Role of human neutrophil peptides in lung inflammation associated with α1-antitrypsin deficiency

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Submitted 3 April 2003; accepted in final form 28 October 2003

Spencer, L. Terry, Gregorino Paone, Peter M. Krein, Farshid N. Rouhani, Jesus Rivera-Nieves, and Mark L. Brantly. Role of human neutrophil peptides in lung inflammation associated with α1-antitrypsin deficiency. Am J Physiol Lung Cell Mol Physiol 286: L514–L520, 2004. First published October 31, 2003; 10.1152/ajplung.00099.2003.—Individuals with α1-antitrypsin (α1-AT) deficiency are at risk for early-onset destructive lung disease as a result of insufficient lower respiratory tract α1-AT and an increased burden of neutrophil products such as elastase. Human neutrophil peptides (HNP), the most abundant protein component of neutrophil azurophilic granules, represent another potential inflammatory component in lung disease characterized by increased numbers of activated or deteriorating neutrophils. The purpose of this study was to determine the role of HNP in lower respiratory tract inflammation and destruction occurring in α1-AT-deficient individuals. α1-AT-deficient individuals (n = 33) and healthy control subjects (n = 21) were evaluated by bronchoalveolar lavage. HNP concentrations were significantly higher in α1-AT-deficient individuals (1.976 ± 692 vs. 29 ± 12 nM, P < 0.0001), and levels correlated with markers of neutrophil-mediated lung inflammation. In vitro, HNP produced a dose-dependent cytotoxic effect on alveolar macrophages and stimulated production of the potent neutrophil chemokine-activator leukotriene B4 and interleukin-8 by alveolar macrophages, with a 6- to 10-fold increase in chemokine-activator production over negative control cultures (P < 0.05). A synergistic effect was noted between HNP and neutrophil elastase with regard to leukotriene B4 production. Importantly, the proinflammatory effects of HNP were blocked by α1-AT. HNP likely play an important role in amplifying and maintaining neutrophil-mediated inflammation in the lungs.

pulmonary emphysema; granulocytes; alveolar macrophages; serpins

LOW SERUM LEVELS of α1-antitrypsin (α1-AT) and an increased risk of obstructive lung disease in early adulthood characterize α1-AT deficiency, an autosomal hereditary disorder. Central to the development of lung inflammation and destruction in α1-AT-deficient individuals is the accumulation of neutrophils in the lower respiratory tract. Normally nearly absent, neutrophils are the predominant inflammatory cell in the airways of individuals with α1-AT deficiency (11). These cells release a variety of destructive enzymes into the airways, including proteases, toxic oxygen metabolites, nitric oxide, and phospholipases. Human neutrophil peptides (HNP), also known as α-defensins, are another major product of activated and deteriorating neutrophils. These 3.5-kDa, 29- to 35-amino acid cationic peptides are cysteine rich and have a β-sheet structural conformation (9). HNP types 1–4 are abundant in human neutrophils, constituting 5% of all neutrophil proteins and 30–50% of the total protein content of the azurophilic granules (20). Although they lack enzymatic activity, they possess a wide range of antimicrobial and cytotoxic capabilities. Whereas antimicrobial activity primarily takes place in neutrophil vacuoles after phagocytosis, activated human neutrophils also release up to 10% of their defensin content extracellularly (8).

α1-AT is a serine protease inhibitor with potent anti-inflammatory properties. Its major function is thought to be inhibition of the deleterious effects of neutrophil proteases, particularly those associated with neutrophil elastase. α1-AT deficiency was chosen as a model to investigate organ-specific and unopposed effects of HNP because it has also been shown that, in vitro, α1-AT binds to HNP molecules and neutralizes their cytotoxic effects on bronchial epithelial cells (17, 22). A relative imbalance of α1-AT and proteases (in favor of an increased burden of proteases) has been demonstrated in other neutrophil-mediated lung diseases such as cystic fibrosis (4) and emphysema not related to congenital α1-AT deficiency (7, 23), suggesting that α1-AT plays a critical role in modulating the effects of HNP and neutrophil products in a number of inflammatory lung diseases. In this context, the presence of adequate local concentrations of α1-AT may be critical for the maintenance of lower respiratory tract homeostasis, particularly in the presence of neutrophils releasing toxic factors.

In this study, we evaluated the cytotoxic and inflammatory activities of HNP in α1-AT-deficient individuals with mild pulmonary function impairment. We demonstrate that lower respiratory tract HNP levels are significantly increased in α1-AT-deficient patients compared with healthy subjects and that HNP are cytotoxic to alveolar macrophages at concentrations similar to those measured in the airways of a small number of α1-AT-deficient individuals. HNP also stimulate...
alveolar macrophages to release the potent neutrophil chemoattractants leukotriene B$_4$ (LTB$_4$) and interleukin-8 (IL-8). Our studies suggest that HNP-mediated chemoattractant production contributes to the chronic neutrophil burden in the lower respiratory tract that is characteristic of α$_1$-AT-deficient individuals with lung function impairment. HNP appear to be important neutrophil-derived factors with the capacity to injure lung tissue and amplify the inflammatory response in the absence of adequate levels of α$_1$-AT. These findings are likely relevant to systemic neutrophil-mediated inflammatory processes as well as localized inflammation in other organ systems.

**METHODS**

**Subjects.** A total of 33 α$_1$-AT-deficient individuals (16 men and 17 women, 46 ± 2 yr of age) were studied at the University of Florida and the National Institutes of Health Clinical Center as part of institutional review board-approved protocols. Informed consent was obtained from all participants. Homozygous Z-type α$_1$-AT deficiency occurred in 32 individuals, and the remaining individual carried the at-risk phenotype Pi ZM$_{ba}$fl.$\lambda$. The patients had mild lung disease, with an average forced expiratory volume in 1 s (FEV$_1$) of 87 ± 4%, forced vital capacity of 100 ± 4%, and diffusion capacity of 97 ± 5% of predicted values. Thirteen individuals (39%) met criteria for obstructive lung disease by FEV$_1$ criteria (<80% of predicted). None were smokers at the time of study. Twelve individuals were ex-smokers; time since cessation was 0.8–38 yr. Despite their normal lung function indexes, the majority of subjects had emphysematous changes on chest CT. In the few patients receiving it, intravenous α$_1$-AT augmentation therapy was discontinued ≥3 wk before participation in the study. The mean baseline serum α$_1$-AT level of 6.2 ± 2.2 μM confirmed the clearance of previously administered exogenous protein. Six subjects were using inhaled steroids at the time of study entry. All subjects were at their baseline state of health at the time of bronchoscopy, without clinical signs of pulmonary infection, exacerbation of chronic obstructive lung disease, or uncontrolled asthma. As a control population, we studied 21 healthy nonsmoking individuals without α$_1$-AT deficiency (14 men and 7 women, 37 ± 3 yr of age). These individuals had a mean serum α$_1$-AT level of 28.1 ± 1.1 μM and normal lung function (102 ± 4% of predicted FEV$_1$), α$_1$-AT protease inhibitor types were determined by isoelectric focusing of the serum, and serum α$_1$-AT levels were determined by nephelometry (Behring Diagnostics, Marburg, Germany) using a purified α$_1$-AT standard (5).

Subjects underwent pulmonary function testing within 2 days before bronchoscopy. Spirometry was performed and evaluated according to American Thoracic Society standards (1).

**Bronchoalveolar lavage (BAL) was performed on all study subjects. Briefly, after local anesthesia with lidocaine, a fiber-optic bronchoscope was wedged in the right middle lobe or lingula using direct visualization, and five 20-ml aliquots of sterile saline solution were injected and immediately recovered by gentle suction. The BAL fluid was filtered through surgical gauze to remove mucus plugs and cellular debris. After filtration, an aliquot of fluid from each lobe was removed for cell counts and cytospin preparation. Cell counts were performed using a hemocytometer, cytospots were prepared using a Cytospin II (Shandon Instruments, Cheshire, UK), and differential cell counts were performed by light microscopy after treatment with Diff-Quik stain solutions (Allegiance, McGraw Park, IL). The remaining BAL fluid was centrifuged at 300 g for 15 min at 4°C. The supernatant was removed and stored at −70°C until protein quantification assays were performed. Cell pellets were resuspended in serum-free RPMI 1640 culture medium with penicillin (250 U/ml) and streptomycin (250 U/ml, RPMI + P/S; Biofluid, Rockville, MD). Cell viability was calculated before and immediately after each experiment by trypan blue exclusion. Because recovered BAL fluid consists of the instilled saline in addition to the cellular and soluble components of the lower respiratory tract epithelial lining fluid (ELF), quantitative assessment of HNP and other soluble markers of inflammation, as well as inter subject comparison, is complicated by variable dilution of the ELF. As a molecule that is freely diffusible between the ELF and plasma, urea is used as an intra-alveolar marker to determine the degree of ELF dilution. If it is assumed that the plasma urea concentration is approximately equal to the ELF concentration (19), measurement of urea in plasma in BAL fluid allowed calculation of the ELF volume obtained in each BAL, and all BAL measurements are expressed as ELF concentrations.

**Determination of BAL HNP concentration.** HNP concentration in BAL fluid was determined using a slight modification of a previously described enzyme-linked immunoassay (18). Immulon-2 plates (Dynatech, Chantilly, VA) were coated overnight at room temperature with mouse anti-HNP monoclonal antibody (Bachem, Torrance, CA) suspended in 0.1 M carbonate buffer, pH 9.6. The plates were washed four times in PBS-Tween and blocked with 1% gelatin in 20 mM Tris-HCl and 500 mM NaCl, pH 7.5 (TBS), for 1 h at room temperature. A standard curve was prepared using synthesized HNP (Bachem) diluted in TBS at 0.01% hexadecyltrimethylammonium bromide (CETAB; Fluka Chemicals, St. Louis, MO). The plates were applied directly to the Immulon plates, and end-point 1:2 dilutions were performed using TBS + CETAB diluent. After 2 h of incubation, the plates were washed and reconfigured as described above. Polyclonal rabbit anti-HNP antiserum (Domcoft, Lovettsville, VA) diluted 1:500 in TBS and CETAB was added to the wells and incubated for 1.5 h. After they were washed, the plates were incubated for 1 h in goat anti-rabbit IgG horseradish peroxidase-conjugated antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN) diluted 1:1,000 in TBS + 1% gelatin. The plates were developed by addition of 100 μl of O-phenylenediamine (Sigma, St. Louis, MO), 0.2 mg/ml in 20 mM citrate buffer, pH 4.7, containing 30% H$_2$O$_2$ (0.25 μg/ml). The reaction was stopped by the addition of 2.5 N H$_2$SO$_4$, and the plates were read at 492 nm using a SPECTRAmax plate reader (Molecular Devices, Sunnyvale, CA). Sample concentrations were calculated using best-of-fit curve of the standards, and values were corrected for BAL fluid dilution to give ELF concentration of HNP. This ELISA has an interassay reproducibility of >95% and was sensitive to 0.78 nM HNP.

Issues specific to the quantification of HNP in biological specimens include cationicity and the tendency of the peptides to form dimers. The high cationicity of HNP can affect the sensitivity of ELISA by nonspecific binding to plastics, for example, and is partially mitigated by the use of the detergent CETAB. The tendency of HNP to form dimers can also alter the measured levels in BAL fluid, but the use of a polyclonal and a monoclonal antibody in the sandwich immunoassay likely offers sufficiently broad specificity for accurate quantification. To address these issues, additional methods were utilized as confirmation of BAL ELISA analysis.

**Identification of HNP by HPLC and Western blot analysis.** The presence of HNP in BAL fluid, previously determined by ELISA, was confirmed by reverse-phase HPLC and Western blotting. BAL fluid obtained from an α$_1$-AT-deficient individual and from a healthy control subject was concentrated by evaporation under nitrogen. Reverse-phase HPLC analysis was performed using a Hewlett Packard 1100 chromatograph (Waldbrom, Germany). BAL concentrate was equilibrated with 0.1% trifluoroacetic acid (TFA)-acidified water and injected into an HPLC column (Zorbax SBC-8). HNP were separated from other BAL proteins by gradient elution in 100% buffer (0.1% TFA-H$_2$O) to 50% buffer (0.85% TFA-acetonitrile) over 45 min. The sample was monitored at 280 nm (24). The proteins were recovered in tricine-SDS sample buffer, and electrophoresed through a 4–20% tricine-SDS gel. Proteins were transferred to a nitrocellulose membrane (Novex, San Diego, CA) at 22 V for 2 h in
transfer buffer (12 mM Tris-base and 96 mM glycine in 20% methanol, pH 8.3).

After transfer, the nitrocellulose sheet was washed in phosphate-buffered saline (PBS), pH 7.4 (GIBCO BRL, Grand Island, NY), containing 0.05% polyoxyethyleneboron monolaurate-Tween 20 (Sigma) and blocked overnight in 1% blocking buffer (Tropix, Bedford, MA) in PBS-0.3% Tween. The membrane was incubated for 2 h with rabbit polyclonal anti-HNP antibody (Bachem) diluted 1:1,000 in blocking buffer. After it was washed in PBS-0.3% Tween, the membrane was incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Boehringer Mannheim) diluted 1:1,000 in blocking buffer. Binding of the secondary antibody was detected using a chemiluminescence detection system (Amersham, Arlington Heights, IL).

Quantitation of antiprotease and inflammatory markers in BAL fluid and cell culture supernatants. Quantitative measurements of α1-AT and neutrophil elastase were performed using indirect sandwich immunoassay. IL-8 levels were measured by commercial quantitative sandwich ELISA (R & D Systems, Minneapolis, MN) according to the manufacturer’s instructions using a SPECTRAMax plate reader and SoftmaxPRO software (Molecular Devices).

Before determination of LTB4 concentrations, potentially cross-reacting material was removed by extraction of eicosanoids with C18 reverse-phase HPLC, Western blot analysis, and immunoassay. Before determination of LTB4 concentrations, potentially cross-reacting material was removed by extraction of eicosanoids with C18 reverse-phase HPLC, Western blot analysis, and immunoassay. Before determination of LTB4 concentrations, potentially cross-reacting material was removed by extraction of eicosanoids with C18 reverse-phase HPLC, Western blot analysis, and immunoassay. Before determination of LTB4 concentrations, potentially cross-reacting material was removed by extraction of eicosanoids with C18 reverse-phase HPLC, Western blot analysis, and immunoassay. Before determination of LTB4 concentrations, potentially cross-reacting material was removed by extraction of eicosanoids with C18 reverse-phase HPLC, Western blot analysis, and immunoassay. Before determination of LTB4 concentrations, potentially cross-reacting material was removed by extraction of eicosanoids with C18 reverse-phase HPLC, Western blot analysis, and immunoassay.

Cell culture. Alveolar macrophages obtained from normal subjects and α1-AT-deficient individuals were utilized to evaluate LTB4 and IL-8 responses after exposure to HNP at concentrations approximating those measured in the lower respiratory tract. To isolate alveolar macrophages, BAL fluid cell pellets were resuspended in RPMI + P/S at 1.0×10^6 viable macrophages/ml and plated in duplicate in tissue culture plates (Costar, Cambridge, MA). Cells were incubated for 2 h at 37°C in the presence of 5% CO2 to allow macrophage adherence and then washed three times with RPMI + P/S. The resulting alveolar macrophage population was >95% viable by trypan blue exclusion. Cells were cultured at 37°C in 5% CO2 in the presence of increasing concentrations of HNP ([25, 500, 1,625, and 2,500 nM]). To study possible interactions between HNP and neutrophil elastase on LTB4 and IL-8 release from alveolar macrophages, we cultured the cells in the presence of HNP and neutrophil elastase (Alters Research and Technology, Athens, GA). To study possible modulation of alveolar macrophage chemotactant production by α1-AT, we cultured cells in the presence of the average concentration of α1-AT (Athens Research and Technology) detected in the ELF of normal individuals (2,200 nM). Aliquots were removed at 5 h and centrifuged at 3,000 g for 5 min, and the supernatants were frozen at –20°C for 3 h and then stored at –70°C until assayed for LTB4 or IL-8 production. As controls we used alveolar macrophages incubated in medium alone or medium plus neutrophil elastase or α1-AT incubated in the absence of alveolar macrophages for the same period and in the same conditions and processed identically.

Cell viability evaluated after each experiment did not differ between experimental conditions. Because of the high number of cells required for this experiment, these analyses were performed in a limited number of subjects (6 healthy controls and 7 α1-AT-deficient individuals).

Cytotoxicity assay. HNP-mediated human alveolar macrophage cytotoxicity was studied with a chromium release assay. Alveolar macrophages obtained from α1-AT-deficient individuals (n = 5) and control subjects (n = 4) were resuspended at 2×10^6 cells/ml in RPMI + P/S containing 0.5 mCi of 51Cr (NEN Dupont, Boston, MA) for 1 h at 37°C in 5% CO2. The unincorporated 51Cr was removed from the cells by three washes in RPMI. Cells (2×10^6/well) were then incubated at 37°C in 5% CO2 with serial dilutions of HNP (3,500–28,000 nM). Cells in medium alone and cells lysed with Triton X-100 (Research Products International, Delta Lane, IL) were used to calculate spontaneous and maximum 51Cr release, respectively. After an 18-h incubation, 51Cr in the supernatant was measured using a gamma counter (TiterTek, Huntsville, AL), and the percent lysis was calculated by the following equation: (cpm_exp – cpm_spont) / (cpm_max – cpm_spont) × 100, where cpm_exp, cpm_spont, and cpm_max represent experimental, spontaneous, and maximal counts per minute, respectively. All experiments were repeated in triplicate.

Statistical evaluation. Values are means ± SE. Control and subject ELF values were evaluated using unpaired t-test or a Mann-Whitney rank test if data were found to deviate from a Gaussian distribution by the Kolmogorov-Smirnov test. Comparisons of alveolar macrophage LTB4 and IL-8 production from normal and α1-AT-deficient individuals were determined using a Mann-Whitney rank test. Wilcoxon’s rank test was used for intragroup comparisons. Correlation coefficients were determined using Spearman’s rank test. All tests were performed using the statistics programs Prism 3.0 (Graphpad Software) or StatView 5.0 (SAS Institute).

RESULTS

HNP levels in the lower respiratory tract. HNP were demonstrated in the BAL fluid of α1-AT-deficient individuals by reverse-phase HPLC, Western blot analysis, and immunoassay. Although no peaks of protein were noted over 15–20 min of elution of concentrated BAL fluid in a normal individual, an α1-AT-deficient individual with increased ELF neutrophils demonstrated a peak at 18–20 min of elution, which corresponds to a similar peak when normal BAL fluid was spiked with synthesized HNP types 1 and 2 (Fig. 1, A–C). The amplitude of the peak detected in the α1-AT-deficient individual’s BAL fluid was specifically increased by the addition of synthesized HNP types 1 and 2 (Fig. 1D). Western blot analysis of the 15–20 min elution time-pooled fractions from the α1-AT-deficient individual (Fig. 1, inset, lane 2) demonstrated an immunoreactive 4-kDa protein similar to synthesized HNP types 1 and 2 in lane 1 and partially purified neutrophil extract in lane 4. This observation contrasts with the lack of an immunoreactive 4-kDa band in lane 3, representing a similar pooled fraction from a normal BAL fluid sample (Fig. 1, inset). The mean ELF concentration of HNP as determined by ELISA was 68-fold greater in α1-AT-deficient individuals than in healthy control subjects (1,976 ± 692 vs. 29 ± 12 nM, P < 0.0001; Fig. 2).

The mean percentage of neutrophils in the BAL fluid was significantly higher in α1-AT-deficient individuals than in healthy controls (10.1 ± 2.7 vs. 10.0 ± 0.1, P = 0.0099). Consistent with this higher burden of neutrophils in the lower airways, neutrophil elastase levels were also higher in α1-AT-deficient individuals (192.5 ± 71.70 vs. 129.0 ± 5,500 nM, P = 0.05). Lower respiratory tract HNP levels significantly correlated with the degree of neutrophil burden as expressed by the percentage of BAL neutrophils and the ELF neutrophil concentration, as well as neutrophil elastase levels (Table 1).
HNP-mediated cytotoxicity. A 51 Cr release assay was used to determine the cytotoxic effect of HNP on alveolar macrophages from healthy control (n = 4) and α1-AT-deficient individuals (n = 5). This demonstrated cellular cytotoxicity proportional to the amount of HNP in the medium over a wide range of concentrations (Fig. 3). There were no significant differences in the cytotoxic effect of HNP on alveolar macrophages from normal or α1-AT-deficient individuals. Extrapolation of the in vitro cytotoxicity assay findings to in vivo circumstances revealed that only 6 of the 33 α1-AT-deficient individuals and none of the healthy control subjects had HNP ELF concentrations that induced cytotoxicity in alveolar macrophages under the assay conditions. In this context, exposure of alveolar macrophages to the average ELF concentration of HNP among α1-AT-deficient individuals (1,976 ± 692 nM) would result in cytotoxicity in <5% of the cells.

Dose-response effect of HNP on alveolar macrophage chemotactrant production. Alveolar macrophages harvested from healthy subjects and α1-AT-deficient individuals incubated with increasing concentrations of HNP demonstrated a proportional increase in LTB4 production (Fig. 4). At 24 h,

Table 1. Relation of ELF HNP levels to markers of lower respiratory tract neutrophil burden

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<tr>
<th>HNP*</th>
<th>%PMN†</th>
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Correlation coefficients were determined by Spearman’s rank test. HNP, human neutrophil peptides; PMN, polymorphonuclear leukocyte. *Epithelial lining fluid (ELF) concentration. †Percentage of total bronchoalveolar lavage cells. ‡Cells per milliliter of ELF.

**HNP-mediated cytotoxicity.** A 51Cr release assay was used to determine the cytotoxic effect of HNP on alveolar macrophages from healthy control (n = 4) and α1-AT-deficient individuals (n = 5). This demonstrated cellular cytotoxicity proportional to the amount of HNP in the medium over a wide range of concentrations (Fig. 3). There were no significant differences in the cytotoxic effect of HNP on alveolar macrophages from normal or α1-AT-deficient individuals. Extrapolation of the in vitro cytotoxicity assay findings to in vivo circumstances revealed that only 6 of the 33 α1-AT-deficient individuals and none of the healthy control subjects had HNP ELF concentrations that induced cytotoxicity in alveolar macrophages under the assay conditions. In this context, exposure of alveolar macrophages to the average ELF concentration of HNP among α1-AT-deficient individuals (1,976 ± 692 nM) would result in cytotoxicity in <5% of the cells.

**Dose-response effect of HNP on alveolar macrophage chemotactrant production.** Alveolar macrophages harvested from healthy subjects and α1-AT-deficient individuals incubated with increasing concentrations of HNP demonstrated a proportional increase in LTB4 production (Fig. 4). At 24 h,
there was a significant increase in LTB₄ production by alveolar macrophages from control subjects and α₁-AT-deficient individuals incubated with 2,500 nM HNP (a concentration of HNP at the level of detectable HNP-induced alveolar macrophage cytotoxicity). Although alveolar macrophages from α₁-AT-deficient individuals exposed to 2,500 nM HNP tended to produce more LTB₄ at 1 and at 24 h than control subjects, this difference was not significant for the separate groups.

Production of IL-8 increased in alveolar macrophages from α₁-AT-deficient individuals 24 h after incubation with HNP (Fig. 5). Production of IL-8 at baseline and after incubation with increasing doses of HNP was consistently two to four times greater in alveolar macrophages from normal subjects than in those from deficient individuals (data not shown).

Effect of neutrophil elastase and α₁-AT on HNP-induced chemoattractant production. To evaluate the effect of HNP on alveolar macrophage production of LTB₄ in the typical in vivo milieu of α₁-AT-deficient individuals, alveolar macrophages isolated from healthy control and α₁-AT-deficient individuals were studied using neutrophil elastase and HNP concentrations approximating the observed levels in the ELF of α₁-AT-deficient individuals (220 and 1,625 nM, respectively). The modulating effect of α₁-AT was evaluated using concentrations approximating the mean ELF level observed in healthy control subjects (2,200 nM).
Consistent with the dose-response experiments, HNP alone significantly increased LTB₄ and IL-8 production over baseline in alveolar macrophages isolated from α₁-AT-deficient individuals after 24 h of incubation. Neutrophil elastase alone did not significantly increase LTB₄ or IL-8 production in alveolar macrophages isolated from α₁-AT-deficient individuals but significantly increased LTB₄ in normal alveolar macrophages (data not shown). The addition of HNP plus neutrophil elastase significantly increased LTB₄ production over baseline in α₁-AT-deficient (Fig. 5) and normal alveolar macrophages. The synergistic effect of HNP and neutrophil elastase was significantly increased over that seen with HNP alone and over the effects of neutrophil elastase alone in normal alveolar macrophages. The neutrophil elastase-HNP enhancement of LTB₄ production was fourfold greater in alveolar macrophages isolated from α₁-AT-deficient individuals than in normal alveolar macrophages. Because of high variability in cellular response within each group, the difference did not reach statistical significance. In contrast to the synergistic effect with regard to cellular LTB₄ production, less production of IL-8 resulted from the combination of neutrophil elastase and HNP than from either treatment alone (Fig. 5).

Importantly, the addition of α₁-AT completely prevented HNP from increasing LTB₄ production by alveolar macrophages isolated from α₁-AT-deficient individuals (Fig. 5) and from control subjects (data not shown). This was also true for the combined effect of neutrophil elastase and HNP with regard to LTB₄ production. α₁-AT also blocked HNP-induced IL-8 production by normal and α₁-AT-deficient alveolar macrophages.

**DISCUSSION**

Antimicrobial peptides are a key component of the human innate host defense system. Those produced by neutrophils play an important role in microbial killing in the intracellular environment but also possess properties that are potentially harmful to host tissue when released extracellularly. In this study, we evaluated the role of HNP in chronic inflammatory lung disease associated with α₁-AT deficiency. We observed that this neutrophil product is significantly elevated in the lower respiratory tract of these patients and that its levels correlate with other markers of neutrophil-mediated inflammation. At concentrations approximating those measured in the lower respiratory tract of a small subset of α₁-AT-deficient individuals, HNP were found to be cytotoxic to alveolar macrophages in vitro. Perhaps more importantly, HNP stimulated the production of potent neutrophil chemoattractants by alveolar macrophages. These data provide novel evidence that HNP play a significant role in lung disease associated with α₁-AT deficiency, a finding with potential relevance to other neutrophil-mediated lung disease as well as inflammatory conditions in other organ systems characterized by neutrophil activation.

HNP are abundant in neutrophils and lack enzymatic activity. Their cationic and amphiphilic characteristics facilitate interactions with microbial surface structures, and their mechanism of action involves the formation of membrane pores that alter microbial membrane permeability and internal homeostasis (14). Although the majority of this activity takes place in neutrophil vacuoles after phagocytosis, activated and deteriorating neutrophils represent a source of extracellular HNP. Because HNP production is constitutive and regulated only by the intrinsic maturation program of the cell (10), the accumulation of large numbers of activated neutrophils at the surface of the airway or other tissues can result in high local concentrations of HNP. Along with the significant elevation of HNP in the lower respiratory tract of individuals with α₁-AT deficiency, we noted a strong correlation of HNP levels with the burden of neutrophils and inflammatory markers, demonstrating that HNP measurement may prove to be a useful biomarker of disease progression and severity.

The cytotoxic potential of HNP was first noted in tumor cell targets (13). Subsequent in vitro assays demonstrated that neutrophil granule extracts are cytotoxic to pulmonary epithelial cells and pulmonary fibroblasts in a dose-dependent manner and that the cytotoxicity of the granule extracts is specifically due to their HNP content (16). Our study demonstrates that the cytotoxic potential of HNP extends to alveolar macrophages. However, in this group of 33 individuals with mild lung dysfunction, only 6 had HNP levels that induced alveolar macrophage cytotoxicity under the assay conditions. Because of the in vitro dose-dependent response of alveolar macrophages, the cytotoxic effects of HNP may play a greater role with regard to lung damage and host cell defense in patients with a higher burden of extracellular HNP in the lower airways. This requires further study, because the in vivo cytotoxic effects of HNP may be partially mitigated by α₁-AT and by the binding of highly cationic HNP to anionic molecules such as mucopolysaccharides and nucleic acids.

Although HNP levels measured in a minority of patients approached concentrations noted to cause alveolar macrophage cytotoxicity, at lower concentrations the primary effect of unbound HNP appears to be perpetuation of the inflammatory response. HNP may play a significant role in the maintenance of chronic inflammation, as evidenced by a dose-dependent relation between HNP and alveolar macrophage production of the potent chemoattractants LTB₄ and IL-8. Importantly, HNP-stimulated IL-8 and LTB₄ release by alveolar macrophages was completely abrogated by the addition of α₁-AT to the culture supernatant. The presence of adequate local concentrations of α₁-AT may therefore be critical in controlling the harmful effects of extracellular HNP in an environment of activated neutrophils.

In addition to the inflammatory effects of HNP alone, we noted a significant synergistic effect between HNP and neutrophil elastase in the stimulation of alveolar macrophage LTB₄ production, an effect also reversed by the addition of α₁-AT. In contrast, the combination of HNP and neutrophil elastase resulted in lower levels of IL-8 production by alveolar macrophages than by control cells or HNP and neutrophil elastase alone, although the difference was not statistically significant. This finding is consistent with the finding of a previous study that reported inhibitory interactions between HNP and neutrophil elastase with respect to pulmonary epithelial cell production of IL-8 (22). These contrasting observations between stimulation and inhibition of alveolar macrophage LTB₄ and IL-8 production, respectively, warrant further study, as do the in vivo implications of our findings as they relate to perpetuation of the inflammatory cycle in this disease.

Consistent with the findings of other investigators (11), we found that, in normal alveolar macrophages, neutrophil elastase...
alone significantly increased LT \(_B\) secretion. In contrast, we found that neutrophil elastase alone did not increase LT \(_B\) production in alveolar macrophages isolated from \(\alpha_1\)-AT-deficient individuals. Neutrophil elastase is bound and internalized by alveolar macrophages via nonspecific low-affinity receptors (6, 15). Although this was not the focus of the present investigation, our finding warrants further focus on differences that may exist between normal and \(\alpha_1\)-AT-deficient alveolar macrophages with regard to surface receptor expression or subsequent activation of arachidonic acid pathways after neutrophil elastase endocytosis.

In summary, we have demonstrated that HNP are significantly elevated in the lower respiratory tract of \(\alpha_1\)-AT-deficient individuals. At concentrations approximating those measured in this group of patients with mild lung disease, HNP exhibited cytotoxic but primarily inflammatory properties when incubated with alveolar macrophages, effects that were abrogated by the addition of \(\alpha_1\)-AT. Neutrophils are normally in low abundance and are not activated in the lower respiratory tract. HNP appear to contribute to inflammation and tissue destruction in the lower respiratory tract of \(\alpha_1\)-AT-deficient individuals by promotion of neutrophil accumulation as well as by direct injury of lung cells. It is anticipated that the in vitro findings regarding HNP effects are not specific to \(\alpha_1\)-AT deficiency. In fact, the findings may be relevant to a number of lung diseases characterized by chronic neutrophil-mediated inflammation in which elevated levels of HNP have been reported, such as cystic fibrosis (21), pulmonary tuberculosis (2), and diffuse panbronchiolitis (3). HNP are likely key components in the promotion and maintenance of chronic lung inflammation and may be useful markers of neutrophil burden and activation.

**ACKNOWLEDGMENTS**

The authors thank Dr. Tomas Ganz for advice regarding HNP analysis and Dr. Joyce M. Koenig for critical reading of the manuscript.

**GRANTS**

This work was supported by National Heart, Lung, and Blood Institute Grant 1K23 HL-04213 (L. T. Spencer) and Grants 1Z01 HL-002538 and 1K24 HL-04456001AA2-N (M. L. Brantly), General Clinical Research Center Grant RR-00082, and the Alpha-1 Foundation.

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