INFECTION AND IMMUNITY, Nov. 1971, p. 519-524

Vol. 4, No. 5
Printed in U.S.A.

Experimental Salmonellosis: Hypersensitivity to Cell Wall Lipopolysaccharide and Anti-Infectious Resistance of Mice Infected with *Salmonella*

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Received for publication 17 June 1971

Mice infected with various strains of *Salmonella enteritidis* and *S. typhimurium* were found to be more sensitive to the cell wall lipopolysaccharide (LPS) extracted from certain strains of *Salmonella* than noninfected mice. This hypersensitivity was induced by those smooth or rough strains which possessed a polysaccharide chain longer than that of a glucoseless mutant. The major antigenic group participating in this hypersensitivity was presumed to be in rough core polysaccharide sequence because fractions containing either O side chain or LPS of a heptoseless mutant were ineffective in provoking a hypersensitivity reaction. Conditions for the induction and the phases of development of this hypersensitivity to LPS and for anti-infectious resistance were shown to be different. Present and previously obtained results suggest that the antigens participating in each of these two conditions were different.

In mouse salmonellosis, immunization with suitable strains of live bacteria confers on mice an anti-infectious immunity against the infection with virulent *Salmonella* (8, 10). On the other hand, this immunization also makes these mice more sensitive to the lethal action of endotoxin extracted from the *Salmonella* spp. than non-immunized mice (12). However, it was difficult to induce such an increased susceptibility by administration of killed vaccines or endotoxin to mice.

Hyperreactivity to endotoxin has also been observed in animals infected with heterologous bacteria such as *Brucella* (1) and *Mycobacterium tuberculosis* (16). In this case, the increased sensitivity was thought not to be a hypersensitivity based on the immunologically specific reaction (16). On the other hand, Stetson (15) has tried to explain the reaction to endotoxin by referring it to hypersensitivity of host, which might be sensitized naturally with cross-reactive antigens. Watson and Kim (17, 18) suggested the presence of naturally acquired hypersensitivity to endotoxin in adult animals, observing a lower susceptibility of young animals to endotoxin. They presumed that the hypersensitivity of adults was acquired as a result of subclinical infections or contact with the intestinal flora, and that a common antigen in the endotoxin of various microorganisms participated in the hypersensitive reaction because of its cross-reactivity. In this point of view, it was thought that there may be an indication of hypersensitivity in the increased susceptibility to endotoxin in *Salmonella*-infected mice. If this assumption is the case, there must be a specific antigenic determinant active in the hypersensitivity reaction in endotoxin. The present paper will indicate the presence of such an antigenic determinant in cell wall lipopolysaccharide (LPS) of *Salmonella*.

Furthermore, the hypersensitivity or increased susceptibility to endotoxin is thought to be a factor which affects the immune resistance against challenge infection. For the elucidation of the mechanism of acquired antimicrobial resistance in mouse salmonellosis, it is important to know the role of the hypersensitivity to *Salmonella* endotoxin.

MATERIALS AND METHODS

Animals. Mice of DDN strain (4 weeks old) weighing 15 to 18 g were used. The basic maintenance conditions were already reported (6). Mice used for the experiments were weaned from mothers, which were given tap water per os containing colistin methanesulfonate (100 μg/ml) and gentamicin (50 μg/ml). Each group of 10 mice was kept in a cage containing sterilized wood shavings and was given autoclaved mouse diet (MF, Oriental Yeast Co., Tokyo, Japan), and drug-free water. Natural infection with *Salmonella* was checked by cul-

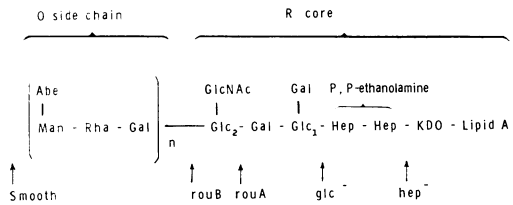

FIG. 1. *Proposed structure of S. typhimurium LPS (13). Gal, galactose; Rha, rhamnose; Man, mannose; Abe, abequose; Glc, glucose; GlcNAc, N-acetylglucosamine; Hep, glycero-mannoheptose; KDO, 3-deoxyoctulosonate. Arrow indicates the distal end of LPS chain of each strain.*

turing their feces before and after the experimental infection.

Bacterial strains. *Escherichia coli* O.111, *Aerobacter aerogenes* M7, a smooth strain of *S. enteritidis* 116-54, and its rough variant, SER, were our stock strains (6). *S. typhimurium* smooth wild-type LT2 and a series of rough and semirough mutants derived from this strain were kindly donated by Hiroshi Nikaido, University of California, Berkeley. Possible LPS structures of these mutants have been reported (Fig. 1) by Nikaido (13). The virulence and immunizing potencies of these bacteria have been reported elsewhere (6).

Preparation of antigens. Bacteria were cultivated by methods described previously (7). Heat-killed vaccines were prepared by heating 0.5 mg of the bacterial suspension per ml in 0.85% NaCl for 20 min at 70 C.

LPS was extracted from smooth and rough strains with 90% phenol containing 0.001 M disodium ethylenediaminetetraacetate (EDTA). After an overnight dialysis against distilled water and concentration by evaporation, 10 μg of deoxyribonuclease (Worthington Biochemicals Co., Freehold, N.J.) and 100 μg of ribonuclease T1 (Sankyo, Co., Tokyo, Japan) in 1 ml of 0.001 M $MgCl_2$ were added to 10 ml of the extract, and the mixture was allowed to stand for 1 hr at room temperature and then dialyzed against 0.0001 M $MgCl_2$ overnight at 4 C. The LPS was precipitated and washed by centrifugation at 100,000 $\times$ *g* for 120 min, and the final precipitate was dissolved in 0.001 M EDTA. After centrifugation of this solution at 7,000 $\times$ *g* for 10 min, the supernatant fluid was dialyzed against 0.85% NaCl and stored at 4 C.

The O side chain (2) and O side chain-lipid complex (19) were prepared from the TV160 strain. Polysaccharide was extracted from harvested bacteria with 90% phenol. After overnight dialysis and nucelease treatments, LPS was removed from the extract by centrifugation at 100,000 $\times$ *g* for 120 min. The materials in the supernatant fluid were fractionated by chromatography by the methods described in a previous paper (7). A fraction containing the O side chain was obtained by the elution of diethylaminoethyl (DEAE) cellulose column with 0.005 M NaCl-0.001 M phosphate buffer (*p*H 7.0). This fraction was further passed through a carboxymethyl (CM) cellulose column equilibrated with the same buffered saline, and the fraction recovered in the void volume was collected. This fraction gave a strong positive precipitin reaction with antisera prepared by immunizing rabbits with heated LT2 and adsorbed with his-rfb388 strain (anti-LT2 serum), and gave the negative reaction with antiserum against TV160 adsorbed with LT2 (anti-TV160 serum). This fraction contained 77% hexose (calculated as glucose). From the infrared adsorption spectrum, this material was thought to contain no lipid.

The O side chain-lipid complex was found in a fraction which was eluted with 0.05 M NaCl-0.01 M phosphate buffer (*p*H 7.0), and not eluted with 0.01 M NaCl-0.002 M phosphate buffer (*p*H 7.0) in DEAE cellulose chromatography. This fraction was also passed through a CM cellulose column and concentrated by evaporation. The precipitin reaction of this fraction with anti-LT2 serum was positive and that with anti-TV160 serum was negative. A precipitate was formed by heating this fraction for 10 min at 60 C at *p*H 8.0. A supernatant fluid of the heated material was found to contain polysaccharide which had the same chromatographic characteristics as the O side chain. The precipitate was soluble in chloroform-ethanol (1:1). The strong absorptions at 2,910 and 2,850 per cm in the infrared spectrum suggested a lipid nature for this precipitate.

Chemical and serological techniques. Methods described in a previous paper (7) were used for the determination of protein and various sugars. Infrared absorption was measured by a method previously described (5). The precipitin test of serially diluted antigen solution was carried out by the conventional overlay method in tubes and diffusion method in agar gel.

Infection and immunization. Generally, 10^{-5} mg (approximately 4×10^4 viable cells) of bacteria was given by intravenous injection to each mouse. In the case of LT2 and 116-54 infection, the intravenous inoculation was followed by treatment with kanamycin which saved the host from death and thus anti-infectious immunity was conferred, as well as maintaining viable bacteria at a certain level in the host (14). LPS was administered to mice by intravenous injection from one to three or even nine times at suitable intervals (2 to 5 days). Some mice received intramuscular injection with LPS or heat-killed bacteria with 0.1 ml of Freund's complete adjuvant. Anti-infectious resistance was measured by methods described previously (8).

Measurement of sensitivity to antigens. Various amounts of antigenic materials were administered to infected mice by an intravenous injection. Usually, 10 mice were given injection with each of three to five different doses of antigen. According to the method of Litchfield and Wilcoxon (11), 50% lethal dose (LD_{50}) and its confidence limits were calculated from the death rate recorded 48 hr after the infection, and the relative sensitivity was drawn from these values.

RESULTS

Sensitivity to smooth LPS. Mice were infected with various strains of bacteria, and the sensitivity to the LPS of smooth strains of *Salmonella*

TABLE 1. *Sensitivity of infected mice to lipopolysaccharide (LPS) extracted from smooth strains of Salmonella*

Mice infected with[a]		LPS extracted from	LD_{50} (μg)	Confidence limits ($P = 0.05$)
Salmonella enteritidis 116-54	S	116-54	19	11–32
S. enteritidis SER	R	116-54	27	17–45
S. typhimurium LT2	S	116-54	21	14–34
S. typhimurium his-rfb388	rouB	116-54	31	21–57
S. typhimurium TV160	rouA	116-54	27	14–52
S. typhimurium SL1102	hep^-	116-54	96	56–155
Escherichia coli 0:111	S	116-54	121	74–187
E. coli 0:111R	R	116-54	110	57–190
Noninfected control		116-54	118	70–201
S. typhimurium LT2	S	LT2	18	11–30
S. typhimurium his-rfb388	rouB	LT2	14	11–29
Aerobacter aerogenes M7	S	LT2	>170	
Noninfected control		LT2	181	104–315

[a] In each group tested, 27 to 60 mice were intravenously injected with various amounts of LPS 15 days after the infection.

TABLE 2. *Sensitivity of infected mice to lipopolysaccharide (LPS) extracted from rough strains of Salmonella*

Mice infected with[a]		LPS extracted from	LD_{50} (μg)	Confidence limits ($P = 0.05$)
Salmonella enteritidis SER	R	SER	42	24–73
Noninfected control		SER	163	123–217
S. enteritidis SER	R	his-rfb388	31	14–65
S. typhimurium LT2	S	his-rfb388	27	13–55
S. typhimurium his-rfb388	rouB	his-rfb388	33	15–73
S. typhimurium SL1004	glc_1^-	his-rfb388	>187	
Noninfected control		his-rfb388	255	181–360

[a] In each group tested, 29 to 63 mice were intravenously injected with various amounts of LPS 14 days after the infection.

was measured. Three repeated tests on the sensitivity of his-rfb388 infected mice to 116-54 LPS gave LD_{50} values of 26, 31, and 48 μg at 14, 15, and 20 days after the infection, respectively. As shown in Table 1, hypersensitivity to the LPS of smooth *Salmonella* was conferred by infection with *Salmonella* which was pathogenic for mice, whereas nonpathogenic bacteria such as *Escherichia coli* and *Aerobacter aerogenes* did not sensitize the mice. It was noted that heterologous *Salmonella* also caused a hypersensitive state to the LPS. Rough strains of *Salmonella* also sensitized the mice, whereas rough *E. coli* strain did not.

The mean survival time of the mice which were infected previously and then given LPS was 8 hr. Bacteria in the liver and spleen of these mice were enumerated at the time of death, and counts of 1.2×10^2 to 8.4×10^6 per g of tissue were obtained from the animals tested. This fact indicated that the death of the infected mice by the administration of LPS was not due to a septicemia caused by the aggressive effect of LPS, because the bacterial number was approximately 10^2 to 10^6 per g of tissue 2 or 3 weeks after the immunizing infection with rough strains, but it reached 8×10^8 to 10^9 per g of tissue at the time of death from infection with virulent *Salmonella* (4; *unpublished data*).

Sensitivity to rough LPS. Those mice sensitized by infection with rough or smooth strains of *Salmonella* were hypersensitive to LPS extracted from certain strains of rough mutants (Table 2). The sensitized mice were shown to have their sensitivity to LPS from rouB or rouA strains, which have a complete or part of the rough core-polysaccharide chain in their cell walls. However, they were not sensitive to the LPS of a heptoseless mutant lacking the rough core. They become rather resistant to this LPS at a later stage of infection (Table 3). These results indicated that the antigenic determinant concerning the hyper-

TABLE 3. *Sensitivity of infected mice to lipopolysaccharide (LPS) of heptoseless strain of Salmonella*

Mice infected with		Time after infection (day)	LPS extracted from	LD_{50} (μg)	Confidence limits ($P = 0.05$)
S. typhimurium SL1102	hep$^-$	40	SL1102	1730	
S. enteritidis SER	R	14	SL1102	215	
		31	SL1102	>1000	
		40	SL1102	1620	
Noninfected controls			SL1102	273	163–341

TABLE 4. *Sensitivity of infected mice to O side chain of Salmonella*

Mice infected with		Fractions administered[a]	LD_{50} (μg)
Salmonella typhimurium LT2	S	O side chain	>1,050
S. typhimurium his-rfb388	rouB	O side chain	>1,050
Noninfected controls		O side chain	>1,050
S. typhimurium LT2	S	O side chain-lipid	>540
Noninfected controls		O side chain-lipid	>540

[a] Mice were given these fractions intravenously 14 days after the infection.

sensitivity reaction was in the rough core-polysaccharide but not in the lipid A of LPS.

Sensitivity to O side chain. Mice infected with smooth or rough strains were not killed by injection with 1,050 μg of O side chain part of LPS as shown in Table 4. Furthermore, a fraction containing the O-side chain-lipid complex was also shown to be ineffective. The amount of hexose in the O-side chain and O-side chain-lipid fraction injected was 22 and 6 times more than that contained in the LD_{50} of the smooth LPS, respectively. Although no deaths were recorded in the noninfected control group, the infected mice were shown to be insensitive to these amounts of O-side chain and O-side chain-lipid complexes.

Conditions affecting the development of hypersensitivity. The sensitivity to LPS developed at the second week and decreased within 4 weeks of infection. On the other hand, the resistance to challenge infection increased gradually 3 to 5 weeks after the immunizing infection (Fig. 2). Administration with a suitable amount of heat-killed bacteria with Freund's complete adjuvant was found to give a hypersensitivity to LPS to mice, but it did not confer resistance against infection with the virulent *Salmonella* even several weeks after the administration. These differences in the conditions for the development of hypersensitivity and anti-infectious resistance suggest that both states were produced by different factors existing in bacterial cells.

DISCUSSION

Watson and Kim (17, 18) speculated that there were two interdependent activities in endotoxin molecule: the intrinsic or primary toxicity was associated with the lipid portion of the molecule, and the secondary toxicity resulted from the acquisition of hypersensitivity of the host to some portion of the molecule. Our present results show that mice gained resistance to LPS of heptoseless mutant after an immunizing infection with *Salmonella*. This fact may be consistent with their observation of lowered febrile response to LPS of the heptoseless mutant in immunized rabbits (9). Furthermore, present results, indicating the specific participation of an antigenic determinant in the rough core of the LPS in the hypersensitivity reaction, support their assumption of secondary toxicity to LPS caused by hypersensitivity of host. The sensitized mice were not affected by the administration of fractions containing O side chains. The molecular size of the isolated O-side chain or O-side chain-lipid complexes is big enough to be precipitated by O-specific antiserum (*see above*), but it might be too small to be involved in the hypersensitivity reaction. It is plausible, from the present results, that the antigen concerned with the hypersensitivity reaction does not include the O side chain of smooth LPS. The insensitivity of infected animals to the LPS of heptoseless mutants excludes the possible participation of lipid A in the reaction. From these reasons, the main antigenic component of the LPS concerning the hypersensitivity may be in the polysaccharide sequence from glucose-1 to glucose-2 of the rough core (Fig. 1). The fact that the hypersensitivity was induced by infection with those strains, which has LPS longer than that of glu-

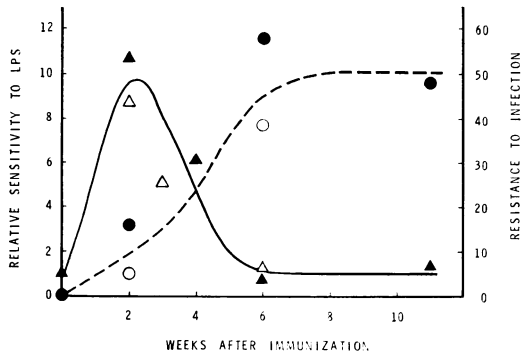

FIG. 2. *Sensitivity to LPS and resistance to infection of mice immunized with live baceteria. Mice immunized by infection with 10^{-5} mg of S. enteritidis SER or S. typhimurium his-rfb388 were challenged with LPS or live bacteria of a virulent strain of S. enteritidis, 116-54. The relative sensitivity to 116-54 LPS was expressed as LD_{50} in controls/LD_{50} in experimental group. The anti-infectious resistance was expressed as per cent survival 30 days after challenge infection with 10^{-7} mg of 116-54. LPS sensitivity of mice immunized with SER (▲) or his-rfb388 (△); anti-infectious resistance of mice immunized with SER (●) or his-rfb388 (○).*

cose-1-less mutant (Table 2), does not conflict with this assumption.

It was shown previously that the anti-infectious immunity in mouse salmonellosis was conferred basically by live bacteria possessing a wall polysaccharide chain longer than that of the glucose-1-less mutant (6). The coincidence of the rough polysaccharide chain of mutant strains concerning the protecting potency as well as the ability to induce hypersensitivity suggests the participation of an identical antigenic determinant. Some authors have speculated that, in the infection with facultative intracellular parasites including mouse salmonellosis, infection with live bacteria induced a specific hypersensitive state of old tuberculin type which in turn nonspecifically activated the host cells, and this cell activation caused the anti-infectious resistance (3). However, it was shown in the present experiments that the developmental phases of the LPS hypersensitivity and anti-infectious resistance were different and that the hypersensitivity was produced by administration with LPS or killed cells, whereas these materials were not able to confer anti-infectious resistance even by multiple injections (8). Therefore, it seems unlikely that the anti-infectious resistance was caused by a hypersensitivity reaction to LPS which is induced by live bacteria although the hypersensitivity to any component of bacteria other than LPS may concern the establishment of anti-infectious resistance.

TABLE 5. *Sensitivity to lipopolysaccharide (LPS) and resistance to infection of immunized mice*

Immunization			Sensitivity to LPS[b]		Resistance to infection[c] (% survival)
Materials	Dose[a] (μg)	Time of injection	LD_{50} (μg)	Confidence limits	
Live 116-54	0.0001	1	30	16–58	79
Live SER	0.01	1	31	14–65	53
Live LT2	0.01	1	27	13–55	93
Killed LT2	10	3	130		5
Killed LT2 with adjuvant	10	3	45	28–72	0
Rfb LPS	2	9	62		0
Rfb LPS with adjuvant	5	3	35		5
Nonimmunized controls			255	181–360	0

[a] Each mouse was administered this dose at each injection at suitable intervals. The interval between the first and last administrations was 12 days.

[b] Sensitivity of 22 to 60 mice in each group to his-rfb388 LPS was measured 14 to 16 days after the first immunization.

[c] Resistance of 20 to 38 mice in each group to 116-54 infection was measured 30 days after the first immunization.

By using macrophage-migration inhibition and foot-pad swelling as test systems, hypersensitive states have been observed in mouse salmonellosis, and antigens other than LPS have been found (3). The comparison of these hypersensitivities and hyper-reactivity remains to be studied.

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