Therapeutic Efficacy of a Polymyxin B-Dextran 70 Conjugate in Experimental Model of Endotoxemia

SCOTT E. BUCKLIN,[1] PHILIP LAKE,[2] LENNART LÖGDBERG,[2] AND DAVID C. MORRISON[3]*

Department of Microbiology, Molecular Genetics, and Immunology[1] and Cancer Center,[3] University of Kansas Medical Center, Kansas City, Kansas 66160, and Receptor Mechanism Group, Preclinical Research Department, Sandoz Research Institute, East Hanover, New Jersey 07936[2]

Numerous studies have suggested that lipopolysaccharide (LPS), a major component of the cell wall of gram-negative bacteria, is responsible for the initiation of gram-negative septic shock. Previously, others have designed therapeutic regimens to target the biologically active lipid A region of LPS by either neutralization of the biological properties of LPS or enhancement of clearance of this molecule. One such compound capable of neutralizing lipid A is the antibiotic polymyxin B. However, the clinical utility of polymyxin B is limited by its toxicity. We therefore covalently conjugated this antibiotic to the high-molecular-weight polysaccharide dextran 70, resulting in reduced toxicity of polymyxin B but retention of its endotoxin-neutralizing ability. The studies described in this report were designed to test the in vivo efficacy of this compound in an experimental animal model of gram-negative septic shock. Mice were administered graded doses of *Escherichia coli* or *Pseudomonas aeruginosa* along with D-galactosamine and the antibiotic imipenem. We had previously determined that antibiotic chemotherapy provides significant protection against *E. coli*-mediated lethality with smaller doses of bacteria; however, the antibiotic does not provide protection against larger doses of bacteria, but it is effective at killing the bacterial inoculum in vivo. Administration of the polymyxin B-dextran 70 conjugate provided significant protection when given with an antibiotic but was not effective by itself. A requirement for a pretreatment period prior to *E. coli* challenge was shown to depend upon the bacterial challenge dose. In other studies using this D-galactosamine sensitization model, we demonstrated that the lipid A-specific conjugate had no effect on lethality caused by *Staphylococcus aureus* or tumor necrosis factor alpha. The results of these studies indicate that this compound is effective in preventing lethal gram-negative septic shock in mice and may be useful as a potential therapeutic agent in humans as well.

Although advances in antibiotic chemotherapy have allowed medical practitioners to successfully treat many infections caused by gram-negative bacteria, mortality associated with gram-negative sepsis ranges between 30 and 50% (3, 17, 31). Therefore, successful eradication of the bacterial infection does not always result in a successful clinical outcome. Alternative therapies have been designed to target one of the major constituents of the gram-negative cell wall, the endotoxic lipopolysaccharide (LPS), which has been implicated as a contributing factor in the pathogenesis of gram-negative sepsis, (reviewed in reference 20). When injected into human volunteers, purified LPS induces many of the pathophysiologic features seen clinically during gram-negative sepsis (5, 18). Additional indirect evidence suggesting that LPS can initiate gram-negative sepsis includes in vitro stimulation of human monocytes with LPS, which results in the production of multiple proinflammatory mediators often found in the circulation of septic patients (22). Furthermore, when experimental animals are challenged with purified LPS, therapeutic targeting of either the LPS component or the mediators induced by endotoxin can result in protection of these animals from LPS-induced lethality (1, 11). For example, if antiendotoxin monoclonal antibodies are administered prior to a lethal LPS challenge, experimental animals can be protected against its lethal effects by several putative mechanisms, including enhanced clearance and direct neutralization. Additionally, neutralizing monoclonal antibodies, or receptor antagonists, directed against the proinflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukins 1, 6, and gamma interferon can protect animals against LPS-induced lethality (2, 10, 16, 30). Current evidence suggests that, at least in experimental animals, a combination of antiendotoxin immunotherapy and anticytokine immunotherapy may be synergistic in preventing LPS lethality (8, 24). In addition, recently published studies by our laboratory have provided evidence that targeting both the cytokine (gamma interferon) and its receptor with neutralizing monoclonal antibodies can lead to synergy in their ability to protect animals from a lethal LPS challenge (4).

Current evidence suggests that endotoxin can be released from the gram-negative bacterial cell wall during a gram-negative infection by several pathways, including interactions with serum proteins or antibiotics (14, 29). However, the relative importance of endotoxin release versus bacterial proliferation during gram-negative septic shock has not been fully defined. We have developed an animal model allowing us to examine the contribution of antibiotic-released endotoxin to the development of lethal septic shock. This model has employed modifications of the D-galactosamine-induced hypersensitivity to LPS initially characterized by Galanos et al. (13). In this model, mice are administered a lethal dose of gram-negative bacteria intraperitoneally (approximately 4×10^4 *Escherichia coli* CFU) along with a cell wall-active antibiotic. Control mice are given saline. We have shown that antibiotic chemotherapy provides significant protection compared with

saline treatment of animals given small doses of bacteria; however, lethality still occurs in antibiotic-treated animals given larger doses of bacteria. We have demonstrated that with these large doses of bacteria, the antibiotic is nevertheless still rapidly bactericidal. These findings do not suggest that lethality is due to bacterial proliferation per se but rather implicate a role for microbe-derived LPS. Evidence in support of the hypothesis that antibiotic-treated mice die of endotoxemia rather than overwhelming infection derives from additional studies with LPS-hyporesponsive C3H/HeJ mice. When C3H/HeJ mice are given antibiotics, the dose of *E. coli* required for lethality is much greater than that required for C3H/HeJ mice given saline. Additionally, LPS-responsive mice are protected approximately 8-fold by antibiotic therapy while LPS-resistant mice are protected by at least 30-fold. The differential responses in LPS-sensitive versus LPS-hyporesponsive mice given antibiotics indicate that LPS contributes to lethality in these groups. With these results in mind, this experimental model has allowed us to test potential therapeutic intervention strategies that target LPS and to determine their efficacy in the treatment of gram-negative septic shock.

One agent that has long been known to target LPS specifically and provide protection against LPS lethality is polymyxin B (PMX). PMX is a cationic cyclic polypeptide antibiotic with the ability to bind with high affinity to lipid A, the biologically active portion of LPS, and neutralize its biological activity (19, 21). Other antibiotics of this class include colistin and tyrocidine, both of which can neutralize LPS to some extent (6). The neutralizing effect of PMX on LPS activity was observed as early as 1956 by Neter (23). When LPS was incubated with PMX, the agglutination of erythrocytes normally induced by LPS was inhibited. Since then, others have demonstrated that PMX can inhibit LPS lethality in chicken embryos, mice, rabbits, and dogs (12, 25, 27). PMX inhibition of the toxic properties of LPS, such as the local Shwartzman reaction, disseminated intravascular coagulation, and other hemodynamic perturbations have also been described (7, 26).

Although it seems, therefore, that PMX might serve as an ideal therapeutic agent to treat gram-negative sepsis, the attendant nephrotoxicity of this agent has limited its use in humans. It is currently thought that the lipid solubility of PMX allows its insertion into the membranes of renal epithelial cells in the proximal tubule of the nephron (28). In some of the most severe cases, acute tubular necrosis has been observed histologically. The precise biochemical basis for this toxicity is currently being studied and may involve interruption of membrane permeability or lysosomal integrity in renal cortical cells (28).

Attempts have been made to decrease the toxicity of PMX without removing its antiendotoxin activity. In one study, deacylation of PMX resulted in significantly decreased toxicity, but its antiendotoxin activity was also reduced by approximately 63-fold (9). As an alternative approach to capitalize upon the potent neutralizing properties of PMX, the conjugation of PMX to dextran 70 (DXT) has been explored. It has been previously determined that the intrinsic toxicity of PMX is reduced by conjugation to DXT (15). In the studies reported here, we investigated whether this compound could function in vivo to inhibit lethality in the experimental mouse endotoxemia-bacteremia model described above.

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MATERIALS AND METHODS

Reagents. Pyrogen-free saline (Baxter, Deerfield, Ill.) was used as a diluent for all reagents. D-Galactosamine was purchased from Sigma Chemical Company, St. Louis, Mo., and was administered intraperitoneally at a dose of 20 mg per mouse. The cell wall-active antibiotic imipenem-cilastatin sodium (Merck and Co., West Point, Pa.) was administered at a dose of 500 μg per mouse along with D-galactosamine. Both the PMX-DXT conjugate and DXT were provided by Sandoz Research Institute, East Hanover, N.J. (15). The concentration of the PMX component of the conjugate was 2.49 mg/ml with 38.1 mg of PMX per g of DXT. All dosage calculations were based upon the PMX component. When DXT was administered alone, the dose corresponded to the DXT component of the conjugate. PMX sulfate was purchased from the Upjohn Company (Kalamazoo, Mich.). The TNF-α used in these studies was purchased from Genzyme Corporation (Cambridge, Mass.). MacConkey agar was purchased from Difco Laboratories (Detroit, Mich.). Trypticase soy broth was purchased from Becton Dickinson Microbiology Systems (Cockeysville, Md.).

Bacteria. The organisms used in these studies included *E. coli* O111:B4 (generously provided by List Biological Laboratories), *Pseudomonas aeruginosa* MB3286 (Merck and Co.), and a clinical isolate of *Staphylococcus aureus*. A stock culture of *E. coli* was maintained on MacConkey agar at 4°C. Stock cultures of *P. aeruginosa* and *S. aureus* were maintained on Trypticase soy agar. An isolated colony was used to aseptically inoculate 50 ml of Trypticase soy broth. The broth was then incubated at 37°C overnight in an orbital shaker at 200 rpm. The next day, 0.2 ml of the overnight cultures was used to inoculate 50 ml of fresh Trypticase soy broth. This culture was then incubated at 37°C while shaken at 250 rpm until the optical density at 650 nm was 0.65, which corresponds to a bacterial density of approximately 5.0×10^8 CFU/ml. This was determined by growing the bacteria and monitoring CFU per milliliter as a function of optical density. This density corresponds to a portion of the log-phase of growth of this organism in Trypticase soy broth. Serial dilutions of this culture were performed with saline until the appropriate concentration of bacteria was obtained for challenge.

Mice. Outbred female CF-1 mice (Charles River Laboratories, Wilmington, Mass.) 6 to 10 weeks old and weighing approximately 25 g were used for all experiments. The mice were housed in the American Association of Laboratory Animal Care-accredited University of Kansas Medical Center vivarium and fed ad libitum. Prior to experimentation, all mice were allowed at least 5 days of acclimation following shipment.

Lethality model. The model used for the PMX-DXT conjugate studies involved intraperitoneal administration of a lethal dose of gram-negative bacteria (*E. coli* O111:B4 or *P. aeruginosa* MB3286) or gram-positive organisms *S. aureus* along with the sensitizing agent D-galactosamine (20 mg per mouse) and the antibiotic imipenem (20 mg/kg; Merck and Co.) to outbred female CF-1 mice either before or after administration of either DXT; the PMX-DXT conjugate, or PMX. In some experiments, saline was administered instead of imipenem. All compounds were administered intraperitoneally. Previous studies with this model have demonstrated that the bacteria are rapidly killed, within 15 min of challenge, by the antibiotic treatment. Mice given no antibiotics rapidly develop bacteremia, which persists until death of the animal. Lethality was then monitored for the subsequent 48 h.

In vitro test for synergy between imipenem and the PMX-DXT conjugate. Various twofold dilutions of both imipenem and the PMX-DXT conjugate or DXT were prepared in a 96-well microtiter plate (Costar, Cambridge, Mass.) with Trypticase soy broth as the diluent. The volume of antibiotic-containing broth was 100 μl. A 100-μl volume of a suspension of 10^6 CFU of *E. coli* per ml was added to the antibiotic or DXT mixture. The plates were then incubated overnight at 37°C. The optical density at 630 nm was measured with a Dynatech MR5000 plate reader (Dynatech Laboratories, Inc., Chantilly, Va.).

RESULTS

As described in the introduction, mice sensitized with D-galactosamine are sensitive to *E. coli* infection with an approximate 50% lethal dose of 4×10^4 CFU. Coadministration of the cell wall-active antibiotic imipenem provides significant protection, with an approximately eightfold shift in the 50% lethal dose. At a microbial dose of 5×10^5 CFU, antibiotic-treated mice still die despite the absence of detectable CFU in either the circulation of peritoneal lavages. We have hypothesized that this lethality is the result of endotoxemia.

To assess the potential protective efficacy of the antiendotoxin compound PMX-DXT, we have adopted an experimental model in which sufficiently large bacterial doses are employed such that antibiotic treatment is no longer efficacious. For these studies, mice were given D-galactosamine and 5×10^5 *E. coli* CFU intraperitoneally along with either saline or imipenem (20 mg/kg). Fifteen minutes later, separate groups of mice were administered either the PMX-DXT compound or,

TABLE 1. Efficacy of a PMX-DXT conjugate in protection of mice against *E. coli* lethality

E. coli challenge dose (CFU)[a]	Imipenem[b]	Treatment[c]	Lethality[d] (no. dead/total)			
			Expt 1	Expt 2	Total	%
5×10^5	−	DXT	8/10	6/10	14/20	70
5×10^5	−	PMX-DXT		6/10	6/10	60
5×10^5	+	DXT	9/10	8/10	17/20	85
5×10^5	+	PMX-DXT	1/10	3/10	4/20	20[e]
1×10^6	+	DXT[f]		9/10	9/10	90
1×10^6	+	PMX-DXT[f]		8/10	8/10	80

[a] Mice were administered *E. coli* intraperitoneally along with 20 mg of D-galactosamine per kg.
[b] Imipenem (20 mg/kg) was administered along with the *E. coli*.
[c] Mice were given either DXT or the PMX-DXT conjugate intraperitoneally at 1 mg/kg 15 min after challenge with *E. coli*.
[d] Lethality was monitored for 48 h following the challenge.
[e] $P < 0.05$ versus all other groups by Fisher's exact test. $P < 0.01$ versus the DXT control with imipenem.
[f] Mice were given 5 mg of either compound per kg.

TABLE 2. PMX-DXT conjugate protects mice against lethality due to gram-negative bacteria but not gram-positive bacteria or TNF-α

Challenge[a] (dose)	Treatment[b]	Lethality[c] (%)
E. coli (1×10^6 CFU)	DXT + imipenem	8/10 (80)
	PMX-DXT + imipenem	0/10[d] (0)[d]
E. coli (5×10^6 CFU)	DXT + imipenem	8/8 (100)
	PMX-DXT + imipenem	1/8[d] (13)[d]
P. aeruginosa (1×10^6 CFU)	DXT + imipenem	15/16 (94)
	PMX-DXT + imipenem	1/16[d] (6)[d]
S. aureus (7.5×10^7 CFU)	DXT + imipenem	5/8 (63)
	PMX-DXT + imipenem	4/8 (50)
TNF-α (5 μg)	DXT	5/8 (63)
	PMX-DXT	6/8 (75)

[a] Challenge organisms or TNF-α were administered intraperitoneally along with 20 mg of D-galactosamine per kg. The antibiotic was not included in the TNF-α-treated group.
[b] Mice were given the conjugate or dextran 5 min prior to challenge.
[c] Lethality was monitored for 48 h following the challenge. The data shown are no. of dead mice/total.
[d] $P < 0.01$ versus the control by Fisher's exact test.

as a control, unconjugated DXT and lethality was monitored over the next 48 h. The results of several experiments (Table 1) indicate that the compound significantly protected animals against lethal *E. coli* infection, but only if imipenem was also administered. These results suggest, therefore, that the PMX-DXT conjugate was not effective against lethality due to the bacteremia but significantly reduced lethality due to endotoxemia.

Although the conjugate was not protective by itself, indicating that it did not have significant antibacterial activity alone, it was possible that the conjugate could augment the microbicidal activity of imipenem. We performed a checkerboard titration of both imipenem and the conjugate in vitro to determine if the conjugate could affect the microbicidal activity of imipenem. The results of these studies (Fig. 1) demonstrate that addition of imipenem and the conjugate at a ratio of 4:1,
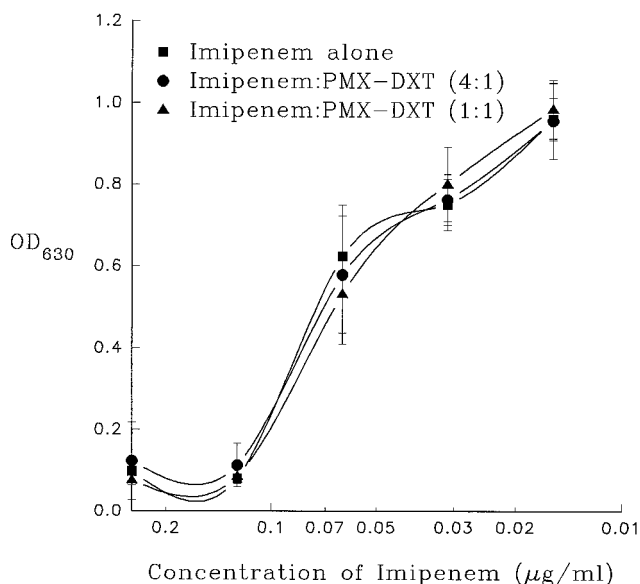

FIG. 1. Examination of potential synergy between imipenem and the PMX-DXT conjugate. Various imipenem-conjugate combinations were prepared by serial dilution of both compounds. After overnight incubation at 37°C, the optical density at 630 nm (OD$_{630}$) was measured. The results are combined from two independent experiments performed in duplicate.

the ratio of concentrations used in the in vivo experiments, did not enhance the microbicidal activity. When the imipenem-conjugate ratio was increased to 1:1, the conjugate still had no effect on the activity of imipenem. Therefore, these results are consistent with the hypothesis that the conjugate did not synergize with imipenem in antimicrobial activity and did truly act as an anti-LPS reagent rather than an antimicrobial agent.

When the doses of both the conjugate and *E. coli* were increased to 5 mg/kg and 10^6 CFU per mouse, respectively, protection was not observed when the conjugate was given 15 min postchallenge. We therefore tested whether the conjugate might afford protection if given before challenge when the larger doses of bacteria were used. For these experiments, the conjugate was administered 5 min before challenge with either 10^6 or 5×10^6 CFU of *E. coli* and imipenem. The results (Table 2) demonstrate that PMX-DXT was highly effective in significantly reducing lethality, even with these relatively large doses of bacteria. Therefore, under conditions of acute bacteremia, the timing of anti-LPS therapy may be of key significance for the desired outcome. These data indicate that the 50% lethal dose was increased by at least an order of magnitude in mice given the compound over that observed in control mice.

To be effective in a clinical setting, the conjugate should also be effective against other gram-negative organisms. We therefore tested the conjugate for protection against experimental *Pseudomonas* septic shock. The results (Table 2) demonstrate that mice can be almost completely protected when given the conjugate 5 min prior to a lethal *Pseudomonas* challenge. Therefore, the conjugate exhibits a range of effectiveness against gram-negative microbes. Since the conjugate was expected to be lipid A specific, experiments were performed to examine the efficacy of this compound against lethality due to a gram-positive organism or the cytokine TNF-α, both of which are known to be lethal when given to D-galactosamine-sensitized mice. Table 2 shows the results of these control experiments involving a lethal challenge with *S. aureus* or TNF-α. As anticipated, the lipid A-neutralizing conjugate was not effective in reducing lethality due to gram-positive organisms of TNF-α.

One potential problem with using anti-LPS or anticytokine

TABLE 3. Functional half-life of the PMX-DXT conjugate

Treatment[a]	Time given (h) prechallenge	Lethality (no. dead/total)[b]			
		Expt 1	Expt 2	Total	%
DXT	2	8/10		8/10	80
PMX	2	1/10		1/10	10[c]
PMX-DXT	2	0/10		0/10	0[c]
DXT	5	5/5	4/4	9/9	100
PMX	5	2/10	1/8	3/18	17[c]
PMX-DXT	5	9/10	7/8	16/18	89

[a] Mice were administered PMX-DXT (5 mg/kg), PMX (5 mg/kg), or DXT. At 2 or 5 h later, mice were challenged with 5×10^5 CFU of *E. coli* and imipenem (20 mg/kg).
[b] Lethality was monitored for 48 h following the challenge.
[c] $P < 0.01$ by Fisher's exact test.

strategies could be the need for continuous infusion of the compound to achieve clinical efficacy. To determine if this could be the case for the PMX-DXT conjugate, experiments were designed to examine the functional half-life of the conjugate in comparison with the parent compound, PMX. To estimate the pharmacokinetics of PMX-DXT, mice were treated with either native PMX, DXT, or PMX-DXT either 2 or 5 h before challenge with *E. coli* and an antibiotic. As shown by the data in Table 3, although both PMX and the conjugate were both protective when given 2 h prior to the *E. coli* challenge, significant protection was not observed at 5 h for the conjugate or DXT alone. However, native PMX still provided significant protection when given 5 h prior to a challenge.

DISCUSSION

The results of these studies indicate that the PMX-DXT conjugate was effective in reducing lethality in this mouse model of endotoxemia. However, protection was dependent upon coadministration of the cell wall-active antibiotic imipenem. We have previously determined that the antibiotic is rapidly bactericidal against large doses of bacteria but does not protect experimental animals from lethality. The ability of the conjugate, which has been designed to neutralize the endotoxic effects of LPS, to protect mice under these conditions suggests that LPS is a major mediator of lethality in this experimental sepsis model. Additionally, we have found that an anti-LPS monoclonal antibody also protects mice in this model, thereby supporting a role for microbe-derived LPS as a mediator of lethality (unpublished results). The observation that the compound appeared to be ineffective in protecting mice against bacteria alone with no antibiotic treatment also suggests that the antimicrobial activity of the conjugate most likely does not contribute to protection in this model. Therefore, these studies provide a further rationale for a combination of antibiotic and anti-LPS therapy in the treatment of gram-negative sepsis.

Although many gram-negative bacteria are antigenically diverse, the lipid A moiety of LPS is highly conserved, making it a favorable target for anti-LPS therapy. Therefore, it became necessary to ensure that the compound would protect mice against different gram-negative bacteria. We have, in this respect, been able to demonstrate protective efficacy in both *E. coli* and *Pseudomonas* infections. Since PMX is lipid A specific, it would be anticipated that PMX-DXT would not be able to protect against lethality due to other causes, such as a gram-positive organism or TNF-α, and this has been confirmed experimentally. Together, the findings that protection occurred against several gram-negative bacteria, but not gram-positive

bacteria or TNF-α, suggest that lethality in mice challenged with gram-negative bacteria is due to LPS and that LPS is the target of the PMX-DXT conjugate.

Many experimental models involving lethal challenge and treatment with anti-LPS or anticytokine strategies require a pretreatment time, a factor that may limit their clinical success. With small doses of bacteria, a pretreatment time was not required for the conjugate to provide protection. However, when the dose of bacteria was increased to 10^6 CFU, the conjugate was no longer protective when given 15 min postchallenge. These results may indicate that with large bacterial inocula, the antibiotic causes rapid release of LPS, resulting in the interaction with LPS receptor or binding proteins within the first 15 min, thereby initiating a cascade of proinflammatory events that are not reversible by the conjugate. With smaller doses of bacteria, the antibiotic is still bactericidal but the decreased concentration of LPS in the circulation allows the interaction of LPS with the conjugate prior to the interaction with LPS receptors or binding proteins. The protective efficacy of the conjugate can be restored when large bacterial challenge doses are used if it is given 5 min prior to challenge. Pretreatment with the compound may give it an advantage in being in the circulation to interact with and neutralize LPS before the LPS binds to receptors or is internalized by cells.

When examining the functional half-life of this compound, we found that it could be given 2 h prior to challenge and still be effective. However, native PMX was still protective when given 5 h before challenge but the conjugate was not. One potential mechanism to explain these results may be the ability of native PMX to bind to membranes, thereby extending its physiologic half-life. PMX conjugated to dextran would not be likely to have this ability. In any case, the toxicity of the conjugate is greatly decreased by conjugation to DXT, thus allowing an increase in the dose of the compound given. However, increasing the dose of native PMX would lead to PMX-induced lethality without LPS challenge.

We conclude that the PMX-DXT conjugate is efficacious in protecting mice against lethal endotoxemia. This ability was specific for gram-negative organisms, did not extend to gram-positive organisms or TNF-α, and was dependent upon coadministration of imipenem with the bacteria. The conjugate was effective when given before challenge, but a pretreatment time was not required with smaller doses of bacteria. Together, these results support the hypothesis that lethality in this mouse model is due to LPS from antibiotic-treated bacteria rather than bacterial proliferation.

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