

# Molecular requirements for B-lymphocyte activation by *Escherichia coli* lipopolysaccharide

(mitogenic glycolipids/ $\beta$ -hydroxymyristate/2,3-diacetylglucosamine 1-phosphate/C3H/HeJ mice/lipid A receptors)

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**ABSTRACT** Certain *Escherichia coli* mutants altered in phosphatidylglycerol metabolism accumulate fatty acyl derivatives of glucosamine 1-phosphate. Especially prominent is 2,3-diacetylglucosamine 1-phosphate (previously designated lipid X), which may be an early precursor of lipid A. We have examined the activity of lipid X ( $M_r = 711.9$ ) and several related compounds as mitogens towards mouse lymphocytes. As judged by labeling with [*methyl*-<sup>3</sup>H]thymidine, lipid X is mitogenic, and it mimics the properties of lipopolysaccharide and lipid A. The following evidence suggests that lipid X exerts its effects by a route similar to that of lipopolysaccharide: (i) lymphocytes from C3H/HeJ mice, which are unresponsive to lipopolysaccharide, are also not stimulated by lipid X; (ii) polymyxin B abrogates lymphocyte stimulation by lipid X; and (iii) lipid X induces the proliferation and maturation of lymphocytes to antibody-producing plaque-forming cells. Selective removal of the ester-linked hydroxymyristate moiety at position 3 totally abolishes mitogenic activity. Other phospholipids, such as phosphatidic acid, CDP-diglyceride, phosphatidylcholine, and lysophosphatidylcholine, have no activity as mitogens. If lipid X and lipid A induce by common mechanism(s) B-lymphocyte proliferation, then it follows from structural comparison that the reducing-end subunit of lipid A is the minimal structural requirement for this activity. Because the structure of lipid X is completely defined, biochemical and pharmacological dissection of B-cell activation by lipopolysaccharide should now be possible.

The outer membrane of Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium* consists of proteins, phospholipids, and lipopolysaccharide (1, 2). The latter substance is localized almost exclusively on the outer surface of the outer membrane (3). It accounts for many of the immunological and endotoxic properties of Gram-negative organisms (4). Although the complete structure of lipopolysaccharide is unknown, it consists of three domains (4). The outer sugars, which are highly variable, give rise to the O-antigenic determinants. The core sugars are relatively conserved and may be involved in cell penetration by certain bacteriophages. The lipid A molecule (which is highly conserved between species) functions as a hydrophobic anchor holding lipopolysaccharide in place. Because lipopolysaccharide represents several percent of the dry weight of Gram-negative bacteria (4), it follows that lipid A (and not phospholipid) must account for most of the outer leaflet of the outer membrane.

The complete structure and biosynthesis of lipid A are unknown. The current representation (5) of the smallest lipid A unit is shown in Fig. 1. As indicated by the distribution of R and R', there is uncertainty surrounding the locations of the fatty acid esters. Some forms of lipid A are further derivatized

with aminoarabinose and ethanolamine, but the function of these is unknown (6, 7).

Lipopolysaccharide (specifically the lipid A moiety) exerts many striking pathophysiological effects on mammalian organisms (4). These include the induction of endotoxic shock, pyrogenicity, complement activation, adjuvant activity, and B-lymphocyte mitogen activity. The molecular features of lipid A responsible for these actions are not well defined.

We have discovered and established (8) the complete structure of two acylated monosaccharides that accumulate in certain phosphatidylglycerol-deficient mutants of *E. coli* at non-permissive temperatures. The simplest and best characterized of these is lipid X (Fig. 1), which consists of glucosamine 1-phosphate derivatized with an amide-linked and an ester-linked hydroxymyristate at positions 2 and 3, respectively. Lipid X may be an early precursor of lipid A, and its existence leads us to postulate the model for lipid A shown in Fig. 2 (8). Because lipid X is easy to purify and can be manipulated further by controlled chemical degradation (8), we have examined its ability to activate mouse lymphocytes. We have found that X resembles intact lipopolysaccharide by this criterion and that the ester-linked hydroxymyristate at position 3 is crucial for this biological function. The results suggest that lipid X, like lipid A (9, 10), is a B-cell mitogen.

## MATERIALS AND METHODS

**Materials.** [*methyl*-<sup>3</sup>H]Thymidine was purchased from New England Nuclear. *E. coli* lipopolysaccharide W 055:B5 was obtained from Difco. This material contained less than 1% phospholipid or protein contaminants. Dimyristoyl phosphatidylcholine, egg-derived lysophosphatidylcholine, concanavalin A, and polymyxin B were obtained from Sigma. All other phospholipids were from Serdary Research Laboratories (London, ON, Canada). Fetal bovine serum (lot 2B076) from M. A. Bioproducts (Walkersville, MD) produced little or no lymphocyte activation on its own. Dulbecco's minimal essential culture medium was purchased from GIBCO.

Lipids X and Y were isolated from *E. coli* strain MN7 by using a modification of the method of Nishijima and Raetz (11). Evidence for purity and proof of structure will be presented elsewhere (8). The mild alkaline hydrolysis product of lipid X (which retains the N-linked but not the O-linked hydroxymyristate residue) was obtained under standard conditions for deacylation of phospholipids (11). Lipid Y\* was obtained from lipid Y by treatment with triethylamine (12) for 3 hr at 100°C. Dephosphorylated lipid X was generated by mild acid treatment (0.1 M HCl at 100°C for 60 min). A lipid A derivative previously designated TLC-3 (which lacks the sugar 1-phosphate moiety at the reducing end) was generated as described (12). Microspheres [polystyrene divinylbenzene beads, (5.7 ± 1.5)- $\mu$ m di-

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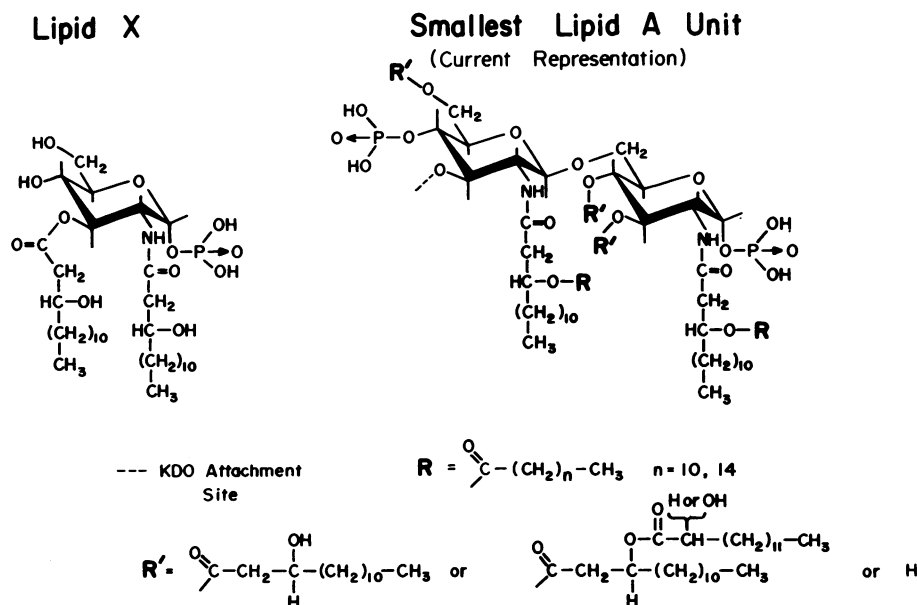


FIG. 1. Structure of lipid X and its relationship to lipid A. The smallest lipid A unit (lacking phosphoethanolamine and aminoarabinose) is the currently accepted representation discussed by Wollenweber *et al.* (5). Ketodeoxyoctulosonate (KDO) is assumed to be attached at position 3' in this model.

ameter; Dow, Indianapolis, IN] coated with lipopolysaccharide or dephosphorylated lipid X were prepared by published methods (13).

**Mitogenesis Experiments.** C57BL/10 and C3H/HeJ mice, originally from The Jackson Laboratory, were propagated for 8–9 generations in the colony of C. E. Hayes. Prior to use, animals were anesthetized with ether, and the spleens were excised immediately. After opening the splenic capsule, the cells were suspended in 10 ml of Dulbecco's minimal essential medium, washed twice with the same medium, and then resuspended at  $5 \times 10^6$  cells per ml in the same medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM minimal essential medium nones-

sential amino acids, 10 mM HEPES, 50  $\mu\text{M}$  2-mercaptoethanol, and 100 units of penicillin G and 100  $\mu\text{g}$  of streptomycin per ml. Multiple wells of a 96-well Costar microtiter dish were seeded with 0.1 ml of this cell suspension ( $5 \times 10^5$  cells per well). Next, an additional 0.1 ml of Dulbecco's minimal essential medium containing an appropriate amount of test mitogen was added, as indicated in the figure legends. After addition of mitogen, the dishes were incubated for 2 days at 37°C and 100% humidity in 7.5%  $\text{CO}_2/92.5\%$  air. Finally, [*methyl*- $^3\text{H}$ ]thymidine was added at 1  $\mu\text{Ci}$  per well (1 Ci =  $3.7 \times 10^{10}$  Bq), and the cells were incubated at 37°C for an additional 6 hr. Cells were washed free of medium and excess thymidine by using 0.9% NaCl with a Bellco microharvester. The washed cells were retained on Bellco glass fiber filter strips, and radioactivity incorporated into the cells was quantitated by liquid scintillation counting. Each mitogen concentration was tested in triplicate wells, and the standard deviation of the measurement was approximately  $\pm 10\%$ . Induction of plaque-forming cells by various mitogen preparations was quantitated by the method of Cunningham and Szenberg (14), with a monolayer of sheep erythrocytes (Flow Laboratories) as the indicator.

## RESULTS

**Mitogenic Effect of the  $\text{CHCl}_3$ -Soluble Fraction from Mutant MN7.** Membrane phospholipids were extracted under acidic conditions (11) from a strain of *E. coli* that is wild type with respect to its membrane lipids or from mutant MN7, which accumulates lipid X. The crude lipids were dried under  $\text{N}_2$  to remove  $\text{CHCl}_3$ , suspended in 1 mM EDTA adjusted to pH 6 with NaOH, and dispersed at a concentration of 1–2 mg/ml by sonic irradiation for 5 min at 25°C in a bath sonicator. *E. coli* lipopolysaccharide was dissolved in 1 mM EDTA in a similar manner. Next, triplicate sets of mouse lymphocyte cultures were incubated for 48 hr with increasing amounts of added phospholipid as described in the legend to Fig. 3. After this, the cells were labeled for 6 hr with [*methyl*- $^3\text{H}$ ]thymidine, and incorporation of  $^3\text{H}$  into DNA was determined.

Phospholipid dispersions derived from MN7 stimulated lymphocyte proliferation to a much greater extent than did similar preparations from strains of *E. coli* with a wild-type lipid com-

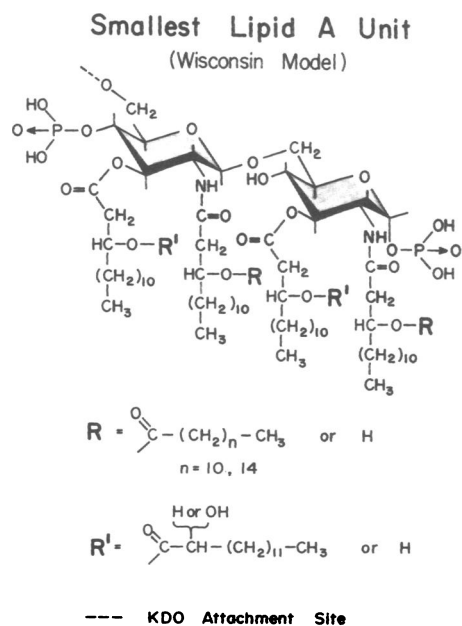


FIG. 2. Postulated structure of lipid A based on the discovery of lipid X (Wisconsin Model). Lipid X is assumed to be a precursor of both the reducing and nonreducing ends of lipid A (8). This would place ketodeoxyoctulosonate (KDO) in position 6'.

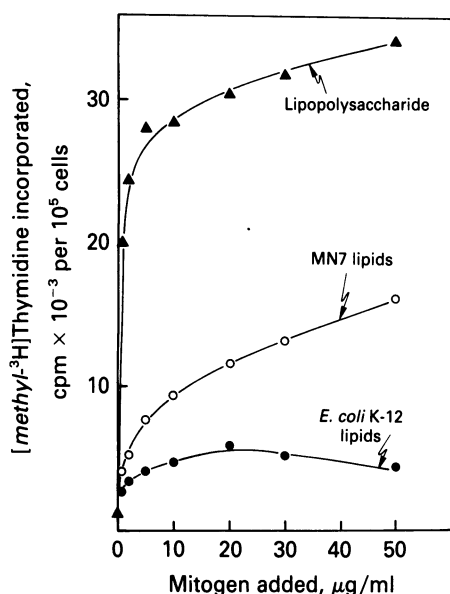


FIG. 3. Mitogenic factor in the  $\text{CHCl}_3$ -soluble fraction of mutant MN7 cells. Lipopolysaccharide or crude phospholipids (dried out of  $\text{CHCl}_3$ ) were suspended in sterile 1 mM EDTA (pH 6) at 1–2 mg/ml. Stable dispersions were prepared by ultrasonic irradiation for 5 min at  $25^\circ\text{C}$  in a bath sonicator (Laboratory Supplies, Hicksville, NY). Cells in microtiter dishes (see text) were incubated for 48 hr in the presence of various levels of test mitogen. The volume change caused by the addition of mitogen to each well did not exceed 5%, and separate controls showed that EDTA by itself had no effect at this level and did not inhibit proliferation (not shown). After 48 hr, cells were labeled with [ $^3\text{H}$ ]thymidine as described.

position (Fig. 3). The stimulation of cells by commercial *E. coli* lipopolysaccharide is shown for comparison. The results suggest that there is an additional mitogenic factor in the  $\text{CHCl}_3$ -soluble fraction of strain MN7 that is not present in the wild type. The main difference between MN7 and wild-type *E. coli* lipids has been attributed previously to the presence of glycolipids X

and Y in the mutant at levels that are 300-fold (or more) greater than normal (11).

**Mitogenic Activity of Purified Lipid X.** The predominant glycolipid that accumulates in MN7 mutant is a diacylglycerolamine 1-phosphate, substituted with  $\beta$ -hydroxymyristate at positions 2 and 3 (Fig. 1). We have purified and characterized this material (8). It is readily dispersed in 1 mM EDTA (pH 6) at concentrations of 1–2 mg/ml, especially with mild ultrasonic irradiation or brief heating to  $60^\circ\text{C}$ .

Dispersions of lipid X resembled commercial lipopolysaccharide in their ability to stimulate lymphocyte proliferation (Fig. 4A). However, unlike the commercial lipopolysaccharide, this activity now can be attributed directly to a highly purified and structurally defined molecule. Phosphatidic acid, which is structurally similar to lipid X, had no mitogenic activity. Phosphatidylcholine, sphingomyelin, phosphatidylinositol, cardiolipin, phosphatidylserine, CDP-diglyceride, lysophosphatidylcholine, lysophosphatidylethanolamine, and lysophosphatidic acid were also ineffective. Polymyxin B (50  $\mu\text{g/ml}$ ), which inhibits lipopolysaccharide-induced mitogenesis (15), also abolished lymphocyte stimulation by lipid X (not shown).

To further demonstrate that lipid X and lipopolysaccharide exert their effects by a common mechanism(s), we examined splenic lymphocytes from C3H/HeJ mice. These are unresponsive to lipopolysaccharide (16–18), presumably because they lack a membrane receptor (or enzyme) that recognizes this molecule (19). C3H/HeJ lymphocytes also responded poorly (if at all) to lipid X (Fig. 4B). However, they were activated readily by the T-cell mitogen (20) concanavalin A (Fig. 4) or by the purified protein derivative of tuberculin (21), which function by different mechanisms (20, 21). T cells do not seem to be required for lipid X mitogenesis because splenic lymphocytes from *nude* athymic mice responded well to lipid X and lipopolysaccharide but not to concanavalin A (not shown).

**Molecular Requirements for Mitogenicity.** Mild alkaline hydrolysis of lipopolysaccharide abolishes its mitogenic activity (9). Because the locations of the ester-linked fatty acids in lipid X are not precisely known (Figs. 1 and 2), it is unclear which

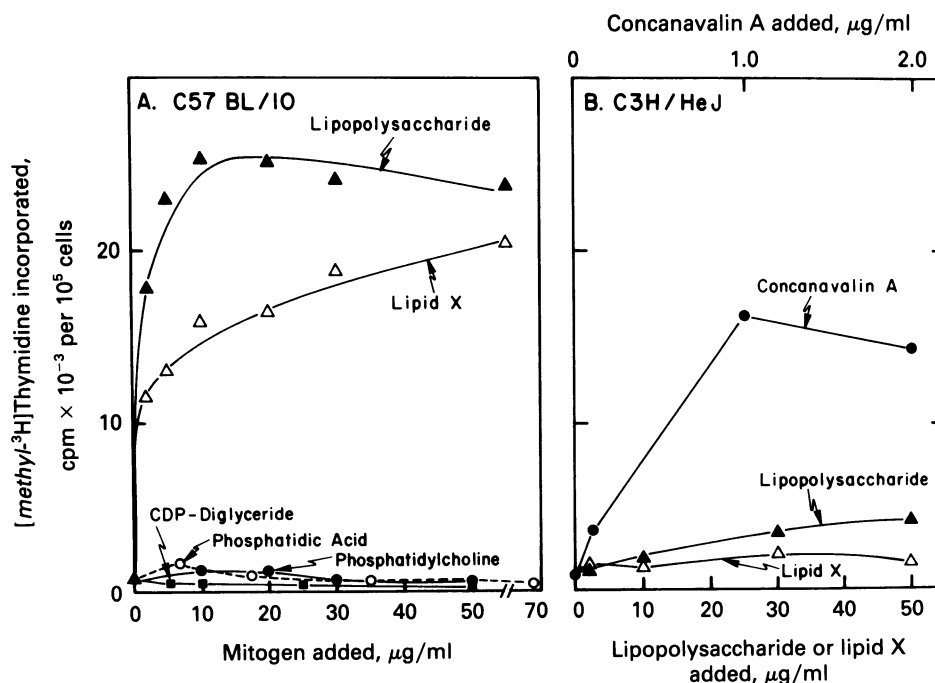


FIG. 4. Mitogenic activity of pure lipid X on proliferation of lymphocytes from two mouse strains. Dispersions of lipid X and other phospholipids were prepared as described in Fig. 3. Concanavalin A was freely soluble in water and was not subjected to sonic irradiation.

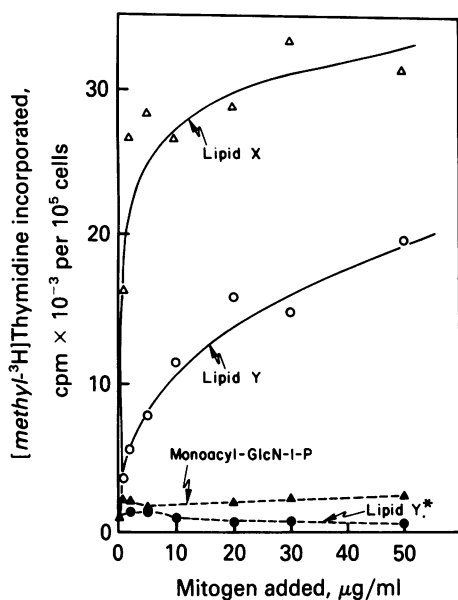


FIG. 5. Ester-linked hydroxymyristate at position 3 is required for B-cell stimulation by lipids X and Y. Lipids were dispersed as described in Fig. 3.

particular ester linkage is required. The very fact that lipid X has strong mitogenic activity implies that the  $\beta$ -hydroxymyristate esterified at position 3 of this molecule must mediate this function. To prove this, we subjected lipid X to mild alkaline hydrolysis (11, 22) and isolated a monoacylglucosamine 1-phosphate derivative bearing only the amide-linked  $\beta$ -hydroxymyristate. Removal of the esterified hydroxymyristate completely abolished the B-cell proliferation (Fig. 5). Simultaneous addition of equimolar  $\beta$ -hydroxymyristate and monoacylglucosamine 1-phosphate (not shown) or of glucosamine 1-phosphate plus  $\beta$ -hydroxymyristate (not shown) did not cause significant proliferation.

In addition to lipid X, mutant MN7 accumulates a compound designated lipid Y, especially after 3 hr at 42°C (11). This material is similar to lipid X (Fig. 1) but has an additional esterified palmitate moiety on the  $\beta$ -hydroxyl group of the N-linked hydroxymyristate (unpublished data). Lipid Y is much less soluble in H<sub>2</sub>O than is lipid X but can still be dispersed at 1 mg/ml by sonic irradiation at pH 6. This material was also mitogenic, though somewhat less under these conditions, possibly because of poor solubility (Fig. 5). Selective removal of the 3-O-linked  $\beta$ -hydroxymyristate from the glucosamine ring of lipid Y with triethylamine (12) gave rise to a derivative that we designated Y\*. This substance retains the esterified palmitate and therefore is very similar to lipid X in its physical properties (unpublished data). However, lipid Y\* was completely inactive as a mitogen (Fig. 5). This result confirms that lymphocytes somehow recognize the  $\beta$ -hydroxymyristate ester linkage at position 3 of lipids X and Y and that this linkage must be intact for induction of cell proliferation.

Mild acid treatment can be used to remove the 1-phosphate moiety from lipid X, leaving the two fatty acid residues in place (8). The resulting substance, dephosphorylated lipid X, was very insoluble in H<sub>2</sub>O and could not be dispersed by sonic irradiation. Fine suspensions of dephosphorylated lipid X or preparations immobilized on hydrophobic beads did cause slight cell proliferation (not shown). The finding that dephosphorylated lipid X retained significant biological activity strongly suggests that the sugar 1-phosphate moiety is not obligatory for the mitogenic response. This conclusion is further supported by the

Table 1. Stimulation of plaque-forming cells by incubation of mouse spleen cells with various mitogens

Mitogen added during preincubation	Plaque-forming units per 10 <sup>6</sup> spleen cells
None	5
<i>E. coli</i> lipopolysaccharide, 20 $\mu$ g/ml	60
50 $\mu$ g/ml	70
Lipid X, 20 $\mu$ g/ml	45
50 $\mu$ g/ml	80
Lipid Y, 50 $\mu$ g/ml	40
Lipid Y*, 50 $\mu$ g/ml	0
Dephosphorylated lipid X, 50 $\mu$ g/ml	30

Mitogen dispersions (1–2 mg/ml) were prepared by mild sonication and were incubated at the concentrations indicated with splenic lymphocytes for 48 hr prior to assay for plaque-forming cells on a monolayer of sheep erythrocytes (14). Values shown are averages of duplicate determinations; the standard deviation is approximately  $\pm 5$  plaque-forming units.

observation (not shown) that TLC-3, a lipid-A derivative from *S. typhimurium* G30/C21 lacking the 1-phosphate residue (12), also was fully mitogenic under the same conditions as those pertaining to Figs. 3–5.

**Formation of Plaque-Forming Cells.** We used a minor modification of the method of Cunningham and Szenberg (14) to detect the stimulation of plaque-forming cells by *E. coli* lipopolysaccharide, lipid X, or other preparations. All derivatives that stimulated radioactive thymidine incorporation (Figs. 3–5) also increased the incidence of antibody-producing cells (Table 1) significantly. These results show that lipid X, lipid Y, and perhaps also dephosphorylated lipid X all stimulate true lymphocyte proliferation and that the observed increase in [*methyl*-<sup>3</sup>H]thymidine incorporation is not a radiochemical artifact.

## DISCUSSION

Because the complete covalent structure of lipid A is not known (4), it has been difficult to elucidate the molecular mechanisms by which lipid A (or lipopolysaccharide) triggers diverse physiological responses, such as B-cell proliferation or endotoxic shock (4). The discovery of biologically active monosaccharide lipid A fragments with defined structures (8) makes it possible to explore lipid A function at a biochemical level. As shown in Figs. 3–5 and Table 1, the lipid X molecule retains many of the properties of intact lipopolysaccharide with respect to the induction of B-lymphocyte proliferation. In this case the O-acyl-linked  $\beta$ -hydroxymyristate at position 3 of the glucosamine ring (Fig. 1) is critical for function, while the phosphate moiety may enhance biological activity by increasing the solubility of the acylated sugar.

Does lipid X possess other activities (4) normally associated with lipopolysaccharide? Sheep can respond to intravenous injection of lipid X in a manner that resembles the pathophysiological effects of Gram-negative endotoxin (unpublished data), including both the characteristic pulmonary hypertension and the increased lung lymph flow. However, considerably higher doses are required than with lipid A disaccharides. Further, the limulus lysate assay for Gram-negative endotoxin is positive with lipid X under certain conditions, and removal of the O- and acyl-linked hydroxymyristate abolishes clot-forming activity (unpublished data). On the other hand, lipid X appears to be relatively nontoxic as judged by the chicken embryo lethality test (CELD<sub>50</sub> > 10  $\mu$ g, unpublished data). It would appear that many of the biological activities of lipid A are mediated by the sugar-linked O-acylated hydroxymyristate at position 3 and that

activity is further enhanced by formation of the lipid A disaccharide. It should be of great interest to synthesize a series of lipid X analogs and to explore the molecular pharmacology of endotoxin function.

The existence of autosomally recessive mutations (as in the C3H/HeJ mice), which confer resistance both to induction of B-cell proliferation and to endotoxic shock, argues for a common biochemical mechanism (18). We imagine that lipid X (or lipopolysaccharide) interacts with or inserts itself into the cell membrane and subsequently binds to a membrane receptor (or enzyme), which recognizes the ester linkages at position 3. After this common step, a cascade of events is triggered that, in the case of B cells, results in proliferation and maturation. Because the receptor (or recognition enzyme) is presumably missing in the C3H/HeJ mice, an analysis of lipid X binding and metabolism by C3H/HeJ lymphocytes might reveal the underlying biochemical lesion.

How does the binding of lipid X or lipopolysaccharide to a membrane protein bring about a global proliferative response? Because of the importance of the ester-linked hydroxymyristate and its relative chemical lability in the presence of triethylamine, we hypothesize that this moiety may play a more direct role. It is well established that many plasma membrane proteins (including the transferrin receptor and the vesicular stomatitis virus glycoprotein) are covalently modified with esterified fatty acids (23). In the case of vesicular stomatitis virus, viral mutations that interfere with the acylation of the glycoprotein may in some instances prevent its export from the endoplasmic reticulum to the cell surface (24). If acylation is important for the function of certain membrane proteins, then perhaps an alteration of their acylation pattern by lipid X or lipopolysaccharide might influence a large number of cellular parameters, including cyclic nucleotide levels as studied by Watson (25). In principle, lipid X could participate directly as a fatty acyl donor because transacylation reactions involving membrane lipids are well documented (26, 27). It should be possible to test this hypothesis directly with appropriate radiochemical preparations of lipid X. Whatever the mechanism, we doubt that changes in membrane fluidity are involved (28), because lipid Y\*, which is very similar to lipid X in structure and solubility, has no mitogenic activity (Fig. 5).

Other polyclonal B cell mitogens have been described that are totally different in structure from lipid X. These substances include dextran sulfate and poly(I)-poly(C) (29). Perhaps, these agents function at steps beyond the putative membrane receptor required for the action of lipid X or lipopolysaccharide. One must also consider the possibility that the activation of specific B-cell clones by true antigens shares some common molecular components with lipid X-mediated mitogenesis and that macrophages participate in lipid X action. However, lipid X (like lipopolysaccharide) appears to bypass the T-cell requirement (30, 31) for activation of specific B cells because lipid X activates splenic lymphocytes from *nude* athymic mice.

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