Antimicrobial Agents and Chemotherapy, June 1991, p. 1153–1159
0066-4804/91/061153-07$02.00/0
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Vol. 35, No. 6

β-Lactam Antibiotics Potentiate Magainin 2 Antimicrobial Activity In Vitro and In Vivo

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Received 13 November 1990/Accepted 22 March 1991

The ability of magainin 2 to augment antibiotic therapy was examined. Susceptibility to magainin 2 was determined on *Escherichia coli* incubated in the presence and absence of sublethal concentrations of antibiotics both in vitro and in vivo. Experiments in buffer and normal human serum revealed that *E. coli* exposed to sublethal amounts of cefepime, a β-lactam antibiotic, was significantly more susceptible to the antimicrobial activity of magainin 2. Bacteria incubated with subinhibitory concentrations of other β-lactam type antibiotics, but not amikacin (an aminoglycoside) or ciprofloxacin (a quinolone), were also more susceptible to magainin 2 in normal human serum. Bacteria were less susceptible to magainin 2 when they were examined in heat-inactivated serum. Complement was shown to be required for magainin 2 activity in serum by using C8-deficient sera. The combination of magainin 2 and cefepime was shown to be more antimicrobial in normal human serum for a variety of bacterial strains. Magainin 2 was completely inactive as a therapeutic agent when it was administered alone (2 mg per mouse) but significantly increased the survival of mice when it was administered with a low level of cefepime.

The response to antibiotic treatment in neutropenic patients is poor (18). Efforts to improve the clinical response to antibiotic treatment in these patients include therapies designed to augment the host immune system (9, 20) as well as the continuing search for more potent antibacterial agents (15). The inability of an antibiotic to eradicate a bacterial infection in the absence of an intact host defense system can lead to bacterial persistence (1). This phenomenon, as recently described by Bryan (1), may represent an unappreciated cause of partial or complete therapeutic failure in these patients. Bacteria that persist after antibiotic treatment display relatively minor changes in antibiotic susceptibility (1, 24), and one possible consequence of bacterial persistence is that bacteria are injured but not killed by the antibiotic. A consensus is developing in the literature that antibiotic-injured bacteria are more susceptible to both polymorphonuclear cell-dependent (12, 28) and polymorphonuclear cell-independent (27, 32) killing mechanisms. It is therefore tempting to speculate that antibiotic-injured bacteria may be adequately handled by immunosufficient hosts but not by immunocompromised patients (29). We are attempting to improve the clinical response to antibiotic treatment for neutropenic patients by investigating therapies that would kill antibiotic-injured bacteria. Such approaches could theoretically mimic the host role in antibiotic therapy of immune deficient patients.

Subinhibitory levels of antibiotics are routinely used to examine how antibiotics may injure bacteria in the absence of bactericidal action (11, 29). One effect that we have recently shown is that the serum susceptibility of *Pseudomonas aeruginosa* is significantly increased when cells were incubated in serum with sublethal concentrations of cefepime, a β-lactam-type antibiotic (5). The analysis showed that the terminal complement complex was necessary for enhanced antibiotic killing and that the β-lactam-altered bacterial cell surface allowed more terminal complement complex deposition (5). The increase in complement deposition suggested to us, on the basis of the previous work of Joiner (16) and others (38), that β-lactam-altered bacteria might be more susceptible to agents whose action is directed at the bacterial inner membrane.

A group of novel oligopeptide antibiotics which act at the inner bacterial membrane has been described recently (19, 37). These peptides are obtained from a wide variety of different sources and include magainins (39), cecropins (30), sarcotoxins (26), and other similar peptides (2, 10). Some of these peptides have been shown to uncouple oxidative phosphorylation from ATP synthesis (25, 36). Consistent with this mechanism, other peptides have been shown to form channels in artificial membrane systems (3, 4). In the past, the therapeutic application of many peptide antibiotics was severely limited by their lack of specificity (8). In contrast, the cationic peptides mentioned above demonstrate a selective toxicity for bacteria (3, 23). These peptides represent novel agents for the treatment of systemic bacterial infections. However, their in vitro activities are highly susceptible to assay conditions (6), making it difficult to determine whether their potencies are sufficient for in vivo use.

In this study, the effects of one of these peptides, magainin 2 (39), was examined against *Escherichia coli* grown with and without antibiotics. Bacteria grown with sublethal concentrations of cefepime, a β-lactam antibiotic, were more susceptible to the antimicrobial activity of magainin 2 both in buffer and in serum. Remarkably, the terminal complement complex was required for magainin 2 activity in human serum. In addition, in a mouse model of *E. coli* infection, magainin 2 was completely inactive as a therapeutic agent alone but significantly increased the survival of mice when it was administered with cefepime for the treatment of this systemic bacterial infection.

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MATERIALS AND METHODS

Reagents and buffers. The following buffers were used: gelatin-Veronal buffer with the addition of 0.1% glucose (GVB++ [17]) and phosphate-buffered saline (PBS). Cefepime {7-[(Z)-2-(2-aminothiazol-4-yl)-2-methoxyiminoacetamido]-3-(1-methlypyrrolidinio)methyl-3-cephem-4-carboxylate} and other antibiotics were obtained from Robert Kessler, Bristol-Myers Squibb Co., Wallingford, Conn., as standard laboratory powders. Magainin 2 (Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser) was synthesized by solid-phase synthesis (22) on an Applied Biosystems model 430A peptide synthesizer by using Boc/benzyl-based protection. The assembled peptide resin was treated by the low/high hydrogen fluoride procedure (31), and the cleaved deprotected peptide was purified by high-performance liquid chromatography on a Dynamax C-8 column (Rainin). Highly purified magainin 2 was characterized by analytical high-performance liquid chromatography and amino acid analysis. Mueller-Hinton base was obtained from BBL Microbiological Systems, Cockeysville, Md.

Bacterial strains and culture conditions. *E. coli* ATCC 25922 was used for most of the experiments and was obtained from the American Type Culture Collection. Other *E. coli* strains were A011 obtained from Harborview Medical Center, Seattle, Wash., and a K1 capsule isolate H16 from the Walter Reed Army Institute of Research, Washington, D.C. In addition, *Klebsiella pneumoniae* ATCC 13883 and an *Enterobacter cloacae* strain (G444) obtained from the Veterans Administration Hospital, Seattle, Wash., were examined. Strains were examined for purity, were properly identified, and were then stored at −70°C. Each week new cultures were made from the frozen bacterial stocks to avoid repetitive subculturing. All strains were grown in adjusted Mueller-Hinton broth containing 50 mg of CaCl per liter and 25 mg of MgCl per liter (17).

Serum. Blood was aseptically collected from five healthy volunteers and allowed to clot at room temperature for 1 h. After 20 min of incubation at 4°C, the blood was centrifuged for 15 min at 1,500 ×*g*. Serum was removed, pooled, and then stored at −70°C in small amounts. Shortly before use the pooled normal human sera (NHS) was thawed and kept on ice until it was mixed with bacteria for the serum bactericidal assay. Where indicated, serum complement was inactivated by heating it at 56°C for 30 min (H-NHS). Several different pooled serum sources were used in the course of these investigations, and essentially the same results were obtained with each pool. However, since small differences in the amount or rate of bacterial killing were noticed with different serum pools, all comparisons of antibiotic activity in serum were made with the same serum pool. Some experiments used C8-depleted sera and purified C8, which were both obtained from Cytotech, San Diego, Calif.

Determination of the MIC. MICs were determined by the microdilution plate dilution method by using adjusted Mueller-Hinton broth (17) with a final inoculum density of 7.5×10^5 CFU/ml obtained from mid-exponential-phase cells. Plates were incubated for 18 h at 37°C. At least three different determinations were performed with each antibiotic for each strain.

Serum bactericidal assay. Bacteria inoculated from overnight cultures were grown to the mid-exponential phase with or without antibiotic (optical density at 660 nm, between 0.35 and 0.5; approximately 2×10^8 to 3×10^8 cells per ml for cells grown with cefepime and 9×10^8 cells per ml for cells grown without cefepime). Cells were diluted without washing to 5×10^4 cells per ml in GVB++, and 0.05 ml of the cell suspension was added to serum in a 1.5-ml Eppendorf vial. For those cells pregrown in antibiotic, the same amount of antibiotic that was present during growth was added for both the preparation and examination of antimicrobial activity. Appropriate dilutions of NHS or H-NHS were prepared beforehand in GVB++ to a volume of 0.2 ml. Antibiotics, when present, were added to the serum mixture before the addition of bacteria. The total reaction mixture was 0.25 ml. The number of bacteria added to serum was determined by adding 0.05 ml of the bacterial suspension to a tube containing only GVB++ and plating at the beginning of the assay. The reaction mixtures were then rotated at 37°C for 3 h. In most experiments, 25-μl samples were removed at intervals for plate count analysis (in triplicate) by the pour plate method by using Trypticase soy agar. By this method, bacterial numbers between 4.3 and 3.08 $\log_{10}$ CFU/ml could be accurately obtained (30 to 500 colonies per plate). The number of CFU was determined after overnight incubation.

The potential for error caused by drug carry-over effects was eliminated by inclusion of appropriate controls. Inclusion of magainin 2 and cefepime in H-NHS and C8-deficient sera eliminated the need for drug removal procedures, since significant antimicrobial activity was detected only in NHS and C8-replenished sera. In addition, possible drug carry-over effects of magainin 2, cefepime, or the combination of the two would not influence the interpretation of the cation titration experiment (see Fig. 1), since each variable was examined at all divalent cation concentrations (see legend to Fig. 1).

Mouse protection experiments. All studies were performed with male CrL:CF1 mice obtained from Harlen Sprague-Dawley (Indianapolis, Ind.). Neutropenia was induced in two separate groups of mice (weight, 16 to 18 or 18 to 20 g) by subcutaneous injection of 250 mg of cyclophosphamide (Mead Johnson) per kg of body weight. Examination of blood revealed that the level of circulating leukocytes declined to 10% of normal by day 4 after injection and remained depressed for the following 2 days. Mice were challenged intraperitoneally with a log-phase bacterial suspension in PBS (0.2 ml) on day 4 after cyclophosphamide administration. The survival of the mice was monitored for 10 days following bacterial challenge, and the 90% lethal dose was determined by Probit analysis (7). Mice were given 2×10^4 (1× the 90% lethal dose) *E. coli* organisms.

To determine the protective efficacy of peptides, antibiotics, or combinations, mice were divided into four different groups and given injections of (i) PBS, (ii) magainin 2 (2 mg per mouse), (iii) cefepime (0.2 mg/kg), or (iv) a combination of cefepime and peptide (0.2 mg of cefepime per kg and 2 mg of magainin 2 per mouse) that were combined prior to injection. All injections were made in PBS (0.2 ml) and were administered intramuscularly, with each injection given in separate legs 1 and 3.5 h after bacterial challenge. The survival of the mice was monitored for 10 days, and the results were analyzed by the Fisher exact test for $n < 20$ mice and by chi-square analysis for $n > 20$ mice (21).

RESULTS

Activities of magainin 2 and cefepime in buffer. Magainin 2 was synthesized from the published sequence (39), and its antibacterial activity against *E. coli*, grown with and without cefepime, was examined in buffer (Fig. 1). A concentration of cefepime was chosen that had no effect on cell viability in

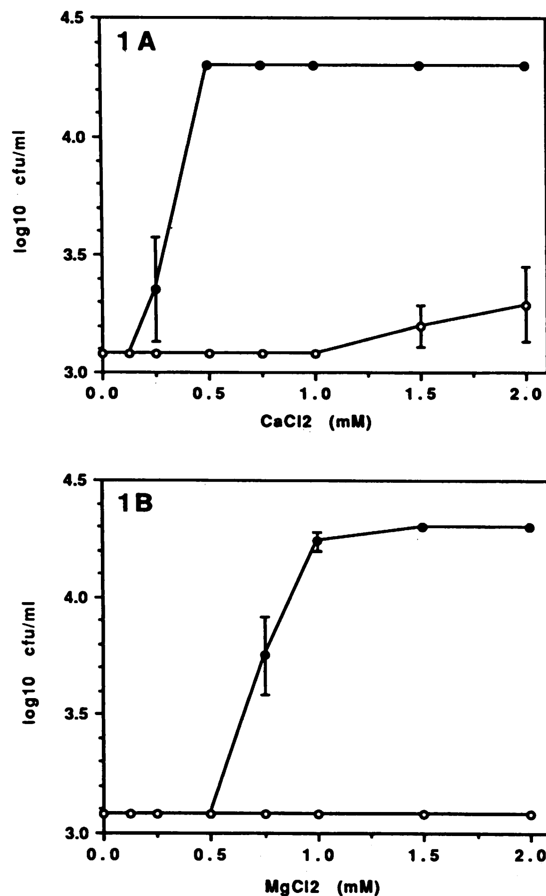

FIG. 1. Calcium and magnesium inhibition of magainin 2 antimicrobial activity with *E. coli*. *E. coli* ATCC 25922 was grown to the mid-logarithmic phase without (untreated) or with (cefepime altered) one-quarter the MIC (0.016 μg/ml) of cefepime. Bacteria ($\log_{10}$ CFU/ml of 3.93 ± 0.045 for untreated and 3.73 ± 0.15 for cefepime-altered bacteria) were added to GVB++ containing either no magainin 2 or 200 μg of magainin 2 per ml. Various amounts of either calcium chloride (A) or magnesium chloride (B) were added to tubes containing magainin 2, and the $\log_{10}$ CFU per milliliter was determined after 180 min. Values greater than 4.3 or less than 3.08 $\log_{10}$ CFU/ml are plotted as their respective minimum or maximum values. Greater than 4.3 $\log_{10}$ CFU/ml was observed for samples (both with and without cefepime) which did not contain magainin 2 (data not shown). Each assay was performed in triplicate on three separate occasions, and the means ±1 standard deviation are shown for those points which fell in the detectable range. ●, untreated *E. coli*; ○, cefepime-altered *E. coli*.

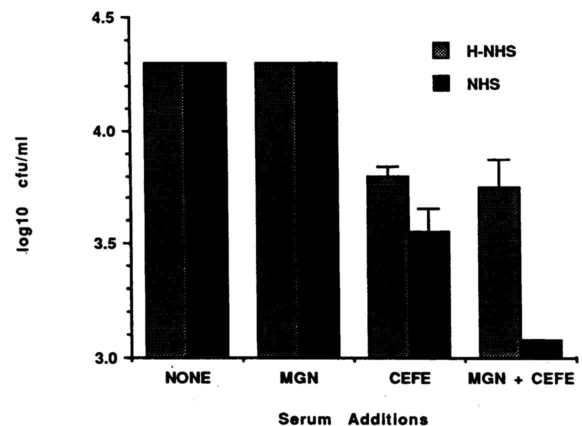

FIG. 2. Susceptibility of *E. coli* to magainin 2 killing in human serum. *E. coli* ATCC 25922 was grown to the mid-logarithmic phase without or with one-quarter the MIC (0.016 μg/ml) of cefepime. Bacteria were added to both H-NHS and NHS. The serum (40%) contained no additions (NONE); 200 μg of magainin 2 (MGN) per ml; one-quarter the MIC of cefepime (CEFE); or 200 μg of magainin 2 per ml and one-quarter the MIC of cefepime (MGN + CEFE). Viable counts were determined at the time of bacterial addition to serum (input without cefepime, 3.98 ± 0.021 $\log_{10}$ CFU/ml; with cefepime, 4.02 ± 0.02 $\log_{10}$ CFU/ml) and after 3 h for each sample. Values greater than 4.3 or less than 3.08 $\log_{10}$ CFU/ml are plotted as their respective minimum or maximum values. Each assay was performed in triplicate on three separate occasions with same serum source. The means ±1 standard deviation are shown.

the assay (see legend to Fig. 1). It was suspected that divalent cations might play an important role in determining the susceptibilities of the bacterial cells to magainin 2 because of the well-known antagonism between antimicrobial cationic agents and Mg or Ca ions (13). Magainin 2 activity was examined in buffer that contained either no added divalent cations or various levels of exogenously added $CaCl_2$ or $MgCl_2$ increasing to levels slightly over those normally found in human serum (35). When concentrations of less than 0.25 mM $CaCl_2$ or 0.5 mM $MgCl_2$ were examined, magainin 2 reduced the bacterial input by greater than 1 log unit for both untreated and cefepime-altered bacteria. However, increasing concentrations of either calcium chloride or magnesium chloride resulted in a significant inhibition of magainin 2 antibacterial activity when untreated bacteria were examined (Fig. 1). In contrast, cefepime-altered bacteria remained more susceptible to the antimicrobial activity of magainin 2 throughout the divalent cation range used in the experiment. Therefore, as the divalent cation concentration was increased, magainin 2 demonstrated increased antimicrobial activity on cefepime-altered cells when compared with that on untreated controls.

Activities of magainin 2 and cefepime in serum. The ability of low levels of cefepime to augment the antimicrobial activity of magainin 2 was next examined in pooled NHS. The antimicrobial activity of magainin 2 was determined in both NHS and H-NHS against untreated and cefepime-altered bacteria (Fig. 2). When bacteria not exposed to low levels of cefepime were examined, there was a slight increase in survival in 40% NHS and H-NHS with or without magainin 2 (200 μg/ml) treatment. In contrast, the survival of cefepime-altered bacteria decreased slightly but significantly in NHS when compared with that in H-NHS ($\log_{10}$ CFU/ml of 3.5 ± 0.10 for NHS compared with 3.8 ± 0.04 for H-NHS; $P < 0.01$, two-sample t test). Remarkably, however, the cefepime-altered cells were significantly more susceptible to the antimicrobial activity of magainin 2 when examined in NHS compared with that when they were examined in H-NHS ($\log_{10}$ CFU/ml of 2.8 ± 0.08 for NHS compared with 3.7 ± 0.11 for H-NHS; $P < 0.001$, two-sample t test). Magainin 2 had no significant effect on the survival of cefepime-altered bacteria in H-NHS. The data showed that bacteria incubated with low levels of cefepime are more susceptible to the antimicrobial activity of magainin 2 in serum; however, a heat-sensitive serum component was required.

Studies with C8-deficient sera showed that the terminal complement complex significantly enhanced the antimicrobial activity of magainin 2 in NHS (Fig. 3). In the absence of

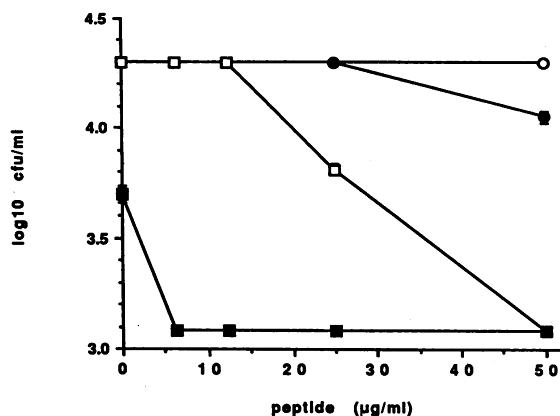

FIG. 3. Complement requirement for the antimicrobial activity of magainin 2 in serum. *E. coli* was prepared as described in the text and the legend to Fig. 1. Bacteria were added to a set of tubes that contained either 40% human serum specifically depleted of human complement protein C8 (no C8) or 40% human serum depleted of C8 to which purified C8 was added back to 50 µg/ml. Cefepime, when present, was added to 0.016 µg/ml. Each set of tubes also contained 0, 6.25, 12.5, 25, or 50 µg of magainin 2 per ml. Viable counts were determined at the time of bacterial addition to serum (3.97 ± 0.037 $\log_{10}$ CFU/ml for untreated bacteria and 3.99 ± 0.02 $\log_{10}$ CFU/ml for cefepime-altered bacteria) and after 3 h (as shown). Values greater than 4.3 or less than 3.08 $\log_{10}$ CFU/ml are plotted as their respective minimum or maximum values. The assay was performed in triplicate; the means ±1 intraassay standard deviation are shown. ○, no C8 or cefepime; ●, cefepime but no C8; □, C8 but no cefepime; ■, C8 and cefepime.

a complete terminal complement complex (no C8), there was no reduction in the bacterial input when untreated or cefepime-altered bacteria were examined (a bacteriostatic effect was observed with cefepime-altered cells at 50 µg of magainin 2 per ml). When sera was reconstituted with physiological levels of purified C8, a significant reduction in the $\log_{10}$ CFU per milliliter was observed for both untreated and cefepime-altered bacteria ($P < 0.001$, two-sample *t* test). Consistent with our earlier observations, cefepime-altered bacteria were more susceptible to the antimicrobial activity of magainin 2 than untreated bacteria in human sera were. However, in contrast to our previous data (Fig. 2), untreated bacteria were also susceptible to magainin killing in serum. Further investigation revealed that this difference is most likely due to the serum source and may reflect different cation contents in NHS and the C8-absorbed sera. These data demonstrated that the antimicrobial activity of magainin 2 is greatly potentiated by both serum complement and low levels of cefepime.

Combinations of magainin 2 and other antibiotics in serum. The ability of other antibiotics to potentiate the antimicrobial activity of magainin 2 in both H-NHS and NHS was determined. *E. coli* ATCC 25922 was grown to the mid-logarithmic phase and was examined with and without various concentrations of amikacin, ciprofloxacin, cefaloridine, or imipenem. For each antibiotic a range of concentrations was examined that allowed detection of any potential increase in magainin 2 antimicrobial activity. The antibiotic concentrations examined ranged from amounts at which no reduction in the $\log_{10}$ CFU per milliliter was observed after 3 h (compared with that of a non-antibiotic-treated control) to increasing amounts at which greater than 1-log-unit kill in both H-NHS and NHS serum was obtained. Cells were added to 40% NHS and 40% H-NHS, and after 180 min the $\log_{10}$ CFU per milliliter was determined for samples with and without magainin 2 (200 µg/ml) at each antibiotic concentration. There was no significant difference in the $\log_{10}$ CFU per milliliter with or without magainin 2 in either H-NHS or NHS when amikacin or ciprofloxacin was examined. In contrast, cells grown and assayed with 2 µg of cephaloridine per ml (one-half the MIC) (bacterial input was 3.84 ± 0.02 $\log_{10}$ CFU/ml) showed a significant difference in the $\log_{10}$ CFU per milliliter with or without magainin 2 in both H-NHS and NHS ($\log_{10}$ CFU/ml of 3.3 ± 0.04 with magainin 2 for H-NHS compared with 3.9 ± 0.034 without magainin 2; $\log_{10}$ CFU/ml of <3.0 with magainin 2 for NHS compared with 4.0 ± 0.04 without magainin 2; $P < 0.001$, two-sample *t* test). The increase in killing observed in NHS compared with that observed in H-NHS was also significant ($P < 0.01$, two-sample *t* test) and was similar to that previously observed with cefepime (Fig. 2). Cells grown with subinhibitory concentrations of imipenem also displayed optimal magainin killing in NHS when compared with that in H-NHS. Cells grown with 0.125 µg of imipenem per ml (1/16th the MIC) and assayed in H-NHS (bacterial input, 4.13 ± 0.03 $\log_{10}$ CFU/ml) increased in number ($\log_{10}$ CFU/ml of >4.3 with or without magainin 2). In contrast, when assayed in NHS, these cells were significantly more susceptible to magainin 2 ($\log_{10}$ CFU/ml of 3.0 ± 0.01 with magainin 2 for NHS compared with 4.0 ± 0.01 without magainin 2; $P < 0.001$, two-sample *t* test). The data showed that bacteria incubated with low levels of the β-lactam antibiotics (cephaloridine and imipenem) but not an aminoglycoside (amikacin) or a quinolone (ciprofloxacin) were more susceptible to the antimicrobial activity of magainin 2 in serum. In addition, similar to what was observed with cefepime (Fig. 2), the antimicrobial activity of magainin 2 was potentiated by NHS.

Magainin 2 and cefepime activity against other bacteria. A variety of different bacterial strains exposed to low levels of cefepime were more susceptible to the antimicrobial activity of magainin 2 in NHS. Strains were first examined for their susceptibilities to NHS. A concentration of serum (up to 40%) which did not significantly reduce the bacterial input was chosen. Magainin 2 was added to NHS, and antimicrobial activity was determined with untreated and cefepime-altered bacteria. In addition, the antimicrobial activity of magainin 2 was examined in H-NHS with cefepime-altered bacteria (Table 1). In the absence of cefepime, magainin 2 (200 µg/ml) had little or no effect on the $\log_{10}$ CFU per milliliter with three of the four strains examined. This was similar to what was observed with *E. coli* ATCC 25922 (Fig. 2). It was not determined whether the lower serum concentration used for *K. pneumoniae* influenced its susceptibility to magainin 2. In contrast to the data obtained in serum without antibiotic, the antimicrobial activity of magainin 2 was significantly enhanced ($P < 0.001$, two-sample-*t* test) in NHS with each bacterial strain when cefepime was present (Table 1). It should be noted that 400 µg of magainin 2 per ml was used for *E. cloacae* with cefepime. Therefore, a direct comparison with the data obtained without cefepime cannot be made with this strain. These studies showed that with three of the four strains examined, the antimicrobial activity of magainin 2 was potentiated by NHS.

Mouse protection experiments. In vivo protection experiments showed that magainin 2, when administered with a low level of cefepime, significantly increased the survival of mice given a lethal infection of *E. coli* H16. Neutropenic mice were challenged with 2×10^4 *E. coli* H16 organisms by intraperitoneal injection and were divided into four separate

TABLE 1. Susceptibilities of a variety of bacteria to magainin 2 killing in human serum[a]

Serum content	Log_{10} CFU/ml			
	E. coli A011	*E. coli* H16	*E. cloacae*	*K. pneumoniae*
No cefepime				
Input	4.03 ± 0.03	4.03 ± 0.04	4.07 ± 0.02	3.98 ± 0.01
No MGN	5.40 ± 0.06	5.37 ± 0.02	5.82 ± 0.02	4.30 ± 0.01
With MGN	5.47 ± 0.01	5.31 ± 0.03	5.78 ± 0.01	3.42 ± 0.02
Plus cefepime[b]				
Input	4.18 ± 0.01	4.07 ± 0.02	4.07 ± 0.01[c]	4.02 ± 0.03
No MGN	4.59 ± 0.03	4.14 ± 0.01	>4.3	4.03 ± 0.01
With MGN	2.74 ± 0.09	3.49 ± 0.01	3.31 ± 0.02[c]	<3.08
H-NHS with MGN[d]	>4.3	>4.3	3.7 ± 0.03[c]	<3.08

[a] Bacterial cells were added to NHS (input) with and without 200 μg of magainin 2 (MGN) per ml. After 180 min, dilutions were performed and the $\log_{10}$ CFU per milliliter was determined. Values represent the mean and one intraassay standard deviation. The following NHS concentrations were used: *E. coli* A011, 20%; *E. coli* H16 and *E. cloacae*, 40%; *K. pneumonia*, 5%.

[b] Cefepime concentration for *E. coli* A011; 0.016 μg/ml; *E. coli* H16, 0.008 μg/ml; *E. cloacae*, 0.008 μg/ml; and *K. pneumonia*, 0.016 μg/ml.

[c] A total of 400 μg of magainin 2 per ml was used for *E. cloacae* plus cefepime.

[d] These samples contained magainin 2 and cefepime and were examined in H-NHS.

treatment groups, and their survivals were monitored for 10 days (Fig. 4). The majority of mice that were administered PBS (29 dead of 32 total) or magainin 2 (31 dead of 34 total) died within 3 days after the bacterial challenge. Although higher doses of magainin 2 might be protective, they were not examined in this study. Another group was given a suboptimal dose of cefepime and had slightly better survival (18.5% survival; 7 alive of 39 total), although this did not represent a significant increase ($P < 0.5$, chi-square test) when compared with the control mice that were administered PBS. However, when magainin 2 and cefepime were coadministered, there was a significant increase ($P < 0.001$, chi-square test) in the survival of the mice (62.5% survival; 24 alive of 39 total). The combination of magainin 2 and low-level cefepime treatment resulted in a significant increase in the survival of the mice when compared with the survival after either treatment alone.

In similar experiments, combinations of amikacin (an aminogycoside) and magainin 2 were examined in vivo. After determining a suboptimal dose of amikacin and dividing the mice into four groups, the following results were obtained: PBS alone, 0 alive of 8 total; magainin 2 (two injections of 1 mg per mouse), 0 alive of 9 total; amikacin (two injections of 3 mg/kg), 3 alive of 10 total; a combination of magainin 2 and amikacin (at the doses given above), 1 alive of 10 total. No increase in the survival of the mice was observed when a suboptimal dose of amikacin was combined with magainin 2. This was consistent with the in vitro data (see above) and supports the hypothesis that β-lactam antibiotics may be unique in their interactions with magainin 2 and serum.

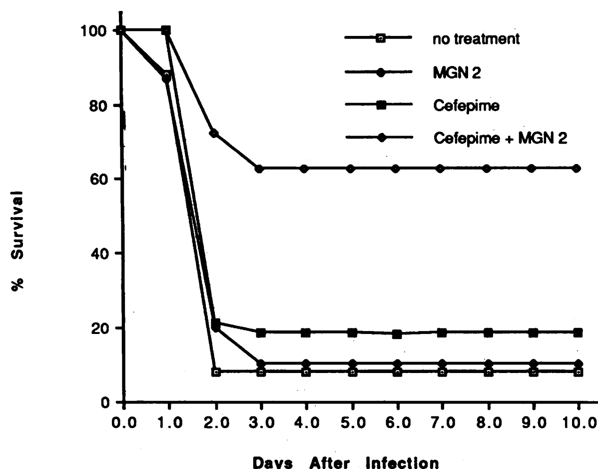

FIG. 4. Effect of magainin 2 and cefepime for the treatment of a systemic *E. coli* infection. Mice were challenged with 2×10^4 *E. coli* H16 organisms and divided into the four treatment groups described in the text and indicated in the figure. The number of mice in each group was as follows: no treatment, $n = 32$; magainin 2, $n = 34$; cefepime, $n = 39$; and cefepime plus magainin 2, $n = 39$. The results represent the sums of four separate experiments. In each experiment, statistically significant protection ($P < 0.05$, Fisher exact test) was obtained with the combination of magainin 2 and cefepime.

DISCUSSION

Therapies designed to augment an antibiotic's action by killing antibiotic-altered bacteria might be useful clinically. It is possible that a number of therapeutic failures in neutropenic patients are due to minor changes in the antibiotic susceptibilities of the bacteria which occur during treatment (1). This suggested to us that at least transient forms of antibiotic-altered bacteria may be present in vivo. Presumably, immunocompromised patients cannot eradicate these bacteria.

Our previous observation (5) that bacterial susceptibility to complement protein C9 deposition was altered by low levels of cefepime prompted us to examine the combination of cefepime and magainin 2. The antimicrobial peptide magainin 2 was chosen since both magainin 2 and the terminal complement complex are believed to act at the bacterial inner membrane (37, 38), and this peptide is representative of a new group of peptides that have potential for the treatment of systemic infections (30, 39).

In vitro susceptibility assays in buffer revealed that both magnesium chloride and calcium chloride completely inhibited the antimicrobial activity of magainin 2 when untreated bacteria were examined. Consistent with this observation is the fact that the antimicrobial activity of another group of similar peptides called cecropins has been shown to be

significantly increased when EDTA is present in the test medium (6). These studies are both consistent with other studies that have shown that a variety of cationic compounds and antibiotics are inhibited by divalent cations (13, 14). In contrast, the antagonistic effect of divalent cations on the antimicrobial activity of magainin 2 was significantly lessened when cefepime-altered bacteria were examined. It has been proposed that the site of divalent cation inhibition of other cationic agents is at the outer membrane (13). If this is correct, then it is possible that β-lactam antibiotics alter existing or create new sites in the outer membrane for peptide entry that are less susceptible to cation inhibition.

Complement significantly increased the potency of magainin 2 in serum. Experiments with C8-deficient sera demonstrated that magainin 2 and complement act together to kill bacteria. The results obtained with the bactericidal combination of complement and magainin 2 are similar to previous observations made by Vaara and Vaara (33), in which a synergistic bactericidal combination of polymyxin B nonapeptide and human complement was demonstrated. In a separate study (34), a variety of different peptides have been shown to have a disorganizing effect on the outer membrane which increased the bacteria's susceptibility to complement. In this study, it was found that cefepime further enhanced bacterial susceptibility to complement and magainin 2. In C8-deficient sera replenished with C8, magainin 2 was more potent against cefepime-altered cells, and furthermore, in NHS cefepime was required for magainin 2 activity. We suspect that the difference in potency observed in replenished sera, NHS, and buffer reflect the susceptibility of magainin 2 to experimental conditions.

Optimal antimicrobial activity in serum, then, requires three components: complement, magainin 2, and cefepime. No potentiation of the antimicrobial activity of magainin 2 was observed in serum with either ciprofloxacin (a quinolone) or amikacin (an aminoglycoside). The possibility that serum components, divalent cations, or other factors may have interfered with a potential interaction of ciprofloxacin or amikacin and magainin 2 was not examined. However, the increase in susceptibility to both magainin 2 and complement by β-lactam-altered bacteria is consistent with the hypothesis that β-lactam antibiotics create sites on the bacterial cell surface that allow more complement deposition (5) and facilitate access to the inner bacterial membrane. It is not known whether these are the same or different sites, and further experiments are necessary to more fully understand the interaction among complement, magainin 2, and cefepime.

The results from the mouse protection studies encourage further evaluation of therapies directed at antibiotic-altered bacteria. When administered alone, neither cefepime nor magainin 2 protected mice from the lethal effects of the bacterial infection. The combination of these agents, however, resulted in highly significant protection. The amount of cefepime administered in the animal protection experiments determined the survival rate for the mice. Administration of low doses of cefepime was necessary to determine whether the addition of magainin 2 would significantly improve survival. Low doses of cefepime may mimic the clinical situation in which insufficient concentrations of the β-lactam get to its bacterial target. The failure of high concentrations of magainin 2 to protect mice is consistent with its lack of antimicrobial activity in NHS when it was examined with untreated bacteria.

The ability of magainin 2 and cefepime to increase the survival of mice given a lethal infection of *E. coli* offers promise for use of these types of peptides as adjuncts to β-lactam antibiotic therapy. Results of the experiments in animals suggest that cefepime-altered forms of the bacteria are killed by magainin 2 in vivo. Further support of this was obtained when no increase in survival was observed in mouse protection experiments with magainin 2 and amikacin.

Further work is necessary to determine whether magainin 2 or similar peptides will be useful adjuncts to antibiotic therapy in neutropenic patients. Our in vivo work is consistent with the in vitro data and supports the idea that elimination of bacteria that are injured but not killed by antibiotics may be beneficial. The utility of the approach is dependent upon the presence of antibiotic-altered forms of bacteria in vivo. Their presence or role in therapy is currently uncertain (1). We suspect that this therapy is not appropriate for positive function resistance (1) (high level), since the change in antibiotic susceptibility would be too great to allow alteration of the bacteria in the absence of killing.

ACKNOWLEDGMENTS

We thank Leah Lipsich and Perry Fell for critical reading of the manuscript and helpful suggestions; Marvin Gorman for critical reading, keen advice, and encouragement; and Ana Wieman for help in manuscript preparation.

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