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Report

Antiendotoxin Activity of Lipid A Analogues: Requirements of the Chemical Structure[1]

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Lipid X, a monosaccharide precursor of lipid A, has been found to prevent death in animals given a lethal dose of endotoxin, but the mechanism of this protective effect is unknown. We previously reported that lipid X blocks endotoxin-induced priming of human neutrophils in a manner consistent with competitive inhibition. To determine the molecular requirements for this antiendotoxin activity, we studied several derivatives of lipid X using the neutrophil priming assay. Neutrophil priming was quantitated by measuring stimulated superoxide (O_2^-) release. The removal of either acyl group from lipid X or even the simple change of the amide to an ester linkage at C2 of the glucosamide ring resulted in a marked loss of antagonism. Monosaccharide analogues, structurally related to native lipid A by the presence of acyloxyacyl side chains, demonstrated marked inhibition of endotoxin-induced priming at low concentrations but an endotoxin-like, priming effect at high concentrations. The addition of a phosphate group at position 4 of the sugar moiety was the only modification studied so far that produced a pure antagonist with increased antiendotoxin activity. Demonstration of these structural requirements for the antiendotoxin activity of lipid A analogues supports the hypothesis that this effect may be mediated via specific cellular binding sites. Lipid X derivatives may be useful for studying the interaction of endotoxin with cells and their antiendotoxin activity may prove beneficial in the treatment of septicemia.

KEY WORDS: endotoxin; lipopolysaccharide; lipid A; lipid X; neutrophil; superoxide.

INTRODUCTION

Endotoxin, a lipopolysaccharide (LPS) found in the outer membrane of gram-negative bacteria, has been implicated in the pathogenesis of septic shock (1), a syndrome associated with a high mortality despite appropriate antibiotics (2,3). Lipid X (N^2,O^3-diacyl-glucosamine-1-phosphate) is a monosaccharide precursor in the biosynthetic pathway of lipid A, the toxic moiety of endotoxin (4). This lipid A part-structure has been shown to protect mice (5) and sheep (6) from lethal LPS challenge, but the mechanism of this beneficial effect remains unknown.

Previously we demonstrated that lipid X can block LPS-induced priming of human neutrophils for an enhanced respiratory burst (7). This antiendotoxin effect was found to be dose dependent. Increasing concentrations of lipid X shifted the LPS dose–response curve of neutrophils rightward without preventing maximal priming at higher LPS concentrations, a finding suggestive of competitive inhibition. Further, lipid X inhibited LPS-induced priming by a direct interaction with the neutrophil, in contrast to polymyxin B, which neutralized LPS by binding to it (7).

In this investigation, we studied closely related analogues of lipid X in the neutrophil priming assay (7,8) to determine the molecular requirements for anti-LPS activity. Representative derivatives of lipid X were found to have loss of inhibitory activity, enhanced inhibitory activity, or concentration-dependent mixed (blocking and priming) activity, depending on the specific alteration in the chemical structure. These preliminary results suggest that these compounds may be useful in characterizing LPS–cell interactions. Further, the antiendotoxin activity of lipid X derivatives may have efficacy in the treatment of gram-negative sepsis.

MATERIALS AND METHODS

Human neutrophils were isolated from healthy donors using dextran sedimentation and Ficoll–Hypaque centrifugation. The neutrophils, suspended in Hanks' balanced salt solution (HBSS; Gibco, Grand Island, NY) without calcium and magnesium (−), were then incubated in a shaking water

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bath at 37°C for 60 min with increasing concentrations of lipid X or a lipid X derivative in either the presence or the absence of LPS (10 ng/ml) from *Salmonella minnesota* Re595 (List Biological Laboratories, Campbell, CA), as previously described (7). This concentration of LPS has been shown to result in a near-maximal priming effect for most neutrophil donors (7,8). Stock solutions of LPS and the analogues (1 mg/ml) were made in sterile pyrogen-free water, sonicated for 4 min at maximum output (Model 16-850, Virtis Co., Gardiner, NY), and stored in small aliquots at −70°C. Aliqots were thawed, sonicated for an additional 2 min, and adjusted to the required concentration in HBSS (−) when used. Each analogue in individual experiments was run concurrently with lipid X using neutrophils from the same donor. The lipid X and lipid X derivatives used in these experiments were synthesized and prepared by Sandoz Forschungsinstitut using previously described methods (9).

Following incubation, trypan blue exclusion showed the cells to be >92% viable, and the absence of aggregation was confirmed microscopically. Lipid X and some of its derivatives could not be used at high concentrations (10 μ*M*) because this caused neutrophil clumping, and O_2^- release by clumped neutrophils cannot be accurately assessed. This clumping of cells was considered to be a nonspecific membrane effect since examination of it in an aggregometer disclosed a very slow increase in light transmittance over 60 min, and not a characteristic stimulated aggregation response (7). Further, high concentrations of lipid X did not induce a respiratory burst.

N-Formyl-methionyl-leucyl-phenylalanine (FMLP)-stimulated O_2^- production was determined after incubation by previously described methods based on superoxide dismutase-inhibitable reduction of cytochrome *c* (7,8). The nanomoles of O_2^- released per 10^6 neutrophils was calculated from the peak absorption change by using the extinction coefficient for cytochrome *c* of $2.1 \times 10^4\ M^{-1}\ cm^{-1}$ (7). Superoxide production for each sample was then converted to the percent priming effect as described in Fig. 2. The best linear, unbiased estimate for the relative potency of an analogue compared to lipid X was determined using an analysis of covariance for parallel line assays (a standard parallel-line assay method could not be used since the data were paired by experiment).

RESULTS

The 3-hydroxytetradecanoate residues of lipid X (Fig. 1) were found to be important structures in regard to the anti-LPS activity of the molecule. Removing this group from the third carbon atom (C3) of the glucosamine ring or shortening it to an acetyl residue at C2 resulted in nearly complete loss of anti-LPS activity (Figs. 2a, data shown for N^2-acetyl,O^3-acyl-glucosamine-1-phosphate). Even simply changing the amide to an ester linkage at C2 (O^2,O^3-diacylglucose-1-phosphate) reduced the ability of the compound to inhibit LPS-induced neutrophil priming (Fig. 2b) by a factor of 2.7 (see Methods). Analogues with one or both of the 3-hydroxytetradecanoate residues replaced by nonhydroxylated fatty acids of the same length did not have altered anti-LPS activity compared to lipid X (data not shown).

Acyloxyacyl analogues, formed by substitution of either

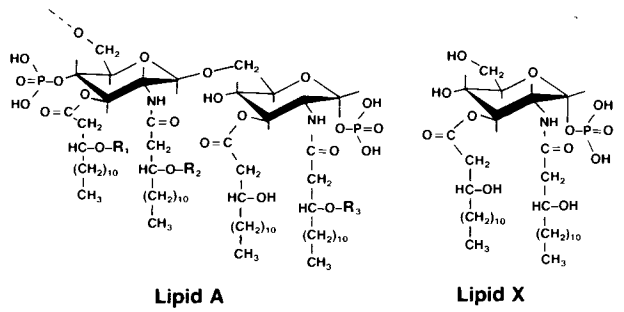

Fig. 1. Chemical structure of lipid A and lipid X. Lipid X is a monosaccharide precursor in the biosynthetic pathway of lipid A. Depending on the source of lipid A, R_1 is usually a lauroyl, R_2 a myristoyl, and R_3 either a hydrogen or a palmitoyl moiety.

hydroxyl group of the fatty acids with a tetradecanoate residue, enhanced the anti-LPS activity of the monosaccharide at low concentrations but resulted in marked agonism of priming (LPS-like effect) at high concentrations (Fig. 2c, data shown for N^2-acetyl,O^3-acyl-glucosamine-1-phosphate). Only one of the derivatives studied, N^2,N^3-diacyl-glucosamine-1,4-bisphosphate, was found to be a "pure antagonist" with greater activity than the parent compound, lipid X (Fig. 2d). This bisphosphate analogue had a relative potency of 3.9 compared to lipid X (see Methods).

DISCUSSION

These data support the hypothesis that the inhibitory effect of lipid X analogues on LPS-induced priming of human neutrophils is mediated by cellular binding sites that exhibit structural specificity. Although there is evidence for the existence of LPS receptors that mediate the cellular effects of endotoxin, definitive proof and characterization of these binding sites have not been accomplished (10). Direct binding studies using Scatchard analysis have been hampered by the nonspecific, high-affinity binding of LPS to mammalian cell membranes (10). Lipid A part-structures, such as the monosaccharide derivatives studied here, may be useful in better characterizing the interaction between endotoxin and cell membranes. Further, the anti-LPS effect of some of these analogues may provide a novel approach to managing the acute toxicity of gram-negative infections.

The mechanism of LPS-induced neutrophil priming remains unknown. Resting levels of intracellular calcium are not changed by LPS priming (11). The translocation of cytosolic enzymes involved in stimulus–response coupling to the cell membrane by myristoylation or other means has been proposed as a possible explanation for the priming phenomena (7). Protein kinase C (PKC), a potential candidate for such an interaction, was recently found to be activated directly by lipid A in intact platelets (12), but this occurred only at concentrations more than 10,000 times higher than that of the LPS used in our studies. Further, there is no evidence, at present, that PKC is accessible to endotoxic lipid A in whole cells (12). Interestingly, lipid X was shown to block PKC activation by both lipid A and phorbol 12-myristate 13-acetate (PMA), suggesting that all three compounds have a common site of action (12). In our assay system, however, neither lipid X nor its derivatives block

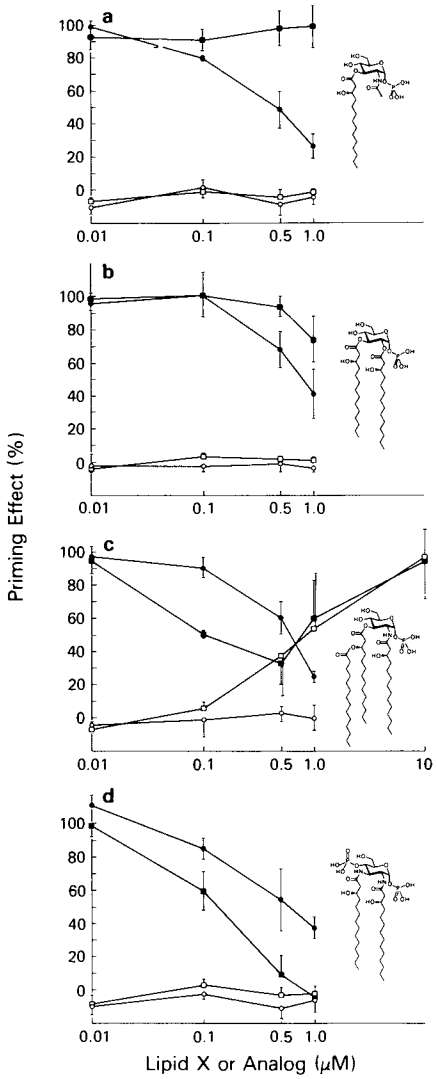

Fig. 2. The effect of increasing concentrations of lipid X derivatives on neutrophil priming as determined by FMLP-stimulated O_2^- release. The structure of each derivative tested is shown to the right of its corresponding graph: (a) N^2-acetyl,O^3-acyl-glucosamine-1-phosphate ($N=4$); (b) O^2,O^3-diacyl-glucose-1-phosphate ($N=3$); (c) N^2-acyl,O^3- acyloxyacyl-glucosamine-1-phosphate ($N=3$); (d) N^2,N^3-diacyl-glucosamine-1,4- bisphosphate ($N=3$). Results are shown as the mean ± SE. Each analogue (□) is run concurrently with lipid X (○) for comparison, using neutrophils from the same donor. Filled symbols (■, ●) denote the presence of LPS (10 ng/ml), during the incubation. The 0% and the 100% priming limits on the graphs are determined for each set of experiments by the amount of O_2^- produced by unprimed neutrophils (C_B) incubated in pyrogen-free buffer and fully primed neutrophils (C_E) incubated with endotoxin (10 ng/ml), respectively. The percentage priming effect for each test sample (X) was calculated from the formula $[1 - [C_E - X/C_E - C_B)] \times 100$. None of the derivatives tested interfered with FMLP- or PMA (500 ng/ml, data not shown)-stimulated O_2^- release in the absence of LPS (7).

FMLP- or PMA-stimulated O_2^- generation in the absence of LPS, arguing against a direct action on PKC at the phorbol ester binding site (7). In addition, very high concentrations of lipid X were required to block the activation of platelet PKC (12). This may have caused nonspecific membrane effects as evidenced by the neutrophil clumping that occurred in our studies at high concentrations. For these reasons, although priming and its inhibition may occur via binding to PKC, there are arguments against this as a primary mechanism and interactions at other sites cannot be discounted. Recently, priming in macrophages was associated with the myristoylation of a specific PKC substrate and its translocation to the cell membrane, thus underscoring the complexity of this system and the need for further investigation (13).

The importance of neutrophil priming has not been established *in vivo*. This action of LPS, however, occurs in the same range of endotoxin concentrations found in patients with septicemia (14) and in a dose range similar to other important cellular actions of LPS, such as the release of tumor necrosis factor (15). Further, neutrophil activation during sepsis has been implicated in the development of multiple organ failure (16), a major cause of death in human septic shock (17). *In vivo* evidence in rabbits suggests that primed neutrophils may "overreact" in the setting of an inflammatory stimulus or infection, causing untoward damage to host tissue (18).

Alteration of lipid X, by converting the C2 amide linkage to an ester linkage or by removing either acyl group, markedly decreased its ability to inhibit LPS-induced priming of neutrophils. This suggests that these structures are important to the antiendotoxin activity of the molecule. Monosaccharide derivatives containing an acyloxyacyl structure were found to have partial agonist activity at high concentrations. It is of interest that this moiety is present in native LPS and that native LPS can be partially detoxified by acyloxyacyl hydrolases (19). Compared to lipid X, acyloxyacyl analogues also exhibited increased antagonism of LPS at low concentrations, an effect possibly related to the increased hydrophobicity of the analogue. The most potent "pure antagonist" studied so far, however, has an additional phosphate group that makes it more hydrophilic, suggesting that differences in anti-LPS activity between derivatives cannot be explained simply by changes in solubility.

In summary, our results suggest that the inhibition of LPS-induced priming of human neutrophils by monosaccharide analogues of lipid A is mediated by interactions which display structural specificity. Certain derivatives of lipid X may have advantages over whole lipid A in studying the cellular sites that mediate the biological effects of LPS. Further, the preliminary observations reported here provide the basis for the development of new anti-LPS agents that may prove useful in the treatment of sepsis.

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