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Immunogenicity of the Ribosomal Fraction of *Salmonella typhimurium*: Analysis of Humoral Immunity

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The ribosomal fraction prepared from *Salmonella typhimurium* LT2 was further purified by gel filtration of Sepharose 4B and afforded excellent protection against homologous challenge. The highly effective immunogens were composed of several fractions which could give different types of protection to mice. The first type of protection was heat-labile antigens which could induce humoral immunity, and the second type of protection was heat-stable antigens capable of evoking cellular resistance in mice. The former were different from O-antigens and the latter were free of endotoxin and rich in ribonucleic acid. The third type of protection was heat-resistant substances of cell wall components, which were mainly composed of O-antigens. The high immunogenicity observed in this study could be obtained only by the heat-stable antigens rich in ribonucleic acid, and the immunity conferred by this kind of antigen was due to the cellular type of protection.

Many investigations have already revealed the efficiency of ribosomal fractions prepared from *Salmonella typhimurium* (3, 6, 7, 8, 13, 15–17) as effective immunogens against experimental salmonellosis. However, the immunogenic moiety of ribosomal fractions still remains controversial, and it is also unknown how it works in vivo to evoke the immune response against salmonellosis. Several kinds of immunogenic moieties have been reported from different laboratories. Venneman and co-workers (16, 17) suggested that the immunogenic moiety may be a heat-stable, ribonuclease-resistant ribonucleic acid (RNA) or an RNA-protein complex, whereas Johnson (3) reported the protective immunity to be a ribosomal protein. Smith and Bigley (13) defined the protective antigen to be an ethanol-precipitated RNA preparation. By use of polyacrylamide gel electrophoresis, Houchens and Wright (13) showed protective immunity conferred by a ribosomal fraction rich in proteins. In contrast to these results, Eisenstein (1) and Medina et al. (9) have shown that O-antigen contaminates both RNA and protein ribosomal extract and is responsible for at least part of their strain-specific protective activity. The efficiency of ribosomal vaccine has never been evaluated without discussing the contamination of O-antigen during the isolation of the ribosomal subfractions. A minimum amount of contaminating carbohydrate can act as a superantigen when it forms a complex of the RNA-O-antigen (1). Many studies have been conducted on the role of O-antigen contamination (4, 11, 14). Recently, Misfeldt and Johnson (10) have reported that endotoxin cannot totally account for the effectiveness of ribosomal vaccines prepared from *S. typhimurium*. They used four strains of inbred mice with different sensitivities to *Salmonella* infection and endotoxin in their investigation, which suggested that some additional antigens, such as membrane components, endotoxin, and ribosomal proteins, should be closely examined for a better understanding of the immunogenic moiety of *Salmonella* ribosomes. In our previous report (8), the immunity conferred by a ribosomal fraction prepared from *S. typhimurium* was composed of both cellular and humoral factors and was due to the cooperation of the effective immunogen localized with ribosomal substance and O-antigen contaminated in the preparation. Therefore, this study was undertaken to clarify the immunogenic moiety in connection with the role of O-antigen contamination by the analysis of humoral immunity evoked by ribosomal fraction.

MATERIALS AND METHODS

Animals. ddy female mice of 4 or 5 weeks, weighing 15 to 20 g, were used in all experiments. Mice were housed 10/cage, and standard laboratory food and water were provided ad libitum.

Organisms. *S. typhimurium* LT2 was generously provided by K. Saito, Keio University, and used throughout this investigation. The intraperitoneal 50% lethal dose of the strain for mice was 10^3 organisms, maintained by several passages through mice. Organisms suspended in skim milk (Difco) were kept at −70°C and were cultured on brain heart infusion agar (Difco) at 37°C for 18 h to ensure the viability before use.

Preparation. *S. typhimurium* LT2 was grown in nutrient broth (Difco) at 37°C for 12 h. Organisms were harvested by centrifugation at 5,000 rpm, and the cells were washed three times in 10^{-2} M phosphate buffer (PB), pH 7.1. Crude ribosomal fraction was prepared from the washed cells by the method previously described (8) as shown in Fig. 1. The pellet finally obtained from the centrifugation at 15,000 × *g* was suspended in 4 ml of 10^{-2} M PB (pH 7.1) containing 10^{-4} M Mg^{2+} and designated as crude ribosomal fraction, and it was kept at −70°C. Crude ribosomal fraction diluted to the concentration of immunizing dose was filtered through a membrane filter (Millipore Corp., Bedford, Mass.; Swinny adapter, type HA, 0.45-μm grid) for sterilization before immunization.

Purification of ribosomal vaccines. A 1.0-ml portion of crude ribosomal fraction was fractionated on Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden; 3.5- by 95-cm column) equilibrated with 10^{-2} M PB, pH 7.1, containing 10^{-4} M Mg^{2+} (buffer A). Effluent was collected in 10-ml portions, and absorbance at 260 nm was measured on a Hitachi model 101 recording spectrophotometer. Another elution was carried out with a different buffer system consisting of 10^{-2} M PB (pH 7.1) containing 10^{-4} M Mg^{2+} and 0.1% (wt/vol) sodium dodecyl sulfate (SDS) (buffer B). Gel filtration by Sepharose 4B equilibrated with buffer A was employed for the further purification of crude ribosomal fraction. Elution was carried out with buffer A and the eluant was collected in 5-ml portions.

Immunization and challenge. Groups of 30 mice were given 2 injections of crude ribosomal fraction intraperitoneally in a volume of 0.1 ml (2 optical density at 260 nm [OD_{260}] units) 4 days apart. Each fraction separated by Sepharose 4B filtration was filtered through a membrane filter (Millipore Corp.) for sterilization and aseptically concentrated five times against polyethylene glycol for the purpose of immunization. Groups of 30 mice were immunized intraperitoneally with 0.2 ml of the concentrated fractions. Each mouse received two injections of equal concentration of ribosomal fraction 4 days apart. To prepare the antiserum against crude ribosomal fraction, we gave 100 mice three injections of crude ribosomal fraction intraperitoneally in a volume of 0.1 ml (2 OD_{260} units) 4 days apart. All mice immunized with antigens were bled or challenged 7 days after final immunization. The amount of vaccine was standardized by estimating RNA content from OD_{260} nm. The standard amount calculated from purified yeast RNA (Sigma) equates 1 OD_{260} unit to 100 μg of RNA. For preparation of antiserum against O-antigen of *S. typhimurium*, we gave each mouse 3 intraperitoneal injections of lipopolysaccharide (LPS) (1 μg of LPS prepared from *S. typhimurium*; Difco control 612420) 4 days apart.

Washed packed cells
| Suspended in 20 ml of 10^{-2} M PB, pH 7.1, containing 10^{-2} M $MgCl_2$, 0.25% SDS, and 2 μg of DNase/ml; broken by cell homogenizer (TÜBINGEN) with glass beads

Cell extract
| Centrifuged at 27,000 × *g* for 15 min at 4°C

Top four-fifths of the supernatant fluid
| Centrifuged at 45,000 × *g* for 20 min at 4°C

Top four-fifths of the supernatant fluid
| Centrifuged at 150,000 × *g* for 3 h at 4°C

Sediment
| Suspended in 10^{-2} M PB (pH 7.1) containing 10^{-4} M $MgCl_2$ (buffer A) and refrigerated overnight in an ice bath to separate SDS by precipitation; centrifuged at 35,000 × *g* for 15 min to remove the precipitated SDS

Upper three-fourths of the supernatant fluid
| Centrifuged at 150,000 × *g* for 3 h at 4°C after sterilization by membrane filter

Sediment
Suspended in buffer A; designated as crude ribosomal fraction

FIG. 1. *Procedure for the preparation of crude ribosomal fraction.*

Serum fractionation. Pooled serum was obtained from two groups of mice described here as either LPS immunized or ribosomal fraction immunized. Mouse serum was fractionated on a column of Sephadex G-200 equilibrated with buffer A. Pooled fractions were prepared and concentrated by ultrafiltration to about one-fifth the original volumes. The pools were grouped as indicated in Fig. 2 and 3, and then the passive hemagglutination (PHA) test was used to determine which contained reactivity for LPS and crude and purified ribosomal fractions as previously described (8). A bactericidal assay was done as follows. A 0.025-ml amount of each fraction or pool was serially (twofold) diluted in brain heart infusion broth by using microtiter plates. The assay organisms were suspended in brain heart infusion broth diluted with saline (broth-saline = 1:9), and 0.025 ml of the suspension containing 10^6 organisms was added to each well. After addition of 0.025 ml of brain heart infusion broth into each well, the incubation was carried out at 37°C for 2 h. One loopful of the reaction mixture of each fraction was expressed by the maximum dilution resulting in a 50% killing of inoculated bacteria. Percent inhibition of bactericidal activity of fractionated serum was employed by use of LPS and purified ribosomal fraction. After serial dilution of serum, 10^{-3} ml of purified ribosomal fraction or 10^{-3} ml of LPS (10^{-3} μg) was added into each well, and the microtiter plate was gently shaken before the bacterial suspension and culture medium were added. Percent inhibition in the presence of antigen was calculated as follows: Percent inhibition = [1 − (bactericidal index with antigen/bactericidal index without antigen)] × 100.

Heat stability of ribosomal fraction. Purified ribosomal fraction was heated at 60 and 80°C for 30 min, respectively, and the immunogenic activity was examined.

Immunoelectrophoresis. Pools obtained by Sephadex G-200 chromatography were examined by immunoelectrophoresis with anti-mouse immunoglobulin G (IgG), IgM, and IgA sera (Fuji Zouki, Tokyo).

RESULTS

Fractionation and bactericidal assay of serum. The elution profiles of mouse serum

chromatographed on a column of Sephadex G-200 are illustrated in Fig. 2 and 3. Figure 2 shows the pooled serum from mice immunized with crude ribosomal fraction, and Fig. 3 is obtained from chromatography of LPS-immunized mouse serum. Antibodies against crude ribosomal fraction were usually found in both pools I and II. Pool I was free of antibodies to both LPS and heat-killed whole cells of *S. typhimurium* by the PHA test (Table 1). Neither IgG nor IgA was demonstrable in pool I, and both were present in pool II on the basis of immunoelectrophoresis. The single immunoglobulin was identified as IgM with specific antiserum. This antibody was susceptible to treatment with 2-mercaptoethanol, whereas antibody detected in pool II was stable and no fall in serum titer was demonstrated. The bactericidal assay revealed the significance of bactericidal activity against *S. typhimurium* LT2 in pool II. The Sephadex separation pattern of the pooled serum was similar between the LPS-immunized and the ribosomal fraction-immunized groups. Pool IV was free of both IgG and IgA, and bactericidal activity was not significant in pool IV and V, despite the higher titer of antibodies to LPS and heat-killed whole cells.

Immunological assay of ribosomal fraction. On Sephadex G-200 gel filtration (Fig. 4), a single sharp peak was obtained with buffer A and a diffuse one was obtained with buffer B. Fractions ranging from 33 to 36, at which two elution curves overlapped each other, were concentrated to the original volume, dialyzed against buffer A, and applied to a column of Sepharose 4B. A single peak was obtained with fractions 10 to 15 (Fig. 5). Table 2 summarizes the immunogenic characteristics of these fractions. Fractions 12 and 13 were rich in RNA and free of endotoxin detected by the *Limulus* lysate coagulation test previously described (8). Fraction 12 yielded 100% protection to mice, and fraction 13 gave 90% protection. Although fraction 11 was able to raise antibodies to ribosomal fraction and yield a significant bactericidal activity of mouse serum, it gave only 60% protection to mice. Fractions 11 and 12 were able to inhibit the bactericidal activity of pooled serum from mice immunized with ribosomal fraction, but fraction 13 was unable to do so.

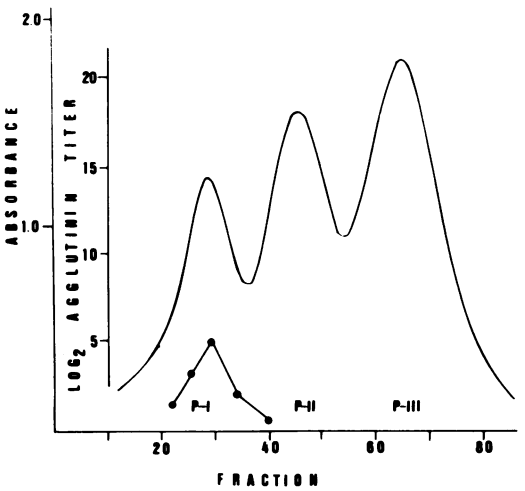

FIG. 2. *Sephadex G-200 gel filtration of pooled sera from mice immunized with crude ribosomal fraction. OD_{280} (———); agglutinin titer (●——●). Pooled region I(P-I) is fractions 22 to 32; region II(P-II) is fractions 42 to 52; and region III(P-III) is fractions 60 to 70.*

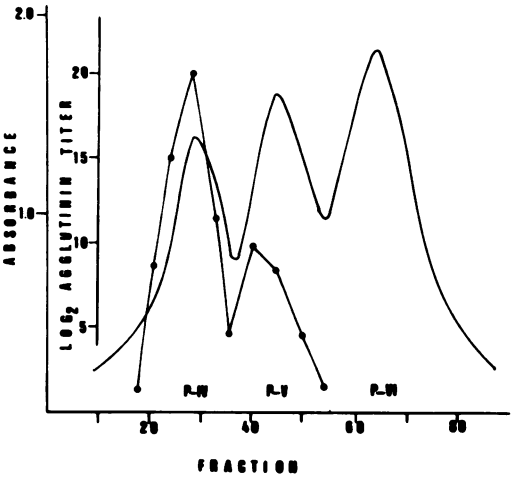

FIG. 3. *Sephadex G-200 gel filtration of pooled sera from mice immunized with LPS. OD_{280} (———); agglutinin titer (●——●). Pooled region IV(P-IV) is fractions 24 to 34; region V(P-V) is fractions 42 to 52; and region VI(P-VI) is fractions 62 to 72.*

TABLE 1. *Comparison of immunological activities of fractions obtained by Sephadex G-200 gel filtration*

Pool	Bactericidal index (log_2)	PHA titer (log_2)[a]		
		LPS	CRF[b]	PRF[c]
P-I	7	3	6	3
P-II	12	ND[d]	10	8
P-III	ND	ND	ND	ND
P-IV	4	12	2	ND
P-V	2	5	1	ND
P-VI	ND	ND	ND	ND

[a] Mean titer of 15 fractions in each pool.
[b] CRF, Crude ribosomal fraction.
[c] PRF, Purified ribosomal fraction.
[d] ND, Not determined.

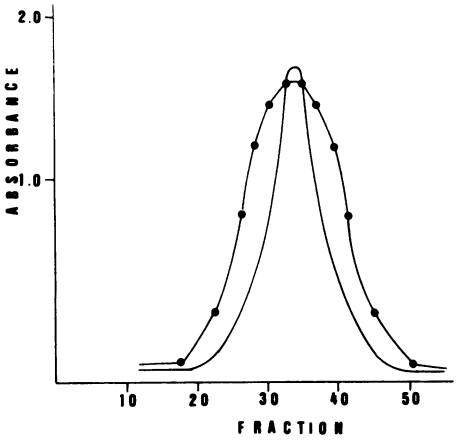

FIG. 4. *Sephadex G-200 gel filtration of crude ribosomal fraction. Elution was carried out with buffer A (———) and buffer B (●——●) at OD_{260}. Fractions 33 to 36 were pooled and further purified by Sepharose 4B gel filtration after being concentrated to original volume.*

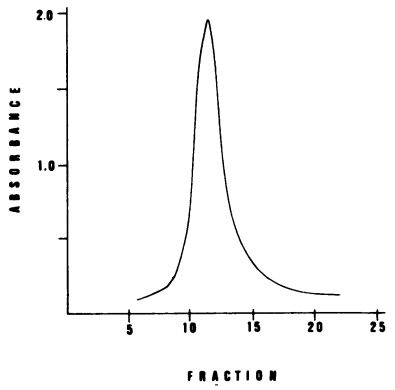

FIG. 5. *Sepharose 4B gel filtration of fractions 33 to 36 obtained by Sephadex G-200 gel filtration. Elution was carried out with buffer A, and fractions of 5 ml were collected (at OD_{260}).*

Cells and serum transfer experiments. Peritoneal cells and serum from mice immunized with each fraction obtained by gel filtration on Sepharose 4B were tested for the ability to transfer the observed immunity. Both cellular and humoral immunities were passively transferred to recipient mice (Table 3), and peritoneal cells of mice immunized with purified ribosomal fraction transferred significant protection to yield more than 50% survival rate at 60 days postchallenge. Prolongation of survival was possible by transfer of pooled serum from mice immunized with fraction 11, but all mice died at 60 days postchallenge; whereas peritoneal cells from mice immunized with fractions 12 and 13, respectively, were able to give the recipient mice

TABLE 2. *Immunogenic characteristics of fraction separated by gel filtration of Sepharose 4B*

Fraction	OD ratio (260/280)	*Limulus* lysate coagulation	Immunogenicity (%)[a]	% Inhibition[b]	Bactericidal index[c]	PHA[d]
10	0.98	+	0	0	2	ND[e]
11	1.98	+	60	80	10	8
12	2.72	−	100	30	4	5
13	2.31	−	90	0	4	4
14	1.01	+	0	0	ND	ND
15	0.88	+	0	0	ND	ND
30	0.65	+	0	0	ND	ND

[a] Mean percent survival at 40 days postchallenge. Each group consisted of 30 mice. Mice were challenged intraperitoneally with 1,000 50% lethal doses of *S. typhimurium* LT2 after immunization.

[b] Of bactericidal activity of pooled sera from mice immunized with purified ribosomal fraction.

[c] Of pooled sera from mice immunized with each fraction.

[d] Antibody titer to purified ribosomal fraction of pooled sera from mice immunized with each fraction.

[e] ND, Not determined.

TABLE 3. *Passive transfer of resistance with peritoneal cells and serum*[a]

Fraction[b]	Survival rate (%)[c] after challenge[d]		
	20 days	40 days	60 days
Cells			
9	0	0	0
10	50	20	0
11	60	30	10
12	70	50	50
13	60	50	50
14	20	0	0
15	0	0	0
PRF	70	60	60
Serum			
9	0	0	0
10	40	10	0
11	80	30	0
12	30	10	0
13	20	0	0
14	0	0	0
15	0	0	0
PRF	90	40	0

[a] Peritoneal cells and serum were collected from immunized mice and were transferred to recipients as described in the text.

[b] Each fraction and PRF (purified ribosomal fraction) contained 200 μg/injection, and two injections were given to all mice 4 days apart.

[c] Mean percent survival; each group consisted of 30 mice. $P \leq 0.025$, significantly different from yeast RNA-immunized controls.

[d] Mice were challenged intraperitoneally with 1,000 50% lethal doses of *S. typhimurium* LT2.

50% survival rate at 60 days postchallenge.

Heat stability of ribosomal preparations. Purified ribosomal fraction was heated at 60 and 80°C for 30 min, respectively, and was tested for the ability to confer protection on mice (Fig. 6). Although survival rate at 40 days postchallenge was not affected by heat treatment of purified ribosomal fraction at 60°C (Table 4), bactericidal activity of pool II was slightly lessened. Heat treatment of purified ribosomal fraction at 80°C affected bactericidality of pool II, whereas such treatment had no effect on that of pool I. Survival rate still remained 80% at 40 days postchallenge even if purified ribosomal fraction was heated at 80°C. The inhibition test showed that purified ribosomal fraction heated at 60°C inhibited the bactericidal activity of pool II, while the bactericidal index of neither pool I nor pool II was affected by ribosomal preparation heated at 80°C (Fig. 7).

DISCUSSION

In the experiments of chromatographic separation of pooled serum from the ribosomal fraction-immunized mice, bactericidal activity was significant in pool II, which is mainly equivalent to that of IgG. Antibodies against ribosomal fraction were observed in pool II with a significant titer, whereas no antibodies were found against LPS. Although no agglutinins could be demonstrable against heat-killed whole cells of

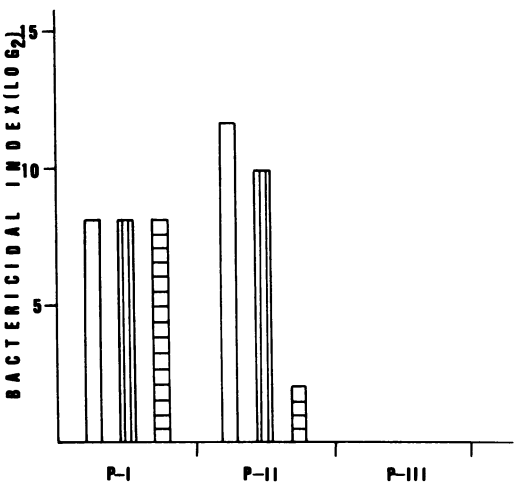

FIG. 6. *Effect of heat treatment on the immunogenicity of purified ribosomal fraction (PRF). P-I, P-II, and P-III were obtained by Sephadex G-200 gel filtration of immune mouse serum as described in Fig. 2. Mice were immunized with nontreated PRF (□), PRF heated at 60°C (▥), and PRF heated at 80°C (▤). Pooled sera of each group were tested for bactericidal activity against S. typhimurium LT2.*

TABLE 4. *Effect of heating on the immunogenicity of purified ribosomal fraction (PRF).*

Temp[a] (°C)	% Survival at 40 days[b]
60	100[c]
80	80[c]
Untreated	100[c]

[a] PRF was heated for 30 min. PRF (200 μg) was given intraperitoneally to each mouse.

[b] Each group consisted of 30 mice. Each mouse was challenged intraperitoneally with 1,000 50% lethal doses of *S. typhimurium* LT2 1 week after immunization.

[c] $P < 0.01$.

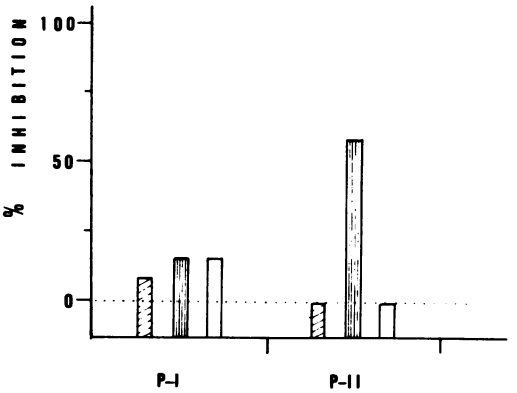

FIG. 7. *Percent inhibition of bactericidal activity of P-I and P-II by LPS (▨), and PRF heated at 60°C (▥) and at 80°C (□).*

S. typhimurium in pool II, it is not clear whether our ribosomal preparation contained any surface components of organisms. The PHA test showed a high titer of antibody to LPS in pools I and II, and antibodies to ribosomal fraction were negligible in both pools. Undoubtedly, the bactericidal activity of serum should correlate with the titer of antibodies to ribosomal fraction. Bactericidal activity observed in pooled serum of ribosomal fraction-immunized mice was heat stable and group specific as previously reported (8). In this study heat stability of ribosomal fraction was examined. Pool II was still bactericidal when purified ribosomal fraction was heated at 60°C before immunization. Heat treatment of purified ribosomal fraction at 80°C before immunization affected the bactericidal activity of pool II with a significant decline in antibody titer to purified ribosomal fraction. Bactericidal activity found in pool II was affected not by heating at 60°C but by heating at 80°C in accordance with the remarkable decline in antibody titer of mouse serum against ribosomal fraction. This phenomenon coincides with the result of another experiment that revealed the inability of purified

ribosomal fraction heated at 80°C to inhibit bactericidal activity of pool II. It is interesting that the survival rate at 40 days postchallenge remained at 80% despite the fall of the serum bactericidal activity. Another most probable explanation is that heat-stable antigens inducing cellular protection exist in the ribosomal fraction. The latter explanation coincides with the results reported by Misfeldt and Johnson (10) indicating that an additional antigen acts as the protective immunogen in their experiments. By chromatographic separation of crude ribosomal fraction on a column of Sepharose 4B, these effective fractions could be eluted, and fractions 12 and 13 were free of endotoxin at least on the basis of the *Limulus* lysate coagulation test. These fractions were rich in RNA and yielded significant protection which gave mice a more than 90% survival rate at 40 days postchallenge. Pooled sera from mice immunized with fraction 11 were found to be bactericidal to *S. typhimurium* with a moderate rise of antibody titer to purified ribosomal fraction. The immunogenicity conferred by fraction 11, however, was poor. On the other hand, fractions 12 and 13 gave a more than 90% survival rate at 40 days postchallenge to mice whose sera possessed low bactericidal activity. In the study with ribosomal antigens, the most commonly used experimental protocols employ an intraperitoneal route of infection in which antibodies developed against a minimum of contaminating cell wall fractions might be expected to exert a maximal protective effect (18). The result obtained from our previous study (8) showed our ribosomal fraction could be immunogenic even if it is parenterally administered. The role of contaminating O-antigens has been considered that of the protective antigens in ribosomal fraction, but it might play a minor role in the immunogenicity of ribosomal vaccines. In addition to our experiments, there was a report that the O-antigens from *Salmonella*, either in combination with isolated cell walls or incorporated with adjuvants, failed to induce the level of immunity by *Salmonella* ribosomal vaccines. In this study a fair amount of O-antigens existed in a crude ribosomal fraction, but there was no difference found in the immunogenicity between the crude fraction and Sepharose 4B fractions 12 and 13 in which endotoxin was not detected by the *Limulus* lysate test. The *Limulus* lysate test is probably the most sensitive measure for endotoxin, but it lacks specificity. Although the *Limulus* lysate test was negative in fractions 12 and 13, it cannot be said that these fractions were free from O-antigens. In this study the *Limulus* lysate test was employed to show that O-antigens existing in these fractions were present in amounts too small for detection by conventional analytical methods. It is important to recognize that a total amount of O-antigens present in fractions 12 and 13 was less than that found in fractions capable of evoking immune response. Recently a mutant of strain LT2 unable to synthesize O-antigen without galactose in the medium was used to prepare ribosomal vaccine (5), and it was shown that this kind of vaccine failed to induce protective immunity in mice. We have already confirmed that ribosomal vaccines derived from complete rough mutants of strain LT2 were not protective. At the same time O-antigens prepared from *S. typhimurium* were not protective when administered by incorporating them into ribosomal fractions derived from a rough mutant of strain LT2 (unpublished data). Our results disagree with those of Eisenstein (3), Hoops et al. (4), and Lim et al. (5). This difference can be explained by the hypothesis that rough and cell wall-deficient mutants of strain LT2 might lose the ability to synthesize protective antigens attached to ribosomes as they become more rough (8). Especially enzymes related to the synthesis of specific sugars of O-antigens seem to have close relations to the synthesis of protective antigens in ribosomal fraction. In our ribosomal fraction these kinds of protective antigens are heat stable, able to induce cellular immunity, and seem to be bound to ribosomes existing in fractions 12 and 13. Studies are in progress to fully isolate and characterize the heat-stable immunogens by the method of electrophoretic separation.

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