Temporin A Alone and in Combination with Imipenem Reduces Lethality in a Mouse Model of Staphylococcal Sepsis

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Background. Morbidity and mortality from staphylococcal toxic shock remain high, despite the availability of antibiotics to which the microorganism is sensitive.

Methods. In in vitro experiments, the ability of temporin A to inhibit lipoteichoic acid–induced production of tumor necrosis factor (TNF)–α and nitric oxide (NO) was determined. Also, mouse models of staphylococcal sepsis were used to evaluate the efficacy of temporin A alone and in combination with imipenem. BALB/c mice were injected intravenously with live Staphylococcus aureus or heat-killed cells and then received either isotonic sodium chloride solution, 2 mg/kg temporin A, 7 mg/kg imipenem, or 2 mg/kg temporin A in combination with 7 mg/kg imipenem immediately and 6 h after challenge. The main outcome measures were lethality rates, plasma bacterial counts, and plasma TNF-α and interleukin (IL)-6 levels.

Results. The in vitro experiments showed that temporin A did not cause TNF-α or NO release. In the in vivo experiments with live bacteria, both compounds reduced lethality rates and bacterial growth. Imipenem exhibited the highest efficacy. The combination-treated group had significantly lower bacterial counts than did the singly-treated groups and the lowest lethality rates. In the experiments with heat-killed cells, only temporin A demonstrated significant efficacy with respect to lethality and reduction of plasma TNF-α and IL-6 levels.

Discussion. This study shows that temporin A can reduce the stimulatory effects of bacterial cell components and suggests that it may be beneficial in the treatment of severe staphylococcal sepsis.

Over the past several years, the frequency of Staphylococcus aureus sepsis has increased dramatically, and it is still associated with a high mortality [1–4]. In addition, in recent years there has been a continuous progression in the percentage of S. aureus strains that are resistant to methicillin, not only in hospitals but also in intensive care settings [5–7]. S. aureus is a unique pathogen because of its virulent properties, but few data are available on the microbial factors involved in the induction of septic shock by gram-positive organisms [8, 9]. One possibility is that cell-wall components or secreted exotoxins can induce the cytokine cascade in the same way as lipopolysaccharides (LPSs) from gram-negative organisms [10–14]. In gram-positive bacteria, the cell wall contains peptidoglycan (PG) and lipoteichoic acid (LTA), which synergize to cause shock and organ failure in animals and to activate human blood to release proinflammatory cytokines such as tumor necrosis factor (TNF)–α, interleukin (IL)–1, and IL-6 [10–12, 14]. Superantigens include a class of certain bacterial and viral proteins that exhibit highly potent lymphocyte-transforming (mitogenic) activity toward human and other mammalian T cells. S. aureus exotoxins possess superantigenic properties that arise from cross-linking antigen-presenting cells and T cells, binding to the outer region of major histocompatibility complex class II antigen-presenting cells outside the classical antigen-binding groove and concomitant-
ly binding in their native form to Vβ-specific determinants on T cell receptors [11, 13, 15]. The resulting complex triggers the activation of the targeted T cells and leads to the in vivo and in vitro release of large amounts of various cytokines and other effectors by immune cells; in vivo, this leads to the systemic inflammatory response syndrome and the consequent inability of the host to regulate the inflammatory response, which eventually leads to organ failure and death [11, 13, 15].

Despite their structural differences, PG and LTA both activate macrophages and polymorphonuclear leukocytes by binding to CD14, a surface receptor anchored in the outer leaflet of the plasma membrane. It cannot induce cellular activation without a transmembrane signal transducing coreceptor. Recent studies have led to the identification of Toll-like receptor 4 as the coreceptor for LPS [16, 17]. LPS-CD14 complexes initiate intracellular signaling reactions by binding to specific receptors on macrophages and other cells and subsequently activating transcription factors that mediate the production of proinflammatory cytokines such as TNF, IL-1, IL-6, IL-8, platelet-activating factor, arachidonic acid metabolites, erythropoietin, and endothelin. These pathophysiological phenomena can lead to endothelial damage and hemodynamic and metabolic derangements. With the identification of the systemic inflammatory response to infection and of microbial products as a major component of the pathogenesis of septic shock, several studies have focused on modulating the activation of this complex cascade of mediators [16]. For this reason, compounds that bind to bacterial components and ablate their ability to bind to CD14 could be important candidates for use as anti-inflammatory agents. It has been shown that LTA has both a polyanionic and a lipodic nature and, similarly to LPS, is therefore able to interact with polycationic peptides. Moreover, recent experiments have shown that synthetic peptides corresponding to different regions of various superantigens can directly block the interaction of staphylococcal exotoxins and thus prevent the proliferative inflammatory responses these antigens usually generate [16].

The antimicrobial peptides are recognized as an important component of the innate immune response in most multicellular organisms and are used by animals to effectively deal with microbes in their environment. Over the past few years, their potential roles have further expanded. These effector molecules of innate immunity are endowed with direct antimicrobial activity and LPS-binding capacity and are being evaluated as possible alternatives to conventional antibiotics [18, 19]. Amphibian skin is a rich source of antimicrobial peptides. These molecules are produced and stored in dermal structures called "granular glands," which release their contents on the skin of the frog after adrenergic stimulation or injury. Other cationic peptides are expressed in the cells of the gastric mucosa and intestinal tract [20].

Among these molecules, temporins (A–H, K, and L), a group of peptides isolated from the skin of the European red frog *Rana temporaria,* are a family of linear, 10–13-residue-long peptides with a net positive charge and are amidated C-terminus antimicrobial peptides. They have shown activity against clinically important gram-positive cocci, including multidrug-resistant staphylococci and vancomycin-resistant *Enterococcus faecium.* Temporin A is a basic, highly hydrophobic, antimicrobial peptide amide (FLPLIGRVLSGIL-NH₂) that, like the other temporins, is active against clinically important antibiotic-resistant gram-positive cocci [21–23].

To test further the therapeutic value of temporin A, in the present study we analyzed the ability of this peptide to inhibit the production of TNF-α, IL-6, and NO by murine macrophages stimulated with LTA and its capacity to protect mice alone and in combination with imipenem in models of staphylococcal sepsis. As inducers of sepsis, live *S. aureus* ATCC 25923 or heat-killed cells of the same strain were used. Lethality rates, plasma bacterial counts, and plasma TNF-α and IL-6 levels were evaluated.

**MATERIALS AND METHODS**

**Organisms.** The commercially available quality-control strain of *S. aureus* ATCC 25923 (Oxoid Italia) was used.

**Mice.** Male BALB/c mice weighing 25–33 g were used for all experiments. Throughout the study, all mice were housed in individual cages under constant temperature (22°C) and humidity with a 12-h light/dark cycle and had access to chow and water ad libitum. The study was approved by the animal research ethics committee of the INRCA IRRCS, Polytechnic University of Marche.

**Reagents.** LTA from *S. aureus* (Sigma-Aldrich) was re-suspended in endotoxin-free water, aliquotted, and stored at −20°C for short periods. LPS contamination of the LTA preparation was <2 ng/mL, as determined by the Limulus assay from BioWhittaker.

**Agents.** Temporin A was synthesized manually by the solid-phase method with the fluorenylmethoxy carbonyl/tert-butyl (Fmoc/But) procedure (Faculty of Pharmacy, Medical University of Gdansk, Poland) and was purified by reversed-phase (Vydac C-18; 10 × 250 mm) high-pressure liquid chromatography (HPLC) with a Knauer K501 2-pump system. The product was analyzed by HPLC and matrix-assisted laser-desorption ionization mass spectrometry [24]. Serial dilutions of the peptide were prepared (1) in cell culture medium on RAW 264.7 cells, for the in vitro assays; (2) in 0.01% acetic acid containing 0.2% bovine serum albumin in polypropylene tubes, for the in vitro susceptibility tests; and (3) in physiological saline, for the in vivo experiments. Imipenem powder (Merck Sharp & Dohme) was diluted in accordance with the manufacturer’s specifications.
Solutions were made fresh on the day of assay. The concentration range assayed for each compound was 0.25–256 mg/L.

**Supernatant cytokine levels in RAW 264.7 cell culture.** The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection. Cells were plated in 24-well dishes (Sigma-Aldrich) at $1 \times 10^6$ cells/well in the above-specified medium and was incubated overnight at 37°C in 5% CO$_2$. RPMI 1640 was aspirated from cells grown overnight and replaced with fresh medium. Cells were incubated with LTA from S. aureus at 37°C in 5% CO$_2$, in either the absence or the presence of 0.5–5 μmol/L temporin A. The latter was added at the same time as was LTA. After a 24-h incubation, the supernatants were removed and tested for TNF-α level by ELISA (Walter Occhiena Srl), in accordance with the manufacturer’s specifications. All samples were run in duplicate. The lower limit of detection for TNF-α was <0.025 ng/mL.

**NO production.** RAW 264.7 cells were cultured as described above. The level of NO in the supernatant was estimated on the basis of the accumulation of the stable NO metabolite nitrite after stimulation with Griess reagent (Molecular Probes), in accordance with the manufacturer’s specifications.

**Susceptibility testing and interaction experiments.** Susceptibilities were determined in accordance with the procedures outlined by the NCCLS [25]. Experiments were performed in triplicate. Interaction experiments were performed by a checkerboard titration method with 96-well polypropylene microtiter plates. The fractional inhibitory concentration (FIC) index for combinations of 2 antimicrobials was calculated according to the following equation: FIC index = $FICA + FICB = A/MIC_A + B/MIC_B$, where $A$ and $B$ are the respective MICs of drug A and drug B in the combination, MIC$_A$ and MIC$_B$ are the respective 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 indices were interpreted as follows: <0.5, synergy; 0.5–4.0, indifferent; and >4.0, antagonism [26].

**Preparation of the inoculum.** S. aureus ATCC 25923 was grown overnight at 37°C in brain-heart infusion broth. The log phase of growth was established by quantitative cultures: aliquots of the bacterial suspension were serially diluted, and a 0.1-mL volume of each dilution was spread on blood agar plates and cultured for 48 h at 35°C for enumeration of developed colonies. When bacteria were in the log phase of growth, the suspension was centrifuged for 15 min at 1000 g, the supernatant was discarded, and the bacteria were resuspended and diluted in sterile saline to a concentration of $\sim 1 \times 10^7$ cfu/mL. Heat-killed S. aureus was prepared by boiling for 10 min and then sonicating the bacterial suspension in PBS containing $\sim 2.5 \times 10^9$ cells/mL for 1 min. The efficacy of the heat treatment was confirmed by culturing the bacteria overnight, to ensure that there was no growth.

**Implantation of inoculum.** All mice were anesthetized by an intramuscular injection of ketamine (30 mg/kg of body weight). Mice were injected via the tail vein with 0.2 mL of the above-described bacterial suspensions ($2.0 \times 10^4$ cfu of live S. aureus [model 1] or $5.0 \times 10^4$ heat-killed cells [model 2]) on day 0 and were monitored for 72 h.

**Antibiotic therapy.** The mice were randomized to receive via the tail vein either isotonic sodium chloride solution (control), 2 mg/kg temporin A, 7 mg/kg imipenem, or 2 mg/kg temporin A combined with 7 mg/kg imipenem immediately (model 1a and 2a) or 6 h (model 1b and 2b) after bacterial
challenge. Each group included 20 mice. After administration of the antibiotics, the mice were returned to individual cages and were monitored for the subsequent 72 h. The end points of the study were lethality rates (model 1), plasma bacterial counts (model 1), and plasma TNF-α and IL-6 levels (model 2). For each model, toxicity was evaluated on the basis of the presence of any drug-related adverse effects, such as local signs of inflammation, anorexia, weight loss, vomiting, diarrhea, fever (rectal temperature >38°C), hypothermia (rectal temperature <36°C), and behavioral alterations. In particular, to evaluate the physiological effects of temporin A, leukocyte count, rectal temperature, pulse, blood pressure, and breathing rate were monitored in a supplementary group of temporin A–treated mice that had not been infected.

Measurement of plasma antibiotic levels. Preventive experiments were performed to measure plasma antibiotic levels in uninfected mice. Blood samples were obtained via the tail vein from 25 mice (5 mice for each agent) at 30, 60, 120, 240, and 720 min after a single intravenous (iv) dose of either 2 mg/kg temporin A or 7 mg/kg imipenem and were pooled for analysis of the levels in plasma. The levels were determined by a bioassay that used Bacillus subtilis ATCC 6633 grown on tryptic soy agar as the indicator organism. The plates were read after incubation for 18 h at 30°C.

Evaluation of treatment. Blood samples for culture were obtained via the tail vein by aseptic percutaneous puncture 24 h after bacterial challenge. The mice that died before this time were not considered for the cultures. To perform quantitative bacterial cultures, blood samples were serially diluted, and a 0.1-mL volume of each dilution was spread on blood agar plates and cultured at 35°C for 48 h for enumeration of developed colonies. The limit of detection was <1 log₁₀ cfu/mL. The bacterial isolates were identified by a biochemical assay.

Measurement of plasma TNF-α and IL-6 levels. For determination of plasma TNF-α and IL-6 levels (model 2), blood samples were collected from the tail vein 0, 6, 12, 24, and 48 h after injection. IL-6 and TNF-α levels were measured by ELISA, in accordance with the manufacturer’s specifications. The lower limits of detection for IL-6 and TNF-α were 12 pg/mL and 0.05 ng/mL, respectively.

Statistical analysis. Lethality rates between groups were compared by Fisher’s exact test. Data from quantitative blood cultures are presented as means ± SDs, and statistical comparisons between groups were made by analysis of variance (ANOVA). Post hoc comparisons were performed by Bonferroni’s test. Plasma TNF-α and IL-6 levels, also presented as means ± SDs, were compared between groups by ANOVA. Significance was accepted when \( P \leq .05 \).

RESULTS

Cytokine and NO production. The murine macrophage cell line RAW 264.7 was used. At the concentrations tested (0.5–5 \( \mu \)mol/L), temporin A alone did not cause the release of either TNF-α or NO, which are known to contribute substantially to the pathogenesis of septic shock. TNF-α levels increased 532–956-fold after stimulation with 5 \( \mu \)g/mL LTA, compared with that for untreated cells, and were found to significantly decrease in the presence of temporin A (75.0% ± 3.8% inhibition at 0.5 \( \mu \)mol/L peptide), with a near-complete inhibition (97.3% ± 0.6%) at 5 \( \mu \)mol/L peptide. TNF-α levels ranged from 0.066 to 0.107 ng/mL in the supernatants of cells cultured with no
Susceptibility testing. *S. aureus* ATCC 25923 showed susceptibility to temporin A and imipenem, which exhibited MICs of 2.00 and 0.50 mg/L, respectively. Synergy between temporin A and imipenem, which had an FIC index of 0.312, was observed.

**Model 1a.** In this model, mice received via the tail vein 2.0 × 10^6 cfu of live *S. aureus* ATCC 25923 and antibiotic treatment immediately after bacterial challenge. The lethality rate for the control mice was 100% within 72 h. In contrast, both compounds demonstrated an in vivo efficacy significantly higher than that of the isotonic sodium chloride solution (*P* < .05)—specifically, lethality rates of 25% and 20% were observed for the mice treated with either temporin A or imipenem alone, respectively. Combination experiments showed the lowest lethality rate; temporin A administered in combination with imipenem produced a rate of 10%. Quantitative blood culture for the control mice showed a plasma bacterial count of 4.8 × 10^6 ± 0.5 × 10^6 cfu/mL. All treatment exhibited an antimicrobial activity significantly higher than that of the isotonic sodium chloride solution. In the singly-treated mice, imipenem treatment resulted in the lowest plasma bacterial counts (2.6 × 10^3 ± 0.6 × 10^3 cfu/mL). Temporin A showed an antibacterial activity (plasma bacterial count, 3.0 × 10^7 ± 0.3 × 10^6 cfu/mL) comparable to that observed for imipenem. The combination-treated group had significantly lower plasma bacterial counts (5.8 × 10^5 ± 1.2 × 10^5) than did the singly-treated groups (*P* < .05) (table 1).

**Model 1b.** In this model, mice received via the tail vein 2.0 × 10^6 cfu of live *S. aureus* and antibiotic treatment 6 h after bacterial challenge. With respect to lethality rates, the impact of administration of drugs 6 h after bacterial challenge was comparable to that observed in the groups treated immediately after challenge (table 1).

**Model 2a.** In this model, mice received via the tail vein 5.0 × 10^6 heat-killed *S. aureus* cells and antibiotic treatment immediately after bacterial challenge. As is shown in figure 2A and 2B, peak plasma TNF-α and IL-6 levels were observed 6 and 12 h, respectively, after iv administration of 0.2 mL of heat-killed cells. Treatment with temporin A, both alone and in combination with imipenem, resulted in a marked decrease (*P* < .05) in plasma TNF-α and IL-6 levels, compared with those in the control mice and in the mice that received imipenem alone. Finally, no substantial increase in cytokine levels was observed in the plasma of the control mice and of the mice that received imipenem alone.

**Model 2b.** In this model, mice received via the tail vein 5.0 × 10^6 heat-killed *S. aureus* cells and antibiotic treatment 6 h after bacterial challenge. A constant increase in plasma TNF-α and IL-6 levels was observed in the control mice and in the mice that received imipenem alone, whereas a constant decrease was observed in the mice that received temporin A alone and in combination with imipenem 6 h after bacterial challenge (figure 3A and 3B). The mice that received temporin A, both...
Figure 3. Mouse plasma interleukin (IL)-6 levels after challenge with heat-killed *Staphylococcus aureus* cells. Shown are the effects of 2 mg/kg temporin A (TMP-A), 7 mg/kg imipenem (IMP), and 2 mg/kg TMP-A in combination with 7 mg/kg IMP administered intravenously immediately (A) and 6 h (B) after injection of 0.2 mL of saline solution containing $5.0 \times 10^8$ heat-killed *S. aureus* ATCC 25923 cells. Each group included 20 mice; data are means ± SDs. Treatment with TMP-A, both alone and in combination with IMP, resulted in a marked decrease ($P<.05$) in plasma IL-6 levels, compared with those in the control mice and in the mice that received IMP alone.

alone and in combination with imipenem, had significant reductions in cytokine levels, compared with those in the control mice and the mice that received imipenem alone.

**Toxicity.** None of the mice showed clinical evidence of drug-related adverse effects, such as local signs of inflammation, anorexia, weight loss, vomiting, diarrhea, fever, hypothermia, and behavioral alterations.

**Measurement of plasma antibiotic levels.** Thirty minutes after a single iv injection, temporin A and imipenem reached their peak levels of 4.0 mg/L and 26.8 mg/L, respectively (figure 4).

**DISCUSSION**

The complexities of the pathogenesis of sepsis continue to be understood only incompletely [12, 14, 27]. Recently, a novel approach to the treatment of superantigen-mediated disease has been reported that involves the administration of antagonist peptides that are able to inhibit the induction of human Th1-type cytokine gene expression by bacterial superantigens. Furthermore, researchers have identified highly conserved amino acid homology in 2 distinct regions of all staphylococcal toxin families and have created peptides from these regions to enhance their immunogenicity in animals, but the value of this type of intervention for human diseases is not completely known [16, 28, 29]. Although the prospect of compounds by which superantigenicity can be switched off is exciting, there have been many false dawns in the field of sepsis research. Animal studies of superantigen-mediated human disease have well-recognized limitations. The mouse model, although one of the best established systems for the study of toxic shock, requires far higher doses of superantigen than are needed to induce shock in humans, as well as prior “sensitization” of the animal with the hepatotoxin d-galactosamine. However, it is well known that other mechanisms are involved in the pathogenesis of sepsis. That antimicrobial peptides possess not only the ability to bind to LTA but also the capacity to reduce the production of TNF-α in response to it has been described [30].

In the present study, model 1 was used to test the effect of temporin A on lethality and plasma bacterial count; in this model, live *S. aureus* were inoculated to induce sepsis. To test the ability of the peptide to inhibit cytokine release, model 2 was used, in which heat-killed and sonicated *S. aureus* were inoculated. The agents were administered either immediately or 6 h after bacterial challenge, the latter to mimic the clinical situation in which an interval between the onset of sepsis and the initiation of therapy is present. Our in vitro experiments showed that temporin A inhibits both the LTA-induced release of TNF-α by RAW 264.7 cells and the production of NO. In
model 1, the results showed that drug efficacy was slightly affected by the time of treatment, even though the highest lethality rates were observed in the mice treated 6 h after bacterial challenge. In this model, the most efficacious drug for the improvement of survival was imipenem. This finding can be explained by the potent antimicrobial activity of this antibiotic, despite its capacity to induce the release of soluble cell-wall components from staphylococci, including such components as PG, LTA, and toxins [31, 32]. Our in vivo results for imipenem were similar to those reported in other studies, which have found that its potential to induce endotoxin depends on its affinity for the penicillin-binding proteins (PBPs). Imipenem has a high affinity for PBP-2, which causes conversion of the bacteria into round cells with a loss of viability but without extensive cell-wall degradation [33].

Although temporin A lacks the potency of imipenem, it produced lethality rates comparable to those of the antibiotic and a greater ability to inhibit the release of TNF-α and IL-6. It has been suggested that temporin A can induce the migration of human monocytes, neutrophils, and macrophages. It can selectively stimulate the chemotaxis of phagocytic leukocytes by use of human formyl peptide receptor–like 1 and can elicit the infiltration of neutrophils and monocytes into the injection site of mice [23].

When temporin A was combined with imipenem, an interesting synergy was observed that resulted in an increase in survival. In previous investigations, antimicrobial peptides have been shown to have the capacity to synergize with antibiotics. The mechanism of this positive interaction remains largely unknown, although it might be due to the contemporaneous effect of temporin A and imipenem on PG. The peptide might act by inserting itself into the cytoplasmic membrane and triggering the activity of bacterial murein hydrolases, resulting in damage or degradation of PG and lysis of the cell [16, 19, 34]. The probable role of temporin A in such synergy is its rapid permeabilization of the bacterial membrane, which allows enhanced penetration and activity of imipenem. Compared with other antimicrobial peptides, temporin A shows a strong antistaphylococcal activity and positive interaction with β-lactams [21, 22, 35]. In the present study, none of the mice showed clinical evidence of drug-related adverse effects, and no changes in physiological parameters were observed in the supplementary group of uninfected mice that received temporin A; nevertheless, other reports have demonstrated its potential hemolytic property [36]. For this reason, further investigations are needed to elucidate this concern. Its antimicrobial activity and its ability to inhibit the LTA-induced release of cytokines—as well as its synergistic interaction with imipenem—suggest that temporin A is a promising candidate for the treatment of staphylococcal toxic shock.

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