Antiendotoxin Activity of Cationic Peptide Antimicrobial Agents

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The endotoxin from gram-negative bacteria consists of a molecule lipopolysaccharide (LPS) which can be shed by bacteria during antimicrobial therapy. A resulting syndrome, endotoxic shock, is a leading cause of death in the developed world. Thus, there is great interest in the development of antimicrobial agents which can reverse rather than promote sepsis, especially given the recent disappointing clinical performance of antiendotoxin therapies. We describe here two small cationic peptides, MBI-27 and MBI-28, which have both antiendotoxic and antibacterial activities in vitro and in vivo in animal models. We had previously demonstrated that these peptides bind to LPS with an affinity equivalent to that of polymyxin B. Consistent with this, the peptides blocked the ability of LPS and intact cells to induce the endotoxic shock mediator, tumor necrosis factor (TNF), upon incubation with the RAW 264.7 murine macrophage cell line. MBI-28 was equivalent to polymyxin B in its ability to block LPS induction of TNF by this cell line, even when added 60 min after the TNF stimulus. Furthermore, MBI-28 offered significant protection in a galactosamine-sensitized mouse model of lethal endotoxic shock. This protection correlated with the ability of MBI-28 to reduce LPS-induced circulating TNF by nearly 90% in this mouse model. Both MBI-27 and MBI-28 demonstrated antibacterial activity against gram-negative bacteria in vitro and in vivo against Pseudomonas aeruginosa infections in neutropenic mice.

Systemic disease associated with the presence of pathogenic microorganisms or their toxins in the blood (i.e., sepsis) is the 13th leading cause of death in the United States, where it accounts for an estimated $5 billion to $10 billion in annual health care expenditures. Gram-negative bacteria are often associated with this disease, and their pathogenesis is in part related to the release of an outer membrane component, endotoxin (7, 37). Endotoxin is classically a lipopolysaccharide (LPS)-protein complex, although the endotoxic activity is contained entirely within the lipid A portion of LPS (8). Symptoms associated with the presence of circulating endotoxin include fever, hypertension, and, under more extreme cases, endotoxic shock (9). It is known that many antibiotics stimulate the release of endotoxin and thus stimulate the occurrence of such symptoms (11, 31, 36). Indeed, even those patients cured of bacterial infections are at immediate risk from endotoxic shock. Therefore, there is substantial interest in identifying novel strategies to overcome endotoxic shock, especially given the somewhat disappointing results obtained with certain other new therapies (17, 41, 42, 32, 33).

The physiological mechanism whereby endotoxin exerts its effect on humans involves the release of cytokines, of which tumor necrosis factor (TNF) appears to be the most significant one. Experimental data have demonstrated that (i) circulating TNF can be detected in animals that have been administered lethal doses of endotoxin (4), (ii) injection of TNF into experimental animals recreates the symptoms of endotoxin injection (4, 5, 36), and (iii) treatment of laboratory animals with anti-TNF antibodies reverses the lethal effects of endotoxin (5, 36).

In the present study, we were interested in investigating a new class of antibiotics that could neutralize rather than enhance endotoxemia and turned our attention to small cationic peptides that are ubiquitous in nature as components of non-specific defenses against microorganisms (19). These peptides can be produced recombinantly (29), making them the first genetically engineered antibiotics. Two 26- to 28-amino-acid α-helical peptides, MBI-27 (formerly CEME) and MBI-28 (CEMA), derived from parts of silk moth cecropin and bee melittin peptides, were produced with reasonable activity against gram-negative bacteria (28, 30). Both were demonstrated to bind to purified and whole-cell LPS from Pseudomonas aeruginosa by a dansyl polymyxin displacement assay, although MBI-28 with two additional positively charged lysines at its carboxy terminus was clearly superior (28). With the results reported in this paper, we demonstrate that these two molecules also have antiendotoxin activity.

MATERIALS AND METHODS

Cationic peptides. Cationic peptides were synthesized at the University of British Columbia service facility. The sequence of MBI-27 was KWKLFKKIGGAVKLTLTGLPALKLTK and that of MBI-28 was KWKLFKKIGGAVKLTLTGLPALKLTK by the one-letter amino acid code (28). For some experiments, recombinant cationic peptides (29) were utilized. Recombinant peptides were produced in a Staphylococcus aureus protein A fusion system with the plasmid pRIT5 (Pharmacia). Peptides were purified by reverse-phase high-performance liquid chromatography or fast protein liquid chromatography and were apparently homogeneous. MIC determinations were performed by standard broth dilution methods in microtiter trays with cation-adjusted Mueller-Hinton broth as the medium (2).

Bacteria and LPS. Bacteria included the Bort strain of Escherichia coli (6) that was originally derived from a case of neonatal meningitis, and which is therefore considered a relevant pathogenic strain to study, and the O111:B4 strain of E. coli (kindly provided by D. Morrison), which is a standard source of endotoxin (20). In addition, P. aeruginosa PA01 strain H103, P. aeruginosa M2, P. aeruginosa K799, E. coli UB1005, Enterobacter cloacae 218S, Salmonella typhimurium MS7953s, Klebsiella pneumoniae ATCC 13883, Xanthomonas malophilia ATCC 13637, and Acinetobacter calcoaceticus 8193 from our laboratory stock collection were employed. LPS was obtained from E. coli Bort by the technique of Westphal and Jann (40) and from P. aeruginosa H103 by the method of Darveau and Hancock (14). E. coli O111:B4 LPS was purchased from Sigma.

TNF induction in macrophage cell lines. The murine cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, Md.) and...
maintained and passaged as described previously (20). TNF induction experiments with LPS were performed as described by Kelly et al. (20). Briefly, Dulbecco’s modified Eagle medium was aspirated from RAW 264.7 cells grown overnight in 24-well tissue culture plates after seeding with 10^4 cells per ml per well and replaced with fresh medium. LPS at a final concentration of 100 ng/ml was incubated with the cells for 6 h at 37°C in 5% CO₂, prior to a TNF assay. At the same time as LPS addition, or after prescribed intervals, cationic peptides or polymyxin B was added at a final concentration of 2 to 50 μg/ml. All assays were performed three times with similar results.

To ensure that the cationic peptides were interacting with the LPS rather than the cell lines, 20 μg of MBI-28 per ml was added to macrophage cell lines as described above, and after incubation at 37°C in 5% CO₂ for 60 min, the supernatant was removed and added to a second well of untreated RAW 264.7 cells. The peptide-treated RAW cells were washed three times with Hanks balanced salt solution, and fresh medium was added. One hundred nanograms of E. coli Bort LPS per ml was then added to both the peptide-treated cells and those reconstituted with supernatant, and these wells were assayed for TNF production after 6 h of incubation at 37°C in 5% CO₂. All assays were performed three times with similar results.

Control assays were performed to demonstrate that peptides, at the highest concentrations utilized, did not induce TNF and were not cytotoxic as judged by trypan blue exclusion and continued adherence of RAW 264.7 cells.

**TNF assays.** TNF was measured in cell culture supernatants and mouse serum on the basis of cytotoxicity for L929 fibroblast cells (20). Periodic controls in which cytotoxicity was neutralized with monoclonal antibodies against TNF-α and TNF-β (antibodies IP400 and 1221-00; Genzyme Corp., Cambridge, Mass.) indicated that TNF was solely responsible for cytotoxicity. TNF activity was expressed in units as the reciprocal of the dilution of TNF that caused 50% cytotoxicity of L929 cells, as computed with the ELISA+ program (Meddata Inc. New York, N.Y.). In our hands, 1 U of TNF corresponded to 62.5 pg of recombinant murine TNF (Genzyme) per ml.

**Animal models.** Endotoxemia was induced by intraperitoneal injection of 10 or 20 μg of E. coli O111:B4 LPS in phosphate-buffered saline (PBS; pH 7.2) into galactosamine-sensitized 8- to 10-week-old female CD-1 mice (15, 16). In experiments involving peptides, 50 to 200 μg of sterilized water was injected at separate intraperitoneal sites within 10 min of LPS injection. In survival experiments, survival was monitored at 24 and 48 h postinjection. In experimental mice, TNF blood was withdrawn by cardiac puncture at timed intervals up to 8 h after the injections and allowed to clot prior to measurement of TNF in the serum.

To measure the antibacterial effect of peptides, a similar strategy was employed. Female CD-1 mice were rendered neutropenic with three injections of cyclophosphamide as described previously (12). A 90 to 100% lethal dose (LD₉₀/₁₀₀) of P. aeruginosa M2 (approximately 10⁷ organisms per mouse) was injected intraperitoneally, and in the single-dose experiments, 100 μg of PBS or sterile water containing 200 μg (8.7 mg/kg) of a cationic peptide was injected immediately afterwards. In the two-dose experiments, peptide was injected at 1 and 14 h after the initiation of bacterial infection. Survival was recorded at 24 and 48 h. After 48 h, no additional deaths occurred up to 1 week subsequent to bacterial challenge.
TABLE 3. Effect of peptides on the production of TNF by the RAW 264.7 macrophage cell line exposed to purified E. coli O111:B4 LPS or LPS-protein complexes secreted from intact E. coli O111:B4 cells

<table>
<thead>
<tr>
<th>LPS source</th>
<th>TNF induced (U/ml [% of control])a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No peptide controlb</td>
</tr>
<tr>
<td>Purified LPS, 100 ng</td>
<td></td>
</tr>
<tr>
<td>Viable E. coli O111:B4 cells</td>
<td></td>
</tr>
<tr>
<td>105</td>
<td>9,802 ± 573</td>
</tr>
<tr>
<td>106</td>
<td>12,313 ± 999</td>
</tr>
<tr>
<td>107</td>
<td>31,438 ± 3,299</td>
</tr>
<tr>
<td>108</td>
<td>48,176 ± 1,411</td>
</tr>
</tbody>
</table>

a. Typical results from three separately performed experiments are reported. Cells were treated with 20 µg of peptide or polymyxin B (P×B).
b. The background TNF produced by the RAW cells alone was 28 ± 2 U/ml. The TNF produced by MBI-28 (20 µg) alone was 50 ± 3 U/ml. This was not subtracted from the background.
c. ND, not done since polymyxin B causes cell lysis.

MBI-28 was comparable to 10.74 µM polymyxin B in its ability to block, by approximately 100% for both, induction of TNF induced by E. coli O111:B4 and E. coli Bort LPS. Figure 1 demonstrates that both polymyxin B and MBI-28 could block LPS induction of TNF by 81 and 95%, respectively, when added up to 60 min after LPS addition to RAW 264.7 cells.

In human and animal infections, LPS is probably not released as a purified molecule but rather in association with other bacterial cell components. To mimic this situation, we employed a model system in which the LPS-protein complexes, which are naturally released from bacteria, were used as a stimulus to induce TNF production in RAW 264.7 cells. Viable cells of E. coli Bort and O111:B4 or P. aeruginosa H103 were incubated in a transwell filter unit in which a 0.2-µm-pore-size membrane filter separated the intact bacteria from the compartment containing the RAW 264.7 cells. Control experiments without peptides added indicated a high level of TNF induction comparable to that due to LPS stimulation shown in Table 3. Addition of 20 µg of peptide per ml to 105, 106, or 107 viable E. coli O111:B4 cells (Table 3) or E. coli Bort cells (data not shown) reduced the TNF levels by 90 to 96%. Addition of 20 µg of MBI-28 per ml to 105 viable P. aeruginosa cells similarly resulted in a 96% reduction of TNF induction.

Blocking of TNF induction and lethality due to LPS injection into galactosamine-sensitized mice. Mice are naturally resistant to LPS. Galanos et al. (16) were able to demonstrate that injection of galactosamine into mice lowers this natural resistance, probably by its action on the liver (15), rendering mice hypersensitive to amounts of LPS as low as 1 µg or less. A dose of 10 to 20 µg of LPS injected intraperitoneally caused 100% lethality within 24 h. In contrast, when MBI-28 was injected at around the same time as LPS but at a separate site in the peritoneum, it protected mice in a dose-dependent fashion (Fig. 2). A dose of 200 µg of MBI-28 (approximately 8.7 mg/kg) protected 78% of mice (P < 0.05 by Fisher’s exact test). Consistent with its lower affinity for LPS (28), MBI-27 showed a reduced but also dose-dependent ability to protect mice against lethal endotoxic shock. Preincubation of MBI-27 with LPS prior to injection resulted in 100% protection, with the difference in protection presumably reflecting the requirement for the peptides to find, bind, and neutralize LPS when injected at separate sites in the mouse peritoneum. Control experiments indicated that the peptides themselves did not cause lethality in mice at the highest concentrations utilized here.

There is a considerable body of evidence that indicates that TNF is a major mediator in endotoxic shock. To determine whether the peptides were exerting their effects in animals by suppressing TNF induction, we monitored TNF induction in response to injection of 10 µg of E. coli O111:B4 into galactosamine-treated CD-1 mice. As demonstrated by others, LPS injection led to a typical delayed increase in serum TNF levels that peaked approximately 90 min after LPS introduction (Fig. 3). However, when 200 µg of MBI-28 was injected immediately after the LPS, similar kinetics of TNF induction were observed but serum TNF levels were reduced by 89%.

DISCUSSION

The data presented here clearly illustrate the potential of cationic antibacterial peptides in overcoming lethal endotoxemia. Both MBI-27 and MBI-28 are able to bind to LPS (28, 30) and were shown here to prevent its ability to induce a TNF response with both a macrophage tissue culture cell line and galactosamine-sensitized mice. As a consequence, the peptides...
protected against lethal endotoxemia in vivo. Other candidate antiendotoxin therapies have progressed to clinical trials in recent years, including antiendotoxin monoclonal antibodies (18, 42) and anti-TNF antibodies (41), with disappointing results. An anti-TNF monoclonal antibody was recently tested in a large phase II/III clinical trial (41). Unfortunately, the trial indicated that anti-TNF was not a valid treatment when administered to patients presenting with bacteremia and organ dysfunction, and, furthermore, failed to demonstrate any proven benefit even in the subgroup of patients presenting with endotoxic shock. However, one study was able to show limited success in reducing mortality in the early stages of sepsis (1). Current research in the area of anti-TNF therapy is focused on the use of soluble TNF receptors to block the action of circulating TNF in endotoxic shock (26, 38).

Along with the development of anti-TNF strategies, considerable effort is being devoted to the development of antiendotoxin strategies (9). This has resulted in clinical trials being conducted on two separate antiendotoxin monoclonal antibodies, namely, the human monoclonal antiendotoxin HA-1A (42) and the murine monoclonal antiendotoxin E-5 (17). Unfortunately these antiendotoxins did not prove to be beneficial in a bacteremic and/or endotoxic population of patients. As a result, both products were refused product licensure by the U.S. Food and Drug Administration. Criticisms surrounding the studies conducted on these first-generation antiendotoxins have included their inability to bind to LPS in its myriad of forms (39) and their inability to block the induction of TNF by LPS (3, 12, 39). It follows that any studies conducted on new antiendotoxic shock therapeutic agents must address these issues.

Currently, endotoxin binding molecules other than antibodies are being investigated as potential therapies against endotoxic shock. The most advanced of these is bactericidal/permeability increasing protein (BPI) (23, 24), particularly, a recombinant form corresponding to the first 199 amino acids of human BPI (BPI199). This form shows no significant homology to MBI-28, although it does show some homology to the natural cationic peptide cecropin (22). BPI is a cationic protein with weak antibacterial activity from granules of human and rabbit neutrophils (21). BPI has been shown to be superior to the monoclonal antibodies described above in its abilities to bind a variety of LPS molecules and to block LPS induction of TNF in vitro and in vivo. However, BPI has demonstrated mixed results in protection studies (32, 33), and its weak antibacterial activity suggests it may be nonoptimal. E5531 is a potent antagonist of endotoxin which has shown potential in both in vivo and in vitro systems (10).

On the basis of a comparison of the results reported here with those in the literature, we believe that there is cause for cautious optimism regarding the prospects of these small cationic peptides. For example, polymyxin B, a small cyclic cationic peptide with a lipid tail, is considered the most potent antiendotoxin agent identified to date, in terms of both its affinity for binding to endotoxin (LPS) and its ability to interfere with the biological activities of LPS. As such, it is frequently used as the standard against which novel antiendotoxin compounds are measured, although its toxicity precludes its use in systemic therapy of humans. For this reason, it is significant that, compared with polymyxin B, MBI-27 and MBI-28...
showed similar binding affinities for LPS (28) and similar abilities to block induction of TNF in cell lines. In contrast, BPI at 0.83 μM resulted in only 50% inhibition in TNF production in response to LPS in one study (33), whereas 10 μg of polymyxin B per ml reduced TNF induction by 90%.

It is significant that the cationic peptides discussed here are antibacterial in nature. Although they lack the potency of many of the recently introduced β-lactams and quinolones against the most susceptible organisms, they do have certain potential advantages. First, both β-lactams (31, 34) and quinolones (11, 34) are known to promote endotoxin release, and hence there is a risk of endotoxia. In contrast, the cationic peptides actually block endotoxia. Indeed, their ability to protect neutropenic mice against P. aeruginosa given via the intraperitoneal route may in part reflect endotoxin neutralization in vivo and potential asset lies in the potential enhancer activity of cationic peptides whereby the peptides demonstrate synergy or additive activities with conventional antibiotics (19, 28). Thus, one can envision their use in combination with conventional antibiotics to increase killing and, at the same time, neutralize LPS released by these antibiotics. We are currently investigating these issues with a view to designing peptides with enhanced efficacy.

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REFERENCES


