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Cutting Edge: Cationic Antimicrobial Peptides Block the Binding of Lipopolysaccharide (LPS) to LPS Binding Protein

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We investigated the mechanism by which cationic antimicrobial peptides block the activation of macrophages by LPS. The initial step in LPS signaling is the transfer of LPS to CD14 by LPS binding protein (LBP). Because many cationic antimicrobial peptides bind LPS, we asked whether these peptides block the binding of LPS to LBP. Using an assay that measures the binding of LPS to immobilized LBP, we show for the first time that a variety of structurally diverse cationic antimicrobial peptides block the interaction of LPS with LBP. The relative ability of different cationic peptides to block the binding of LPS to LBP correlated with their ability to block LPS-induced TNF-α production by the RAW 264.7 macrophage cell line. The Journal of Immunology, 2000, 164: 549–553.

Inflammation and sepsis caused by Gram-negative bacteria are due primarily to LPS that is released from the bacterial outer membrane. Treatment of Gram-negative bacterial infections would be greatly aided by substances that can effectively block LPS-induced production of inflammatory mediators. Cationic antimicrobial peptides are an evolutionarily ancient component of the innate immune system that block many of the actions of LPS (1). In mammals, these peptides are found in the blood, in secretions, and in neutrophil granules. In vitro, naturally occurring cationic peptides, as well as synthetic analogues block the ability of LPS to stimulate the production of TNF-α, IL-6, and other inflammatory mediators (2–9). Moreover, studies in mice have shown that cationic peptides can block endotoxin-induced TNF-α release and reduce the mortality associated with endotoxia in the galactosamine-sensitized mouse model (2, 4, 6, 9). Although several cationic peptides have been shown to bind LPS (2, 7, 10), little is known about how they block the biological effects of LPS.

The mechanism by which LPS activates macrophages is now understood in some detail. LPS binding protein (LBP), an acute-phase reactant that is present in the blood, binds LPS, extracts it from micelles, and transfers it to CD14, a protein that exists as a soluble form in blood and as a GPI-linked molecule on the surface of monocytes and macrophages. LPS-CD14 complexes are thought to initiate intracellular signaling reactions by binding to Toll-like receptors (TLRs) on macrophages and other cells (11). TLR4 appears to be required for LPS to initiate signaling and to induce inflammatory responses. Inbred strains of mice with loss-of-function mutations in TLR4 do not respond to LPS (12, 13). LPS-CD14 complexes activate the NF-κB transcription factor as well as the extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinases, all of which mediate the production of inflammatory cytokines (11, 14–16). By facilitating the initial step in LPS signaling, the binding of LPS to CD14, LBP greatly enhances the efficiency of LPS signaling such that 100-fold less LPS is required to trigger cytokine secretion (17).

Because cationic antimicrobial peptides that block LPS-induced macrophage activation can bind to LPS (2, 4, 5, 10, 18–20), we hypothesized that these cationic peptides act by blocking the binding of LPS to LBP. To test this, we used an ELISA-type assay that measures the ability of biotinylated LPS to bind to immobilized LBP. Using this assay, we analyzed the ability of structurally diverse cationic peptides to block the binding of LPS to LBP. We show that peptides belonging to all of the major structural groups of antimicrobial peptides can block the binding of LPS to LBP in the assay. In general, the relative ability of the different peptides to block the binding of LPS to LBP in vitro correlated with their ability to block LPS-induced production of TNF-α by RAW 264.7 cells. Thus, the ability of cationic peptides to block macrophage activation by LPS may be due in large part to their ability to block the binding of LPS to LBP and prevent the LBP-mediated transfer of LPS to CD14.
Materials and Methods

Reagents

_Escherichia coli_ O55:B5 LPS was purchased from Sigma (St. Louis, MO) and biotinylated using biotin-LC-hydrazide (Pierce, Rockford, IL) as described previously (21). CEMA, CP29, CP208, bacterecin (Bac) 2A-NH₂, indolicidin, and CP11CN were synthesized at the Nucleic Acid/Protein Synthesis Unit at the University of British Columbia as described previously (7). Gramicidin S and human neutrophil peptide-1 (HNP-1) were purchased from Sigma. Human β-defensin-2 (HBD-2) was kindly provided by Dr. T. Ganz (University of California, Los Angeles, CA). The amino acid sequences of these peptides are shown in Table I.

Purification of recombinant LBP

Chinese hamster ovary cells expressing human recombinant LBP were a gift from Dr. P. Tobias. The recombinant LBP was purified from the culture medium as described previously (21) and its concentration determined by ELISA (22).

Measurement of LPS-LBP interactions

The anti-LBP mAb HM14 (21), which recognizes human LBP as well as LBP-LPS complexes, was diluted to 10 μg/ml in PBS and adsorbed onto 96-well Nunc MaxiSorp ELISA plates (Nunc, Rochester, NY) overnight at 4°C. Plates were blocked at room temperature for 1 h with PBS/1% BSA and washed with 0.1% Tween 20 in dH₂O. Recombinant LBP (50 ng/ml) diluted in PBS/0.1% BSA was added to the plates for 1.5 h at room temperature. After washing the plates, biotinylated LPS was added in the presence of the CP29 peptide 10 times as much LPS was required to yield the same amount of LPS bound to LBP compared with when the peptide was not present. CP29 reduced the binding of LPS to LBP almost completely when 45 ng/ml LPS was added to the well and by >80% when 450 ng/ml LPS was added to the well. Thus, the CP29 cationic peptide strongly inhibits the binding of LPS to LBP.

The dose-response and kinetic characteristics for the cationic peptide inhibition of LPS binding to LBP are shown in Fig. 2. In these experiments, we analyzed the inhibition of the LPS-LBP interaction by the CP29 peptide as well as by polymyxin B, a

Table I. _Peptides used in this study_  

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Structure</th>
<th>Amino Acid Sequence</th>
</tr>
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<tbody>
<tr>
<td>HBD-2</td>
<td>β-sheet</td>
<td>TCLKSGAICHVPFCPRRYKQIGTGCGLPGTKCCKKP</td>
</tr>
<tr>
<td>HNP-1</td>
<td>β-sheet</td>
<td>ACYCRIPACIAGERRYGTCIYQGRLWAFC</td>
</tr>
<tr>
<td>Gramicidin S</td>
<td>β-structured</td>
<td>Loop</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>Cyclic</td>
<td>Lipopeptide</td>
</tr>
<tr>
<td>Indolicidin</td>
<td>Extended</td>
<td>ILPWKWPWPPRR-RH₂</td>
</tr>
<tr>
<td>CP11CN</td>
<td>Extended</td>
<td>ILKWWPPWPRR-NH₂</td>
</tr>
<tr>
<td>Bac 2A-NH₂</td>
<td>Linear</td>
<td>RLARIVVIRVAR-NH₂</td>
</tr>
<tr>
<td>CEMA⁵</td>
<td>α-helical</td>
<td>LKWFKKKIGAVLKVTGLPALKTLK</td>
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<tr>
<td>CP29³</td>
<td>α-helical</td>
<td>LWKSFLKLTTAVKVLTLTGLPALS</td>
</tr>
<tr>
<td>CP208⁴</td>
<td>α-helical</td>
<td>KKSFIKLTSKAVSVLTTAKPLISS</td>
</tr>
</tbody>
</table>

⁴ Single letter amino acid code: O, ornithine; B, diaminobutyrate; Fᵢ, the D enantiomer of phenylalanine.

⁵ Synthetic peptides.

⁶ Sequence in parentheses is cyclized.

Results

Cationic antimicrobial peptides block the interaction of LPS with LBP

The mechanism by which cationic antimicrobial peptides block the activation of macrophages by LPS has not been elucidated. We hypothesized that they block the first step in the process, the interaction of LPS with LBP. To test this, we used an assay that measures the binding of biotinylated LPS to immobilized LBP. Fig. 1 shows that amounts of LPS ranging from 10 to 500 ng/ml produced a linear binding curve. Because previous studies showed that the CP29 cecropin:melittin hybrid cationic antimicrobial peptide effectively inhibited LPS-induced TNF-α production by RAW 264.7 cells (7), we asked whether this peptide could block the binding of biotinylated LPS to LBP in this assay. When 10 μg/ml (3.4 μM) CP29 was added at the same time as LPS, it substantially reduced the ability of the LPS to bind to LBP (Fig. 1). The dose-response curve for LPS binding was shifted ~10-fold, i.e., in the presence of the CP29 peptide 10 times as much LPS was required to yield the same amount of LPS bound to LBP compared with when the peptide was not present. CP29 reduced the binding of LPS to LBP almost completely when 45 ng/ml LPS was added to the well and by >80% when 450 ng/ml LPS was added to the well.

FIGURE 1. Inhibition of the binding of biotinylated LPS to immobilized LBP by CP29. Biotinylated _E. coli_ O55:B5 LPS (○) or biotinylated LPS plus 10 μg/ml (3.4 μM) CP29 (●) were added to wells containing recombinant LBP immobilized using anti-LBP Abs. Binding of the biotinylated LPS to the immobilized LBP was detected using HRP-conjugated streptavidin. The values represent the mean and SE for the averages of duplicate samples from three independent experiments. Where no error bars are shown, they were smaller than the symbols.
cationic antibiotic that causes substantial inhibition of LPS-induced TNF-α production (see Fig. 3B). Fig. 2A shows that as little as 100 ng/ml polymyxin B (70 nM) or 10 ng/ml CP29 (3.4 nM) could markedly inhibit the binding of LPS to LBP when 45 ng/ml LPS was added to the wells. The ability of polymyxin B to inhibit the LPS-LBP interaction leveled off at 60–70% inhibition when 1–10 μg/ml (0.7–7 μM) polymyxin B was added to the wells, whereas increasing the amount of CP29 from 1 μg/ml (0.34 μM) to 10 μg/ml (3.4 μM) resulted in greater inhibition, with 10 μg/ml CP29 causing nearly complete inhibition of the LPS-LBP interaction, as seen also in Fig. 1. Despite these differences, it is clear that both CP29 and polymyxin B are potent inhibitors of the LPS-LBP interaction.

We also observed that preincubating the LPS with CP29 or polymyxin B before adding it to the immobilized LBP substantially increased the ability of these peptides to block the LPS-LBP interaction (Fig. 2A). At least 10-fold less CP29 or polymyxin B was required to block the LPS-LBP interaction when the peptide was mixed with the LPS for 30 min before adding the LPS to the immobilized LBP-coated wells. This observation is consistent with our previous findings that CP29, polymyxin B, and other cationic peptides bind to LPS (7) and suggests that this interaction prevents LPS from binding to LBP.

While CP29 and polymyxin B can block the interaction of LPS with LBP, they cannot effectively disrupt the binding of LPS to LBP once it has occurred. Fig. 2B shows that when these peptides were added to the LBP-coated wells 20 min after the LPS, they were no longer able to substantially reduce the binding of LPS to LBP.

Structurally different cationic antimicrobial peptides inhibit the LPS-LBP interaction

Antimicrobial peptides have a wide variety of secondary structures (1, 3). For this study, peptides belonging to different structural groups were tested for their ability to inhibit LPS-LBP interaction. We tested 1) the human defensins HNP-1 and HBD-2, which have rigid β-sheet structures; 2) indolicidin and the indolicidin variant, CP11CN, which have an extended structure with β-turn elements (23); 3) Bac 2A-NH₂ which is a linear form of the loop peptide bactenecin (24); 4) the CP29, CEMA, and CP208 synthetic melittin:cecropin hybrids, which are amphipathic α-helical peptides (2,
We compared the abilities of cationic peptides belonging to these different structural groups to inhibit the LPS-LBP interaction (Fig. 3A). At the same time, we compared their abilities to block LPS-induced TNF-α production by the RAW 264.7 macrophage cell line (Fig. 3B). CP29 and CEMA, synthetic α-helical peptides, were the most effective peptides at blocking both the LPS-LBP interaction and the production of TNF-α by RAW 264.7 cells. They consistently caused 85–95% inhibition of the LPS-LBP interaction while reducing LPS-induced TNF-α production by >75%. In contrast to CP29, CP208, an α-helical peptide that is related to CP29 but that lacks the tryptophan found at the N terminus of CP29, had little effect on the LPS-LBP interaction. Consistent with this observation, this peptide did not significantly reduce LPS-induced TNF-α production. We have previously shown that CP208 binds LPS poorly compared with CP29 and CEMA and that it has little antimicrobial activity (7). Thus, the ability of the α-helical cationic peptides to inhibit LPS-LBP interactions depends on structural features in addition to their positive charges.

Of the other structural groups of cationic peptides, Bac 2A-NH₂, gramicidin S, and polymyxin B all caused significant (55–80%) inhibition of the LPS-LBP interaction (Fig. 3A). For gramicidin S and polymyxin B, this correlated with their ability to block LPS-induced TNF-α production by 60–80% (Fig. 3B). In contrast, Bac 2A-NH₂ repeatedly caused only a modest (~30%) inhibition of TNF-α production even though it inhibited the LPS-LBP interaction by 75%. The human neutrophil peptide α-defensin HNP-1 and the human β-defensin HBD-2, which belong to the β-sheet class of cationic antimicrobial peptides, both exhibited only a modest ability (~40% inhibition) to block the LPS-LBP interaction, and this correlated with their modest ability (25–40% inhibition) to block LPS-induced TNF-α production. Indolicidin and the indolicidin variant CP11CN both significantly inhibited the LPS-LBP interaction (~50% inhibition), but to a lesser extent than CP29. This correlated with their lower ability to inhibit TNF-α production (~40% inhibition) as compared with CP29. Thus, with the exception of Bac 2A-NH₂, the ability of different cationic peptides to inhibit LPS-induced TNF-α production correlated well with their ability to block the binding of LPS to LBP. The strong correlation (R² = 0.921) between these two properties is illustrated in Fig. 3C. Moreover, the nearly 1:1 correlation between the inhibition of LPS-LBP interaction and the inhibition of TNF-α release suggests that the ability of the peptides to block the LPS-LBP interaction may be a major mechanism by which they block the ability of LPS to activate macrophages.

**FIGURE 3.** Inhibition of the LPS-LBP interaction and LPS-induced TNF-α production by structurally different cationic peptides. A, Biotinylated LPS (45 ng/ml) was added to wells with LBP in the presence or absence of 10 μg/ml of the indicated cationic peptides. The peptides were added to the wells at the same time as the LPS. In the absence of peptides, the binding of the biotinylated LPS to the immobilized LBP yielded an OD₅₅₀ of 1.1–1.7. The data are expressed as % inhibition of LPS binding by the peptides. The values represent the mean and SE for three independent experiments. B, RAW 264.7 cells were incubated with 100 ng/ml E. coli O55:B5 LPS in the presence or absence of 20 μg/ml of the indicated peptides for 6 h. TNF-α was measured by ELISA. TNF-α production by unstimulated RAW 264.7 cells was always <0.3 ng/ml, while the LPS stimulation routinely resulted in 2.4–3.2 ng/ml TNF-α in the culture supernatant. The LPS-stimulated value was used as 100% and the data are represented as % inhibition of LPS-stimulated TNF-α production by the peptides. Note that the peptides did not affect the production of TNF-α by unstimulated RAW 264.7 cells. The values represent the mean and SE for three to four independent experiments. C, The data from A and B were graphed together as an XY scatter plot. The numbers represent the peptides as follows: 1, CP208; 2, HNP-1; 3, Bac 2A-NH₂; 4, HBD-2; 5, indolicidin; 6, CP11CN; 7, gramicidin S; 8, polymyxin B; 9, CEMA; 10, CP29. With the values for Bac 2A-NH₂ omitted, the coefficient of correlation (R²) between the peptide-induced inhibition of the LPS-LBP interaction and the inhibition of TNF-α release was 0.921. The slope of the best fit line was 0.965, indicating that there was nearly a 1:1 correlation between the inhibition of the LPS-LBP interaction and the inhibition of TNF-α release.
Discussion
In this report, we investigated the mechanism by which cationic antimicrobial peptides block the activation of macrophages by LPS. Using an assay that measures the binding of biotinylated LPS to immobilized LBP, we show for the first time that structurally diverse antimicrobial peptides can all block the interaction of LPS with LBP. Of the peptides tested, the α-helical peptide CP29 had the best ability to inhibit both the LPS-LBP interaction and LPS-induced TNF-α secretion by macrophages. It significantly inhibited the LPS-LBP interaction at concentrations as low as 10 ng/ml (3.4 nM), and 10 μg/ml (3.4 μM) CP29 caused nearly complete inhibition of the LPS-LBP interaction. The other cationic peptides tested including Bac 2A-NH₂, the indolicidins, and gramicidin S also inhibited both the LPS-LBP interaction and LPS-induced TNF-α production. Bac 2A-NH₂ was different from the other peptides in that it caused moderate inhibition of LPS-LBP interaction but had only a minor effect on LPS-induced TNF-α production. However, in general, the relative ability of different peptides to block the binding of LPS to LBP correlated strongly with their ability to block LPS-induced TNF-α production by RAW 264.7 cells. Thus, the ability of cationic peptides to block macrophage activation by LPS may be due in large part to their ability to block the binding of LPS to LBP. This would presumably block the transfer of LPS to CD14 by LBP, greatly decreasing the ability of LPS to activate macrophages.

LeRoy et al. recently showed that mAbs that block either the binding of LPS to LBP or the binding of LBP to CD14 are potent inhibitors of LPS toxicity in vivo and also block LPS-induced TNF production by RAW 264.7 cells (25). Together with our data, these results argue that inhibiting the LPS-LBP interaction is likely to be a very specific and efficient way to reduce or prevent LPS-induced inflammatory responses. The use of cationic peptides, in combination with anti-LBP mAbs, may be a very potent anti-endotoxin treatment. Our results also suggest that LPS-induced production of cationic peptides (26) may limit the magnitude of inflammatory responses by preventing further LPS signaling.

Acknowledgments
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References