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Relation of Polysaccharide Content to Some Biological Properties of Endotoxins from Mutants of *Salmonella typhimurium*

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ABSTRACT

KESSEL, R. W. I. (Rutgers, The State University, New Brunswick, N.J.), HENRY H. FREEDMAN, AND WERNER BRAUN. Relation of polysaccharide content to some biological properties of endotoxins from mutants of *Salmonella typhimurium*. J. Bacteriol. 92:592–596. 1966.—Endotoxins were extracted by the phenol-water procedure from a variety of *Salmonella typhimurium* mutants with known differences in the composition of their cell wall polysaccharides. The lethality of these preparations for mice proved to be correlated with the complexity of the polysaccharide: endotoxin from the smooth parent strain and from rough strains with several sugars attached to the heptose-phosphate backbone were of high toxicity, whereas endotoxin from a mutant possessing only glucose attached to the heptose-phosphate backbone was less toxic, and endotoxin from a mutant possessing the backbone only was least toxic. All of these mutants yielded endotoxins that were equally capable of protecting mice against subsequent challenge with *Pseudomonas aeruginosa*. Material obtained from a heptoseless mutant by the phenol-water method proved to be neither toxic nor protective. The apparent dissociation of biological properties that can be achieved with the aid of endotoxin preparations from certain mutants is discussed in terms of possible mechanisms.

The availability of *Salmonella typhimurium* mutants with known mutational blocks in the biosynthesis of cell-wall lipopolysaccharides (6, 8, 9, 10) provided an opportunity to investigate some of the biological properties of endotoxin preparations isolated from such mutants. Others previously tested the lethal effect of killed cells and of endotoxins from some mutant strains (7). They reported that endotoxins derived from a number of mutant strains differ in yield per cell but, when adjusted to equal weight, show no difference in lethality for mice. In these earlier investigations, no data were collected in regard to the capability of endotoxin preparation derived from different mutants to increase host resistance to gram-negative infections ("protection"). In the present study, both lethal effects and the protective activity against *Pseudomonas aeruginosa* infections were determined in mice for endotoxins derived from wild-type *S. typhimurium* cells and from several mutants thereof with known differences in cell wall constitution (Fig. 1.)

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MATERIALS AND METHODS

The strains employed are listed in Table 1. All of the cultures were checked for colonial morphology; since Nikaido's LT2 contained two colony types, this culture was purified by picking a single colony isolate of the smoother type.

Endotoxins from these mutants were extracted by the Westphal phenol-water method (12) from cells grown for 24 hr on nutrient agar. Differences in the carbohydrate nature of the lipopolysaccharides extracted from the mutant strains were confirmed by the Dische methods (2), and all preparations were examined for absorption at 260 mμ. A commercial *Escherichia coli* O127:B8 endotoxin (Difco), extracted by the Boivin procedure, was employed as a reference standard for bioassays.

Lethality tests were performed as follows. Female, HaICR mice weighing 20 ± 0.5 g were held at 37 C for 4 hr, at which time graded amounts of the endotoxins were injected intraperitoneally (0.2 ml). The animals were held at 37 C for an additional 24 hr, and deaths

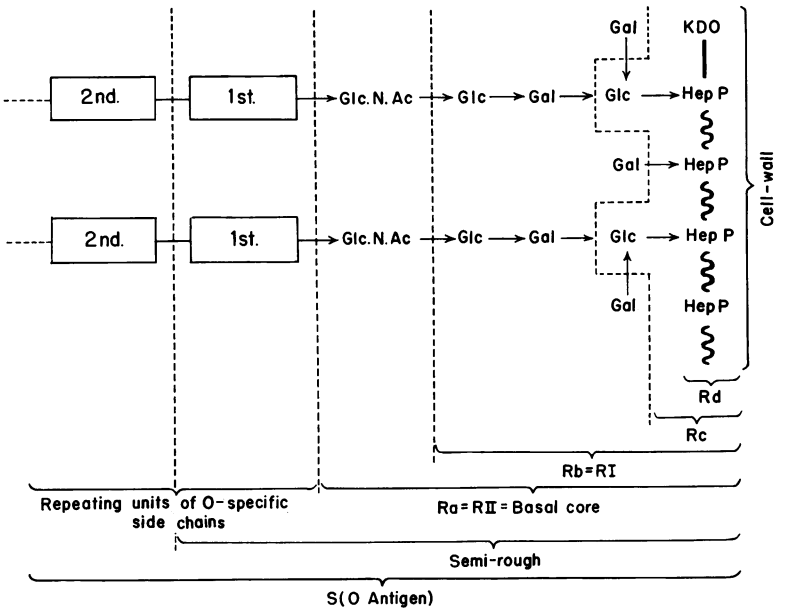

FIG. 1. *Suggested structure of Salmonella O and R antigens. The dotted lines indicate where biosynthesis is blocked in different R mutants derived from S strains; the respective R mutants (chemotypes Ra, Rb, Rc, etc.) synthesize only the part of the O antigen that is on the right side of the respective dotted line. Depending on the chemotype (or serotype), the repeating units of the O-specific side chains contain sugars such as mannose, rhamnose, galactose, and abequose. The semirough mutant contains only one repeating unit in the polysaccharide of its cell wall. Hep = heptose; KDO = 2-keto-3-deoxyoctonate; Glc = glucose; Gal = galactose; Glc.N.Ac. = N-acetyl-glucosamine (6).*

TABLE 1. *Description and sources of Salmonella typhimurium mutants employed*

Designation	Strain no.	Cell wall composition	Source
S	LT2	Heptose-P backbone, basal core, and side chains	Nikaido (8)
S	LT2 (698)	Heptose-P backbone, basal core, and side chains	Stocker via Rothfield (9)
Ra (RII)	TV-119	Lacks O-specific side chains	Nikaido (8)
Rb (RI)	TV-148	Lacks side chains and glucosamine	Nikaido (8)
Rb (RI)	TV-161	Lacks side chains and glucosamine	Nikaido (8)
Rc	LT2-M1	Uridine diphosphate galactose-4-epimeraseless	Nikaido (8)
Rc	G-30	Uridine diphosphate galactose-4-epimeraseless	From LT2-Zinder via Rothfield (9)
Rd	SL-1032	Heptose-P backbone only	From LT2 (698) via Osborn (9)
—	G-30/C21	Heptoseless (2-keto-3-deoxy-octonate and lipid only)	Osborne (9)

were recorded at 1, 2, 4, 6, and 24 hr after injection. (No deaths were ever observed in the first 2 hr.)

Protection tests were carried out with mice held at room temperature, injected intraperitoneally (0.2 ml) with graded amounts of endotoxin 24 hr prior to challenge with 20 LD_{50} of *P. aeruginosa* 25-A2 (2×10^8, intraperitoneally in 0.2 ml). Deaths were recorded daily for 4 days.

In any given experiment, protectivity and lethality tests were always carried out in parallel with mice from a single batch and with 10 mice per group. The LD_{50} values were calculated from the regression lines.

RESULTS

Preliminary tests on lethality and protectivity utilized all of Nikaido's strains cited in Table 1.

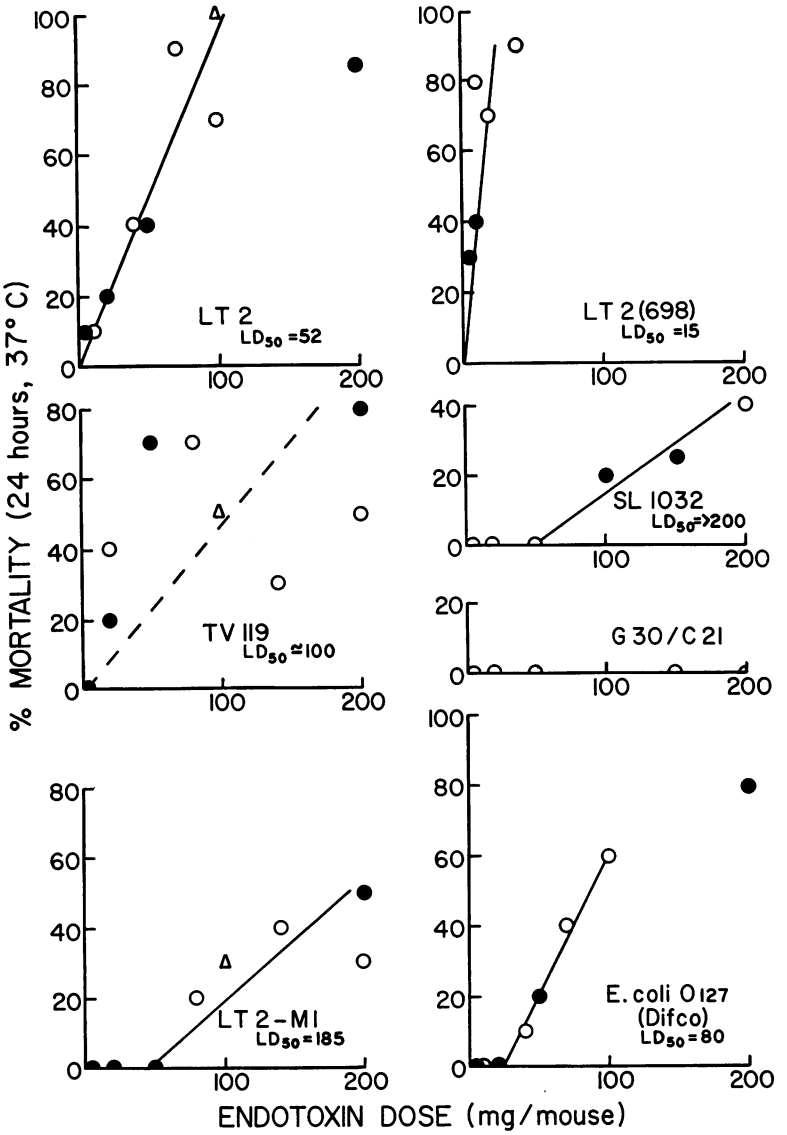

FIG. 2. *Lethality of endotoxins from mutants of Salmonella typhimurium for mice held at 37 C. See text and Fig. 1 for descriptions of the strains employed.*

TABLE 2. *Yields and properties of endotoxins prepared from Salmonella typhimurium LT2, TV-119, and LT2-M1*

Strain	Total bacterial yield[a] (viable)	Endotoxin recovered	Presence (+) or absence (−) of hexoses			Optical density (260 mμ)[b]
			Glucose	Galactose	Mannose	
		mg				
LT2	3.4×10^{12}	105	+	+	+	0.162
TV-119	4.1×10^{12}	83	+	+	−	0.500
LT2-M1	10.9×10^{12}	59	+	−	−	0.550

[a] After washing of cells harvested from 24 hr of growth on nutrient agar in Blake bottles.
[b] Concentration of samples: 62.5 μg/ml.

TABLE 3. *Effect of endotoxins isolated from Salmonella typhimurium LT2, TV-119, LT2-M1, LT2(698), SL1032, and G30/C21 and from Escherichia coli O127 on resistance to infection with Pseudomonas aeruginosa (1.8 × 10⁸, intraperitoneally)*[a]

Dose employed (μg/mouse)	Source of endotoxin																
	LT2				TV-119			LT2-M1				LT2 (698)	SL 1032	G30/C21	*E. coli* O127		
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2	Expt 3	Expt 4				Expt 1	Expt 2	Expt 3[b]	Expt 4
20	—	—	—	—	—	—	—	—	—	—	—	—	—	—	90	—	—	—
10	90	100	80	—	100	100	90	90	100	100	—	—	—	—	80	90	78	—
2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	50	—	—	—
1	80	70	70	80	100	90	100	90	80	90	80	90	100	0	—	80	22	100
0.1	—	60	80	40	—	70	90	—	80	70	70	70	40	0	—	80	22	20
0.01	—	40	40	10	—	70	100	—	100	60	30	50	0	0	—	60	0	0
0.001	—	—	30	30	—	—	70	—	—	40	0	20	0	10	—	—	11	10
0.0001	—	—	—	10	—	—	—	—	—	—	10	10	0	10	—	—	—	30
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Results are expressed as the per cent survival at 4 days.
[b] Nine mice per group. All other groups were of 10 mice.

These tests revealed similar biological properties for all the strains tested, except that endotoxin from LT2-M1 (Rc) appeared to be far less toxic than endotoxins from the other strains. Accordingly, this strain and strain TV-119 (Ra), as well as the wild-type S parent strain LT2, were selected for further investigation. Data regarding yields and chemical properties of endotoxins from these strains are listed in Table 2. Lethality tests with such endotoxins confirmed that the uridine diphosphate - galactose (UDPgal) - 4 - epimeraseless Rc mutant (LT2-M1) yielded an endotoxin with significantly less lethality for mice than either the parental wild type or the galactose- and glucosamine-containing Ra mutant, TV-119 (Fig. 2). There was an approximately fourfold difference in LD_{50} between LT2-M1 and LT2 (the data do not permit a comparable calculation of LD_{50} for TV-119). In addition, it was found that endotoxin from the Rd mutant (SL1032) was of extremely low toxicity (Fig. 2). Extracts of the heptoseless mutant (G30/C21) produced no toxicity even at 200 μg per mouse (Fig. 2).

Table 3 summarizes data on the endotoxin-elicited increase in resistance to infection with *P. aeruginosa*. Except for the extract from the heptoseless mutant (G30/C21), which failed to produce any protection, no significant differences in protectivity were noted in several separate trials. The lack of a clear dose response, such as is apparent in the data of Table 3, is frequently encountered in such tests with bacterial endotoxins (11).

DISCUSSION

It is apparent that there are significant differences in mouse lethality of endotoxins from related mutant types and that these differences are correlated with the complexity of the respective cell wall polysaccharides. No toxic effects were encountered with material extracted by the phenol-water procedure from heptoseless mutants, and relatively low toxicity characterized endotoxins from mutants that possessed the heptose-phosphate backbone only or from mutants that possessed this backbone plus glucose. Toxicity is more pronounced when additional sugars are part of the polysaccharide molecule of the mutant type. Since endotoxin from the Rothfield LT2(698) strain displayed greater toxicity than the Nikaido LT2 endotoxin and since the "heptose only" mutant (SL1032) originated from Rothfield's LT2(698), whereas LT2-M1 was derived from Nikaido's LT2, the data suggest that the toxicity of the more "naked" SL1032 may be less than the glucose-containing material from LT2-M1. It is perhaps noteworthy that an independently derived UDPgal-4-epimeraseless mutant, derived from another wild strain, also displayed low toxicity, which suggests that the differences are mutant-dependent rather than strain-dependent.

It is well established that, for any given preparative method, individual batches of endotoxin derived from the very same strain can differ considerably in their toxicity for mice. However, it should be noted that the differences in LD_{50} observed with the preparations derived from some of the mutants here tested are significantly greater than the slight differences that were found in the present study among individual endotoxin batches from the same mutant.

To work with available amounts of endotoxins, all of the lethality titrations reported here were

with mice that had been exposed to 37 C to elevate their sensitivity to endotoxins (3). However, there are no known reasons to assume that the differences noted under these environmental conditions are not also reflecting differences obtainable with larger amounts of endotoxin at lower ambient temperatures.

In regard to the ability to protect against subsequent infection with *P. aeruginosa*, all preparations, except that from the heptoseless mutant (G30/C21), performed equally well. (Preliminary studies indicate that, as far as materials from TV-119 and LT2-M1 are concerned, the same results are obtained with *S. enteriditis* or *S. typhosa* TY2 as challenge agent.) The possibility remains that some differences in protective capacities might be detected among endotoxins from different *Salmonella* mutants under conditions where dose-response curves are steeper than in the present experiments. However, to judge from the data available here, it would appear that a dissociation of toxic and protective effects may be achieved with the aid of endotoxins from certain sugar-deficient mutant strains.

The basis for such a dissociation is still obscure. In view of prior evidence for the triggering of endotoxin-elicited events by antigen-antibody reactions on cell surfaces (1, 4, 5), it can be suggested that either the extent of such triggering reaction or the target cell involved may differ as a function of the polysaccharide constitution of the endotoxin used. It may well be that a dissociation of various biological properties can be accomplished more effectively by the bacterium than by the chemist.

ACKNOWLEDGMENT

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ADDENDUM

After completion of this manuscript, we learned about the data by Y. B. Kim and D. W. Watson (Bacteriol. Proc., p. 50, 1966), who failed to detect any significant differences in lethality between *S. minnesota* S and a heptoseless mutant derived therefrom. It remains to be established whether the difference in the results obtained by Kim and Watson and those reported in the present publication are due to species and strain difference or involve differences in extraction procedures.

LITERATURE CITED

1. BRAUN, W., AND R. W. I. KESSEL. 1964. Cytotoxicity of endotoxins in relation to their effects on host resistance, p. 397–409. *In* M. Landy and W. Braun [ed.] Bacterial endotoxins. Rutgers Univ. Press, New Brunswick, N.J.
2. DISCHE, Z. 1955. New color reactions for the determination of sugars in polysaccharides. Methods Biochem. Analy. **2**:313–358.
3. KASS, E. H., R. P. ATWOOD, AND P. J. PORTER. 1964. Observations on the locus of lethal action of bacterial endotoxin, p. 595–601. *In* M. Landy and W. Braun [ed.], Bacterial endotoxins. Rutgers Univ. Press, New Brunswick, N.J.
4. KESSEL, R. W. I., AND W. BRAUN. 1965. Cytotoxicity of endotoxin *in vitro*. Effects on macrophages from normal guinea pigs. Australian J. Exptl. Biol. Med. Sci. **43**:511–522.
5. KESSEL, R. W. I., W. BRAUN, AND O. J. PLESCIA. 1966. Endotoxin cytotoxicity: role of cell-associated antibody. Proc. Soc. Exptl. Biol. Med. **121**:449–452.
6. LÜDERITZ, O., A. M. STAUB, AND O. WESTPHAL. 1966. Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae*. Bacteriol. Rev. **30**:192–255.
7. NAKANO, M. 1962. Mutants of *Salmonella* with unusually low toxicity for mice. Nature **196**:-1118–1119.
8. NIKAIDO, H., K. NIKAIDO, T. V. SUBBAIAH, AND B. A. D. STOCKER. 1964. Rough mutants of *Salmonella typhimurium*. III. Enzymatic synthesis of nucleotide-sugar compounds. Nature **201**:1301–1302.
9. OSBORN, M. J., S. M. ROSEN, L. ROTHFIELD, L. D. ZELEZNICK, AND B. L. HORECKER. 1964. Lipopolysaccharide of the gram-negative cell wall. Science **145**:783–789
10. SUBBAIAH, T. V., AND B. A. D. STOCKER. 1964. Rough mutants of *Salmonella typhimurium*. I. Genetics. Nature **201**:1298–1299
11. SULTZER, B. M., AND H. H. FREEDMAN. 1962. The stimulation of non-specific host resistance to infection by chemically modified endotoxin. J. Exptl. Med. **116**:943–956.
12. WESTPHAL, O. 1957. Pyrogens, p. 115–220. *In* G. D. Springer [ed.], Polysaccharides in biology, 2nd conference, Josiah Macy, Jr. Foundation, New York.