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Local Skin Response in Mice Induced by a Single Intradermal Injection of Bacterial Lipopolysaccharide and Lipid A

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Dermal inflammation and hemorrhagic necrosis induced by bacterial lipopolysaccharide (LPS) and lipid A were studied in mice. In ddY mice, a single intradermal injection of *Salmonella typhimurium* S-form LPS and lipid A into the abdominal dermis elicited an edematous change due to an increase in local vascular permeability 12 h postinjection, followed by hemorrhagic necrosis from 24 to 72 h. This skin reaction was also induced in a dose-dependent manner by S-form LPS, R-mutant LPS, and lipid A of *S. typhimurium* and *Escherichia coli*, but not by polysaccharide from *Salmonella* S-form LPS. The dermal inflammation-inducing activities of LPS and lipid A were roughly in the following order (from highest to lowest): Re-form LPS, Rc-form LPS and lipid A, Ra-form LPS, and S-form LPS. These results suggest that the lipid A portion of the LPS molecule is responsible for the skin reaction. In C3H/HeN mice, Re-form LPS and lipid A induced the same intensity of skin reaction as that in ddY mice. In C3H/HeJ mice, which have a low response to LPS, Re-LPS and lipid A did not induce any hemorrhagic response but showed a distinct edematous change. Although hemorrhagic necrosis and edematous changes could be explained by quantitative differences in skin lesions, the other possible explanation is that hemorrhagic necrosis and the increase in local vascular permeability are induced by different mechanisms, only one of which depends on the regulation of the *lps* gene.

Lipopolysaccharides (LPS) from the outer membrane of gram-negative bacteria and their biological active moiety, lipid A, elicit a wide spectrum of biological effects in many animal species (21). Among many pathophysiological changes induced by LPS, the Shwartzman reaction (20, 25) in rabbits has been well known for about 50 years as an LPS-induced skin reaction. Recent approaches by analysis of LPS, especially lipid A and R-core polysaccharide, and by chemical synthesis of lipid A (22), have elucidated the structural requirement for the biological activities of LPS, and the Shwartzman reaction is one of the biological effects which has the highest structural requirement (4, 5, 15, 16). Although there were earlier studies concerned with skin reactions in mice (9, 13, 29), there is, unfortunately, no known report from the past 15 years concerning this reaction or its relationship to the structure of LPS. Our previous investigation (29) demonstrated that a single intradermal (i.d.) injection of LPS in mice induced local edematous change due to increased vascular permeability and necrosis. In this study, we have focused on this reaction once more and attempted to establish the local skin reaction in mice as one of the parameters of the biological activity of LPS.

MATERIALS AND METHODS

Mice. Six-week-old mice of the following strains were obtained: ddY mice from Shizuoka Agricultural Co. for Laboratory Animals, Hamamatsu, Japan, and C3H/HeN and C3H/HeJ mice from Nihon Clea, Tokyo, Japan. All mice were used within 7 to 11 weeks of age.

LPS and related compounds. *Salmonella typhimurium* LT2 and *Salmonella minnesota* R595 were cultivated in nutrient broth at 37°C for 16 h with vigorous shaking. The cells, collected by continuous centrifugation (15,000 × *g*) after heating the cultures at 120°C for 20 min, were washed three times with distilled water and then acetone dried. LPS of *S. typhimurium* LT2 (S form) and *S. minnesota* R595 (Re mutant) were extracted from their acetone-dried cells with hot phenol-water (27) and with phenol-chloroform–petroleum ether (6), respectively, and purified by repeated ultracentrifugation (six times at 105,000 × *g* for 3 h) and by treatment with ribonuclease (8). LPS yields were 3.3 and 3.1% (wt/wt) of the dried cells of *S. typhimurium* LT2 and *S. minnesota* R595, respectively. Lipid A and polysaccharide components of the LPS of *S. typhimurium* LT2 were prepared as follows: LPS (600 mg) was hydrolyzed in 5% acetic acid (100 ml) at 100°C for 1.5 h (7). The lipid A component was precipitated by centrifugation (10,000 × *g* for 15 min), washed with water, and freeze-dried (157 mg). After evaporation to dryness, the supernatant was subjected to gel chromatography by using a Sephadex G-50 column (36 by 1,000 mm) which was eluted with pyridine–acetic acid–water (10:4:1,000). The effluent was monitored with a refractoindex monitor (RID 6A; Shimadzu, Kyoto, Japan), and the fractions (3 ml) were analyzed for total carbohydrate according to the phenol-sulfuric acid method (2). Three peaks were observed, with the first (101 mg) corresponding to the polysaccharide component (the O-specific polysaccharide side chain attached to the core stub) of the LPS, the second (132 mg) corresponding to core oligosaccharide, and the third (90 mg) corresponding to mono- or disaccharide and *ortho*-phosphate, which were released during the acid hydrolysis. LPS of *S. minnesota* R60 (Ra mutant) was kindly supplied by C. Galanos (Max-Planck-Institut für Immunologie, Freiburg, Federal Republic of Germany). LPS of *Esch-*

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erichia coli 0111:B4 (S form), LPS of *S. minnesota*, LPS of *E. coli* J5 (Rc mutant), and lipid A from *E. coli* J5 were purchased from Ribi Immunochemical Research, Inc., Hamilton, Mont. LPS and polysaccharide samples were reconstituted at 1.0 mg/ml in sterile pyrogen-free saline (Ohtsuka Pharmaceutical Co., Tokushima, Japan), and lipid A samples were reconstituted at 500 μg/ml in saline with 0.025% (vol/vol) triethylamine. All further dilutions were accomplished in the pyrogen-free saline.

Skin reactions. These procedures were performed as described previously (29) with some modifications. The sample solution in a volume of 30 to 80 μl was injected i.d. in one, mainly the right, side of the murine abdominal region. Each mouse also received the same volume of pyrogen-free saline or 0.025% triethylamine in saline i.d. on the other side of the abdominal area. Forty-eight hours after i.d. injection, 200 μg of Evans blue (Merck, Darmstadt, Federal Republic of Germany) in 100 μl of saline was intravenously injected, and the mouse was sacrificed 30 min after the Evans blue injection. Sections of skin from the injection sites were inspected from the external and the inner layers of dermis. Reaction intensities were classified as described in Results. Some sections of skin were fixed in formalin and prepared for microscopic examination.

Statistical analyses. Data were statistically analyzed by two approaches: one was the determination of the 50% effective doses (ED_{50}s) needed to induce hemorrhagic lesions, which were estimated by Probit analysis (3), and the other was a two-way analysis of variance (10), by which significant differences between two samples can be tested. In the two-way analysis of variance, reaction intensities were coded numerically, such as code 0 to 3 for 0− to 3+, respectively.

RESULTS

Classification and appearance of skin reactions. Through preliminary observations, we have classified the intensity of dermal responses according to the following criteria: 3+, a lesion (with hemorrhagic necrosis) with a diameter of more than 1 mm; 2+, a lesion (with hemorrhagic necrosis) with a diameter of 1 mm or less; 1+, a blue spot (due to a leak of Evans blue into the extravascular space) with a diameter of more than 2 mm and no hemorrhagic necrosis; and 0−, a blue spot with a diameter of 2 mm or less and no hemorrhagic necrosis.

The assessment of skin reactions was carried out by macroscopical observation of the inner layer of dermis. As described previously (29), the administration of Evans blue before the sacrificing of mice allowed for an objective and quantitative assessment of the increase in local vascular permeability. A typical hemorrhage and necrosis (3+) induced by lipid A is shown in Fig. 1. The largest hemorrhagic region was 12 by 12 mm, and the size of a 3+ lesion was frequently 3 to 5 mm in diameter. The microscopic finding of a hemorrhagic region indicates the early stage of nonspecific inflammation (Fig. 2), as manifested by a hemorrhage, marked infiltration of neutrophilic granulocytes, thrombosis in small blood vessels, and swelling and alteration of subcutaneous muscle layer. The hemorrhagic lesions which were judged as 2+ showed petechial hemorrhages 0.5 to 0.7 mm in diameter and could be easily distinguished from the 3+ hemorrhagic lesions which showed massive hemorrhaging.

Time course of skin reactions. Dermal responses induced by *S. typhimurium* S-form LPS and lipid A were evaluated at various hours after injection (Table 1). An edematous change (1+) had already developed at 12 h postinjection, and the peaks of skin reactions were observed at 24 to 48 h and at 48 to 72 h. These local lesions were reduced by the end of the experiment. In addition, the hemorrhagic lesions which were observed only at 24 h after the injection of S-form LPS showed diffused and petechial hemorrhaging, although the three positive skin regions (of 5 regions, i.e., 60% of the mice) were judged as 3+. These hemorrhages appeared to be different from those of other positive regions, which showed massive hemorrhaging, and were occasionally observed with the injection of S-form LPS but not Re-form LPS and lipid A. In further experiments, 24 or 48 h after the i.d. injection of S-form LPS or lipid A (10 μg per mouse), 300 μg of S-form LPS was intravenously injected. This second injection, however, did not induce more severe hemorrhagic lesions than those from a single injection, as with the local Shwartzman phenomenon. In the following experiments, each dermal response was thus observed 48 h after a single i.d. injection of the sample.

Differences in the skin reaction-inducing activities among LPS and its compounds. Skin reactions induced by S-form LPS, lipid A, and the polysaccharide fraction of S-form LPS were examined. The actual data are shown in Table 2 (for the ED_{50}s of stimulants, see Table 4). All preparations of S-form LPS, R-mutant LPS, and lipid A induced local inflammatory and hemorrhagic lesions in ddY mice; the intensities of dermal responses, however, varied among preparations of LPS and lipid A. R-mutant LPS had a fairly potent effect on the skin reaction. The activity of R-mutant LPS appears to decrease with an increase in the length of the oligosaccharide portion ($P < 0.01$, between Ra- and Re-mutant LPS). Lipid A preparations also showed strong activity, although their activity was significantly weaker than that of Re-form LPS ($P < 0.01$). S-form LPS showed a still weaker activity than R-mutant LPS ($P < 0.01$) and lipid A (a comparison of ED_{50}s was not significant [$P < 0.05$]), with a varying of response. On the other hand, the polysaccharide from S-form LPS did not induce any inflammatory change in murine skin even at the high dose of 80 μg ($P < 0.01$, between the polysaccharide and S-form LPS). Furthermore, the polysaccharide does not appear to inhibit the skin reaction, because the mixture of Re-form LPS (10 μg) and polysaccharide (10 μg) also induced the same intensity of hemorrhagic lesions, 3+, as those induced by only Re-form LPS ($n = 3$).

LPS- and lipid A-induced skin reactions in LPS-low-response C3H/HeJ mice. C3H/HeJ mice are resistant to many of the biological effects of LPS, which are due to the lipid A moiety (23, 26). We therefore examined whether LPS and lipid A could induce local dermal inflammation in C3H/HeJ mice. As shown in Table 3, Re-form LPS and lipid A induced skin reactions in LPS-sensitive C3H/HeN mice, which are histocompatible with C3H/HeJ mice. The intensities of hemorrhagic responses were similar to those in ddY mice (Table 4), and the intensities of skin responses were not significantly different between these strains, in which the same stimulant was injected. In LPS-low-response C3H/HeJ mice, S-form LPS, Re-form LPS, and lipid A did not induce any hemorrhagic response at a dose of 40 μg or less, but they did induce distinct edematous changes (1+) even at 1.3 μg of Re-form LPS, at 5 μg of lipid A, and at 5 μg of S-form LPS. In further experiments, the time kinetics of skin reactions in C3H/HeJ mice were observed. Re-form LPS was injected i.d. in C3H/HeJ mice at a high dose of 40 μg, and skin responses were evaluated at 24, 48, and 72 h postinjection ($n = 4$ or 5). Re-form LPS did not induce any hemorrhagic change at 24 to 72 h postinjection.

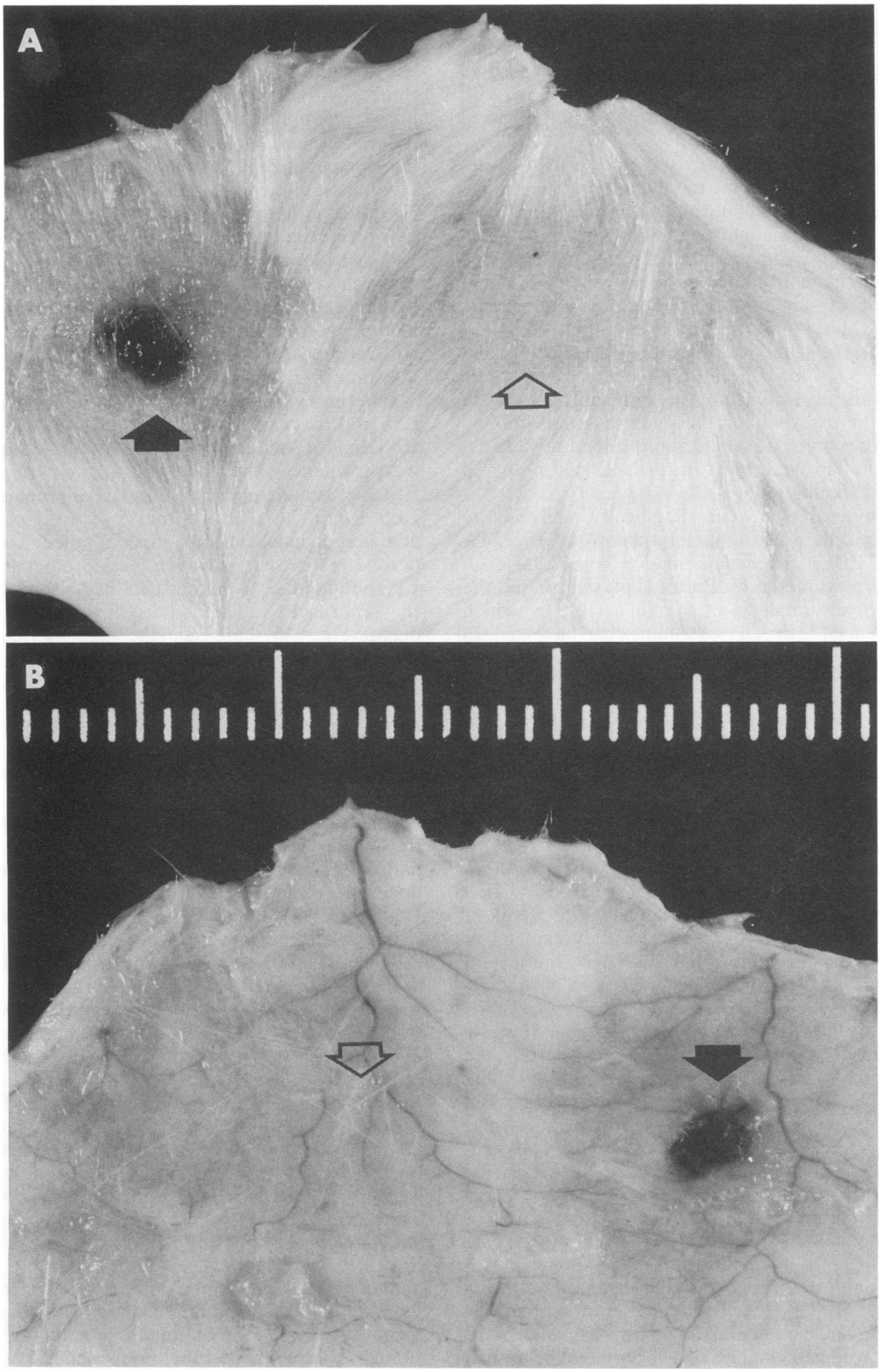

FIG. 1. Exterior (A) and inner (B) views of hemorrhagic skin lesions 48 h after i.d. injection of 20 μg of *S. typhimurium* LT2 lipid A (closed arrows) or 20 μg of 0.025% triethylamine in saline in the control site (open arrows).

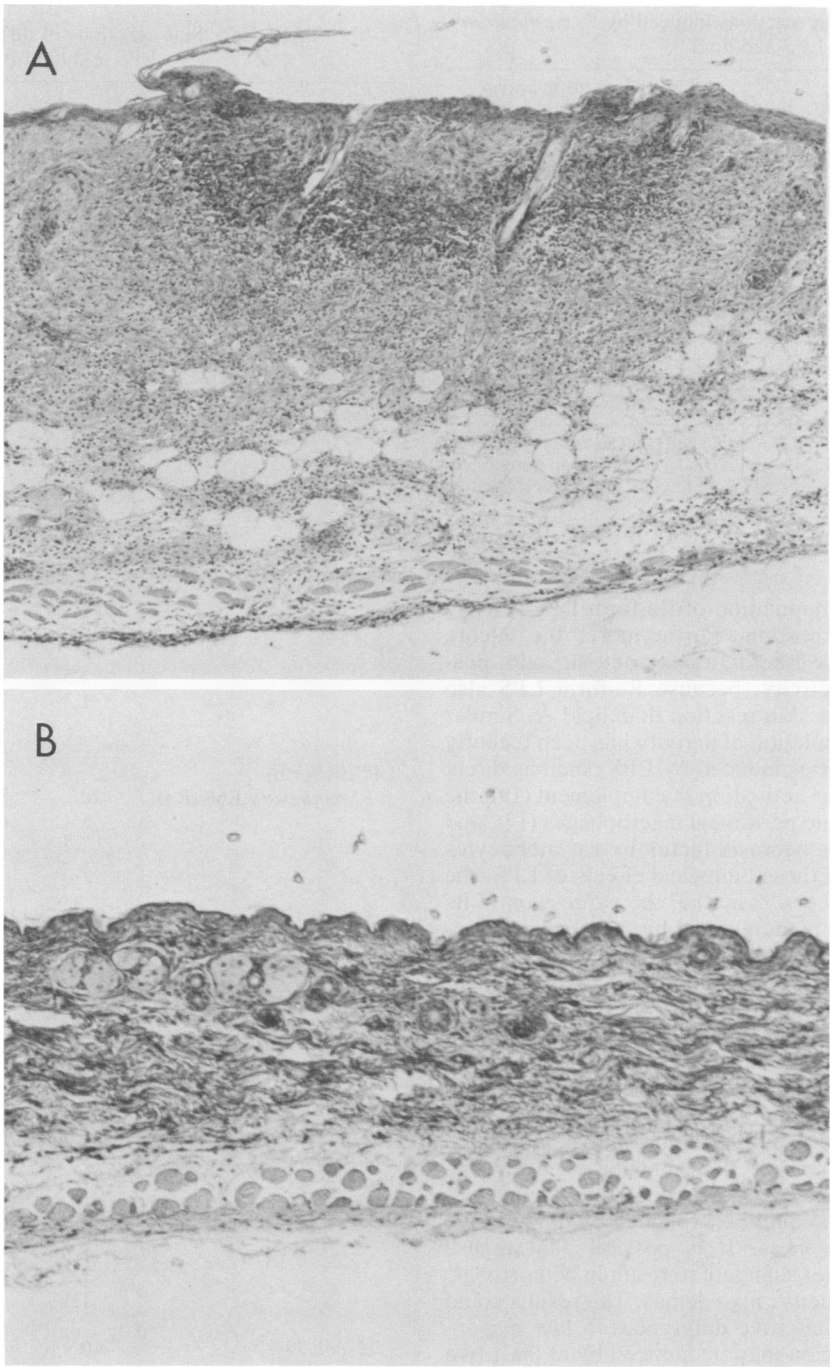

FIG. 2. (A) Histopathology of hemorrhagic skin lesions 48 h after injection of lipid A as described in the legend to Fig. 1; (B) histology of the control site. Hematoxylin and eosin staining were used. Magnification, ×130.

DISCUSSION

In this paper, we have shown that a single i.d. injection of bacterial LPS in mice induces an increase in vascular permeability, followed by hemorrhagic necrosis in the dermis. This reaction could be induced by the lipid A portion of the LPS molecule, but not by the polysaccharide fraction from S-form LPS, thus suggesting that the lipid A portion is involved in this reaction. This conclusion is supported by the observation that Re-mutant LPS and lipid A did not induce the hemorrhagic reaction in LPS-low-response C3H/HeJ mice, because these mice are hyporesponsive to most biological effects of LPS that are due to the lipid A moiety (23).

The activity of LPS preparations seems to decrease by increasing the length of the polysaccharide portion of the LPS molecule; thus Re-form LPS had the strongest activity, followed by Rc-form LPS, Ra-form LPS, and S-form LPS. This negative correlation between the activity and the length of a polysaccharide chain could be explained by the ratio of the amount of the lipid A portion to the remaining whole LPS preparation. Another possibility is that the correlation may

TABLE 1. Kinetics of skin reactions induced by *S. typhimurium* LT2 LPS and lipid A[a]

Stimulant	Time (h) postinjection	% Mice with the following dermal responses[b]:			
		3+	2+	+	−
S-form LPS	12	0	0	80	20
	24	60	40	0	0
	48	60	0	40	0
	72	0	0	40	60
Lipid A	12	0	0	100	0
	24	0	60	40	0
	48	80	0	20	0
	72	60	40	0	0
	120	20	0	80	0

[a] Ten micrograms of LPS or lipid A was injected i.d. The local reaction was evaluated at various hours postinjection. Five mice in each group were tested.
[b] Dermal responses were evaluated according to criteria described in Results.

be due to the negative modulation of Re-form LPS activity by the polysaccharide portion. Furthermore, the R-core polysaccharide, probably 2-keto-3-deoxy-octonic acid, positively modulates the activity, because Re-form LPS also had stronger capacity for skin reaction than lipid A. Similar positive or negative modulation of activity has been recently reported for some activities induced by LPS, such as direct hemagglutination (14), the activation of complement (19), the release of LTC_4 by murine peritoneal macrophages (17), and the production of tumor necrosis factor by rat monocytes (24). Although, for all of these biological effects of LPS, the presence of lipid A has been essential, the expression of its activity has appeared to be modulated by the oligo-polysaccharide portion. The molecular mechanisms of this modulation remain unclear but will be elucidated by the determination of the physicochemical properties of LPS and lipid A (22), including the supramolecular conformation in several solutions, and by the identification of LPS receptors on target cells (18) and the functional properties of the receptors.

Re-form LPS and lipid A did not induce any hemorrhagic lesions in LPS-low-response C3H/HeJ mice even at relatively high doses, but they induced edematous changes at low doses. These results could be explained by quantitative differences in responsiveness. It is possible that mouse responses to LPS were not sufficient to result in hemorrhagic necrosis and that LPS elicits only edemas. The results could also be explained by qualitative differences in low responsiveness. This skin reaction may be induced by at least two different mechanisms; in other words, hemorrhagic lesions and edematous changes are different reactions. C3H/HeJ mice are resistant to most of the biological effects of LPS, which are thought to be mediated by host cells (23). Nevertheless, some of the effects, e.g., the activation of complement (1), can be induced by LPS in the same C3H/HeJ mice. The local edematous change induced by LPS injection may be a response to the activation of complement, whereas the hemorrhagic reaction may be related to the activation of target cells, such as macrophages and intravascular endothelial cells. In order to clarify the relationship between hemorrhagic and edematous changes, we are planning to do further experiments, such as inhibition of skin reactions by antibodies against cytokines and by antagonists against complement.

TABLE 2. Skin reactions of ddY mice induced by LPS and lipid A[a]

Stimulant	Dose (μg/mouse)	% Mice with the following dermal responses[b]				No. of mice tested
		3+	2+	+	−	
LPS (S form)						
S. minnesota	40	100	0	0	0	3
	20	100	0	0	0	5
	10	100	0	0	0	5
	5	40	0	60	0	5
	2.5	20	0	80	0	5
	1.3	0	20	60	20	5
S. typhimurium LT2	40	67	17	17	0	6
	20	50	0	33	17	6
	10	60	0	40	0	10
	5	20	20	60	0	5
	2.5	20	20	60	0	5
	1.3	0	20	80	0	5
E. coli 0111:B4	40	80	0	20	0	5
	20	50	17	17	17	6
	10	60	40	0	0	5
	5	50	17	34	0	6
	2.5	20	20	60	0	5
	1.3	20	20	20	40	5
LPS (R form)						
S. minnesota R60 (Ra)	10	83	0	17	0	6
	5	60	0	40	0	5
	2.5	0	40	40	20	5
	1.3	0	40	20	40	5
E. coli J5 (Rc)	10	100	0	0	0	6
	5	80	0	20	0	5
	2.5	40	0	60	0	5
	1.3	40	0	40	20	5
S. minnesota R595 (Re)	10	100	0	0	0	5
	5	100	0	0	0	5
	2.5	100	0	0	0	5
	1.3	20	60	20	0	5
Lipid A						
S. typhimurium LT2	20	100	0	0	0	5
	10	90	5	0	5	21
	5	43	43	0	14	14
	2.5	10	50	30	10	10
	1.3	7	28	21	43	14
E. coli J5	10	40	40	20	0	10
	5	20	70	10	0	10
	2.5	20	20	60	0	5
	1.3	0	40	60	0	5
Polysaccharide (*S. typhimurium* LT2)	80	0	0	0	100	5
	40	0	0	0	100	5
Saline (pyrogene free)		0	0	0	100	164
0.025% triethylamine in saline		0	0	0	100	94

[a] LPS or related compounds were injected i.d. The local reaction was evaluated 48 h postinjection.
[b] Dermal responses were evaluated according to criteria described in Results.

TABLE 3. Skin reactions of LPS-low-response mice induced by S-form LPS, Re-form LPS, and lipid A[a]

Mouse strain and stimulant	Dose (μg/mouse)	% Mice with the following dermal responses[b]				No. of mice tested
		3+	2+	+	−	
C3H/HeJ						
S-form LPS (*S. minnesota*)	40	0	0	100	0	5
	20	0	0	80	20	5
	10	0	0	40	60	5
	5	0	0	20	80	5
	2.5	0	0	20	80	5
	1.3	0	0	20	80	5
Re-form LPS (*S. minnesota* R595)	40	0	0	100	0	5
	10	0	0	100	0	5
	5	0	0	100	0	5
	2.5	0	0	80	20	5
	1.3	0	0	40	60	5
Lipid A (*S. typhimurium* LT2)	10	0	0	67	33	9
	5	0	0	100	0	5
	2.5	0	0	0	100	5
	1.3	0	0	0	100	5
C3H/HeN						
S-form LPS (*S. minnesota*)	40	100	0	0	0	3
	20	100	0	0	0	5
	10	80	20	0	0	5
	5	20	20	60	0	5
	2.5	0	0	80	20	5
	1.3	0	0	80	20	5
Re-form LPS (*S. minnesota* R595)	10	100	0	0	0	5
	5	100	0	0	0	5
	2.5	80	20	0	0	5
	1.3	40	0	60	0	5
Lipid A (*S. typhimurium* LT2)	10	60	10	20	10	10
	5	40	20	40	0	5
	2.5	0	80	20	0	5
	1.3	0	60	40	0	5

[a] LPS or related compounds were injected i.d. The local reaction was evaluated 48 h postinjection.

[b] Dermal responses were evaluated according to criteria described in Results.

TABLE 4. Dose requirements of LPS and lipid A to induce hemorrhagic necrosis

Mouse strain and stimulant	ED_{50} (μg)[a]
ddY mice	
S-form LPS	
S. minnesota	5.1
S. typhimurium LT2	5.3
E. coli 0111:B4	4.7
R-form LPS	
S. minnesota R60 (Ra)	3.1
E. coli J5 (Rc)	2.1
S. minnesota R595 (Re)	<1.3
Natural lipid A	
S. typhimurium LT2	1.9
E. coli J5	2.5
Synthetic lipid A	
506 (*E. coli*)[b]	<1.3
406 (precursor Ia)[b]	>10.0
Polysaccharide (*S. typhimurium* LT2)	>80.0
C3H/HeN	
S-form LPS (*S. minnesota*)	5.1
R-form LPS (*S. minnesota* R595 [Re])	1.3
Lipid A (*S. typhimurium* LT2)	2.5
C3H/HeJ	
S-form LPS (*S. minnesota*)	>40.0
R-form LPS (*S. minnesota* R595 [Re])	>40.0
Lipid A (*S. typhimurium* LT2)	>10.0

[a] ED_{50}s of samples to induce hemorrhagic lesions (2+ and 3+) were estimated by Probit analysis (2) from the results shown in Tables 2 and 3.

[b] ED_{50}s of these compounds were estimated as described elsewhere (28 [Table 5]).

Skin reactions may provide a good tool to clarify the structural requirement for biological activities of LPS, especially of R-mutant LPS and lipid A. By using this bioassay, we have already examined the activities of two kinds of synthetic compounds of lipid A and its derivative (28), which were synthesized by Imoto et al. (11, 12). ED_{50}s of these compounds are shown in Table 4. The activity of synthetic *E. coli*-type lipid A (compound 506), which induced the skin reaction in mice, was slightly stronger than that of natural lipid A (though not significant [$P > 0.05$]), whereas the activity of a synthetic counterpart of a lipid A precursor (compound 406) is considerably less than those of compound 506 and natural lipid A ($P < 0.01$). Nevertheless, compound 406 induced an increase in vascular permeability at a low dose. In further experiments, deacylated Re-form LPS induced edematous change but hardly induced hemorrhagic necrosis (14a). Although these results could also be explained by quantitative differences in activities, the results, including the data concerning LPS low-response C3H/HeJ mice in these studies, are consistent with the hypothesis that the hemorrhagic lesions and edematous changes are induced by different mechanisms, only one of which depends on the regulation of the *lps* gene. The former mechanism requires a restricted LPS structure, similar to that required for the Shwartzman reaction in rabbits, and the latter does not require so restricted an LPS structure.

Recent chemical analyses and the organic synthesis of LPS and lipid A offer information on the structures of several LPS preparations. The skin reactions discussed in this report will provide further information on the mechanisms of LPS action and on the structural requirements for the biological activities of LPS.

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