ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2003, p. 3901–3906 Vol. 47, No. 12
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.12.3901–3906.2003


# Novispirin G10-Induced Lung Toxicity in a *Klebsiella pneumoniae* Infection Model

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Received 5 June 2003/Returned for modification 19 August 2003/Accepted 9 September 2003

**Mammalian cathelicidins are a class of innate antimicrobial peptides isolated from leukocytes and epithelial cells that aid host defense against bacterial infections. Synthetic analogs of cathelicidins offer the promise of potent broad-spectrum antimicrobial efficacy. We developed a combined lung infection and ex vivo whole-blood assay model to characterize the toxicity and efficacy of synthetic cathelicidin-derived peptides. Male C57BL/6 mice were administered saline or *Klebsiella pneumoniae* by intratracheal instillation. Five hours later, the *Klebsiella*-infected mice were instilled with saline, tobramycin (1 mg/kg of body weight or 10 mg/kg), novispirin G10 (0.4 mg/kg), or a combination of tobramycin (1 mg/kg) and G10 (0.4 mg/kg). At 24 h, bronchoalveolar lavage fluid (BAL) was collected for analysis of culturable bacteria and for markers of inflammation and lung toxicity. Blood samples were analyzed for circulating cytokines. Recovery of *Klebsiella* from the lung, recruitment of neutrophils, and production of interleukin-6 (IL-6) in BAL samples were highly correlated ($r = 0.68$ and 0.84, respectively; $P < 0.01$). Animals treated with G10 or G10 plus tobramycin had increased hemoglobin ($P < 0.001$) and protein ($P < 0.001$) levels compared to those for *Klebsiella*-infected or tobramycin-alone-treated animals. The levels of circulating IL-6 in mice infected with *Klebsiella* were 1000- to 10,000-fold higher than in the noninfected controls. The highest levels of IL-6 were measured in mice given G10 alone or in combination with tobramycin. These studies demonstrated that G10 was relatively nontoxic in saline-treated mice but was highly toxic in mice infected with *Klebsiella*. This finding establishes the importance of investigating candidate antimicrobial agents in an in vivo infection model.**

The search for new and effective antimicrobial agents to treat bacterial or fungal infections has focused in recent years on components of the innate defense systems of plants and animals. Peptides isolated and purified from phagocytic cells have been especially promising as agents against multidrug-resistant bacteria and special-niche bacteria associated with chronic conditions such as cystic fibrosis (6, 7, 10, 14, 17, 27). A class of antimicrobial peptides that has been particularly well studied is the mammalian-derived cathelicidins. These peptides share a highly conserved N-terminal domain identical to a cathelin protein (hence the class name) but are structurally diverse at the C terminus, which also determines the antimicrobial activity of the peptide (14, 27, 28, 29). Lipid A binding and endotoxin neutralization are also determined by the C terminus (2, 5, 11, 12, 15, 18, 29).

The members of one class of promising antimicrobial peptides resemble the 18 amino acids of the N terminus of SMAP-29, derived from sheep neutrophils (16, 19). While showing a broad spectrum of antimicrobial activity and low toxicity in ovine models, the parent compound, SMAP-29, exhibits unacceptably high hemolytic and cytotoxic activity in human cells (1, 16). A series of substituted peptides has been assembled to circumvent the toxic potential while retaining antimicrobial activity. These substituted peptides are termed ovispirins; the structures and functional three-dimensional properties of these peptides are given by Sawai et al. (16) and Tack et al. (23). One peptide in this series, novispirin G10 (in which glycine is substituted at position 10 for isoleucine), has been shown to be significantly less hemolytic for human cells while retaining high antimicrobial activity against gram-negative and gram-positive bacteria (16, 21, 22).

Few in vivo studies have been reported for the ovispirins and novispirins. Brogden and colleagues (1) tested the efficacy of SMAP-29 in an ovine model of pulmonary infection by using *Mannheimia haemolytica*. Steinstraesser and colleagues (21) examined the activity of novispirin G10 in a burned skin model by using *Pseudomonas aeruginosa*. We wished to establish a mouse model of subacute bacterial lung infection in order to examine novispirin G10 alone and in combination with an established pulmonary therapeutic agent in the absence of foreign substances to prolong bacterial seeding (e.g., agarose beads). Our hypothesis was that the novispirin G10 would reduce lung toxicity by binding lipopolysaccharide (LPS) released by gram-negative cells during treatment of the infection. For our model, we chose *Klebsiella pneumoniae*, an organism known to elicit a strong cytokine response in mouse lung (8). The efficacy of the test antimicrobial peptide was compared to that of tobramycin sulfate, a standard antibiotic for the treatment of pulmonary infections.

## MATERIALS AND METHODS

**Cathelicidin.** A synthetic analogue of cathelicidin deduced from sheep myeloid mRNA (SMAP) was provided by Brian Tack (Department of Microbiology, University of Iowa, Iowa City). Novispirin G10 has a molecular weight of

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204.8 and is truncated at 18 amino acids (KNLRRIIRKGIHIIKKYG) (16). The peptide was supplied at a concentration of 850 μg/ml in 0.02% acetic acid.

**Endotoxin binding assay.** Endotoxin was measured by the kinetic chromogenic *Limulus* amebocyte lysate (LAL) assay (Kinetic-QCL; BioWhittaker, Inc., Walkersville, Md.) as previously described (24). Cathelicidins bind to lipopolysaccharide (LPS) at the lipid A moiety and extinguish endotoxic activity but do not affect the LAL assay itself (11). Accordingly, dilutions of novispirin G10 were added to pyrogen-free water (BioWhittaker, Inc.) containing 175 EU of endotoxin (*Escherichia coli* O55:B5; BioWhittaker, Inc.) per ml. The concentration of endotoxin was confirmed by activity in the LAL assay. The 50% endotoxin binding capacity was calculated from a regression of the recovered endotoxic activity against the spiked activity.

**MICs and MBCs.** *K. pneumoniae* (ATCC43816) was obtained from the American Type Culture Collection (Manassas, Va.). The MIC of the peptide was measured by using a modification of the protocol of Steinberg and Lehrer (20). Briefly, the bacterial culture was inoculated into tryptic soy broth (Difco, Franklin Lakes, N.J.) and grown overnight at 37°C. Aliquots of the overnight culture were inoculated into fresh tryptic soy broth and grown to log phase in a shaking water bath (2 to 3 h). The bacteria were centrifuged at 3,000 × *g* for 10 min, washed, and resuspended in 10 mM phosphate-buffered saline (pH 7.4). Dilutions of novispirin G10 were made in 0.01% acetic acid–0.1% BSA, and 50-μl aliquots were dispensed in duplicate into wells of a 96-well cell culture plate (Costar 3595). Washed bacteria were added to the peptide dilutions or buffers (final concentrations, $2 \times 10^5$ CFU/ml) and preincubated for 3 h at 37°C. At the end of the preincubation period, 100 μl of 2× concentrated Mueller-Hinton broth (MHB) (BBL; Becton Dickinson and Co., Cockeysville, Md.) was added to each well, and the wells were incubated for a further 16 h. After incubation, the optical densities of the wells were determined at 650 nm ($OD_{650}$) by using a SPECTRAmax 340 microplate spectrophotometer (Molecular Devices, Sunnyvale, Calif.). The positive-control wells contained MHB inoculated with bacteria, and the negative-control wells contained MHB without bacteria. The lowest concentration of antimicrobial agent showing ≤ 50% growth control was considered to be the MIC. Aliquots from wells with no growth were plated onto tryptic soy agar (TSA; Difco) and incubated overnight. The minimal bactericidal concentration (MBC) was determined to be the lowest dilution concentration that showed no growth on TSA. The MIC and MBC of tobramycin sulfate (Eli Lilly & Co., Indianapolis, Ind.) were determined for *K. pneumoniae* by using both a standard broth microdilution assay (13) and by the method described above for antimicrobial peptides.

A checkerboard assay was used to test for synergistic interaction between G10 and tobramycin sulfate (3). The fractional inhibitory concentration (FIC) of the combined antibiotics was determined by using the following relationship: FIC = (lowest concentration of antibiotic A/MIC of A) + (lowest concentration of antibiotic B/MIC of B).

**Pulmonary toxicity models.** The University of Iowa Institutional Animal Care and Use Committee approved all experimental protocols. Eight-week-old male C57BL/6 mice were quarantined for 2 weeks in the Inhalation Toxicology Facility vivarium before treatment.

The in vivo pulmonary toxicity of G10 and tobramycin was tested by intratracheal instillation. The mice were anaesthetized with inhaled ether and restrained, ventral side up, on a reclined support. A Jelco 19-mm catheter (Johnson & Johnson, Arlington, Tex.) was guided into the trachea of the recumbent mouse. One hundred microliters of antibiotic or saline control was transferred to a 1-ml syringe by micropipette. The syringe was then attached to the catheter hub, and the fluid was gently expelled into the catheter, where it was aspirated by the mouse. The mouse was placed in a recovery jar before being returned to its cage. Food (Purina mouse chow) and water were available ad libitum.

Before necropsy, the mice were anaesthetized with ether and euthanized by cervical dislocation. A total of 4 ml of pyrogen-free saline (1 ml per wash) was introduced through a cannula inserted into the trachea. The saline and associated bronchoalveolar fluid (BAL) were collected by gravity drain and then centrifuged, after which the supernatant was divided into aliquots for biochemical characterization. The cell pellet was prepared for cell count and differential staining as previously described (25).

**Pulmonary infection model.** Single colonies of *K. pneumoniae* grown for 16 h on TSA were emulsified into pyrogen-free saline, and the turbidity of the suspensions was measured at $OD_{360}$. Mouse lungs were instilled with *Klebsiella* as described above as part of an established infection model (8). The concentrations of instilled *Klebsiella* organisms were checked by spread plating the inocula on TSA and ranged from 8.27 to 8.72 $\log_{10}$. Five hours after the introduction of the *Klebsiella* suspension, the mice were instilled with one of five treatment regimens: saline (control), G10 (0.4 mg/kg), tobramycin (1 mg/kg), tobramycin (1 mg/kg) plus G10 (0.4 mg/kg), or tobramycin (10 mg/kg). Necropsies were performed, and the BAL was collected at 24 h postinstillation. At the inocula concentration chosen, *Klebsiella* was recovered from the lungs of instilled mice for up to 72 h without mortality.

**Pulmonary endotoxin stimulation model.** In order to examine the role of the gram-negative cell membrane in a noninfectious model, a suspension of *Klebsiella* was prepared as above and pasteurized at 70°C for 1 h. One hundred microliters of the pasteurized culture was plated on TSA to ensure that 99.9% of the cells were nonviable. The cell suspension was adjusted to the same optical density and instilled as described for the infection model. At 5 h postinstillation, the mice were instilled intratracheally with saline, G10 (0.4 mg/kg), or tobramycin (1 mg/kg) plus G10 (0.4 mg/kg). Necropsies were performed at 24 h, and BAL was collected. Comparison groups of mice were instilled with commercial endotoxin prepared from *E. coli* O111:B4 (BioWhittaker, Inc.) at two concentrations, 10 mg/kg and 40 mg/kg. Necropsies were performed at 5 and 24 h postinstillation.

**Measures of response.** Inflammation of the lung was assessed by leukocytosis and neutrophil recruitment and by the release of the proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α). Increased permeability of the lung was assessed by measurement of hemoglobin and albumin concentrations in the BAL. Colonization of the lung was assessed by the continuing presence of *Klebsiella*, indicative of a failure to clear the infection.

The cell pellet of the centrifuged BAL was resuspended in 150 μl of Hanks' buffered salt solution (Cellgro Mediatech, Inc., Herndon, Va.). Twenty-five microliters of the suspension was diluted 1:20 and dispensed into 3% acetic acid to lyse the erythrocytes. The leukocyte suspension was counted by using a hemocytometer at 400× magnification. Fifty microliters of the remaining cell suspension was centrifuged onto microscope slides by using a cytofuge (StatSpin, Norwood, Mass.) and stained with Diff Quick (Dade Behring AG, VWR) for differential cell counting.

Cytokine concentrations in BAL fluid were determined by using commercial enzyme-linked immunosorbent assay kits (IL-6 and TNF-α DuoSet; R&D Systems, Minneapolis, Minn.). The assays were measured at $OD_{450}$ with a correction wavelength of $OD_{540}$. BAL albumin was measured by using a colorimetric assay against a bovine serum albumin (BSA) standard curve (Microprotein-PR; Sigma, St. Louis, Mo.). The concentration of hemoglobin was determined by a spectrophotometric scan at $OD_{380}$, $OD_{415}$, and $OD_{450}$ (26) by using a BSA standard curve.

Aliquots of uncentrifuged BAL were plated onto TSA to determine the concentration of surviving *Klebsiella*. To test the effect of the BAL fluid itself on the antibiotics, the MICs of tobramycin and G10 were determined by using the protocol outlined above with the following modification: BAL from mice instilled with saline, pasteurized *Klebsiella*, G10 alone, or pasteurized *Klebsiella* plus G10 was used as the diluent for the antibiotics. A control MIC using the normal diluent, 0.01% acetic acid–0.1% BSA, was determined on the same microtiter plate.

**Plasma cytokines.** At the time of necropsy, blood was collected by cardiac puncture into a 1-ml syringe containing 75 μl of heparin. The blood from all mice in the same treatment group was pooled and measured, and an equal volume of pyrogen-free saline was added. The blood was divided into 900-μl aliquots and incubated for 5 h at 37°C in 5% $CO_2$. The samples were gently centrifuged in a microfuge to pellet the cells. The supernatant was stored at −85°C until analyzed for cytokine concentration as described for the BAL fluid.

**Statistical analyses.** The data were analyzed using SPSS (Chicago, Ill.) version 10 software for Windows. The distributions of the data were examined. The distribution of the cytokine concentrations (IL-6 and TNF-α) was approximately log normal and was transformed to the base 10 logarithm for use in parametric statistics. Tests of significance for multiple comparisons were performed by using the post hoc procedure of Scheffé (4).

## RESULTS

The 50% endotoxin binding capacity of novispirin G10 was 14.2 μg/ml, which was within the range of values obtained from the parent SMAP and related derived compounds. The MIC of tobramycin for *K. pneumoniae* ATCC 43816 was 0.78 μg/ml with a traditional microdilution assay and 1 μg/ml with the modified Steinberg and Lehrer protocol for antimicrobial peptides. The MIC of G10 was 6.25 μg/ml (Table 1).

The combination of tobramycin and G10 reduced the MIC of both. The calculated FIC index was 0.37, which indicated a weak synergism. The addition of BAL to the dilutions of tobramycin

TABLE 1. MIC and MBC of tobramycin and G10 for *Klebsiella pneumoniae*

| Milieu | MIC (MBC) (μg/ml) | |
|---|---|---|
| | Tobramycin | G10 |
| Mueller-Hinton broth | 1 (1) | 6.25 (100) |
| BAL from pasteurized *Klebsiella*-treated mice | 1 (1) | 25 (100) |
| BAL from pasteurized G10-treated mice | 1 (1) | 25 (100) |
| BAL from mice treated with pasteurized *Klebsiella* + tobramycin (1 mg/kg) + G10 (0.4 mg/kg) | 1 (1) | 100 (100) |

did not change the MIC for *Klebsiella*. However, this was not the case for G10. As indicated in Table 1, the MIC was fourfold higher (25 μg/ml) when the BAL from mice treated with pasteurized *Klebsiella* or G10 alone was used as the diluent of G10 for the 3-h preincubation. The MIC was eightfold higher (100 μg/ml) when the MIC was determined by using as the diluent BAL from mice treated with the mixture of pasteurized *Klebsiella* plus tobramycin (1 mg/kg) plus G10 (0.4 mg/kg).

When administered to uninfected mice, neither the peptide diluent (0.01% acetic acid–0.1% BSA) G10 nor tobramycin was inflammatory as measured by neutrophil recruitment to the lung or production of the proinflammatory cytokines IL-6 and TNF-α (Table 2). There was no evidence of toxicity with these treatments, as assessed by the concentration of hemoglobin or protein in the BAL fluid, compared to that for the naïve controls or the controls instilled with saline (Table 2). The combination of G10 and tobramycin was not toxic or inflammatory.

Response markers examined in the BAL of mice infected with viable *K. pneumoniae* are summarized in Table 3. Mice infected with *Klebsiella* and then treated with saline (treatment control) demonstrated leukocytosis with 29.9% neutrophils in the lavage and significantly elevated TNF-α. In these mice, the instilled *Klebsiella* had not been killed and yielded $7 \times 10^3$ CFU/ml (3.84 log units). Infected mice treated with tobramycin at 1 mg/kg had comparable levels of infection, whereas treatment at the higher dose of 10 mg/kg reduced the bacterial recovery by nearly two log units. When infected mice were treated with G10 alone or in combination with low-dose tobramycin, higher concentrations of *Klebsiella* were recovered and strong evidence of inflammation and lung toxicity emerged. Treatment with G10 resulted in increased permeability of the lung, as evidenced by increased hemoglobin and protein in the BAL. This effect was significantly greater than for the naïve mice and the infected mice treated with saline. The instillation of *Klebsiella* itself resulted in inflammation, but this effect was exacerbated by the administration of G10 (Table 3). In order to determine if infection was necessary for this effect, mice were instilled with pasteurized *Klebsiella*, followed by the treatments described above. This experiment showed that killed bacteria could induce inflammation and some toxicity, which novispirin G10 failed to alleviate (Table 4). High doses of bacterial LPS with or without added G10 did not produce the high degree of inflammation and toxicity seen with lung infection and G10.

In a further set of experiments, blood was drawn by cardiac puncture at the time of necropsy. Blood from mice within the same treatment group was pooled, diluted, and separated into replicate tubes. Results for the IL-6 and TNF-α concentrations in blood are reported in Table 5. In this series, the blood of mice infected intratracheally with *Klebsiella* had significantly elevated levels of both IL-6 and TNF-α (Table 5). However, there was a profound increase in the concentration of the cytokine IL-6 in blood drawn from infected mice treated with G10 alone or in combination with tobramycin, an effect which was not seen in mice instilled only with LPS, a noninvasive control stimulant ($P < 0.001$). This finding suggests that in the infection model the cathelicidin, G10, produced a strong lung response and profound systemic inflammation that was not observed with 10 mg/kg of tobramycin, with LPS, or with infection and saline treatment.

## DISCUSSION

We have shown that in vitro measures of efficacy do not predict the in vivo case for one antimicrobial peptide, novispi-

TABLE 2. Analysis of BAL fluid from noninfected mice

| Compound | No. of mice | % PMN[a] [mean (SD)][g] | Il-6 (pg/ml) [GM[b] (GSD)[c]] | TNF-α (pg/ml) [GM (GSD)] | Hemoglobin (μg/ml) [mean (SD)] | Protein (μg/ml) [mean (SD)] |
|---|---|---|---|---|---|---|
| None[d] | 17 | 1.9 (1.8) | 17.1 (2.2) | 16.1 (1.9) | 9.8 (7.6) | 50.4 (16.2) |
| Saline | 6 | 1.7 (1.7) | 19.1 (2.9) | 24.9 (2.4) | 11.4 (15.6) | 36.8 (16.6) |
| Peptide diluent[e] | 2 | 2.0 (1.4) | 14.6 (2.4) | 87.1 (1.1) | 18.6 (8.7) | 39.5 (15) |
| G10 | | | | | | |
| 0.03 mg/kg | 2 | 1.0 (1.4) | 45.7 (1.2) | 114.8[h] (1.2) | 10.0 (4.2) | 52.9 (0.2) |
| 0.34 mg/kg | 2 | 0.5 (0.7) | 16.2 (2.8) | 105.9[h] (1.8) | < 10 | 29.1 (12) |
| 3.4 mg/kg | 2 | 1.5 (0.7) | 34.7 (2.5) | 162.2[h] (1.6) | 14.7 (2.3) | 70.7 (65) |
| Tobramycin | | | | | | |
| 10 mg/kg | 4 | 3.5 (1.9) | 11.6 (1.6) | NT[f] | < 10 | 56.3 (6.0) |
| Tobramycin (1 mg/kg) + G10 (0.4 mg/kg) | 4 | 1.5 (1) | 9.6 (1.6) | 27.5 (1.6) | 7.7 (5.4) | 70.7 (4.0) |

[a] Percentage of polymorphonuclear leukocytes (PMN) by differential count.
[b] GM, geometric mean.
[c] GSD, geometric standard deviation.
[d] Control (naïve), mice not instilled.
[e] Control, mice instilled with 100 μl of 0.9% pyrogen-free saline.
[f] NT, not tested.
[g] SD, standard deviation.
[h] $P = 0.001$ compared to results for naïve control mice.

TABLE 3. Summary of pulmonary inflammation or toxicity responses in the BAL of *Klebsiella*-infected mice[a]

| Group | No. of mice | Neutrophilia % PMN[c] [mean (SD)][d] | Il-6 (pg/ml) [GM (GSD)][e] | TNF-α (pg/ml) [GM (GSD)] | Hemoglobin (μg/ml) [mean (SD)] | Protein (μg/ml) [mean (SD)] | *K. pneumoniae* recovery[b] (CFU/ml) [GM (GSD)] |
|---|---|---|---|---|---|---|---|
| Naïve | 23 | 1.8 (1.6) | 20.7 (2.0) | 17.6 (1.6) | 11.8 (9.2) | 47.5 (15.7) | 0 (0) |
| *Klebsiella*-infected, treated with saline | 17 | 29.9* (26.9) | 67.9 (7.5) | 65 (6.8) | 33.1 (39.4) | 130.5 (169) | $6.9 \times 10^3$* (794) |
| *Klebsiella*-infected, treated with tobramycin (1 mg/kg) | 9 | 23.9 (23.5) | 105 (11.1) | 80 (7.9) | 87.5 (121) | 176.7 (178) | $7.9 \times 10^3$* (1259) |
| *Klebsiella*-infected, treated with G10 (0.4 mg/kg) | 9 | 23.6 (23.4) | 398* (18.5) | 277* (9.4) | 189*† (222) | 357.3* (373) | $4.9 \times 10^4$* (5011) |
| *Klebsiella*-infected, treated with tobramycin (1 mg/kg) + G10 (0.4 mg/kg) | 9 | 53* (24.4) | 684* (6.9) | 525* (4.9) | 63.5 (47) | 235.9 (127) | $3.5 \times 10^6$* (63) |
| *Klebsiella*-infected, treated with tobramycin (10 mg/kg) | 9 | 20.7 (21.1) | 29.4£ (4.6) | 29£ (3.7) | 13.8§ (20.2) | 67.3§ (41.8) | $8.9 \times 10^1$£ (501) |

[a] Statistical comparisons reference significant differences ($P \leq 0.05$) compared to the treatment group indicated by the symbol. The symbols *, †, ‡, §, and £ refer to comparisons made by the Scheffé post hoc procedure.
[b] *K. pneumoniae* recovered from BAL at necropsy.
[c] % PMN, percentage of polymorphonuclear leukocytes by differential count.
[d] SD, standard deviation.
[e] GM, geometric mean; GSD, geometric standard deviation.

rin G10. This peptide is significantly less toxic than its parent compounds, SMAP-29 or ovispirin-1. Sawai and colleagues (16) showed a ten-fold reduction in the lytic action of novispirin G10 compared to that for SMAP-29. Steinstraesser and colleagues (21) showed lower cytotoxicity in A549 human lung epithelial cells for novispirin G10 than for other novispirins (T7, G7, and T10) or ovispirin-1. This compound had previously shown efficacy against an ovine pathogen in an ovine pulmonary infection model.

However, when this peptide was used alone or in combination with standard treatment for gram-negative bacterial lung infection, the resulting toxicity was unexpected and profound. Interestingly, the antecedents of this reaction were noted but downplayed in previous work. For example, several studies have shown that novispirin G10 is inhibited by calcium or magnesium ions in test broth (9, 21; Lehrer, personal communication), but because the peptide is active in a dose-dependent fashion, the reduction in efficacy did not extinguish its antimicrobial properties beyond therapeutic ranges. In addition, other studies have shown novispirin G10 to be sensitive to inhibition by the presence of serum. Steinstraesser and colleagues (21) found that the addition of serum in the gel underlay of the plate inhibition test increased the apparent MIC by 10-fold for *P. aeruginosa*. In a previous study, we confirmed the reduction in effect for *P. aeruginosa* but also found that the addition of (bovine) serum completely inhibited the bactericidal action of novispirin G10 for *K. pneumoniae*.

In in vivo models, related compounds, CAP18 (rabbit derived peptide), for example, instilled alone (5 μg) resulted in significant lung edema. When CAP18 was mixed with *P. aeruginosa* prior to intratracheal instillation in mice, the bacterial counts in the lung homogenates were not significantly decreased compared to those for the lungs instilled with bacteria alone (15). In Steinstraesser's burned skin model, peripheral blood samples showed that the numbers of white blood cells and neutrophils were significantly increased in the novispirin G10-treated group compared to those for the controls, supporting our findings with BAL and circulating blood. However,

TABLE 4. BAL following intratracheal instillation of killed *Klebsiella pneumoniae* or endotoxin[a]

| Group | No. of mice | Neutrophilia % PMN [Mean (SD)][b] | Il-6 (pg/ml) [GM (GSD)][c] | TNF-α (pg/ml) [GM (GSD)] | Hemoglobin (μg/ml) [Mean (SD)] | Protein (μg/ml) [Mean (SD)] |
|---|---|---|---|---|---|---|
| Naïve | 23 | 1.8 (1.6) | 20.7 (2.0) | 17.6 (1.6) | 11.8 (9.2) | 47.5 (15.7) |
| Killed *Klebsiella*, treated with saline | 6 | 80.2* (13.9) | 218.4* (3.5) | 193.4* (2.4) | 227.6* (370) | 100.9* (24) |
| Killed *Klebsiella*, treated with G10 (0.4 mg/kg) | 6 | 49.7* (26.6) | 196.8* (4.9) | 140.9* (2.6) | 178.7 (118) | 172.5*† (81.5) |
| Killed *Klebsiella*, treated with tobramycin (1 mg/kg) + G10 (0.4 mg/kg) | 5 | 54.2* (25.8) | 87.1 (3.5) | 94.0 (2.7) | 83.8 (52.7) | 95.3‡ (43) |
| LPS (40 mg/kg) | 5 | 37.2*† (41.5) | 62.5 (4.5) | 79.8 (4.1) | < 10 | 68.8‡ (24.0) |
| LPS (40 mg/kg), sham treated with G10 (0.4 mg/kg) | 10 | 28.7*† (24.2) | 72.8 (3.1) | 28.1 (4.3) | 17.2 (28.0) | 75.0‡ (17.2) |

[a] Statistical comparisons reference significant differences ($P \leq 0.05$) compared to the treatment group indicated by the symbol. The symbols *, †, ‡, §, and £ refer to comparisons made by the Scheffé post hoc procedure.
[b] SD, standard deviation.
[c] GM, geometric mean; GSD, geometric standard deviation.

TABLE 5. Circulating cytokine concentration in the blood of mice intratracheally-instilled with *Klebsiella* or LPS[a]

| Group | No. of samples[b] | Concn (pg/ml) [GM (GSD)][c] in plasma | |
|---|---|---|---|
| | | IL-6 | TNF-α |
| Naïve | 6 | <15 (0) | <15 (0) |
| *Klebsiella*-infected, treated with saline | 3 | 2951* (1.5) | 39.8* (4.1) |
| *Klebsiella*-infected, treated with tobramycin (1 mg/kg) | 4 | 5473* (1.7) | 71.7* (1.1) |
| *Klebsiella*-infected, treated with G10 (0.4 mg/kg) | 4 | 41792*†‡ (1.1) | 204.9*† (1.1) |
| *Klebsiella*-infected, treated with tobramycin (1 mg/kg) + G10 (0.4 mg/kg) | 2 | 13316*†‡§ (1.8) | 121.3*†‡ (1.5) |
| *Klebsiella*-infected, treated with tobramycin (10 mg/kg) | 3 | 499*†‡§£ (1.4) | 77.8*£ (1.2) |
| LPS (40 mg/kg) | 2 | 452*†‡§£ (1.1) | 13.5§ (2.2) |
| LPS (10 mg/kg) | 2 | 68.4*†‡§£¥# (11.5) | <15‡§£¥ (0) |

[a] Statistical comparisons reference significant differences ($P \leq 0.05$) compared to the treatment group indicated by the symbol. The symbols *, †, ‡, §, £, ¥, and # refer to comparisons made by the Scheffé post hoc proced ure.
[b] The number of replicate aliquots of blood pooled from mice in the same treatment group.
[c] GM, geometric mean; GSD, geometric standard deviation.

this cytotoxicity may be limited to interspecies differences between peptide and host, as Brogden and colleagues (1) showed reduced inflammation in lambs infected with *M. haemolytica* treated with SMAP-29 compared to that for lambs with infection but no treatment.

Previous studies have shown that cathelicidin mixed with LPS or bacteria prior to instillation reduced lung damage (15). However, these are not sufficient models to use if the intent is to reduce the number of bacteria in a fulminant lung infection. In the model presented here, a gram-negative bacterial pathogen that would resist clearance for 48 to 72 h in mouse lung was used to approximate a chronic infection. Two treatment concentrations of tobramycin were used in building our pulmonary model. Tobramycin at 1 mg/kg was chosen to be suboptimal to allow observations of synergy between the aminoglycoside and the peptide. Tobramycin at 10 mg/kg was a therapeutic dose. The concentration of G10 was chosen based on the synergistic MIC and on previous work of Travis et al. (27). The treatment outcomes between the regimens was significant (3.9 $\log_{10}$ bacteria recovered at necropsy for tobramycin at 1 mg/kg versus 1.9 $\log_{10}$ for tobramycin at 10 mg/kg; $P = 0.08$). The unique contribution of G10 in combination therapy was to bind to LPS, thereby reducing the endotoxic potential of the gram-negative bacteria for the host.

Contrary to expectations, the addition of G10 to the treatment regimen increased both the inflammatory and toxic responses of the host. IL-6 and TNF-α are proinflammatory cytokines that are sensitive to endotoxin stimulation, and if unregulated, they contribute to pulmonary disease in gram-negative infections. These cytokines were clearly elevated in the BAL of mice treated with either G10 alone or G10 in combination with tobramycin. In addition, the lung epithelium was more permeable in the infection model when G10 was added as part of the treatment. This effect was also seen when killed *Klebsiella* were present in the lung. The mechanism for this action and whether it is an effect residing with the neutrophils are not known at present but deserve further study. One intriguing observation was that the BAL of mice instilled with killed *Klebsiella* in combination with G10 and tobramycin appeared to have elevated MICs of G10, consistent with observations for cation and serum inhibition of the peptide. The products of the profound inflammatory response in the mouse lung similarly blocked the action of G10 in vitro. The IL-6 concentration in the lungs was higher when killed *Klebsiella* was used as the stimulant (218 pg/ml) than when endotoxin was used (62.5 pg/ml), but the addition of a sham G10 treatment to either killed *Klebsiella* or LPS stimulation did not elevate the levels of cytokine further.

The response for circulating cytokines was very similar to that for pulmonary cytokines, but the difference in response to the two stimulants was much greater, particularly for IL-6. The concentration of circulating IL-6 was elevated after challenge of mouse lung with purified endotoxin, but the concentration was 10,000-fold lower than that after challenge with the killed *Klebsiella*.

In summary, a mouse pulmonary infection model was developed that can be used as an early in vivo test of the efficacy and toxicity of antimicrobial peptides and should be included in the work of developing these compounds. The study was limited to the testing of one peptide in a mouse model; however, this peptide underwent screening in vitro with other SMAP- and CAP-derived compounds, and at no stage of the in vitro investigation was there any indication that G10 would prove to be toxic in vivo. In addition, G10 was chosen for the model based on its apparent lack of action on erythrocytes in preliminary screening of its hemolytic index. Further work is planned to examine the mechanism for the effects observed in this study.

## ACKNOWLEDGMENTS

We thank Marsha O'Neill and Brittany Goodenow for their technical assistance.

This study was supported in part by Environmental Health Sciences Research Center grant NIEHS P30 ES05605. K.H.B. was the recipient of a postdoctoral fellowship from the Canadian Institutes of Health Research.

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