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Endotoxin (Lipopolysaccharide) Neutralization by Innate Immunity Host-Defense Peptides

*PEPTIDE PROPERTIES AND PLAUSIBLE MODES OF ACTION**

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Binding of lipopolysaccharide (LPS) to macrophages results in proinflammatory cytokine secretion. In extreme cases it leads to endotoxic shock. A few innate immunity antimicrobial peptides (AMPs) neutralize LPS activity. However, the underlying mechanism and properties of the peptides are not yet clear. Toward meeting this goal we investigated four AMPs and their fluorescently labeled analogs. These AMPs varied in composition, length, structure, and selectivity toward cells. The list included human LL-37 (37-mer), magainin (24-mer), a 15-mer amphipathic α-helix, and its D,L-amino acid structurally altered analog. The peptides were investigated for their ability to inhibit LPS-mediated cytokine release from RAW264.7 and bone marrow-derived primary macrophages, to bind LPS in solution, and when LPS is already bound to macrophages (fluorescence spectroscopy and confocal microscopy), to compete with LPS for its binding site on the CD14 receptor (flow cytometry) and affect LPS oligomerization. We conclude that a strong binding of a peptide to LPS aggregates accompanied by aggregate dissociation prevents LPS from binding to the carrier protein lipopolysaccharide-binding protein, or alternatively to its receptor, and hence inhibits cytokine secretion.

Lipopolysaccharide (LPS),[2] also termed endotoxin, is an integral structural component of the outer membrane of Gram-negative bacteria (1). LPS is released from the bacteria during cell division, cell death, or in particular, as a result of antibiotic treatment against bacterial infection (2, 3). Upon its release, LPS is recognized by mononuclear phagocytes (monocytes and macrophages), which are part of the innate immunity of the host, and activates them. This results in an increase in their phagocytic activity and significantly enhances the secretion of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and others (4–6). Although pro-inflammatory cytokine secretion is essential for the development of the local inflammatory response, an unbalanced and overproduction of such cytokines may lead to septic shock characterized by endothelial damage, loss of vascular tone, coagulopathy, and multiple system organ failure, often resulting in death (7, 8). The activation mechanism of macrophages by LPS starts when LPS (through its toxic entity, lipid A) binds with LPS-binding protein (LBP), accelerating the binding of LPS to CD14, the primary receptor of LPS, which is expressed mainly on macrophages (9–11). The LPS-CD14 complex initiates intracellular signaling by interacting with the transmembrane protein Toll-like receptor-4 (TLR-4), which activates the NF-κB transcription factor, resulting in the production and secretion of pro-inflammatory cytokines (12–16).

In an attempt to understand the mechanism of macrophage stimulation by LPS, two major approaches have been reported. The first one utilized LPS receptor antagonists including anti-CD14 antibodies, anti-LBP antibodies, and lipid A analogs, all of which bind to essential components participating in the signaling mechanism (17, 18). The second approach utilized LPS blockers such as anti-lipid A antibodies and modified liposomes, both of which bind to LPS itself and prevent its ability to activate the macrophages (19, 20). Note, however, that although these studies have helped us to understand the steps involved in LPS neutralization, the approaches used could not deal with the bacteria from which the LPS is derived (19).

Recent studies have shown that a few antimicrobial peptides (AMPs) also have the potential to neutralize LPS-induced endotoxic effects. These peptides are important components of the innate defense system of all species of life (21). They are produced in large quantities at the site of infection and/or inflammation and act rapidly to clear microbes (22). Several AMPs prevent LPS-dependent cytokine induction in macrophages and block sepsis in animal models (23–27). These studies also showed a direct correlation between the ability of AMPs to bind LPS and their antimicrobial activity. Furthermore, it has been shown that other factors are involved, such as peptide hydrophobicity and amphipathicity and a defined structure (28–30).

To better understand the mechanism by which AMPs block LPS-dependent cytokine induction and the peptide properties required for this activity, we investigated four AMPs and their fluorescently labeled analogs. The peptides vary in their amino acid composition, length, structure, and selectivity toward different cells. The list includes two native antimicrobial peptides, human LL-37 (37-mer) and magainin (24-mer), as well as a 15-mer amphipathic α-helical peptide composed of Leu and Lys, and its D,L-amino acid diastereomer, a structurally altered analog. The peptides were investigated for their ability to inhibit LPS-induced cytokine release and to bind LPS in solution and when bound to macrophages and affect its oligomerization, as well as to compete with LPS on its binding site on the CD14 receptor and LBP. The results are discussed in view of the essential properties of a particular peptide required for LPS detoxification, as well as plausible modes of action.

EXPERIMENTAL PROCEDURES

Materials—Rink amide MBHA resin and Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) amino acids were purchased from Calbiochem-Novabiochem. Other reagents used for peptide synthesis included trifluoroacetic acid (Sigma), *N*,*N*-diisopropylethylamine (DIEA, Aldrich), methylene chloride (peptide synthesis grade, Bio-Lab), dimethylform-

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[2] The abbreviations used are: LPS, lipopolysaccharide; AMPs, antimicrobial peptides; LBP, lipopolysaccharide-binding protein; RP-HPLC, reverse-phase high performance liquid chromatography; NLLSQ, nonlinear least-squares; TNF, tumor necrosis factor; IL, interleukin; DMEM, Dulbecco's modified Eagle's medium; RT, reverse transcription; FITC, fluorescein isothiocyanate; NBD, 7-nitrobenz-2-oxa-1,3-diazole-4-yl; MBHA, 4-methylbenzhydrylamine.

amide (peptide synthesis grade, Bio-Lab), and benzotriazolyl-*n*-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP, Sigma). Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal calf serum, L-glutamine, penicillin-streptomycin, amphotericin B antibiotics, and nonessential amino acid solution (1:100) were supplied by Biological Industries, Beit Haemek, Israel. Bovine serum was purchased from Hyclone. TRI-reagent, RedTaq™ DNA polymerase, glyceraldehyde-3-phosphate dehydrogenase, IL-6, and TNF-α primers for PCR, oligo(dT) for RT-PCR, lipopolysaccharide from *Escherichia coli* 0111:B4, and fluorescein isothiocyanate (FITC)-conjugated lipopolysaccharide were supplied by Sigma. dNTP mix (10 μM) was supplied by Promega. SuperScript RNase H^- reverse transcriptase was purchased from Invitrogen.

Peptide Synthesis and Purification—The peptides were synthesized by a solid phase method on rink amide MBHA resin (0.05 meq) by using an ABI 433A automatic peptide synthesizer (Applied Biosystems). Labeling of the N terminus of the peptides with 5,6-carboxytetramethylrhodamine succinimidyl ester and 7-nitrobenz-2-oxa-1,3-diazole-4-yl (NBD) was done on the resin-bound peptide as described previously (31). The resin-bound peptides were cleaved from the resins by trifluoroacetic acid, washed with dry ether, and extracted with 30% acetonitrile/water. Trifluoroacetic acid cleavage of the peptides bound to rink amide MBHA resin, which resulted in C terminus-amidated peptides. Each crude peptide contained one major peak, as revealed by RP-HPLC, that was 50–70% pure by weight. The peptides were further purified by RP-HPLC on a C_{18} reverse-phase Bio-Rad semipreparative column (250 × 10 mm, 300-Å pore size, 5-μm particle size). The column was eluted in 40 min by using a linear gradient of 20–60% acetonitrile in water, both containing 0.1% trifluoroacetic acid (v/v), at a flow rate of 1.8 ml/min. The purified peptides were shown to be homogeneous (>98%) by analytical HPLC. The peptides were further subjected to amino acid analysis and electrospray mass spectroscopy to confirm their composition and molecular weight.

Antibacterial Activity of the Peptides—The antibacterial activity of the peptides was examined in sterile 96-well plates (Nunc F96 microtiter plates) in a final volume of 100 μl as follows. Aliquots (50 μl) of a suspension containing bacteria (midlog phase) at a concentration of 106 colony-forming units/ml in culture medium (LB medium) were added to 50 μl of water containing the peptide in serial 2-fold dilutions in water. Inhibition of growth was determined by measuring the absorbance at 600 nm with a Microplate autoreader El309 (Bio-Tek Instruments) after an incubation of 18–20 h at 37 °C. Antibacterial activities were expressed as the minimal inhibitory concentration, the concentration at which 100% inhibition of growth was observed after 18–20 h of incubation. Two strains of Gram-negative bacteria were used: *E. coli* D21 and *Acinetobacter baumannii* (ATCC 19606).

Primary Murine Bone Marrow Macrophage Cultures—Primary cultures of murine bone marrow macrophages were established with cells harvested from the femurs and tibias of 8-week-old BALB/c mice. The marrow cells were flushed from the bones with phosphate-buffered saline. Following centrifugation, the cells were resuspended to a final concentration of 10^6 cell/ml in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 5% horse serum, 15% L-sup (supplement from the medium of L-929 cells that produces macrophage-stimulating factor), 1% L-glutamine, 1% penicillin-streptomycin, and 1% sodium pyruvate. Cells were seeded in 35-mm-diameter plastic bacterial culture dishes for 1 week at 37 °C. Nonadherent cells were then removed by washing the adherent cells and were cultured in 96-well plates, 5×10^5 cells/well.

Cytotoxicity Assays (XTT Proliferation Assay)—RAW264.7 murine macrophages were grown in DMEM supplemented with 10% fetal calf serum and antibiotics at 37 °C in a humidified atmosphere at 5% CO_2 and 95% air (unless mentioned otherwise). Cells were passaged twice a week. A 96-well plate (Falcon) was used for this assay. Each well contained cell suspension (90 μl) in medium with 10% bovine serum (10^4 RAW264.7 murine macrophage cells/well). Wells in the first and second columns served as blanks (medium only) and 100% survival controls (cells and medium only), respectively. Dulbecco's phosphate-buffered saline (10 μl) was added to the first and second columns, and various concentrations of peptides (10 μl each), freshly prepared from the stock solution (1 mg/ml in water), were added to the remaining columns (two wells for each concentration of peptide solution). The plate was then incubated for 3 h before adding to each well 50 μl of XTT reaction solution (sodium 3′-[1-(phenyl-aminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate and *N*-methyl dibenzopyrazine methyl sulfate, mixed in a proportion of 50:1). The optical density was read at 450 nm wavelength in an enzyme-linked immunosorbent assay plate reader after a 2-h incubation of the plates with XTT (37 °C and 5% CO_2 + 95% air). Cell viability was determined relative to the control, and final results were recorded. The results were confirmed using replications in at least three independent experiments. The LC_{50} value for each peptide was obtained from the curve of cell viability *versus* the concentration of peptide and was taken from the concentration at which cell viability was 50%.

The Effect of AMPs on LPS-FITC Binding to RAW264.7 Cells Using Flow Cytometry—RAW264.7 cells (10^6 cells) were incubated for 30 min at 37 °C with LPS-FITC (1 μg/ml) in the presence or absence of the peptides (1 and 10 μM final concentration) in DMEM supplemented with 10% bovine serum. Preincubated cells were washed with phosphate-buffered saline, and then the peptides were added for another 30 min and then washed again. After the wash, the binding of LPS-FITC to RAW264.7 cells was analyzed by flow cytometry.

Effect of Peptides on LPS-dependent Cytokine Induction—RAW264.7 cells were plated in six-well plates (10^6 cells/well) and cultured overnight. Cells were stimulated without (negative control) or with 10 ng/ml LPS in the presence or absence of 10 μM peptide in DMEM supplemented with 10% bovine serum for 3 h. After stimulation, the cells were detached from the wells and washed once with phosphate-buffered saline. Total RNA was extracted using TRI® reagent according to the manufacturer's instructions. The quality and quantity of the RNA samples were determined. The RNA samples were used as a template in RT-PCR. The cDNA products were amplified in the presence of 3′ and 5′ primers (TNF-α, 5′-TCTCAGCCTCTTCTCATTCC-3′ and 5′-GTCCCAGCATCTTGTGTTTC-3′; IL-6, 5′-CCAGAAACCGCTATGAAGTTCC-3′ and 5′-TAGCCACTCCTTCTGTGACTCC-3′). A housekeeping gene, *GAPDH* (5′-CCATCAACGACCCCTTCATTGAC-3′ and 5′-GGATGACCTTGCCCACAGCCTTG-3′), was amplified in each experiment to evaluate the extraction procedure and to eliminate the possibility of genomic DNA contamination. The cycling condition steps for the amplification reaction consisted of a single 2-min heating step at 94 °C followed by 30 cycles (for IL-6) or 20 cycles (for TNF-α) of denaturation at 94 °C for 1 min, annealing at 61 °C (for IL-6) or 56 °C (for TNF-α and glyceraldehyde-3-phosphate dehydrogenase) for 1 min, and an extension at 72 °C for 1 min. After the last cycle, the reaction mixture was incubated at 72 °C for 10 min and then cooled to 4 °C. The PCR-amplified products were electophoresed and visualized on 1% agarose gel. The intensities of the amplified products were analyzed by a MultiImager Fluor-S™ image analysis system (Bio-Rad).

TABLE 1
Designations, sequences, and retention times of the peptides investigated

Peptide designation	Sequence[a]	RP-HPLC retention time
		min
Amphipathic-L	L KLLKKLLKKLLKLL-NH_2	34.45
Amphipathic-D	LK**L**LK**K**L**L**K**K**LL**K**LL-NH_2	22.02
Magainin	GIGKFLHSAKKWGKAFVGEIMNS-NH_2	22.4
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-NH_2	35.8

[a] Underlined and bold amino acids are D-enantiomers. All of the peptides are amidated in their C termini.

Evaluation of TNF-α Release from RAW264.7 Macrophages and Bone Marrow-derived Primary Macrophages—RAW264.7 macrophages were cultured overnight in 96-wells plate (2.5×10^5 cells/well). The medium was then removed followed by the addition to each well of fresh DMEM supplemented with 5% of bovine serum. The cells were stimulated with LPS (10 ng/ml final concentration) in the presence of 0.5, 1, 5, and 10 μM amphipathic-L, amphipathic-D, LL-37, or magainin. In another experiment, bone marrow-derived macrophages (5×10^5 cells/well) were stimulated with 100 ng/ml LPS in the presence of 0.5, 1, 5, and 10 μM amphipathic-L and magainin. Cells that were stimulated with LPS alone, and untreated cells served as controls. The cells were incubated for 6 h at 37 °C after which samples of the medium from each treatment were collected. TNF-α concentration in the samples was evaluated using a mouse TNF-α enzyme-linked immunosorbent assay kit according to the manufacturer's protocol (ELISA, Biosource). All experiments were done in duplicate.

Binding Assay—The experiments were conducted as described previously (32). Briefly, LPS (estimated molecular weight of 4000 g/mol) solution (20 ng/μl double distilled water) was added successively to 0.5 μM NBD-labeled peptide (amphipathic-L, amphipathic-D, magainin, and LL-37, all dissolved in double distilled water). The changes in NBD emission (530 nm; Δ*F*) were monitored as a function of the LPS/peptide molar ratio using a SLM-Aminco® luminescence spectrometer with excitation set at 467 nm (8-nm slit) until the system reached equilibrium. To account for the background, the emission of both double distilled water and LPS alone at the same wavelength was monitored. The changes in the probe emission represented the amount of LPS bound to the peptide, because NBD is known to change its emission in a hydrophobic environment (32), thereby enabling us to evaluate the LPS binding to the peptides. Our system reached binding equilibrium (F_{max}) at a certain LPS/peptide ratio, and therefore the affinity constant could be calculated from the relationship between the equilibrium level of NBD-labeled peptide emission and the LPS concentration, using a steady-state affinity model. The affinity constants were thus determined by nonlinear least-squares (NLLSQ) and Scatchard plot. The NLLSQ fitting was done using the following equation,

$$Y(x) = K_a \times X \times F_{max}/(1 + K_a \times X) \qquad \text{(Eq. 1)}$$

where *X* is the LPS concentration, ΔF_{max} is the maximal difference in the emission of NBD-labeled peptide before and after the addition of LPS (it represents the maximum LPS bound or the equilibrium-binding response), and K_a is the affinity constant. In the Scatchard plot, $\Delta F/F_{max}$ values were plotted against $(\Delta F/F_{max})/L_{free}$. $\Delta F/F_{max}$ represents the number of moles of LPS bound, and $(\Delta F/F_{max})/L_{free}$ represents the ratio of the bound to free LPS.

Effect of Peptides on LPS-FITC Aggregates—The assay was done as described previously (33). Briefly, LPS-FITC (0.5 μg/ml) was treated with increasing concentrations of the peptides. The changes in the emission of FITC (515 nm) were monitored by using a SLM-Aminco® luminescence spectrometer with excitation set at 488 nm (8-nm slit). The emissions of both double distilled water and the peptides alone were taken as a background level. Dissociation of the aggregates results in an increase in the fluorescence of FITC because of dequenching (34). The changes in emission were tracked until the system reached equilibrium.

Co-localization and Visualization of Peptides and LPS Binding to Cells Using Fluorescence Confocal Microscopy—RAW264.7 cells (10^4 cells/chamber) were plated in an 8-chamber Lab-Tek-chambered coverglass system (Nagle Nunc) and cultured overnight. The cells were washed and incubated for 30 min at 37 °C with LPS-FITC (100 μg/ml) in DMEM supplemented with 10% bovine serum. After incubation, the cells were washed and incubated under the same conditions with rhodamine-labeled peptides (0.1 μM). Nonincubated cells and cells that were incubated only with LPS-FITC or only with rhodamine-labeled peptides were used as controls. The cells were washed and covered with fresh medium. The co-localization and visualization of the rhodamine-labeled peptides and LPS-FITC on the cells was analyzed using fluorescence confocal microscopy.

RESULTS

Antimicrobial Activity and Cytotoxicity—Four AMPs were synthesized and investigated. The list includes human LL-37, magainin, a model 15-mer peptide composed of only Lys and Leu, and its D,L-amino acid analog (Table 1). The 15-mer was designed to form an amphipathic structure as depicted in a Schiffer and Edmundson wheel structure (not shown) (35). The data reveal that all AMPs are active against both the Gram-positive and Gram-negative bacteria strains tested (Table 2). The fluorescently labeled peptides preserved the activity of the unlabeled peptides (data not shown). The peptides were not toxic to macrophages at the concentrations tested in the LPS detoxification assay.

Inhibition of the Binding of LPS-FITC to RAW264.7 Macrophages—RAW264.7 macrophages were incubated with LPS-FITC (1 μg/ml) in the presence or absence of peptides. Untreated cells served as a negative control. The results shown in Fig. 1*A* (10 μM peptide) and quantified in Fig. 1*B* (1 and 10 μM peptides) reveal that LL-37, amphipathic-L, and amphipathic-D inhibit LPS-FITC binding to the macrophages in a dose-dependent manner, whereas magainin did not significantly inhibit LPS-FITC binding to the cells. Amphipathic-L was the most active peptide and reduced the binding of LPS by 35 and 90% at 1 and 10 μM, respectively, whereas amphipathic-D was active only at 10 μM.

Inhibition of LPS-mediated Cytokines Expression—RAW264.7 macrophages were stimulated with LPS (10 ng/ml) with or without the peptides (10 μM). Total RNA extraction served as a template for RT-PCR to determine the inhibitory effect of the peptides on TNF-α and the IL-6 mRNA level. The results, shown in Fig. 2, reveal that besides magainin, which was only slightly active, all other peptides were potent inhibitors. Quantitative analysis showed that amphipathic-L and LL-37 caused a marked decrease in mRNA expression, at times much below the basal level, whereas amphipathic-D brought it back to the basal level. These results correlate with the potency of the peptides to inhibit the binding of LPS to macrophages (Fig. 1) but do not correlate with their antimicrobial activity, their tendency to form an α-helical structure, or

TABLE 2
Different properties of AMPs

Peptide designation	Antibacterial activity[a]		LPS binding (K_a)	Estimated effect on LPS oligomers[b]	LPS neutralization by AMPs	
	E. coli D21	*A. baumannii*			Added before LPS binding	Added after LPS binding
	μM		M^{-1}			
LL-37	50	6	$1.5 (\pm 0.1) \times 10^7$	+++	+	+
Magainin	3	3	$3.1 (\pm 0.8) \times 10^7$	+/−	−	−
Amphipathic-L	50	12	$2.2 (\pm 0.3) \times 0^7$	+++	+	−
Amphipathic-D	6	6	$2.1 (\pm 0.2) \times 10^7$	++	+	−

[a] Minimum inhibitory concentration is given in μM.
[b] The effect was estimated based on the increase in the fluorescence of FITC after treatment with the most active AMPs (*i.e.* LL-37 and amphipathic-L).

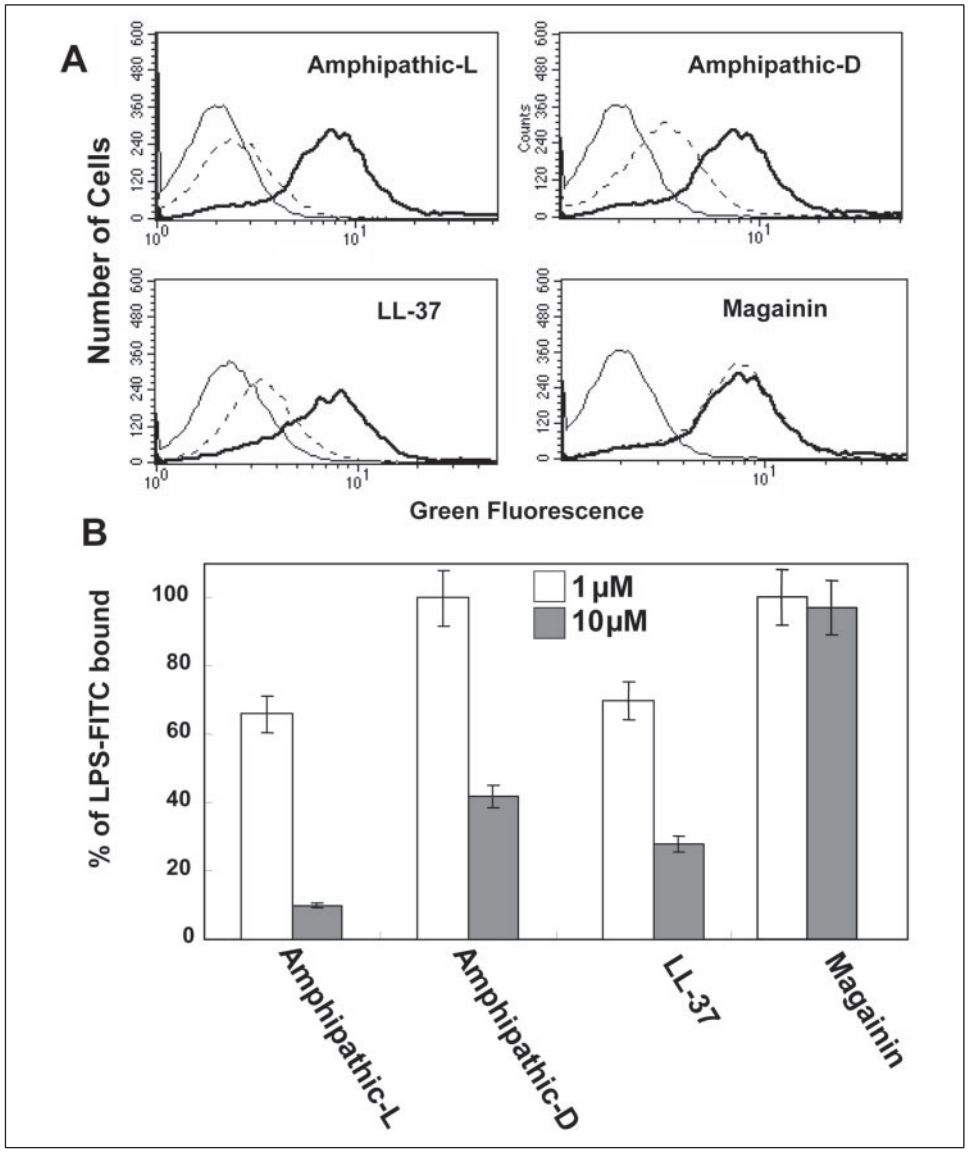

FIGURE 1. **Effects of the peptides on the binding of LPS-FITC to RAW264.7 macrophages.** *A*, RAW264.7 macrophages were incubated with LPS-FITC (1 μg/ml) in the absence (*thick line*) or presence (*dashed line*) of AMPs (10 μM). The cells were washed, and the binding of LPS-FITC was analyzed by flow cytometry. Background was taken by using untreated macrophages (*thin line*). *B*, quantification of the data shown in *A* for 1 and 10 μM peptide.

the extent of their binding to LPS (which will be discussed in the following paragraphs). Note that in some assays different amounts of LPS-FITC were used depending on the sensitivities of these assays. However, because saturation of binding to LPS for all the peptides is at an ~1:1 molar ratio (see paragraph on the binding of the peptides to LPS), all the experiments were done under conditions of a large excess of peptides compared with LPS.

Inhibition of TNF-α Secretion—We used a TNF-α enzyme-linked immunosorbent assay kit to evaluate TNF-α concentration in the cell medium after stimulation with LPS (10 ng/ml and 100 ng/ml for RAW264.7 and bone marrow primary macrophages, respectively) in the presence of different concentrations of AMPs. Fig. 3*A* shows a dose-dependent effect, which reveals activities similar to those obtained in the RT-PCR experiments. The three active peptides at a concentration of 10 μM brought the TNF concentration close to the basal level (36 ± 5 pg/ml). Amphipathic-L, the most active peptide, was evaluated together with the inactive magainin on inhibition of TNF-α secretion from LPS-stimulated bone marrow primary macrophages (Fig. 3*B*). Amphipathic-L (5 μM), but not magainin, was able to bring back the TNF-α level to that of the basal concentration (6 ± 5 pg/ml). Note that

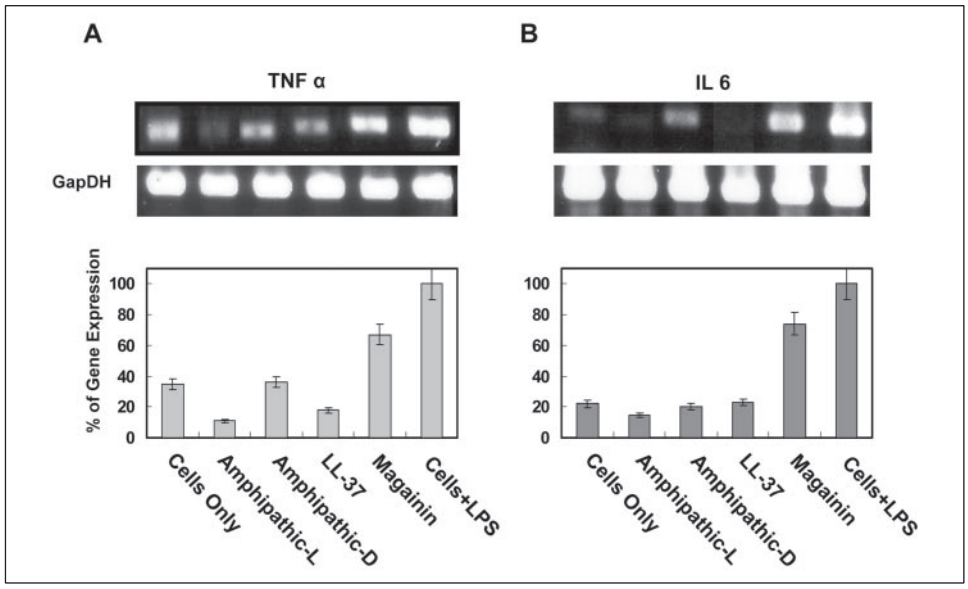

FIGURE 2. **Effects of the peptides on LPS-induced TNF-α (*A*) and IL-6 (*B*) mRNA expression.** Cells were stimulated with LPS (10 ng/ml) in the presence or absence of peptides. The photomicrograph shows a representative experiment of three experiments that gave similar results. The density of each PCR product for IL-6 and TNF-α was normalized against the control.

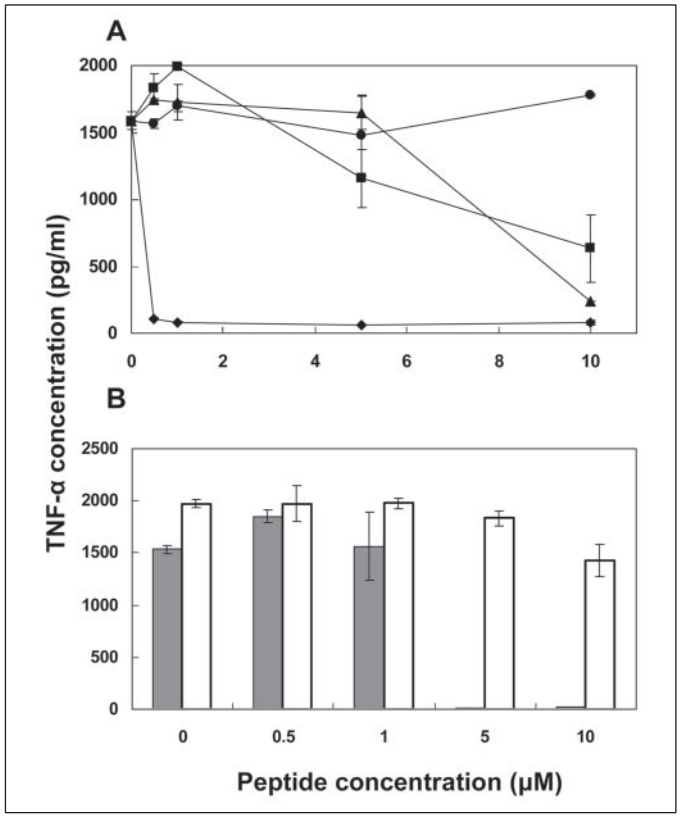

FIGURE 3. **The effect of the peptides on TNF-α secretion from RAW264.7 macrophages (*A*) and bone marrow-derived macrophages (*B*).** *A*, RAW264.7 cells were stimulated with LPS (10 ng/ml) and treated with 0.5, 1, 5, and 10 μM amphipathic-L (*diamonds*), amphipathic-D (*squares*), magainin (*circles*), and LL-37 (*triangles*). *B*, bone marrow primary macrophages were stimulated with 100 ng/ml LPS and treated with 0.5, 1, 5, and 10 μM amphipathic-L (*gray*) and magainin (*white*). All experiments were preformed in duplicates.

the activity of amphipathic-L in this experiment is 10-fold less than in RAW264.7 macrophages. This is in agreement with the 10-fold LPS required to stimulate the primary macrophages compared with RAW264.7.

Binding of Peptides to LPS in Solution—One of the mechanisms by which antimicrobial peptides are believed to neutralize LPS is by directly binding to LPS, thus preventing its binding to LBP (29). We performed binding experiments by using NBD-labeled peptides as described previously (32, 36). The emission change of the NBD-labeled peptides was followed as a function of LPS concentration; the result, shown in Fig. 4, correlates LPS:peptide molar ratio to the changes in NBD emission (ΔF), which is represented in arbitrary units. The change in the emission represents the amount of peptide bound to LPS, because NBD fluorescence depends upon the hydrophobicity of its environment. The affinity constants (K_a) were determined by NLLSQ fitting and were found to be high and similar for all of the peptides ($K_a \approx 2.0 \times 10^7\ M^{-1}$, Table 2) despite the finding that magainin is biologically inactive. These constants were similar to the constants that were calculated from the Scatchard plot (Fig. 4*B*). These data indicate that the detoxification activity of a peptide is not only due to their ability to bind LPS.

Binding of Peptides to Macrophages in the Absence or Presence of LPS—We visualized the binding of rhodamine-labeled peptides to macrophages alone or after their incubation with LPS-FITC by using fluorescence confocal microscopy. Incubation of the macrophages with labeled peptides (0.1 μM) alone revealed that amphipathic-L and LL-37 bound to the cells much better than amphipathic-D and magainin, and the peptides could also penetrate the cells (Fig. 5*A*). This is in agreement with their relative hydrophobicities reflected in RP-HPLC retention times (Table 1). However, when rhodamine-labeled peptides were incubated with the macrophages only after LPS-FITC binding and washing, all of the peptides (Fig. 5*B*, colored in *red*) bound strongly and co-localized predominantly with LPS (Fig. 5*B*, colored in *green*). Co-localization was not only restricted to the cell membrane, because peptides were localized together with LPS inside the cell as well. LPS is known to internalize together with CD14 upon binding (37). These results are in agreement with the high affinity of the peptides to LPS (Table 2), which is about 100–1000-fold higher than their affinity to the phospholipids comprising cell membranes (38). On the basis of these data we can rule out the possibility that binding to macrophage membrane alone or to macrophage-bound LPS is a mechanism of LPS neutralization.

The Ability of Peptides to Remove LPS Bound to CD14—LL-37 has been shown to compete with LPS binding to its primary receptor CD14 (30, 39). To investigate whether this feature is shared by all peptides, we preincubated the macrophages with LPS-FITC, washed them, and only then treated the macrophages with the peptides. The results, shown in Fig. 6, reveal that none of the peptides besides LL-37 could significantly

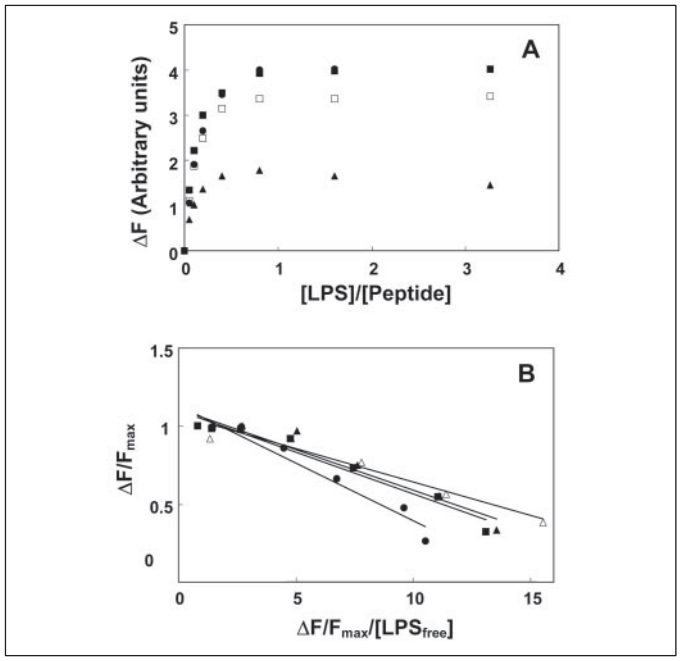

FIGURE 4. **Binding of LPS to AMPs.** *A*, the changes in fluorescence intensity of the NBD-labeled peptides (ΔF) as a function of the peptide:lipid molar ratio. NBD-amphipatic-L (*filled triangle*), NBD-amphipatic-D (*filled square*), NBD-magainin (*empty triangle*), and NBD-LL-37 (*filled circle*) (0.5 μM) were titrated with LPS. The results are the mean of three independent experiments with a standard deviation of $\pm$10%. *B*, Scatchard plot analysis of AMPs binding to LPS. $\Delta F/F_{max}$ values were plotted against $(\Delta F/F_{max})/L_{free}$. $\Delta F/F_{max}$ represents the number of moles of LPS bound, and $(\Delta F/F_{max})/L_{free}$ represents the ratio of bound to free LPS.

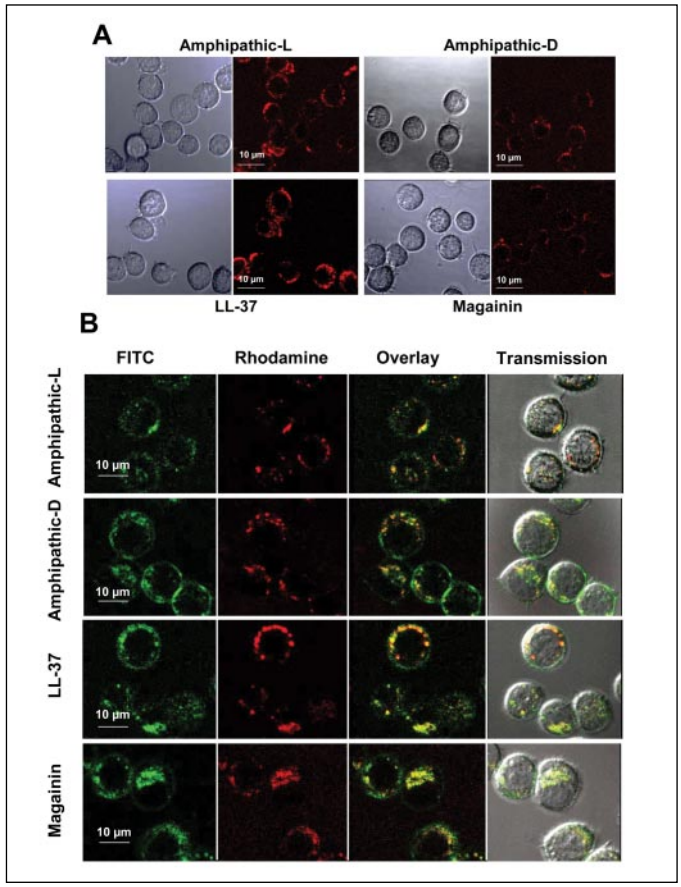

FIGURE 5. **Confocal laser-scanning microscopy images.** *A*, RAW264.7 macrophage cells treated with 0.1 μM rhodamine-labeled peptides. *B*, RAW264.7 macrophage cells preincubated with FITC-LPS (100 μg/ml) and after washing were treated with rhodamine-labeled peptides (0.1 μM). The first column shows the LPS-FITC fluorescence signal, the second column shows the rhodamine-labeled peptide signal, the third column shows the overlay of the two probes enabling one to visualize co-localization (reflected in *yellow*), and the fourth column shows the localization of the probes on the cell.

displace LPS after its binding to CD14. These data suggest a different mode of action for the other peptides, which does not involve receptor binding. This is in line with previous studies suggesting that LL-37 neutralizes LPS by directly binding to LPS and also by competing with it on its receptor, CD14 (30).

Effect of Peptides on LPS-FITC Aggregates—LPS forms aggregates in aqueous suspension (40), which are believed to monomerize by LBP upon their transfer to CD14 (11). We tested the potential of the peptides to dissociate LPS-FITC aggregates and whether this property correlates with LPS detoxification. The fluorescence of LPS-FITC aggregates is self-quenched but should increase when the aggregates dissociate, because of dequenching. The dose-dependent effect of the peptides on LPS-FITC fluorescence is shown in Fig. 7. The data reveal that all the peptides other than magainin have a strong effect on the aggregation of LPS-FITC; the order of their effect is amphipathic-L $\approx$ LL-37 $>$ Amphipathic-D $\gg$ magainin. This order is in agreement with their LPS neutralizing activity (Figs. 1–3). Interestingly, there are three types of effects. First, with amphipathic-L and amphipathic-D, there is an "all or none" effect. The peptides become fully active only above a threshold concentration ($\sim$1 μM for amphipathic-L and $\sim$10 μM for amphipathic-D). This threshold is in agreement with the finding that amphipathic-L is already active at 1 μM, whereas amphpathic-D is active only at 10 μM (Fig. 1). Second, with LL-37 there is a gradual increase in the effect up to 100 μM, the maximal concentration tested. Finally, in the case of magainin there is a slight increase in the effect only at about 10 μM, which diminishes as the concentration increases, the reason for which is not clear.

DISCUSSION

We investigated the correlation between the antimicrobial activity of AMPs and their ability to neutralize LPS, as well as the possible mechanisms of LPS neutralization. The important conclusion is that a strong binding of a peptide to LPS aggregates accompanied by aggregate dissociation prevents LPS from binding to the carrier protein LBP, or alternatively to its receptor, and hence inhibits cytokine secretion. This conclusion is based on the characterization of four antimicrobial peptides, all of which have potent antimicrobial activity but differ in composition, structure, and ability to neutralize LPS (Tables 1 and 2).

To prevent macrophage activation by LPS, a peptide needs to prevent the binding of LPS to the CD14 receptor (6, 14, 41). Indeed, we show first by using flow cytometry that all of the peptides except magainin block LPS-FITC from binding to macrophages (Fig. 1). These data correlate with the potency of the peptides to reduce the level of mRNA expression of the two cytokines IL-6 and TNF-α (Fig. 2), as well as to prevent TNF-α secretion from both RAW264.7 and primary macrophages (Fig. 3).

Mechanisms by Which a Peptide Can Prevent LPS Biding to Macrophages

Direct Interaction with LPS in Solution or When Bound to Macrophages—Our binding experiments show that all the peptides bind LPS in solution with high and similar affinity ($\sim 2 \times 10^7$ M^{-1}) and with a stoichiometry of $\sim$1:1 between LPS and each peptide (Fig. 4, Table 2). Furthermore, confocal microscopy indicates that all the peptides bind to LPS even when it is already prebound to the macrophages,

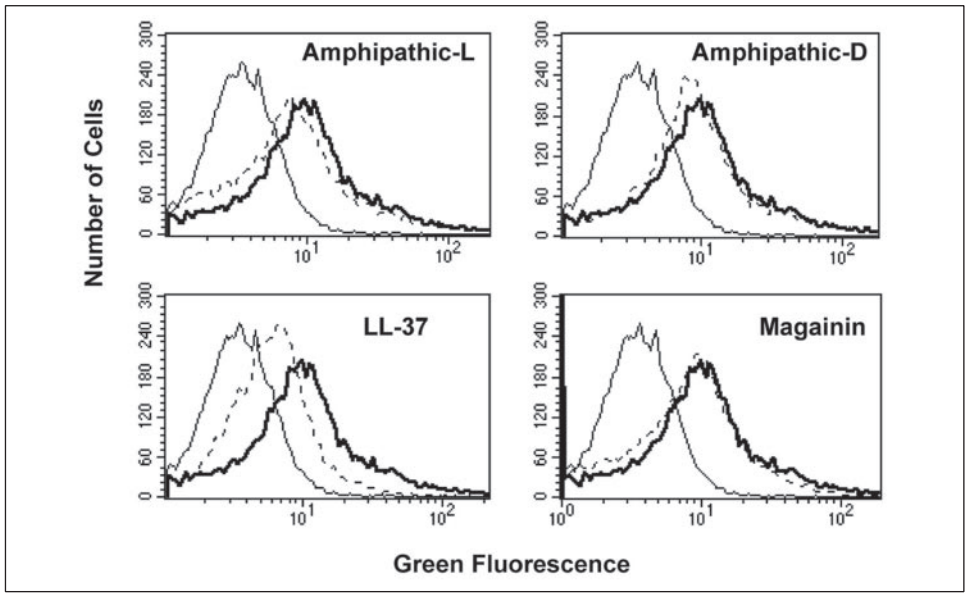

FIGURE 6. **Effects of the peptides on the binding of LPS-FITC to RAW264.7 macrophages that were preincubated with LPS-FITC.** RAW264.7 macrophages were incubated with LPS-FITC (1 μg/ml) in the absence of peptides (*thick line*). The cells were washed, peptides were then added, and the LPS-FITC that remained bound to the cells was analyzed by flow cytometry (*dashed line*). Background was taken by using untreated macrophages (*thin line*). Maximum LPS-FITC binding was obtained by treating the cell only with LPS-FITC.

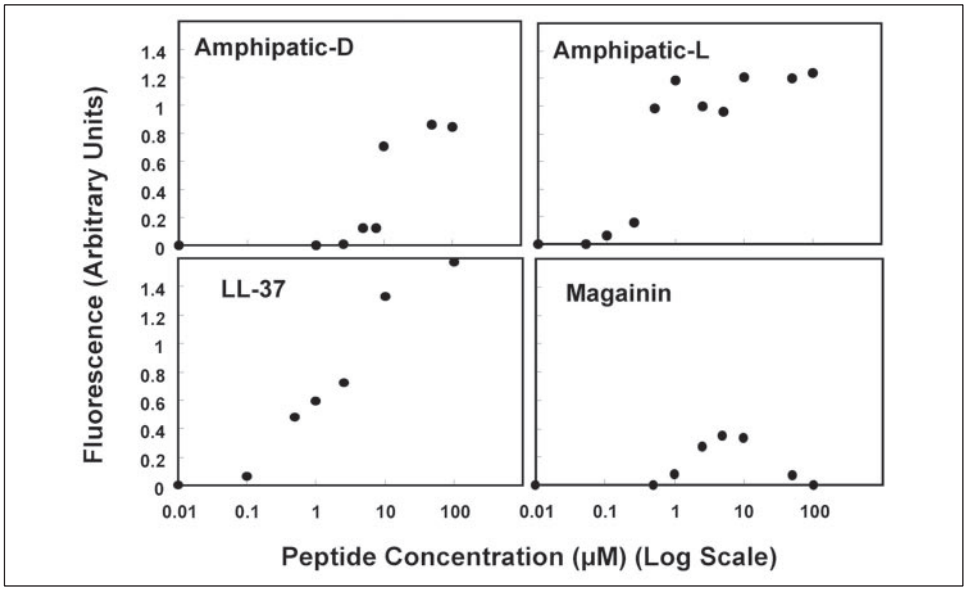

FIGURE 7. **The relationship between peptide concentration and the LPS-FITC aggregation state.** LPS-FITC (0.5 μg/ml) was treated with increasing concentrations of peptides. The change in FITC emission after each treatment was monitored until emission reached equilibrium. The increase in emission reflected the change in the LPS-FITC aggregational state. FITC increases its emission when the distance between its monomers increases due to self-quenching. Experiments were repeated three times with 5% variations.

either on the membrane or when internalized (Fig. 5*B*). However, when added to macrophages alone under similar conditions, only LL-37 and amphipathic-L are bound (Fig. 5*A*), in agreement with the hydrophobicity of the peptides (Table 1). These data do not correlate with the LPS-neutralizing activities but correlate with the antimicrobial activities of the peptides (Figs. 1 and 2). We conclude that strong binding of a peptide to LPS is not sufficient to block LPS biological activity. Note that Scott *et al.* (26) found a correlation between the ability of a series of peptides to bind *E. coli* O111:B4 LPS and their antimicrobial activity and ability to block LPS-stimulated TNF-α and IL-6 production.

Competition between the Peptides and LPS on the Binding Site within the CD14 Receptor—Flow cytometry experiments reveal that only LL-37 competes with LPS-FITC, which is already bound to its receptor (Fig. 6), suggesting that only this peptide and LPS share a similar binding site within CD14. Note, that LL-37 does not remove completely LPS from its receptor, indicating that this mechanism is not the only mechanism of LPS detoxification by LL-37. These results point out that this interaction is sequence-dependent and is not shared by all LPS-neutralizing peptides. In support of this premise, Nagaoka *et al.* (30, 39) found that LL-37, CAP18, and CAP11 peptides, which belong to the cathelicidine family, bind to the cell surface CD14 and inhibit the binding of FITC-LPS to the cells.

Competition between the Peptides and LPS on the Binding site within LPB—Studies on the mechanism by which AMPs inhibit LPS-induced release of cytokines have suggested that direct binding of the peptides to LPS takes place, making it unavailable to LBP, rather than interaction of the peptides with LPB itself (29, 30, 42). Here we found that the peptides did not bind LBP, as revealed by the incubation of their fluorescently labeled analogs with serum prior to separation on SDS-PAGE (data not shown).

Dissociation of LPS Aggregates by the Peptides—We found that only the three LPS-detoxifying peptides (Figs. 2 and 3) could dissociate LPS aggregates (Fig. 6). In addition, the binding assay revealed about a 1:1 ratio between LPS and the peptide bound (Fig. 4). Amphipathic-L and LL-37 are the most active peptides followed by amphipathic-D and magainin. Interestingly, the dose-response profiles show different behaviors: LL-37 shows a linear dose-dependent curve, amphipathic-L

and Amphipathic-D become fully active only above a threshold concentration, and magainin shows a slight activity only at a narrow range of concentrations (Fig. 6).

Conclusion

The properties of the peptides, as summarized in Table 2, demonstrate that the ability of AMPs to strongly bind to LPS and to exhibit antimicrobial activity are not sufficient to neutralize LPS-induced macrophage activation. To do so, the peptides also need to dissociate the LPS aggregates. However, some AMPs can, in addition, compete with LPS on its binding site within the CD14 receptor, as in the case of the cathelicidine family. Interestingly, once a peptide is active, even major structural alterations caused by the incorporation of several D-amino acids do not abolish this function. Finally, the finding that diastereomeric antimicrobial peptides are active in neutralizing LPS, together with previous studies demonstrating the advantages of such diastereomers for *in vivo* applications (43, 44), makes them potential candidates to kill bacteria and at the same time detoxify LPS.

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