Interferon Production in Mice by Cell Wall Mutants of *Salmonella typhimurium*

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The interferon response elicited by *Salmonella typhimurium* mutants in mice is not dependent on the presence of a complete cell wall lipopolysaccharide. In fact, a mutant (G30/C21) which has lost all the polysaccharide side chains and sugars of the O antigen and contains only 2-keto-3-deoxyoctonate and lipid is indistinguishable in its interferon-stimulating ability from the wild type which possesses a complete O antigen with polysaccharide side chains.

Intravenous injection of gram-negative bacteria or bacterial endotoxin into mice is followed by the rapid appearance in the circulation of an inhibitor indistinguishable in its action from virus-induced interferon (7). These results have been duplicated in rabbits (1). In mice, endotoxin from *Escherichia coli* produces peak levels of circulating interferon about 2 hr after injection; evidence has been obtained that this interferon is preformed and is released from cells with which the endotoxin reacts (10, 11).

To determine which chemical structure of complex endotoxin preparations is responsible for the release of interferon in mice, experiments were carried out with *Salmonella typhimurium* mutants with known mutational blocks in the biosynthesis of cell wall lipopolysaccharides (3, 5, 6, 8). Nakano (4) demonstrated that endotoxins derived from mutant strains differed in yield per cell, but showed no significant differences in lethality for mice when adjusted to equal weight. Kessel et al. (2) reported that endotoxins from *S. typhimurium* mutants with deficiencies in the polysaccharide content of their cell walls were equally capable of protecting mice against subsequent challenge with *Pseudomonas aeruginosa*. Studies were therefore undertaken to test both the lethal effects and the interferon-stimulating ability of wild-type *S. typhimurium* cells and of several mutants with known deficiencies in cell wall composition.

MATERIALS AND METHODS

S. typhimurium mutants were obtained through the courtesy of Werner Braun. The description of the cell wall composition of the wild and mutant organisms listed in Table 1 is taken from the compilation of Kessel et al. (2). Bacteria were grown for 24 hr on tryptose-phosphate agar and were suspended in 0.15 M NaCl. After being filtered through sterile filter paper,

the suspensions were adjusted to a standard turbidity and viable counts were made by plating dilutions of the suspensions on tryptose-phosphate agar. The bacteria were killed by placing the suspensions in a boiling-water bath for 5 min or by heating at 56°C for 1 hr.

Endotoxin prepared by the Boivin method from *E. coli* O:113 was generously provided by A. I. Braude.

Interferon was produced in mice injected intravenously with 0.1 ml of the appropriate dilutions of killed bacteria and bled 2 hr after inoculation. Pooled plasmas from 10 mice were tested for interferon content in L-cell cultures by a plaque reduction method with vesicular stomatitis virus (VSV) as the challenge virus. The details of interferon assay have been recorded (12).

RESULTS

Infectivity of wild-type and mutant S. typhimurium cells. Nakano established (4) that mutants with complete or deficient polysaccharide content of the cell wall are markedly different in infectivity for the mouse; the smooth (S) wild type is highly infective and the rough (R) mutants with deficient cell walls are not infective. To check the identity of the different mutants used in this study, groups of 10 mice were injected intraperitoneally with 1.0 ml of decimal dilutions of living wild-type and mutant cells from 24-hr agar cultures. Deaths were recorded for a 2-week period, and LD₅₀ doses were determined by probit analysis. The results of these experiments (Table 2) indicate that the mutants being tested have infectivity characteristics consistent with their cell wall composition.

Interferon production in mice by different mutants of S. typhimurium. Results obtained by the intravenous injection of killed cells, as described above, show (Table 3) that there was no significant difference in the ability of the wild-type and mutant cells of *S. typhimurium* to produce inter-

TABLE 1. Description of *Salmonella typhimurium* strains (2)

Designation	Strain	Cell wall composition
S (wild type)	LT2	Heptose-phosphate backbone, basal core, and side chains (complete O antigen)
S (wild type)	LT2 (698)	Heptose-phosphate backbone, basal core, and side chains (complete O antigen)
Ra	TV-119	Lacks O-specific side chains
Rb	TV-161	Lacks O-specific side chains and glucosamine
Rc	LT2-M1	Uridine diphosphate galactose-4-epimeraseless
Rd	SL-1032	Heptose-phosphate backbone only
—	G-30/C21	Heptoseless (2-keto-3-deoxyoctonate and lipid only)

TABLE 2. Infectivity of different strains of *Salmonella typhimurium* for the mouse

Strain	LD ₅₀ of live bacteria (Log ₁₀ bacteria per mouse)	
	Intraperitoneal injection	Intravenous injection
(S) LT2	<3.6	<3.6
(S) LT2 (698)	<3.7	<3.7
(Rc) LT2-M1	7.4	7.5
(Rd) SL-1032	>8.9	>8.9
(—) G-30/C21	>8.7	>8.7

feron in mice. The differences seen are within the experimental error of the method for interferon assay. Control experiments, which are not recorded, showed that peak interferon titers in plasmas were reached at 2 hr after injection of the different mutants, the typical "endotoxin-type" of response (7).

Hyporeactivity produced by mutants of S. typhimurium and by E. coli endotoxin. Previous reports from this laboratory (11) showed that prior treatment of mice with *E. coli* endotoxin eliminated the characteristic appearance of interferon which followed endotoxin inoculation into untreated mice. An experiment was carried out in which wild-type and mutant *S. typhimurium* cells having markedly different cell wall compositions were injected into mice. The ability of these mice to produce interferon when injected 2 days later with the same and different strains or with endotoxin was determined. Large groups of mice were in-

TABLE 3. Interferon production in mice by different strains of *Salmonella typhimurium*

Designation	Strain	No. of killed bacteria injected ^a	Interferon titer of plasma at 2 hr
S	LT2	6.4×10^7	300
		6.4×10^6	280
		6.4×10^5	32
Ra	TV-119	1.8×10^7	130
		1.8×10^6	64
		1.8×10^5	16
Rb	TV-161	1.7×10^7	310
		1.7×10^6	280
		1.7×10^5	16
Rc	LT2-M1	1.7×10^7	270
		1.7×10^6	140
		1.7×10^5	20
Rd	SL-1032	5.2×10^7	170
		5.2×10^6	100
		5.2×10^5	30
—	G-30/C21	1.2×10^7	450
		1.2×10^6	140
		1.2×10^5	16
Uninoculated control mice			<16

^a Cultures (24 hr) on tryptose-phosphate-agar were suspended in saline, filtered through filter paper, and adjusted to standard turbidity. Viable counts were then made, and the bacteria were killed by heating at 56 C for 1 hr. Mice were injected intravenously with 0.1 ml of the appropriate dilutions of killed bacteria.

jected with heat-killed cells of wild (LT2) and mutant (SL-1032 or G-30/C21) types. Another group of mice was given 100 µg of *E. coli* endotoxin. Two days later, each group of mice was challenged with a wider range of mutants and with endotoxin. The results in Table 4 show that the hyporeactive state was induced by all the *S. typhimurium* strains and by endotoxin to an equal degree. No signs of mutant or endotoxin specificity were observed.

Comparison of interferon production by different S. typhimurium mutants in BCG-infected and control mice. Previously published results showed that infection with *M. tuberculosis* strain BCG increases the reactivity of mice to *E. coli* endotoxin when measured by interferon response (11) as well as by lethality (9). No enhancing effect of BCG infection on interferon production by viruses (11) or statolon (12) was observed.

Further attempts were made to demonstrate

TABLE 4. *Cross-hyporeactivity produced by different strains of Salmonella typhimurium and Escherichia coli O113 endotoxin*

1st injection (day 0)	Interferon titer of plasma 2 hr after 2nd injection on day 2					
	(S) LT2 (2.4×10^7) ^a	(Rb) TV-161 (2.0×10^7)	(Rc) LT2-M1 (1.8×10^7)	(Rd) SL-1032 (3.0×10^7)	(-)G-30/C21 (1.7×10^7)	Endotoxin (100 µg)
Saline (0.1 ml).....	1,200	1,100	500	500	700	700
(S) LT2 (3.4×10^7) ^a	24	<16	16	20	25	25
(Rd) SL-1032 (3.5×10^7).....	60	28	60	60	50	60
(-) G-30/C21 (2.4×10^7).....	40	40	<16	20	20	60
<i>E. coli</i> endotoxin (100 µg).....	60	28	25	25	20	50

^a Number of heat-killed cells injected, based on viable counts before heating.

TABLE 5. *Influence of pretreatment with cycloheximide on interferon production in mice by different strains of Salmonella typhimurium and by Escherichia coli endotoxin*

Pretreatment of mice	Time of bleed- ing (hr)	Interferon titer of plasmas of mice injected with					
		(S) LT2 (5.0×10^7) ^a	(Rc) LT2-M1 (6.6×10^7)	(Rd) SL-1032 (4.6×10^7)	(-)G-30/C21 (3.1×10^7)	<i>E. coli</i> en- dotoxin (50 µg)	CH only
Saline (1.0 ml intraperitoneally)	2	640	350	130	110	150	
	6	160	100	40	<32	45	
	10	150	85	40	<32	<32	
Cycloheximide (244 mg/kg intra- peritoneally)	2	330	180	150	150	130	64
	6	2,800	2,500	2,000	1,100	3,200	400
	10	5,000	5,500	4,500	4,000	5,000	400

^a Number of heat-killed cells injected, based on viable counts before heating.

that the release of interferon by *S. typhimurium* strains with complete and deficient cell walls was an example of the "endotoxin-type" of response. Mice infected with tubercle bacilli were injected with the different mutants and with *E. coli* endotoxin. Mice were infected intravenously with 0.1 mg of a 10-day culture of BCG grown in a modified Dubos-Middlebrook medium. Fourteen days later, LD₅₀ determinations made with *E. coli* endotoxin demonstrated that the infected mice were hyper-reactive to the lethal effects of endotoxin; the LD₅₀ of endotoxin in uninfected and infected mice was 350 and 1.5 µg, respectively. In addition, groups of infected and control mice were injected intravenously with approximately 5×10^7 heat-killed cells of different mutants or with 20 µg of *E. coli* endotoxin and were bled 2 hr later. Interferon assays of pooled plasmas showed that four- to eightfold more interferon was produced in BCG-infected mice than in uninfected control mice. These results again demonstrated the "endotoxin-type" of interferon response produced by wild-type and deficient strains of *S. typhimurium*.

Effect of inhibition of protein synthesis on interferon production in mice injected with S. typhimurium mutants. It was demonstrated previously (13) that doses of cycloheximide which effectively

inhibited protein synthesis in the mouse enhanced the release of interferon after injection of *E. coli* endotoxin. In contrast, interferon production by viruses was eliminated by blockade of protein synthesis.

To confirm the evidence above that *S. typhimurium* mutants with deficient cell walls effect the release of preformed interferon, the following experiment was performed. Groups of mice were injected with saline (1.0 ml) or cycloheximide (244 mg/kg in 1.0 ml of saline) intraperitoneally. This dose of cycloheximide reduces protein synthesis by more than 95% in all organs of the mouse (13). One hour later, heat-killed cells of wild-type and mutant *S. typhimurium* were injected intravenously. Endotoxin (50 µg) from *E. coli* was used as a control stimulus. The data presented in Table 5 show that, in control mice treated with saline, the typical peak interferon titer at 2 hr was recorded in animals injected with the mutants or with endotoxin. In marked contrast, treatment with cycloheximide produced an enhancement of interferon titer as well as a shift to a later peak time with all the *Salmonella* strains and with endotoxin. These results further confirm that the "endotoxin-type" response was produced by each of the *Salmonella* strains tested.

DISCUSSION

The finding that the interferon response elicited by *S. typhimurium* mutants in mice is not dependent on the presence of a complete cell wall lipopolysaccharide is consistent with the findings of Nakano (4). Nakano showed that the lipopolysaccharide from epimeraseless mutants, in spite of the absence of various sugars other than glucose, showed the same degree of toxicity as the wild-type, indicating that the polysaccharide part of endotoxin was not directly related to the toxicity. Contradictory to this conclusion are the data obtained by Kessel and his colleagues (2), who noted that the lethality of endotoxins separated from the cell walls of *S. typhimurium* mutants could be correlated with the complexity of the polysaccharide. Endotoxin from the wild (S) type and from mutants 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. In contrast, all of these mutants yielded endotoxins that were equally capable of protecting mice against a subsequent challenge with *P. aeruginosa*. Endotoxin prepared from a heptoseless mutant containing only 2-keto-3-deoxyoctonate and lipid proved to be neither toxic nor protective.

It is interesting that, as far as interferon production in mice is concerned, the latter mutant (G30/C21) is indistinguishable from the wild type which contains a complete O antigen with polysaccharide side chains. It thus seems that it is not the polysaccharide or heptose-phosphate backbone portion of endotoxin which stimulates interferon production.

The possibility should not be overlooked that despite mutational blocks in synthesis of complete O-antigen, certain polysaccharide components could be present but not incorporated into the cell wall of the deficient mutants. These components could be responsible for the interferon-stimulating effect of whole cells of mutants with major deficiencies in cell wall composition. Experiments now underway are designed for the preparation and chemical fractionation of lipopolysaccharides from wild-type and deficient strains of *S. typhimurium* in an attempt to understand the exact chemical portion of endotoxin, or other bacterial components, which stimulates interferon production in animals.

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