COMPARISON OF STRUCTURALLY RELATED PEPTIDES IN THEIR
ANTIMICROBIAL AND ANTI-ENDOTOXIC ACTIVITY

by

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We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

June 1998

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Vancouver, Canada

Date Sept 8/98
ABSTRACT
Sepsis, a serious problem in the developed world, is often associated with Gram negative bacteria. Their pathogenesis is in part related to the release of an outer membrane component, endotoxin. There is great interest in the development of an anti-endotoxin due to the lack of an effective therapy. In this study a series of α-helical cationic peptides, based on hybrids of the moth peptide cecropin and the bee peptide melittin was created using as the parent peptides, MBI-27 and MBI-28. MBI-27 and MBI-28 had already been shown to have antimicrobial and anti-endotoxin activity. From these two peptides along with CP26 and CP29 (more amphipathic in nature), a series of 31 variants with small amino acid changes were designed. The antimicrobial activity of the peptides was studied by MIC determination, killing assays and inner membrane permeabilization studies. The peptides were also tested for their ability to bind LPS and block production of IL-6 and TNF by LPS-stimulated macrophages. Changes made to the variant peptides did not improve upon the antimicrobial activity of MBI-28. Many of the peptides lost Gram-positive activity and some of the peptides lost all antimicrobial activity. The addition of Mg²⁺ had a partial inhibitory effect on the killing of E. coli and P. aeruginosa by CP26 and MBI-28 as well as inhibiting inner membrane permeabilization of E.coli by CP26. The peptides were found to be non-toxic to the macrophage cells under the conditions tested. The peptides were found to inhibit TNF production measured by the L cell assay (bio-active TNF) to a larger extent than that tested by ELISA, although the trends were generally the same. The peptide effect on IL-6 production was very similar to that seen with the TNF ELISA data. The peptides that were good antimicrobials and had high binding affinity for E.coli LPS also were able to inhibit production of IL-6 and
TNF by LPS-stimulated macrophages. Some of the peptides that had no antimicrobial activity and higher LPS binding affinities were not as effective in preventing production of cytokines although this was not always the case. There are a large number of factors involved in what makes a good peptide; length and charge but not hydrophobicity of the peptides were found to play a role.
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<thead>
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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide-m-chlorophenyl hydrazone</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GPI</td>
<td>glycerophosphatidylinositol</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LBP</td>
<td>lipopolysaccharide binding protein</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium blue</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NPN</td>
<td>1-N-phenylnaphthylamine</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ONPG</td>
<td>o-nitrophenyl-β-D-galactoside</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PMB</td>
<td>polymyxin B</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear granulocyte</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor</td>
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</table>
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1 INTRODUCTION

1.1 Cationic Peptides

Antibiotics produced by microorganisms have long been used to fight many diseases caused by bacteria, fungi and yeast. Within the past several decades, however, a new generation of antimicrobial agents, termed "peptide antibiotics", has been discovered. Zeya and Spitznagel (1966) isolated the first peptide antibiotics from mammalian polymorphonuclear leukocytes (PMN) and noted their cationic nature. Since then, further investigation has led to the discovery of many different peptides from a wide range of animal, plant, and bacterial species. These peptides are found as components of non-specific defenses against microorganisms. The secondary structure of these antimicrobial peptides include β-sheets, amphipathic α helices, extended helices and loops formed by disulfide bridges. Two naturally occurring peptides that form α-helices are cecropin A (silk moth) and melittin (bee).

Boman et al (1989a) created the first hybrid peptides that contained sequences from both cecropin A and melittin. The peptides were the same size as melittin, and contained different combinations of the hydrophobic and hydrophilic regions of cecropin A and melittin. They synthesized a peptide consisting of the first 13 amino acids of cecropin A followed by the first 13 amino acids of melittin. This peptide was 100-fold more active against S.aureus than cecropin A and had antimalarial activity that was 10-fold better than cecropin B (Boman et al., 1989a). Unlike melittin, however, this cecropin-like peptide possessed no hemolytic activity.
Piers et al (1993) produced MBI-27 (formerly known as CEME), which contains the first eight amino acids of cecropin and the first eighteen amino acids of melittin, recombinantly using a bacterial expression system. MBI-27 and its derivative MBI-28 (formerly known as CEMA, and containing two additional charges on the C terminus) were found to have antimicrobial activity against Gram negative bacteria as well as a high affinity for bacterial endotoxin (LPS) (Piers et al., 1994). These hybrid peptides were also found to have endotoxin neutralizing activity in murine macrophages and in mice (Gough et al., 1996). Since the α-helical nature of the peptides was considered important for their activity, amino acid changes were made to the peptides in order to create a more amphipathic molecule. This resulted in the peptides CP26 and CP29. From these four peptides, a series of 31 variants with small amino acid changes were designed in order to study structure/function relationships.

1.2 Action of Peptides on Bacteria

MBI-27 and MBI-28 were found to not only be active antimicrobial agents but they were also able to permeabilize the outer membrane of P. aeruginosa to lysozyme and 1-N-phenylnaphthlyamine (NPN) (Piers et al., 1994). The peptides are thought to cross the outer membrane by self-promoted uptake (Hancock et al., 1995). In this process, the peptides interact with the outer membranes of Gram-negative bacteria and competitively displace divalent cations (Mg\(^{2+}\) or Ca\(^{2+}\)) from their binding sites on LPS. This causes a distortion of the outer membrane allowing the peptides to pass. Although it is known that the peptides subsequently cause a general collapse of membrane integrity with a resulting
loss of the cytoplasmic permeability barrier (Hancock et al., 1995), the exact nature of the mechanism of killing is not known (Silvestro et al., 1997).

1.3 Endotoxic Shock

Systemic disease associated with the presence of pathogenic microorganisms or their toxins in the blood (i.e. septicaemia) affects hundreds of thousands of North Americans annually. Characteristic clinical manifestations associated with the presence of circulating endotoxin include fever, and/or hypothermia, tachypnea, tachycardia, reduced or elevated circulating PMNs, hypotension, and, multi-organ hypoperfusion. 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 (Brandtzaeg, P. et al., 1989; Van Deventer S.J.H. et al., 1988). Endotoxin is classically a lipopolysaccharide (LPS) - protein complex, although its endotoxic activity is entirely contained within the lipid A portion of LPS (Budavari S. et al., 1989). LPS is an amphipathic molecule consisting of three domains: a) the hydrophilic O-antigen side chain that extends outward from the cell, b) a core polysaccharide region consisting of various conserved components, and c) the hydrophobic lipid A tail which anchors the molecule into the membrane. Lipid A generally consists of a 4-phosphoglucomaminyl-(1-6)-glucosamine-1-phosphate backbone (Wilkinson, 1983) to which four to six saturated fatty acids are either ester or amide linked (Karunaratne et al., 1992). These linkages can either be directly on the sugar backbone, or through the hydroxyl groups of other fatty acid chains (Kropinski et al., 1985).
Endotoxic shock is often initiated by microbial infection. A series of host inflammatory responses, designed to protect the host from infection, is often initiated by the bacteria or its products (e.g., LPS). This response can involve the complement and coagulation pathways, as well as cellular mediation systems, both of which result in the generation of a variety of inflammatory mediators. In endotoxic shock, these mediators themselves act as stimuli for production of additional inflammatory mediators, triggering a cascade amplification response, and resulting systemic inflammation and tissue destruction.

The interaction of LPS with immune cells usually involves an acute-phase reactant protein, LPS-binding protein (LBP). LBP is a 60 kD glycoprotein that forms high-affinity complexes with LPS (Tobias et al., 1986). LBP has been found to enhance LPS-macrophage interaction but it is not absolutely necessary. Endotoxin interacts with specific receptors on the surface of host inflammatory cells. The most studied of these receptors is CD14, a 55 kD protein which can be membrane-bound or soluble (Tobias et al., 1994). CD14 is anchored in the cell membrane of monocytes, macrophages, and polymorphonuclear granulocytes (PMNs) as a glycerophosphatidylinositol (GPI)-tailed protein that lacks an intracellular signalling domain. Both GPI-anchored CD14 and soluble CD14 bind LPS and in both cases the LPS-CD14 interactions are markedly facilitated by LBP, which acts as a transfer protein (Tobias et al., 1986). Binding of LPS to GPI-anchored CD14 also results in rapid translocation of LPS from the cell membrane to intracellular locations (Gallay et al., 1993). The pathway by which LPS binding to CD14 results in intracellular signalling is unknown. The most widely held hypothesis is that CD14 acts as a carrier molecule, presenting LPS to a 'signalling' receptor (Lynn,
At high concentrations of LPS, monocytes and neutrophils can respond through a LBP/CD14-independent pathway (Lynn et al., 1993). LPS has also been found to bind to complement receptor type 3 (CR3) (Ingalls et al., 1995) and the acetylated LDL receptor (AcLDL) on macrophages (Hampton et al., 1991).

1.4 Mediators of Endotoxic Shock

The physiological mechanism whereby endotoxin exerts its affect on man involves the release of cytokines; the most thoroughly studied cytokines include TNF-α, IL-1, IL-6, IL-8, IL-10, IL-15, IFN-α/β, TGF-β, MIP-1, and GSF. TNF-α exhibits immense diversity of cellular responses mediated through membrane receptors present on nearly all cell types. TNF-α plays a significant role in endotoxic shock. Experimental data has demonstrated that (i) circulating TNF can be detected in animals administered lethal doses of endotoxin (Beutler et al., 1985), (ii) injection of TNF into animals induces symptoms of endotoxemia (Beutler et al., 1985a, 1985b, Tracey et al., 1987) and (iii) treatment of animals with anti-TNF antibody reverses the lethal effects of endotoxin (Beutler et al., 1985b, Tracey et al., 1987).

Two types of TNF-α receptors have been recognized, p55 and p75. The p55 receptor is ubiquitous, whereas the p75 receptor is restricted to cells of hematopoietic origin (Smith, 1990: Loetscher, 1990). Following TNF-receptor binding, an increase in membrane fluidity and permeability, subsequent calcium influx into the cytoplasm and enhanced cAMP levels and protein kinase activity have been observed. There appears to be no intrinsic biological activity specific to TNF-α and its receptors. Rather, different cells types can respond very differently to TNF-α due to subtle tissue-specific differences in
TNF-α signal assimilation (Schutze S., 1988). As a consequence, the biological activities of TNF-α cover a broad spectrum of action, including activation of macrophages and granulocytes, activation of T and B lymphocytes and inhibition of hematopoiesis.

The pathogenesis of endotoxic shock also involves other cytokines. IL-1, IL-6 as well as TNF play a critical role in inducing the acute phase response in the liver. IL-1 and IL-6 induce fever, which favours effective host defences. As an inflammatory mediator, IL-6 is involved in lymphocyte activation and increased antibody production. Some cytokines have a regulatory role such as IL-10 which has a protective effect in the inflammatory process.

1.5 Clinical Significance

There is substantial interest in identifying novel strategies to overcome endotoxic shock due to the lack of an effective therapy. Many new strategies, including neutralizing antibodies and various endotoxin-binding factors, have been tested with mixed results. An anti-TNF monoclonal antibody was tested in a large phase II/III clinical trial (Wherry et al., 1993). Unfortunately, the trial indicated that anti-TNF was not a valid treatment when administered to patients presenting with bacteraemia 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 (Abraham et al., 1995). Current research in the area of anti-TNF therapy is focussed on the use of soluble TNF receptors to block the action of circulating TNF in endotoxic shock. Constructs of both p75 and p55 have proceeded to clinical trials in which p75 failed to prevent mortality and may
have even increased mortality at high doses (Fisher et al., 1996). A phase II clinical trial of p55 sTNFR revealed a trend towards decreased mortality at the highest dose used (The Ro45-208 Study Group, 1996).

Since other cytokines play a role in the pathogenesis of sepsis, antagonists to these cytokines have also been developed. Since a natural inhibitor to IL-1 was detected in a patients with arthritis (Prier et al., 1987; Malyak et al., 1993), it has also been studied for its potential therapeutic value. Paris et al. (1995) found that the injection of IL-1 receptor antagonist and sTNFR with LPS did not significantly alter the inflammatory response.

Since endotoxin is implicated in a large proportion of septicemia cases, tremendous interest and effort has been devoted to the development of anti-endotoxin strategies (Burd et al., 1992). This has resulted in clinical trials being conducted on anti-endotoxin monoclonal antibodies namely, the human monoclonal anti-endotoxin HA-1A (Ziegler et al., 1991) and the murine monoclonal anti-endotoxin E-5 (Greenman et al., 1991). Unfortunately these anti-endotoxins 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 Drugs Administration. Criticisms surrounding the studies conducted on these first generation anti-endotoxins have included their inability to bind to LPS in its myriad of forms (Warren et al., 1993), and their inability to block the induction of TNF by LPS (Baumgartner et al., 1990, Cross et al., 1994, Warren et al., 1993). It follows that any research being conducted on new anti-endotoxic 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) (Marra et al., 1990, 1992). BPI is a cationic protein, with weak antibacterial activity, from the granules of human and rabbit neutrophils (Levy et al., 1994). BPI has been shown to be superior to the above monoclonal antibodies in its abilities to bind a variety of LPS molecules, and block LPS induction of TNF \textit{in vitro} and \textit{in vivo}. However BPI has demonstrated mixed results in protection studies (Rogy et al., 1994a, 1994b), and its weak antibacterial activity suggests it may be non optimal. Other LPS binding proteins include CAP-7, CAP-18, CAP-37, and P-15.

The discovery of the importance of the CD14 receptor led to new avenues of research. These include anti-CD14 antibodies and a soluble form of the CD14 receptor. The anti-CD14 antibodies have been shown to inhibit the secretion of TNF by myeloid cells (Haziot et al., 1993). Goyert et al (1996) produced a recombinant form of the soluble receptor (rsCD14) using a Baculovirus expression system and found it was effective in inhibiting the secretion of TNF in murine cells and preventing death and secretion of TNF in a murine endotoxic shock model.

1.6 Aims of this Study

The goal of this thesis was to study a series of cationic $\alpha$-helical peptides and their activity against Gram-Negative bacteria, and their effect on LPS-stimulated macrophages. It was of interest to determine which peptide characteristics affected peptide activity and what makes a good antimicrobial and good anti-endotoxin.
2 METHODS AND MATERIALS

2.1 Bacterial Strains and Growth Conditions

All strains used in this study are listed in Table 1. Most strains were grown on Mueller-Hinton supplemented with 1.5% (w/v) agar. The minimum inhibitory concentration (MIC) assays were also done in this medium. The following strains, *P. aeruginosa* K799 (parent of Z61), *P. aeruginosa* Z61 (antibiotic supersusceptible), *Escherichia coli* UB1005 (parent of DC2), *Escherichia coli* DC2 (antibiotic supersusceptible), *Salmonella typhimurium* 14028s (parent of MS7953s), and *Salmonella typhimurium* MS7953s (defensin supersusceptible), are from our laboratory stock collection and were employed for MIC determinations of the peptides. *E. coli* 0111:B4 (smooth) and *E. coli* J5 (rough mutant of 0111:B4) LPS were purchased from Sigma.

2.2 Design and Production of Peptides

The cationic peptides were synthesized at the University of British Columbia service facility by fmoc [(N-(9-fluorenyl) methoxycarbonyl)] chemistry. The amino acid sequences of the peptides are found in Table 2 using the one letter amino acid code. MBI-27 contains the first eight amino acids of cecropin and the first eighteen amino acids of melittin. MBI-28 has two additional positive charges (lysines) on the C-terminus. MBI-8 and MBI-20 are the result of splitting MBI-28 into an 8 aa and a 20 aa peptide to determine if the carboxy terminus or the N-terminus were responsible for activity. MBI-8 was predicted also give an idea about the role of peptide length.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>UB1005</td>
<td>parent for DC2</td>
<td>Richmond, et al., 1976</td>
</tr>
<tr>
<td>DC2</td>
<td>antibiotic supersusceptible mutant</td>
<td>Richmond, et al., 1976</td>
</tr>
<tr>
<td>ML-35</td>
<td>lactose permease-deficient mutant with constitutive cytoplasmic β-galactosidase</td>
<td>E. Ruby, USC</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K799 (H187)</td>
<td>wildtype isolate; parent for Z61</td>
<td>Angus, et al., 1982</td>
</tr>
<tr>
<td>Z61 (H188)</td>
<td>antibiotic supersusceptible mutant</td>
<td>Angus, et al., 1982</td>
</tr>
<tr>
<td><strong>S. typhimurium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C587 (14028s)</td>
<td>parent of C610</td>
<td>Fields, et al., 1989</td>
</tr>
<tr>
<td>C610 (MS7953s)</td>
<td>phoP / phoQ mutant; defensin sensitive</td>
<td>Fields, et al., 1989</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RN4220 (K147)</td>
<td>methicillin resistant, clinical isolate</td>
<td>Kreiswirth, et al., 1983</td>
</tr>
<tr>
<td>SAP0017</td>
<td>methicillin resistant, clinical isolate</td>
<td>T. Chow, BC</td>
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**Table II:** Peptide Amino Acid Sequences

<table>
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<tr>
<th>Peptide</th>
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<th>Length</th>
<th>Charge</th>
<th>Hydro. (%)</th>
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<td>MBI-28</td>
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<td>+2</td>
<td>70</td>
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<td>MBI-8</td>
<td>KWKLF KKI</td>
<td>8</td>
<td>+4</td>
<td>50</td>
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<td>CM4</td>
<td>KWKLF KKIGIGAVLKVLTTGLPALKT</td>
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<td>+7</td>
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<td>+6</td>
<td>65</td>
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<td>27</td>
<td>+7</td>
<td>63</td>
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<td>CM7</td>
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<td>29</td>
<td>+7</td>
<td>66</td>
</tr>
<tr>
<td>CPα2</td>
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<td>+9</td>
<td>60</td>
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CP26 and CP29 (Karunarate and Hancock PCT patent, 1995) were designed to be more α-helical in nature than MBI-27 and MBI-28 and were designed to conform to an Edmundson helical wheel (to create amphipathic α-helices), placing hydrophilic residues on one side of the peptide and hydrophobic residues on the other side. It was also possible that peptides with decreased activity would be found. This can be instructive since it could inform us more about the importance of certain amino acids, and provide a negative control. Many of the peptides in the CM series I designed are the result of single amino acid changes made to CP26 and MBI-28 changing the position of a charged amino acid or adding more charged residues. The peptides were divided into three series based on similar amino acid sequences. The first series (C1) contains peptides based on MBI-27. The amino acid sequences of these peptides are very conserved. CPα3 varies the most from the other peptides with the loss of several amino acids. The C2 series contains peptides based on CP26 and CP29. The N-terminal sequence, KWKSFIKK, is very conserved among these peptides. The rationale behind these peptides was that this sequence, as found in CP26 and CP29, was important for conferring antimicrobial activity. The peptides in the C3 series contain peptides were designed to conform to an Edmundson helical wheel although MBI-21A9, MBI-21A10, and MBI-21A11 are shorter and not as amphipathic. The MBI-21A series were taken from a patent application (Karunarate and Hancock PCT patent, 1995). The presence of positively charged amino acid (lysine or arginine) at positions 11, 14, 15 and 21 was proposed to be very important for antimicrobial activity since it placed these charge residues on the polar face of the α-
helix. It was hoped that by making conservative changes in the middle residues of such peptides, an enhancement in antimicrobial activity could be achieved.

2.3 Determination of Minimum Inhibitory Concentration Assay

The MIC of each peptide for a range of microorganisms was determined by the broth dilution method (Amsterdam, 1991). Briefly, cells were grown overnight at 37°C in Mueller-Hinton and diluted 1 in 10,000 in the same medium to give concentrations of about 10^4-10^5 colony-forming units/ml. Serial dilutions of each peptide were made in Mueller-Hinton medium in 96-well polypropylene microtitre plates (Costar). Each well was then inoculated with 10μl of the test organism. Samples of the bacterial inoculum were plated to ensure they were within the proper range. The MIC was determined after overnight incubation of the plates at 37°C. The microtitre plates were scored for growth in the wells and the MIC was taken as the lowest peptide concentration at which growth was inhibited.

2.4 Killing Assay

This assay was performed based on a method described previously (Lehrer et al., 1983). Briefly, reactions were carried out in 100 μl volumes and contained 10^6 CFU of E.coli UB1005 or P.aeruginosa H187 in a low strength buffer (10mM potassium phosphate, pH 7.4), and different concentrations of CP26 or MBI-28. After incubation for 5 to 60 minutes at 37°C, samples of the bacteria were removed, diluted and plated to obtain a viable count after overnight growth.
2.5 **Inner Membrane Permeabilization Assay**

Inner membrane permeability was determined by measurement in *E.coli* ML-35 of β-galactosidase activity using o-nitrophenyl-β-D-galactoside (ONPG) as a substrate, as described previously (Falla et al., 1996; Lehrer et al., 1989). Logarithmic phase bacteria were washed in 10 mM sodium phosphate (pH 7.4) and resuspended in 0.75 ml of the same buffer containing 1.5 mM ONPG. At time 0, increasing amounts of CP26 or MBI-28 were added. The production of o-nitrophenyl over time was monitored spectrophotometrically at 420 nm.

2.6 **Determination of Peptide- LPS Binding Affinity**

The relative binding affinity of each peptide for LPS was determined using the assay described previously by Moore et al (1986). The fluorescence was measured in a Perkin-Elmer 650-10S fluorescence spectrophotometer with an excitation wavelength of 340 nm and an emission wavelength of 485 nm using slit widths of 5 nm. A concentration of dansyl polymyxin B giving 90-100% maximum fluorescence (2.5 μm) was chosen and used in the experiments. Dansyl polymyxin B and *E.coli* O111:B4 or J5 LPS (300 μg/mL) were mixed in 1 ml of 5mM HEPES (pH 7.2). This resulted in >90% of maximum fluorescence as measured by the fluorescence spectrophotometer. The decrease in fluorescence due to dansyl polymyxin B displacement was recorded upon the progressive addition of aliquots of 5-10 μl of each of the peptides. Addition of the peptide continued until it resulted in only a small (<5%) decrease in fluorescence. The data were plotted as the % displacement of the dansyl polymyxin B as a function of peptide concentration. The relative affinities of the peptides for LPS were determined by
calculating the $I_{50}$ values directly from the graph. The $I_{50}$ value represented the concentration of peptide that resulted in 50% maximal displacement of dansyl polymyxin B from the LPS.

2.7 Cell Maintenance

The RAW 264.7 macrophage cell line and the L929 cell line (TNF-sensitive mouse fibroblasts) were obtained from ATCC (Rockville, MD). Both cell lines were maintained in DMEM (Dulbecco's modified Eagle medium) complete medium, which is DMEM (GIBCO Laboratories, Grand Island, N.Y.) supplemented 10% heat-inactivated fetal bovine serum, 2.4 mM L-glutamine, 60 U/mL penicillin, and 60 µg/mL streptomycin (GIBCO). The cell lines were incubated at 37°C and 5% CO$_2$ in 175 cm$^2$ cell culture flasks (Costar, Cambridge, MA) and passaged once a week. A flask with a confluent monolayer of RAW macrophage cells was treated with 10 mL cell dissociation medium (Sigma, St. Louis, MO) at 37°C for 10 minutes and L929 fibroblasts were treated with Trypsin-EDTA (GIBCO) at 37°C for 5 minutes to dissociate cells from the wall of flasks. The detached cells were transferred to a 50 mL centrifuge tube containing 30 mL DMEM complete medium and then centrifuged. The supernatant was discarded and the cells were resuspended in DMEM complete medium. Viable cells were counted with trypan blue exclusion using a hemocytometer (American Scientific Products, McGraw Park, IL). A 175 cm$^2$ flask was seeded with $10^6$ viable cells and 45 mL of DMEM complete medium and incubated at 37°C in 5% CO$_2$ for 7 days.
2.8 Assessment of Macrophage Viability

Trypan blue staining and visible adherence of the macrophages were used after the experiments to determine if the peptide had caused visible damage to the cells. The peptides were also tested to determine if by themselves they were stimulating the macrophage cells to produce TNFα. The peptides (20 μg/mL) were incubated with the macrophage cells for 6 hours at which point the supernatant was removed and tested for presence of TNF-α.

2.9 Cytokine Production in RAW 264.7 Macrophages

The RAW macrophage cells were maintained and passaged as described in the above section. TNF induction experiments with LPS were performed as described by Kelly et al (1991). Macrophages were seeded in 24 well plates (Costar) at a density of 10⁶ cells per well in DMEM complete medium and incubated at 37°C in 5 % CO₂ overnight. DMEM was aspirated from RAW 264.7 cells grown overnight and replaced with fresh medium. For experiments testing cytokine production, LPS at a final concentration of 100 ng/ ml, was incubated with the cells for 6 hr at 37°C in 5% CO₂. At the same time as LPS addition, cationic peptides were added at final concentrations of 20 μg/ml. 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.
2.10 Assessment of TNF-α Production by RAW Macrophages

2.10.1 TNF-α Bioassay

TNF was measured in cell culture supernatants on the basis of cytotoxicity for L929 fibroblast cells (Kelly et al., 1991). Periodic controls in which cytotoxicity was neutralized with monoclonal antibodies against TNF-α and TNF-β (Antibodies IP400 and 1221-00; Genzyme Corp., Cambridge, MA) 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. One unit of TNF corresponded to 62.5 pg/ml of recombinant murine TNF (Genzyme Corp.).

2.10.2 TNF-α ELISA

The concentration of TNF-α in the macrophage supernatants was measured with an ELISA (R & D Systems, Minneapolis, MA) kit. The manufacturer’s directions were followed. The samples were diluted 1:10 in calibrator diluent in order to fall into the range of the standard curve. The plates were read immediately following addition of stop solution with a Molecular Devices plate reader at an absorbance of A₅₅₀ minus the reference A₆₉₀.

2.11 Assessment of IL-6 Production by RAW Macrophages

The concentration of IL-6 in the macrophage supernatants was measured with an ELISA (Endogen) kit. The manufacturer’s directions were followed. The samples were diluted 1:5 in culture media (samples with LPS alone were diluted 1:10) in order to fall into the range of the standard curve. The plates were read with a Molecular Devices plate reader at an absorbance of A₅₅₀ – the reference A₆₉₀.
3 RESULTS

3.1 Antimicrobial Activity of the Peptides.

3.1.1 Introduction

The antimicrobial properties of MBI-27 and MBI-28 against Gram-positive and Gram-negative bacteria have been previously documented (Gough et al., 1996). This data showed that both peptides have a broad range of activity at concentrations ranging from 1 to 16 µg/ml. They have similar activities against the different types of bacteria tested, including the life-threatening pathogens commonly associated with septicemia and endotoxic shock. In this chapter the antimicrobial activity of MBI-28 and CP26 (a more amphipathic peptide) was compared with those of the peptide variants. It was of interest to correlate changes in antimicrobial activity to those amino acid changes made to the peptide in order to gain insight into the structure/function relationships of the peptides.

3.1.2 Minimum Inhibitory Concentrations (MIC) of the Peptides.

The peptide variants were tested for their antimicrobial activity against a panel of Gram-negative and Gram-positive bacteria (Materials and Methods, section 2.1; Table I). The results, shown as an average of three experiments, are found in Tables III, IV, and V. CP26 and CP29, which were designed to have increased antimicrobial activity due to their amphipathic nature, had reduced activity against Gram-positive bacteria compared to MBI-27 and MBI-28. Generally none of the peptide variants had higher activity then MBI-28 except CM7 which was equivalent. Many of the peptides did not have useful Gram-positive activities but retained Gram-negative activity. Certain shorter peptides
Table III: MIC (µg/mL) of the Cl Peptide Series

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Ec: Escherichia coli UB1005; Ec': Escherichia coli DC2; Pa: Pseudomonas aeruginosa K799 (or H187); Pa': Pseudomonas aeruginosa Z61 (or H188); St: Salmonella typhimurium 14028s; St': Salmonella typhimurium MS7953s; Sa': Staphylococcus aureus SAP0017; Sa'': Staphylococcus aureus RN4220; and Ef: Enterococcus facealis
Table IV: MIC (µg/mL) of the C2 Peptide Series

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Table V: MIC (μg/mL) of the C3 Peptide Series

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Ec: *Escherichia coli* UB1005; Ec': *Escherichia coli* DC2; Pa: *Pseudomonas aeruginosa* K799 (or H187); Pa': *Pseudomonas aeruginosa* Z61 (or H188); St: *Salmonella typhimurium* 14028s; St': *Salmonella typhimurium* MS7953s; Sa': *Staphylococcus aureus* SAP0017; Sa^2: *Staphylococcus aureus* RN4220; and Ef: *Enterococcus facealis*
MBI-20, MBI-8 (Table III), 19 (Table IV), 13 and 14 (Table V) had lost activity against all the bacteria tested.

The peptides were generally more effective against the antibiotic supersusceptible mutants (*P. aeruginosa* Z61, *E. coli* DC2, and *S. typhimurium* MS7953s) as compared to their parent strains. The *P. aeruginosa* strain Z61 has an outer membrane alteration resulting in increased susceptibility to most antibiotics (Angus et al., 1982). Studies on *E. coli* DC2 have shown that there is decrease in the esterification of the LPS molecules, which makes them more negatively charged than the parent strain (Rocque et al., 1988). An increase in accessible binding sites for the peptides would allow better interaction and explain the increased the susceptibility of the organism to the peptides. The *S. typhimurium* defensin supersusceptible strain has a mutation in the *phoP* / *phoQ* two-component virulence regulon (Fields et al., 1989). The PhoQ protein is a transmembrane sensor kinase that contains an anionic domain predicted to be in the periplasmic region (Miller et al., 1989). Characterization of *phoP* and *phoQ* mutants revealed that defensin supersusceptibility was conferred by the lack of synthesis of PhoP-activated genes (Miller et al., 1990) and was related to LPS alterations (Guo et al., 1997).

In the C1 peptide series none of the peptide variants were better than MBI-28. MBI-8 and MBI-20 had no antimicrobial activity against the bacteria tested. CM4, CM7 and CPc2, peptides with one to four amino acid changes, had similar activity to MBI-28. CM7 differed from MBI-28 only by the addition of leucine to the N-terminus and CM4 had an additional leucine at the C-terminus. CPc2, (more positively charged than MBI-28) had an additional lysine at the C-terminus and an isoleucine and lysine at the N-
terminus. CM6, CM5, and CPα3 all had lost activity against the Gram-positive strains. CM5 is missing the KW sequence at the N-terminus. CM6 is only missing a leucine at the C-terminus but this affected its Gram-positive activity although not Gram-negative activity. CPα3 had two positive charges on both the N- and C-terminus although it is missing 6 amino acids at the C-terminus.

The peptides in the C2 series all contained KSFIK (or a slight variation) at the N-terminus usually after the KW. Peptide 11 differed from peptide 10 by a lysine-serine substitution in the previously mentioned sequence. This change restored some of the Gram-negative activity to peptide 11. This same change did not effect the activity of CM3 which already had good Gram-negative activity. CM1, CM2 and CM3 had very similar sequences to CP26 but different C-termini. This did not effect Gram-negative activity but slightly decreased activity against *S. aureus* RN4220. In the C2 series, none the peptides were significantly better then CP26 although CP29, 16, and 12 had slightly better Gram-positive activity. Peptides 12 and CP29 had the same C-terminus sequence (PALIS) and both had very similar Gram-positive activity that was better than CP26 with a different C-terminus (PLISS). Peptides 10 and 11 (no Gram-positive activity) also had the PALIS the C-terminus but the middle amino acids had been altered. Peptide 19 which was a short and had low charge (+4) had no activity against both Gram-negative and Gram-positive bacteria.

In the C3 series, many of the variant peptides lost all activity against Gram-positive bacteria, except MBI-21A1. MBI-21A1 was also the only peptide with reasonable activity against Gram-negative bacteria. The peptides in this series were the only ones in
this study that had KKWW at the N-terminus which must not preclude Gram-negative activity since 21A1 had reasonable activity. MBI-21A2, which differed from MBI-21A1 by two amino acids (alanine and glutamine), had significantly reduced activity. MBI-21A5, MBI-21A6, and MBI-21A7 had similar amino acid sequences. MBI-21A7 and MBI-21A6 had the same activity against all the bacteria except S. typhimurium in which case MBI-21A7 was better. These peptides only differed by one amino acid; MBI-21A7 had an alanine instead of a lysine near the C-terminus so it was also not as charged.

Alteration of the N-terminus of the parent peptides appeared to have more effect on Gram-negative activity of the peptides than alteration of the C-terminus. Specifically the presence of the K and W amino acids, although the N-terminus alone was not responsible for activity since MBI-8 (contains 8 N-terminus residues of MBI-28) demonstrated no antimicrobial activity against any of the bacteria tested. MBI-20, which did not have the 8 amino acids of the MBI-28 N-terminus also had no activity. CM5 which had the KW removed from the N-terminus had decreased activity against all bacteria tested. Addition of a hydrophobic residue to the KWK sequence at the N-terminus did not affect activity (CM7).

3.1.3 Killing of E. coli and P. aeruginosa by CP26 and MBI-28.

The antimicrobial activity of peptides MBI-28 and CP26, was examined by a killing assay (Lehrer et al., 1983) in which 10^7 CFU of E. coli or P. aeruginosa were incubated with different concentrations of the peptides. After time points from 5 to 60 minutes, samples of the bacteria were diluted and plated to obtain a viable count. The data was plotted as the log of CFU/mL as a function of time (Figures 1, 2, 3 and 4). The
experiments were done three times and the data for one representative experiment is shown for each figure. In Figure 1, the results of CP26 killing of *P. aeruginosa* are shown. CP26 (8 μg/mL) was able to kill 100% of the bacteria (Figure 1). The addition of 1 mM Mg\(^{2+}\) significantly reduced this effect by more than 6 orders of magnitude. In Figure 2, MBI-28 (24 μg/ml) was also shown to completely kill *P. aeruginosa*, although the addition of 1mM Mg\(^{2+}\) had a significant inhibitory effect, decreasing the killing of *P. aeruginosa* by more than 3 log orders. The antimicrobial effect of the peptides was very rapid, occurring within minutes of addition to bacteria. It is interesting that both peptides could completely kill *P. aeruginosa*, but not *E. coli* (Figure 3,4) for which they had a lower minimum inhibitory concentration.

3.1.4 Inner Membrane Permeabilization of *E. coli* by CP26 and MBI-28.

The target of many cationic peptides is proposed to be the cytoplasmic membrane, and the depolarization of this membrane by such peptides leads to the dissolution of the electrical potential gradient (\(\Delta \Psi\)) and results in cell death, presumably through the loss of membrane integrity. This has been demonstrated for a range of cationic peptides including the magainins (Juretic et al., 1989) and defensins (Cociancich et al., 1993). Loss of cytoplasmic membrane integrity has been followed by the unmasking of cytoplasmic β-galactosidase (increased accessibility of a normally impermeable substrate) in *E. coli*, which is the basis for this assay. In Figures 5, 6, and 7, the parent peptides, CP26 and MBI-28 were utilized in this assay to examine the inner membrane permeabilization of *E. coli* by the peptides. The rate of permeabilization by MBI-28, as seen in Figure 5, was dependent on peptide concentration. With increasing peptide
Figure 1: Killing of *P. aeruginosa* H187 by CP26.

*P. aeruginosa* H187 (*10^7* CFU/mL) was incubated with 8 μg/mL (2 x MIC) of CP26 for 30 or 60 minutes before plating the bacteria out for viability counts.
Figure 2: Killing of *P. aeruginosa* H187 by MBI-28.

*P. aeruginosa* H103 (10⁷ CFU/mL) was incubated with 24 μg/mL (8 x MIC) of CP26 for 5 to 60 minutes before plating the bacteria out for viability counts.
**Figure 3**: Killing of *E. coli* UB1005 by CP26.

*E. coli* UB1005 (10^7 CFU/mL) was incubated with 8 µg/mL (6 x MIC) of CP26 for 5 to 60 minutes before plating the bacteria out for viability counts.
Figure 4: Killing of *E.coli* UB1005 by MBI-28.

*E.coli* UB1005 (10⁷ CFU/mL) was incubated with 24 μg/mL (8 x MIC) of CP26 for 5 to 60 minutes before plating the bacteria out for viability counts.
Figure 5: Inner membrane permeabilization of *E. coli* ML-35 by CP26.

Permeabilization was determined by measuring cytoplasmic β-galactosidase activity spectrophotometrically at 420nm as assessed by hydrolysis of the normally impermeable ONPG. *E. coli* (10⁶) were resuspended in 10 mM sodium phosphate buffer, pH 7.5, containing 100 mM NaCl and 1.5 mM substrate. The effects of CP26 at concentrations of 2 µg/mL, 4 µg/mL, 8 µg/mL and 16 µg/mL are presented.
concentration there was decreasing lag time. This was also seen with CP26 in Figure 6. The activity of the peptides against the inner membrane occurred at concentrations as low as 2 μg/ml, although, CP26 had a slower rate of permeabilization than MBI-28 at lower concentrations, especially at 2 μg/ml. In Figure 7, the effects of Mg$^{2+}$ and CCCP (carbonyl cyanide-m-chlorophenyl hydrazone) on inner membrane permeabilization of *E. coli* by CP26 are seen. Pretreatment of bacteria with 100 μM CCCP, which blocks membrane potential, did not significantly reduce permeabilization by the peptide in stark contrast to results observed with the peptide indolicidin (Falla et al, 1996). The addition of Mg$^{2+}$ did significantly reduce the permeabilization of *E. coli* by CP26. This correlates well with the killing assay data in Figure 2, in which Mg$^{2+}$ reduced the killing of *E. coli* by CP26 by several log orders.

### 3.1.5 Binding Affinity of the Peptides for *E. coli* O111:B4 and J5 LPS

To investigate the binding of the peptides to LPS, dansyl polymyxin displacement assays were performed using *E. coli* O111:B4 and J5 LPS. Piers et al. (1994) tested MBI-27 and MBI-28 with *P. aeruginosa* H103 LPS and found their binding affinity (expressed in molar terms) to be similar to polymyxin B. Since *E. coli* O111:B4 and J5, a smooth and rough strain, are commonly used in endotoxin studies, they were also used in the following studies. Dansyl polymyxin B has been shown to bind LPS, resulting in enhanced fluorescence of the dansyl group (Moore et al., 1984). This property led to the development of an assay for determining the LPS-binding affinities of cationic antibiotics (Materials and Methods, section 2.6; Moore et al., 1986). Dansyl polymyxin B was added to a sample of LPS until approximately 90% of the binding sites were occupied as
Figure 6: Inner membrane permeabilization of *E. coli* ML-35 by MBI-28.

Permeabilization was determined by measuring cytoplasmic β-galactosidase activity spectrophotometrically at 420nm as assessed by hydrolysis of the normally impermeable ONPG. *E. coli* ($10^6$) were resuspended in 10 mM sodium phosphate buffer, pH 7.5, containing 100 mM NaCl and 1.5 mM substrate. The effects of MBI-28 at concentrations of 2 µg/mL, 4 µg/mL, 8 µg/mL and 16 µg/mL are presented.
Figure 7: Inhibition by Mg\textsuperscript{2+} of inner membrane permeabilization of *E. coli* ML-35 by CP26.

Permeabilization was determined by measuring cytoplasmic β-galactosidase activity spectrophotometrically at 420nm as assessed by hydrolysis of the normally impermeable ONPG. *E. coli* (10\textsuperscript{6}) were resuspended in 10 mM sodium phosphate buffer, pH 7.5, containing 100 mM NaCl and 1.5 mM substrate. The effects of CP26 at a concentration of 4 μg/mL with 5 mM Mg\textsuperscript{2+} is presented.
indicated by 90% of maximal fluorescence enhancement. Cationic peptides were then titrated in and the displacement of dansyl polymyxin B was monitored by a decrease in fluorescence. The data was plotted as the percent dansyl polymyxin B displacement as a function of compound concentration. Polymyxin B (no dansyl group) is used as a standard to which the peptides are compared. The data is shown as the average of three experiments. The \( I_{50} \) values for the binding affinities of the peptides to \( E. coli \) O111:B4 and J5 LPS are found in Table VI.

The influence of peptide concentration on the displacement of dansyl-polymyxin B binding from \( E. coli \) O111:B4 LPS by C1 series peptides is found in Figure 8. Peptides CM7, CM4 and MBI-28 all had similar high-affinity binding to O111:B4 LPS as shown by the graph and Table VI (\( I_{50} \) values: 12, 14, 11 \( \mu \)g/mL). Since the data in the graph was expressed in \( \mu \)g/mL, and since MBI-28 has a 3 fold higher molecular weight than polymyxin B, these data confirm the conclusions of Piers et al. (1994) that the two compounds have similar affinity for LPS. MBI-27 also bound well but at higher concentrations (\( I_{50}=20 \mu \)g/mL).

The LPS binding of the C2 peptide series is graphed in Figure 9. These peptides showed a large range in ability to displace dansyl polymyxin B. CP29, CM2, CM3 and 16 had the best binding affinities as shown by Figure 9 and Table VI (\( I_{50}:16, 14, 15 \)). These peptides all had good antimicrobial activity against \( E. coli \) bacteria. Peptides 21A14, 10, 11 and 19 had poor binding affinities (\( I_{50}: 50, 40, 32, 30 \)). Peptides, 10 and 11 but not 19 had good antimicrobial activity against Gram-negative bacteria. The data for the C3 series are shown in Figure 10. Data for CP29 was plotted together with the peptides to
Table VI: \(I_{50}\) Values (in \(\mu g/ml\)) for Peptides Against \textit{E. coli} O111:B4 and J5 LPS.

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<th>\textit{E. coli} J5 LPS</th>
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Figure 8: Inhibition of dansyl-polymyxin B (DPX) binding to *E. coli* O111:B4 LPS by C1 series peptides.

Peptides were titrated into cuvettes containing LPS that had DPX bound to it. Displacement of the DPX from the LPS was measured as a decrease in fluorescence.
Figure 9: Inhibition of dansyl-polymyxin B (DPX) binding to *E. coli* O111:B4 LPS by C2 peptides.

Peptides were titrated into cuvettes containing LPS that had DPX bound to it. Displacement of the DPX from the LPS was measured as a decrease in fluorescence.
Figure 10: Inhibition of dansyl-polymyxin B (DPX) binding to *E. coli* O111:B4 LPS by C3 peptides.

Peptides were titrated into cuvettes containing LPS that had DPX bound to it. Displacement of the DPX from the LPS was measured as a decrease in fluorescence.
aid in comparison with the other peptide series. CP29 had a much lower binding affinity than any of the C3 peptides. Many of the peptides (21A9, 21A10, 21A11, 13, 14, and 15) had similar binding affinities for O111:B4 LPS as shown by the graph and the I\textsubscript{50} values (Table VI). MBI-21A6 and MBI-21A7 had slightly lower affinities and MBI-21A5 had a very low binding affinity (I\textsubscript{50} = 52 \textmu g, Table VI) for \textit{E. coli} O111:B4 LPS.

The inhibition of dansyl-polymyxin B binding to rough \textit{E. coli} J5 LPS by C1 peptides is shown in Figure 11. MBI-28, CM4 and CM7 had high binding affinities for \textit{E. coli} J5 LPS as seen with \textit{E. coli} O111:B4 LPS. The I\textsubscript{50} values in Table VI demonstrated that the C1 peptides had the same binding affinities to O111:B4 and J5 LPS (with the exception of CM5). CM5 had poor binding affinity as seen in the figure and in Table VI (I\textsubscript{50}=62\textmu g).

3.1.6 Summary

The peptides varied greatly in their abilities to kill Gram-negative and Gram-positive bacteria. Certain alterations (eg. removal of KW) at the N-terminus of MBI-28 had a negative effect on antimicrobial activity. None of the peptide variants had significantly better antimicrobial activity than MBI-28 or CP26. An effect of peptide charge on antibacterial activity was seen by comparing the MIC against \textit{E. coli} of peptides with their charges. Peptides with a net positive charge over 7 had better activity than those with a lower charge. CP26 and MBI-28 were able to kill all \textit{P. aeruginosa} but not \textit{E. coli} in a killing assay. This killing was partially inhibited by Mg\textsuperscript{2+}. These peptides also showed concentration dependent permeabilization of the inner membrane of \textit{E. coli} that
**Figure 11:** Inhibition of dansyl-polymyxin B (DPX) binding to *E. coli* J5 LPS by C1 peptides.

Peptides were titrated into cuvettes containing LPS that had DPX bound to it. Displacement of the DPX from the LPS was measured as a decrease in fluorescence.
was inhibited by the addition of Mg$$^{2+}$$. The studied peptides showed a large variation in binding affinities to \textit{E.coli} O111:B4 and J5 LPS. The C1 peptides had the same binding affinities for \textit{E. coli} J5 and O111:B4 LPS suggesting that LPS side chains do not play a significant role in peptide-LPS binding. Peptides with good binding affinities did not all have good antimicrobial activity (eg. 17). Peptides with relatively low binding affinities ($I_{50}>30\mu g$) to O111:B4 LPS had poor antimicrobial activity (ie. CM5, 19, 21A14, 10, 11, 21A5, 13 and 14).
3.2 Anti-Endotoxin Activity of the Peptides

3.2.1 Introduction

The peptides, MBI-27 and MBI-28, have previously demonstrated endotoxin neutralizing ability in macrophage cells and a murine model of endotoxic shock (Gough et al., 1996). MBI-28 was shown to reduce levels of circulating TNF by over 90% and prevent mortality (by 80%) in a murine model of endotoxic shock (Gough et al., 1996). In this chapter, the peptide variants were tested for their ability to neutralize the effects of *E. coli* LPS on murine macrophage cell lines. Macrophages and macrophage cell lines produce cytokines in response to LPS stimuli, namely, TNF and IL-6. This chapter focuses on the peptides' ability to block production of these cytokines in response to an LPS stimuli.

3.2.2 Peptide Effect on Macrophages

The macrophage cells were examined visibly for signs of lifting off the wells and with trypan blue staining to determine if the peptides were having a detrimental affect on the macrophages. There was no significant change in the wells with macrophages incubated for 6 hours with medium alone or with 20 μg/mL of the peptides.

To demonstrate that the peptides were not by themselves stimulating TNF secretion by the macrophage cells, 20 μg/mL of peptide was incubated with the RAW macrophage cells for 6 hours. The levels of TNF were measured by the L929 cell cytotoxicity assay. The peptides on their own resulted in production of only 12 – 21 U/mL of TNF. These values were not significantly higher than with medium alone (14 ± 4 U/mL). To show that these macrophage cells were capable of producing TNF, a control was run with 100
ng of \textit{E. coli} O111:B4 LPS. After a 6 hour incubation with macrophage cells, LPS induced the cells to secrete an average of $4697 \pm 481$ U/mL of TNF.

3.2.3 Peptide Effect on TNF-\(\alpha\) Production

Control experiments indicated that the cationic peptides were non-toxic for this macrophage cell line at the highest concentrations employed in these experiments (Section 3.2.2). The TNF produced by the macrophage cells was measured in two ways, ELISA and L929 cell cytotoxic assay. The ELISA assay measured all TNF-\(\alpha\) found in the supernatants tested, whereas the L929 cytotoxic assay measured only bio-active TNF-\(\alpha\) or TNF that was toxic to the L929 TNF-sensitive fibroblast cells.

The effect of the different peptides on \textit{E. coli} O111:B4 LPS stimulated RAW 264.7 macrophage cells as measured by ELISA and L cell assay was compared. The peptides varied greatly in their ability to inhibit the induction of TNF secretion by macrophage cells. The data in these graphs is presented as the amount of TNF produced by the peptide + LPS divided by the amount of TNF produced by LPS alone multiplied by 100 (\% TNF). Figure 12, demonstrates the effect of the C1 series peptides on the rough mutant of O111:B4, \textit{E. coli} J5 LPS. All of these peptides inhibited greater then 60\% of the TNF induced by LPS. The results presented in Figure 13 include peptides from the C2 and C3 series and their effects on \textit{E. coli} J5 LPS-stimulated macrophages.

ELISA and L cell assay results for the effects of C1 series peptides on O111:B4 LPS-induced TNF production in macrophages are shown in Figure 14. The L cell assay results in this Figure demonstrated a similar pattern to that seen in Figure 12 for J5 LPS, with the
Figure 12: Effect of C1 series peptides on % TNF produced by E.coli J5 LPS-stimulated macrophages as measured by ELISA.

RAW macrophages were stimulated with 100 ng of *E.coli* J5 LPS and 20 µg/mL peptide for 6 hours. The supernatant was then tested for TNF by ELISA (solid bars). The data shown is the average of three experiments.
Figure 13: Effect of C2 and C3 peptides on % TNF produced by *E.coli* J5 LPS-stimulated macrophages as measured by ELISA.

RAW macrophages were stimulated with 100 ng of *E.coli* J5 LPS and 20 µg/mL peptide for 6 hours. The supernatant was then tested for TNF by ELISA (solid bars). The data shown is the average of three experiments.
exception of CM7 which showed a lower inhibition of TNF induced by J5 LPS. The results from the ELISA method in Figure 14, demonstrated that the inhibition of LPS-induced TNF production by the peptides was consistently lower than when measured by the L cell assay (with the exception of CM7). This seems reasonable since ELISA is measuring all of the TNF-α, bio-active or not. The results with the less active peptides (MBI-8, MBI-20, CM5, CM6, and CPα3) showed a larger variability between assays. For example, CPα3 inhibited LPS-stimulated TNF production by 15% as assessed by ELISA and 63 % according to the L cell assay. None of the peptide variants were better than the parent peptides, MBI-27 and MBI-28, although CM4, CM7 and CPα2 had similar activity. The active peptides (MBI-27, MBI-28, CM4, CM7 and CPα2) were similar to polymyxin B (PMB) in their ability to reduce LPS-stimulated production of TNF.

The effects of the C2 series peptides on O111:B4 LPS stimulated TNF produced by macrophages, as measured by ELISA and L cell assay, are presented in Figure 15. The peptides were generally more effective at inhibiting TNF production by O111:B4 LPS stimulated macrophages compared to J5 LPS stimulated cells. Peptides 12 and 26 had very good activity, resembling that of their parent peptides, CP26 and CP29. There was a large variance, in TNF production as measured by ELISA and the L cell assay, for peptides, 17, 18, 19 and somewhat for peptides 10 and 11.

It is interesting to note that with the C3 series of peptides, 21A5 inhibited TNF production by up to 90% and yet had very poor LPS binding affinity to O111:B4 LPS (Figure 10). Conversely, peptides 13 and 14 had a very small inhibitory effect on TNF production (both about 20%). These peptides had no antimicrobial activity and had a
Figure 14: Effect of C1 series peptides on % TNF produced by *E.coli* O111:B4 LPS-stimulated macrophages as measured by ELISA and L cell assay.

RAW macrophages were stimulated with 100 ng of *E.coli* O111:B4 LPS and 20 µg/mL peptide for 6 hours. The supernatant was then tested for TNF by ELISA (open bars) and the L cell assay (solid bars). The data presented above is the average of three experiments.
Figure 15: Effect of C2 series peptides on % TNF produced by *E. coli* O111:B4 LPS-stimulated macrophages as measured by ELISA and L cell assay.

RAW macrophages were stimulated with 100 ng of *E. coli* O111:B4 LPS and 20 μg/mL peptide for 6 hours. The supernatant was then tested for TNF by ELISA (open bars) and the L cell assay (solid bars). The data presented above is the average of three experiments.
Figure 16: Effect of C3 series peptides on % TNF produced by *E. coli* O111:B4 LPS-stimulated macrophages as measured by ELISA and L cell assay.

RAW macrophages were stimulated with 100 ng of *E. coli* O111:B4 LPS and 20 μg/mL peptide for 6 hours. The supernatant was then tested for TNF by ELISA (open bars) and L cell assay (solid bars). The data presented above is the average of three experiments.
low binding affinity for O111:B4 LPS (displace about 30% of dansyl polymyxin B, \( I_{50} \): 32,31).

3.2.4 Peptide Effect on IL-6 Production

The effect of the peptides on production of IL-6 by LPS-stimulated macrophages was examined using an ELISA assay. The effect of the cationic peptides on *E. coli* O111:B4 LPS-induced IL-6 production in the macrophages is shown in Figures 17, 18 and 19. The results are shown as % IL-6 produced which represents the amount of IL-6 produced in the presence of peptide divided by the amount of IL-6 with LPS alone multiplied by 100.

The peptides in the C1 series had large variances in their ability to inhibit the macrophage production of IL-6 in the presence of LPS (Figure 17). In this series, CM4, CM7, and CPα2 (88%, 95%, and 94 % inhibition) were all better than their parent peptides MBI-27 (76% inhibition) and MBI-28 (82% inhibition). MBI-8, MBI-20, and CPα3 had little activity in that the IL-6 production by the macrophages was not much different than with LPS alone. These results correlated well with the effect of peptides on TNF production. MBI-8, MBI-20, and CPα3 were also the least effective in inhibiting TNF production in the presence of LPS.

In the C2 series (Figure 18), CP29, 12 and 16 had the largest effect on IL-6 production (% inhibition). CP26, CM1, CM2 and CM3 were also very effective with only 5-9% production of IL-6. Most peptides (10, 17, 18 and 19) had little effect (about 10-20%). These results demonstrate that small amino acid changes can have a large effect. For
Figure 17: Effect of C1 series peptides on % IL-6 produced by E.coli O111:B4 LPS-stimulated macrophages as measured by ELISA.

RAW macrophages were stimulated with 100 ng of E.coli O111:B4 LPS and 20 μg/mL peptide for 6 hours. The supernatant was then tested for IL-6 by ELISA (solid bars). The data presented above is the average of three experiments.
Figure 18: Effect of C2 series peptides on % IL-6 produced by *E. coli* O111:B4 LPS-stimulated macrophages as measured by ELISA.

RAW macrophages were stimulated with 100 ng of *E. coli* O111:B4 LPS and 20 μg/mL peptide for 6 hours. The supernatant was then tested for IL-6 by ELISA (solid bars). The data presented above is the average of three experiments.
**Figure 19:** Effect of C3 series peptides on % IL-6 produced by *E. coli* O111:B4 LPS-stimulated macrophages as measured by ELISA.

RAW macrophages were stimulated with 100 ng of *E. coli* O111:B4 LPS and 20 μg/mL peptide for 6 hours. The supernatant was then tested for IL-6 by ELISA (solid bars). The data presented above is the average of three experiments.
example, peptide 16 was very active in inhibiting LPS-stimulated production of IL-6 by 97% but peptide 17 had lost all activity (0% inhibition). Only the tryptophan at the N terminus had been removed from peptide 17 compared to peptide 16. The results of the effect of the C3 series of peptides on IL-6 production is shown in Figure 19. Again a similar pattern to that seen with TNF production was observed. Peptides, 21A9, 13, 14 and 15 had the least effect on LPS-stimulated macrophages. The peptides 21A2, 21A5, 21A6 and 21A7 all had similar activity to CP29. The activity of MBI-21A5 was interesting since it had little antimicrobial activity (Table V) and a low LPS binding affinity (Figure 10). It did however demonstrate up to 90% inhibition of TNF produced by O111:B4 LPS-stimulated macrophages (Figure 16). The data for this peptide suggests that factors additional to LPS binding are important in the blockage of cytokine production.

3.2.5 Summary
The peptides were found to be non-toxic to the macrophage cells under the conditions tested. The peptides were found to inhibit LPS-stimulated TNF production as measured by the L cell assay (bio-active TNF) to a larger extent than that tested by ELISA, although the trends were generally the same. The peptide effect on IL-6 production was very similar to that seen with the TNF ELISA data. Peptides that were effective in blocking TNF production also blocked IL-6 production suggesting the possibility of a similar mechanism of action. The peptides (MBI-28, CM4, CM7, CPα2, CP26, CP29, 12, and 16) that were good antimicrobials and had high binding affinity for E.coli LPS also were able to inhibit production of IL-6 and TNF by LPS-stimulated macrophages.
Some of the peptides (13, 14, 15, 21A14, and 19) that had no antimicrobial activity and higher LPS binding affinities were not as effective in preventing production of cytokines although this was not always the case. It appears that although the best peptides have good antimicrobial, anti-endotoxin and LPS binding affinity, these factors are not always linked.
Cationic peptides have become well known for their broad range antimicrobial activity and more recently for anti-endotoxin activity. Both properties are important for the treatment of endotoxic shock. The cationic peptides used in this study are based on the cecropin/melittin hybrid, MBI-27, and its C-terminally-modified derivative MBI-28. MBI-27 and MBI-28 have previously been shown to bind to LPS (Piers et al., 1994) and prevent its ability to induce a TNF response using both a macrophage tissue culture cell line and galactosamine-sensitized mice (Gough et al., 1996). These results led to this study on the efficacy of a series of related cationic peptides. The purpose of this study was to look at how amino acid changes to the peptides affected their activity. The amino acid changes made to the peptides affected their charge, length, and hydrophobicity. CP26 and CP29 were designed to be more amphipathic and therefore to have better activity than MBI-27 and MBI-28. From these peptides, a series of variants was created.

Many of the peptides studied here exhibited antibacterial activity against a wide variety of bacteria. The peptides were generally more effective against Gram-negative bacteria than Gram-positive bacteria. The peptides were most effective against the *E. coli* with the exception of 13, 14, and 19 which had no activity against any of the bacteria tested (MICs > 64 μg/mL) and MBI-20 and MBI-8 which were effective only against the antibiotic supersusceptible strain *E. coli* DC2. There was no correlation (*E. coli*: r=0.21, *P. aeruginosa*: r=0.37, *S. aureus*: r=0.41, *S. typhimurium* r=0.39 by linear regression analysis) found between hydrophobicity of the peptides and antimicrobial activity when the MIC results of the peptides were plotted against the % hydrophobicity of the peptides.
There was however some correlation with peptide charge and antimicrobial activity against *E. coli* (r=0.6 by linear regression), *P. aeruginosa* (r=0.65), and *S. typhimurium* (r=0.71). Similar results were seen with peptide length. The 8 amino acid peptide, MBI-8, as mentioned previously was only active against a supersusceptible strain of *E. coli* suggesting that the peptides probably have to be above a certain length to be effective. Other short peptides that had no significant antimicrobial activity were 18, 19, 21A9, 21A10 and 21A14. There were also correlations found between the MICs of the peptides against different bacteria, for example, *S. typhimurium* and *P. aeruginosa* (r=0.88) suggesting that the peptides have a similar mechanism of interaction with these bacteria. Interestingly, a correlation (r=0.88) was also seen between the MIC results of the peptides against *S. aureus* and *P. aeruginosa*.

MBI-28 was found to be the best peptide for broad spectrum antimicrobial activity. Although peptides with minor amino acid changes from MBI-28 (CM4, CM7, and CPα2) still had good Gram-negative activity, CM4 and CPα2 had decreased Gram-positive activity. The changes made to the C-terminus in CM4 and CPα2 decreased Gram-positive activity. CM4 only had an additional leucine in the carboxy terminus and CPα2 had 2 additional lysines, one in the N-terminus and one in the C-terminus. It is possible that the C-terminus is important in the action of the peptides on Gram-positive bacteria and it is sensitive to small changes as there was no correlation (r=0.44) seen between overall peptide charge and MICs of the peptides against *S. aureus*. There was also no correlation found between hydrophobicity (r=0.41) and Gram-positive activity. There may be some correlation (r=0.56) between peptide length and Gram-positive activity. As
with the peptide action on Gram-negative bacteria these peptides probably need a certain length to form the proper structure and be effective.

Peptides 12 and 16, variants of CP29 and CP26 (12 has the first 19 amino acids of CP26 and the last 5 amino acids of CP29) had similar activity to their parent peptides but certainly this combination did not improve activity. Peptides in this series (CM1, CM2, and CM3) also demonstrated that changes to the C-terminus can cause a loss of Gram-positive activity. The changes made to 13, 14, and 19 diminished all antimicrobial activity of the peptides. The N-terminus of peptides 13 and 14 had a KKWW motif but this could not account for loss of activity since it did not diminish activity of MBI-21A1 and MBI-21A2 which also had this motif. Peptides 13 and 14 were the only peptides to have had a QTLAQ motif at the C-terminus which could be detrimental to antimicrobial activity although their low charge and short length could also be factors. This could also be the case with peptide 19 which had only 18 amino acids and a low charge (+4).

The MBI-21A peptides of the C3 series had little antimicrobial activity against Gram-negative and Gram-positive bacteria with the exception of MBI-21A1 which was active against Gram-negative bacteria. Peptides MBI-21A1 and MBI-21A2 differ by only two amino acids at positions 7 and 9 but the latter had reduced antimicrobial activity. Interestingly, valine had been substituted for alanine at position 7 (both hydrophobic residues) and at position 9, serine had been replaced with glutamine (both hydrophilic residues) in MBI-21A2. Overall these two peptides had the same charge, length and hydrophobicity. These peptides have SVLK at the carboxy terminus and have greater
activity than MBI-21A5 - MBI-21A11 which shared a SNV or SNIV amino acid sequence near the carboxy terminus.

The killing assays done with MBI-28 and CP26 shed some light on the time frame required for the peptides to kill *E. coli* and *P. aeruginosa*. It is interesting that the both peptides were able to kill all the *P. aeruginosa* but not quite all of the *E. coli* despite the fact that the MIC for both peptides was lower with *E. coli*. The majority of the killing occurred done within the first 5 minutes in both cases. Mg$^{2+}$ had an inhibitory effect on bacterial killing by the peptides. This was also seen with the inner membrane permeabilization assays. However the influence of Mg$^{2+}$ on outer membrane uptake would be the most likely explanation for this effect.

Inner membrane permeabilization of *E. coli* by CP26 and MBI-28, as assayed by measuring access to cytoplasmic β-galactosidase, occurred with minimal lag (cf Indolicidin; Falla et al., 1996). Permeabilization by CP26 was inhibited by the addition of Mg$^{2+}$ as seen with the killing assay. The addition of the uncoupler CCCP did not have an effect on permeabilization as seen with indolicidin (Falla et al., 1996) suggesting that these peptides have different mechanisms of action. Indolicidin has also been proposed to cross the outer membrane via the self-promoted uptake pathway but structurally it is very different from the α-helical peptides in this study. The maximal rate of permeabilization was faster than that observed for the cationic defensins HNP-1 to HNP-3 (Lehrer et al., 1989), the bactenecins Bac5 and Bac7 (Skerlavaj et al., 1990) and indolicidin (Falla et al., 1996). Results from both the killing assay and the inner
membrane permeabilization assay demonstrate the action of the α-helical peptides on Gram-negative bacteria is very fast.

The self-promoted uptake model was proposed as the mechanism of uptake across the outer membrane for the cecropin/melittin hybrid, MBI-27 (Piers et al., 1994). MBI-27 is proposed to interact with the negatively charged sites on LPS molecules that are normally occupied by Mg\(^{2+}\) ions. These Mg\(^{2+}\) ions form stabilizing cross bridges between adjacent LPS molecules. The LPS binding capabilities of the peptides were compared using dansyl polymyxin B as a probe. The observations that many of the peptides that had lost LPS binding capability also had decreased antimicrobial activity and that Mg\(^{2+}\) inhibits antimicrobial activity would support the self-promoted uptake pathway as the mechanism of action. There was some correlation \((r=0.69)\) seen between the MICs of the peptides against \(P. aeruginosa\) and peptide : LPS binding affinity affinities when MIC results were plotted against \(I_{50}\) values. However, there was no clear correlation \((r=0.18)\) between antimicrobial activity of the peptides against \(E. coli\) and their LPS binding. It should be noted that strain of \(E. coli\) used for MICs is different from that strain which the LPS was obtained. Interestingly there was some correlation \((r=0.68)\) with \(S. aureus\) MIC results and LPS binding affinity of the peptides. As of yet there is no proposed mechanism of action on Gram-positive bacteria which do not have LPS molecules as part of their structure.

The peptides were found to be non-toxic to macrophage cells as judged by trypan blue staining. When the peptides were incubated alone with the macrophage cells they induced very low levels of TNF, similar to that seen with media alone, suggesting that the
peptides by themselves did not induce TNF. In previous studies intraperitoneal injection of MBI-27 and MBI-28 (at therapeutic concentrations) into mice did not cause any visible signs of distress or cause abnormal secretion of TNF (Gough et al., 1996).

In studies done with CP26 and MBI-28, the peptides were able to inhibit TNF secretion even when added 30 or 60 minutes after LPS (Gough et al., 1996 and unpublished data). It is known that LPS is internalized in macrophage cells very quickly (Lynn et al, 1998), so that CP26 and MBI-28 were probably not only binding to LPS and preventing interaction with the macrophage cells but also effecting the macrophage cells’ ability to secrete TNF. The peptides could also bind to any LPS that is recycled and prevent repeated secretion of TNF. There is always the possibility that the peptides interfered at the cell membrane level. There is not a lot known about how these peptides affect eukaryotic cells. In preliminary experiments, it was found that the peptides precipitate DNA most likely due to their cationic nature, but whether the peptides are getting inside the macrophage cells and having an effect on cell functioning is unknown.

The production of TNF by LPS-stimulated macrophages as measured by ELISA and the L cell assay demonstrated similar patterns of peptide activity. There was a fairly good correlation (r=0.74) when the ELISA data was plotted against the L cell assay data. The ELISA results were generally higher than the L cell assay results, most likely due to ELISA measuring all the TNF in the supernatant and the L cell assay only measuring bio-active TNF. This implies that not all the TNF produced by the macrophages is bio-active. There were a few peptides that had much higher levels of TNF inhibition when measured by the L cell assay (MBI-8, MBI-20, 17, 18, 19 and 15). When E. coli J5 rough LPS was
used to stimulate the macrophage cell line, the peptides had a similar pattern of activity to that seen with O111:B4 smooth LPS, although there was no correlation seen when the ELISA and L cell TNF results were plotted against TNF produced by J5 stimulated macrophages. It seems unclear whether the O-polysaccharide side chain missing from *E. coli* J5 affects interaction with peptides and macrophage cells although it did not affect peptide-LPS binding.

When plotting the TNF results against MIC results, a higher correlation was seen with the ELISA data (r=0.77) than with the L cell results (r=0.56). The MIC results for *P. aeruginosa* (r=0.77) and *S. typhimurium* (r=0.72) had the highest correlation to the results of TNF ELISA experiments. A correlation was also seen with TNF L cell results. The lower correlation (r=0.65) seen with *E.coli* strain UB1005 suggested that this strain does not yield MIC data that is compatible with the TNF experiments and future experiments should use the *E. coli* O111:B4 strain for MICs.

In each of the peptides series, there was a large range of antimicrobial and anti-endotoxin activity. The ability of the peptides to inhibit TNF production ranged from 0% to 99%. Addition of a leucine to the N- or C-terminus did not significantly alter activity as seen with CM1 and CM4. The KWK sequence of the N-terminus appeared to be important for antimicrobial, anti-endotoxin and LPS binding affinity as seen with the weakly active peptides, CM5 (no KW) and peptide 17 (no W). Other studies have found that the KWKLFKK sequence from cecropin and the VLKVL from melittin are important for treating bacterial keratitis (Nos-Barbera et al., 1997). The N-terminal sequence arrangement KKWW did not significantly reduce activity. It appears that although the
presence of lysine and tryptophan at the N-terminus is necessary for activity, the
arrangement of the amino acids can vary. Another example of this is provided by MBI-
21A14, which lacked this sequence and had reduced antimicrobial activity.

The peptides that were effective in blocking LPS-stimulated production of TNF were also
effective in blocking IL-6 production. Peptide inhibition of IL-6 production ranged from
98% to 2% demonstrating that the peptides affected the secretion of more than just one
mediator of sepsis. There was a very strong correlation \( (r=0.94) \) between IL-6 activity and
TNF ELISA activity suggesting the possibility of a general mechanism of action. There
was some correlation seen between peptide length and TNF-suppressing activity \( (r=0.67) \)
and IL-6-suppressing activity \( (r=0.62) \), but not with peptide charge or hydrophobicity.
Interestingly there was no correlation seen with LPS binding affinity and TNF or IL-6-
suppressing activity, although the most active peptides (MBI28, CM7, CP26, CP29, and
16) had good antimicrobial and anti-endotoxin activity, as well as LPS binding affinity.
There were also some peptides that had good binding affinities for LPS but were not as
active at preventing the secretion of TNF or IL-6. For example, CM6 had the same
binding affinity as CP26 but it only suppressed TNF by 66% and IL-6 by 47%, while
CP26 inhibited TNF by 81% and IL-6 by 90%. It appears there were other factors
besides LPS binding that contributed to the peptides’ ability to be a good anti-endotoxin.
This suggests that the peptides do more than interact with the LPS to prevent binding to
macrophage cells. There are several important factors involved in the activity of the
peptides that should be taken into account including the 3D structure of the peptide, the
positioning of charges and hydrophobic residues, and also the peptide’s ability to form α-helices.

Based on comparison of the results reported here with those in the literature, there is cause for cautious optimism regarding the prospects for these small cationic peptides as effective agents for treating endotoxic shock. For example, polymyxin B, a small cyclic cationic peptide with a fatty acyl tail, is considered the most potent anti-endotoxin agent identified to date, both in terms of its affinity for binding to the lipid A portion of LPS and its ability to interfere with the biological activities of LPS. As such it is frequently used as the standard against which novel anti-endotoxin compounds are measured, although its toxicity precludes its use in systemic therapy of humans. A less toxic version of polymyxin B, polymyxin B nonapeptide (lacking the fatty acyl tail), was found to be less effective at inhibiting LPS (Danner et al., 1989). Another less toxic version of polymyxin B (polymyxin B conjugated with dextran 70) was found to still have antibacterial and anti-endotoxin activity (Buklin et al., 1995). In comparison to polymyxin B, MBI-27 and MBI-28 showed similar binding affinity for P. aeruginosa H103 LPS (Piers et al., 1994) and also E. coli O111:B4 and J5 LPS (when looking at μM ratios). Many of the peptide variants including MBI-28 and CP26 had similar abilities to block induction of TNF and IL-6 in macrophage cell lines. In contrast, BPI (neutrophil granule protein) at 0.83 μM resulted in only 50% inhibition in TNF production in response to LPS in one study (Rogy et al., 1994), whereas 10 μg/ml of polymyxin B reduced TNF induction by 90%. More recent studies with a recombinant N-terminal fragment of BPI to have demonstrated that is has better anti-endotoxin abilities than the
whole protein (Kohn et al., 1993). However, the recombinant BPI has a short half-life and requires continuous infusion. This problem was solved by a chimeric construct of 21 amino acids of the N-terminus of BPI fused to the Fc portion of human IgG. In human volunteers rBPI\textsubscript{23} abolished the physiological response to endotoxin challenge and Phase II/III clinical trials of rBPI\textsubscript{23} are in progress including a multi-national placebo-controlled trial in meningococcaemia (Lynn et al., 1998).

It is significant that the some of the cationic peptides discussed here are antibacterial in nature. Although they lack the potency of many of the recent β-lactams and quinolones against the most susceptible organisms, they do have certain potential advantages. First both β-lactams (Prins et al., 1995 and Shenep et al., 1985) and quinolones (Cohen et al., 1985 and Shenep et al., 1985) are known to promote endotoxin release and hence there is a risk of endotoxaemia. In contrast, the cationic peptides actually block endotoxaemia. MBI-28 has been shown to protect neutropenic mice against \textit{P.aeruginosa} given via the intraperitoneal route, and this may in part reflect endotoxin neutralization \textit{in vivo}. A second potential asset lies in the enhancer activity of cationic peptides, whereby the peptides demonstrate synergy or additive activities with conventional antibiotics (Hancock et al., 1995). 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.
REFERENCES


