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Protection of Mice against Lethal Endotoxemia by a Lipid A Precursor

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Lipid X, the major biosynthetic precursor of lipid A, has recently been described. Although lipid X is a mitogen and coagulates the *Limulus* amebocyte lysate, we found that it is not lethal for mice, even when given in large doses (2×10^6 μg/kg). Furthermore, lipid X was found to give partial protection against a 100% lethal dose of endotoxin, even if the lipid X was given as late as 6 h after endotoxin challenge.

Bacteremia with gram-negative organisms is associated with high mortality and morbidity (29). The lipopolysaccharide (LPS) component of the outer membrane of these bacteria, known as endotoxin (27), reproduces much of the pathophysiology associated with gram-negative sepsis (1, 7). Recently, the correct structure of the toxic portion of LPS, lipid A (27), has been elucidated (Fig. 1) (2, 9, 13, 17–19, 22–24). This was greatly facilitated by the discovery of lipid X (Fig. 1), a novel monosaccharide precursor of lipid A that accumulates in certain phospholipid mutants of *Escherichia coli* (2, 13, 17, 19, 23, 24). Discovery of the biologic activities of lipid A precursors and derivatives has rapidly followed (5, 10, 11, 25, 28). We have found that lipid X isolated from *E. coli* is mitogenic for murine B cells (18), clotted the *Limulus* amebocyte lysate (16), and caused transient pulmonary hypertension with mild permeability changes in sheep (3; Burhop et al., Fed. Proc. **42**:4781, 1983). However, even large doses of *E. coli* lipid X (1,000 μg/kg) did not cause lethality or serious morbidity in sheep (3) or mice (see below). Since lipid X is a substructure of lipid A, we hypothesized that it might protect against lethal endotoxemia and examined this possibility in mice.

To test for toxicity of lipid X itself, C57BL/10 mice were challenged with 750, 2,000, or 5,000 μg intraperitoneally (12 mice), or with 750, 1,500, or 3,000 μg intravenously (8 mice). In all experiments, lipid X was dissolved at 7.5 to 10 mg/ml in physiological saline titrated to pH 8 with Tris. Of 20 mice, 19 lived. Consequently, lipid X appeared to be as nontoxic in mice as in sheep.

In preliminary testing, the lethal dose of our *E. coli* endotoxin preparation was determined with 40 mice that were challenged intravenously. The endotoxin was prepared from *E. coli* O111:B4 by the Westphal method (Sigma Chemical Co., St. Louis, Mo.), and 8- to 10-week-old, C57BL/10 mice, each weighing 20 to 25 g, were obtained from Jackson Laboratory, Bar Harbor, Maine. The mice were anesthetized with ether and injected intravenously with a total volume of 0.05 to 0.2 ml of endotoxin via the retro-orbital plexus. Endotoxin was dissolved in sterile, phosphate-buffered saline. All deaths occurred within 72 h of challenge; however, survivors were observed for at least 7 days. The dose that killed 100% of the mice was 250 μg.

The dose of lipid X that protects 50% of mice from a 100% lethal dose of endotoxin was calculated by the method of Reed and Muench (20). The data are shown in Table 1. To determine whether the time interval between endotoxin challenge and lipid X administration would alter the 50% protection dose, lipid X was given 30 s, 2 h, 4 h, or 6 h after

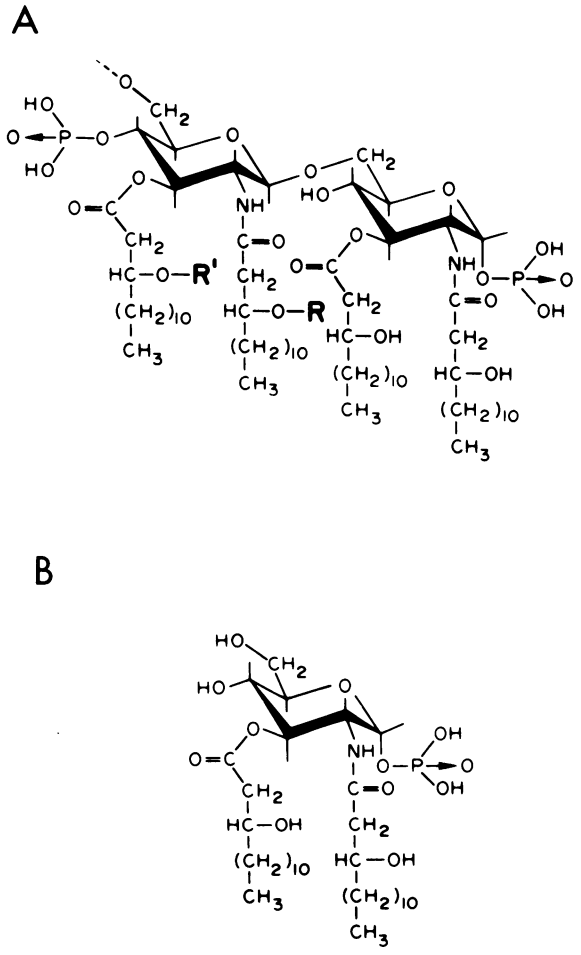

FIG. 1. Covalent structure of mature lipid A (A) and its relation to the monosaccharide precursor lipid X (B). The discovery of lipid X and its role in lipid A biosynthesis are reviewed in references 2, 13, 17–19 and 22–24.

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TABLE 1. Mortality as a function of dose and time of lipid X administration

Dose of lipid X (μg/mouse)[a]	No. of mice alive or dead at time (h)[b] of lipid X administration (250 μg)							
	0		2		4		6	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
0	1	11	0	4	0	6	0	12
100	3	3	1	5	1	5	2	4
200	3	3	1	5	2	4	1	5
400	5	1	4	2	2	4	1	5
800	5	0	4	2	4	2	2	4
1,200	5	1	5	0	4	2	3	3
1,600	5	0	5	0	4	2	3	3

[a] Both lipid X and endotoxin were given via the tail vein to C57BL/10 mice weighing 20 to 25 g.
[b] Times are relative to time of endotoxin challenge. There was approximately 30 to 60 s between the endotoxin challenge and the administration of lipid X for time zero.

endotoxin challenge. The 50% protection doses were: 0 h, 140 μg; 2 h, 350 μg; 4 h, 520 μg; 6 h, 1,100 μg. When the data are analyzed by chi-squared analysis, using the Yates correction (lipid-X-treated versus untreated), the differences were highly significant for all time points ($P < 0.01$).

In another experiment designed to further define the relationship between time of LPS challenge and lipid X administration, mice were given 750 μg of lipid X from 72 h before through 4 h after endotoxin challenge with either one or two 100% lethal doses (Table 2). Control mice received Tris-buffered saline and anesthesia. With the lower endotoxin challenge dose, 81% of mice survived if treated with lipid X from 1 h before through 4 h after LPS challenge. With the higher LPS dose, 41% of mice receiving lipid X from 6 h before endotoxin challenge through 1 h after LPS challenge survived. At both challenge doses of endotoxin, the group of mice that received lipid X shortly after endotoxin challenge showed higher mortality than the group on either side. Perhaps this is because those animals were anesthetized twice within a short time and received the toxic endotoxin challenge before receiving lipid X. After we observed this, eight mice were anesthetized twice within 15 min and given lipid X during the second period of anesthesia. None of these mice died.

Particularly striking was the reversal of lethal endotoxicity at times as late as 6 h after endotoxin challenge, although this was demonstrated only at the lower endotoxin dose. By 4 h after 250 μg of LPS, the mice had stopped normal behavior; i.e., nesting had stopped, little spontaneous activity occurred, and the animals were shaking. Prevention of lethal endotoxicity by the use of glucocorticoids, prostaglandins, naloxone, pressors, fluid resuscitation, or anti-endotoxin antibody is contingent upon their being given before or shortly after the administration of the endotoxin challenge (4, 6, 8, 12, 14, 15, 26, 30). Lipid X prevented lethal endotoxicity, even when given 4 to 6 h after endotoxin, but this was not so apparent with 500 μg of LPS (Table 2).

Although the mechanism of protection by lipid X is unknown, the simplest explanation is competition for a common target molecule. Other possibilities include termination of endotoxin action by allosteric or noncompetitive interactions, release of an extra- or intracellular inhibitor(s), or rapid elaboration of a protective substance(s), e.g., protein C (F. B. Taylor, Clin. Res. 42:566A, 1985). We are in the process of examining other lipid A precursors for their ability to protect mice against lethal endotoxemia. The data presented offer hope that a specific, nontoxic inhibitor of endotoxin might be found among the endotoxin precursor molecules or their derivatives.

TABLE 2. Mortality and time to death as a function of the timing of lipid X administration and LPS challenge dose

Time (h) of lipid X administration[a]	Mortality of mice		Time (h) to death	
	% Dead	No. Dead/total	Mean	Range
250-μg LPS challenge[b]				
No lipid X[c]	100	12/12	22	10–52
−72 to −2	95	19/20	26	14–40
−1	25	1/4	22	NA[d]
−0.5 to 0	0	0/12	NA	NA
0.25	100	4/4	50	30–58
0.5 to 1	25	2/8	46	40–52
2	25	1/4	48	NA
4	0	0/4	NA	NA
500-μg LPS challenge				
No lipid X	100	11/11	18	15–27
−72 to −2	83	15/18	21	16–44
−1	33	1/3	40	NA
−0.5 to 0	60	6/10	28	24–36
0.25	100	3/3	38	24–44
1	50	2/4	25	20–30
2	100	4/4	26	20–38
4	100	4/4	22	20–26

[a] The times of lipid X injection (750 μg) are given relative to the LPS challenge, which is designated time zero. Both LPS and lipid X were given via the retro-orbital plexus.
[b] The LPSchallenge represents the 100% lethal dose (250 μg) or twice the 100% lethal dose (500 μg).
[c] Control mice were anesthetized and given saline instead of lipid X. One or two mice were anesthetized at each time point.
[d] NA, Not applicable.

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Volume 52, no. 3, p. 905: The affiliation line should read as printed above.

Page 905, abstract, line 3: "doses (2×10^6 μg/kg)" should read "doses (2×10^5 μg/kg)."

Page 906, col. 1, line 3: "Yates correction" should read "Yates' correction."