INFECTION AND IMMUNITY, Dec. 1996, p. 4922–4927 Vol. 64, No. 12
0019-9567/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Antiendotoxin Activity of Cationic Peptide Antimicrobial Agents

MONISHA GOUGH,[1] ROBERT E. W. HANCOCK,[1]* AND NIAMH M. KELLY[2]

Departments of Microbiology and Immunology[1] and Pathology and Laboratory Medicine,[2]
University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3

Received 7 May 1996/Returned for modification 7 August 1996/Accepted 30 August 1996

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., septicemia) 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 nonspecific 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 KWKLFKKIGI GAVLKVLTTGLPALIS and that of MBI-28 was KWKLFKKIGIGAVLKVLT TGLPALKLTK 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* PAO1 strain H103, *P. aeruginosa* M2, *P. aeruginosa* K799, *E. coli* UB1005, *Enterobacter cloacae* 218S, *Salmonella typhimurium* MS7953s, *Klebsiella pneumoniae* ATCC 13883, *Xanthomonas maltophilia* 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

---

* Corresponding author. Phone: (604) 822 2682. Fax: (604) 822 6938. Electronic mail address: Bob@cbdn.ca.

TABLE 1. Activity of MBI-27 and MBI-28 against a variety of gram-negative bacteria

Strain	MIC (μg/ml)	
	MBI-27	MBI-28
Escherichia coli UB1005	4	4
Escherichia coli Bort	6	8
Escherichia coli O111:B4	4	4
Enterobacter cloacae 218S	4	4
Salmonella typhimurium MS7953s	4	4
Klebsiella pneumoniae ATCC 13883	8	12
Pseudomonas aeruginosa H103	16	8
Pseudomonas aeruginosa K799	4.8[a]	2.8[a]
Xanthomonas maltophilia ATCC 13637	8	12
Acinetobacter calcoaceticus 8193	2	1

[a] MIC data taken from reference 28.

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 10^6 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% CO_2 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% CO_2 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% CO_2.

To examine the effects on whole bacterium induction of TNF, bacteria grown in Luria broth overnight (approximately 10^8/ml) and diluted to 10^5, 10^6, or 10^7 organisms per ml and RAW 264.7 cells were each added to separate compartments of a transwell diffusion chamber, which contained a 0.2-μm-pore-size membrane filter separating the two compartments. Peptides were added, when appropriate, to the bacterium-containing compartment at a final concentration of 20 μg/ml. TNF levels were measured after 6 h of incubation at 37°C in 5% CO_2. 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. Endotoxic shock 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 in 100 μl of sterile 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 experiments measuring 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 (LD_{90-100}) of *P. aeruginosa* M2 (approximately 10^2 organisms per mouse) was injected intraperitoneally, and in the single-dose experiments, 100 μl 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.

RESULTS

Antibacterial activity of cationic peptides. We confirmed and extended previous results showing that MBI-27 and MBI-28 were able to kill a broad range of gram-negative bacteria, with MICs ranging from 1 to 16 μg/ml (Table 1). These bacteria included many of the life-threatening gram-negative pathogens associated with septicemia and endotoxic shock. Consistent with these in vitro data, in a neutropenic mouse model, both MBI-27 and MBI-28 showed an ability to significantly protect animals against an LD_{90} dose of the mouse-pathogenic *P. aeruginosa* strain M2 (Table 2), under conditions established to mimic the acute-endotoxin therapeutic regime (single-dose studies) and with MBI-28 by using a two-dose therapeutic procedure.

Blockage of TNF induction in the RAW 264.7 macrophage cell line. RAW 264.7 cells have been demonstrated previously to induce TNF in cell cultures in response to LPS addition and thus represent an in vitro model of LPS-mediated TNF induction. Control experiments indicated that the cationic peptides were nontoxic for this macrophage cell line at the highest concentrations employed in these experiments. Both MBI-27 and MBI-28 were reproducibly able to inhibit the induction by *E. coli* O111:B4 LPS of TNF secretion by RAW 264.7 cells (Table 3). As little as 2 μg of peptide per ml reduced TNF induction by more than 50%, whereas 20 to 50 μg of peptide per ml caused almost complete blockage of TNF induction. Virtually the same results were obtained with *E. coli* Bort LPS and with *P. aeruginosa* H103 LPS. To demonstrate that this was due to the interaction of the peptides with LPS rather than the macrophages, MBI-28 at 50 μg/ml was preincubated with RAW 264.7 cells for 60 min and then the supernatant was removed. Addition of fresh medium and 100 ng of *E. coli* O111:B4 LPS per ml led to a very high level of induction of TNF (19,062 ± 1,388 U/ml), indicating that the peptides had not repressed the ability of the RAW 264.7 to respond to LPS induction. In contrast, the supernatants from these preincubations (which presumably contained the peptides) when added to fresh RAW 264.7 cells were able to suppress the ability of these cells to induce TNF in response to LPS stimulation, producing only 4,450 ± 520 U/ml.

The kinetics of blocking of TNF induction by MBI-28 at 20 μg/ml were also examined and compared with that of blocking by polymyxin B. Polymyxin B has been demonstrated to have a very high affinity for binding to LPS (27, 28, 30) and an ability to block many of the biological activities of LPS (18, 35). Previous data indicated that MBI-28 had a high affinity for LPS very similar to that of polymyxin B, as judged by dansyl polymyxin displacement assays (28). Consistent with this, 5.52 μM

TABLE 2. Influence of cationic peptides on survival of neutropenic mice challenged intraperitoneally with *P. aeruginosa* M2

Mouse group receiving:	Survival[a] (%)	p[b]
No peptide	2/30 (6.7)	
MBI-27, 200 μg[c]	8/30 (26.7)	0.04
MBI-28		
200 μg[c]	13/30 (43.3)	0.0011
200 μg[d]	11/20 (55.0)	0.0002

[a] Number of mice surviving/total number.

[b] By Fisher's exact test.

[c] These groups were given a single injection of peptide. The data reported for these groups are a compilation of three experiments.

[d] This group was given two injections of MBI-28 at 2 h and 12 h after the addition of *P. aeruginosa*. The data reported for this group are a compilation of the results of two experiments.

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

LPS source	TNF induced (U/ml [% of control])[a]			
	No peptide control[b]	MBI-27 treated	MBI-28 treated	P×B treated
Purified LPS, 100 ng	9,802 ± 573	566 ± 14 (5.8)	566 ± 69 (5.8)	573 ± 4 (5.8)
Viable *E. coli* O111:B4 cells				
10^5	12,313 ± 999	486 ± 40 (3.9)	552 ± 61 (4.5)	ND[c]
10^6	31,438 ± 3,299	1,653 ± 114 (5.3)	1,892 ± 1,370 (6.0)	ND
10^7	48,176 ± 1,411	4,569 ± 675 (5.9)	4,126 ± 3,890 (8.5)	ND

[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 10^5, 10^6, or 10^7 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 10^6 viable *P. aeruginosa* cells similarly resulted in a 96% reduction of TNF induction.

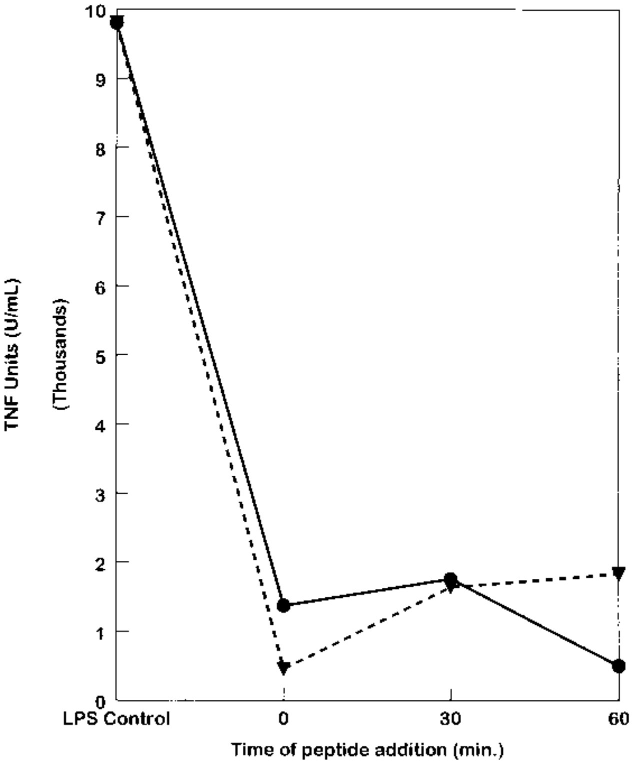

FIG. 1. Suppression by MBI-28 and polymyxin B of *E. coli* O111:B4 LPS-induced TNF in the RAW macrophage cell line. MBI-28 (5.52 μM) (●) or 10.74 μM polymyxin B (▼) was added at the same time as LPS and 30 min and 60 min post-LPS addition. Results are presented as the mean of TNF levels in the macrophage supernatant.

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

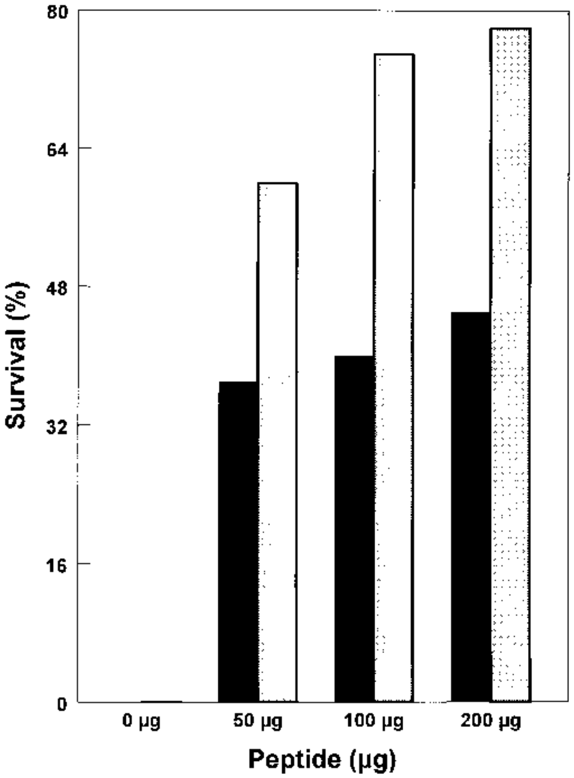

FIG. 2. Protective effect of cationic peptides against lethal endotoxemia in galactosamine-sensitized mice. Endotoxemia was induced with 10 µg of *E. coli* O111:B4 LPS injected into the peritoneum. Subsequently, 50, 100, or 200 µg (2.2, 4.4, and 8.7 mg/kg) of MBI-27 (solid bar) or MBI-28 (shaded bar) was injected into a separate site in the peritoneum. Three mice were assessed for each experimental point. Mortality was observed after 24 h.

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 (BPI_{23}). 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

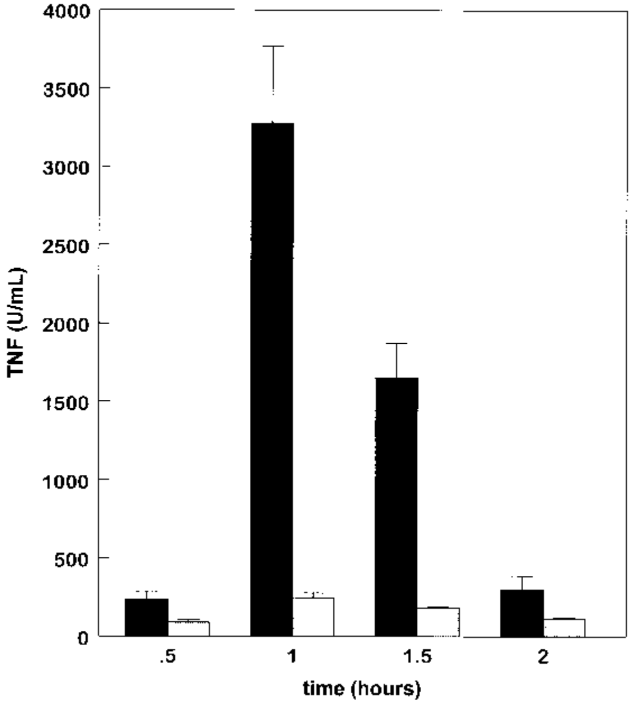

FIG. 3. Suppression by MBI-28 of an LPS-induced circulating TNF response in galactosamine-sensitized mice. Endotoxemia was induced by intraperitoneal injection of 10 µg of *E. coli* O111:B4 LPS (solid bar) and half of the mice also received 200 µg of MBI-28 injected into a separate location in the mouse peritoneum at time zero (shaded bar). At the time points shown, blood was taken and TNF was measured in the L929 cytotoxicity assay. Results are presented as the means ± standard deviations of TNF levels in mice.

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 endotoxemia. In contrast, the cationic peptides actually block endotoxemia. Indeed, their ability to protect neutropenic mice against *P. aeruginosa* given via the intraperitoneal route may in part reflect endotoxin neutralization in vivo. A second 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.

ACKNOWLEDGMENTS

This research was financially supported by the Canadian Bacterial Diseases Network, with additional funding from Micrologix Biotech Inc.

REFERENCES

1. **Abraham, E., R. Wunderink, H. Silverman, T. M. Perl, S. Nasraway, H. Levy, R. Bone, R. P. Wenzel, R. Balk, R. Allred, J. E. Pennington, and J. C. Wherry.** 1995. Efficacy and safety of monoclonal antibody to human tumor necrosis factor α in patients with sepsis syndrome. JAMA **273:**934–941.
2. **Amsterdam, D.** 1991. Susceptibility testing of antimicrobials in liquid media, p. 72–78. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. Williams & Wilkins Co., Baltimore.
3. **Baumgartner, J. D., D. Heumann, J. Gerain, P. Weinbreck, G. E. Grau, and M. P. Glauser.** 1990. Association between protective efficacy of anti-lipopolysaccharide (LPS) antibodies and suppression of LPS-induced tumor necrosis factor and interleukin 6. J. Exp. Med. **171:**889–896.
4. **Beutler, B., I. W. Milsark, and A. Cerami.** 1985. Cachectin/tumor necrosis factor: production, distribution, and metabolic fate *in vivo*. J. Immunol. **135:**3972–3977.
5. **Beutler, B., I. W. Milsark, and A. Cerami.** 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science **229:**869–871.
6. **Bortolussi, R., P. Ferrieri, B. Björkstén, and P. G. Quie.** 1979. Capsular K1 polysaccharide of *Escherichia coli*: relationship to virulence in newborn rats and resistance to phagocytosis. Infect. Immun. **25:**293–298.
7. **Brandtzaeg, P., P. Kierulf, P. Gaustrad, A. Skulberg, J. N. Bruun, S. Halvorsen, and E. Sorensen.** 1989. Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease. J. Infect. Dis. **159:**195–204.
8. **Budavari, S., M. O'Neil, A. Smith, and P. E. Heckelman.** 1989. Merck Index, 11th ed. Merck and Co., Inc., Rahway, N.J.
9. **Burd, R. S., C. S. Cody, and E. L. Dunn (ed.).** 1992. Immunotherapy of gram-negative bacterial sepsis. R. G. Landes Co., Austin, Tex.
10. **Christ, W. J., O. Asano, A. L. C. Robidoux, M. Perez, Y. Wang, G. R. Dubuc, W. E. Gavin, L. D. Hawkins, P. D. McGuinness, M. A. Mullarkey, M. D. Lewis, Y. Kishi, T. Kawata, J. R. Bristol, J. R. Rose, D. P. Rossignol, S. Kobayashi, I. Hishinuma, A. Kimura, N. Asakawa, K. Katayama, and I. Yamatsu.** 1995. E5531, a pure endotoxin antagonist of high potency. Science **268:**80–83.
11. **Cohen, J., and J. S. McConnell.** 1985. Antibiotic-induced endotoxin release. Lancet **ii:**1069–1070.
12. **Cross, A. S., and S. Opal.** 1994. Therapeutic intervention in sepsis with antibody to endotoxin: is there a future? Endotox. Res. **1:**57–61.
13. **Cryz, S. J., Jr., E. Furer, and R. Germanier.** 1983. Simple model for the study of *Pseudomonas aeruginosa* infections in leukopenic mice. Infect. Immun. **39:**1067–1071.
14. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium*. J. Bacteriol. **155:**831–838.
15. **Freudenberg, M. A., O. Keppler, and C. Galanos.** 1986. Requirement for lipopolysaccharide response in macrophages in galactosamine-induced sensitization to endotoxin. Infect. Immun. **51:**891–897.
16. **Galanos, C., M. A. Freudenberg, and W. Reutter.** 1979. Galactosamine induced sensitization to the lethal effects of endotoxin. Proc. Natl. Acad. Sci. USA **76:**5939–5943.
17. **Greenman, R. L., R. M. Schein, M. A. Martin, R. P. Wenzel, N. R. MacIntyre, G. Emmanuel, H. Chmel, K. B. Kohler, M. McCarthy, J. Plouffe, J. A. Russell, and the XOMA Sepsis Study Group.** 1991. A controlled clinical trial of E5 murine monoclonal IgM antibody to endotoxin in the treatment of gram-negative sepsis. JAMA **266:**1097–1102.
18. **Hanasawa, K., T. Tani, and M. Kodama.** 1989. New approach to endotoxic and septic shock by means of polymyxin B immobilized fiber. Surg. Gynecol. Obstet. **168:**323–331.
19. **Hancock, R. E. W., T. Falla, and M. H. Brown.** 1995. Cationic bacterial peptide, p. 135–175. *In* R. K. Poole (ed.), Advances in microbial physiology. Academic Press Ltd., London.
20. **Kelly, N. M., L. Young, and A. S. Cross.** 1991. Differential induction of tumor necrosis factor by bacteria expressing rough and smooth lipopolysaccharide phenotypes. Infect. Immun. **59:**4491–4496.
21. **Levy, O., C. E. Ooi, J. Weiss, R. I. Lehrer, and P. Elsbach.** 1994. Individual and synergistic effects of rabbit granulocyte proteins on *Escherichia coli*. J Clin. Invest. **94:**672–682.
22. **Little, R. G., D. N. Kelner, E. Lim, D. J. Burke, and P. J. Conlon.** 1994. Functional domains of recombinant bactericidal/permeability increasing protein (rBPI$_{23}$). J. Biol. Chem. **269:**1865–1872.
23. **Marra, M. N., C. G. Wilde, M. S. Collins, J. L. Snable, M. B. Thornton, and R. W. Scott.** 1992. The role of bactericidal/permeability-increasing protein as a natural inhibitor of bacterial endotoxin. J. Immunol. **148:**532–537.
24. **Marra, M. N., C. G. Wilde, J. E. Griffith, J. L. Snable, and R. W. Scott.** 1990. Bactericidal/permeability-increasing protein has endotoxin neutralizing activity. J. Immunol. **144:**662–666.
25. **Mathison, J. C., E. Wolfson, and R. J. Ulevitch.** 1988. Participation of tumor necrosis factor in the mediation of gram-negative bacteria lipopolysaccharide-induced injury in rabbits. J. Clin. Invest. **88:**1925–1937.
26. **Mohler, K. M., D. S. Torrance, C. A. Smith, R. G. Goodwin, K. E. Stremler, V. P. Fung, H. Madani, and M. B. Widmer.** 1993. Soluble tumor necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoxemia and function simultaneously as both TNF carriers and TNF antagonists. J. Immunol. **151:**1548–1561.
27. **Moore, R. A., N. C. Bates, and R. E. W. Hancock.** 1986. Interaction of polycationic antibiotics with *Pseudomonas aeruginosa* lipopolysaccharide and lipid A studied by using dansyl-polymyxin. Antimicrob. Agents Chemother. **29:**496–500.
28. **Piers, K. L., M. H. Brown, and R. E. W. Hancock.** 1994. Improvement of outer membrane-permeabilizing lipopolysaccharide-binding activities of an antimicrobial cationic peptide by c-terminal modification. Antimicrob. Agents Chemother. **38:**2311–2316.
29. **Piers, K. L., M. H. Brown, and R. E. W. Hancock.** 1993. Recombinant DNA procedures for producing small antimicrobial cationic peptides in bacteria. Gene **134:**7–13.
30. **Piers, K. L., and R. E. W. Hancock.** 1994. The interaction of a recombinant cecropin/melittin hybrid peptide with the outer membrane of *Pseudomonas aeruginosa*. Mol. Microbiol. **12:**951–958.
31. **Prins, J. M., E. J. Kuijper, M. L. Mevissen, P. Speelman, and S. J. Van Deventer.** 1995. Release of tumor necrosis factor and interleukin 6 during antibiotic killing of *Escherichia coli* in whole blood: influence of antibiotic class, antibiotic concentration, and presence of septic serum. Infect. Immun. **63:**2236–2242.
32. **Rogy, M. A., L. L. Moldawer, H. S. Oldenburg, W. A. Thompson, W. J. Montegut, S. A. Stackpole, A. Kumar, M. A. Palladino, M. N. Marra, and S. F. Lowry.** 1994. Anti-endotoxin therapy in primate bacteremia with HA-1A and BPI. Ann Surg. **220:**77–85.
33. **Rogy, M. A., H. S. Oldenburg, S. E. Calvano, W. J. Montegut, S. A. Stackpole, K. J. Van Zee, M. N. Marra, R. W. Scott, J. J. Seilhammer, L. L. Moldawer, and S. F. Lowry.** 1994. The role of bacterial/permeability-increasing protein in the treatment of primate bacteremia and septic shock. J. Clin. Immunol. **14:**120–133.
34. **Shenep, J. L., R. P. Barton, and K. A. Mogan.** 1985. Role of antibiotic class in the rate of liberation of endotoxin during therapy for experimental gram-negative bacterial sepsis. J. Infect. Dis. **151:**1012–1018.
35. **Stokes, D. C., J. L. Shenep, M. Fishman, W. K. Hildner, G. K. Bysani, and K. Rufus.** 1989. Polymyxin B prevents lipopolysaccharide-induced release of tumor necrosis factor from alveolar macrophages. J. Infect. Dis. **160:**52–57.
36. **Tracey, K. J., Y. Fong, D. Hesse, K. R. Manogue, A. T. Lee, G. C. Kuo, S. F. Lowry, and A. Cerami.** 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature (London) **330:**662–664.
37. **Van Deventer, S. J. H., H. R. Buller, J. W. TenCate, A. Sturk, and W. Paul.** 1988. Endotoxemia: an early predictor of septicaemia in febrile patients. Lancet **i:**605–608.
38. **Van Zee, K. J., T. Kohno, E. Fischer, C. S. Rock, L. L. Moldawer, and S. F.**

Lowry. 1992. Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis factor *in vitro* and *in vivo*. Proc. Natl. Acad. Sci. USA **89:**4845–4849.

39. **Warren, H. S., S. F. Amato, C. Fitting, K. M. Black, P. M. Loiselle, M. S. Pasternack, and J. M. Cavaillon.** 1993. Assessment of ability of murine and human anti-lipid A monoclonal antibodies to bind and neutralize lipopolysaccharide. J. Exp. Med. **177:**89–97.
40. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further application of the procedure, p. 83–91. *In* R. L. Whistler (ed.), Methods in carbohydrate chemistry, vol. 5. Academic Press, Inc., New York.
41. **Wherry, J., R. Wenzel, E. Abraham, R. Allred, R. Balk, R. Bone, H. Levy, S. Nasraway, T. Perl, H. Silverman, R. Wnderink, and the TNF MAb Study Group.** 1993. A controlled randomized double blind clinical trial of monoclonal antibody to tumor necrosis factor (TNF MAb) in patients with sepsis syndrome. Chest **104:**3S.
42. **Ziegler, E. J., C. J. Fisher, Jr., C. L. Sprung, R. C. Straube, J. C. Sadoff, G. E. Foulke, C. H. Wortel, M. P. Fink, R. P. Dellinger, N. N. H. Teng, I. E. Allen, H. J. Berger, G. L. Knatterud, A. F. LoBuglio, C. R. Smith, and the HA-1A Sepsis Study Group.** 1991. Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin: a randomized, double-blind, placebo-controlled trial. N. Engl. J. Med. **324:**429–432.

Editor: R. E. McCallum