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Effects of Intratracheal Administration of Novispirin G10 on a Rat Model of Mucoid *Pseudomonas aeruginosa* Lung Infection

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Chronic *Pseudomonas aeruginosa* lung infection is a major problem for patients with cystic fibrosis (CF). The biofilm mode of growth of the pathogen makes it highly resistant to antibiotic treatment, and this is especially pronounced with mucoid strains. In this study, novispirin G10, a synthetic antimicrobial peptide patterned loosely on sheep myeloid antimicrobial peptide 29, was tested in a rat model of mucoid *P. aeruginosa* lung infection. *P. aeruginosa* NH57388A, a mucoid strain isolated from a CF patient, was mixed with the alginate produced by the bacterium itself and adjusted to a concentration of 10^{10} CFU/ml. Each rat received 10^9 CFU of bacteria intratracheally in the left lung to establish lung infection. At 0 and 3 h post *P. aeruginosa* infection, the treated group of rats received novispirin G10 (0.1 mg/ml, 0.1 ml/rat) intratracheally, whereas the control group received vehicle treatment only. The animals were sacrificed on days 3, 5, 7, and 10 after challenge for evaluation of various parameters. On day 5, 50% of the rats in the treated group had cleared the bacteria from the lungs, whereas in the control group, none of the rats cleared the pathogen ($P < 0.03$). The average bacterial loads remaining in the lungs of treated rats on days 3 and 5 were more than 170- and 330-fold lower than in the control groups ($P < 0.0005$ and $P < 0.0003$). In accordance, the macroscopic and microscopic lung pathology was also significantly milder in the treated group compared to the control group ($P < 0.0002$). Lung cytokine responses in the treated group were significantly lower than in the control group. The results suggest that novispirin G10 might be useful in treating antibiotic-resistant *P. aeruginosa* lung infections.

Pseudomonas aeruginosa is an important opportunistic pathogen responsible for nosocomial infections (2), burn infections (1, 31), and chronic lung infections in cystic fibrosis (CF) patients (11). *P. aeruginosa* lung infections are difficult to eradicate in CF patients, even with intensive antibiotic treatment, probably due to the concurrent presence of multidrug-resistant strains (5) and their biofilm mode of growth (11). Most *P. aeruginosa* strains isolated from the lungs of chronically infected CF patients have a typical mucoid phenotype due to overproduction of alginate. For the organism, this may be a survival strategy and/or an adaptation to the environments (8, 11).

In recent years, peptides possessing potent antimicrobial properties have attracted considerable attention. Such antimicrobial peptides have been isolated from a diversity of organisms, including vertebrates, plants, insects, and bacteria (16). Sheep myeloid antimicrobial peptide 29, an alpha-helical peptide isolated from sheep neutrophil leukocytes, has a broad and potent spectrum of antimicrobial activity (19, 21). Ovispirin 1 and novispirin G10 are octadecapeptide derivatives of sheep myeloid antimicrobial peptide 29 that possess substantial activity against gram-positive and gram-negative bacteria (19, 28). Although novispirin G10 differs from ovispirin 1 only by containing an isoleucine-to-glycine substitution at residue position 10, this modification leads to significantly lower in vitro cytotoxicity (4, 28). It has been demonstrated that novispirin G10 can effectively kill *P. aeruginosa* in burn wounds (28). However, the effects of the peptide on *P. aeruginosa* lung infections are still unknown. In the present study, novispirin G10 was tested in a rat model with mucoid *P. aeruginosa* lung infection mimicking the infections seen in CF patients.

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MATERIALS AND METHODS

Cloning, expression, and purification of novispirin G10. Novispirin G10 (KN LRRIIRKGIHIIKKYG) was produced recombinantly in *Escherichia coli* as inclusion bodies. This was achieved through gene fusion with a fragment of KSI (ketosteroid isomerase) using the commercially available plasmid pET31b+ (www.novagen.com). To facilitate downstream processing and recovery of novispirin G10, the novispirin G10 gene was preceded by an aspartate-proline (DP) dipeptide, followed by an aspartate-glutamate dipeptide (DE). Thus, the gene fusion construct encodes KSI-D-P-D-E-novispirin G10. The DP dipeptide is prone to mild acid hydrolysis at elevated temperatures (7, 13), and the glutamic acid residue located immediately upstream of novispirin G10 is a substrate of a glutamyl endopeptidase I.

The expression plasmid was constructed using the following oligonucleotides encoding novispirin G10: 5′-ATT ATT CAG ATG CTG GAT CCG GAC GAA AAA AAC CTG CGT CGC ATT ATC CGC AAA GGC ATC CAT ATC ATT AAA AAA TAT GGC TAA TAA CTC GAG ATT ATT-3′ and 5′-AAT AAT CTC GAG TTA TTA GCC ATA TTT TTT AAT GAT ATG GAT GCC TTT GCG GAT AAT GCG ACG CAG GTT TTT TTC GTC CGG ATC CAG CAT CTG AAT AAT-3′. Enzymatic digestion of introduced flanking restriction endonuclease sites (AlwNI/AvaI) enabled cloning of the synthetic gene as a fusion construct in pET31b+ (standard procedures as described by the manufacturer [New England Biolabs Inc., Ipswich, MA]). All standard protocols have been described elsewhere (18).

Recombinant pET31b+ was transformed into *E. coli* Novablue as described by the manufacturer (Novagen). Plasmids were recovered by QIAprep Mini Columns (QIAGEN Inc., Valencia, CA) and sequenced by automated sequencing using plasmid-specific primers 5′-TGC TAG TTA TTG CTC AGC GG-3′ and 5′-ACC GTA GTT GCG CCC ATC G-3′. Recombinant plasmid was transformed in *E. coli* BLR-DE3 according to the manufacturer's (Novagen) recom-

mendations. Bacteria were cultivated in LB medium to an optical density at 600 nm of ~0.8, and recombinant protein synthesis was initiated by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Upon 3 h of induction, bacteria were harvested, resuspended in 1/10 volume of buffer A (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8), and lysed by pressure disruption (1.5×10^5 Pa). D-P-D-E-novispirin G10 is located in inclusion bodies, which were isolated by centrifugation at 24,000 × *g*, 2 h. The resulting inclusion body pellet was washed twice in buffer B (50 mM Tris-HCl, 10 mM EDTA, 0.5% Triton X-100, 100 mM NaCl, pH 8). All standard protocols have been described elsewhere (18).

P-D-E-novispirin G10 was liberated from the inclusion bodies by hot acid hydrolysis, which cleaves specifically between Asp-Pro. This sequence was introduced between the KSI fusion partner and N terminally to the synthetic gene encoding novispirin G10. For hot acid hydrolysis, inclusion bodies were resuspended in 100 mM sodium phosphate (pH 2.3) and incubated overnight at 85°C. The resulting supernatant contained P-D-E-novispirin G10. The sample was neutralized by adding 100 mM sodium phosphate (pH 12.3). The peptide was maturated with glutamyl endopeptidase I from *Bacillus licheniformis* (30) produced by Novozymes A/S.

The maturated octadecapeptide was purified by cation-exchange chromatography, as follows. The novispirin G10-glutamyl endopeptidase mixture was adjusted to pH 6 and 7 mS/cm prior to application to a CM-Sephadex column (Amersham Biosciences). Novispirin G10 was eluted with a 0 to 1 M NaCl gradient in 50 mM malonic acid (pH 6) over 10 column volumes. The fractions were analyzed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometric analysis (for novispirin identification) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (for high-molecular-weight impurity detection). The selected fractions were pooled and desalted on a Sephadex G10 column (Amersham Biosciences) equilibrated with phosphate-buffered saline (PBS), pH 7.4 (50 mM phosphate buffer, 150 mM NaCl). Again, the fractions were analyzed by MALDI-TOF mass spectrometric analysis and pooled. The novispirin G10 pool was analyzed for purity by SDS-PAGE and reverse-phase high-performance liquid chromatography (Jupiter 5-μm C_{18} 300Å column; 150 by 2 mm; 50 min; gradient of 8 to 80% acetonitrile in 0.1% trifluoroacetic acid; flow rate, 0.15 ml/min; λ, 214 nm). The identity of the peptide was determined by automated N-terminal sequencing using a Procise automatic sequencer (Applied Biosystems Division, Perkin-Elmer), and the concentration was determined by amino acid analysis performed on a Biochrom 20 Plus (Biochrom Ltd.).

MALDI-TOF mass spectrometric analysis. A 0.5-ml sample was spotted onto a stainless steel target plate. One-half microliter of 10-mg/ml 4 hydroxy-α-cyanocinnapinic acid in 50% acetonitrile–0.1% trifluoroacetic acid was subsequently mixed with the sample on the target plate, and the sample spots were allowed to dry at room temperature before analysis (dried-droplet method). MALDI-TOF analyses were performed on a Voyager DE Pro mass spectrometer with delayed-extraction technology and a 337-nm N_2 laser producing 3-ns pulses with a repetition rate of 20 Hz (Applied Biosystems). The instrument was run in reflective mode with positive ionization. External calibration was applied using CalMix2 (Applied Biosystems).

Antibacterial activity of peptide. MICs of peptide were determined strictly according to Clinical and Laboratory Standards Institute guidelines. Briefly, suspensions of *P. aeruginosa* (5×10^5 CFU/ml) in cation-adjusted Mueller-Hinton broth (MHB) were challenged with serial dilutions of the peptide. The MIC was determined as the lowest peptide concentration that was able to inhibit visible growth of the bacteria.

Additionally, a previously published radial diffusion assay protocol was applied to assess antimicrobial activity (17). The MEC (minimum effective concentration, i.e., the concentration at which the drug starts having an effect on the bacteria) was determined. Target bacteria (5×10^6 CFU) were added to 10 ml of underlay agarose (1% low-electroendosmosis agarose, 0.03% Trypticase soy broth, 10 mM sodium phosphate, pH 7.4, 37°C). This suspension was solidified on an INTEGRID petri dish (Becton Dickinson Labware, Franklin Lakes, NJ). A 3-mm Gel Puncher (Amersham Pharmacia Biotech, Uppsala, Sweden) was used to make wells in the underlay agarose. Twofold dilutions of the peptide were added to the wells, and plates were incubated at 37°C for 3 h. An overlay (LB medium, 7.5% agar) was poured on top, and the plate was incubated overnight. Antimicrobial activity was seen as bacterial clearing zones around the wells. The diameters of the clearing zones (multiple dilutions of the peptide) were used to determine the MECs using linear regression. To determine the bactericidal kinetics, we applied peptide (at 2.3 times the MIC) to *P. aeruginosa* in MHB (~5×10^5 CFU/ml). At three time points (30 min, 90 min, and 24 h), 10-fold dilutions were prepared in MHB and immediately plated onto tryptic soy broth agar plates. Colony counts were made to determine the number of CFU per milliliter. Gentamicin sulfate (catalog no. 190057; ICN Biomedicals, Inc., Aurora, OH) was included for comparison.

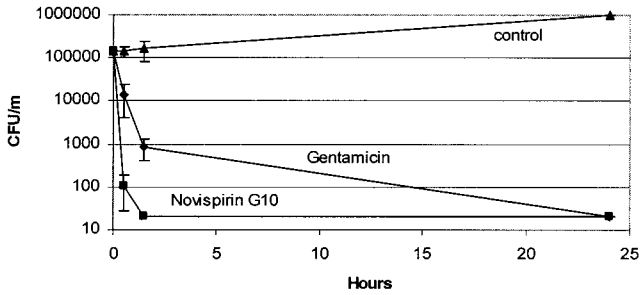

FIG. 1. Killing kinetics against *P. aeruginosa* ATCC 27853. Gentamicin was assayed at a final concentration of 4 μg/ml (~4 times the MIC), whereas novispirin G10 was applied at 300 μg/ml (~2.3 times the MIC). The detectable range was 20 to 10^6 CFU/ml. Vertical error bars depict 2 standard deviations of each measurement (repeated four times).

Bacterial strains. Mucoid *P. aeruginosa* NH57388A, a clinical isolate from a CF patient, was used in this study (10). The strain overproduces alginate steadily due to a mutation in *mucA* (a deletion of a C residue at position 170), and it lacks the repeating O polysaccharide corresponding to semirough lipopolysaccharide (10). The bacterial strain has an intact quorum-sensing system (10). *P. aeruginosa* ATCC 27853 was purchased from the American Typing Culture Collection. This is a nonmucoid bacterial strain.

Preparation of bacteria for challenge (10). *P. aeruginosa* NH57388A was cultured in ox broth with 1% glycerol at 37°C for 28 h with shaking (170 rpm). Bacterial cells were harvested by centrifugation (23,000 × *g*, 30 min, at 4°C) and resuspended in 2 ml of fresh ox broth for bacterial concentration detection (CFU per milliliter). The alginate in the supernatant was precipitated by addition of 3 volumes of 99% ethanol (4°C). The cotton-like alginate precipitate was harvested and washed three times in sterile saline. The alginate was then resuspended in 20 ml of sterile saline (0.85% NaCl) and whipped vigorously on a magnetic stirrer until a homogeneous suspension was formed. The alginate suspension was finally adjusted to a concentration of approximately 11 mg/ml. The bacteria were then adjusted in the alginate suspension to yield 10^{10} CFU/ml. The yield was confirmed by colony counts.

Animal. Seven-week-old healthy female Lewis rats (Charles River, Würzburg, Germany) with body weights of approximately 150 g were used. The rats were divided into treated and control groups, with each group containing 60 rats.

Challenge and novispirin G10 administration. At the time of challenge, all rats were anesthetized by subcutaneous injection of a 1:1 mixture of etomidate (Janssen, Birkerød, Denmark) and midazolam (Roche, Hvidovre, Denmark) at a dose of 1.5 ml/kg of body weight and tracheotomized (14, 24). One-tenth milliliter of the alginate suspension of *P. aeruginosa* (10^{10} CFU/ml) was instilled intratracheally into the lower left lung of each animal. As soon as this challenge was finished, 0.1 ml of novispirin G10 (0.1 mg/ml) in sterile saline was instilled into the lower left lung of each treated rat, whereas the control animal received only sterile saline intratracheally. Three hours later, all animals received the anesthetic at two-thirds of the previous dose and experienced intratracheal drug or saline administration in the left lung again. The incision was then sutured with silk thread and healed without any complications. On days 3, 5, 7, and 10 after challenge, the rats were sacrificed by injection of 20% pentobarbital (DAK, Copenhagen, Denmark) at 3 ml/kg of body weight, and lungs were removed from thoracic cavities aseptically for further investigation.

Macroscopic pathology of the lungs. Macroscopic lung pathology was expressed as the lung index of macroscopic pathology (LIMP), which was calculated by dividing the area of the left lung showing pathological changes by the total area of the whole lung (24, 32). In addition, the gross pathological changes in the lungs were also assigned the following scores, according to the severity of the inflammation (24, 26): I, normal lungs; II, swollen lungs, hyperemia, and small atelectasis (<10 mm^2); III, pleural adhesions and atelectasis (<40 mm^2); and IV, abscesses, large atelectasis, and hemorrhages. The evaluation was done in a double-blind way to avoid bias.

Histopathology of the lungs. Lung histological examination was performed as described previously (14, 24). The cellular alterations were classified as acute or chronic inflammation by a scoring system based on the proportions of neutrophils (PMNs) and mononuclear leukocytes (MNs) in the inflammatory foci.

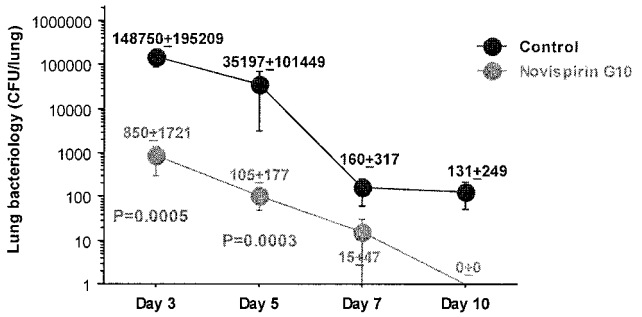

FIG. 2. Comparison of lung bacteriology between novispirin G10-treated and placebo control groups at different time points (mean ± standard error; treated with the Mann-Whitney U test).

Acute inflammation was defined as an inflammatory infiltration in which PMNs were predominant (≥90% PMNs with ≤10% MNs), whereas chronic inflammation was defined as a preponderance of MNs (≥90% with ≤10% PMNs), which included lymphocytes and plasma cells, and the presence of granulomas.

Lung bacteriology. Ten random entire lung samples from each group of rats at each time point were prepared for quantitative bacteriological examination. Each lung was mixed with 5 ml cold sterile PBS, and the mixture was homogenized in a blender. Appropriately diluted samples were plated on blue agar plates (a modified Conradi-Drigalsky medium selective for gram-negative rods and containing lactose, pH 7 · 0; State Serum Institute, Copenhagen, Denmark) to determine the number of bacterial CFU after 28 to 30 h of incubation at 37°C.

Lung cytokines. Concentrations of gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-4 (IL-4), IL-6, and IL-10 in the lung supernatants from the homogenate described above were determined using enzyme-linked immunosorbent assay kits (Nordic BioSite). Standard curves were constructed for IFN-γ (range, 31.25 to 1,000 pg/ml; sensitivity, <10 pg/ml), TNF-α (range, 31.25 to 1,000 pg/ml; sensitivity, <20 pg/ml), IL-4 (range, 31.24 to 1,000 pg/ml; sensitivity, <15 pg/ml), IL-6 (range, 31.3 to 2,000 pg/ml; sensitivity, 19 pg/ml), and IL-10 (range, 15.6 to 1,000 pg/ml; sensitivity, <5 pg/ml). The optical density of each sample was plotted on the standard curve of the respective cytokine enzyme-linked immunosorbent assay plate to obtain cytokine concentrations.

Statistical analyses. The categorical data were analyzed by the χ^2 test. Both the Mann-Whitney U test and analysis of variance (ANOVA) were used to compare the data obtained from groups in this study. Statistical analyses were carried out using Statview, a computer program for statistics, from Abacus Concepts, Inc. (www.abacus.com).

RESULTS

Recombinant novispirin G10. The identity of novispirin G10 was confirmed by automatic Edman degradation and MALDI-TOF mass spectrometric analysis. The obtained amino acid sequence was identical to the theoretical sequence, and the measured monoisotopic mass matched the expected mass within 0.01%. Reverse-phase high-performance liquid chromatography analysis determined a purity higher than 90% at λ = 214 nm. No significant impurities were detected by SDS-PAGE analysis or MALDI-TOF mass spectrometric analysis (data not shown).

TABLE 1. Percentages of animals that cleared the bacteria from their lungs

Treatment or parameter	% of rats that cleared bacteria[a]			
	Day 3 (n1 = n2 = 10)	Day 5 (n1 = n2 = 10)	Day 7 (n1 = n2 = 10)	Day 10 (n1 = 10, n2 = 9)
Novispirin G10	30	50	90	100
Placebo control	0	0	60	66.67
P value (χ^2 test)	NS[b]	<0.03	NS	<0.05

[a] n1 and n2 are the animal numbers in the novispirin G10-treated and control groups.
[b] NS, not significant.

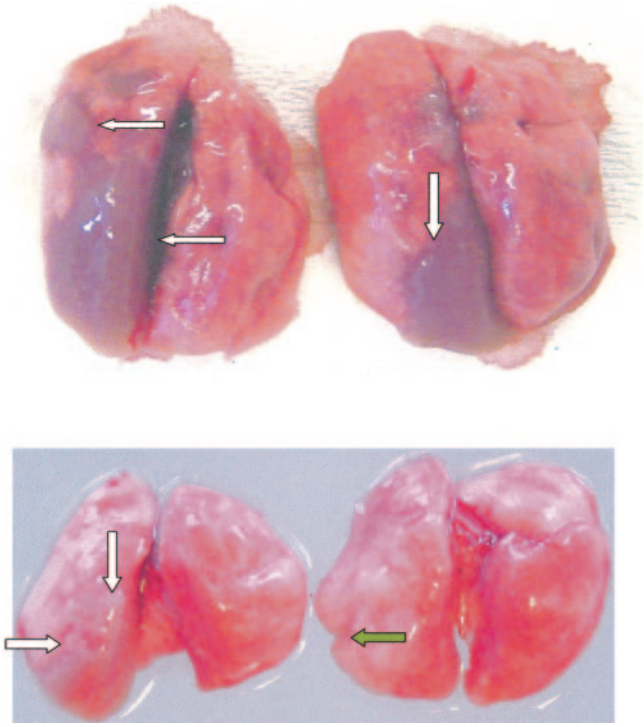

FIG. 3. Comparison of macroscopic lung pathology. Control lungs are on the left, and novispirin G10-treated lungs are on the right. On day 3 (top), significant lung consolidations were seen in both groups (indicated by arrows). However, on day 7 (bottom), significant lung consolidation was still seen in the control group (white arrows), whereas in the novispirin G10-treated group, only small atelectasis was seen (green arrow).

Antibacterial activities of novispirin G10. The MIC of novispirin G10 against both *P. aeruginosa* NH57388A and ATCC 27853 was 128 μg/ml, and the MEC was 2 μg/ml. Novispirin is highly basic, and its antimicrobial activities are influenced by the polyanions and divalent cations found in MHB. Indeed, removal of the polyanions present in MHB reduced the MIC of novispirin 10-fold or more (data not shown).

Under low-nutrient conditions, novispirin G10 was highly active against the nonmucoid (ATCC 27853) and mucoid (NH57388A) isolates of *P. aeruginosa*.

The bactericidal kinetics of *P. aeruginosa* ATCC 27853 treated with novispirin G10 are presented in Fig. 1. A rapid bactericidal effect (3-log reduction in the number of CFU per milliliter) was seen within 30 min of incubation. Upon 90 min of incubation with novispirin G10, no bacteria could be recovered (<20 CFU/ml). The comparator antibiotic, gentamicin (at four times the MIC, 4 μg/ml) effected only a 1-log reduction in the number of CFU per milliliter within 30 min.

Lung bacterial counts. The pulmonary bacterial counts in the novispirin G10-treated group were significantly lower than those in the placebo control group on days 3 and 5 post bacterial challenge (Fig. 2). The bacterial counts in the treated

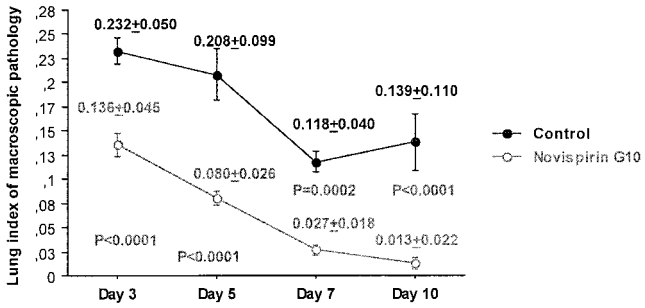

FIG. 4. Comparison of LIMP scores at different time points between novispirin G10-treated and placebo control groups (mean ± standard error; treated with the Mann-Whitney U test and ANOVA).

group on days 3 and 5 were more than 170- and 330-fold lower than those in the placebo control group (Mann-Whitney U test, $P = 0.0005$ and $P = 0.0003$), respectively. ANOVA indicates a significantly lower lung bacterial count on day 3 in the novispirin G10-treated group ($P = 0.0277$) compared to the control group. There were significantly more rats in the novispirin G10-treated group that cleared the bacteria from their lungs on days 5 and 10 compared with the placebo control group (Table 1).

Lung pathology. (i) Macroscopic pathology. Lung consolidation (on days 3 and 5) and atelectasis (on days 7 and 10) could be seen in both groups (Fig. 3). The macroscopic lung pathology in the placebo control group was dominated by a large area of lung consolidation, lung adhesion, or abscess, whereas in the novispirin G10-treated group, it was dominated by lung atelectasis without indications of abscess or adhesion.

(ii) LIMP. Significantly lower LIMP scores were found in the novispirin G10-treated rats at all four time points compared with the placebo control group (Fig. 4). Lower LIMP scores indicate less lung pathology.

(iii) Macroscopic lung scoring. The lung pathology scoring in the novispirin G10-treated group showed mainly milder lung pathology (scores, 1 to 3). In contrast, the placebo control group was predominated by more severe lung pathology (score, 4). The differences were statistically significant (Table 2).

Histopathology. The histopathology in the placebo control group was dominated by significant infiltration of neutrophils into the lung tissues (focal acute inflammation seen on day 3) and pulmonary edema (seen on days 5 and 10 postchallenge; Fig. 5). However, in the novispirin G10-treated group, the lung pathology changed quickly from focal chronic inflammation (on day 3) to interstitial inflammation (on day 5) and nearly normal (on day 10) (Fig. 5).

Lung cytokine responses. (i) IFN-γ. Compared with the placebo control group, the lung IFN-γ response on day 3 was significantly lower in the novispirin G10-treated group (Table 3). However, no significant differences were seen on days 5 and 7.

(ii) TNF-α. Significantly lower TNF-α responses were found in the novispirin G10-treated group on days 3 and 5 compared with the placebo control group (Table 3). No obvious difference was found on day 7 between the two groups.

(iii) IL-4, IL-6, and IL-10. Lower pulmonary IL-4, IL-6, and IL-10 responses were seen in the novispirin G10-treated group on day 3 (Table 3), whereas no significant differences were found on days 5 and 7.

Mortality. After an intratracheal bacterial challenge, a few animals could die of severe pneumonia on day 2 or 3 postchallenge. Autopsies showed that more than 60% of the lungs of each dead rat became acutely consolidated and the animals became significantly dehydrated. These findings indicated that those animals might have died from respiratory failure and infectious shock.

Two or three rats died in each group after the challenge. There was no significant difference in mortality between the two groups.

DISCUSSION

Antibiotic resistance is a common and serious clinical problem in the treatment of chronic *P. aeruginosa* lung infection. The *P. aeruginosa* genome is as large as 6.3×10^6 bp and contains 8.4% regulatory genes (29), affording the bacterium excellent environmental adaptability. By changing the activity of the genes involved in metabolism, material transport, efflux of organic compounds, mutating genes, and biofilm formation, the bacteria can modulate their sensitivity to antimicrobial agents and host defense strategies (3, 6, 12, 20, 29). The biofilm model of growth of the pathogen enables it to significantly resist the effects of both antibiotics and host immune defense mechanisms (6, 11, 12). Numerous searches have been made for new and efficacious antimicrobial agents (15, 16, 19, 21, 28, 34), alternative therapeutical drugs (22–27), and compounds that attenuate the virulence of the pathogen (9, 33) to help solve the problem. Novispirin G10, the product of one of these searches, has been demonstrated to rapidly kill both gram-positive and gram-negative bacteria, including mucoid strains

TABLE 2. Macroscopic lung scores at different time points after intratracheal challenge and novispirin G10 administration

Treatment or parameter	% of rats[a]											
	Day 3 (n1 = n2 = 14)			Day 5 (n1 = n2 = 14)			Day 7 (n1 = n2 = 11)			Day 10 (n1 = 14, n2 = 12)		
	I + II[c]	III	IV	I + II	III	IV	I + II	III	IV	I + II	III	IV
Novispirin G10	0	21.4	78.6	28.6	71.4	0	54.5	45.5	0	78.6	21.4	0
Placebo control	0	0	100	0	0	100	0	36.4	63.6	8.3	33.3	58.3
P value (χ^2 test)	NS[b]	NS	NS	NS	<0.001	<0.001	<0.005	NS	<0.005	<0.001	NS	<0.001

[a] n1 and n2 are the animal numbers in the novispirin G10-treated and control groups.
[b] NS, not significant.
[c] See Materials and Methods.

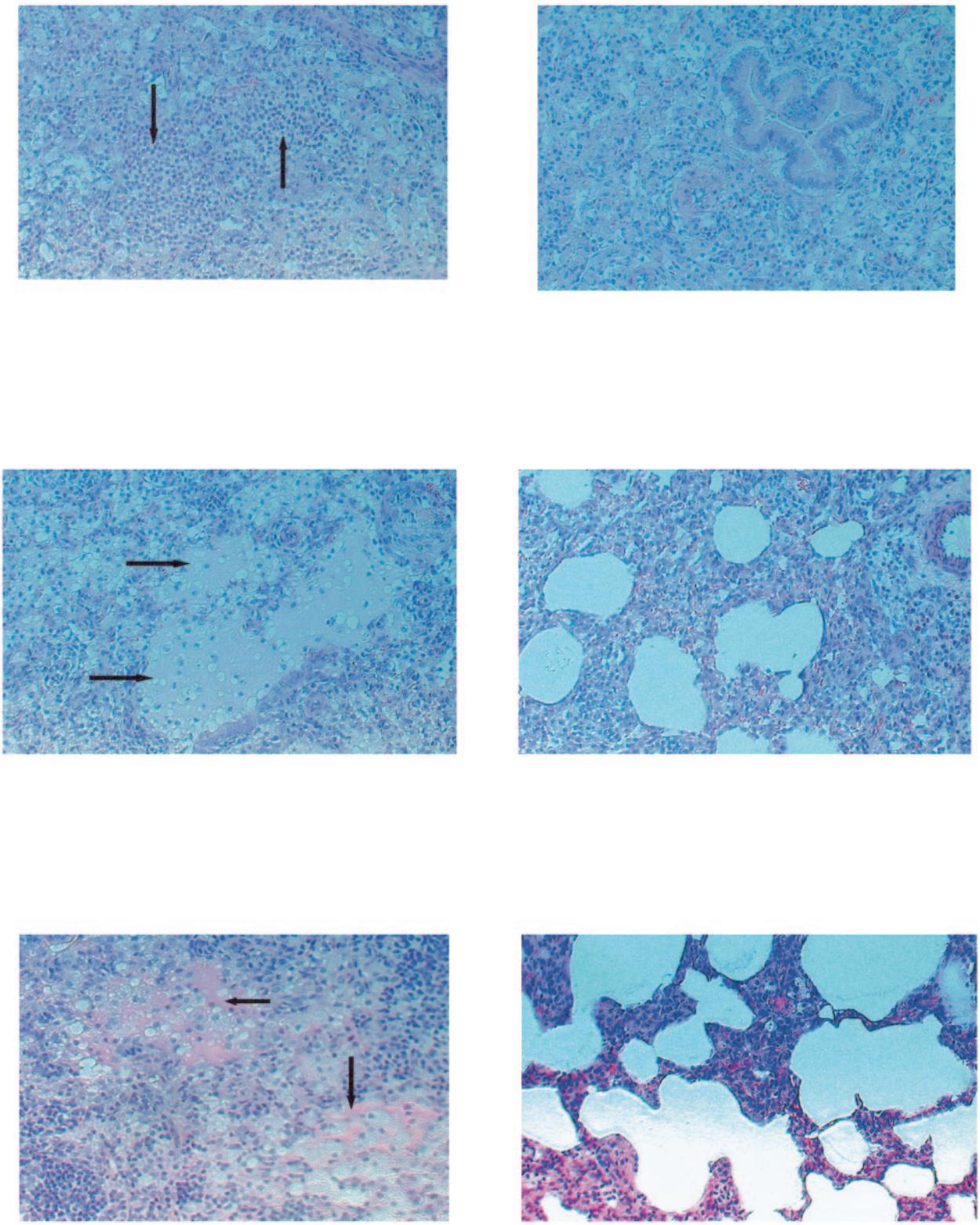

FIG. 5. Photomicrographs of lung sections from the placebo control group (left panel) and the novispirin G10-treated group (right panel) on day 3 (upper), day 5 (middle), and day 10 (lower). Arrows indicate neutrophil infiltration of lung tissue or pulmonary edema. Hematoxylin-and-eosin staining; magnification, ×200.

TABLE 3. Pulmonary cytokine responses at different time points post lung bacterial challenge with and without treatment

Cytokine	Mean cytokine response ± SD[a]					
	Day 3		Day 5		Day 7	
	Novispirin	Control	Novispirin	Control	Novispirin	Control
IFN-γ	65.29 ± 28.58[c]	115.88 ± 42.99	116.61 ± 42.08	114.12 ± 11.86	79.91 ± 16.22	86.96 ± 26.20
TNF-α	159.21 ± 23.81[d]	219.22 ± 48.82	128.11 ± 19.04[d]	178.52 ± 38.05	121.76 ± 19.63	140.90 ± 36.78
IL-4	34.49 ± 4.49[b]	42.45 ± 8.42	45.32 ± 5.91	47.96 ± 4.54	37.34 ± 5.92	52.87 ± 38.99
IL-6	74.08 ± 10.01[b]	91.05 ± 23.36	65.23 ± 11.11	66.97 ± 11.71	61.54 ± 11.01	56.91 ± 12.95
IL-10	51.67 ± 2.63[e]	70.45 ± 6.84	57.76 ± 7.58	62.87 ± 8.47	51.31 ± 2.88	53.68 ± 8.97

[a] n1 = n2 = 10.
[b] Compared with the control group (ANOVA), $P < 0.05$.
[c] Compared with the control group (ANOVA), $P < 0.01$.
[d] Compared with the control group (ANOVA), $P < 0.005$.
[e] Compared with the control group (ANOVA), $P < 0.001$.

of *P. aeruginosa*, in vitro and to kill multiresistant nonmucoid *P. aeruginosa* in burn wounds (19, 28).

In the present study, novispirin G10 effectively killed our mucoid and nonmucoid strains of the gram-negative bacterium *P. aeruginosa* in vitro, in accordance with the literature (4, 19, 28, 34). Our animal studies showed that two intratracheal administrations of novispirin G10 given within 3 h post intratracheal bacterial challenge dramatically decreased the bacterial load in the lungs during the early time points (days 3 and 5) relative to the placebo control group. The model is therefore characterized as antimicrobial prophylaxis and early treatment of the infection. The significantly faster bacterial clearance seen in the peptide-treated group suggests a powerful bactericidal effect of the peptide in vivo in the lungs. Significantly milder lung pathology and quicker recovery were seen in the peptide-treated group, presumably reflecting the significantly earlier bacterial killing due to novispirin G10 treatment. Lung cytokine determination showed that novispirin G10 treatment led to significantly lower local cytokine responses on day 3, including IFN-γ, TNF-α, IL-4, IL-6, and IL-10, compared with the placebo control group. The cytokine responses are perfectly consistent with the earlier bacterial killing and milder lung inflammatory responses seen in the peptide-treated group.

In the present study, the local inflammatory response was rendered milder and the immune response less strong. Probably the bacteria were cleared more rapidly by novispirin G10. In these experiments, all rats received 10^9 CFU of *P. aeruginosa* intratracheally on day 0. Seventy-two hours later (day 3), the average lung bacterial load in the control group was 1.5×10^5, whereas the average bacterial load in the peptide-treated group was only 8.5×10^2 CFU, more than 99% lower than in the control group. On day 5, the lung bacterial load in both groups decreased further but the load in the control group was 335 times higher than that in the novispirin G10-treated group. Apparently, the great difference in lung bacterial loads after the peptide treatment greatly reduced both lung pathology and pulmonary cytokine responses.

The concentration of novispirin G10 used in the present study (0.1 mg/kg, 100 μg/ml) was either around the MIC (128 μg/ml) or significantly above the MEC (2 μg/ml). However, neither the MIC nor the MEC is predictive of the in vivo efficacy of novispirin G10 at the site of infection. Factors such as the alveolar distribution of the peptide solution, the reduction of the solute volume (and concomitant concentration of the peptide), the rate of peptide clearance, and the precise physical-chemical conditions present in the lungs will influence the antimicrobial activity of novispirin G10. The relatively low peptide concentration and a bland vehicle (saline) were chosen to minimize lung toxicity that could impact on the pathology and delay recovery from the bacterial infection.

Compared to the other members of the ovispirin family, novispirin G10 possesses significantly lower cytotoxicity (19, 28). However, Bartlett and colleagues recently reported that novispirin G10 induced lung toxicity in the C57BL/6 mouse model of *Klebsiella pneumoniae* lung infection (4). Although we cannot exclude the possibility that the murine lung is more susceptible to damage by novispirin G10 than the rat lung is, at least three technical differences between their protocol and that used in the present study may have caused the pulmonary toxicity they reported. The first of these relates to the novispirin G10 dosage—theirs being about 10 times higher than ours, both in concentration and on a milligram-per-kilogram basis. The second relates to the respective vehicles used to deliver novispirin G10. We used PBS, but Bartlett et al. dissolved the peptide in 0.01% acetic acid–0.1% bovine serum albumin—a formulation that had a pH of 3.70 when recently tested in our laboratory. Indeed, Table 2 of their report indicates that their peptide-free vehicle caused a fourfold increase in the TNF-α concentration in bronchoalveolar lavage fluid, suggesting that it induced pulmonary injury. Finally, whereas the "established infection model" they cited had used an intratracheal challenge of 10^2 CFU of *K. pneumoniae*, the challenges used by Bartlett et al. exceeded 10^8 CFU/mouse.

In our own pilot study, novispirin G10 at concentrations of 20 mg/ml, 5 mg/ml, and 1 mg/ml led to significant lung toxicity (data not shown). However, novispirin G10 at a concentration of 100 μg/ml did not result in significant pathological lung changes and did not negatively affect the recovery of the lungs from a bacterial infection.

Our results suggest that intratracheal administration of novispirin G10 efficiently reduced the loads of viable mucoid *P. aeruginosa* in the animal lungs, which led to much milder lung pathology and more rapid clearance than would otherwise have occurred. Novispirin G10 deserves further investigation as a potentially safe and effective alternative or adjuvant for treating mucoid *P. aeruginosa* lung infections in CF patients, as well as for treating other, peripheral infections.

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