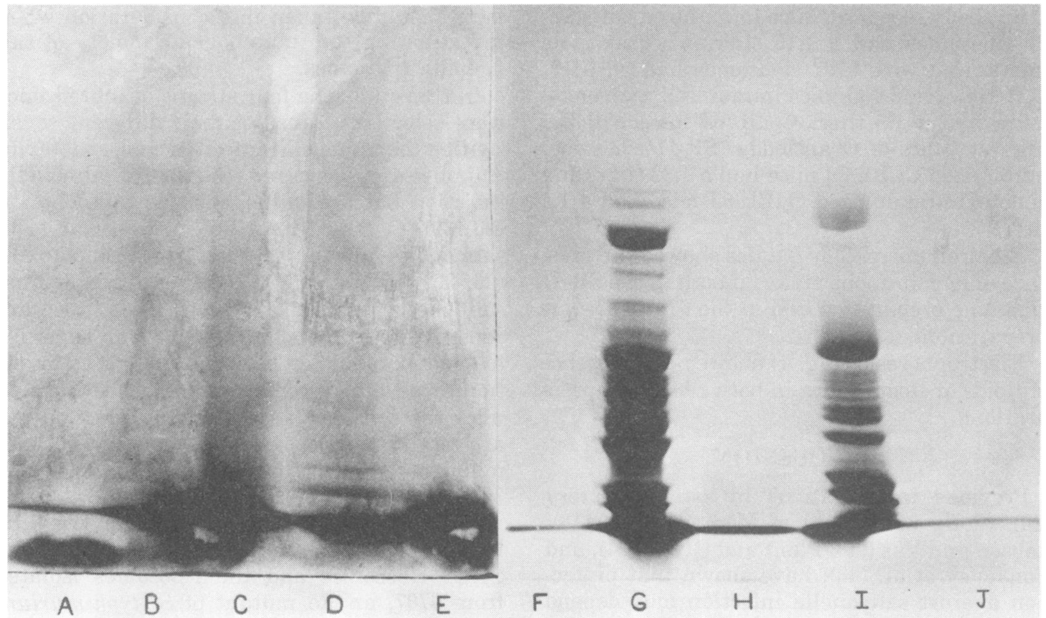

FIG. 1. *Sodium dodecyl sulfate-gel electrophoresis of endotoxin and ribosomal preparations. Columns A through E were stained with Sudan black B for the presence of lipids and lipoproteins. Columns F through J were stained with Coomassie brilliant blue for proteins. Column A, lipid A; column B, SR-11 ribosomes; column C, SR-11 endotoxin; column D, 6707 ribosomes; columns E and F, LT2 endotoxin; column G, 6707 ribosomes; column H, SR-11 endotoxin; column I, SR-11 ribosomes; and column J, lipid A.*

tion induced by the SR-11 ribosomes in the C_3H/HeJ mice was lower than that achieved in the A/J and C_3H/He_{Dub} mice. $C_{57}BL$/6J mice could not be protected against salmonella infection by either SR-11 ribosomes or 6707 ribosomes. The data obtained from the experiment in which C_3H/HeJ mice, which are nonresponders to endotoxin, were significantly protected by SR-11 ribosomes would lend some evidence to the concept that endotoxin may not be totally responsible for the protective immunity induced by salmonella ribosomes.

Purified endotoxin preparations from *S. typhimurium* SR-11 prepared by the phenol-water extraction method could induce protection only in the A/J mice. This protection may be similar to that induced by polyinosinic acid:polycytidylic acid in the A/J mice in the studies of Medina et al. (18). C_3H/He_{Dub} mice were not significantly protected by the endotoxin, but the levels were increased as the dosage levels increased. The resistance may be due to the antibody levels against the endotoxin, since Borel (4) has shown that most C_3H mice are able to produce antibodies against *Escherichia coli* endotoxin (with the exception of the C_3H/HeJ mice, which are nonresponders to the effects of endotoxin). The inability to protect $C_{57}BL$/6J mice with endotoxin against infection agrees with our previous investigations, which showed that only a live avirulent strain of *Salmonella* can induce significant levels of protection in $C_{57}BL$/6J mice against salmonella infection (20).

The results obtained with ribosomes prepared from 6707, an Re mutant of *S. typhimurium* LT2, contradict those obtained by Eisenstein (8) and Hoops et al. (11). These investigators concluded from their data that O antigens must be present for the vaccine to be effective. These variations in results may be explained by differences in the immune responses of the strains of mice used in their investigation. There are several explanations for the inability of 6707 ribosomes to protect C_3H/HeJ mice against salmonella infection. First, there may be basic differences between SR-11 ribosomes and 6707 ribosomes in their protein content. Another explanation may be that ribosomes from the Re mutant lack the necessary "contaminant" that enables the ribosomes to be completely protective (10, 19, 22, 32, 36). Since Re mutant bacteria have only 2-keto-3-deoxy sugar and lipid A of the whole LPS molecule, somatic proteins or some other additional substances associated with endotoxin may be lacking (10, 22, 36). C_3H/HeJ mice have been shown to be nonresponsive to phenol-ex-

tracted endotoxin, but are responsive to endotoxin preparations that contain protein (9, 22, 32, 26). Ribosomes prepared from the Re mutant may lack these proteins as contaminants.

Results in this study from the biological tests for endotoxin indicate that small amounts of LPS are present in some of the vaccines. However, as the experiments with the lead-sensitized mice show (Table 4), the assay may not be specific for endotoxin. Ribosomes prepared from rough colonies of pneumococci, along with RNA prepared from pneumococcal ribosomes, were also toxic to lead-sensitized mice. These preliminary results indicate that the lead-sensitized mouse assay may not be specific for endotoxin.

Passive hemagglutination studies confirmed the finding that O antigens are not completely responsible for the protective immunity induced by salmonella ribosomes. Antibodies to the O antigen were lacking in all four inbred mouse strains when immunized with the Re mutant ribosomes. This is to be expected since Re mutants lacked the O antigenic region. All mouse strains except for C_3H/HeJ mice showed antibodies against O antigen when immunized with SR-11 ribosomes, yet $C_{57}BL$/6J mice, which had the highest titers against endotoxin, were not protected against salmonella infection by the ribosomes. C_3H/HeJ mice, which were unable to produce antibodies against O antigens, showed no titers to endotoxin, yet SR-11 ribosomes were significantly protective in the C_3H/HeJ mice. Therefore, an additional antigen must be responsible for the protective immunity induced by salmonella SR-11 ribosomes.

Endotoxin alone is not protective for C_3H/He_{Dub} and C_3H/HeJ mice, yet these strains showed significant levels of protection when immunized with SR-11 ribosomes. Protection with ribosomes prepared from the Re mutant rules out O antigenic groups as the protective immunogen. Talcott et al. (38) and Gürtler and Rank (9) have shown that C_3H/HeJ mice are resistant to the action of LPS and lipid A in relation to their toxicity, antibody induction, and adjuvanticity. Therefore, the immunity induced by SR-11 ribosomes cannot be accounted for by either LPS or lipid A. In addition, neither LPS nor lipid A (6, 31) is capable of acting as an adjuvant in C_3H/HeJ mice (38). Our experimental results indicate that some additional antigen acts as the protective immunogen of salmonella SR-11 ribosomes in C_3H/HeJ mice.

The results presented in this paper should serve as the basis for investigating such components as membrane components, endotoxin protein, and ribosomal proteins to lead to a better understanding of the immunogenic moiety of salmonella ribosomes.

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LITERATURE CITED

1. Ames, B. N., F. D. Lee, and W. E. Durston. 1973. An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc. Natl. Acad. Sci. U.S.A. 70:782-786.
2. Ashwell, G. 1957. Colorimetric analysis of sugars. Methods Enzymol. 3:73-105.
3. Berry, L. J., G. N. Douglas, P. Hoops, and N. E. Prather. 1975. The background of immunization against salmonellosis, p. 388-398. *In* E. Neter and F. Milgrom (ed.), Proceedings of the Fourth International Convocation on Immunology. Karger, Basel.
4. Borel, J. F. 1974. Comparison of the immune response to sheep erythrocytes, tetanus toxoid and endotoxin in different strains of mice. Agents Actions 4:277-285.
5. Chedid, L., M. Parant, C. Damais, F. Parant, D. Guy, and A. Galell. 1976. Failure of endotoxin to increase nonspecific resistance to infection of lipopolysaccharide low-responder mice. Infect. Immun. 13:722-727.
6. Chiller, J. M., B. J. Skidmore, D. C. Morrison, and W. O. Weigle. 1973. Relationship of the structure of bacterial lipopolysaccharides to its function in mitogenesis and adjuvanticity. Proc. Natl. Acad. Sci. U.S.A. 70:2129-2133.
7. Dische, A. 1955. Color reaction of nucleic components, p. 285-306. *In* E. Chargaff and J. N. Davidson (ed.), The nucleic acids, vol. 1. Academic Press Inc., New York.
8. Eisenstein, T. K. 1975. Evidence for O antigens as the antigenic determinants in "ribosomal" vaccines prepared from *Salmonella*. Infect. Immun. 12:364-377.
9. Gürtler, L. G., and W. R. Rank. 1976. Genetic differences between endotoxin-sensitive and resistant C_3H mice. Intraperitoneal cell response to lipopolysaccharide and prostaglandins. Z. Immunitaetsforsch. 151:420-429.
10. Hill, A. W., K. G. Hibbitt, and A. Shears. 1973. The stimulation by endotoxin of the nonspecific resistance of mice to bacterial infection. Br. J. Exp. Pathol. 55:194-202.
11. Hoops, P., N. E. Prather, L. J. Berry, and J. M. Ravel. 1976. Evidence for an extrinsic immunogen in effective ribosomal vaccines from *Salmonella typhimurium*. Infect. Immun. 13:1184-1192.
12. Houchens, D. P., and G. L. Wright, Jr. 1973. Immunity of *Salmonella typhimurium* infection: characterization of antigens in active protection by polyacrylamide gel electrophoresis. Infect. Immun. 7:507-511.
13. Janda, J., and E. Work. 1971. A colorimetric estimation of lipopolysaccharides. FEBS Lett. 16:343-345.
14. Johnson, W. 1972. Ribosomal vaccines. I. Immunogenicity of ribosomal fractions isolated from *Salmonella typhimurium* and *Yersinia pestis*. Infect. Immun. 5:947-952.
15. Johnson, W. 1973. Ribosomal vaccines. II. Specificity of the immune response to ribosomal ribonucleic acid and protein isolated from *Salmonella typhimurium*. Infect. Immun. 8:395-399.

16. **Laemmli, U. F.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
18. **Medina, S., S. I. Vas, and H. G. Robson.** 1975. Effect of nonspecific stimulation on the defense mechanisms of inbred mice. J. Immunol. 114:1720-1725.
19. **Melchers, F., V. Braun, and C. Galanos.** 1975. The lipoprotein of the outer membrane of *Escherichia coli*: a B-lymphocyte mitogen. J. Exp. Med. 142:473-482.
20. **Misfeldt, M. L., and W. Johnson.** 1976. Variability of protection in inbred mice induced by a ribosomal vaccine prepared from *Salmonella typhimurium*. Infect. Immun. 14:652-659.
21. **Molinari, J. L., and C. Larralde.** 1974. Acquired immunity to murine typhoid induced in mice with fractions of *Salmonella typhimurium*. Rev. Latinoam. Microbiol. 16:189-197.
22. **Morrison, D. C., S. J. Betz, and D. M. Jacobs.** 1976. Isolation of a lipid A bound polypeptide responsible for "LPS-initiated" mitogenesis of C_3H/HeJ spleen cells. J. Exp. Med. 144:840-846.
23. **Nowotny, A.** 1969. Basic exercises in immunochemistry, p. 144. Springer Verlag, New York.
24. **Plant, J., and A. A. Glynn.** 1976. Genetics of resistance to infection with *Salmonella typhimurium* in mice. J. Infect. Dis. 33:72-78.
25. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27:493-497.
26. **Robson, H. G., and S. I. Vas.** 1972. Resistance of inbred mice to *Salmonella typhimurium*. J. Infect. Dis. 126:38-386.
27. **Rudbach, J. A.** 1971. Molecular immunogenicity of bacterial lipopolysaccharide antigens: establishing a quantitative system. J. Immunol. 106:993-1001.
28. **Russell, R. R. B., and K. G. Johnson.** 1975. SDS-polyacrylamide gel electrophoresis of lipopolysaccharides. Can. J. Microbiol. 21:2013-2018.
29. **Selye, H., B. Tuchweber, and L. Bertók.** 1966. Effect of lead acetate on the susceptibility of rats to bacterial endotoxins. J. Bacteriol. 91:884-890.
30. **Siegel, S.** 1956. The Fisher exact probability test, p. 96-104. *In* S. Siegel (ed.), Nonparametric statistics for the behavioral sciences. McGraw-Hill Book Co., Inc., New York.
31. **Skidmore, B. J., J. M. Chiller, D. C. Morrison, and W. O. Weigle.** 1975. Immunologic properties of bacterial lipopolysaccharide (LPS): correlation between the mitogenic, adjuvant, and immunogenic activities. J. Immunol. 114:770-775.
32. **Skidmore, B. J., D. C. Morrison, J. M. Chiller, and W. O. Weigle.** 1975. Immunologic properties of bacterial lipopolysaccharides (LPS). II. The unresponsiveness of C_3H/HeJ mouse spleen cells to LPS-induced mitogenesis is dependent on the method used to extract LPS. J. Exp. Med. 142:1488-1508.
33. **Smith, R. A., and N. J. Bigley.** 1972. Ribonucleic acid-protein fractions of virulent *Salmonella typhimurium* as protective immunogens. Infect. Immun. 6:377-383.
34. **Smith, R. A., and N. J. Bigley.** 1972. Detection of delayed hypersensitivity in mice injected with ribonucleic acid-protein fractions of *Salmonella typhimurium*. Infect. Immun. 6:384-389.
35. **Sultzer, B. M.** 1969. Genetic factors in leucocyte responses to endotoxin: further studies in mice. J. Immunol. 103:32-38.
36. **Sultzer, B. M., and G. W. Goodman.** 1976. Endotoxin protein: a B-cell mitogen and polyclonal activator of C_3H/HeJ lymphocytes. J. Exp. Med. 144:821-827.
37. **Takagi, A., and H. Namba.** 1965. Immunological studies on cell fractions of *Salmonella enteritidis*. Yonago Acta Med. 9:224-240.
38. **Talcott, J. A., D. B. Ness, and F. C. Grumet.** 1975. Adjuvant effect of LPS on in vivo antibody response: genetic defect in C_3H/HeJ mice. Immunogenetics 2:507-515.
39. **Vas, S. I., R. S. Roy, and H. G. Robson.** 1973. Endotoxin sensitivity of inbred mouse strains. Can. J. Microbiol. 19:767-769.
40. **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.
41. **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.
42. **Venneman, M. R., N. J. Bigley, and L. J. Berry.** 1970. Immunogenicity of ribonucleic acid preparations obtained from *Salmonella typhimurium*. Infect. Immun. 1:574-582.
43. **VonJeney, N., E. Günther, and K. Jann.** 1977. Mitogenic stimulation of murine spleen cells: relation to susceptibility to *Salmonella* infection. Infect. Immun. 15:26-33.
44. **Waravdekar, V. S., and L. D. Saslaw.** 1959. A sensitive colorimetric method for the estimation of 2-deoxy sugars with the use of the malonaldehyde-thiobarbituric acid reaction. J. Biol. Chem. 234:1945-1950.
45. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further application of the procedure, p. 83-91. *In* R. L. Whistler and M. L. Wolfrom (ed.), Methods in carbohydrate chemistry, vol. 5. Academic Press Inc., New York.