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Synergistic Effect of a Recombinant N-Terminal Fragment of Bactericidal/Permeability-Increasing Protein and Cefamandole in Treatment of Rabbit Gram-Negative Sepsis

YUE LIN,* WILLIAM J. LEACH, AND W. STEVE AMMONS

Department of Pharmacology, Xoma Corporation, Berkeley, California 94710

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As a consequence of their bactericidal actions, many antibiotics cause the release of endotoxin, a primary mediator of gram-negative sepsis. Bactericidal/permeability-increasing protein (BPI) has bactericidal activity and neutralizes endotoxin in vitro and in vivo. We sought to examine the effect of a recombinant N-terminal fragment of BPI ($rBPI_{21}$) in conjunction with cefamandole, a cephalosporin antibiotic, in the treatment of *Escherichia coli* bacteremia and septic shock in rabbits. Cefamandole (100 mg/kg of body weight) was injected intravenously. This was followed by simultaneous 10-min infusions of *E. coli* O7:K1 (9×10^9 CFU/kg) and $rBPI_{21}$ (10 mg/kg). $rBPI_{21}$ was continuously infused for an additional 110 min at 10 mg/kg/h. The administration of $rBPI_{21}$ in conjunction with the administration of cefamandole prevented the cefamandole-induced increase of free endotoxin in plasma, accelerated bacterial clearance, ameliorated cardiopulmonary dysfunction, and thereby, prevented death, whereas neither agent alone was protective in this animal model. The efficacy of the combined treatment with $rBPI_{21}$ and cefamandole suggests a synergistic interaction between the two agents. The data indicate that $rBPI_{21}$ may be useful in conjunction with traditional antibiotic therapy.

Despite the prompt administration of contemporary bactericidal antibiotics, septic shock secondary to overwhelming bacterial infections remains a major cause of mortality in critically ill patients (3, 19). As a consequence of their bactericidal actions, many antibiotics cause the release of endotoxin, a known primary mediator of gram-negative septic shock (3, 5–9). Antibiotic-induced endotoxin liberation has been demonstrated during the treatment of human septicemia (6, 37) and in rabbits infected experimentally with *Escherichia coli* (37, 39, 41). Furthermore, endotoxin liberated during antibiotic therapy is believed to be correlated with cardiovascular and pulmonary dysfunction in septic shock (7, 8, 16, 32, 34). In view of the significant role that endotoxin may play in the pathogenesis of sepsis, especially during antibiotic treatment, much effort has been spent identifying new therapeutic agents that neutralize endotoxin activity (for a review, see reference 40).

Among the substances with lipopolysaccharide (LPS)-neutralizing activity is bactericidal/permeability-increasing protein (BPI). BPI is a cationic protein found in human neutrophil azurophilic granules (44, 45). BPI is bactericidal toward gram-negative bacteria (46) and neutralizes the biological activities of endotoxin (25, 26, 29, 30). The 25-kDa N-terminal portion of the molecule contains the biological activity of the holoprotein (29, 30). Previous studies have demonstrated that a recombinant 23-kDa N-terminal fragment of BPI, $rBPI_{23}$, binds LPS and neutralizes the biological activity of LPS in vitro (10, 11, 15, 27, 28) and in vivo (2, 18, 21, 22, 43). Furthermore, $rBPI_{23}$ prevented death and enhanced bacterial clearance in mouse models of gram-negative bacteremia (17, 20). Recently, $rBPI_{21}$, a recombinant N-terminal fragment of BPI that is closely related to $rBPI_{23}$, was shown to have efficacy similar to that of $rBPI_{23}$ against gram-negative sepsis (1). Here, we report the cooperative effects of $rBPI_{21}$ and cefamandole in the treatment of acute *E. coli* bacteremia and septic shock in rabbits.

(This study was presented in part at the 34th Interscience Conference on Antimicrobial Agents and Chemotherapy, Orlando, Fla., 4 to 7 October 1994.)

MATERIALS AND METHODS

The present study was approved by an institutional animal care and use committee, and the experiments described herein were performed in accordance with the guidelines of the National Institutes of Health.

Reagents. $rBPI_{21}$ is a recombinant N-terminal protein produced from a construct encoding the first 193 amino acids of human BPI. In addition, an alanine residue was substituted for a cysteine residue at position 132. $rBPI_{21}$ was cloned and expressed in CHO-K1 cells and was purified by cation-exchange chromatography (4, 14). $rBPI_{21}$ binds to the same spectrum of LPS types that $rBPI_{23}$ does, the unmodified N-terminal fragment, and has inhibitory effects similar to those of $rBPI_{23}$ on LPS-induced cytokine production in whole human blood (31). $rBPI_{21}$ was formulated in a buffer containing 5 mM sodium citrate, 150 mM sodium chloride (pH 5.0), and stabilizing surfactants (vehicle). Cefamandole naftate (Mandol; Eli Lilly & Company, Indianapolis, Ind.) was prepared in phosphate-buffered saline (PBS).

Bacterial dose preparation. Log-phase *E. coli* O7:K1 (ATCC 23503) was suspended in sterile PBS to a concentration of 10^{10} CFU/ml. The concentration was initially estimated from a standard curve by measuring the optical density at 570 nm and was later verified by counting the numbers of CFU from dilutions that had been cultured onto tryptic soy agar plates and incubated overnight at 37°C. The actual bacterial doses ranged from 8.8×10^9 to 9.2×10^9 CFU/kg (see Table 1).

Animal preparations and surgery. Thirty-four adult male New Zealand White rabbits (Charles River Laboratories, St. Constant, Canada) weighing between 1.8 and 2.3 kg were used in the study. The animals were fasted for 24 h before use. Each rabbit was anesthetized and catheterized as described previously (22). In short, a left femoral arterial catheter was implanted for blood pressure determination and blood sample collection. To measure cardiac output, a catheter was implanted in the right jugular vein for administration of the thermal dilution injectate (PBS), and a 3.5-French thermistor-tipped catheter (Columbus Instruments, Columbus, Ohio) was placed in the right carotid artery. Proper placement of the injectate catheter and the thermistor-tipped catheter was confirmed by observing the characteristic thermal dilution curve. The catheters were exteriorized at the base of the neck. In addition, an angiocath (20 gauge by 1.25 in. [3.18 cm]; Becton Dickinson Vascular Access, Sandy, Utah) was implanted in the marginal ear vein for administration of the bacteria. The animals were allowed to recover from the surgery until all hemodynamic and blood gas parameters stabilized within the normal range.

Experimental protocol. As shown in Fig. 1, all animals received a 10-min

* Corresponding author. Mailing address: Department of Pharmacology, Xoma Corporation, 2910 Seventh St., Berkeley, CA 94710. Phone: (510) 644-1170. Fax: (510) 841-7805.

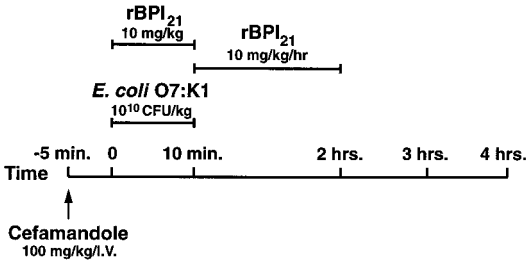

FIG. 1. Experimental design. I.V., intravenous.

infusion of *E. coli* O7:K1 via the ear vein. Cefamandole was slowly injected intravenously at a dose of 100 mg/kg of body weight prior to bacterial challenge. $rBPI_{21}$ was infused via the right jugular vein at a dose of 10 mg/kg during the first 10 min. This was followed by a continuous infusion at 10 mg/kg/h for an additional 110 min.

The animals were divided into four groups according to the treatment that they received. The vehicle control group received saline and $rBPI_{21}$ vehicle, the $rBPI_{21}$ group received saline and $rBPI_{21}$, the cefamandole group received cefamandole and $rBPI_{21}$ vehicle, and the cefamandole–$rBPI_{21}$ group received both cefamandole and $rBPI_{21}$.

Plasma endotoxin assay. Plasma samples were filtered through a sterile 0.2-μm-pore-size syringe filter (Whatman) to remove the bacteria (39) and were stored at −70°C until they were assayed. The filtered plasma samples were randomly plated and cultured onto tryptic soy agar plates, and the plates were incubated overnight at 37°C to verify that the filtering process removed all bacteria. Free endotoxin levels in the plasma samples were determined by a modified *Limulus* amebocyte lysate chromogenic assay (Pyrochrome; Associates of Cape Cod). The microplates (PyroPlate; Associates of Cape Cod) were incubated for 24 min at 37°C, and the optical density at 405 nm was measured on an enzyme-linked immunosorbent assay reader (Vmax Kinetic Microplate Reader; Molecular Devices). A standard curve with *E. coli* O113 endotoxin (Associates of Cape Cod) was used to calibrate each assay. Endotoxin concentrations were calculated from the linear part of the standard curve.

Bacterial clearance. Tenfold serial dilutions of an aliquot of each blood sample were made in sterile PBS. A 100-μl aliquot of each dilution was cultured onto tryptic soy agar, the plates were incubated overnight at 37°C, and the numbers of CFU were counted. The results were expressed as the number of CFU per milliliter of blood.

Measurement of physiological parameters. Preliminary experiments demonstrated that $rBPI_{21}$, or vehicle, when it was administered in the same manner to unchallenged rabbits, did not affect their hemodynamic, respiratory, or metabolic parameters.

(i) Hemodynamics. Mean arterial blood pressure, cardiac output, and heart rate were monitored continuously throughout the experiments and were recorded every 30 min and at the end of the bacterial infusion. Cardiac output was determined by the thermodilution technique as described previously (22). Cardiac output measurements were always performed in duplicate, and each injection consisted of 900 μl of PBS at room temperature. The cardiac index (CI) was then calculated as the cardiac output per kilogram of body weight.

(ii) Blood gas analysis. Blood gas parameters were determined every 30 min by using a Ciba-Corning blood gas system (model 278; Ciba-Corning Diagnostics Corp., Medfield, Mass.). The blood gas system directly measures blood pH, partial pressure of CO_2, and partial pressure of O_2 (pO_2). Other parameters including the alveolar-arterial oxygen gradient, standard HCO_3^-, and in vivo base excess were calculated by using the formulas provided by Ciba-Corning Diagnostic Corp.

(iii) Glucose and lactate assays. The levels of glucose and lactate in plasma were determined with a glucose–L-lactate analyzer (2300 STAT; Yellow Springs Instruments, Yellow Springs, Ohio).

Statistical analysis. Data are reported as means ± standard errors of the means and were analyzed with SAS/STAT software program (SAS Institute Inc., Cary, N.C.). The general linear models procedure was used for repeated measures analysis of variance, and Tukey's studentized range test was used when F ratios exceeded the critical value ($P < 0.05$) (36). Survival data were compared by using the chi-square statistic.

RESULTS

Bacterial clearance. Bacterial counts in blood decreased in all four groups during the first 120 min and remained relatively stable thereafter (Fig. 2A). Compared with the vehicle control group, the bacterial count was nearly 1 log lower in the $rBPI_{21}$ group, 2 logs lower in the cefamandole group, and approximately 2.5 logs lower in the cefamandole–$rBPI_{21}$ group at the end of the study. The cefamandole–$rBPI_{21}$ group had significantly lower counts than the other groups at 30 and 60 min.

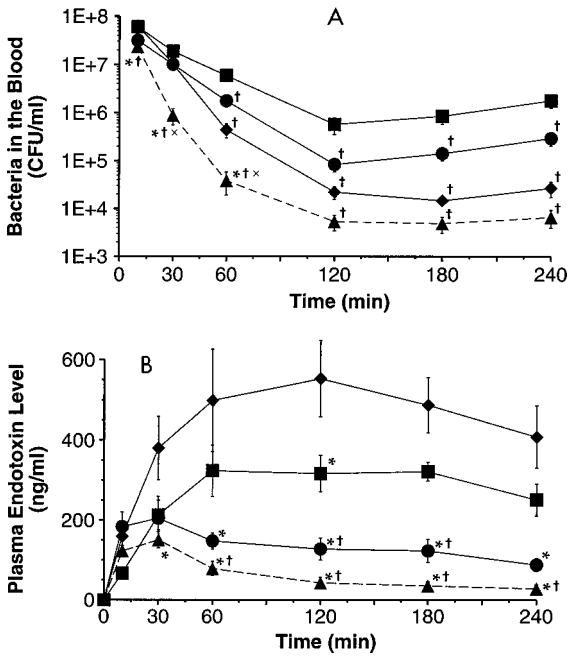

FIG. 2. (A) Effects of $rBPI_{21}$ and/or cefamandole on bacterial clearance from blood. *, $P < 0.05$ versus cefamandole group; †, $P < 0.05$ versus vehicle control group; x, $P < 0.05$ versus $rBPI_{21}$ group. (B) Effects of $rBPI_{21}$ and/or cefamandole on plasma endotoxin levels. *, $P < 0.05$ versus cefamandole group; †, $P < 0.05$ versus vehicle control group; x, $P > 0.05$ versus $rBPI_{21}$ group. ◆, cefamandole ($n = 8$); ●, $rBPI_{21}$ ($n = 7$); ▲, cefamandole–$rBPI_{21}$ ($n = 8$); ■, vehicle control group ($n = 8$). All P values were determined by Tukey's test.

Plasma endotoxin levels. The free endotoxin levels in plasma increased in all animal groups at the end of the infusion of bacteria (Fig. 2B). The animals treated with $rBPI_{21}$ had less than half of the endotoxin levels observed in the vehicle control group at 60 min and beyond. The cefamandole group had the highest plasma endotoxin levels among the four groups, reaching as high as 553 ± 95 ng/ml at 120 min. The lowest levels of endotoxin in plasma were found in the cefamandole–$rBPI_{21}$ group at all the time points after 30 min.

Hemodynamics. The mean arterial blood pressure declined in all four groups after bacterial challenge (Fig. 3A). The decline in the cefamandole–$rBPI_{21}$ group, however, was significantly less than those in the other groups.

CI fell rapidly to about 50% of the prechallenge level by the end of the bacterial infusion in all groups except the cefamandole–$rBPI_{21}$ group. The CIs for rabbits treated with both agents were significantly higher than those for rabbits in the other groups during the first half of the study and were significantly higher than those for rabbits in the cefamandole group for the entire study (Fig. 3B).

Blood gases and metabolism. The pO_2 fell 18% in both vehicle control and $rBPI_{21}$ groups and 35% in the cefamandole group during the first 30 min (Fig. 4A). The pO_2 then recovered and returned to the baseline levels in these groups except in the cefamandole group. In contrast, the pO_2 in the cefamandole–$rBPI_{21}$ group remained above 90 mm Hg for the entire study.

The alveolar-arterial oxygen gradient rose markedly in the vehicle control, $rBPI_{21}$, and cefamandole groups during the first 30 min (Fig. 4B). The alveolar-arterial oxygen gradient in

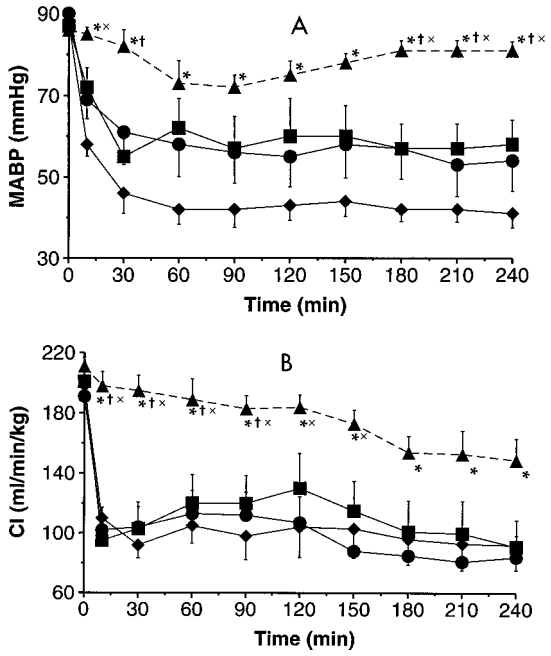

FIG. 3. (A) Effects of $rBPI_{21}$ and/or cefamandole on mean arterial blood pressure (MABP). *, $P < 0.05$ versus cefamandole group; †, $P < 0.05$ versus vehicle control group; x, $P < 0.05$ versus $rBPI_{21}$ group. ◆, cefamandole ($n = 8$); ●, $rBPI_{21}$ ($n = 8$); ▲, cefamandole–$rBPI_{21}$ ($n = 8$); ■, vehicle control group ($n = 8$). (B) Effects of $rBPI_{21}$ and/or cefamandole on CI. *, $P < 0.05$ versus cefamandole group; †, $P < 0.05$ versus vehicle control group; x, $P < 0.05$ versus $rBPI_{21}$ group. ◆, cefamandole ($n = 7$); ●, $rBPI_{21}$ ($n = 8$); ▲, cefamandole–$rBPI_{21}$ ($n = 8$); ■, vehicle control group ($n = 7$). All P values were determined by Tukey's test.

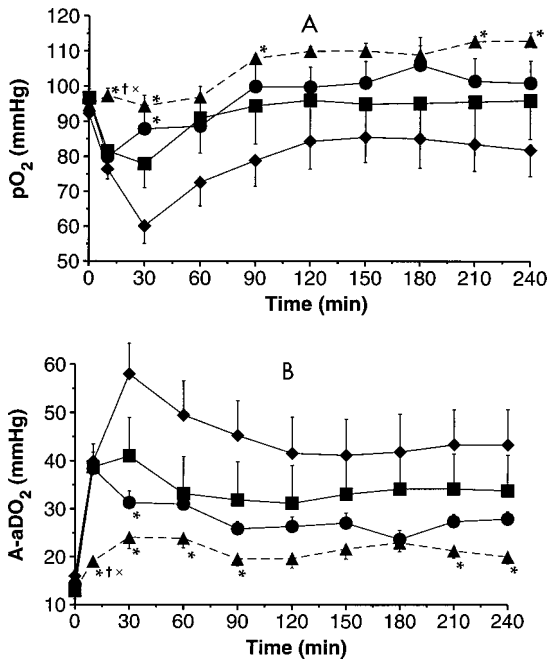

FIG. 4. (A) Effects of $rBPI_{21}$ and/or cefamandole on arterial oxygen tension (pO_2). *, $P < 0.05$ versus cefamandole group; †, $P < 0.05$ versus vehicle control group; x, $P < 0.05$ versus $rBPI_{21}$ group. ◆, cefamandole ($n = 8$); ●, $rBPI_{21}$ ($n = 7$); ▲, cefamandole–$rBPI_{21}$ ($n = 8$); ■, vehicle control group ($n = 8$). (B) Effects of $rBPI_{21}$ and/or cefamandole on calculated alveolar-arterial oxygen gradient ($A\text{-}aDO_2$). *, $P < 0.05$ versus cefamandole group; †, $P < 0.05$ versus vehicle control group; x, $P < 0.05$ versus $rBPI_{21}$ group. ◆, cefamandole ($n = 8$); ●, $rBPI_{21}$ ($n = 7$); ▲, cefamandole–$rBPI_{21}$ ($n = 8$); ■, vehicle control group ($n = 8$). All P values were determined by Tukey's test.

the cefamandole–$rBPI_{21}$ group was significantly lower than that in the cefamandole group at most time points and was significantly lower than that in the vehicle control or $rBPI_{21}$ group at 30 min.

Arterial blood pH fell moderately and stayed above 7.30 in the vehicle control group for the entire study, but it fell to as low as 7.17 ± 0.05 in the cefamandole group (Fig. 5A). The pH in the cefamandole–$rBPI_{21}$ group, however, remained within the normal range throughout the experiment.

Plasma lactate levels increased gradually in all four groups after bacterial challenge (Fig. 5B). The values in the cefamandole–$rBPI_{21}$ group were significantly lower than those in the other groups at various time points. Other arterial blood gas parameters, such as the arterial HCO_3^- and base excess, also changed gradually with time in all four groups. However, there was no significant difference between the groups (data not shown).

Lethality of bacterial challenge. A number of animals died before the end of the 4-h study (Table 1). Most of these animals died in the last hour (180 to 240 min) of cardiopulmonary dysfunction and acidosis. Neither $rBPI_{21}$ nor cefamandole alone improved the survival rate. Nonetheless, the combined treatment with cefamandole and $rBPI_{21}$ completely prevented death.

DISCUSSION

Antibiotic-induced endotoxin liberation during the treatment of gram-negative sepsis has been demonstrated in animal models (5, 34, 37, 39) and in humans (5, 38). Cephalosporin antibiotics disrupt the cell walls of susceptible bacteria (23, 42), which results in endotoxin liberation (for a review, see reference 33). In the present study, cefamandole was able to eliminate approximately 99% of the circulating bacteria (Fig. 2A). Nonetheless, this bactericidal effect was associated with a strong increase in the level of free endotoxin in the plasma of rabbits in the cefamandole group (Fig. 2B). In this group, there also appeared to be an exacerbation of multiple system dysfunction (Fig. 3 to 5). These results strongly support previous findings by other investigators indicating that antibiotic therapy during severe sepsis can cause additional endotoxin release in animals (5, 34, 37, 39), as well as in humans (5, 38), thereby exacerbating shock (12, 13, 34). Furthermore, the cefamandole group also had the highest mortality rate (75%) among the four groups (Table 1). The data suggest that free endotoxin levels in the blood, rather than the count of viable bacteria, may be more closely related to, if not the cause of, the high mortality in this acute bacteremia model.

Among endotoxin-neutralizing agents other than BPI, polymyxin B (9) and ENP (a recombinant endotoxin-neutralizing protein) (35) have been studied in rabbit peritonitis models. These agents were demonstrated to improve some physiological responses or to reduce circulating endotoxin levels when they were used in conjunction with antibiotic therapies. However, they failed to improve survival. In the present study, $rBPI_{21}$, when given in conjunction with cefamandole, appeared to have accelerated the clearance of bacteria from the blood (Fig. 2A), prevented the cefamandole-induced increase in the level of free endotoxin in plasma (Fig. 2B), and ameliorated multiple system dysfunction (Fig. 3 to 5). Moreover, the combined treatment with $rBPI_{21}$ and cefamandole prevented cir-

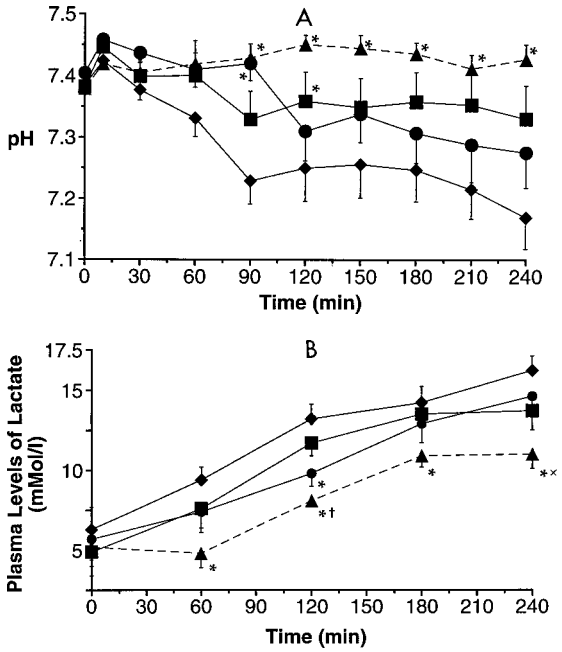

FIG. 5. (A) Effects of $rBPI_{21}$ and/or cefamandole on arterial blood pH. *, $P < 0.05$ versus cefamandole group. ◆, cefamandole ($n = 8$); ●, $rBPI_{21}$ ($n = 7$); ▲, cefamandole–$rBPI_{21}$ ($n = 8$); ■, vehicle control group ($n = 8$). (B) Effects of $rBPI_{21}$ and/or cefamandole on plasma lactate level. *, $P < 0.05$ versus cefamandole group; †, $P < 0.05$ versus vehicle control group; x, $P < 0.05$ versus $rBPI_{21}$ group. ◆, cefamandole ($n = 7$); ●, $rBPI_{21}$ ($n = 8$); ▲, cefamandole–$rBPI_{21}$ ($n = 8$); ■, vehicle control group ($n = 7$). All P values were determined by Tukey's test.

culatory shock and subsequent death, whereas neither agent alone protected the animals.

The ability of $rBPI_{23}$ to bind to and neutralize endotoxin, to inhibit LPS-induced production of cytokines, and to prevent LPS-induced neutrophil adherence to endothelial cells has been well characterized in vitro (10, 11, 15, 28). $rBPI_{23}$ and/or $rBPI_{21}$ were also protective in animal models of sepsis and endotoxemia (2, 20, 21). $rBPI_{23}$ reduced cytokine production in endotoxic mice (18) and prevented LPS-induced multiple organ dysfunction (1, 2, 33, 34, 43). In the present study, $rBPI_{21}$ alone significantly improved bacterial clearance and reduced plasma endotoxin levels, but it failed to prevent multiple system dysfunction or subsequent death. We believe that the results of the present study do not contradict previous findings, given the uniqueness of this animal model. The purpose of the present investigation was to assess the cooperative effects of two agents, $rBPI_{21}$ and cefamandole. Thus, the model was designed to achieve a state of septic shock that was so overwhelming that neither agent alone would be effective in protecting the animals. This animal model also differs from most clinical cases of septicemia in that an extremely high dose of bacteria was introduced in a very short period and that antibiotic was given prior to bacteremia in order to achieve maximum killing of the bacteria and endotoxin liberation in early reversible stages of multiple system dysfunction. Therefore, it is noteworthy that in this extreme and acute model of bacteremia, the therapeutic effects achieved probably reflect substantial alterations in the pathophysiology associated with the bacterial infusion.

TABLE 1. Survival of rabbits challenged with bacteria

Group	Body wt (kg)	Bacterial dose CFU/kg [10^9])	No. of rabbits that survived/total no. of rabbits
Cefamandole	2.2 ± 0.08	8.8 ± 0.5	2/8
Cefamandole–$rBPI_{21}$	2.1 ± 0.05	9.0 ± 0.6	9/9[a]
$RBPI_{21}$	2.2 ± 0.08	9.2 ± 0.4	4/8
Vehicle control	2.1 ± 0.06	9.2 ± 0.3	4/9

[a] $P < 0.01$ versus each of the other three groups.

The mechanisms underlining the cooperative effects of $rBPI_{21}$ and cefamandole are not clear. The efficacy data suggest a synergistic interaction between the two agents. For instance, the combined treatment prevented the dramatic decline in CI, whereas either agent alone had no effect (Fig. 3B). One possible mechanism may be attributed to the complementary effects of the two agents. Cefamandole markedly reduced the counts of circulating bacteria. Therefore, significantly fewer bacteria or less bound LPSs which are located on the surface of the bacterial wall were available for $rBPI_{21}$ to bind to. As a result, more free $rBPI_{21}$ molecules were available to neutralize free endotoxin. Our data strongly support this mechanism, in that both bacterial clearance and free endotoxin levels in plasma were the lowest in the cefamandole–$rBPI_{21}$ group. Another possible mechanism which may contribute partly to the cooperative effects of the two agents is that the two agents may facilitate or accelerate each other's action in terms of penetrating the cell wall and binding to target sites. For instance, the damaging effect of $rBPI_{21}$ on the bacterial wall and cytoplasmic membrane (24) may facilitate cefamandole's penetration of the cell wall and promote the subsequent binding of cefamandole to the penicillin-binding proteins located on the bacterial cytoplasmic membrane, which in turn expedite the inhibition of bacterial wall synthesis by cefamandole (23, 42), or vice versa. Presumably, this cooperative interaction leads to an enhancement of bacterial killing, particularly in the early stage of the treatment, as indicated in Fig. 2A. It is, however, not clear to what extent the enhancement of bacterial clearance may contribute to the protective effect observed in the combined treatment group.

In summary, the administration of $rBPI_{21}$ in conjunction with cefamandole completely prevented the cefamandole-induced increase of free endotoxin in plasma, accelerated bacterial clearance, ameliorated cardiopulmonary dysfunction, and thereby, prevented death. The efficacy of the combined treatment with $rBPI_{21}$ and cefamandole suggests a synergistic interaction between the two agents. This synergistic protective effect is most likely due to the ability of $rBPI_{21}$ to neutralize the endotoxin released as a result of cefamandole treatment and/or its ability to enhance the bactericidal activity of cefamandole. The results suggest that $rBPI_{21}$ may be beneficial when it is used in conjunction with traditional antibiotic therapy to protect against endotoxemia and cardiovascular and pulmonary dysfunction of septic shock.

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