Evaluation of Antimicrobial and Lipopolysaccharide-Neutralizing Effects of a Synthetic CAP18 Fragment against *Pseudomonas aeruginosa* in a Mouse Model

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CAP18 (cationic antimicrobial protein; 18 kDa) is a neutrophil-derived protein that can bind to and inhibit various activities of lipopolysaccharide (LPS). The 37 C-terminal amino acids of CAP18 make up the LPS-binding domain. A truncated 32-amino-acid C-terminal fragment of CAP18 had potent activity against *Pseudomonas aeruginosa* in vitro. We studied the antimicrobial and LPS-neutralizing effects of this synthetic truncated CAP18 peptide (CAP18106–137) on lung injury in mice infected with cytotoxic *P. aeruginosa*. To determine its maximal effect, the CAP18106–137 peptide was mixed with bacteria just prior to tracheal instillation, and lung injury was evaluated by determining the amount of leakage of an alveolar protein tracer (125I-albumin) into the circulation and by the quantification of lung edema. The lung injury caused by the instillation of 5 × 10^5 CFU of *P. aeruginosa* was significantly reduced by the concomitant instillation of CAP18106–137. However, the administration of CAP18106–137 alone, without bacteria, induced lung edema, suggesting that it has some toxicity. Also, the peptide did not significantly reduce the number of bacteria that had been simultaneously instilled, nor did it significantly improve the survival of the infected mice. The addition of CAP18106–137 to aztreonam along with the bacteria did decrease the level of antibiotic-induced release of inflammatory mediators including tumor necrosis factor alpha, interleukin-6, and nitric oxide and also improved the survival of the mice. Therefore, more investigations are needed to confirm the toxicities and the therapeutic benefits of CAP18106–137 as an adjunctive therapy to antibiotics in the treatment of infections caused by gram-negative bacteria.

It is well known that lipopolysaccharide (LPS), the outer membrane component of gram-negative bacteria, is an extremely biologically active substance and has a key role in the pathogenesis of the sepsis syndrome; circulating LPS induces the release of potent inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and IL-6 (1). Although antibiotics can kill gram-negative bacteria, the administration of antibiotics does not neutralize the LPS released from the outer membranes of the dying bacteria (32). This release of LPS can actually increase lung injury and lead to the sepsis syndrome (4). Therefore, a drug that neutralizes LPS may be a reasonable additional therapy to antibiotic therapy for infections caused by gram-negative bacteria (26).

CAP18 is a lipopolysaccharide-binding protein first isolated from rabbit granulocytes (12, 13). The protein is composed of two domains: an N-terminal portion with an unknown function and a C-terminal fragment of 37 amino acids that has LPS-binding activity (17). The C-terminal fragment also has potent antimicrobial activity against both gram-negative and gram-positive bacteria. Experiments with synthetic 37-amino acid C-terminal fragments of rabbit CAP18 also showed broad antimicrobial activity (19). This 37-amino-acid C-terminal fragment inhibited the release of inflammatory mediators from macrophages and decreased the rate of mortality among mice that had received lethal quantities of LPS (18, 19). A truncated 32-amino acid C-terminal fragment of CAP18 was found to have even more potent antibacterial activity (18–20) and was also found to protect pigs given lethal quantities of LPS (2) and to improve survival in the endotoxia model with mice sensitized with D-galactosamine (16).

Bacterial pneumonia is a leading cause of mortality among critically ill patients (9, 28, 34, 36). *Pseudomonas aeruginosa* is the most common gram-negative bacterium associated with nosocomial pneumonia (6, 14). Despite the use of potent antibiotics and intensive-care support, the management of patients with nosocomial pneumonia due to *P. aeruginosa* still results in sepsis, adult respiratory distress syndrome, multisystem organ failure, shock, and death (5, 27).

We investigated the antimicrobial and LPS-neutralizing effects of the truncated synthetic 32-amino acid C-terminal peptide of rabbit CAP18 (CAP18106–137) on *P. aeruginosa* in a mouse model. We mixed the synthetic CAP18 fragments with the bacteria to determine the maximal effects of the drug. By instilling the bacterial solution with antibiotics, we attempted to compare the maximal effect of the antibiotic in this model. Finally, both the CAP18 peptide and the antibiotic were mixed with the bacteria. Both treatments were compared as to the quantity of lung injury caused by the bacteria alone and CAP18 peptide alone. More beneficial effects were seen when the CAP18 peptide was instilled with the antibiotic and with the bacteria; the CAP18 peptide attenuated the inflammation and the lung injury caused by the antibiotic therapy that killed the *P. aeruginosa* bacteria.

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**MATERIALS AND METHODS**

**Laboratory animals.** Eight- to 12-week-old male BALB/c mice were purchased from Simonsen Laboratories (Gilroy, Calif.). Animals were housed in cages with filter tops under specific-pathogen-free conditions. Sterile food and water were given ad libitum. All experiments were done in compliance with the Animal Care Committee rules at The University of California, San Francisco, and all protocols were approved.

**Reagents and antibodies.** Synthetic CAP18 C-terminal peptide (CAP18_{106-137}) was made as reported previously (18). Aztreonam was purchased from E. R. Squibb & Sons, Inc. All antibodies and recombinant cytokines used in the measurement of the cytokine concentrations were purchased from Pharmingen, San Diego, Calif. These materials included: recombinant murine TNF-α, rat anti-mouse TNF-α monoclonal antibody (MP6-XT22; rat immunoglobulin G1 [IgG1]), biotin-conjugated rabbit anti-mouse TNF-α polyclonal antibody, recombinant murine IL-6, rat anti-mouse IL-6 monoclonal antibody (MP5-20F3; rat IgG1), and biotin-conjugated rat anti-mouse IL-6 monoclonal antibody (MP5-32C11; rat IgG2a).

**P. aeruginosa strains and culture conditions.** The wild-type strain *P. aeruginosa* PA103 was generously provided by Dara W. Frank (Medical College of Wisconsin, Milwaukee) and was stored as a bacterial stock at −70°C in a 10% sterile skim milk solution. Bacteria from this frozen stock were streaked onto Trypticase soy agar plates and grown in a deferrated dialyse of Trypticase soy at 33°C for 13 h in a shaking incubator. Cultures were centrifuged at 8,500 *g* for 5 min, and the bacterial pellet was washed twice in phosphate-buffered saline (PBS) and diluted into the appropriate number of CFU per milliliter in PBS, as determined by spectrophotometry. The quantity of bacteria was confirmed by sequential dilutions and overnight culture on sheep blood agar plates.

**In vitro bactericidal assay.** Twenty microliters of CAP18_{106-137} was added to 180 µl of bacterial suspension and this mixture was incubated at 37°C for 1 h. Then, 100 µl of this reaction mixture was plated onto an agar plate. After 24 h of incubation at 37°C, the numbers of CFU of live bacteria were determined.

**Intratracheal instillation of bacteria.** Bacterial solutions with or without antibiotics were administered to mice by an intratracheal technique. The mice were briefly anesthetized with inhaled methoxyflurane (Metofane; Pitman-Moore, Mundelein, Ill.) and then were placed in a supine position at a head-up angle of approximately 30°. Fifty microliters of the solution was instilled slowly into the lungs through a modified animal feeding needle (24 gauge; Popper & Sons, Inc., New Hyde Park, N.Y.) that had been inserted into the trachea via the mouth. The syringe was weighed prior to and after tracheal instillation to quantitate the exact amount instilled into each mouse.

The alveolar instillate consisted of 0.05 µCi of 125I-albumin labeled human serum albumin (Merck-Frosst, Quebec, Canada) added to the bacterial solution. The total radioactivity (in counts per minute per gram) of the instillate was measured in a gamma counter (Auto-Gamma, model 5550; Packard, Downers Grove, Ill.). CAP18_{106-137}, aztreonam, or both were added to the bacterial instillate just prior to tracheal instillation in the treated groups. Four hours after instillation, the mice were anesthetized with pentobarbital (2.0 mg intraperitoneally) and final weights were recorded. The hearts were removed and the lungs were cut into small pieces. The lungs, tracheas, opharynges, stomachs, and livers were harvested and the levels of radioactivity in these samples were measured. The percentage of the remaining radioactive albumin in the lung was measured.

**Quantification of bacterium-induced lung injury.** The quantity of 125I-albumin that had entered the circulation was calculated by multiplying the counts measured in the terminal blood sample (per milliliter) by the blood volume (body weight × 0.07) (38). The lungs were homogenized and placed in preweighed aluminum pans and dried in an oven at 80°C for 3 days to calculate the wet weight-to-dry weight ratios (wet/dry ratio) as described previously (38). Each experimental group except the control group had five mice. The control group, into which PBS had been instilled, had three mice. As we have shown previously, we can consistently cause a predictable quantity of lung injury with a specific dose of *P. aeruginosa* for a defined interval (29, 30). The wet/dry ratio of the lungs was used because it is a well-accepted index of lung edema (38).

**Bacterial cultures of the lungs.** The lungs were homogenized in sterile containers with sterile water (see above). Lung homogenates were sequentially diluted and plated on sheep blood agar plates for quantitative assessment of the remaining bacteria in the lungs.

**BAL.** Another set of mice was used for bronchoalveolar lavage (BAL). Four hours after bacteriological instillation, mouse lungs were lavaged with 1 ml of PBS–0.1% bovine serum albumin (BSA) solution. BAL fluid was centrifuged and filtered (syringe filter; pore size, 0.4 µm; Corning, Cambridge, Mass.) for cytokine enzyme-linked immunosorbent assay and for measurement of the nitrite concentration.

**Measurements of endotoxin and cytokine concentrations.** Chromogenic quantitative endotoxin assay was done with plasma and BAL fluids according to the manufacturer’s protocol (Pyrochrome; Cape Cod Inc., Falmouth, Mass.). Enzyme-linked immunosorbent assays were performed for TNF-α and IL-6. Microtiter plates (Easy wash; Corning, Cambridge, Mass.) were coated overnight at 4°C with 2 µg/ml of coating antibody (2 µg/ml of coating solution, which in turn consisted of 0.1 M NaHCO₃ [pH 8.2]). The plates were then washed twice with PBS with 0.05% Tween 20 (Sigma Chemical Co., St. Louis, Mo.), blocked with 200 µl of 3% BSA–PBS for 2 h at room temperature, and washed twice with PBS–0.05% Tween 20. Standards and samples were added to the plates (100 µl/well), and the plates were incubated at room temperature for 4 h. The plates were washed four times and 50 µl of biotin-conjugated developing antibody (1 µl/ml in 3% BSA–PBS) was added. The plates were incubated at room temperature for 45 min and washed six times, and 100 µl of streptavidin–alkaline phosphatase (2 µg/ml in 3% BSA–PBS; Jackson ImmunoResearch Laboratories, West Grove, Pa.) was added. The plates were incubated at 37°C for 45 min, washed six times, and developed with 100 µl of developing solution (Sigma 104; p-nitrophenyl phospho- phate) dissolved in 0.1 M alkaline buffer solution (Sigma 221; 2-amino-2-methyl-1-propanol buffer [1.5 M; pH 10.3]). The optical densities of the plates were read at 405 nm with a Spectramax microplate reader (Molecular Devices, Menlo Park, Calif.). Sample concentrations were calculated by comparison with standard curves by using recombinant murine cytokines.

**Measurement of nitric oxide metabolites.** The Griess reagent was prepared by mixing equal volumes of sulfanilamide (2.5% phosphoric acid) and naphthylethylen diamine dihydrochloride (0.1%). A total of 100 µl of reagent was mixed with 100 µl of supernatant, and the mixture was incubated for 30 min in the dark. The absorbance at 550 nm was then measured with the microplate reader. Nitrite (NO₂⁻) was quantitated by using NaNO₂ as a standard.

**Statistical analysis.** The Mantel-Cox log-rank test was used for survival analysis. One-way analysis of variance was used, as was the Bonferroni correction, for all other comparisons. Significance was accepted as a *P* value of <0.05.

**RESULTS**

**CAP18_{106-137} peptide inhibits the growth of *P. aeruginosa*.** Figure 1 demonstrates the in vitro antimicrobial activity of
instillation of CAP18106–137 alone into mice led to the production of lung edema, as reflected by high lung wet/dry ratios. These findings suggest that CAP18106–137 itself can cause lung edema.

Bactericidal effect of CAP18106–137 in an in vivo environment. The number of bacteria remaining in the lungs 4 h after the instillation of CAP18 along with the bacteria was measured quantitatively (Fig. 3). Bacterial numbers were decreased, but not significantly, by the addition of high doses of CAP18106–137 to the instillates.

The addition of aztreonam increases lung injury. Figure 4 shows the resultant lung injury that occurred after the administration of strain PA103 with CAP18106–137 and/or aztreonam. Because 100 μg of aztreonam per ml for 1 h inhibited the growth of 10^7 CFU/ml of PA103 in vitro, we decided to add a higher concentration of aztreonam (5 mg/ml) to bacterial instillates that contained 10^7 CFU/ml of PA103. Five micrograms of CAP18106–137, 0.5 mg of aztreonam, or both were mixed with 5 × 10^6 CFU of PA103 1 min prior to instillation into the airspace. Both the leakage of the alveolar tracer (125I-albumin) from the lung to the bloodstream and the wet/dry ratios decreased significantly if CAP18106–137 was added to the bacterial instillate. The administration of aztreonam to the bacteria significantly increased the wet/dry ratio of the lung. If CAP18106–137 was added along with the aztreonam, the leakage of the alveolar protein tracer (125I-albumin) into the circulation decreased, but not significantly. Bacterial numbers were decreased, although not significantly, by the addition either of aztreonam or of CAP18106–137 to the instillate (Fig. 5).

Effect of addition of CAP18106–137 on endotoxin levels and release of inflammatory mediators. Because CAP18106–137 has been reported to bind to LPS, we evaluated the effect of the addition of CAP18106–137, in combination with aztreonam, on endotoxin activity and the release of inflammatory mediators. BAL was performed 4 h after instillation. Figure 6 shows the concentration of endotoxin in the plasma and in the BAL fluids obtained 4 h after instillation. The animals into which CAP18106–137 with bacteria was instilled had a tendency to have lower levels of endotoxin in their plasma and in their BAL fluids, although there was no statistical difference compared to these levels in the animals into which bacteria alone or bacteria, CAP18, and antibiotics were instilled. Figure 7 shows the concentration of TNF-α, IL-6, and NO_2^- in the plasma and in the BAL fluids 4 h after instillation. When 0.5
mg of aztreonam alone was added to the bacterial instillate, high concentrations of TNF-α were detected both in the plasma and in the BAL fluids. The addition of 5 μg of CAP18 to the bacterial instillate with aztreonam decreased the concentrations of TNF-α and IL-6 in both plasma and BAL fluids. The concentration of NO\textsubscript{2} in BAL fluids also was not significantly different among the groups but had changes similar to those for TNF-α and IL-6.

Effect of CAP18 on survival. The survival of mice after instillation of 5 × 10\textsuperscript{5} CFU of PA103 was examined after the four different experimental interventions: bacteria in PBS (i) without antibiotics, (ii) with CAP18, (iii) with aztreonam, or (iv) with both CAP18 and aztreonam (Fig. 8). None of the mice receiving the bacteria alone survived. The addition of both CAP18 and aztreonam to the bacteria improved the mortality, but not significantly. None of the mice which received aztreonam in their bacterial instillates survived for more than 72 h. In contrast, all of the mice which received both the CAP18 and aztreonam in their bacterial instillates survived for a week and were then killed.

**DISCUSSION**

Most animal species, from insects to humans, have developed a diverse array of peptide defense systems to counter microbial invasion and infection (3, 21). Polymorphonuclear neutrophils contain a number of proteins and peptides that have antimicrobial activities. Defensins, basic β-sheet peptides consisting of 29 to 35 amino acids, were originally discovered in the granules of rabbit neutrophils (22). Similar peptides including the tracheal antimicrobial peptide (8) and the lingual antimicrobial peptide (31) have been isolated from bovine epithelium. These peptides have more activity against gram-positive bacteria than gram-negative bacteria, and they also kill mycobacteria and fungi (3, 21). Bactericidal/permeability in-
creasing protein (BPI or CAP57) is a neutrophil primary granule protein (21, 23). In addition to its antibacterial activity against gram-negative bacteria, BPI has been shown to bind to LPS, leading to the abrogation of detrimental host responses to LPS (7, 10, 15, 24, 25, 35). Increases in plasma BPI levels were observed in septic patients and in human volunteers injected with LPS (37).

CAP18 was identified in the azurophilic granules of neutrophils and was purified on the basis of CAP18's ability to agglutinate erythrocytes coated with LPS, especially lipid A (12, 13, 17). The cloning and sequencing of the cDNA of rabbit CAP18 led to the discovery of a C-terminal 37-amino-acid fragment, designated CAP18106–142, that also had LPS-binding activity (18, 19). A truncated peptide, CAP18106–137, a 5-amino-acid truncated segment of the 37-amino acid fragment, has even greater activity than the parental peptide, CAP18106–142 (19). CAP18106–137 has antimicrobial activity against both gram-negative and gram-positive bacterial strains. Human CAP18 was then cloned, and the truncated human peptide CAP18104–135 was found to inhibit LPS induction of tissue factor at concentrations similar to those previously observed for the rabbit CAP18 peptides (20). Lung injury in guinea pigs due to the administration of LPS was attenuated by the administration of these peptides (33). Lately, synthetic peptide CAP18109–135 improved the survival from peritoneal injections of a relatively nontoxic Pseudomonas strain (PAO1) in an endotoxin model with mice sensitized with D-galactosamine (16). In vitro effects of CAP18 peptide against gram-negative bacteria were reported (19). However, the mechanism of antimicrobial effects of CAP18 is still unknown. Because the CAP18 peptide has a strong positive charge, this peptide may bind to the negatively charged cell surface and could cause cytotoxicity and/or induce an inflammatory response in vivo. To examine the maximal beneficial effects of CAP18 peptide on P. aeruginosa-induced lung injury, we instilled the bacterial solution with the peptide into the lungs of mice and quantitated the lung injury.

The addition of CAP18 peptide to the bacterial instillate significantly decreased the lung epithelial injury and the edema formation caused by the cytotoxic strain P. aeruginosa PA103. The instillation of CAP18 peptide alone (5 μg, 100 μg/ml) did not cause lung epithelial injury but did cause significant lung edema formation. There was a tendency for increased lung edema when larger dosages of CAP18 peptide were administered with the bacteria; perhaps more CAP18 peptide was available to bind to negatively charged tissues and cause lung edema. Although CAP18 peptide inhibits the growth of P. aeruginosa in vitro, the bacterial counts in the lung homogenates were not significantly decreased in the animals instilled with CAP18 peptide and the bacteria compared to the bacterial counts in the animals instilled with bacteria alone. The CAP18 peptide, like BPI, may lead to clinical improvement by suppression of bacterial growth and/or elimination of the toxic effects of bacterial products (24).

Because antibiotics do not neutralize LPS and some have been shown to increase the release of LPS by killing gram-negative bacteria, we instilled the bacterial solution with the peptide into the lungs of mice and quantitated the lung injury.

FIG. 7. Effects of the addition of CAP18106–137 and/or aztreonam on the concentrations of TNF-α, IL-6, and NO₂⁻ in plasma and BAL fluids 4 h after instillation of the bacteria. CAP18106–137 (CAP18; 5 μg) and/or aztreonam (AZT; 0.5 mg) was added in bacterial solution (strain PA103; 5 × 10⁸ CFU), and after 1 min the mixture was instilled into the mouse lung. BAL was performed 4 h after instillation of the bacteria. Data are means ± standard deviations. *P < 0.05 compared with the results for the control group treated with PA103 and PBS (black bar). † and ‡P < 0.05 and P < 0.01, respectively, compared with the results for the group treated with aztreonam (one-way analysis of variance followed by the Bonferroni test). Each group has five mice.

FIG. 8. Survival of mice after instillation of P. aeruginosa mixed with PBS, CAP18106–137, and/or aztreonam. A total of 50 μl of 5 × 10⁸ CFU of strain PA103 with CAP18106–137 (5 μg) and/or aztreonam (0.5 mg) was instilled into each mouse (n = 10 for the control group; n = 5 for each of the other groups). *P < 0.05 compared with the results for the PBS-treated group by the Mantel-Cox log rank test. Symbols: ■ control (PBS); ● aztreonam; △, CAP18106–137; ○, CAP18106–137 and aztreonam.
negative bacteria (32), antibiotic therapy may increase bacterium-induced lung injury. In fact, we found that the addition of aztreonam to the bacterial instillate increased the epithelial injury as well as the lung edema, the inflammatory mediator release, and the mortality of the infected mice. These results suggest that conventional antibiotic therapy alone may increase the release of proinflammatory cytokines and increase lung injury. The addition of CAP18 peptide to the aztreonam in the bacterial instillates ameliorated the lung injury. This result suggests that the CAP18 peptide bound the LPS released by the antibiotic. However, the endotoxin levels measured in the plasma and in the BAL fluids were the highest in the mice which had received the CAP18 peptide along with the bacteria. This result is explained by the fact that the CAP18 peptide binds to the endotoxin, and we are measuring the free and the bound endotoxin. CAP18 peptide also strongly binds to the serum albumin which is in plasma and BAL fluids, as reported for BPI (23, 24) (data not shown). The sample preparation for the LPS assay, a chromogenic substrate assay, may have conditions where free LPS could not be distinguished from LPS bound to CAP18 peptide (16). Animals which received CAP18 peptide along with the aztreonam in their bacterial instillates had significantly lower concentrations of the proinflammatory cytokines TNF-α and IL-6 in their plasma and in their BAL fluids. Although the addition of CAP18 peptide to the bacterial instillates did not improve the rate of survival among the infected mice, the addition of CAP18 peptide to the aztreonam in the bacterial instillates did significantly improve the mortality rate. These results suggest that CAP18 peptide improves the survival of antibiotic-treated mice by binding to the LPS released by antibiotic-induced bacterial killing.

In conclusion, the addition of this form of CAP18 simultaneously with bacteria into the lungs of mice was found to significantly decrease the level of bacterium-induced lung injury. However, the administration of CAP18 peptide alone induced significant lung edema, suggesting that the peptide has toxicity. This peptide did not reduce the number of bacteria in the lungs of the infected animals and did not improve the survival of the animals. The addition of this form of CAP18 was found to improve the lung injury caused by the addition of aztreonam to the bacterial instillate. Finally, the addition of synthetic CAP18 peptide was found to significantly improve the survival of mice exposed to aztreonam-bacterium instillates. The mechanism for this improved survival is most likely the decreased mediator release in the airspaces of the lung caused by the antibiotic-induced release of LPS. These results suggest a possible therapeutic role for CAP18 peptide as an adjunctive therapy in combination with conventional antibiotics in the treatment of lung infections caused by gram-negative bacteria. However, more research is needed because the CAP18 peptide was found to have toxic side effects and was not capable of killing the P. aeruginosa organisms in the lungs of the mice.

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