ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 2006, p. 2478–2486 Vol. 50, No. 7
0066-4804/06/$08.00+0 doi:10.1128/AAC.01553-05


# Interaction of Antimicrobial Peptide Temporin L with Lipopolysaccharide In Vitro and in Experimental Rat Models of Septic Shock Caused by Gram-Negative Bacteria†

Andrea Giacometti,[1] Oscar Cirioni,[1] Roberto Ghiselli,[2] Federico Mocchegiani,[2] Fiorenza Orlando,[3] Carmela Silvestri,[1] Argante Bozzi,[4] Antonio Di Giulio,[4] Carla Luzi,[4] Maria Luisa Mangoni,[5] Donatella Barra,[5] Vittorio Saba,[2] Giorgio Scalise,[1] and Andrea C. Rinaldi[6]*

*Institute of Infectious Diseases and Public Health, University of Ancona, Ancona, Italy[1]; Department of General Surgery, I.N.R.C.A. I.R.R.C.S., University of Ancona, Ancona, Italy[2]; Biotechnology Centre, Research Department, I.N.R.C.A. I.R.R.C.S., Ancona, Italy[3]; Department of Biomedical Sciences and Technologies, University of L'Aquila, L'Aquila, Italy[4]; Department of Biochemical Sciences "A. Rossi Fanelli," S. Andrea Hospital, and CNR Molecular Biology Centre, University of Rome "La Sapienza," Rome, Italy[5]; and Department of Biomedical Sciences and Technologies, University of Cagliari, Monserrato (CA), Italy[6]*

Received 6 December 2005/Returned for modification 12 February 2006/Accepted 8 April 2006

**Sepsis remains a major cause of morbidity and mortality in hospitalized patients, despite intense efforts to improve survival. The primary lead for septic shock results from activation of host effector cells by endotoxin, the lipopolysaccharide (LPS) associated with cell membranes of gram-negative bacteria. For these reasons, the quest for compounds with antiendotoxin properties is actively pursued. We investigated the efficacy of the amphibian skin antimicrobial peptide temporin L in binding *Escherichia coli* LPS in vitro and counteracting its effects in vivo. Temporin L strongly bound to purified *E. coli* LPS and lipid A in vitro, as proven by fluorescent displacement assay, and readily penetrated into *E. coli* LPS monolayers. Furthermore, the killing activity of temporin L against *E. coli* was progressively inhibited by increasing concentrations of LPS added to the medium, further confirming the peptide's affinity for endotoxin. Antimicrobial assays showed that temporin L interacted synergistically with the clinically used β-lactam antibiotics piperacillin and imipenem. Therefore, we characterized the activity of temporin L when combined with imipenem and piperacillin in the prevention of lethality in two rat models of septic shock, measuring bacterial growth in blood and intra-abdominal fluid, endotoxin and tumor necrosis factor alpha (TNF-α) concentrations in plasma, and lethality. With respect to controls and single-drug treatments, the simultaneous administration of temporin L and β-lactams produced the highest antimicrobial activities and the strongest reduction in plasma endotoxin and TNF-α levels, resulting in the highest survival rates.**

Sepsis is a serious clinical problem, and despite intense efforts to improve survival, it remains a major cause of morbidity and mortality in patients hospitalized in intensive care units worldwide (2, 5, 13). The incidence of severe sepsis and septic shock is worryingly on the raise, in part as a result of the growing number of subjects immunocompromised because of medical interventions like organ transplantation or chemotherapy for cancer, or simply because in a globally aging population the number of people with weaker immune systems has expanded (15, 36). The primary cause of septic shock is activation of host effector cells by endotoxin, the lipopolysaccharide (LPS) component of the outer membrane of gram-negative bacteria. Recognition of LPS by the Toll-like receptors on the surface of macrophages permits pathogen sensing by the host and stimulates the innate immune response to contain microbial invasion (3, 4). At the same time, however, LPS signaling may overactivate the body's immune system, igniting a cascade of uncontrolled systemic inflammatory responses that can lead to multiple organ failure and eventually to death (3, 4). Treatment of sepsis relies largely on intravenous administration of antibiotics, but under some circumstances these conventional therapies may promote the further release of LPS from the cell envelope of killed bacteria, exacerbating sepsis itself (10). For this reason, the quest for novel compounds able to neutralize the effects of endotoxin is actively pursued.

Antimicrobial peptides (AMPs) are crucial humoral components of the innate immunity system of virtually all organisms, which they defend from the invasion of attacking pathogens (9, 25, 56). Hundreds of these gene-encoded peptides, usually ranging in size from 12 to 50 residues, have been isolated from bacteria, fungi, plants, and animals, including humans. Specialized, web-based repositories such as AMSDb (http://www.bbcm.univ.trieste.it/~tossi/pag1.htm) and ANTIMIC (6) store data on a vast number of AMPs. Inherently a heterogeneous class, AMPs differ greatly in structure and target a spectrum that includes gram-positive and -negative bacteria, yeasts and fungi, viruses, and protozoa. In several instances, AMPs have also been shown to efficiently kill cancer cells (41). Many AMPs can insert into and damage the cellular membrane as part of their killing mechanism (30, 46), and another body of evidence is growing that suggests that peptides might also cross the membrane and enter the cytoplasm of target cells to act as metabolic inhibitors (8). Besides their obvious role in counteracting

* Corresponding author. Mailing address: Department of Biomedical Sciences and Technologies, University of Cagliari, I-09042 Monserrato (CA), Italy. Phone: 39-070-6754521. Fax: 39-070-6754527. E-mail: rinaldi@unica.it.

† We dedicate this work to the memory of Prof. Augusto Rinaldi, colleague, friend, and guide in life, who prematurely passed away on 7 October 2005.

infections, a range of other biological functions related to host defense have been ascribed to different groups of AMPs, such as the mammalian defensins and cathelicidins, so that AMPs are sometimes also termed host defense peptides (20, 28, 39, 40). In addition to their antimicrobial activities, many AMPs also bind strongly to LPS, a property required for those peptides selective for gram-negative bacteria which must interact with the outer membrane before reaching the cytoplasmic membrane and killing the cell (27). The possibility of developing AMPs as potential antiendotoxin agents has spurred a range of studies on the molecular mechanism of interaction of AMPs with LPS based on membrane model systems (1, 14, 29, 42, 53), as well as studies conducted in animal models to demonstrate the endotoxin-sequestrating and -neutralizing activities of peptides in vivo (12, 19, 21, 22). It is hoped that these investigations will lead to the identification of AMPs with antiendotoxin properties improved with respect to polymyxin B, the prototype of LPS-neutralizing peptides whose toxicity limits its use to nonsystemic applications, and to help in the design of peptides for future therapeutic purposes.

Among AMPs, temporins constitute a family of structurally and evolutionarily related linear and short peptides containing 10 to 14 amino acids. All are α-amidated at their carboxyl-terminal ends, bear a net positive charge at neutral pH, and have the potential to adopt an amphipathic α-helical structure upon interaction with membranes or in a mixed hydrophobic/hydrophilic environment. The first group of temporins were found in 1996 in the skin extracts of the European red frog *Rana temporaria* (49), and since then well more than 40 temporin-like peptides have been isolated, not only from the skin secretions of ranid amphibians but also from wasp venom (51). The consensus sequence of the 30 or so frog-derived temporins is FLPLIASLLSKLL-$NH_2$ (52). The spectrum of antimicrobial activity displayed by temporins is interestingly vast and diverse. They are generally most active against gram-positive bacteria, but some show considerable activity also against gram-negative bacteria and fungal pathogens, including *Candida albicans* and *Batrachochytrium dendrobatidis*, a pathogen associated with global amphibian declines (33, 44, 45, 49). Furthermore, selected temporins have been recently shown to exert a potent killing activity against the human parasitic protozoan *Leishmania* (34). Studies aimed at understanding the killing mechanism of temporins have ascertained that these peptides alter the permeability of bacterial cell membrane in a dose-dependent manner without destroying cell integrity, leading to leakage of cytosolic content and cell death (32). Biophysical investigations have been performed using membrane model systems confirming that temporins bind and permeate membranes with different lipid compositions (33, 44, 58, 59). However, the possibility that temporins might act in vivo by translocating through the bacterial cell membrane and interacting with an intracellular target cannot be excluded at present.

We have recently undergone a research project aimed at assaying the antiendotoxin properties of temporins, choosing temporin L (Fig. 1) as a working model. Indeed, this peptide proved to have the highest antimicrobial potency among tested temporins, especially against gram-negative bacteria, and was therefore subjected to in-depth investigation to understand its mode(s) of action (32, 34, 44, 58, 59). However, no information is currently available for this molecule—and for temporins in general—as for its interactions with the outer membrane of gram-negative bacteria and thus its ability to bind and neutralize endotoxin. To fill this gap, we here characterized the in vitro LPS-binding properties of temporin L by means of biochemical and biophysical assays and investigated its efficacy, when combined with selected β-lactam conventional antibiotics imipenem and piperacillin, in the prevention of lethality in two rat models of septic shock.

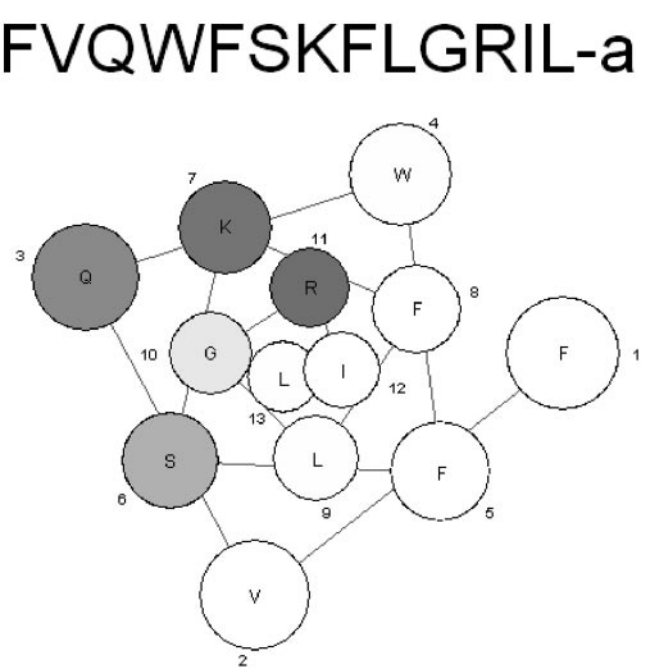


FIG. 1. Temporin L sequence and helical wheel plot. Residue shading is assigned on the basis of the Eisenberg consensus scale of hydrophobicity (16): charged or hydrophilic residues are in gray, and hydrophobic residues are in white. The peptide is amidated at its C terminus.

## MATERIALS AND METHODS

**Reagents and microorganisms.** Synthetic temporin L was purchased from SynPep Corporation (Dublin, Calif.). The purity of the peptide and its sequence and concentration were determined as previously described (33). It was either dissolved in distilled $H_2O$ at 20 times the required maximal concentration or dissolved in 20% ethanol. Successively, for in vitro studies, serial dilutions of the peptide were prepared in 0.01% acetic acid containing 0.2% bovine serum albumin in polypropylene tubes, while for in vivo experiments, it was diluted in physiological saline. LPS from *Escherichia coli* serotype O111:B4 was purchased from Sigma-Aldrich (Milan, Italy), prepared in sterile saline, aliquoted, and stored at −80°C for short periods or directly dissolved in organic solvents as described below. Piperacillin (Wieth Lederle, Aprilia, Italy) and imipenem (Merck, Sharp & Dohme, Milan, Italy) powders were diluted in accordance with the manufacturers' recommendations. Solutions were made fresh on the day of assay. All other chemicals used were of reagent grade. For antimicrobial assays and in vivo experiments, the commercially available quality control strain of *E. coli* ATCC 25922 was used.

**Animals.** Adult male Wistar rats (weight range, 250 to 300 g) were used for all of the experiments. All animals were housed singly in standard cages and had access to chow and water ad libitum throughout the study. The environment was temperature and humidity controlled, with lights on and off at 0630 a.m. and 0630 p.m. The study was approved by the animal research ethics committee of the I.N.R.C.A. I.R.R.C.S., University of Ancona.

**Penetration into LPS monolayers and measurement of LPS-binding activity.** Insertion of temporin L into LPS monolayers spread at an air-buffer (5 mM HEPES, pH 7) interface was monitored by measuring surface pressure ($\pi$) with a Wilhelmy wire attached to a microbalance (DeltaPi; Kibron Inc., Helsinki,

Finland) connected to a personal computer and using circular glass wells (subphase volume, 0.5 ml). After evaporation of LPS solvent (chloroform-methanol-water at 17:7:1) and stabilization of monolayers at different initial surface pressures ($\pi_0$), the peptide (0.1 to 2 μM) was injected into the subphase, and the increase in surface pressure of the LPS film upon intercalation of the peptide dissolved in the subphase was monitored for the next 35 min. The difference between the initial surface pressure and the value observed after the penetration of temporin L into the film was taken as $\Delta\pi$. Measurement of the temporin L ability to bind LPS and lipid A (diphosphoryl; from *E. coli* F583 [Sigma-Aldrich]) was performed by a fluorescent displacement assay using the probe BODIPY TR cadaverine (BC; Molecular Probes, Eugene, Oregon) as described elsewhere (54). All measurements were performed at room temperature.

**In vitro susceptibility testing.** Susceptibility testing was performed by the broth microdilution method according to the procedures outlined by the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) (38). However, since cationic peptides bind polystyrene, polypropylene 96-well plates (Sigma-Aldrich) were used instead of standard polystyrene plates. The MIC was taken as the lowest antibiotic concentration at which observable growth was inhibited. Experiments were performed in triplicate. Exponentially growing bacteria were resuspended in fresh Mueller-Hinton (MH) broth at approximately $10^7$ cells/ml to ensure accurate determination of the 99.9% killing endpoint and exposed to peptide at 4× MIC for 0, 5, 10, 15, 20, 25, 30, 40, 50, and 60 min at 37°C. After these times, samples were serially diluted in 10 mM of sodium HEPES buffer (pH 7.2) to minimize the carryover effect and plated onto MH agar plates to obtain viable colonies. Killing effect was defined as a 3-$\log_{10}$ reduction in vital organisms. In addition, combinations of temporin L with antibiotics of different nature were tested for synergistic effect by a checkerboard titration method. The ranges of drug dilutions used were 0.125 to 64 mg/liter for temporin L and 0.250 to 256 mg/liter for conventional antibiotics. The fractional inhibitory concentration (FIC) index for combinations of two antimicrobials was calculated according to the equation FIC index = $FIC_A + FIC_B = A/MIC_A + B/MIC_B$, where $A$ and $B$ are the MICs of drug A and drug B in the combination, $MIC_A$ and $MIC_B$ are the MICs of drug A and drug B alone, and $FIC_A$ and $FIC_B$ are the FICs of drug A and drug B. The FIC indexes were interpreted as follows: <0.5, synergy; 0.5 to 4.0, indifferent; >4.0, antagonism. The rate of killing of *E. coli* by temporin L was also measured in the presence of LPS. In this case, temporin L (10 μM) was preincubated with *E. coli* O111:B4 LPS (5 to 40 μM) for 30 min at 37°C, and the mixture was then added to a suspension of exponentially growing *E. coli* cells ($1 \times 10^7$/100 μl) in MH broth, incubating at 37°C. Aliquots were then withdrawn at different times, diluted in MH broth, and plated onto MH agar plates for the counting of CFU.

**In vivo experimental design.** Two experimental conditions were studied: (i) intraperitoneal administration of LPS and (ii) *E. coli*-induced peritonitis. Under the first condition, six groups, each containing 20 animals, were anesthetized by an intramuscular injection of ketamine (30 mg/kg of body weight) and injected intraperitoneally with 1.0 mg *E. coli* LPS in a total volume of 500 μl sterile saline. Immediately after injection, animals received intraperitoneally isotonic sodium chloride solution (control group $C_0$), 1 mg/kg temporin L, 20 mg/kg imipenem, or 120 mg/kg piperacillin (the latter two alone or combined with 1 mg/kg temporin L, respectively). Under the second condition, *E. coli* ATCC 25922 cells were grown in brain heart infusion broth. When bacteria were in the log phase of growth, the suspension was centrifuged at 1,000 × *g* for 15 min, the supernatant was discarded, and the bacteria were resuspended and diluted into sterile saline. All animals (six groups, each containing 20 animals) were anesthetized as described above. The abdomen of each animal was shaved and prepared with iodine. The rats received an intraperitoneal inoculum of 1 ml saline containing $2 \times 10^{10}$ CFU of *E. coli*. Immediately after bacterial challenge, animals received intraperitoneally isotonic sodium chloride solution (control group $C_1$), 1 mg/kg temporin L, 20 mg/kg imipenem, and 120 mg/kg piperacillin (the latter two alone or combined with 1 mg/kg temporin L, respectively).

**Evaluation of in vivo treatment.** After treatment, the animals were returned to individual cages and thoroughly examined daily. Depending on the specific experiment, the rate of positivity of blood cultures, quantitation of bacteria in the intra-abdominal fluid, rate of lethality, toxicity, and the levels of plasma endotoxin and tumor necrosis factor alpha (TNF-α) were evaluated. Following treatment, animals were monitored for the subsequent 72 h. For each animal model, toxicity was evaluated on the basis of the presence of any drug-related adverse effects: i.e., local signs of inflammation, anorexia, weight loss, vomiting, diarrhea, fever, and behavioral alterations. In particular, to evaluate the physiologic effects of temporin L, leukocyte count, rectal temperature, pulse, and breathing rate were monitored in a supplementary peptide-treated group without infection or LPS. In all models, the presence of systemic symptoms was defined in analogy to the criteria applied for humans. Each animal was considered to be septic if it satisfied at least two of the following criteria: (i) more than 12,000 or less 4,000 white blood cells per μl, (ii) rectal temperature above 38°C or below 36°C, and (iii) increased pulse rate and finally increased breathing rate. The quantitative bacterial counts in the peritoneal fluid from dead animals (model ii) were performed immediately after death. In the surviving animals, the counts were performed at 72 h postinjection. The surviving animals were killed with chloroform, and blood samples for culture were obtained by aseptic percutaneous transthoracic cardiac puncture. In addition, to perform quantitative evaluations of the bacteria in the intra-abdominal fluid, 10 ml of sterile saline was injected intraperitoneally, samples of the peritoneal lavage fluid were serially diluted, and a 0.1-ml volume of each dilution was spread onto blood agar plates. The limit of detection was ≤1 $\log_{10}$ CFU/ml. The plates were incubated both in air and under anaerobic conditions at 35°C for 48 h.

For determination of endotoxin and TNF-α levels in plasma, 0.2-ml blood samples were collected from the jugular vein after 0, 2, 6, and 12 h after injection into a sterile syringe and transferred to tubes containing EDTA tripotassium salt. During this time, a catheter was placed into the vein and sutured to the back of the rat. Endotoxin concentrations were measured by the commercially available *Limulus* amebocyte lysate test (E-TOXATE; Sigma-Aldrich). Plasma samples were serially diluted twofold with sterile endotoxin-free water and were heat treated for 5 min in a water bath at 75°C to destroy inhibitors that can interfere with the activation. The endotoxin content was determined as described by the manufacturer. Endotoxin standards were tested in each run, and the concentrations of endotoxin in the text samples (in endotoxin units [EU/ml]) were calculated by comparison with the standard curve. TNF-α levels were measured by a commercially available solid-phase sandwich enzyme-linked immunosorbent assay (Nuclear Laser Medicine, S.r.l., Settala, Italy) according to the protocol supplied by the manufacturer. The standards and samples were incubated with a TNF-α antibody coating a 96-well microtiter plate. The wells were washed with buffer and then incubated with biotinylated anti-TNF-α antibody conjugated to streptavidin-peroxidase. This was washed away, and the color was developed in the presence of chromogen (tetramethylbenzidine) substrate. The intensity of the color was measured in a microplate reader (MR 700; Dynatech Laboratories, Guernsey, United Kingdom) by reading the absorbance at 450 nm. The results for the samples were compared to the standard curve to determine the amount of TNF-α present. All samples were run in duplicate. The lower limit of sensitivity for TNF-α by this assay was 0.05 ng/ml. The intra-assay and interassay coefficients of variation were 6.3% and 8.1%, respectively.

**Statistical analysis.** MICs are presented as average values from three independent measurements. Mortality rates and qualitative results for blood cultures between groups were compared by use of Fisher's exact test (significance level fixed at 0.05). TNF-α mean values and quantitative evaluations of the bacteria in the intra-abdominal fluid cultures are presented as means ± standard deviations (SDs) of the mean; statistical comparisons between groups were made by analysis of variance (significance level was fixed at 0.05). Due to the presence of several values below the lower limit of sensitivity, plasma endotoxin levels were compared between groups by Kruskal-Wallis nonparametric test, adjusted for ties; the post hoc comparisons were performed by the Bonferroni method. Each comparison group contained 20 rats. Significance was accepted when the $P$ value was ≤0.05.

## RESULTS

**Endotoxin-binding properties of temporin L. (i) Penetration of temporin L into *E. coli* LPS monolayers.** Recently, monomolecular lipid and LPS films have been increasingly used as suitable model systems to investigate the interactions of a wide range of peptides and proteins with biological membranes of both prokaryotic and eukaryotic origins and with selected components of the bacterial outer membrane (7, 24, 31, 59). We therefore used the monolayer technique to get insights into the ability of temporin L to bind LPS and to mimic its interaction with *E. coli* outer membrane. Temporin L efficiently penetrated into *E. coli* LPS monolayers, as demonstrated by the increase in film surface pressure (Fig. 2). Under experimental conditions, monolayer penetration was dependent on peptide concentration, reaching substantial stability around 1.0 μM temporin L (Fig. 2A), which was therefore selected as the peptide concentration for subsequent experiments. When data

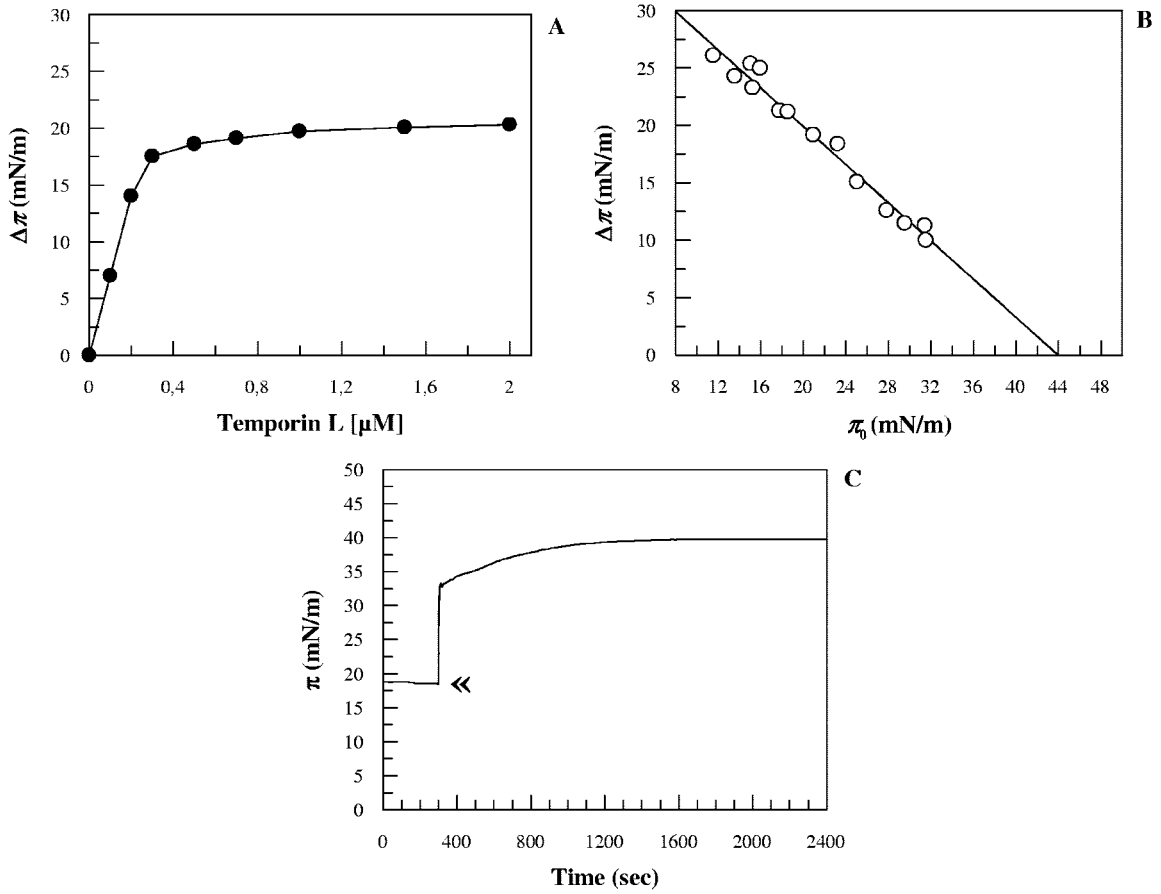


FIG. 2. Insertion of temporin L into *Escherichia coli* LPS monolayers. Increases of surface pressure of *E. coli* LPS monolayers due to the addition of temporin L (dissolved in EtOH 20%) into the subphase are illustrated as a function of peptide concentration (A), at an initial surface pressure varying between 18.7 and 19.3 mN/m, or initial surface pressure (B [with 1.0 μM peptide]). (C) Typical kinetics of surface pressure increase related to temporin L penetration into *E. coli* LPS monolayers ($\pi_0$ = 18.5, with 1.0 μM peptide; an arrow indicates peptide injection into the subphase).

from similar measurements were analyzed in terms of $\Delta\pi$ versus $\pi_0$, the critical surface pressure corresponding to the LPS lateral packing density preventing the intercalation of temporin L into *E. coli* LPS films could be derived by extrapolating the $\Delta\pi$-$\pi_0$ slope to $\Delta\pi = 0$, giving a value of ≈44 mN/m (Fig. 2B). The kinetics of the insertion of the peptide into the LPS monolayer were characterized by a rapid and marked increase in surface pressure that soon followed injection of the protein into the subphase, the lag phase for this process being too short to be measurable with our instrumentation (Fig. 2C). In a typical experiment, within the first 60 s after peptide injection $\pi$ attained slightly over 85% of that recorded at the end of measurement (Fig. 2C). This initial peak was then followed by a slower increase in $\pi$ for approximately the next 18 min, when a plateau was reached, and no more significant variation in $\pi$ was observable for at least the next 15 min. This general kinetics pattern was apparently independent from initial surface pressure and from peptide concentration.

**(ii) Determination of temporin L LPS-binding affinity.** To collect additional information on the temporin L-LPS modes of interaction, we used a fluorescent probe displacement method recently developed by Wood and colleagues (54). The fluorescent probe BC binds LPS, interacting specifically with its toxic center lipid A, probably via salt bridges with its glycosidic phosphate group, and the binding results in a progressive quenching of fluorescence. BC can then be competitively displaced by compounds displaying an affinity for lipid A, with a proportional dequencing of fluorescence. As shown in Fig. 3, temporin L binds to purified *E. coli* LPS and induces a displacement of BC with a quantitative effective displacement ($ED_{50}$) of approximately $1 \times 10^{-5}$ M. Similar results were obtained when purified lipid A was used instead of LPS (not shown).

**Antimicrobial assays. (i) In vitro susceptibility studies.** A preliminary screening was performed to verify whether temporin L interacted synergistically or additively with clinically used antibiotics of different structures against *E. coli* ATCC 25922. In our hands, a strong synergy was observed only when the peptide was combined with the β-lactam antibiotics piperacillin and imipenem (Table 1), with an FIC index equal in both cases to 0.28, and these compounds were thus selected for further studies. According to the broth microdilution method, *E. coli* ATCC 25922 showed different susceptibilities to the compounds tested: the MICs of temporin L, imipenem and piperacillin were 4.00 mg/liter, 0.12 mg/liter, and 0.25 mg/liter, respectively. In vitro time-kill evaluations showed a potent

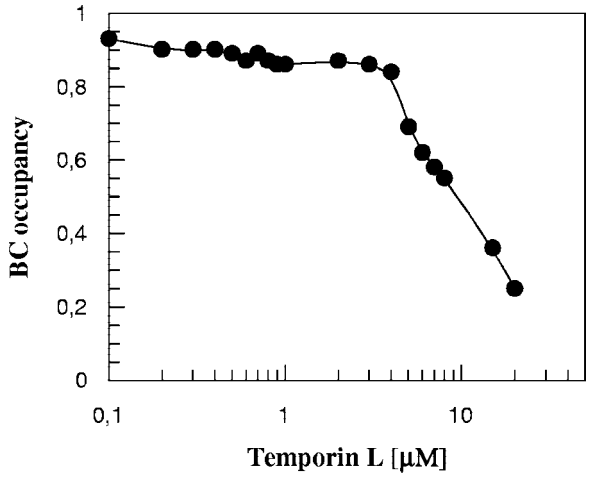


FIG. 3. BC fluorescent displacement assay. The fluorescent probe BC binds LPS, and the binding results in a progressive quenching of fluorescence (54). Temporin L binds *Escherichia coli* LPS, displacing BC from it and causing a proportional dequencing of BC fluorescence. LPS, 10 μg/ml; BC, 10 μM; buffer, 50 mM Tris, pH 7.4. Aliquots of temporin L (at 0.4 mM concentration) were successively added to the cuvette containing the BC-LPS complex, and the fluorescence was recorded. Excitation, 580 nm; emission, 620 nm.

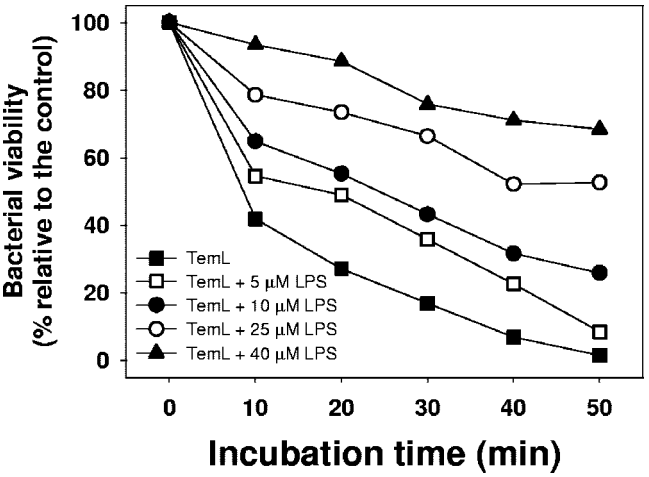


FIG. 4. Effects of LPS on the killing activity of temporin L against *Escherichia coli*. Temporin L (TemL [10 μM]) was preincubated with *E. coli* O111:B4 LPS (5 to 40 μM, solubilized in water) for 30 min at 37°C. These mixtures were then added to exponential-phase bacteria (approximately $10^7$ cells in MH broth) and incubated at 37°C. Aliquots were withdrawn at different times, diluted in MH broth, and plated onto MH agar plates for CFU counting. Data points are means of three independent experiments.

killing activity of temporin L against *E. coli*. In fact, killing by the peptide was shown to be the most rapid of the agents tested, its activity being complete after a 15-min exposure period, whereas killing by imipenem and piperacillin was complete after a 20- to 25-min exposure period (not shown).

**(ii) Effects of LPS on the bactericidal activity of temporin L.** To ascertain whether free LPS might inhibit or otherwise influence the antimicrobial activity of temporin L, we compared the killing effect exerted by the peptide on *E. coli* ATCC 25922 cells to that obtained when the same peptide was preincubated with different amounts of solubilized LPS. The results clearly show a reduction in the bactericidal activity of temporin L as the concentration of LPS increases (Fig. 4). Indeed, pretreatment of temporin L with a fourfold molar excess of LPS abolished the peptide's activity almost completely. This finding can be rationalized by thinking that free LPS tightly binds to temporin L, presumably scavenging it from the solution and reducing the peptide's active concentration, with a parallel decrease in the killing activity. This result also confirms the affinity of the peptide for LPS observed in binding assays (see above).

**In vivo antiendotoxin activity of temporin L. (i) Intraperitoneal administration of LPS.** Temporin L given intraperitoneally immediately after administration of 1.0 mg *E. coli* serotype 0111:B4 LPS resulted in significantly ($P \leq 0.05$) lower plasma endotoxin and TNF-α levels compared with both the control group, $C_0$, and the imipenem- or piperacillin-treated groups, as reported in Table 2. Furthermore, combining temporin L with β-lactams gave the strongest antiendotoxin activity, with the lowest recorded plasma endotoxin and TNF-α levels, although these effects were not statistically significant versus the group treated with temporin L alone (Table 2).

***E. coli*-induced peritonitis.** The efficacy of intraperitoneally administered β-lactams alone or combined with temporin L to counteract *E. coli*-induced peritonitis in a rat model was

TABLE 1. Interaction of conventional antibiotics with temporin L against *E. coli* ATCC 25922

| Compound[a] | Chemical class | FIC index[b] |
|---|---|---|
| Amoxicillin | β-Lactam | 0.75 |
| Ampicillin | β-Lactam | 0.50 |
| Carbenicillin | β-Lactam | 0.44 |
| Cephalosporin C | β-Lactam | 0.50 |
| Chloramphenicol | Chloramphenicol | 0.71 |
| Erythromycin | Macrolide | 0.75 |
| Imipenem | β-Lactam | 0.28 |
| Kanamycin | Aminoglycoside | 0.88 |
| Nalidixic acid | Quinolone | 0.75 |
| Netilmicin | Aminoglycoside | 1.5 |
| Piperacillin | β-Lactam | 0.28 |
| Streptomycin | Aminoglycoside | 0.62 |
| Vancomycin | Glycopeptide | 0.98 |

[a] The ranges of concentrations tested were 0.125 to 64 mg/liter for temporin L and 0.250 to 256 mg/liter for the other antimicrobial compounds.
[b] FIC indexes were interpreted as follows: <0.5, synergy; 0.51 to 4.0, no interaction; >4.0, antagonism.

TABLE 2. Plasma endotoxin and TNF-α levels in a rat model 6 h after intraperitoneal administration of 1.0 mg of *E. coli* O111:B4 LPS

| Treatment[a] | Endotoxin level (EU/ml)[b] | TNF-α level (ng/ml)[b] |
|---|---|---|
| Control group $C_0$ | 0.287 ± 0.12 | 170.2 ± 43.7 |
| TEM-L (1 mg/kg) | 0.038 ± 0.011[c] | 18.3 ± 3.5[c] |
| PIP (120 mg/kg) | 0.295 ± 0.16 | 172.8 ± 44.2 |
| IMP (20 mg/kg) | 0.302 ± 0.19 | 177.2 ± 48.2 |
| PIP (120 mg/kg) + TEM-L (1 mg/kg) | 0.026 ± 0.009[c] | 14.1 ± 2.9[c] |
| IMP (20 mg/kg) + TEM-L (1 mg/kg) | 0.032 ± 0.010[c] | 16.0 ± 3.1[c] |

[a] TEM-L, temporin L; PIP, piperacillin; IMP, imipenem.
[b] Mean ± SD.
[c] $P \leq 0.05$ versus the control group $C_0$ and the piperacillin- and imipenem-treated groups. Each group contained 20 animals.

TABLE 3. Efficacy of intraperitoneal β-lactams alone or combined with temporin L in a rat model of *Escherichia coli*-induced peritonitis[a]

| Treatment[b] | Lethality [no. dead/total (%)][c] | No. positive/total by qualitative blood culture | Fluid abdominal bacterial count (CFU/ml)[d] | Endotoxin level[e] (EU/ml)[d] | TNF-α level[e] (ng/ml)[d] |
|---|---|---|---|---|---|
| Control group $C_1$ | 20/20 (100) | 20/20 | $6.7 \times 10^8 \pm 1.4 \times 10^8$ | $0.323 \pm 0.05$ | $187.9 \pm 65.7$ |
| TEM-L (1 mg/kg) | 4/20 (20)[f] | 4/20[f] | $4.0 \times 10^4 \pm 1.4 \times 10^4$[f] | $0.069 \pm 0.02$[f,g] | $22.6 \pm 2.9$[f,g] |
| PIP (120 mg/kg) | 4/20 (20)[f] | 3/20[f] | $8.0 \times 10^2 \pm 2.3 \times 10^2$[f,h] | $0.356 \pm 0.07$ | $191.2 \pm 60.2$ |
| IMP (20 mg/kg) | 6/20 (30)[f] | 5/20[f] | $7.8 \times 10^2 \pm 3.5 \times 10^2$[f,h] | $0.412 \pm 0.06$ | $193.4 \pm 59.1$ |
| PIP (120 mg/kg) + TEM-L (1 mg/kg) | 2/20 (10)[f,g] | 2/20[f,g] | $3.1 \times 10^1 \pm 0.6 \times 10^1$[f,g,h] | $0.055 \pm 0.01$[f,g] | $18.3 \pm 2.2$[f,g] |
| IMP (20 mg/kg) + TEM-L (1 mg/kg) | 2/20 (10)[f,g] | 2/20[f,g] | $3.8 \times 10^1 \pm 1.2 \times 10^1$[f,g,h] | $0.058 \pm 0.12$[f,g] | $19.2 \pm 2.0$[f,g] |

[a] Rats were administered *E. coli* serotype O111:B4 at a challenge dose of $2 \times 10^{10}$ CFU intraperitoneally along with 1 ml of sterile saline solution.
[b] TEM-L, temporin L; PIP, piperacillin; IMP, imipenem.
[c] Lethality was monitored for 72 h following the challenge.
[d] Mean ± SD.
[e] Endotoxin and TNF-α plasma levels were measured 6 h after treatment.
[f] $P < 0.05$ versus the control group $C_1$.
[g] $P < 0.05$ versus the β-lactam-treated groups.
[h] $P < 0.05$ versus the control group and the temporin L-treated group.

also tested. In this case, the rate of lethality in control group $C_1$ was 100% (Table 3). For groups treated with single-drug regimens (piperacillin, imipenem, or temporin L), all intraperitoneal treatments given immediately after challenge had a better outcome with respect to controls ($P \leq 0.05$). Specifically, survival rates were 80%, 70%, and 80% in the groups treated with temporin L, imipenem, and piperacillin, respectively (Table 3). Bacteriological evaluation showed 100% positive blood and intra-abdominal fluid cultures in control group $C_1$; the average bacterial count in the peritoneal fluid from dead or surviving animals at 72 h was $6.7 \times 10^8 \pm 1.4 \times 10^8$ CFU/ml. Overall, piperacillin and imipenem showed the highest antimicrobial activities and therapeutic efficacies. In fact, there were significant ($P \leq 0.05$) differences in the results for the quantitative bacterial cultures when the data obtained for the β-lactam-treated groups were compared with those obtained for the peptide-treated group. Endotoxin and TNF-α concentrations increased constantly in the control group $C_1$, with mean peak levels achieved at 6 h postinjection (Table 3). Similarly to what was seen for the intraperitoneal administration of LPS (see above), the temporin L-treated group showed significant reduction in plasma endotoxin and TNF-α levels compared to the control and β-lactam-treated groups (Table 3). Treatment with imipenem alone resulted in the highest plasma endotoxin and TNF-α levels. Nevertheless, no significant difference in plasma endotoxin and TNF-α concentrations was observed between the imipenem- and piperacillin-treated groups and control group $C_1$. Combination treatments demonstrated that the simultaneous administration of temporin L and β-lactams produced the highest antimicrobial activities and the strongest (although not statistically significant versus the temporin L alone-treated group) reduction in plasma endotoxin and TNF-α levels, resulting in the highest survival rates (more than 90%). Finally, all agents proved to be nontoxic in our experimental system. Indeed, none of the animals had clinical evidence of drug-related adverse effects, such as local signs of inflammation, anorexia, weight loss, vomiting, diarrhea, fever, and behavioral alterations. No changes in physiological parameters were observed in the supplementary 1-mg/kg temporin L-treated group without infection.

## DISCUSSION

The data reported here clearly illustrate the potential antiendotoxin properties of temporin L. Indeed, this peptide not only proved to have a good affinity for binding to LPS in vitro, but also interfered with its biological activities in two rat models of gram-negative septic shock, protecting the animals against lethal endotoxemia. In particular, the most effective treatment in reducing all of the variables measured in our in vivo systems (mainly bacterial growth inhibition, but also lethality and endotoxemia) was obtained when temporin L was administered intraperitoneally in combination with the conventional β-lactam antibiotics piperacillin and imipenem. It is remarkable that these two antibiotics were the only ones among those tested that displayed a strong synergistic activity with temporin L against *E. coli* ATCC 25922. Not surprisingly, the β-lactams showed an antibacterial potency against *E. coli* significantly higher than that of temporin L, but demonstrated an increase in plasma endotoxin and TNF-α concentrations, a fact already observed by other researchers (10, 43, 48). On the other hand, temporin L was confirmed to be active against gram-negative strains and markedly reduced the levels of both circulating endotoxin and TNF-α compared to any of the other compounds and control, which highlights its double antimicrobial and antiendotoxin activity.

**Endotoxin binding by antimicrobial peptides.** We have produced multiple lines of evidence that temporin L binds LPS, although the modes of this interaction are not fully clear at this stage. The structure and features of the two compounds would support the idea that the binding of temporin L to LPS probably involves a mixture of hydrophobic and ionic interactions. Temporin L has a net cationic charge of +3 at neutral pH—a relatively high value among linear natural AMPs of similar size—and a comparably elevated hydrophobicity and hydrophobic moment (44). LPS is a glycolipid made of a variable and polyanionic polysaccharide portion and a structurally conserved lipid called lipid A with a hydrophilic backbone composed of a β-linked D-glucosamyl (1–6) α-D-glucosamine disaccharide which carries two phosphoryl residues. The polysaccharide and lipid A portion of LPS have therefore the potential to bind temporin L through electrostatic or hydrophobic interactions or a combination of both. The results ob-

tained with the BC fluorescent displacement assay, for example, demonstrate that temporin L displays an affinity for the hydrophilic backbone of lipid A, and it is not surprising that this is much weaker than that displayed by polymyxin B and its analogues (54). In reality, the abundant data on the interaction of temporin L with biological and model lipid membranes provided here and elsewhere indicate that the peptide most likely binds as well to the acyl groups of lipid A, although this binding does not necessarily displace BC from the backbone. So, the assay based on this probe might underestimate to some extent the real affinity of temporin L for LPS/lipid A.

It is interesting to recall that when LPS-carbohydrate mutant strains of *E. coli* were tested for their susceptibility to temporin L, it was found that this increased as the chain length of their LPS polysaccharide moieties decreased (33, 44). This suggests that the peptide's positive residues bind at first the negative charges carried by the outer polysaccharide portion. This interaction probably anchors the peptide to the outer membrane, enabling it to approach the acylic portion of lipid A for hydrophobic interactions and leading to further penetration of the outer membrane (see the description of the "self-promoted uptake model" reported below). However, the interaction with the polysaccharide portion may also hamper or retard the translocation of the peptide to the deeper hydrophobic regions of the outer membrane. Reduction of this curtain of negative charges would most likely facilitate the peptide's access to lipid A and boost its killing activity. Generalizing, it must be stressed that the LPS binding activities of AMPs do not necessarily correlate with their bactericidal potency, and LPS can thus be considered a protective layer, whose role in controlling peptide binding and preventing peptide insertion into the outer membrane of gram-negative bacteria is just starting to receive due attention and some direct experimental support (17, 42).

Besides temporin L and polymyxin B, a number of other antimicrobial peptides, either of natural origin, synthetic analogs, or fragments of LPS binding proteins, have been demonstrated to bind and neutralize endotoxin with an efficiency that made them candidates to be developed as therapeutically effective LPS-controlling drugs, but to date none of the peptides has been approved for clinical application with an indication to treat sepsis (25, 27). The horseshoe crab AMP polyphemusin I and three structural variants penetrated *E. coli* LPS monolayers and significantly inhibited cytokine production by LPS-stimulated macrophages (57). Two other peptides, MBI-27 and MBI-28, derived from parts of silk moth cecropin and bee melittin, have also been shown to bind LPS with an affinity equivalent to that of polymyxin B and to have antiendotoxin activity (23). HLP-2, a peptide arising from human lactofferin, and its synthetic analog, HLP-6, were shown to destabilize the *E. coli* outer membrane and to bind LPS with comparable affinity, as demonstrated by dansyl polymyxin B displacement (11). As mentioned, the high affinity of cationic AMPs for LPS drives their interactions with the bacterial outer membrane and thus the first stage of their antimicrobial activity. According to the current "self-promoted uptake model," cationic peptides bind to the divalent cation binding sites on LPS ($Mg^{2+}$ and $Ca^{2+}$ bind to the anionic charges of LPS under normal conditions and stabilize the outer membrane), distorting the integrity of the outer membrane and increasing its permeability to peptide itself (26). Once they've crossed the outer membrane, the peptides can bind to the lipid bilayer of the cytoplasmic membrane, disturbing its structure and causing cell death, as mentioned. Recent observations indicate that contact with the outer membrane induces folding of the peptides into their final membrane-associated form and that the folded peptides may aggregate into tightly packed "rafts", which in turn would greatly contribute to the disruption of the outer membrane structure (11). According to the information currently in our possession, temporin L and temporin-like peptides could well behave on their gram-negative microbial targets as described by the self-promoted uptake model, but further research work is needed to confirm this aspect.

**Antisepsis therapies.** Current treatment of gram-negative sepsis in critically ill patients is mainly based on the prompt intravenous administration of adequate antimicrobial agents. An initial empirical broad-spectrum antibiotic therapy should be initiated as soon as sepsis is suspected. The decision to begin antibiotic treatment rapidly and without waiting for the complete microbiological documentation of an infection is justified, in the presence of fever and neutropenia, by the high frequency of severe infections with a fulminant course. Alternative pharmaceutical therapies targeting single proinflammatory mediators and/or endotoxin are in development, but despite good results in animal models, their effects in humans—as proven by a number of clinical trials—have been so far disappointing (18). Three main such anti-inflammatory strategies aimed at improving the outcome of septic shock have been investigated, based on the administration of glucocorticoids, the development of endotoxin-directed monoclonal antibodies and other agents capable of binding and neutralizing LPS, or the inhibition of proinflammatory cytokines (e.g., anti-TNF antibodies, interleukin-1 receptor antagonists) (35, 47, 55). Another therapeutic approach with interesting prospects is offered by extracorporeal blood purification, achieved through several distinct techniques (37, 50). Roughly speaking, hemofiltration could be effective during severe septic shock because it permits the unselective removal of endotoxin, cytokines, and inflammatory mediators from the bloodstream.

**Conclusions.** In conclusion, we have demonstrated the LPS-binding properties of temporin L and its protective activity against endotoxemia. These observations reinforce the idea that selected AMPs could be efficiently used as antisepsis agents in vivo in combination with conventional antibiotics to increase killing and neutralize endotoxin as it is released by these compounds. For the future, it would be interesting to explore the antiendotoxin properties of other temporins and synthetic analogs, so to acquire key information needed to assist the design of improved endotoxin-neutralizing temporin-based peptides for therapeutic applications.

## ACKNOWLEDGMENT

This work was supported by funds from the Italian Ministry of Education, University and Research (PRIN 2003).

## REFERENCES

1. **Andra, J., M. Lamata, G. Martinez de Tejada, R. Bartels, M. H. Koch, and K. Brandenburg.** 2004. Cyclic antimicrobial peptides based on *Limulus* antilipopolysaccharide factor for neutralization of lipopolysaccharide. Biochem. Pharmacol. **68:**1297–1307.
2. **Angus, D. C., W. T. Linde-Zwirble, J. Lidicker, G. Clermont, J. Carcillo, and M. R. Pinsky.** 2001. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit. Care Med. **29:**1303–1310.

3. **Beutler, B.** 2004. Innate immunity: an overview. Mol. Immunol. **40:**845–859.
4. **Beutler, B., and E. T. Rietschel.** 2003. Innate immune sensing and its roots: the story of endotoxin. Nat. Rev. Immunol. **3:**169–176.
5. **Bochud, P. Y., and T. Calandra.** 2003. Pathogenesis of sepsis: new concepts and implications for future treatment. Br. Med. J. **326:**262–266.
6. **Brahmachary, M., S. P. T. Krishnan, J. L. Y. Koh, A. M. Khan, S. H. Seah, T. W. Tan, V. Brusic, and V. B. Bajic.** 2004. ANTIMIC: a database of antimicrobial sequences. Nucleic Acids Res. **32:**D586–D589.
7. **Brockman, H. L.** 1999. Lipid monolayers: why use half a membrane to characterize protein-membrane interactions? Curr. Opin. Struct. Biol. **9:**438–443.
8. **Brogden, K. A.** 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. **3:**238–250.
9. **Bulet, P., R. Stöcklin, and L. Menin.** 2004. Anti-microbial peptides: from invertebrates to vertebrates. Immunol. Rev. **198:**169–184.
10. **Byl, B., P. Clevenbergh, A. Kentos, F. Jacobs, A. Marchant, J. L. Vincent, and J. P. Thys.** 2001. Ceftazidime- and imipenem-induced endotoxin release during treatment of gram-negative infections. Eur. J. Clin. Microbiol. Infect. Dis. **20:**804–807.
11. **Chapple, D. S., R. Hussain, C. L. Joannou, R. E. W. Hancock, E. Odell, R. W. Evans, and G. Siligardi.** 2004. Structure and association of human lactoferrin peptides with *Escherichia coli* lipopolysaccharide. Antimicrob. Agents Chemother. **48:**2190–2198.
12. **Ciornei, C. D., T. Sigurdardóttir, A. Schimdtchen, and M. Bodelsson.** 2005. Antimicrobial and chemoattractant activity, lipopolysaccharide neutralization, cytotoxicity, and inhibition by serum of analogs of human cathelicidin LL-37. Antimicrob. Agents Chemother. **49:**2845–2850.
13. **Diekema, D. J., M. A. Pfaller, R. N. Jones, G. V. Doern, P. L. Winokur, A. C. Gales, H. S. Sader, K. Kugler, and M. Beach.** 1997. Survey of bloodstream infections due to gram-negative bacilli: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, and Latin America for the SENTRY Antimicrobial Surveillance Program, 1997. Clin. Infect. Dis. **29:**595–607.
14. **Ding, L., L. Yang, T. M. Weiss, A. J. Waring, R. I. Lehrer, and H. W. Huang.** 2003. Interaction of antimicrobial peptides with lipopolysaccharides. Biochemistry **42:**12251–12259.
15. **Edgeworth, J. D., D. F. Treacher, and S. J. Eykyn.** 1999. A 25-year study of nosocomial bacteremia in an adult intensive care unit. Crit. Care Med. **27:**1421–1428.
16. **Eisenberg, D.** 1984. Three-dimensional structure of membrane and surface proteins. Annu. Rev. Biochem. **53:**595–623.
17. **Farnaud, S., C. Spiller, L. C. Moriarty, A. Patel, V. Gant, E. W. Odell, and R. W. Evans.** 2004. Interactions of lactoferricin-derived peptides with LPS and antimicrobial activity. FEMS Microbiol. Lett. **233:**193–199.
18. **Freeman, B. D., and C. Natanson.** 2000. Anti-inflammatory therapies in sepsis and septic shock. Expert Opin. Investig. Drugs **9:**1651–1663.
19. **Fukumoto, K., I. Nagaoka, A. Yamataka, H. Kobayashi, T. Yanai, Y. Kato, and T. Miyano.** 2005. Effect of antibacterial cathelicidin peptide CAP18/LL-37 on sepsis in neonatal rats. Pediatr. Surg. Int. **21:**20–24.
20. **Ganz, T.** 2003. Defensins: antimicrobial peptides of innate immunity. Nat. Rev. Immunol. **3:**710–720.
21. **Giacometti, A., O. Cirioni, R. Ghiselli, C. Bergnach, F. Orlando, G. D'Amato, F. Moccheggiani, C. Silvestri, M. S. Del Prete, B. Skerlavaj, V. Saba, M. Zanetti, and G. Scalise.** 2004. The antimicrobial peptide BMAP-28 reduces lethality in mouse models of staphylococcal sepsis. Crit. Care Med. **32:**2485–2890.
22. **Giacometti, A., O. Cirioni, R. Ghiselli, F. Moccheggiani, G. D'Amato, R. Circo, F. Orlando, B. Skerlavaj, C. Silvestri, V. Saba, M. Zanetti, and G. Scalise.** 2004. Cathelicidin peptide sheep myeloid antimicrobial peptide-29 prevents endotoxin-induced mortality in rat models of septic shock. Am. J. Respir. Crit. Care Med. **169:**187–194.
23. **Gough, M., R. E. W. Hancock, and N. M. Kelly.** 1996. Antiendotoxin activity of cationic peptide antimicrobial agents. Infect. Immun. **64:**4922–4927.
24. **Gutsmann, T., O. Hagge, J. W. Larrick, U. Seydel, and A. Wiese.** 2001. Interaction of CAP18-derived peptides with membranes made from endotoxins or phospholipids. Biophys. J. **80:**2935–2945.
25. **Hancock, R. E. W.** 2001. Cationic peptides: effectors in innate immunity and novel antimicrobials. Lancet Infect. Dis. **1:**156–164.
26. **Hancock, R. E. W., and D. S. Chapple.** 1999. Peptide antibiotics. Antimicrob. Agents Chemother. **43:**1317–1323.
27. **Jerala, R., and M. Porro.** 2004. Endotoxin neutralizing peptides. Curr. Top. Med. Chem. **4:**1173–1184.
28. **Lehrer, R. I.** 2004. Primate defensins. Nat. Rev. Microbiol. **2:**727–738.
29. **Li, P., T. Wohland, B. Ho, and J. L. Ding.** 2004. Perturbation of lipopolysaccharide (LPS) micelles by sushi 3 (S3) antimicrobial peptide. The importance of an intermolecular disulfide bond in S3 dimer for binding, disruption, and neutralization of LPS. J. Biol. Chem. **279:**50150–50156.
30. **Lohner, K., and S. E. Blondelle.** 2005. Molecular mechanisms of membrane perturbation by antimicrobial peptides and the use of biophysical studies in the design of novel peptide antibiotics. Comb. Chem. High Throughput Screen. **8:**241–256.
31. **Maget-Dana, R.** 1999. The monolayer technique: a potent tool for studying the interfacial properties of antimicrobial and membrane-lytic peptides and their interactions with lipid membranes. Biochim. Biophys. Acta **1462:**109–140.
32. **Mangoni, M. L., D. Barra, M. Simmaco, A. Bozzi, A. Di Giulio, and A. C. Rinaldi.** 2004. Effects of the antimicrobial peptide temporin L on cell morphology, membrane permeability and viability of *Escherichia coli*. Biochem. J. **380:**859–865.
33. **Mangoni, M. L., A. C. Rinaldi, A. Di Giulio, G. Mignogna, A. Bozzi, D. Barra, and M. Simmaco.** 2000. Structure-function relationships of temporins, small antimicrobial peptides from amphibian skin. Eur. J. Biochem. **267:**1447–1454.
34. **Mangoni, M. L., J. M. Saugar, M. Dellisanti, D. Barra, M. Simmaco, and L. Rivas.** 2005. Temporins, small antimicrobial peptides with leishmanicidal activity. J. Biol. Chem. **280:**984–990.
35. **Manocha, S., D. Feinstein, A. Kumar, and A. Kumar.** 2002. Novel therapies for sepsis: antiendotoxin therapies. Expert Opin. Investig. Drugs **11:**1795–1812.
36. **Martino, R., A. Santamaria, L. Muñoz, R. Pericas, A. Altes, G. Prats, and J. Sierra.** 1999. Bacteremia by gram-negative bacilli in patients with hematologic malignancies. Comparison of the clinical presentation and outcome of infections by enterobacteria and non-glucose-fermenting gram-negative bacilli. Acta Haematol. **102:**7–11.
37. **Nalesso, F.** 2005. Plasma filtration adsorption dialysis (PFAD): a new technology for blood purification. Int. J. Artif. Organs **28:**731–738.
38. **National Committee for Clinical Laboratory Standards.** 2001. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 5th ed. Approved standard M7-A5. National Committee for Clinical Laboratory Standards, Wayne, Pa.
39. **Nizet, V., and R. L. Gallo.** 2003. Cathelicidins and innate defense against invasive bacterial infection. Scand. J. Infect. Dis. **35:**670–676.
40. **Oppenheim, J. J., A. Biragyn, L. W. Kwak, and D. Yang.** 2005. Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. Ann. Rheum. Dis. **62:**ii17–ii21.
41. **Papo, N., and Y. Shai.** 2005. Host defense peptides as new weapons in cancer treatment. Cell Mol. Life Sci. **62:**784–790.
42. **Papo, N., and Y. Shai.** 2005. A molecular mechanism for lipopolysaccharide protection of Gram-negative bacteria from antimicrobial peptides. J. Biol. Chem. **280:**10378–10387.
43. **Prins, J. M., E. J. Kuijper, M. L. C. M. Mevissen, P. Speelman, and S. J. H. van Deventer.** 1995. Release of tumor necrosis factor alpha and interleukin 6 during antibiotic killing of *Escherichia coli* in whole blood: influence of antibiotic class, antibiotic concentration, and presence of septic serum. Infect. Immun. **63:**2236–2242.
44. **Rinaldi, A. C., M. L. Mangoni, A. Rufo, C. Luzi, D. Barra, H. X. Zhao, P. K. J. Kinnunen, A. Bozzi, A. Di Giulio, and M. Simmaco.** 2002. Temporin L: antimicrobial, haemolytic and cytotoxic activities, and effects on membrane permeabilization in lipid vesicles. Biochem. J. **367:**91–100.
45. **Rollins-Smith, L. A., C. Carey, J. M. Conlon, L. K. Reinert, J. K. Doersam, T. Bergman, J. Silberring, H. Lankinen, and D. Wade.** 2003. Activities of temporin family peptides against the chytrid fungus (*Batrachochytrium dendrobatidis*) associated with global amphibian declines. Antimicrob. Agents Chemother. **47:**1157–1160.
46. **Shai, Y.** 2002. Mode of action of membrane active antimicrobial peptides. Biopolymers **66:**236–248.
47. **Sharma, V. K., and R. P. Dellinger.** 2003. Recent developments in the treatment of sepsis. Expert Opin. Investig. Drugs **12:**139–152.
48. **Shenep, J. L., R. P. Barton, and K. A. Mogan.** 1985. Role of antibiotic class in the rate of liberation of endotoxin during therapy for experimental gram-negative bacterial sepsis. J. Infect. Dis. **151:**1012–1018.
49. **Simmaco, M., G. Mignogna, S. Canofeni, R. Miele, M. L. Mangoni, and D. Barra.** 1996. Temporins, novel antimicrobial peptides from the European red frog *Rana temporaria*. Eur. J. Biochem. **242:**788–792.
50. **Venkataraman, R., S. Subramanian, and J. A. Kellum.** 2003. Clinical review: extracorporeal blood purification in severe sepsis. Crit. Care **7:**139–145.
51. **Wade, D.** 28 August 2002, posting date. Unambiguous consensus sequences for temporin-like peptides. Internet J. Chem. **5**. [Online.] http://www.ijc.com/articles/2002v5/5/.
52. **Wade, D., A. Silveira, J. Silberring, P. Kuusela, and H. Lankinen.** 2000. Temporin antibiotic peptides: a review and derivation of a consensus sequence. Protein Pept. Lett. **7:**349–357.
53. **Wiese, A., T. Gutsmann, and U. Seydel.** 2003. Towards antibacterial strategies: studies on the mechanisms of interaction between antibacterial peptides and model membranes. J. Endotoxin Res. **9:**67–84.
54. **Wood, S. J., K. A. Miller, and S. A. David.** 2004. Anti-endotoxin agents. 1. Development of a fluorescent probe displacement method optimized for the rapid identification of lipopolysaccharide-binding agents. Combin. Chem. High Throughput Screen. **7:**239–249.
55. **Yethon, J. A., and C. Whitfield.** 2001. Lipopolysaccharide as a target for the

development of novel therapeutics in gram-negative bacteria. Curr. Drug Targets Infect. Disord. **1:**91–106.
56. **Zasloff, M.** 2002. Antimicrobial peptides of multicellular organisms. Nature **415:**389–395.
57. **Zhang, L., M. G. Scott, H. Yan, L. D. Mayer, and R. E. W. Hancock.** 2000. Interaction of polyphemusin I and structural analogs with bacterial membranes, lipopolysaccharide, and lipid monolayers. Biochemistry **39:**14504–14514.
58. **Zhao, H. X., and P. K. J. Kinnunen.** 2002. Binding of the antimicrobial peptide temporin L to liposomes assessed by Trp fluorescence. J. Biol. Chem. **277:**25170–25177.
59. **Zhao, H. X., A. C. Rinaldi, A. Di Giulio, M. Simmaco, and P. K. J. Kinnunen.** 2002. Interactions of the antimicrobial peptides temporins with model biomembranes. Comparison of temporins B and L. Biochemistry **41:** 4425–4436.