Single-Dose Intraperitoneal Magainins Improve Survival in a Gram-Negative-Pathogen Septic Shock Rat Model

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The therapeutic efficacies of three polycationic peptides selected among the class of the magainins (magainin I, magainin II, and magainin II amide), alone and combined with piperacillin, were investigated in a rat model of septic shock. Rats were given an intraperitoneal injection of 2×10^{10} CFU of *Escherichia coli* and randomized to receive intraperitoneally isotonic sodium chloride solution, 60 mg of piperacillin per kg of body weight, and 1 mg of each magainin per kg alone and combined with 60 mg of piperacillin per kg. The main outcome measures were bacterial growth in abdominal exudate and plasma, endotoxin and tumor necrosis factor alpha (TNF- α) concentrations in plasma, and lethality. Treatments with the magainins achieved significant reductions of bacterial growth and plasma endotoxin and TNF- α concentrations. In general, treatments with the combinations of magainins and piperacillin demonstrated the highest efficacies.

Septic shock continues to be a major cause of morbidity and mortality in hospitalized patients (3, 14, 16). The primary cause of shock induced by gram-negative bacteria results, at least in part, from activation of host effector cells by endotoxin, the lipopolysaccharide (LPS) associated with cell membranes of gram-negative bacteria. LPS is composed of an *O*-polysaccharide chain, a core sugar, and a lipophilic fatty acid (1, 2, 9). The lipid A portion of LPS induces expression of cytokine genes through stimulation of receptors on the surfaces of target cells (9). The activated cells (e.g., monocytes and macrophages) secrete large quantities of inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), platelet-activating factor, arachidonic acid metabolites, erythropoietin, endothelin, IL-6, and IL-8 (1, 2, 9, 10). Many agents and methods have been used to treat septic shock, including monoclonal antibodies to endotoxin, IL-1 receptor antagonists, antioxidants, several other agents, and various anti-inflammatory therapies, but these have failed to produce effective results (1, 2, 9, 14, 16).

Magainins are positively charged peptides that were originally isolated from the skin of the African clawed frog (*Xenopus laevis*). Similar to other polycationic peptides, magainins, which are α -helical ionophores, possess two important activities: a broad antimicrobial spectrum and antiendotoxin activity (6, 12). Among the magainins, magainin I (M1 Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Gly-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Lys-Ser), magainin II (M2 Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser), and magainin II amide (M2-NH₂ Gly-Ile-Gly-Lys-Phe-Leu-His-Ala-Ala-Lys-Lys-Phe-Ala-Lys-Ala-

Phe-Val-Ala-Glu-Ile-Met-Asn-Ser-NH₂) have been demonstrated to be nonhemolytic and active against numerous gram-negative and gram-positive bacteria, fungi, and protozoa (5, 13, 15). The helical, amphiphilic structures of the magainins are responsible for their affinities to biological membranes: they dissipate ion gradients, causing membrane perturbation (7, 8). In addition, similar to other cationic peptides, their positively charged molecules bind to LPS and so have antiendotoxin activities, in contrast to other antibiotics, which induce endotoxemia (6). Finally, previous studies showed that magainins increased the survival of mice when the magainins were administered with beta-lactams (4). The aim of the present experimental study was to investigate the therapeutic efficacies of intraperitoneal magainins alone and in combination with a broad-spectrum beta-lactam antibiotic in a rat model of septic shock by evaluating bacterial growth in abdominal exudate and plasma, endotoxin and TNF alpha (TNF- α) concentrations in plasma, and lethality.

MATERIALS AND METHODS

Organisms. The commercially available quality control strain *Escherichia coli* ATCC 25922 was used as the test organism.

Drugs. M1, M2, M2-NH₂, and piperacillin were obtained from Sigma-Aldrich S.r.l. (Milan, Italy). The magainins were dissolved in distilled H₂O at 20 times the required maximal concentrations. Successively, for in vitro studies, serial dilutions of the peptides were prepared in 0.01% acetic acid containing 0.2% bovine serum albumin in polypropylene tubes, while for in vivo experiments, they were diluted in physiological saline. Piperacillin powder was diluted in physiological saline. Solutions were made fresh on the day of assay. The concentration range assayed for each antibiotic was 0.25 to 256 μ g/ml.

Susceptibility testing. Piperacillin susceptibility testing was done by using the broth microdilution method by the procedures outlined by the National Committee for Clinical Laboratory Standards (11). The MIC was taken as the lowest antibiotic concentration at which observable growth was inhibited. However, the MICs of magainins were determined by the procedures recently proposed by Hancock (8) for the testing of antimicrobial peptides. Particularly, since cationic peptides bind to polystyrene, polypropylene 96-well plates (Sigma-Aldrich) were

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substituted for polystyrene plates and were incubated for 18 h at 37°C in air. Experiments were performed in triplicate.

Animals. Adult male Wistar rats (weight range, 250 to 300 g) were used for all the experiments. All animals had access to chow and water ad libitum throughout the study. The study was approved by the animal research ethics committee of the Istituto Nazionale Riposo e Cura Anziani Istituto di Ricovero e Cura a Carattere Scientifico, University of Ancona, Ancona, Italy.

Preparation of inoculum. *E. coli* ATCC 25922 was grown in brain heart infusion broth. When the bacteria were in the logarithmic phase of growth, the suspension was centrifuged at $1,000 \times g$ for 15 min, the supernatant was discarded, and the bacteria were resuspended and diluted in sterile saline to achieve a concentration of 2×10^{10} CFU/ml of saline.

Implantation of inoculum. All animals were anesthetized by intramuscular injection of ketamine (30 mg/kg of body weight). The abdomen of each animal was shaved and prepared with iodine. With the exception of the uninfected control group (group C₀), the rats received an intraperitoneal inoculum of 1 ml saline containing 2×10^{10} CFU of *E. coli* ATCC 25922.

Antibiotic therapy. The antibiotic therapy phase of the experiments determined the therapeutic effects of the drugs tested given intraperitoneally immediately after bacterial challenge. The study included four drug-treated groups; one group each received piperacillin (60 mg/kg), M1 (1 mg/kg), M2 (1 mg/kg), and M2-NH₂ (1 mg/kg). In addition, among three additional groups, one group each received 1 mg of M1, M2, and M2-NH₂ per kg in combination with 60 mg of piperacillin per kg. To establish the lethality of *E. coli* intraperitoneal contamination, the study included an untreated control group (group C₁) and the uninfected control group (group C₀). The animals were returned to individual cages and monitored for the subsequent 48 h. At the end of the study, the rate of positivity of blood cultures, quantitation of the bacteria in the intra-abdominal fluid, and rate of lethality were evaluated.

Plasma endotoxin and TNF- α levels. For determination of endotoxin and TNF- α levels in plasma, blood samples were collected from the jugular vein 0, 60, 120, and 240 min postinjection.

Endotoxin concentrations were measured by the commercially available *Limulus* amoebocyte lysate test (E-TOXATE®; Sigma-Aldrich). Plasma samples were serially twofold diluted 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 (0, 0.015, 0.03, 0.06, 0.125, 0.25, and 0.5 endotoxin units [EU]/ml) were tested in each run, and the concentrations of endotoxin in the test samples 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) by the protocol supplied by the manufacturer. The standards and samples were incubated with a TNF- α antibody-coated 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 tetramethyl benzidine chromogen substrate. The intensity of the color was measured in an MR 700 microplate reader (Dynatech Laboratories, Guernsey, United Kingdom) by reading the absorbance at 450 nm. The concentrations of TNF- α in the samples were determined by comparison with the standard curve. All samples were run in duplicate. The lower limit of sensitivity for TNF- α by this assay was 4 pg/ml. The intra-assay and interassay coefficients of variation were 2.6 and 3.8%, respectively.

Evaluation of treatment. Previous studies with this model have demonstrated that most untreated rats rapidly develop bacteremia, which persists until the death of the animal. In the present study 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 evaluation 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 for enumeration of the colonies that developed. The limit of detection was $<1 \log_{10}$ CFU/ml. The plates were incubated in air at 35°C for 48 h.

Statistical analysis. MICs are presented as the geometric means for three separate experiments. Survival data were compared by the log rank test; qualitative results from blood and intra-abdominal fluid cultures were analyzed by the χ^2 test, Yates' correction, and Fisher's exact test, depending on the sample size. The results of the quantitative evaluation of the bacteria in the intra-abdominal fluid cultures are presented as the means $\pm$ standard deviations of the means; statistical comparisons between groups were made by analysis of variance. Post hoc comparisons were performed by Bonferroni's test. Plasma endotoxin and TNF- α levels were analyzed by the Kruskal-Wallis test; multiple comparisons between groups were performed by the appropriate standard procedure. Each

comparison group contained 15 rats. Significance was accepted when the *P* value was ≤ 0.05 .

RESULTS

In vitro susceptibility. According to the broth microdilution method, *E. coli* ATCC 25922 was similarly susceptible to the magainins tested: the MICs of M1, M2, and M2-NH₂ were 0.50, 0.50, and 0.25 μ g/ml, respectively. The piperacillin MIC was 0.25 μ g/ml.

Animal model. The rate of survival in group C₀, which received only intraperitoneal saline, was 100%. On the contrary, 13 (86.7%) of 15 rats in group C₁ died within 36 h (*P* < 0.05). Bacteriological evaluation showed no infection in animals in group C₀ and 100% positive blood and intra-abdominal fluid cultures in animals in group C₁ (*P* < 0.05). The bacterial counts in the intra-abdominal fluid at the end of 48 h were comparable in all infected rats, averaging $8.8 \times 10^7 \pm 1.6 \times 10^7$ CFU/ml.

All intraperitoneal antibiotic treatments given immediately after challenge were better than no treatment (*P* < 0.05). Treatment with M1, M2, M2-NH₂, and piperacillin gave survival percentages of 73.3, 73.3, 80.0, and 66.7, respectively. There were significant differences in lethality between the three combination treatment groups compared to that for the group that received piperacillin alone. In fact, survival was 100% for all groups treated with a magainin in association with piperacillin.

In any treated group, the presence of a positive blood culture was always detected in conjunction with a positive intra-abdominal fluid culture. No isolated positive blood culture or positive intra-abdominal fluid culture was detected. All positive cultures grew *E. coli* ATCC 25922 in the absence of other concomitant organisms. Similar to the lethality end point, significant differences in qualitative blood and intra-abdominal fluid cultures were demonstrated in the combination treatment group when the results were compared to those for the groups that received magainins and piperacillin alone (*P* < 0.05). Data concerning the lethality and quantitative microbiological evaluation of the effect of any antibiotic treatment are summarized in Table 1. There were also significant differences in the results from the quantitative bacterial cultures when the data obtained for groups that received the magainins combined with piperacillin were compared with those obtained for group C₁ (*P* < 0.05) and the groups treated with piperacillin or one of the magainins alone.

Examination of plasma samples showed significant differences in plasma endotoxin and TNF- α levels between the groups C₀ and C₁ at 60, 120, and 240 min after the challenge. In group C₁, endotoxin and TNF- α concentrations increased constantly, with mean peak levels achieved at 240 min postinjection (0.125 EU and 25.1 pg/ml, respectively). The groups treated with magainins alone and the groups treated with magainins in combination with piperacillin had significant reductions in plasma endotoxin and TNF- α levels compared to those for group C₁ and the piperacillin-treated group. On the contrary, endotoxin and TNF- α levels were significantly increased in the piperacillin-treated group compared with those in group C₁. The results observed at 240 min postinjection are summarized in Table 1.

TABLE 1. Efficacies of intraperitoneal magainins alone and in combination with piperacillin in a rat model of septic shock

Treatment (dose [mg/kg]) ^a	Lethality ^b		Bacterial count (CFU/ml) ^c	Endotoxin level (EU/ml) ^c	TNF- α level (pg/ml) ^c
	No. dead/ total no. tested	%			
No infection (group C ₀)	0/15	0	<10	$\leq 0.015 \pm 0.0$	$\leq 4 \pm 0.0$
No treatment (group C ₁)	13/15	86.7	$8.8 \times 10^7 \pm 1.6 \times 10^7$	0.125 ± 0.015	25.1 ± 2.1
M1 (1)	4/15 ^d	26.7	$2.8 \times 10^3 \pm 0.9 \times 10^{3d}$	$\leq 0.015 \pm 0.0^{d,e}$	$\leq 4 \pm 0.0^{d,e}$
M1 (1) plus PIP (60)	0/15 ^{d,e,f}	0	$<10^{d,e}$	$\leq 0.015 \pm 0.0^{d,e}$	$\leq 4 \pm 0.0^{d,e}$
M2 (1)	4/15 ^d	26.7	$3.2 \times 10^3 \pm 1.1 \times 10^{3d}$	$\leq 0.015 \pm 0.0^{d,e}$	$\leq 4 \pm 0.0^{d,e}$
M2 (1) plus PIP (60)	0/15 ^{d,e,f}	0	$<10^{d,e}$	$\leq 0.015 \pm 0.0^{d,e}$	$\leq 4 \pm 0.0^{d,e}$
M2-NH ₂ (1)	3/15 ^d	20.0	$2.3 \times 10^3 \pm 0.6 \times 10^{3d}$	$\leq 0.015 \pm 0.0^{d,e}$	$\leq 4 \pm 0.0^{d,e}$
M2-NH ₂ (1) plus PIP (60)	0/15 ^{d,e,f}	0	$<10^{d,e}$	$\leq 0.015 \pm 0.0^{d,e}$	$\leq 4 \pm 0.0^{d,e}$
PIP (60)	5/15 ^d	33.3	$2.1 \times 10^3 \pm 0.6 \times 10^{3d}$	$0.250 \pm 0.025^{g,h}$	$29.6 \pm 2.8^{g,h}$

^a Rats were previously administered *E. coli* intraperitoneally along with 1 ml of sterile saline solution. PIP, piperacillin.

^b Lethality was monitored for 48 h following the challenge.

^c Data are means $\pm$ standard deviations.

^d $P < 0.05$ versus group C₁ (significantly lower).

^e $P < 0.05$ versus the piperacillin-treated group (significantly lower).

^f $P < 0.05$ versus the magainin-treated group (significantly lower).

^g $P < 0.05$ versus group C₁ (significantly higher).

^h $P < 0.05$ versus the group treated with a magainins (alone or in combination with piperacillin) (significantly higher).

DISCUSSION

The primary cause of shock after bloodstream infection with gram-negative bacteria is endotoxin. Actually, LPS is a complex macromolecule that is common to the outer membrane and that contains the lipid A responsible for the release of cytokines, such as TNF- α (1, 2, 9, 10, 15). Interestingly, although many gram-negative bacteria are genetically diverse, the lipid A moiety of LPS is highly conserved, making it a favorable target for anti-LPS therapy. One possible approach to addressing therapeutically the problem of gram-negative-pathogen septic shock could be to target LPS itself by the use of agents that can bind to and sequester this potent microbial product, thereby preventing its recognition by effector cells such as macrophages. Nevertheless, current treatments for gram-negative-pathogen septic shock rely on antibiotics to control the infection and support in intensive care units to correct the dysfunctions of cardiovascular, endocrine, and other organ systems. However, it has been suggested that even though the antibiotic is rapidly bactericidal, it does not protect experimental animals from death. Therefore, use of a combination of antibiotic and anti-LPS therapy in the treatment of gram-negative-pathogen septic shock could be useful not only for the treatment of the infection but also for neutralization of the biological effect of the endotoxin. Moreover, it would be interesting to evaluate the potential therapeutic roles of compounds able to inhibit both bacterial growth and the biological activity of the endotoxin. Such an evaluation might be possible as a result of the recent discovery of the antimicrobial polycationic peptides. The magainins are polycationic peptides known to have variable in vitro antibacterial activities (5, 13, 15). Similar to other cationic peptides, they are able to bind to the negatively charged LPSs of gram-negative organisms and so may have antiendotoxin activity, in contrast to other antibiotics, which induce endotoxemia. The present study has described the effects of the intraperitoneal administration of three magainins, M1, M2, and M2-NH₂, alone and in combination with piperacillin, on survival outcome, blood culture results, intra-abdominal fluid bacterial content, and plasma endotoxin and TNF levels in a rat model of gram-negative-pathogen septic shock.

Taken together, the results of the present study indicate that the use of single-dose intraperitoneal magainins can result in significant bacterial growth inhibition, even though the strongest efficacy was reached only upon coadministration of the beta-lactam piperacillin. Furthermore, treatment with the magainins resulted in greater reductions in plasma endotoxin levels than treatment with piperacillin, confirming the double antimicrobial and antiendotoxin activities of the magainins. On the contrary, the intraperitoneal administration of a beta-lactam significantly increased endotoxin and TNF- α levels compared not only with those in the magainin-treated animals but also with those in animals in the control groups, in agreement with the assertion that these clinically used antibiotics can be harmful when administered to treat severe infections caused by gram-negative bacteria, in that they can stimulate the release of endotoxin.

The dual properties of magainins (endotoxin neutralization and bacterial growth inhibition) may give them potential advantages over the conventional clinically used antibiotics; the association of a magainin and a beta-lactam antibiotic can be efficacious in protecting rats against lethal gram-negative-pathogen septic shock. In addition, it is noteworthy that even though no hematologic, chemistry, or histologic studies were performed in the present study, none of the animals died or had clinical evidence of drug-related adverse effects, such as local signs of inflammation, anorexia, vomiting, diarrhea, or behavioral alterations. Nevertheless, there are unanswered concerns about the peptides and their toxicities. For this reason, even though the magainins may be good candidates as lead compounds for the investigation of novel agents with activity against gram-negative-pathogen septic shock, further research based on studies with animals and patients, in which the assets of these peptides will be efficacious, are needed.

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