Biologically Active Endotoxins from *Salmonella* Mutants Deficient in O- and R-Polysaccharides and Heptose

YOON BERM KIM AND DENNIS W. WATSON

Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455

Well-characterized *Salmonella* mutants formerly used in biosynthetic studies of lipopolysaccharides were used to study the toxic portion of the complex endotoxin. Endotoxins prepared from wild types and their mutants were tested for their biological activities, including pyrogenicity, lethality, and immunogenicity. There was little difference either in the endotoxin yields or in the toxicities between endotoxins from the wild-type and O-antigen deficient mutants. Endotoxin containing mostly lipid A and keto-deoxyoctonate (KDO) prepared from the mutant deficient in both O- and R-antigens and the backbone sugar, heptose, was biologically active. Possibly because of the difference in solubility in water, the yield of endotoxin from the heptoseless mutant was about 10% of the wild type. There was complete reciprocal cross-immunity between all endotoxins tested. These observations suggest that the common toxic moiety is not present in the O- and R-polysaccharides or the backbone sugar heptose, but rather is associated with the lipid portion of the molecule which includes mostly lipid A and KDO.

In previous papers in this series (7, 8, 17, 18), evidence was presented for the presence of two interdependent biological activities in the endotoxin molecule. Moreover, acquired resistance to the various toxic reactions of endotoxins, including pyrogenicity and lethality, results from a classical immunological mechanism; this involves 19S antibodies specific for the toxic group common to many endotoxins but distinct from O-specific polysaccharides; in addition, the normally functioning reticuloendothelial system is required (2, 4, 5).

Recently, known mutants of *Salmonella* used in the study of biosynthesis of lipopolysaccharides by the biochemists and geneticists became available to study the immunobiological activities of endotoxins prepared from the various mutants (9, 12–14, 16).

This paper presents additional evidence that O-polysaccharides as well as R-polysaccharides and heptose are not functional in the primary toxicity of endotoxin and suggests that the common toxic moiety of endotoxin is associated with the lipid portion of the molecule which is immunogenic, as reported previously (7, 8, 17, 18).

MATERIALS AND METHODS

Animals. Young adult American Dutch rabbits (3 months old, 1.0 to 1.2 kg) were used for pyrogenicity and cross-immunity assays. Adult Balb/Sy mice (10 weeks old) were used in lethal toxicity assays. All animals were quartered in air-conditioned rooms, about 75 F (23.9 C), and all experiments were performed in an adjoining laboratory at the same temperature as previously described (7, 17). In all experiments, animals were distributed with random sampling procedures by use of a table of random numbers (3).

Preparation of endotoxins. The characteristics of the strains used for the preparation of endotoxin are given in Table 1. *S. typhimurium* wild type Lt2 and its rough mutant TV119 were kindly given to us by M. J. Osborn, Albert Einstein College of Medicine, Bronx, N.Y., (14). *S. minnesota* wild type S and its heptoseless R595 were generously supplied by O. Lüderitz and O. Westphal, Max-Planck-Institut für Immunbiologie, Freiburg, Germany (9). After being checked for purity, these strains were preserved by lyophilization. Inoculum from an individual vial was used for each culture. Bacterial cells were cultured in 2 liters of Trypticase Soy Broth (BBL) for 24 hr at 37 C in 2.8-liter Fernbach flasks in a gyrotory shaker. Contents of 16 flasks were pooled, and the cells were harvested in a Serval continuous-flow centrifuge. The cells were resuspended in pyrogen-free sterile distilled water and recentrifuged; this was repeated and the packed cells were weighed and lyophilized. Endotoxins, with the exception of those from *S. typhosa* 0901 (Difco), were prepared by the hot-phenol water extraction method of Westphal et al. (19, 20). Because of discrepancies in the literature relative to the biological properties of endotoxins from similar R-mutants (6) and because these differences might be accounted for by slight variations in the method of purification, the detailed method used to purify these toxins is pre-

TABLE 1. *Characteristics of strains used for the preparation of endotoxins*

Species	Strain	O-specific polysaccharides			R-common polysaccharides			Backbone	Source
		Sero-group	O-antigen	S-chemo-type	R-sero-type	R-geno-type	R-chemo-type	Chemotype	
Salmonella ty-phimurium	Wild type Lt₂ Mutant TV₁₁₉	B	1, 4, 5, 12 Absent	XIV	R_{II}	rou B	Ra		Osborn et al. (14)
S. minnesota	Wild type S Mutant R₅₉₅	L	21 Absent	II		Absent		Re (hep-tose-less)	Lüderitz et al. (9)
S. typhosa[a]	0901	D	9, 12	XVI					

[a] Lipopolysaccharide (W) obtained from Difco.

sented diagrammatically in Fig. 1. Because the presence or absence of heptose in the purified endotoxin from *S. minnesota* mutant R₅₉₅ (Re) could be used to determine possible back-mutation, the purified endotoxin prepared from this mutant and used in these experiments was assayed for heptose by Otto Lüderitz of the Max-Planck-Institut. The preparation was free of heptose, indicating that the strain had not back-mutated.

Details of the methods for the preparation of stock and working solutions of endotoxins, determination of pyrogenicity [minimal pyrogenic dose, 3 hr (MPD-3)] in rabbits and of lethality in mice, and cross-immunity tests and passive protection experiments have been described previously (7, 8, 17, 18). All the reagents, glassware, and equipment were pyrogen-free and sterile.

RESULTS

Characteristics of endotoxins from Salmonella mutants deficient in O- and R-polysaccharides. As summarized in Table 2, *S. typhimurium* TV₁₁₉ containing R-antigen but deficient in O-antigen (14) yielded toxin in equal quantity and with comparable activity to that of the wild type. Endotoxins from the *S. minnesota* heptoseless mutant R₅₉₅ (Re), deficient in both O- and R-antigens containing mostly lipid A (70%) and keto-deoxyoctonate (KDO 17%) as determined by Lüderitz et al. (9), were biologically active. However, the yield of toxin from the heptoseless mutant was about 10% when compared with that of the wild type S. This could be explained by the decreased solubility of the toxin in water resulting from the high lipid content in the absence of hydrophilic O- and R-polysaccharides; the toxin, therefore, was lost during the isolation procedure especially since the water phase of the phenol-water extract was used. For instance, when R₅₉₅ endotoxin solutions were prepared by first dissolving the toxin in pyridine as in the procedures used to prepare lipid A solution (11, 17, 21), pyrogenic activity

was increased twofold [MPD − 3 = 0.003 μg/kg, intravenous (iv)], and, furthermore, lower molecular weight endotoxin (1,263 mg with a MPD − 3 = 0.03 μg/kg, iv) was lost in the supernatant fluid after ultracentrifugation at 78,000 × g for 60 min.

Representative febrile response curves for titration of pyrogenic activity of endotoxins prepared from *S. minnesota* wild type S and its heptoseless mutant R₅₉₅ are given in Fig. 2 and 3, and the determinations of MPD-3 are given in Fig. 4. The mean febrile response at 3 hr is plotted against the log of the concentration. The regression line is drawn to 1 F, and the quantity of toxin at the intercept represents the MPD-3. Both endotoxins from wild type S and heptoseless mutant R₅₉₅ gave a parallel linear dose-response, and MPD-3 values were 0.0045 μg/kg, iv, and 0.006 μg/kg, iv, respectively.

Lethal toxicity of these two endotoxins was tested in mice by iv and intraperitoneal (ip) routes of injection. The results of duplicate experiments are given in Table 3; LD₅₀ doses of S and R₅₉₅ were 405 μg/kg (iv) or 400 μg/kg (ip) and 825 μg/kg (iv) or 1,100 μg/kg (ip), respectively.

Immunogenicity of endotoxins deficient in O- and R-polysaccharides. The immunogenicity of these endotoxins to the pyrogenic activity of endotoxin was determined in rabbits. For this, five different endotoxins were used to compare the antigenic relationships by reciprocal cross-immunity tests. Representative results are given in Fig. 5 and 6. As shown in Fig. 5, 15 rabbits were immunized with *S. minnesota* S endotoxin with stepwise increasing doses (17) until they were immune to 100 MPD-3 (0.45 μg/kg, iv) of the homologous endotoxin; they were then cross-tested with a 100 MPD-3 dose of heterologous endotoxins, including those from heptoseless mutant R₅₉₅ (0.6 μg/kg, iv), *S. typhimurium* wild type Lt₂ (0.24 μg/kg, iv)

Steps

1. Hot phenol water extraction: *Lyophilized bacteria* (10 g)
Suspended in 350 ml of pyrogen-free sterile distilled water (H_2O) 66 C; added 350 ml of liquefied phenol (88% Mallinckrodt analytical reagent), 66 C; stirred the mixture continuously 20 min, 66 C, and cooled to 5 C; centrifuged at 1,500 × *g* for 30 min, 4 C.

2. Aqueous phase:

Aqueous phase
Pooled; ←————— dialyzed against H_2O, 4 C; concentrated by pervaporation (100 ml); dialyzed against H_2O to remove residual phenol; centrifuged at 1,500 × *g* for 20 min, 4 C.

Phenol phase and remainder
Re-extracted with 300 ml of H_2O at 66 C for 20 min; cooled to 5 C and centrifuged at 1,500 × *g* for 30 min, 4 C

Aqueous phase

Phenol phase and remainder (discard)

3. Alcohol precipitation:

Supernatant fluid
Added 100 mg of sodium acetate and added 10 volumes of cold ethyl alcohol (−20 C); mixed and allowed to stand overnight in refrigerator, 4 C; removed supernatant fluid by suction and collected precipitate; centrifuged at 1,500 × *g* for 20 min, 4 C

Sediment (discard)

4. Purification:

Supernatant fluid (discard)

Precipitate
Dissolved in H_2O (200 ml); centrifuged at 78,000 × *g* for 60 min, 4 C (Spinco 30) (repeated this procedure 5 to 7 times until free of 260 mμ absorbing materials)

Supernatant fluid (discard)*

Sediment
Dissolved in H_2O (200 ml); centrifuged at 1,500 × *g* for 20 min, 4 C

Supernatant fluid
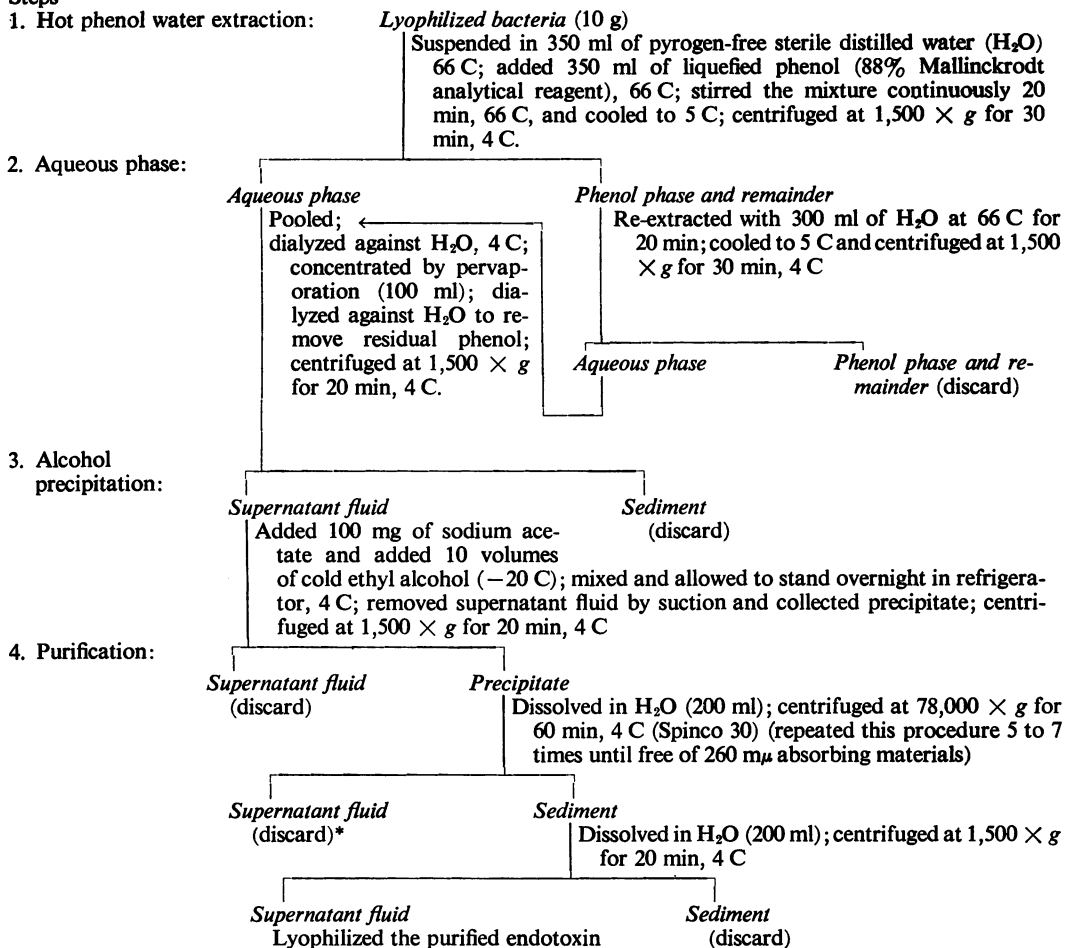Lyophilized the purified endotoxin

Sediment (discard)

FIG. 1. *Preparation of endotoxins. Asterisk represents Salmonella minnesota R_{595} preparation that was dialyzed against sterile distilled water (H_2O) and lyophilized.*

TABLE 2. *Characteristics of endotoxins from Salmonella typhimurium wild type Lt_2 and its mutant TV_{119} (Ra) and S. minnesota wild type S and its heptoseless mutant R_{595} (Re)*

Properties	S. typhimurium		S. minnesota	
	Wild type Lt_2	Mutant TV_{119} (rou B, R_{II} . Ra)	Wild type S	Mutant R_{595} (Re)
Wet weight of cells (g)	187	167	169	115
Dry weight of cells (g)	42	35	35	27
Dry weight (%)	22	21	21	23
Endotoxin (mg)	1,258	1,052	1,490	166 (1,263)[a]
Yield dry weight (%)	3	3	4	0.6 (5.5)[a]
Biological activities				
MPD-3 rabbit (μg/kg, iv)	0.0024	0.0025	0.0045	0.006 (0.03)[a] (0.003)[b]
LD_{50} mice (μg/kg, iv)	350	460	405	825
LD_{50} mice (μg/kg, ip)	325	405	400	1,100
Immunogenicity	+	+	+	+

[a] Low molecular weight endotoxin present in the supernatant fluid of ultracentrifugation at 78,000 × *g* for 60 min (see Fig. 1).

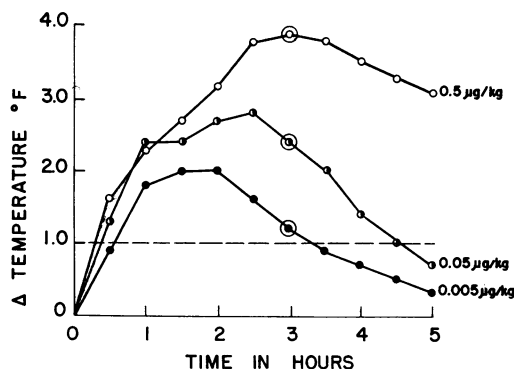[b] Dissolved first in pyridine; see detailed method described to make a solution of lipid A (17).

FIG. 2. *Titration of endotoxin from Salmonella minnesota wild type S for MPD-3. Each curve represents the mean febrile response of five rabbits injected iv with indicated concentrations of endotoxin from S. minnesota wild type S.*
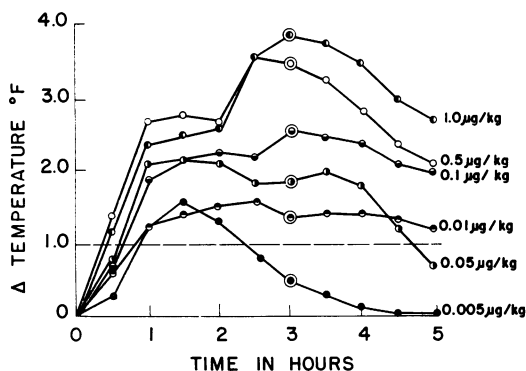

FIG. 3. *Titration of endotoxin from Salmonella minnesota mutant R_{595} for MPD-3. Each curve represents the mean febrile response of five rabbits injected iv with indicated concentrations of endotoxin from S. minnesota mutant R_{595}.*

and its mutant TV_{119} (0.25 µg/kg, iv), and *S. typhosa* 0901 (1.0 µg/kg, iv). There was complete cross-immunity, indicating resistance against a common toxic moiety.

Figure 6 shows that animals immune to mutant R_{595} endotoxin, which is devoid of all O- and R-polysaccharides and backbone sugar heptose, were also immune to its wild-type endotoxin as well as to heterologous endotoxins from *S. typhimurium* wild type Lt_2 or its mutant TV_{119} endotoxin and *S. typhosa* 0901 endotoxin. The reciprocal cross tests with all other toxins also gave complete cross-immunity.

Passive protection in mice against lethal effect of S. minnesota wild type S endotoxin by R_{595} endotoxin-immune rabbit serum. Table 4 gives results showing passive protection against lethal
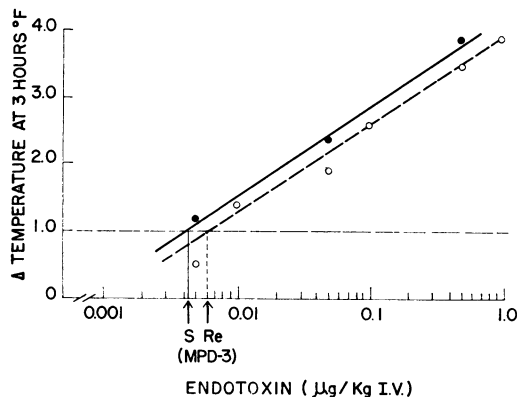

ENDOTOXIN (µg/ Kg I.V.)

FIG. 4. *Determination of MPD-3 of Salmonella minnesota S and mutant R_{595} endotoxins. Each point represents the mean febrile response of five rabbits 3 hr after iv injection with indicated concentrations of endotoxin of S. minnesota wild type S (closed circle) or heptoseless mutants R_{595} (open circle). The linear regression line is drawn to 1 F, and the quantity of toxin at the intercept is the MPD-3; the continuous line for wild type S and the discontinuous line for the mutant R_{595} give MPD-3 values of 0.0045 and 0.006 µg/kg, iv, respectively.*

toxicity of wild-type endotoxin by immune sera from rabbits immunized with endotoxin free of O- and R-polysaccharides. These data also show that the toxic moiety of endotoxin is associated with neither O- and R-polysaccharides nor the backbone sugar, heptose, of the molecule. It appears rather to be with the lipid portion of the molecule which includes lipid A and KDO.

DISCUSSION

Evidence for the presence of two interde pendent activities in the endotoxin molecule has been reported (17, 18). The intrinsic or primary toxicity is associated with the lipid portion of the molecule, and the secondary toxicity results from the acquisition of hypersensitivity of the host to some portion of the molecule, usually as a result of contact with intestinal flora or clinical and subclinical infections. We have continued to stress the interdependence of the primary and secondary toxic activities and the importance of taking into consideration the immunological state of the host in any evaluation of the mechanism of endotoxin action (7, 8, 17, 18). Also, there is unequivocal evidence of a true primary or intrinsic toxicity of endotoxin which is completely independent of antigen-antibody reactions, as demonstrated by the lethal toxicity of endotoxin in germ-free, colostrum-deprived

TABLE 3. *Lethal effect of endotoxins from Salmonella minnesota S and heptoseless mutant R595 (Re) in mice*

Endotoxin (ug/mouse)	Mortality (dead[a]/total)							
	Wild type S				Mutant R595 (Re)			
	iv		ip		iv		ip	
	Expt 1[b]	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
100	0/10	0/10	0/10	0/10				
200	3/10	2/10	2/10	2/10	0/10	0/10	0/10	0/10
400	5/10	6/10	4/10	6/10	2/10	2/10	2/10	1/10
800	7/10	8/10	8/10	7/10	5/10	4/10	4/10	3/10
1,600	9/10	9/10	10/10	9/10	8/10	8/10	5/10	6/10
LD$_{50}$ (μg)	420	390	430	400	800	850	1,050	1,150

[a] Number of deaths determined for 48 hr after the injection.
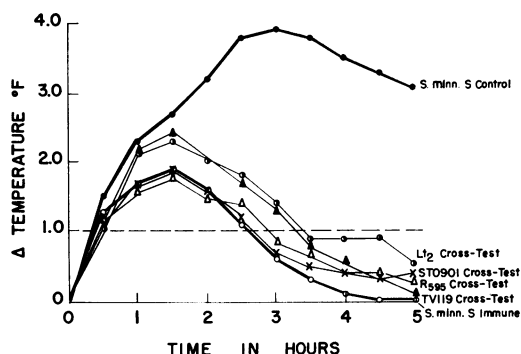[b] Experiments 1 and 2 are duplicate experiments.

FIG. 5. *Cross-immunity between endotoxins. Salmonella minnesota wild-type S endotoxin-immune rabbits tested with its heptoseless mutant R595 endotoxin and heterologous S. typhimurium Lt2 and TV119 and S. typhosa 0901 endotoxins. Each curve represents the mean febrile response of 5 to 15 rabbits injected iv with 100 MPD-3 per kg of endotoxin.*
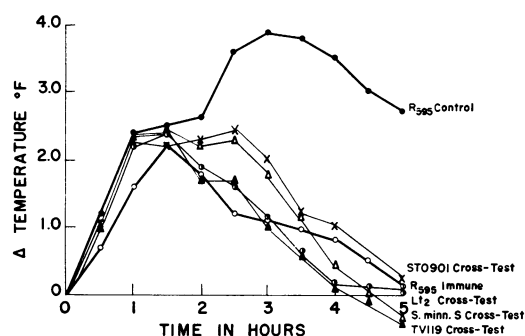

FIG. 6. *Cross-immunity between endotoxins. Salmonella minnesota heptoseless mutant R595 endotoxin-immune rabbits tested with its wild-type S endotoxin and heterologous S. typhimurium Lt2 and TV119 and S. typhosa 0901 endotoxins. Each curve represents the mean febrile response of 5 to 15 rabbits injected iv with 100 MPD-3 per kg of endotoxin.*

piglets lacking antibodies ("immunologically virgin"; 8).

There is significant evidence that the O-specific polysaccharide plays no direct role in primary toxicity. Therefore, antibodies directed against the O-specific determinants (anti-O) play no role in endotoxin immunity (7, 8). Our results support several investigations (1, 10, 22) which preclude the role of anti-O in endotoxin immunity.

More critical experiments have been possible by the use of endotoxins prepared from the mutants deficient in their ability to synthesize O- and R-polysaccharides. These mutants have been isolated and well-characterized by the geneticists and biochemists interested in bio-

synthetic mechanisms (9, 12–14, 16) and therefore become useful tools for determining the nature of the toxic moiety within the complex lipopolysaccharide. Our first experiments involved endotoxin prepared from S. typhimurium mutant TV119 which could not synthesize the O-specific polysaccharide, but contained the R-polysaccharides of the basal core. As summarized in Table 2, this mutant gave yields of active toxin comparable to that produced by the wild type (8), indicating that the O-polysaccharide was not responsible for the toxicity of the endotoxin. Experiments reported here used S. minnesota mutants deficient not only in O-polysaccharide but also in the basal core R-polysaccharide and the backbone sugar, heptose. Although the yield of endotoxin was approximately 10% of

TABLE 4. *Passive protection in mice against lethal effect of Salmonella minnesota S endotoxin by R_{595} endotoxin-immune rabbit serum*

Treatment[a] 24 hr before injection of endotoxin (1.0 ml ip)	Mortality (dead[b]/total) for *S. minnesota* S endotoxin (μg/mouse)						LD$_{50}$ (μg /mouse)	
	400		1,000		2,000			
	iv	ip	iv	ip	iv	ip	iv	ip
Phosphate-buffered saline................	5/10	6/10	9/10	8/10	9/10	10/10	450	400
Normal rabbit serum....	5/10	4/10	7/10	8/10	9/10	9/10	550	560
R_{595} endotoxin-immune rabbit serum..........	0/10	2/10	5/10	2/10	6/10	7/10	1,300	1,350

[a] At 24 hr before injection of endotoxin, 1.0 ml administered ip.
[b] Number of deaths determined for 48 hr after the injection.

that obtained from the wild-type smooth strain, the activity of the endotoxin as determined by pyrogenicity, lethality, and immunogenicity was only slightly less than found for the wild-type endotoxin. This could be explained by the high lipid content without hydrophilic O- and R-polysaccharides which contribute to the solubility of the endotoxins. For instance, when the R_{595} endotoxin solution was prepared by first dissolving in pyridine (11, 17, 21) to give a more stable suspension in 0.5% pyrogen-free dextran buffered saline, the pyrogenic activity was increased twofold (Table 2). The lower yield of R_{595} (Re) endotoxin from this polysaccharide-free mutant can be explained by low solubility in the aqueous phase during the phenol-water extraction procedure, and, because strains are deficient in the carrier polysaccharides, lower molecular weight endotoxins are lost in the supernatant fluid after centrifugation at 78,000 × g, during the procedure used for the purification of endotoxins (Table 2).

Concomitant with the presentation of our studies (Bacteriol. Proc., p. 49, 1966), Kessel et al., using other heptoseless mutants (G-30/C21) derived from *S. typhimurium*, reported (6; Bacteriol. Proc., p. 49, 1966) that material obtained by the phenol-water method was not toxic. As a result, they placed greater emphasis on the role of polysaccharides in the primary mechanism of endotoxin activity. The discrepancy in the results can be explained not only by species differences, but also by possible differences in extraction and isolation or assay procedures (6). As already stressed, as these mutants become deficient in hydrophilic polysaccharide, thereby increasing the ratio of hydrophobic lipid, the procedures involved in extraction, isolation, and assay become more critical.

It seems apparent that the intrinsic or primary toxicity of endotoxin resides with the lipid-KDO

complex which is present in both the wild-type and rough mutants of gram-negative bacteria. It is possible, however, that the hydrophilic O- and R-polysaccharides complexed with the hydrophobic toxic moiety act as a carrier and influence their solubility (21), macromolecular size, and secondary toxicity, depending on the immunological state of the host (15, 17, 18). Because these two biological activities are interdependent in the host-reaction (17, 18), anti-O or anti-R may influence secondarily hyper or hypo reactions to endotoxin.

There is good evidence that the O-specific polysaccharides play a role in infectivity, but are insignificant in the direct toxicity. It is apparent, therefore, that antiendotoxin is distinct from anti-O and, that to protect against endotoxin shock, therefore, requires antibody against the toxic moiety common to most gram-negative bacterial endotoxins.

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