Lipid X Ameliorates Pulmonary Hypertension and Protects Sheep from Death Due to Endotoxin

DOUGLAS T. GOLENBOCK,1 JAMES A. WILL,2,3 CHRISTIAN R. H. RAETZ,4 AND RICHARD A. PROCTOR1,5

Departments of Medicine,1 Veterinary Science,2 Anesthesiology,3 Biochemistry,4 and Medical Microbiology,5 University of Wisconsin, Madison, Wisconsin 53706

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Lipid X (2,3-diaclylgucosamine-1-phosphate) is a novel monosaccharide precursor of lipid A that has some of the physiologic activities of endotoxin but little toxicity. To determine whether lipid X would interfere with the toxic effects of endotoxin, we pretreated sheep with either 100 or 200 μg of lipid X per kg of body weight and then challenged them with a potentially fatal dose of Escherichia coli endotoxin (20 μg/kg). Twenty-one sheep underwent pulmonary artery catheterization and were monitored for changes in pulmonary artery pressure, temperature, pH, partial O₂ pressure, partial CO₂ pressure, blood pressure, and cell counts over 7 h. Overall mortality for control animals was 37% versus 5.3% for pretreated animals. None of the 13 animals pretreated with 100 μg of lipid X per kg died. These differences in survival were significant (P < 0.05). Animals pretreated with 100 μg of lipid X per kg had significantly lower pulmonary artery pressure during both phases 1 and 2 of endotoxin-induced pulmonary artery hypertension. A higher dose of lipid X, 200 μg/kg, produced pulmonary hypertension. Perhaps because lipid X is a subunit of lipid A, lipid X shows a partial pyrogenic effect while also decreasing the pyrogenic activity of complete lipopolysaccharide (LPS). Lipid X did not prevent endotoxin-induced neutropenia or moderate hypotension in response to LPS. Lipid X is a potential prototype compound for a new type of chemotherapy directed at blocking the harmful effects of LPS during bacterial septicemia.

The clinical problem of gram-negative sepsis presents multiple therapeutic challenges beyond the proper selection of antibiotics. While antimicrobial agents often clear the bloodstream rapidly of viable bacteria, sequelae such as acute respiratory distress syndrome, renal failure, and vascular collapse may lead to death (4, 36). The incidence of gram-negative bacteremia in the United States has been estimated at 250,000 per year, with a mortality rate of 20 to 80% (4, 36).

Although death due to gram-negative sepsis is a complex process which is dependent upon multiple factors, much of the pathophysiology associated with gram-negative sepsis is reproduced by endotoxin (lipopolysaccharide [LPS]) (2). Additional evidence suggesting a role for endotoxin in septic shock is that high levels of antibody against the core of endotoxin are associated with enhanced survival in animals and patients with severe, life-threatening gram-negative infection (3, 15, 19, 29). Antiserum directed against the core glycolipid has subsequently been used in humans with some success (37).

Studies of the pathophysiology of endotoxin have led investigators to direct therapy toward controlling the inflammatory response with indomethacin, thromboxane inhibitors, and glucocorticoids. Because endorphins have a putative role in endotoxin shock, narcotic antagonists have also been studied (22). Although animal studies have shown dramatic results using these agents (7, 10, 12, 13), human studies have been limited and the results have often been controversial. Even when optimal antibiotic, fluid, and pressor therapies are used, the mortality rate of at least 20% suggests the need for new therapeutic approaches. Therapy is further complicated by the recent demonstration that some antibiotics can actually liberate endotoxin (30), potentially worsening the outcome.

Recent insights into the structure and biosynthesis of the toxic portion of endotoxin, lipid A (25), may provide new therapeutic avenues. Certain Escherichia coli mutants deficient in phosphatidylglycerol were found to accumulate 2,3-diaclylgucosamine-1-phosphate, in which β-hydroxy-myristoyl moieties are the sole fatty acid substituents (25). The discovery of this monosaccharide, designated lipid X (Fig. 1), helped facilitate the elucidation of the correct structure of lipid A (25, 31). It was subsequently shown that lipid X and a nucleotide derivative of lipid X are condensed to form the disaccharide backbone of lipid A (27).

Studies in our laboratories have demonstrated that lipid X has some of the activities of lipid A and endotoxin but little toxicity (5). Like LPS, it causes gelation of the Limulus amebocyte lysate (16, 23, 32), murine B lymphocyte mitogenesis (25, 26), and macrophage activation (20, 34), though to a much lesser degree than LPS or various lipid A-related disaccharides (11, 17, 33). Lipid X produces a transient rise in pulmonary artery pressure, as well as a modest increase in lung lymph flow, in a biphasic manner similar to that of LPS (5, 9), but lipid X does not cause pulmonary vasculature protein permeability as is seen following LPS challenge. Large doses of lipid X did not kill either sheep (1,000 μg/kg) (5) or mice (200,000 μg/kg) (24), and lipid X is minimally pyrogenic in animals (5, 32).

Because lipid X is a substructure of lipid A, we postulated that it might reduce mortality due to endotoxin challenge by interfering with host-endotoxin interactions. Mice treated with lipid X were protected from a uniformly lethal dose of endotoxin (24), even if the lipid X was administered several...
hours after endotoxin, raising the possibility that lipid X might act therapeutically as well as prophylactically.

Because pathophysiologic parameters are difficult to measure in mice, because endotoxemia has been well characterized in sheep, and because the ovine pulmonary response to endotoxin is similar to that of humans (8, 9), we chose to investigate the effects of lipid X on endotoxemia in adult sheep. We inserted pulmonary and carotid artery catheters to monitor partial arterial pressure (Pap) and mean arterial pressure and monitored the temperature, peripheral leukocyte counts, and arterial blood gases in sheep pretreated with lipid X and subsequently challenged with a potentially lethal dose of E. coli endotoxin. Our results demonstrated that pretreatment with an appropriate dose of lipid X reduced the pulmonary hypertensive response, pyrogenicity, and mortality of endotoxemia in sheep. Thus, lipid X appears to be a promising prototype compound for a new form of therapy against gram-negative sepsis.

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MATERIALS AND METHODS

LPS and lipid X. E. coli (serotype O111:B4) LPS prepared by the phenol-water method was purchased from Sigma Chemical Co. Lipid X was isolated from E. coli MN7 (21) as previously described (31) and found to be greater than 97% pure as judged by 1H nuclear magnetic resonance determination and thin-layer chromatography. The lipid X used in this study, in comparison with a synthetic preparation of lipid X (kindly provided by Frank Unger, Sandoz Pharmaceuticals), was biologically similar in virtually all respects, except that the biologically derived lipid X was a slightly more potent B-cell mitogen than synthetic lipid X (25; unpublished data; see Discussion). lipid X was dissolved in sterile saline at a concentration of 10 mg/ml, titrated to pH 8 with Tris base, and sonicated for 2 min. This solution was then diluted in saline immediately before experimentation so that animals received 100 to 200 μg of lipid X per kg (0.1 ml of saline per kg) or saline alone.

Sheep studies. Thirty-nine mixed-breed sheep were divided prospectively on the basis of weight, sex, and E. coli O111:B4 antibody titer and received either normal saline (n = 19) or lipid X pretreatment at a dose of 100 μg/kg (n = 14) or 200 μg/kg (n = 6) 1 h before endotoxin challenge (20 μg/kg). Pregnant females were excluded from experimentation. Sheep were studied in the following fashion. After 2 h of acclimation to the laboratory surroundings, lipid X or saline was injected. At 1 h after administration of lipid X or a placebo, 20 μg of LPS per kg in 5 to 10 ml of saline was administered as a bolus. The catheter was then flushed with 10 ml of sterile saline. Infusions were via a pulmonary artery catheter over 1 to 2 min; animals in the acute-mortality study received lipid X and LPS through a peripheral catheter in the jugular vein. Animals were observed for 8 h continuously and then placed in a holding room for observation.

Physiology studies. Of the 39 sheep, 21 were catheterized with polyethylene catheters via the external jugular vein and the carotid artery with 2% lidocaine local anesthesia. Sheep were passively restrained in a stanchion throughout the course of each experiment. Animals were provided with water and food ad lib. The patency of the intravenous lines was maintained by intermittent infusion of 10 ml of heparinized saline (1 U/ml). No animal received more than 1,500 U of heparin over an 8-h period.

Of the 21 catheterized sheep, 10 control animals were pretreated with a saline placebo, 5 were pretreated with 100 μg of lipid X per ml, and the remaining 6 sheep were pretreated with 200 μg of lipid X per kg.

Pulmonary artery and blood pressures were continuously measured with pressure transducers manufactured by Gould-Stratham, Inc., and recorded with a Gilson (Middleton, Wis.) six-channel polygraph. All electronic instruments were calibrated before each experiment with a mercury manometer and zeroed every 30 min.

Temperatures were monitored with a rectal electronic thermometer and recorded every 30 min. Cell counts were obtained before pretreatment and before LPS administration, as well as at 1, 3, and 5 h after endotoxin challenge. A 1:100 dilution was made with a commercially available erythrocyte lysing solution (Becton Dickinson and Co.), and leukocyte counts were made with a hemacytometer.

Acute-mortality studies. An acute-mortality study was performed with 18 of 39 sheep after initial results from the physiology study suggested that a dose of lipid X (100 μg/kg) might be protective. All animals were observed for 5 days. Those animals that died underwent immediate necropsy to determine the cause of death. Tissues were fixed in Formalin and stained with hematoxylin and eosin for microscopic examination. Survivors were euthanized after 5 days and necropsied. Tissues were processed in a manner identical to that used for tissues from the mortality study.

Measurement of E. coli O111:B4 LPS antibody. Antibodies against endotoxin were quantitated with a latex agglutination test. Latex beads (Polysciences) 0.9 μm in diameter were washed three times in glycine-buffered saline (GBS), pH 8.4. These were suspended in GBS at a concentration such that a 1:100 dilution had an optical density at 650 nm of 0.45. E. coli O111:B4 LPS was dissolved in GBS at a concentration of 1 mg/ml and mixed volume for volume with the latex suspension. After 1 h of incubation, the beads were washed twice with GBS and once with GBS containing bovine serum albumin (100 mg/100 ml) and sodium azide (50 mg/100 ml). Serum was stored in aliquots at −70°C until testing. E. coli O111:B4 rabbit antiserum (Difco Laboratories) was used as a positive control. As a negative control, latex beads were incubated with albumin, rather than endotoxin, at a concentration of 1 mg/ml. Each sheep serum was tested in each dilution with albumin-coated beads to guard against nonspecific agglutination. Those animals whose serum exhibited nonspecific agglutination were not included for study. The median anti-LPS titer was 1:8 (range, 1:1 to 1:32).

Statistical analysis. Mortality data were analyzed with both
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TABLE 1. Mortality of adult sheep pretreated with lipid X (100 or 200 μg/kg) 1 h before E. coli (O111:B4) endotoxin challengea

<table>
<thead>
<tr>
<th>Outcome</th>
<th>No. (%) of sheep in the following groupsb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 100 μg/kg</td>
</tr>
<tr>
<td>Survival</td>
<td>12 (63)</td>
</tr>
<tr>
<td>Death</td>
<td>7 (37)</td>
</tr>
</tbody>
</table>

a Lipid X concentration, 100 or 200 μg/kg; endotoxin concentration, 20 μg/kg.

b p < 0.05 for combined mortality of pretreated groups versus the control group. P < 0.05 for the control group versus the 100-μg/kg group. P < 0.1 for the control group versus the 200-μg/kg group.

c One animal was deleted from the data because of death due to catheter-induced pulmonary infarction (see the text). Inclusion of this animal would still allow statistical significance at P < 0.05.

d This animal received contaminated lipid X (approximately 10% lipid Y; see the text).

a chi-square test and Fisher's exact test. Pulmonary and blood pressure responses, arterial blood gas, and temperature changes were analyzed with a repeated-measures test of variance (35); for the purpose of analysis, phase 1 of LPS-induced pulmonary hypertension was defined as the time from LPS injection to 1 h after injection, and phase 2 was defined as the subsequent period to completion of the experiment. These times correspond to previous reports (8, 9) and fit with the pulmonary pathophysiologic responses to endotoxins observed in this study. All other data were subjected to repeated-measures tests, as well as paired comparisons (t tests), when appropriate. Analysis was performed by Paul Rasmussen, University of Wisconsin Department of Biostatistics.

RESULTS

The mortality data from 39 endotoxin-challenged sheep are summarized in Table 1. The overall mortality of controls was 37%, compared with an overall mortality of lipid X-pretreated sheep of 5.3%. This difference is significant by both chi-square analysis with the Yates correction and Fisher's exact test at P < 0.05. The dose of 100 μg of lipid X per kg was felt, on the basis of preliminary studies, to be optimal, and indeed, none of 13 animals in this group died. All animals that died showed signs consistent with endotoxin-induced acute respiratory distress syndrome (1): pulmonary edema, leukocyte infiltration of the alveolar spaces, and focal areas of hemorrhage. In addition, one animal had evidence of diffuse intravascular coagulopathy. Microscopic examination of the kidneys revealed occasional areas of acute tubular necrosis and infarct in those animals that died, as well as in survivors that were subsequently euthanized for necropsy. No pulmonary lesions were seen in surviving animals. Since no animal died after 24 h, the contribution of lesions of the kidneys to mortality was negligible.

Data from one sheep were not included in the mortality data because this animal died when the pulmonary artery catheter was sheared off accidentally during removal. Necropsy revealed multiple large pulmonary emboli with massive pulmonary infarction in the right lung. This animal received 100 μg of lipid X per kg. If data from this animal were included in the calculations of significance, the difference between the survival of control animals versus that of pretreated animals would still be significant below the 5% level.

It should also be noted that the animal which died after pretreatment with 200 μg of lipid X per kg received impure material. Since the pulmonary response to this preparation was atypical for animals pretreated with lipid X (5), it was tested for purity. This preparation was contaminated with approximately 10% (by weight) lipid Y (25) and was not used in further experiments. Lipid Y has been found to sensitize C57BL/10 mice to the pathophysiologic effects of endotoxin (unpublished data). Nevertheless, the data from the two animals treated with the contaminated preparation, one of which survived, were included in the calculations. The other preparations of lipid X used in these experiments showed >97% purity.

Figure 2 shows the pulmonary artery pressure responses of sheep pretreated with lipid X at 100 or 200 μg/kg and in controls (endotoxin alone). At a dose of 100 μg/kg, lipid X appeared to decrease the pulmonary hypertensive response to endotoxin significantly. While the peak Ppa was the same for both pretreated and control animals, there was a marked difference in the mean Ppa during phase 1, with lipid X-pretreated animals recovering from high Ppa significantly earlier than control animals (P < 0.05). During phase 2, the Ppa of animals given 100 μg of lipid X per kg was 5 to 9 mm Hg (1 mm Hg = 133.3 Pa) lower than that of control animals (P < 0.03). Thus, at a pretreatment dose of 100 μg of lipid X per kg, endotoxin-induced pulmonary hypertension was significantly ameliorated during both phases 1 and 2 (see reference 9 for a description of these phases). The results of one sheep that had undergone pulmonary artery catheterization (a control sheep treated with normal saline) were not included because technical problems with the catheter did not allow accurate readings. At a pretreatment dose of 200 μg/kg, no significant differences in the hypertensive response to endotoxin could be demonstrated as compared with control animals.

Pretreatment of sheep with lipid X also appeared to diminish the febrile response to endotoxin administration (Fig. 3). Lipid X itself was minimally pyrogenic. Sheep
pretreated with 100 or 200 µg of lipid X per kg had a 0.56 or 0.62°C increase in rectal temperature, respectively, within 1 h of lipid X administration (P < 0.001). Animals pretreated with lipid X showed a biphasic temperature response, while those receiving endotoxin alone had a single temperature peak. Both peaks in lipid X-pretreated sheep were lower than with endotoxin alone. Furthermore, the final differences in temperature between control and lipid X-treated animals were statistically significant at 5 and 6 h postadministration (P < 0.05). For example, although the mean temperature at the beginning of the experiment was slightly lower in the placebo control group, by the end of the experiment, the temperature was 41.03 ± 0.33°C in the control group versus 39.89 ± 0.20°C in the 100-µg/kg group and 40.15 ± 0.26°C in the 200-µg/kg group (P < 0.05).

Both treatment groups became significantly neutropenic after lipid X administration (P < 0.01; Table 2), with 60.6% and 57.8% declines in peripheral leukocyte counts in the 100- and 200-µg/kg groups, respectively. Subsequent administration of endotoxin caused a modest further decline in peripheral leukocyte counts. None of the differences between treatment groups were statistically significant over the 6-h interval.

Arterial blood gas data are listed in Table 3. Statistical analysis of these data revealed no significant trends or differences in responses due to treatment.

The mean arterial pressures of the three treatment groups ranged from 96 to 102 mm Hg at baseline and fell to 65 to 99 mm Hg by 6 h. There were no significant differences between treatment groups.

### DISCUSSION

Pretreatment of adult sheep with lipid X reduced mortality, fever, and pulmonary hypertension following a potentially lethal endotoxin challenge. These observations have therapeutic implications and raise many questions about endotoxin action at the cellular level.

The mild endotoxin-like agonist activities (fever, leukopenia, and transient pulmonary hypertension) demonstrated by lipid X are not surprising, because it is a substructure of lipid A and a major biosynthetic precursor (25, 27). Indeed, the ability of sheep and mice to tolerate relatively large doses of lipid X with only limited toxicity is the surprising observation (5, 24). Disaccharide precursors and other derivatives that more closely resemble mature lipid A are more bioreactive and toxic in comparison with lipid X, as assayed by pyrogenicity (11, 32), local Shwartzman reaction (11, 32), lethality (11, 32, 33), complement activation (11, 33), and *Limulus* amebocyte lysate coagulation (11, 23, 32, 33).

Our sheep experiments clearly show that pretreatment with lipid X interferes with the lethal effects of endotoxin. The most straightforward explanation for the protective efficacy of lipid X is that it competes for a common receptor with endotoxin. The abilities of lipid X to induce leukopenia,

### TABLE 3. Effects of lipid X on arterial blood gasesa

<table>
<thead>
<tr>
<th>Time point (h)</th>
<th>Control (n = 10)</th>
<th>100 µg/kg (n = 4)</th>
<th>200 µg/kg (n = 4)</th>
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<tbody>
<tr>
<td>0</td>
<td>pH 7.50 ± 0.01</td>
<td>7.49 ± 0.01</td>
<td>7.51 ± 0.01</td>
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<tr>
<td></td>
<td>pO₂ 82.63 ± 3.20</td>
<td>88.68 ± 3.23</td>
<td>93.48 ± 4.64</td>
</tr>
<tr>
<td></td>
<td>pCO₂ 30.66 ± 0.92</td>
<td>31.05 ± 1.63</td>
<td>32.88 ± 2.94</td>
</tr>
<tr>
<td>1</td>
<td>pH 7.49 ± 0.02</td>
<td>7.50 ± 0.01</td>
<td>7.50 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>pO₂ 82.86 ± 3.45</td>
<td>91.50 ± 2.01</td>
<td>94.78 ± 2.04</td>
</tr>
<tr>
<td></td>
<td>pCO₂ 31.09 ± 0.82</td>
<td>29.70 ± 3.27</td>
<td>32.98 ± 2.44</td>
</tr>
<tr>
<td>2</td>
<td>pH 7.47 ± 0.02</td>
<td>7.48 ± 0.02</td>
<td>7.49 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>pO₂ 81.06 ± 4.10</td>
<td>96.48 ± 4.55</td>
<td>96.03 ± 2.42</td>
</tr>
<tr>
<td></td>
<td>pCO₂ 30.00 ± 1.11</td>
<td>28.65 ± 3.02</td>
<td>32.05 ± 2.14</td>
</tr>
<tr>
<td>3</td>
<td>pH 7.48 ± 0.02</td>
<td>7.53 ± 0.01</td>
<td>7.51 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>pO₂ 86.88 ± 6.54</td>
<td>94.55 ± 5.63</td>
<td>104.78 ± 2.42</td>
</tr>
<tr>
<td></td>
<td>pCO₂ 30.24 ± 1.76</td>
<td>25.15 ± 0.85</td>
<td>28.93 ± 1.10</td>
</tr>
<tr>
<td>5</td>
<td>pH 7.45 ± 0.03</td>
<td>7.51 ± 0.01</td>
<td>7.46 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>pO₂ 86.23 ± 7.76</td>
<td>88.83 ± 3.47</td>
<td>103.23 ± 2.75</td>
</tr>
<tr>
<td></td>
<td>pCO₂ 31.13 ± 2.41</td>
<td>26.83 ± 1.36</td>
<td>26.37 ± 2.19</td>
</tr>
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</table>

* Relative to injection of LPS (20 µg/kg).

a For the values of pO₂ and pCO₂ are in millimeters of Hg. A repeated-measures test (one-way analysis of variance) showed no significant differences between groups (P > 0.10).

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**FIG. 3.** Effect of lipid X on the febrile response due to administration of endotoxin. The data bars represent mean values ± the standard errors of the means in degrees Celsius for 10 control animals, 5 animals in the 100-µg/kg group, and 6 animals in the 200-µg/kg group. Lipid X itself was slightly pyrogenic (P ≤ 0.05); after LPS administration, control animals experienced a faster rise in temperature (P ≤ 0.05), and a slightly higher peak temperature, and at t = +5 h and t = +6 h they had higher temperatures than did lipid X-treated animals (P ≤ 0.05).
a mild but significant increase in temperature, and transient pulmonary hypertension, as well as B-cell mitogenesis and macrophage activation (17, 20, 26, 34), are consistent with lipid X sharing a common receptor with LPS. Hence, lipid X is an LPS antagonist which also expresses some agonist activities. Work by a number of investigators using cellular systems supports a receptor-mediated mechanism. For example, Danner et al. (R. L. Danner, K. A. Joiner, and J. E. Parillo, J. Clin. Invest., in press) have shown that lipid X prevents priming of polymorphonucleocytes by endotoxin. Bradford Schwartz (University of Wisconsin; personal communication) demonstrated that lipid X blocks LPS-induced production of procoagulant activity by macrophages. Similarly, Carol Sibley et al. (University of Washington, personal communication), using a B-cell tumor line, showed that lipid X inhibited expression of κ chains on the cell surface.

However, other mechanisms, such as secretion of a protective protein in response to lipid X (e.g., protein C [F. B. Taylor, Clin. Res. 42:566A, 1985]) or noncompetitive inhibition of toxic mediator release from effector cells, must also be considered.

Although the mitigating effects of lipid X upon LPS-induced pulmonary hypertension were seen at a dose of 100 μg/kg, animals pretreated with 200 μg of lipid X per kg had pulmonary responses that were similar to those of control animals. However, five of six animals survived a potentially lethal challenge of endotoxin (P < 0.1). Two possible explanations for this paradox follow. (i) Because lipid X has a pulmonary agonist pressor response, higher doses of lipid X cause pulmonary hypertension (6). This may be analogous to morphine antagonism by substances such as nalorphine (14), the administration of which in moderate amounts reverses narcosis but larger doses of which can actually cause central nervous system and respiratory depression. It is unlikely that a biologically derived contaminant accounted for this agonist effect because recent studies using synthetic lipid X showed that this preparation of lipid X had identical pulmonary pressor and cardiovascular effects in comparison with older biological preparations (G. M. Christman, D. T. Golenbock, C. R. H. Raetz, and R. A. Proctor, unpublished data) and, although biologically derived lipid X contains an unidentified mitogenic compound (25), the preparations were similar in all other respects thus far tested, including macrophage activation (34; R. A. Zoeller and C. R. H. Raetz, unpublished data). TNF production (J. K. Chia, M. Pollack, and S. Vogel, personal communication), Limulus lysate activity (Proctor, unpublished data), and blocking effects on polymorphonucleocyte priming (R. L. Danner, personal communication). Second, pulmonary hypertension is only part of the pathophysiology responsible for death due to endotoxin. The pulmonary hypertensive response to lipid X is not accompanied by vascular protein leak (5), whereas sheep dying of endotoxemia demonstrate pulmonary edema and high levels of plasma proteins in the lung-lymph drainage. Consequently, lipid X may prevent death by decreasing vascular permeability. Also, LPS has effects on a variety of other organs. While pulmonary hypertension contributes to the morbidity and mortality associated with endotoxin shock, no single lethal effect has yet been identified. Although the mechanism of lipid X-mediated protection from lethal endotoxemia is uncertain, lipid X may be a potential prototype compound for a new arm of chemotherapeutics against bacterial infection. Chemical synthesis of lipid X and a variety of other endotoxin precursors has been achieved (16, 18); thus, it may be possible to optimize the protective effects relative to endotoxicy by chemical and dose modifications. Ultimately, the high mortality associated with gram-negative sepsis might be reduced by the combination of endotoxin precursors and antibiotics, as well as other supportive measures.

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LITERATURE CITED