Neutrophil defensins mediate acute inflammatory response and lung dysfunction in dose-related fashion

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Zhang, Haibo, Giuliana Porro, Neil Orzech, Brendan Mullen, Mingyao Liu, and Arthur S. Slutsky. Neutrophil defensins mediate acute inflammatory response and lung dysfunction in dose-related fashion. Am J Physiol Lung Cell Mol Physiol 280: L947–L954, 2001.—High concentrations of neutrophil defensins from airway and blood have been reported in patients with inflammatory lung diseases, but their exact role is unclear. We investigated the direct effect of defensins on the lungs of mice. Intratracheal instillation of purified defensins (5–30 mg/kg) induced a progressive reduction in peripheral arterial O2 saturation, increased lung permeability, and enhanced the lung cytochrome c content. These indexes of acute lung dysfunction were associated with an increased total cell number and a significant neutrophil influx into the lung (5.1 ± 0.04% in control vs. 48.6 ± 12.7% in the defensin (30 mg/kg) group, P < 0.05). Elastase concentrations in the bronchoalveolar lavage (BAL) fluids increased from 38 ± 11 ng/ml (control) to 80 ± 4 ng/ml (defensins, P < 0.05). Five hours after defensin instillation, concentrations of tumor necrosis factor-α and macrophage inflammatory protein-2 in BAL fluid were significantly increased. High levels of monocyte chemoattractant protein-1 in BAL fluid and plasma were also found after defensin stimulation. We conclude that intratracheal instillation of defensins causes acute lung inflammation and dysfunction, suggesting that high concentrations of defensins in the airways may play an important role in the pathogenesis of inflammatory lung diseases.

Cytokine; inflammatory lung disease; human neutrophil peptides

HUMAN NEUTROPHIL PEPTIDES 1–3 (HNP-1 to -3), also known as defensins, constitute up to 50% of the total protein content of the azurophilic granules, with the other main protein constituents being cathepsin, elastase, and proteinase 3 (7). Defensins are active against gram-positive and gram-negative bacteria in microbiological medium in vitro (8) and act by permeabilizing both outer and inner lipid membranes of bacteria in a charge- or voltage-dependent manner (8, 19). Human defensins also have been shown to be cytotoxic in vitro using human tumor targets. Optimal lysis was achieved with 25–100 μg/ml defensins after 6 h in various human and murine tumor cell lines (21). However, this effect is not tumor specific; similar concentrations of human defensins also lyse endothelial cells (26), murine thymocytes, and spleen cells (21).

The plasma concentration of defensins in normal volunteers is 0.2–0.3 μg/ml but rises to 170 μg/ml in patients with sepsis (13, 27). Defensin concentrations have been reported to increase by 50-fold in bronchoalveolar lavage (BAL) fluid of patients with acute respiratory distress syndrome (ARDS) compared with normal controls (1). Soong et al. (33) recently reported an increased level of sputum defensins ranging from 300 to >1,600 μg/ml in patients with cystic fibrosis. In addition to the potential antimicrobial effects of defensins, the elevated concentrations of defensins in sepsis, ARDS, and cystic fibrosis may be responsible for initiating inflammatory responses and acute lung injury. A defensin concentration of 100 μg/ml has been shown to increase production of interleukin (IL)-8, a pivotal chemokine, and has been reported to be cytotoxic as assessed by adenine release in a human lung epithelial cell line (A549) in vitro (37). These studies showed that defensins can be injurious in vitro, but there is a paucity of data on the in vivo effects of defensins. We hypothesized that defensins, at the concentrations observed in various lung conditions, might significantly contribute to acute lung dysfunction. Therefore, the purposes of this study were to 1) investigate the role of defensins on acute lung dysfunction as assessed by the measurements of oxygenation, lung mitochondrial cytochrome c, and lung permeability; 2) study the effects of defensins on neutrophil migration and activation as evaluated by elastase activity; and 3) examine the regulatory effects of defensins on the production of pulmonary and systemic cytokines.

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MATERIALS AND METHODS

Isolation of Defensins

Defensins were used as a mixture of HNP-1, -2, and -3 that were isolated from sputum of patients with cystic fibrosis. The technique for purification of defensins has been described previously (11). Briefly, purulent sputum was loaded onto a polyacrylamide gel permeation column and eluted with 5% acetic acid. Defensin-containing fractions were pooled and analyzed for purity by 12.5% acid-urea polyacrylamide gel electrophoresis or 16% tricine-SDS gels and by mass spectrometry. Working defensin solutions were prepared from 4 mg/ml stock solution and a defensin-free vehicle solution was prepared with 0.01% acetic acid.

The lipopolysaccharide (LPS) content of the purified defensins was determined by the assay of Limulus amebocyte lysate (Pyrochrome, Associates of Cape Cod, Falmouth, MA). The LPS concentration was <100 pg/ml in the purified defensin solution.

Mouse

Male C57BL/6 mice (8–10 wk old, 25–28 g; Jackson Laboratories, Bar Harbor, ME) were used in accordance with the institutional animal welfare guidelines of the University of Toronto. The animals were anesthetized with intraperitoneal injection of ketamine (200 mg/kg; Ketalean, Bimeda-MTC Animal Health, Cambridge, ON) and xylazine (10 mg/kg; Rompun, Bayer, Agriculture Division, Animal Health, Etobicoke, ON). The animals were placed on a heating pad to maintain body temperature constant during the surgical preparation and throughout the study period. A tracheotomy was performed and a sterile angiocatheter (Angiocath, 20 gauge, Becton Dickinson Infusion Therapy Systems, Sandy, UT) was inserted into the trachea. Peripheral (tail) arterial O2 saturation was monitored by a veterinary pulse oximeter (model 8600V, Nonin Medical, Minneapolis, MN).

Experimental Protocol

After tracheotomy, the animals were stabilized for 10–15 min in an oxygen chamber. The chamber was made from a 2-liter plastic bottle with an oblong window cut out to accommodate the head of the mouse placed partially within the chamber. Oxygen was introduced through a plastic hose entering the bottle through a hole drilled in its screw-on cap. A flow of oxygen into the bottle at 5–10 l/min was found to be adequate to raise the peripheral arterial O2 saturation of the mice to 100%. Once the peripheral arterial O2 saturation levels were ≥95%, the mice were randomized into one of the following groups of 10 mice each: group 1 served as a vehicle (defensin-free) control; groups 2–6 received defensins at 5.0, 7.5, 15, 22.5, and 30 mg/kg, respectively. In all animals, a total of 50 µl of solution with or without defensins was instilled directly into the trachea through the intratracheal angiocatheter. The instillation was followed by three artificial lung inflations with a 1-ml syringe using a tidal volume of 10 ml/kg to equalize distribution of the solutions in the lung. The syringe was then disconnected from the endotracheal tube, and the spontaneously breathing mice were then placed in the oxygen chamber until peripheral arterial O2 saturation was ≥95%; this usually required 5–10 min. The mice were then removed from the oxygen chamber and monitored for 5 h in room air. Supplemental anesthetic [0.1 ml (ketamine, 70 mg/kg and xylazine, 4 mg/kg)] was given intraperitoneally every 30–40 min.

BAL

On completion of the 5-h study period, the lungs were lavaged in situ with cold sterile physiological saline. Saline was instilled in three 1-ml aliquots and gently withdrawn with a 1-ml syringe. BAL fluid was centrifuged at 300 g for 10 min to pellet cells. Supernatant was collected for the measurements of cytokines and neutrophil elastase activity.

Neutrophil Migration

After centrifugation, the cell pellets of the BAL fluid were resuspended in PBS. Total cell count and viability were determined by trypan blue staining on a grid hemocytometer. Differential cell counts were determined on cytoospin-prepared slides that were stained with Wright-Giemsa stain. The percentage of neutrophils was calculated as the ratio of neutrophils divided by the total number of cells in the BAL fluid sample.

Lung Permeability Assessment

To estimate changes of lung permeability after defensin instillation, the Evans blue dye (EBD) injection technique was employed (2). Five mice of the 10 vehicle controls and 5 of the mice treated with defensins at 15 mg/kg underwent the permeability study. Briefly, animals were administered 20 mg/kg EBD (Sigma, St. Louis, MO) by tail vein injection 30 min before the end of the 5-h experiments. Thirty minutes after EBD injection, blood was sampled by cardiac puncture after the chest was opened. The animals were then exsanguinated by dissecting the abdominal aorta through a midline abdominal incision. To flush the pulmonary vasculature, the right ventricle was punctured with a needle (Angiocath, 21 gauge, Becton Dickinson Infusion Therapy Systems, Sandy, UT) and advanced into the pulmonary artery. The pulmonary vessels were perfused in situ with 3 ml of normal saline while the lung was ventilated with a 1-ml syringe. The lungs were removed, weighed, and then homogenized in formamide (Sigma). EBD was extracted from lung homogenates by incubating samples in the formamide solution at 60°C for 18 h. The tissue suspension was centrifuged at 4,000 g for 30 min. EBD concentration in lung homogenate supernatant was quantitated by a dual-wavelength spectrophotometer at 620 and 740 nm, which allows for correction of contaminating hemoglobin pigments as determined by the following formula: OD620 (EBD) = OD620 − (1.426 × OD740 + 0.030), where OD620 and OD740 are the optical densities at 620 and 740 nm, respectively (34). Concentration of EBD is expressed as nanograms per milligram weight of tissue from generated EBD standard absorbance curves.

Measurement of Lung Cytochrome c

Lung cytochrome c level was quantitated in lung homogenates by an immunoassay kit (Rat/Mouse Cytochrome c Immunoassay, R&D Systems, Minneapolis, MN). Briefly, on the completion of the 5-h study experiment, the lung was excised, weighed (wt), and homogenized in PBS with a homogenizer (Ultra-Turrax T8, IKA Labortechnik, Janke and Kunkel, Staufen, Germany) operated by a power drill at 2,000 rpm six times, each lasting 10–12 s, held in an ice bath. Aliquots of this homogenate were assayed for cytochrome c and measured with a spectrophotometer at 450 nm. The values of cytochrome c were normalized to the lung weight.

Measurement of Elastinolytic Activity

Elastinolytic activity in BAL fluid was determined by measuring the degradation of elastin using an elastase assay kit.
EnzChek; Molecular Probes, Eugene, OR). Elastin was prepared by reductive alkylation of soluble bovine neck ligament elastin that had been labeled with BODIPY FL dye such that the conjugate's fluorescence was quenched. On digestion by elastase, the fluorescence was revealed. The resulting increase in fluorescence was monitored with a fluorescence microplate reader (CytoFluor 2300; Millipore, Bedford, MA) at 530 nm.

Measurement of BAL Fluid Cytokines

Lavage fluid and plasma were assayed for tumor necrosis factor (TNF-α), monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-2. TNF-α and MCP-1 were measured using a polyclonal antibody specific for mouse TNF-α or MCP-1, respectively (Cytoscreen, BioSource, Camarillo, CA) as a capture reagent in a standard sandwich ELISA. MIP-2 concentrations were measured using a rat MIP-2 ELISA kit (Cytoscreen, BioSource) containing antibodies that are cross-reactive to murine MIP-2 as described previously (31).

Lung Histology

At the end of the experiments, the lungs were fixed by immersion in 10% buffered neutral formalin (BDH, Toronto, ON) and processed using standard histological techniques. Morphological integrity was assessed by a pathologist in a blinded fashion.

Statistical Analysis

A one-way analysis of variance followed by Tukey-Kramer test was used for statistical analysis of the data. Differences were considered statistically significant at \( P < 0.05 \). Data are presented as means ± SE.

RESULTS

Evaluation of Lung Dysfunction

To investigate the role of defensins in acute lung dysfunction, defensins at a wide range of concentrations were instilled intratracheally followed by monitoring of oxygenation, estimation of lung permeability changes, and measurement of lung mitochondrial cytochrome c concentrations.

Peripheral arterial \( O_2 \) saturation. We report peripheral arterial \( O_2 \) saturation levels at a 4-h cutoff for all animals studied because 10 mice were injected for the EBD assay at 4.5 h for the lung permeability study, making further peripheral arterial \( O_2 \) saturation measurements impossible in these animals.

In general, peripheral arterial \( O_2 \) saturation levels remained relatively constant in the vehicle-control group throughout the study period, but higher doses of defensins induced a significant reduction in peripheral arterial \( O_2 \) saturation levels (Fig. 1). For a given dose, intratracheal administration of defensins caused a progressive reduction in the peripheral arterial \( O_2 \) saturation level, except for the mice treated with 5 mg/kg that showed a relatively constant peripheral arterial \( O_2 \) saturation. At any given time point, defensins induced a dose-dependent decrease in peripheral arterial \( O_2 \) saturation. The highest doses of defensins (15, 22.5, or 30 mg/kg) resulted in similar reductions of peripheral arterial \( O_2 \) saturation. Based on this study, a dose of 15 mg/kg of defensins was used to assess lung permeability.

Lung permeability. Lung permeability was estimated by EBD technique for the vehicle-control mice and the mice treated with defensins (15 mg/kg). This dose of defensins resulted in a threefold increase in lung accumulation of EBD (2.24 ± 0.60 ng/mg tissue) compared with that in vehicle-control animals (0.74 ± 0.08 ng/mg tissue, \( P < 0.05 \)).

Defensins in Lung Injury

Fig. 1. Intratracheal instillation of defensins reduces peripheral arterial \( O_2 \) saturation (Sp\( O_2 \)). Peripheral arterial \( O_2 \) saturation was monitored by a pulse oximeter in the mice who received the same amount of fluid (0.05 ml intratracheally) containing either vehicle-control buffer (defensin free) or various doses of defensins. \(*P < 0.05\) compared with other groups except the 5 mg/kg group at the same time point. \(**P < 0.05\) compared with 15, 22.5, and 30 mg/kg groups. \(\dagger P < 0.05\) compared with other groups. \(\ddagger P < 0.05\) compared with 22.5 and 30 mg/kg groups.

Fig. 2. Intratracheal instillation of defensins increases lung mitochondrial cytochrome c concentrations. Cytochrome c of lung homogenate from animals 5 h after intratracheal instillation with either vehicle control buffer or defensins (15 and 30 mg/kg) was measured. \(* P < 0.05\) compared with vehicle (control).
Lung cytochrome c. To determine the role of mitochondrial function in acute lung dysfunction induced by defensins, cytochrome c release was measured in lung homogenates. Figure 2 demonstrates that defensins induced an increase in the mitochondrial content of cytochrome c in a dose-dependent fashion.

Lung histology. There were no significant gross lung histological alterations in the vehicle control animals and the animals treated with defensins (15 mg/kg).

Lung Neutrophil Migration and Elastase Activity

To investigate the effect of defensins on neutrophil migration into the lung, differential cell counts were determined from BAL fluid after administration of various doses of defensins. As shown in Fig. 3A, there was an increase in total cell number in the lung, which was associated with an increase in lung neutrophil migration. Figure 3B shows that the neutrophils were 5.1 ± 0.04% of total cells in the vehicle control animals. Intratracheal administration of defensins resulted in a significant neutrophil influx into the alveolar space in a dose-dependent manner (up to 48.6 ± 12.7% in the group treated with defensins at 30 mg/kg) after 5 h.

Elastase concentration in the BAL fluid averaged 38 ± 11 ng/ml in the vehicle control group and increased to 60 ± 6 ng/ml in the group treated with 22.5 mg/kg of defensins and up to 80 ± 4 ng/ml in the group treated with defensins at 30 mg/kg (both P < 0.05, Fig. 4).

TNF-α, MIP-2, and MCP-1 Responses to Defensin Instillation

TNF-α concentrations in BAL fluid were significantly increased 5 h after defensin instillation at doses of defensins >15 mg/kg compared with that in the vehicle control group (236 ± 50 vs. 49 ± 14 pg/ml, P < 0.05; Fig. 5A). TNF-α levels in plasma were somewhat higher in the defensin-treated mice than in the vehicle control group, but the differences were not significant.

MIP-2 concentrations in BAL fluid were significantly increased in a dose-dependent manner after defensin instillation (Fig. 5B). Plasma MIP-2 level increased from 93 ± 10 pg/ml in the vehicle group to 262 ± 23 pg/ml in the group treated with defensins at 7.5 mg/kg (P < 0.05). Although plasma MIP-2 concentrations were increased about 100% with higher doses of defensins, the differences did not reach statistical significance.

MCP-1 levels in BAL fluid significantly increased at a dose of 7.5 mg/kg of defensins and remained high with larger concentrations of defensins. Plasma MCP-1 concentration increased from 218 ± 145 pg/ml in the vehicle group to 683 ± 63 pg/ml in the group treated with 7.5 mg/kg of defensins (P < 0.05) and declined with defensins at 15 and 30 mg/kg (Fig. 5C).
DISCUSSION

Although the effect of defensins has been assessed in vitro, the present study is the first to investigate the direct effects of intratracheal instillation of a broad range of concentrations of defensins on pulmonary inflammatory response and acute lung dysfunction in vivo. We hypothesized that high levels of defensins would be deleterious to the lung and might lead to a pulmonary inflammatory response and tissue damage. A key question in interpreting the physiological and/or clinical significance of our results is how the concentrations of defensins we used compare to values observed in patients.

Clinical Relevance of the Doses of Defensins Used

Neutrophils have ~1,000 granules per cell, and the measured mass of defensins in human neutrophils is about 3–5 μg/10^6 neutrophils. High concentrations of defensins (1–10 mg/ml) are likely to exist in phagocytic vacuoles containing ingested microbes (8). Panyutich et al. (27) found that patients with meningitis infection and sepsis had plasma defensin levels that were extremely high, ranging from 120 to 170,000 ng/ml, compared to a mean concentration of 42 ng/ml in healthy blood donors. Soong et al. (33) reported high sputum defensin levels ranging from 300 to >1,600 μg/ml (the upper detection limit in the study) in patients with cystic fibrosis. Ihi et al. (13) have recently found high defensin concentrations in pleural fluid [13.3 ± 1.9 (SE) mg/ml] in patients with empyema, in BAL fluid (2.0 ± 0.9 mg/ml) in patients with bacterial pneumonia, and in cerebrospinal fluid (3.4 ± 1.2 mg/ml) in patients with bacterial meningitis. Ganz and Lehrer (8) speculated that the local concentrations of defensins in infected tissues are probably much higher, but these have not yet been measured systematically.

Electron microscopy estimates of the thickness of the alveolar surface liquid give an average value of about 0.3 μm in mice (9, 39). Gehr et al. (9) reported morphometric analysis of the alveolar surface area in mouse lung. According to the data they provided, the mean alveolar surface area is ~0.1 m² in a 25-g mouse. Based on these values, the total alveolar surface liquid is about 30 μl in a 25-g mouse. We administered defensins intratracheally in a 50-μl volume to the mice. The doses of defensins we used would thus roughly correspond to an initial concentration ranging from 1 to 10 mg/ml in the lungs of the mice (body wt 25–28 g). This range of concentrations of defensins in the lung is relevant to certain inflammatory lung diseases (8, 13, 23, 33) as previously detailed. Our dose calculation is an estimation. We did not measure the distribution and the concentrations of defensins in the lung in our experiments for two main reasons. First, defensin binding to mammalian target cells begins very rapidly, within 2 min, and then gradually increases and reaches a stable level after 60 min (20). The free defensin level may be very low 5 h after a single instillation. Second, it was impossible to perform multiple BALs on the same animal. A study of the time course of pulmonary defensin levels would be useful in the future. Moreover, the concentrations of defensins in the lung may be altered during fluid absorption and/or defensin metabolism.

We used human neutrophil or α-defensins in the present study. There are several reasons for testing human α-defensins in the mouse, even though murine neutrophils do not express defensins (5). First, there is >60% amino acid homology between human α-defensins and murine crypt cell defensins (6, 12, 14). Second, very high quality α-defensins can be purified from human sputum in sufficient amounts to conduct these experiments. Third, the effect of human neutrophil defensins on murine cells is highly comparable to human cells with respect to cytolyis (21). An intranasal administration of human α-defensins to mice also has been used to study lymphocyte activation by defensins (22).
High Concentrations of Defensins Induce Acute Lung Dysfunction

We demonstrated that intratracheal instillation of defensins at a low concentration, e.g., 5 mg/kg, does not cause significant acute lung dysfunction at 5 h in healthy mice. However, at higher concentrations, defensins induce acute lung dysfunction and biochemical changes as reflected by a decrease in oxygenation, an elevation of mitochondrial cytochrome c levels, and an increase in lung permeability.

Reduced peripheral arterial O2 saturation. An important clinical abnormality in acute lung injury is a decrease in arterial oxygen tension. We used peripheral arterial O2 saturation as our measure of oxygenation because it is noninvasive and we did not want to take multiple blood samples in the mice. Defensins induced a significant and marked decrease in peripheral arterial O2 saturation at higher doses. We did not investigate the mechanism for the decreased peripheral arterial O2 saturation, but it may have been due to a number of factors including ventilation-perfusion mismatches and possibly disruption of the alveolar-capillary membrane. Irrespective of the precise mechanism, the effect was dramatic and rapid at the highest doses.

Increased mitochondrial cytochrome c levels. We measured lung cytochrome c concentrations to look at a cellular signaling response that may reflect NADPH oxidase under in vivo conditions. The increased cytochrome c levels seen in the defensin-treated animals may simply reflect greater cell numbers in the lung. However, it is also possible that defensins, after entering cells, damage mitochondria by interacting with intracellular constituents to modulate NADPH oxidase activity (35, 36). This area deserves further research.

Increased lung permeability. The early phase of acute lung injury is characterized by vascular protein leakage into the airspaces due to increased permeability of the alveolar-capillary barrier (38). Many studies have shown that EBD is a reliable technique for macroscopic evaluation of vascular protein leakage under different conditions (2, 29, 34). Patterson et al. (29) showed EBD-bound albumin to be equivalent or superior to iodinated albumin for determining clearance values across endothelial monolayers. Furthermore, the same group of investigators demonstrated that EBD is a sensitive marker for early protein leak from the vasculature in perfused lung, occurring before detectable lung weight gain (29). Although the present study is the first demonstrating that defensins increase lung permeability in vivo, in another study, it was reported that 72 h after incubation with defensins, the permeability of a tracheal epithelial cell monolayer (as assessed by trypan blue) was increased (33). Using Madin-Darby canine kidney epithelial monolayers, Nygarrd and associates (25, 30) reported that defensins reduce the barrier integrity in a time- and concentration-dependent manner. Ökrent et al. (26) showed that defensins (100–200 μg/ml) increased chromium release from the lung-derived cell lines MRC-5 and A549 and human umbilical vein endothelial cells after 10–20 h of incubation. Cytotoxicity also was reported when 51Cr-labeled A549 cells were stimulated with 100 μg/ml of defensins for ~20 h (37). Taken together, these data suggest that high doses of defensins may damage the two separate barriers in the lung: 1) the alveolar epithelium and 2) the microvascular endothelium.

Lung histology. The lack of significant alterations of lung histology after defensin administration in this study may be related to the length of our experimental protocol. As discussed previously, significant cytotoxicity of defensins was seen in in vitro studies, but the period of exposure to defensins in these studies (26, 37) was much longer than in the present study. Nygaard et al. (25) also reported that human defensins increased epithelial permeability without causing cell lysis as measured by lactate dehydrogenase release in Madin-Darby canine kidney epithelial monolayers. We believe that the lack of histological change is not a sampling artifact despite the demonstrated increase in permeability. In support of this observation, Kaner et al. (16) recently have shown that overexpression of vascular endothelial growth factor increased lung permeability, quantified by the EBD assay and 125I-albumin permeability, without histological alteration apart from some edema. Thus our data suggest that measurements of cytochrome c and lung permeability using the EBD technique are useful tools to detect early acute lung dysfunction induced by defensins.

Defensins Cause Neutrophil Influx Into the Lung Followed by an Enhanced Elastase Activity

At the cellular level, acute lung injury is characterized not only by endothelial and epithelial cell injury but also by the influx and activation of inflammatory neutrophils. The accumulation of neutrophils within the lung is probably dependent on chemotactic gradients established by local generation of chemotactic factors. A number of peptide mediators have been implicated in the recruitment and activation of inflammatory cells within the lung. Van Wetering et al. (37) have shown recently that defensins induce IL-8 synthesis in airway epithelial cells, suggesting that they may contribute to the perpetuation of an inflammatory response by stimulating local chemokine release. Other investigators also have reported that defensins stimulate alveolar macrophage production of the neutrophil chemoattractants leukotriene B4 and IL-8 (28). Subcutaneous injection of defensins in mice also has been shown to induce a neutrophil influx into the peritoneal cavity (40).

Elastase is believed to have an important role in tissue damage in conditions with neutrophil infiltration. There are three potential sources for elastase in the airways: neutrophils, macrophages, and Pseudomonas. Pseudomonas cannot be implicated in the present study conditions, and there was no appreciable accumulation of macrophages in the lungs at 5 h even after high doses of defensins (data not shown). There-
Defensins Induce Lung Cytokine Responses

The involvement of TNF-α in various models of acute lung injury induced by sepsis, acid aspiration, or mechanical ventilation has been well documented (32), suggesting that cytokines mediate the initiation and maintenance of inflammatory lesions. We measured TNF-α concentrations in BAL fluids and plasma samples from the defensin-treated mice. The increased release of TNF-α found in BAL fluid may reflect the initiation of the lung inflammatory cytokine network because TNF-α is considered as an early, central inflammatory cytokine. TNF-α itself can also directly increase the permeability of lung endothelial and epithelial barriers (4, 24).

Neutrophils are attracted into the lung as a result of local production of chemotactic factors, including the neutrophil chemoattractant chemokine IL-8, a member of the C-X-C chemokine family. A previous in vitro study has shown that defensins induce an increased production of IL-8 from cultured lung A549 cells (37). We found that the production of MIP-2, a rodent homolog of IL-8, also was dose dependently increased in the BAL fluid after defensin stimulation. A relatively lower concentration in plasma may reflect the chemotactic gradient between the airspace and lung circulation, resulting in neutrophil migration. This finding suggests that defensins released from activated neutrophils stimulate MIP-2 synthesis in the lung and thus contribute to the local chemokine network, resulting in additional neutrophil influx into the lung.

MCP-1, a C-C chemokine family member, can be derived from monocytes and alveolar macrophages. Blockade of MCP-1 attenuates the development of lung injury (15). MCP-1 may also enhance lung inflammatory responses to other stimuli (10). Because alveolar macrophages are commonly involved in lung inflammation, we measured MCP-1 production. Interestingly, although MCP-1 levels also were increased in BAL fluid and plasma after defensin challenge, they were independent of the doses of defensins used. Moreover, because the MCP-1 levels in BAL fluid and plasma were both elevated, the concentration gradient between the airspace and pulmonary circulation may not be as great as that seen for MIP-2, and, therefore, its chemotactic activity may be limited. Indeed, we found that neutrophils predominated in the BAL fluid and the percentage of monocytes and lymphocytes was not significantly increased.

In summary, a large body of evidence has proven that defensins exert a significant antimicrobial activity. A number of in vitro studies also have shown that defensins are cytotoxic. The present in vivo study demonstrates that defensins at high concentrations may directly induce hypoxemia, impairment of mitochondrial function, an increase in lung permeability, and an elevation of cytokines in the lung. Defensins may thus act as mediators in the early phase of acute inflammatory responses in the lung. These data have potential clinical relevance in the critically ill. Patients with inflammatory lung diseases have significantly elevated concentrations of defensins in the airways and the lungs. In the particular case of cystic fibrosis, because of the high salt concentrations that exist in the airways of these patients, the microbicidal activity of defensins is impaired, and this may contribute to resistant severe infection. However, defensins may still be capable of disrupting the alveolar-capillary barrier, leading to the development of lung injury. Further understanding of the molecular basis of these processes should facilitate the development of selected therapeutic measures.

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