Lipopolysaccharide, a Key Molecule Involved in the Synergism between Temporins in Inhibiting Bacterial Growth and in Endotoxin Neutralization*

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Lipopolysaccharide (LPS) is the major structural component of the outer membrane of Gram-negative bacteria and shields them from a variety of host defense factors, including antimicrobial peptides (AMPs). LPS is also recognized by immune cells as a pathogen-associated molecular pattern and stimulates them to secrete pro-inflammatory cytokines that, in extreme cases, lead to a harmful host response known as septic shock. Previous studies have revealed that a few isoforms of the AMP temporin, produced within the same frog specimen, can synergize to overcome bacterial resistance imposed by the physical barrier of LPS. Here we found that temporins can synergize in neutralizing the LPS-induced macrophage activation. Furthermore, the synergism between temporins, to overcome the protective function of LPS as well as its endotoxic effect, depends on the length of the polysaccharide chain of LPS. Importantly, mode of action studies, using spectroscopic and thermodynamic methods, have pointed out different mechanisms underlying the synergism of temporins in antimicrobial and anti-endotoxin activities. To the best of our knowledge, such a dual synergism between isoforms of AMPs from the same species has not been observed before, and it might explain the ability of such amphibians to resist a large repertoire of microorganisms.

Naturally occurring host-defense antimicrobial peptides (AMPs)2 are produced across virtually all forms of life as a primitive component of their innate immune system and are constitutively or inducibly expressed in response to invasion by pathogens (1–7). Despite differences in their size and sequence, they all share a net positive charge at neutral pH, and fold into amphipathic structures, often after contact with membranes (8, 9). There is compelling evidence that unlike current antibiotics, which interact strongly with specific target molecules such as proteins, many AMPs act by a nonspecific mechanism and kill microbes by disrupting their plasma membrane (3, 10–12). However, before reaching the plasma membrane, they must traverse the cell wall. In Gram-negative bacteria, this cell wall is surrounded by an asymmetric outer membrane (OM) containing primarily the amphiphilic lipopolysaccharide (LPS, endotoxin) in its outer leaflet. The LPS barrier is believed to be stabilized by LPS-associated cations (Mg2+) through salt bridges that neutralize the repulsive forces of adjacent LPS molecules. This leads to the formation of an oriented and tightly cross-linked leaflet that protects bacteria from a variety of host-defense hydrophobic molecules (13, 14), including some AMPs (15–17). Note that for all enterobacterial and most nonenterobacterial strains, LPS is composed of three parts: (i) a hydrophobic moiety, also referred to as lipid A, consisting of fatty acid chains linked to two phosphorylated glucosamine residues; (ii) an oligosaccharide core, covalently bound to the lipid A via ketodeoxyoctoic acid (KDO); and (iii) a hydrophilic O-antigenic domain, composed of repeating saccharide units, which protrudes into the surrounding medium. The composition and the number of the O-antigen repeat units vary among different bacterial species (18, 19).

The immune system has evolved to recognize LPS as a pathogen-associated molecular pattern (PAMP). Upon its recognition, LPS stimulates the innate immune cells, inducing the secretion of pro-inflammatory cytokines (e.g. TNF-α, IL-1, IL-6), mainly by mononuclear cells and macrophages (20–22). Many clinically used antibiotics cause the release of LPS from the microbial cell wall (23, 24), and, as a consequence, give rise to a prolonged activation of the immune cells, which results in Mueller-Hinton medium; MIC, minimal inhibitory concentration; FIC, fractional inhibitory concentration; PBS, phosphate-buffered saline; TNF-α, tumor necrosis factor α; ITIC, isothermal titration calorimetry; QUELS, quasi-elastic light scattering; PIPES, 1,4-piperazinediethanesulfonic acid; IL, interleukin.
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an unbalanced systemic secretion of cytokines. This can rapidly lead to the development of septic shock, which, in extreme cases, may lead to death (25, 26). In contrast with these conventional antibiotics, several AMPs possess dual functions: they kill bacteria and neutralize the endotoxic effect of LPS, although the exact mechanism is not yet well understood (27–30).

Most living organisms produce a single AMP at the site of infection, but some animal species, particularly those from Amphibia, synthesize and secrete different isoforms of the same AMP. Among such isoforms, temporins represent the largest family, with more than 50 members (31). They are among the smallest amphipathic α-helical AMPs found in nature to date (10–14 amino acids) and contain only a few positively charged amino acids (net charge at a neutral pH ranging from 0 to +3) (32, 33). The majority of them act by increasing the permeability of the microbial membrane (34–36). Recently, the physiological significance of the existence of multiple forms of temporins has been addressed and has revealed that temporins A and B strongly synergize in killing Gram-negative bacteria, when each is combined with temporin L (15). Temporin L has also been studied for its potential to suppress the endotoxic effect of LPS (37).

In this study we investigated: (i) the role of the carbohydrate region of LPS in the mechanism underlying the synergistic effect of temporins against Gram-negative bacteria; and (ii) the potential of temporins to synergize in the detoxification of LPS, and whether the underlying molecular mechanism is similar to that of the synergism against Gram-negative bacteria.

Importantly, we found a strong synergism between different isoforms of temporins in the inhibition of TNF-α release from macrophages stimulated with LPS. In addition, our data emphasize a different mode of action for the synergistic effect of these AMPs in antimicrobial and anti-endotoxin activities, which show dependence on the size of the polysaccharide chain of the bacterial LPS.

EXPERIMENTAL PROCEDURES

Materials—Rink amide 4-methyl benzhydrylamine (MBHA) resin and 9-fluorenylmethoxycarbonyl (F-moc)-protected amino acids were obtained from Calbiochem-Novabiochem. Other reagents used for peptide synthesis included trifluoroacetic acid (Sigma), piperidine (Merck), N,N-diisopropylethylamine (DIEA, Sigma), N-hydroxybenzotriazole hydrate (HOBt, Sigma), 2-(1H-benzotriazole-1-yl)-1,1,3,3 tetramethyluronium hexafluorophosphate (HBTU, peptide synthesis grade, Bio-Lab), and dimethylformamide (DMF, peptide synthesis grade, Bio-Lab). Proteinase K and lipopolysaccharides from Escherichia coli O111:B4 (LPS O111:B4) and from E. coli O26:B6 (LPS O26:B6) were purchased from Sigma. All buffers were prepared in double glass-distilled water.

Peptide Synthesis, Fluorescent Labeling, and Purification—Peptides were synthesized by an F-moc solid phase method on Rink amide resin, using an ABI 433A automatic peptide synthesizer. Cleavage of the peptides from the MBHA resin resulted in the amidation of the C terminus. To label the peptides, the F-moc protecting group was removed from the N terminus of the resin-bound peptides, by incubation with piperidine for 12 min, whereas all the other reactive amine groups of the attached peptides were kept protected. The resin-bound peptides were washed twice with DMF, and then treated with rhodamine-N-hydroxysuccinimide (2 equiv), in anhydrous DMF containing 2% DIEA, leading to the formation of a resin-bound N-rhodamine peptide. After 24 h, the resin was washed thoroughly with DMF and then with methylene chloride. The three rhodamine-labeled temporins A (rho-temporin A), B (rho-temporin B), and L (rho-temporin L) were then cleaved from the resin. All the peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) on a C18 reversed-phase Bio-Rad semi-preparative column (250 × 10 mm, 300 Å pore size, 5-μm particle size). The column was eluted with a 40-min linear gradient of 20–60% acetonitrile in water, containing 0.05% trifluoroacetic acid (v/v), at a flow rate of 1.8 ml/min. The purified peptides were further subjected to amino acid analysis and electrospray mass spectrometry to confirm their composition and molecular weights.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)—LPS from E. coli O111:B4 and E. coli O26:B6 were separated by SDS-PAGE with a gel containing 12% acrylamide and 0.5% SDS (38). Samples of 100 μg of LPS were applied to the gel, and LPS bands visualized by immunoblot analysis using anti-LPS core antibodies (mouse IgG, Hycult, Biotechnology, Netherlands).

Antibacterial Activity of the Peptides—Susceptibility testing was performed by the microbroth dilution method according to the procedures outlined by the National Committee for Clinical Laboratory Standards (2001) using sterile 96-well plates. Aliquots (50 μl) of bacterial in mid-log phase at a concentration of 2 × 10⁶ colony-forming units (CFU)/ml in culture medium (Mueller-Hinton, MH) were added to 50 l of MH broth containing the peptide in serial 2-fold dilutions in 20% ethanol. Inhibition of growth was determined by measuring the absorbance at 600 nm with a 450-Bio-Rad Microplate Reader after an incubation of 18–20 h at 30 °C. Antibacterial activities were expressed as the minimal inhibitory concentration (MIC), the concentration of peptide at which 100% inhibition of growth was observed after 18–20 h of incubation. The ranges of peptide dilutions used were 0.15–40 μM for temporin L and 0.2–100 μM for temporins A and B. Synergism between temporins was evaluated by the checkerboard titration method, by adding combinations of two temporins, in a serial 2-fold dilution, to wells of a sterile flat-bottomed 96-well plate, each containing 1 × 10⁶ CFU in a final volume of 100 μl. Briefly, wells in the first row were loaded with temporin L at twice the highest final concentration used in the experiment, and 2-fold dilutions were prepared across rows starting from the first one. Therefore, all wells in the same row kept temporin L at a constant concentration, whereas the concentration of temporin A or B varied in a serial 2-fold dilution. Note that the first column and the last row wells were loaded only with the single peptides (temporin L, A, or B, respectively), in a 2-fold dilution. The fractional inhibitory concentration (FIC) index for combinations of two peptides was calculated according to Equation 1, where A and B are the MICs of drug A and drug B in the com-
bination, MIC_A and MIC_B are the MICs of drug A and drug B alone, FIC_A and FIC_B are the FICs of drug A and drug B, and n is the number of wells per plate used to calculate the FIC. The FIC indices were interpreted as follows (39): FIC ≤ 0.5, synergy; 0.5 < FIC < 1, additivity; 1 ≤ FIC < 4, indifference; FIC ≥ 4, antagonism.

The following Gram-negative bacterial strains were used: E. coli O111:B4, E. coli O26:B6, and the cell wall-defective mutant strains of E. coli D21, i.e. D21 e7, D21 f1, and D21 f2, with shorter LPS carbohydrate chain length (40–42).

The Effect of LPS on the Oligomeric State of the Peptides as Determined by Rhodamine Fluorescence Dequenching Measurements—Rhodamine-labeled peptides (final concentration, 3 μM for temporin A and 1.5 μM for both temporins B and L) were added to 100 μl of phosphate buffered saline (PBS), and changes in the intensity of the fluorescence emission were followed upon the addition of different concentrations of LPS O26:B6, using a microplate counter (Wallac 1420 Victor 3™, Perkin Elmer) with excitation and emission wavelengths set at 485 and 590 nm, respectively. Proteinase K (80 μg/ml in PBS) was then added, and the resulting fluorescence was monitored. An increase in fluorescence indicates that the peptide exists as an oligomer (43). All fluorescence measurements were performed at 30 °C.

Evaluation of TNF-α Release from RAW264.7 Macrophages—RAW264.7 macrophages were cultured overnight in 96-well plates (2 × 10^5 cells/well). The medium was then removed followed by the addition of fresh medium to each well. The cells were stimulated with LPS O111:B4 or LPS O26:B6 (10 ng/ml final concentration), in the presence of 2.5, 5, or 10 μM temporin A, B, or L. In another set of experiments, the effects of all combinations of temporins (A+B, A+L, or B+L, 2.5 μM of each) were tested. Cells that were stimulated with LPS alone and untreated cells served as controls. The cells were incubated for 4 h at 37 °C. Afterwards, samples of the medium from each treatment were collected. Concentrations of pro-inflammatory cytokines (TNF-α, IL-6) in the samples were evaluated using mouse enzyme-linked immunosorbent assay kits according to the manufacturer's protocol (BIOSOURCE (TNF-α); Biologend (IL-6)). All experiments were repeated three times.

Isothermal Titration Calorimetry (ITC)—LPS O26:B6, or LPS O111:B4, was diluted in 20 mM PIPES buffer, pH 7.4, containing 0.14 mM NaCl and 1 mM EDTA, incorporated into the calorimeter cell (1.8 ml) and equilibrated to 38 °C in a MicroCal VP-ITC instrument (MicroCal Inc, Southampton, MA). Solutions of temporins A, B, and L were made at a concentration of 350 μM in matching buffer and placed in the syringe. A volume of 10 μl of peptide (1.9 μM in the cell) was successively titrated into LPS with constant stirring at 300 rpm. Mixtures of temporins A+L, B+L, or A+B, containing 175 μM of each peptide were also injected into LPS in separate experiments. At the end of the titrations, the content of the cell was removed and kept for further processing. LPS is known to be a heterogeneous mixture in nature (38) (see also Fig. 1), making it difficult to establish its molecular weight. In these studies, LPS O26:B6 and LPS O111:B4 were quantified with the Purpald reagent, which is a specific reagent for the detection of aldehydes, using glycerol as a standard (44). The molecular weight for the LPS mono-

mers was determined assuming LPS is a single molecular species, which is very useful as an arbitrary mode of comparison among data found with different amounts of LPS. The obtained values (13,000 and 20,000 kDa for LPS O26:B6 and LPS O111: B4, respectively) were in agreement with those previously reported in the literature (45–47). Data were analyzed and plotted using the program Origin 5.0 and fitted with programs provided by the manufacturer.

Quasi-elastic Light Scattering (QUELS)—To estimate the average size of the LPS particles, QUELS measurements were performed at 38 °C using a BIC 200SM/TurboCorr light scattering instrument (Brookhaven Instruments Corp, Holtsville, NY) measuring the scattered light at 90°. Samples of LPS O26:B6 collected from the calorimeter cell after titration with temporins, as well as from the original LPS suspension, were measured. Measurement of QUELS with LPS O111:B4 were conducted in samples diluted in HEPES buffer, pH 7.4, containing 0.14 mM NaCl, in absence of EDTA. Size distributions obtained by QUELS were analyzed using the Contin method, as provided by the manufacturer.

RESULTS

Synergism between Temporins Toward Bacteria—To investigate the role of the size of the polysaccharide chain of LPS in the molecular mechanism underlying the synergistic effect of temporins A (FLPLGRVLSGIL-NH₂) and B (LLPIVGNNLLKSLL-NH₂) when each is combined with temporin L (FVQWFSKFGL-GRIL-NH₂) against Gram-negative bacteria, we tested the ability of all combinations of these peptides to inhibit the growth of E. coli O111:B4 and E. coli O26:B6. The latter strain is known to have short LPS polysaccharide chains, as indicated both by its low molecular weight bands in SDS-PAGE (Fig. 1) and by its low critical micelle concentration, which is considered to be directly proportional to the polysaccharide chain length of LPS (48). The antimicrobial activity of temporins was also assayed against three cell wall-defective mutant strains of E. coli D21, which have lower amounts of sugar residues in their LPS backbone. The first is E. coli D21 e7, which is devoid of the O-antigen domain and has a shorter outer oligosaccharide core. The second is E. coli D21 f1, lacking the O-antigen and the outer core region, and the third is E. coli D21 f2, carrying the only KDO-lipid A complex (40–42) (Fig. 2). The antimicrobial activity and FIC indices of temporins are illustrated in Table 1.

The data revealed that A+L and B+L pairs exhibited a relatively greater synergistic effect on E. coli O111:B4 (FIC indices of 0.48 and 0.5, respectively) (39, 49, 50) compared with temporins A+B. In contrast, FIC values ranging from 0.56 to 0.9 were found when the same peptide combinations were tested on E. coli O26:B6 and the other bacterial strains. The highest FIC values were obtained toward E. coli D21 f2, indicating the disappearance of a synergistic effect, in parallel to a shortening of the LPS carbohydrate domain.

The Effect of LPS on the Organization of Temporins—To ascertain whether differences in the antimicrobial activity of temporins A+L or B+L against E. coli O111:B4 and E. coli O26:B6 reflected a different organization of the peptides when in contact with LPS with a different length of the polysaccharide region, we studied the effect of purified LPS O26:B6 (with
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short carbohydrate chains) on temporins that were labeled at their N terminus with rhodamine. Rhodamine is a fluorescent probe that is only slightly sensitive to the polarity of its environment and does not interfere with the antibacterial activity of the peptide (data not shown). When rhodamine-labeled monomers are self-associated, and the rhodamine probes are in proximity, the result is self-quenching of the fluorescence emission. However, a significant increase in the fluorescence intensity of the peptide, after treatment with a proteolytic enzyme, indicates that the peptide is self-associated (51).

Here, the fluorescence of the rho-peptides was measured before and after adding different concentrations of LPS. Fig. 3, A, B, and C show the results with rho-temporin A, rho-temporin B, and rho-temporin L, respectively. The addition of increasing concentrations of LPS O26:B6 (arrow at time 0 in Fig. 3) to labeled temporins A or B quickly decreased peptide fluorescence, in a dose-dependent manner, suggesting oligomerization of the two peptides. The strongest effect was achieved when the peptide:LPS ratio was 1:4. The addition of proteinase K (right arrow in Fig. 3) to LPS-treated rho-temporins A and B caused a rapid enhancement of fluorescence up to the initial level measured prior to the addition of LPS, thus confirming the induction of peptide assembly by LPS O26:B6. In contrast, the addition of LPS to rho-temporin L did not significantly affect its fluorescence, but raised it when the peptide:LPS molar ratio increased to 1:4 (Fig. 3C), highlighting a partial disaggregation of this peptide after contact with the endotoxin. Accordingly, proteolytic digestion of LPS-treated rho-temporin L induced an additional increase of fluorescence. Altogether, the overall results with LPS O26:B6 were quite similar to those previously reported for each of these temporins with LPS O111:B4 (15).

The Effect of Unlabeled Temporins on the Fluorescence of Rhodamine-labeled Peptides—To understand the reason for the lack of synergism in the activity of temporins A + L and B + L on E. coli O26:B6, we investigated the effect of temporin L on the oligomerization of A and B in the presence of LPS O26:B6 (Fig. 4) and compared it with that of LPS O111:B4 (15). More specifically, we recorded the fluorescence of each rho-peptide, alone and in combination with equimolar concentrations of each of the unlabeled temporins, before and after the addition of LPS (arrow at time 0 in Fig. 4). Proteinase K was then added after 30–40 min (right arrow in Fig. 4). The data revealed that the fluorescence quenching of rho-temporins A and B, after the addition of LPS O26:B6 (rho-temporin:LPS ratio, 1:4), did not change when the two peptides were combined with an equimolar concentration of the unlabeled A or B (Fig. 4, A and B). Note that the same results were also obtained when the two rhodamine-labeled peptides were mixed with the unlabeled temporin L (Fig. 4, A and B). This is in contrast with the ability of temporin L to prevent the fluorescence quenching of rho-temporins A and B, when in contact with LPS O111:B4 (15). Therefore, these data indicate that temporin L cannot inhibit the oligomerization of A and B, induced by LPS O26:B6. Furthermore, when unlabeled temporin A, B, or L was mixed with rho-temporin L (Fig. 4C), no increase in peptide fluorescence was detected upon the addition of LPS O26:B6, thus supporting the notion that LPS-in-

**TABLE 1**

| Bacterial strains | MIC<sup>a</sup> | FIC index<sup>b</sup> |
|-------------------|-----------------|-----------------------|
|                   | Temp A | Temp B | Temp L | Temp A + L | Temp B + L | Temp A + B |
| E. coli O111:B4   | 100   | 50    | 10     | 0.48      | 0.50       | 0.56       |
| E. coli O26:B6    | 50    | 50    | 10     | 0.56      | 0.61       | 0.56       |
| E. coli D21 e7    | 25    | 25    | 2.5    | 0.56      | 0.58       | 0.56       |
| E. coli D21 f1    | 12.5  | 25    | 2.5    | 0.56      | 0.57       | 0.57       |
| E. coli D21 f2    | 6.25  | 12.5  | 2.5    | 0.66      | 0.90       | 0.56       |

<sup>a</sup> MICs are presented as average values from three independent measurements.

<sup>b</sup> FIC indices were interpreted as follows: FIC ≤ 0.5, synergism; <0.5 FIC < 1, additivity; ≤1 FIC < 4, indifference; FIC ≥ 4.0, antagonism.
duced disaggregation of temporin L did not take place at a peptide:LPS molar ratio lower than 1:4.

Finally, to further verify that the results gained with LPS O111:B4 and LPS O26:B6 were associated with differences in their own biophysical properties (e.g. the length of the polysaccharide chain), we carried out the same experiments with LPS from *E. coli* O55:B5 (LPS O55:B5) bearing a longer O-antigen region (32). The results proved that temporin L hampered the oligomerization of A and B caused by LPS O55:B5 (data not shown), as manifested with LPS O111:B4 (15). These findings underline the importance of long polysaccharide chains of LPS in the synergism between AMPs in the antimicrobial activity.

**Synergism between Temporins in LPS-Detoxification Activity**—We examined the effect of temporins alone, at different concentrations, and when combined with each other, in inhibiting LPS-induced pro-inflammatory cytokines secretion by macrophages. The capacity to suppress TNF-α and IL-6 secretion was found to be significantly higher for temporin L, either when macrophages were stimulated with LPS O26:B6 or LPS O111:B4 (Fig. 5, A and C). In contrast, 10 μM temporins A and B caused about 20% inhibition of TNF-α release by LPS O26:B6-stimulated macrophages, but practically no neutralizing activity was detected when the cells were treated with LPS O111:B4 (Fig. 5A). Interestingly, when temporin L was mixed with temporin A or B, the anti-endotoxin activity (inhibition of TNF-α secretion) was found to be significantly greater than the corresponding additive effect of the single peptides, when mac-
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FIGURE 5. The effect of temporins on TNF-α and IL-6 secretion by LPS-activated macrophages. RAW 264.7 macrophages were stimulated with LPS (10 ng/ml) derived from E. coli O111:B4 or O26:B6 in the presence of 2.5, 5, and 10 μM temporin A, temporin B, or temporin L (A). In another set of experiments, LPS-stimulated macrophages were incubated with 2.5 μM temporins, alone or in combinations of two (A + B, A + L or B + L, 2.5 + 2.5 μM) (B and C). The percentage of inhibition of TNF-α (panel B) or IL-6 (panel C) release was normalized to that of macrophages stimulated with LPS without peptides (0% inhibition). The results are the average of three independent experiments; each experiment was performed in duplicate. Error bars are S.D.

rophones were activated by LPS O26:B6 (Fig. 5B). The subsequent experiments point out to a specific synergistic effect in the inhibition of TNF-α from LPS-O26:B6 stimulated macrophages and that this process is not related to the higher ability of temporin L to interact with the membrane bilayers, and therefore affect the release of any macrophage-produced cytokines. (ii) All three temporins alone at various concentrations (up to 10-fold higher than those used for LPS neutralization) were tested for their ability to kill macrophages and to affect the secretion of IL-6 and TNF-α. The data revealed that all peptides were not toxic toward macrophages, even at the highest concentration used, and that they had no effects on TNF-α as well as on IL-6 release, in the absence of LPS (data not shown). (ii) No synergism was detected in the inhibition of IL-6 secretion from LPS-activated macrophages (Fig. 5C). (iii) Calorimetric studies and light scattering analysis were consistent with the ability of temporin L to synergize with temporins A and B toward LPS O26:B6 and not LPS O111:B4 (see below). Overall, these results support the notion that the synergism of temporins A + L and B + L in the inhibition of TNF-α release is not the result of an unspecific interaction of temporin L with the macrophage cell membrane, but rather a process that depends on the type of peptide combination and LPS used as well as on the cytokine secretion pathway.

Calorimetric Titration for the Binding of Temporins to LPS—To shed light on the molecular mechanism underlying the synergistic effect of temporins on anti-endotoxin activity, we first studied, by ITC, the thermodynamic aspects of the interaction between temporins and the two types of LPS. Several ITC studies dealing with the binding of AMPs to LPS were reported (53–55). To keep conditions similar to those used in the biological assays, we conducted ITC measurements at a physiological temperature (38 °C) at which the species of LPS used are in the liquid crystalline phase. Injection of each temporin into LPS O26:B6 in buffer produced an endothermic biphasic binding curve (Fig. 6, A–C). When equimolar mixtures of temporins A + L or B + L were injected, an exothermic process was obtained, denoting the prevalence of electrostatic peptide-LPS interactions (Fig. 6, D and E). At the end of the titrations, no turbidity was observed in the samples, meaning solubilization of the LPS structure took place (see below). In contrast, when equimolar mixtures of temporins A + B were titrated into LPS O26:B6, an endothermic heat was produced similar to what has been found when individual temporins were injected into LPS, but with a smaller magnitude (Fig. 6F). Overall, there is a large qualitative difference between the results obtained with A + B and those with A + L and B + L. This is in agreement with the findings that A + L and B + L are the only peptide combinations that show a synergistic effect in the neutralization of LPS O26: B6. At 38 °C, the electrostatic attraction of the individual cationic temporins A, B, or L with LPS O26:B6 (which would in itself result in an exothermic process) was superseded by an endothermically driven reaction, probably involving disruption of hydrogen bonds in ordered water molecules around the lipid A backbone. An endothermic biphasic titration was also found when the individual temporins A, B, and L were treated with the gel state of LPS O26:B6 (data at 15 °C, not shown). The balance between the ability of the peptides to interact with the electrostatic and hydrophobic portions of LPS O26:B6 determines whether the overall process will be an entropically or enthalpically driven interaction.

When LPS O111:B4 was titrated at 38 °C with single or mixed temporins, the binding reactions were exothermic in all cases (Fig. 7, A–F), with only tiny differences among them; thus, indicating that strong electrostatic interactions predominate in this LPS species with longer carbohydrate chains.

Quasi-elastic Light Scattering Data Analysis—Analysis of the QUELS data shows that LPS structures can disaggregate in the presence of AMPs to smaller size-mixed populations, or alternatively, that a more compact structure of well-defined size can be generated.

QUELS of LPS O26:B6 disclosed a polydisperse sample with particles having a diameter centered at 235 and 860 nm (Fig. 8A). Titration with a single temporin caused a shift of the average size of LPS to lower values centered at 150–200 nm, but with a high degree of polydispersity (Fig. 8B). When this LPS was titrated with equimolar mixtures of temporins A + L or
B + L, two size-populations of LPS, centered at 85 and 210 nm, respectively, were formed (Fig. 8C), indicating a partial shift of the population to a smaller size. Interestingly, titration with temporins A + B, at equimolar concentration, produced a single polydisperse fraction centered at 190 nm (Fig. 8D). It is striking that this result was quite similar, in size and polydispersity, to what was obtained with each single peptide, and different from what was found for the synergistic mixtures of A + L and B + L, both giving a bimodal distribution (Fig. 8C).

QUELS were also performed with LPS O111:B4, in the absence of EDTA (Fig. 9A). The presence of single temporins as well as combinations of A + L and B + L promoted the formation of a narrow-sized distribution of ~45 nm (Fig. 9, B and C). This resulted from a higher degree of LPS compactness in the overall structure, presumably because of temporins occupying Mg$^{2+}$ binding sites with high affinity and to the displacement of water molecules (52). In contrast, the equimolar mixture of temporins A + B was able to produce a very polydisperse fraction centered at 125 and 519 nm (Fig. 9D). This result suggests that the structure of LPS O111:B4 was affected by the combination of temporins A + B in a different way than individual peptides or their mixtures with temporin L, increasing the polydispersity and particle size of LPS.
DISCUSSION

LPS is a structural component of the outer membrane of Gram-negative bacteria and has two important functions. First, it serves as an efficient barrier to protect bacteria from a variety of hydrophilic and hydrophobic molecules (16, 17). Second, when LPS is recognized by the innate immune system cells, it induces a pro-inflammatory host response, which, in extreme cases, can lead to the development of the clinical syndrome of septic shock (25, 56).

The interesting findings in this study are that temporins can synergize and overcome both the protective function of LPS and its endotoxic effect. Furthermore, these properties depend on the type of LPS surrounding the bacteria. Importantly, the molecular mechanisms governing the two synergistic effects are different.

Synergism in Antimicrobial Activity—A synergistic effect in antimicrobial activity was reported in only a few cases besides temporins. These include the combination of magainin 2 and PGLa from *Xenopus laevis* (57), between different isoforms of dermaseptins (58), cathelicidins, and defensins (59), and between hepcidin and moronecidin, isolated from bass gill tissue (60). However, except for magainin-2 and PGLa, which were found to form heterodimers in solution, and partially for temporins (as explained below), the molecular mechanism...
accounting for these findings is not yet known. We have previously shown that the synergism between temporins A+L and B+L in antibacterial activity is related to the ability of temporin L to hamper the aggregation of A or B within LPS O111:B4, thus allowing their translocation across the bacterial cell wall into the target inner membrane (15). Here we demonstrated that this effect depends upon the type of LPS used, because temporin L loses its ability to disaggregate temporins A and B when in contact with LPS with shorter polysaccharide chains (e.g. LPS O26:B6), compared with LPS with a longer carbohydrate portion (e.g. LPS O111:B4 and LPS O55:B5). However, we cannot rule out the possibility that beside LPS, additional factors (e.g. modifications of peptidoglycan and other cell surface components) contribute to reduce the susceptibility of Gram-negative bacteria to AMPs and perhaps compensate those mechanisms that bacteria use to resist AMPs in general.

**Synergism in Anti-endotoxin Activity**—In this work, we have also demonstrated that all three temporins can suppress the endotoxic effect of LPS, as reflected by their ability to decrease the secretion of TNF-α (a primary mediator of endotoxemia, Ref. 61), by LPS-stimulated macrophages. Such reduction was more evident when macrophages were activated with LPS O26:B6 (Fig. 5A). This is probably related to differences in the size of the polysaccharide chains of the two types of LPS. It is reasonable to assume that the overall shorter saccharidic portion of LPS O26:B6 should make it easier for the peptides to reach and neutralize the endotoxic part of LPS, which is the lipid A. Our ITC measurements revealed that the addition of temporins to LPS O26:B6 gave rise to an initial LPS disaggregation (increase of enthalpy) up to an approximate peptide:LPS molar ratio of 0.2 (Fig. 6). As a consequence, the peptide binding sites on LPS might become more accessible and available to favor an exothermically driven binding of temporins (enthalpy decrease).

Recent studies have suggested that one of the requirements for an AMP to detoxify LPS is its ability to reduce the state of aggregation of the LPS, probably by the formation of small complexes between LPS and the peptide (15). When temporins A+L or B+L were combined, a change in the size of the LPS O26:B6 aggregates was discerned, including the apparent formation of a smaller sized LPS population (Fig. 8) with a distri-
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bution that differs from that found when LPS O26:B6 was treated with each peptide alone or with the combination of temporins A + B. This change in the LPS structure could expose additional Mg$^{2+}$ binding sites, otherwise hidden within the LPS aggregates, so that electrostatic interactions with the cationic peptides would be more favorable. This interpretation is in line with the exothermic nature of the binding reactions of temporins A + L and B + L to LPS O26:B6 (Fig. 6, D and E). Notably, the dissociation of LPS O26:B6 aggregates to smaller vesicles upon contact with equimolar mixtures of A + L or B + L correlated quite well with the synergistic effect of these combinations of peptides in LPS detoxification (Fig. 5B). To the best of our knowledge, this is the first case showing a synergism between AMPs in the LPS neutralizing activity, along with its plausible mode of action.

In contrast, when temporins were added (either alone or in the combinations A + L and B + L) to LPS O111:B4, they were all able to produce exothermic binding reactions (Fig. 7) and an overall shift to a smaller sized structure of LPS. However, an exception was given by the equimolar mixture of temporins A and B, which, differently, increased the polydispersity and particle size of LPS (Fig. 9). Notably, the effects of temporins A + L and B + L on both the enthalpy of reactions as well as on the size of the LPS O111:B4 aggregate was similar to that of the individual temporins. These findings are in agreement with the observation that A + L and B + L pairs did not synergize in the suppression of TNF-α secretion by LPS O111:B4-stimulated macrophages.

Overall, this work reports two important findings. (i) The synergistic effect of temporins in the antimicrobial and anti-endotoxin activities inversely depends on the size of the LPS-carbohydrate chains. The synergistic effect in the microbial killing occurs against bacterial strains endowed with long LPS-polysaccharide chains. In contrast, the synergistic effect in the LPS detoxification occurs with LPS having saccharidic portions with a short length. (ii) The molecular mechanisms underlying the two types of synergism are different. With regard to the antimicrobial activity, it would be based on the ability of the pairs of temporins to disaggregate upon their binding to the bacterial OM, which makes it easier for them to traverse into the inner microbial membrane. The extent of peptide disaggregation is less pronounced with LPS containing short sugar side chains. Such a phenomenon might be hindered by a change in the OM fluidity, which has been shown to decrease significantly as the LPS-polysaccharide region reduces its length and becomes less disordered (62, 63). This can be interpreted in part to result from the reaction between the divalent cations and the negatively charged groups, mainly the phosphate groups of the disaccharide moiety of lipid A. Indeed, this should rigidify the structure of LPS to a higher degree than that of LPS with longer sugar chains, to which Mg$^{2+}$ cations can distribute over a much larger region (64), also increasing the volume occupied by water molecules, in a disordered assembly (63).

With regard to the synergistic effect in the LPS detoxification, it relies on the ability of the pairs of temporins to induce a perturbation of the LPS structure larger than that induced by each individual peptide alone. This process should be more efficient with (i) LPS having short carbohydrate side chains, and (ii) peptide molecules folded in an oligomeric state, which would presumably enhance the disruption of the LPS micelles. This is consistent with previous reports showing that the LPS structure is altered more efficiently by peptide dimers than the respective single monomers (65). Overall, the two synergistic effects between AMPs from the same frog specimen can in part explain the ability of amphibia to protect themselves from infections of a large repertoire of microorganisms as well as their lacking sensitivity to the toxic effects of LPS (66, 67). In addition, these studies should help in the future development of non-single peptide-based therapeutic strategies to kill multidrug-resistant Gram-negative bacteria and/or neutralize the effect of their LPS, subsequently released.

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REFERENCES

1. Boman, H. G. (1995) Annu. Rev. Immunol. 13, 61–92
2. Ganz, T. (2003) Nat. Rev. Immunol. 3, 710–720
3. Zasloff, M. (2002) Nature 415, 389–395
4. Yang, D., Biragyn, A., Hoover, D. M., Lukbowski, J., and Oppenheim, J. J. (2004) Annu. Rev. Immunol. 22, 181–215
5. Hancock, R. E., Brown, K. L., and Mookherjee, N. (2006) Immunobiology 211, 315–322
6. Brown, K. L., and Hancock, R. E. (2006) Curr. Opin. Immunol. 18, 24–30
7. Bulet, P., Stocklin, R., and Menin, L. (2004) Biochemistry 43, 169–184
8. Hancock, R. E., and Rozek, A. (2002) FEBS Lett. 526, 143–149
9. Santarum, N. (2006) Curr. Med. Chem. 13, 679–696
10. Bonten, M. J. N., and Chai, Y. (2002) Biopolymers 66, 236–248
11. Yeaman, M. R., and Yount, N. T. (2003) Pharmacol. Rev. 55, 27–55
12. Lohner, K., and Blondelle, S. E. (2005) Comb. Chem. High Throughput Screen. 8, 241–256
13. Nikaido, H. (1994) Science 264, 382–388
14. Nikaido, H., and Vaara, M. (1985) Microbiol. Rev. 49, 1–32
15. Rosenfeld, Y., Barra, D., Simmaco, M., Shai, Y., and Mangoni, M. L. (2006) J. Biol. Chem. 281, 28565–28574
16. Papo, N., and Shai, Y. (2005) J. Biol. Chem. 280, 10378–10387
17. Sal-man, N., Oren, Z., and Shai, Y. (2002) Biochemistry 41, 11921–11930
18. Trent, M. S., Stead, C. M., Tran, A. X., and Hankins, J. V. (2006) J. Endo-toxin Res. 12, 205–223
19. Rietschel, E. T., Kirikae, T., Schade, F. U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, A. J., Zahringer, U., Seydel, U., Di Padova, F., Schreier, M., and Brade, H. (1994) Faseb J. 8, 217–225
20. Kubo, Y., Fukushima, N., Yoshio, K., Kawasoe, Y., Iriuchi, S., Imao, N., Yasui, Y., Matsu, N., and Akagi, M. (2007) Inflamm. Res. 56, 70–75
21. Gee, K., Kozlovec, M., and Kumar, A. (2003) J. Biol. Chem. 278, 37275–37287
22. Mukhopadhyay, S., Herre, J., Brown, G. D., and Gordon, S. (2004) Immunology 112, 521–530
23. Prins, J. M., Kuijpers, J. M., Mevissen, M. L., Speelman, P., and van Deventer, S. J. (1995) Infect. Immun. 63, 2236–2242
24. Byl, B., Clevenbergh, P., Kentos, A., Jacobs, F., Marchant, A., Vincent, J. L., Brade, H., Ulmer, A. J., Zahringer, U., Seydel, U., Di Padova, F., Schreier, M., and Brade, H. (1994) Faseb J. 8, 217–225
25. Cohen, J. (2002) Nature 420, 885–891
26. Angus, D. C., and Wax, R. S. (2001) Crit. Care Med. 29, S109–116
27. Hancock, R. E., and Diamond, G. (2000) Trends Microbiol. 8, 402–410
28. Scott, M. G., Davidson, D. J., Gold, M. R., Bowdish, D., and Hancock, R. E. (2002) J. Immunol. 169, 3883–3891
29. Bommineni, Y. R., Dai, H., Gong, Y. X., Soulages, J. L., Fernandez, S. C., Desilva, U., Prakash, O., and Zhang, G. (2007) Infect. Immun. 75, 418–428
30. Mookherjee, N., Rehaume, L. M., and Hancock, R. E. (2007) Expert Opin. Ther. Targets 11, 993–1004
Temporin Synergy in Antimicrobial/Anti-endotoxin Activities

31. Mangoni, M. L., Marcellini, H. G., and Simmaco, M. (2007) *J. Pept. Sci.* **13**, 603–613.
32. Simmaco, M., Mignogna, G., Canofeni, S., Miele, R., Mangoni, M. L., and Barra, D. (1996) *Eur. J. Biochem.* **242**, 788–792.
33. Simmaco, M. L. (2006) *Cell Mol. Life Sci.* **63**, 1060–1069.
34. Mangoni, M. L., Rinaldi, A. C., Di Giulio, A., Mignogna, G., Bozzi, A., Barra, D., and Simmaco, M. (2000) *Eur. J. Biochem.* **267**, 1447–1454.
35. Mangoni, M. L., Papo, N., Barra, D., Simmaco, M., Bozzi, A., Di Giulio, A., and Rinaldi, A. C. (2004) *Biochem. J.* **380**, 859–865.
36. Mangoni, M. L., Saugar, J. M., Dellisanti, M., Barra, D., Simmaco, M., and Rivas, L. (2005) *J. Biol. Chem.* **280**, 984–990.
37. Giacometti, A., Cirioni, O., Ghiselli, R., Mocchegiani, F., Orlando, F., Silvestri, C., Bozzi, A., Di Giulio, A., Luzi, C., Mangoni, M. L., Barra, D., Saba, V., Scalise, G., and Rinaldi, A. C. (2006) *Antimicrob. Agents Chemother.* **50**, 2478–2486.
38. Peterson, A. A., and McGroarty, E. J. (1985) *J. Bacteriol.* **162**, 738–745.
39. Lewis, R. E., Diekema, D. J., Messer, S. A., Pfaller, M. A., and Klepser, M. E. (2002) *J. Antimicrob. Chemother.* **49**, 345–351.
40. Boman, H. G., and Monner, D. A. (1975) *J. Bacteriol.* **121**, 455–464.
41. Farnaud, S., Spiller, C., Moriarty, L. C., Patel, A., Gant, V., Odell, E. W., and Evans, R. W. (2004) *FEMS Microbiol. Lett.* **233**, 193–199.
42. Marvin, H. J., ter Beest, M. B., and Witholt, B. (1989) *J. Bacteriol.* **171**, 5262–5267.
43. Papo, N., Oren, Z., Pag, U., Sahl, H. G., and Shai, Y. (2002) *Biochem. J.* **380**, 859–865.
44. Lee, C. H., and Tsai, C. M. (1999) *Anal. Biochem.* **267**, 161–168.
45. Ribi, E., Anacker, R. L., Brown, R., Haskins, W. T., Malmgren, B., Milner, K. C., and Rudbach, J. A. (1966) *J. Bacteriol.* **92**, 1493–1509.
46. McIntire, F. C., Barlow, G. H., Sievert, H. W., Finley, R. A., and Yoo, A. L. (1969) *Biochemistry* **8**, 4063–4067.
47. Srimal, S., Surolia, N., Balasubramanian, S., and Surolia, A. (1996) *Biochem. J.* **315**, 679–686.
48. Aurell, C. A., and Wistrom, A. O. (1998) *Biochem. Biophys. Res. Commun.* **253**, 119–123.
49. Odds, F. C. (2003) *J. Antimicrob. Chemother.* **52**, 1.
50. Pag, U., Oedekoven, M., Papo, N., Oren, Z., Shai, Y., and Sahl, H. G. (2004) *J. Antimicrob. Chemother.* **53**, 230–239.
51. Ghosh, I. K., Shaool, D., Guillaud, P., Cicero, L., Mazier, D., Kustanovich, L., Shai, Y., and Mor, A. (1997) *J. Biol. Chem.* **272**, 31609–31616.
52. Lindberg, B., Lindh, F., and Lonngren, J. (1981) *Carbohydr. Res.* **97**, 105–112.
53. Brandenburg, K., David, A., Howe, J., Koch, M. H., Andra, J., and Garidel, P. (2005) *Biophys. J.* **88**, 1845–1858.
54. Howe, J., Andra, J., Conde, R., Iriarte, M., Garidel, P., Koch, M. H., Gutsmann, T., Moriyo, I., and Brandenburg, K. (2007) *Biophys. J.* **92**, 2796–2805.
55. Andra, J., Howe, J., Garidel, P., Rosslle, M., Richter, W., Leiva-Leon, J., Moriyo, I., Bartels, R., Gutsmann, T., and Brandenburg, K. (2007) *Biochem. J.* **406**, 297–307.
56. Stone, R. (1994) Science **264**, 365–367.
57. Soravia, E., Martini, G., and Zasloff, M. (1998) *FEBS Lett.* **228**, 337–340.
58. Mor, A., Hani, K., and Nicolas, P. (1994) *J. Biol. Chem.* **269**, 31635–31641.
59. Nagaoka, I., Hirota, S., Yomogida, S., Ohwada, A., and Hirata, M. (2000) *Inflamm. Res.* **49**, 73–79.
60. Lauth, X., Babon, J. J., Stannard, J. A., Singh, S., Nizet, V., Carlberg, J. M., Ostland, V. E., Pennington, M. W., Norton, R. S., and Westerman, M. E. (2005) *J. Biol. Chem.* **280**, 9272–9282.
61. Lehmann, V., Freudenberg, M. A., and Galanos, C. (1987) *J. Exp. Med.* **165**, 657–663.
62. Rana, F. R., Macias, E. A., Sultany, C. M., Modrakowski, M. C., and Blazyk, J. (1991) *Biochemistry* **30**, 5858–5866.
63. Tong, J., and McIntosh, T. J. (2004) *Biophys. J.* **86**, 3759–3771.
64. Brandenburg, K., and Seydel, U. (1990) *Eur. J. Biochem.* **191**, 229–236.
65. Li, P., Wohland, T., Ho, B., and Ding, J. L. (2004) *J. Biol. Chem.* **279**, 50150–50156.
66. Berczi, I., Bertok, L., and Bereznai, T. (1966) *Can. J. Microbiol.* **12**, 1070–1071.
67. Iliev, D. B., Roach, J. C., Mackenzie, S., Planas, J. V., and Goetz, F. W. (2005) *FEBS Lett.* **579**, 6519–6528.