Elevated Pleural Fluid Levels of Defensins in Patients With Empyema*

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**Background:** Defensins, also known as human neutrophil peptides, are antimicrobial peptides present in the azurophil granules of neutrophils. We measured their level in pleural effusion in various pulmonary diseases to investigate whether they could be used as a diagnostic marker in the differential diagnosis of specific pleural diseases.

**Patients and participants:** We analyzed pleural effusion samples collected from 61 patients, including 50 exudates (11 with empyema, 3 parapneumonic, 15 tuberculous, 18 neoplastic, 3 miscellaneous) and 11 transudates as controls.

**Measurements:** Defensins were measured by radioimmunoassay and also analyzed by reverse-phase high-performance liquid chromatography. The concentrations of interleukin (IL)-8 and granulocyte colony-stimulating factor (G-CSF) in pleural effusion fluid were measured by enzyme-linked immunosorbent assay to examine the correlation between these cytokines and defensins.

**Results:** The concentration of defensins in all samples of empyema was >5,100 ng/mL and the mean concentration (13,265.8±1,895.2 ng/mL) in these samples was the highest among other groups. The concentration in the other 50 pleural effusion samples tested was <2,800 ng/mL. Defensins were mostly of the mature type in empyema. Pleural effusion levels of IL-8 and G-CSF in patients with empyema were also significantly higher than those in other samples. There was a significant correlation between defensins and IL-8 or G-CSF in pleural effusion fluid (r=0.762, and 0.827, respectively).

**Conclusions:** Our results suggest that the high effusion concentrations of defensins in pleural effusion may constitute an important component of the host defense system or may have a cytotoxic role in empyema. Our results also indicate that the high levels of IL-8 and G-CSF in empyema may indirectly explain the elevated levels of defensins by increasing the number of neutrophils in the pleural space. *(CHEST 1998; 113:788-94)*

**Key words:** defensins; granulocyte colony-stimulating factor; interleukin 8; pleural effusion; pleurisy

**Abbreviations:** G-CSF=granulocyte colony-stimulating factor; IL=interleukin; LDH=lactate dehydrogenase; PMA=phorbol myristate acetate; RIA=radioimmunoassay; RP-HPLC=reverse-phase high-performance liquid chromatography

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Defensins, or human neutrophil peptides, are cationic proteins with antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and certain enveloped viruses. Defensins are arginine- and cysteine-rich antimicrobial peptides, 29 to 34 amino acids long with a molecular weight of approximately 3.5 kd. These peptides have been purified from rabbits, guinea pigs, and human neutrophils. They are the major constituents of the azurophil granules of neutrophils, and represent 5 to 7% of the total cellular protein in human neutrophils. Defensins include a 94-amino acid precursor that produces 75-amino acid prodefensin by cleavage of a signal peptide. Most prodefensin is processed to 56 amino acid intermediates in the bone marrow by preaspartyl proteolytic cleavage, then to mature defensins in peripheral blood neutrophils. Plasma and blood levels of defensins increase during infections. In the presence of defensins at a concentration ranging from 25 to 100 μg/mL, the number of viable organisms was reduced by ≥99% within 30 min.
fensins are present only in cells of neutrophil lineage,10 whereas they may be also involved in the pathogenesis of neutrophil-mediated tissue injury.11

We postulated that the level of defensins is elevated in pleural effusion of patients with pleurisy since a large number of neutrophils are present in the pleural space in pleurisy. To our knowledge, identification and quantification of defensins in pleural effusions have not been investigated previously. We measured pleural effusion levels of defensins in various disorders using radioimmunoassay (RIA) and assessed the diagnostic value of these peptides. We also measured pleural effusion levels of neutrophil-related cytokines, such as interleukin (IL)-8 and granulocyte colony-stimulating factor (G-CSF) and determined the correlation between these cytokines and defensins.

**MATERIALS AND METHODS**

**Patients and Diagnostic Categories**

We studied 61 consecutive patients with pleural effusions who were admitted to our hospital between September 1991 and July 1996. They consisted of 16 women and 45 men, aged 64.0±5.3 years. Thoracentesis was performed in each patient and pleural fluid samples obtained were centrifuged at 500× for 10 min and supernatants were stored at −20°C until analysis. Pleural effusions were categorized as transudates (ratio of pleural fluid to serum total protein concentration <0.5 and ratio of those of lactate dehydrogenase [LDH] <0.6) or exudates (protein ratio >0.5 or LDH ratio >0.6). Exudates were further categorized as either empyemic, parapneumonic, tuberculous, neoplastic, or miscellaneous. Empyema (n=11) was defined as a neutrophilic effusion associated with (1) growth of bacteria on microbiological culture of pleural fluid (n=4), (2) organisms seen on Gram staining of pleural fluid (n=3), or (3) pleural fluid glucose concentration <40 mg/dL in patients with pneumonia (n=4). Parapneumonic effusions (n=5) represented those with glucose concentration >40 mg/dL and no organisms found on Gram staining or no positive pleural fluid culture in patients with pneumonia. Tuberculous effusions (n=15) were defined as those with (1) growth of *Mycobacterium tuberculosis* in cultures from pleural fluid or biopsy specimen (n=3), (2) growth of *M tuberculosis* from sputum (n=2), or (3) recent conversion of tuberculosis skin reactivity, granulomas on pleural biopsy specimen, or response to antituberculosis therapy (n=10). Neoplastic effusions (n=18) were exudates associated with a diagnosis of cancer based on (1) cytoclogic examination of pleural fluid (n=16) or (2) the lung tissue (n=2). Miscellaneous effusions (n=3) included the exudates in which neither infection nor malignancy was found, and an alternative diagnosis was made: pneumothorax (n=2) and POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes) (n=1). Transudates (n=11) were associated with congestive heart failure (n=9), nephrotic syndrome (n=1), and liver cirrhosis (n=1). No bacterial, mycobacterial, or fungal growth was noted in the cultures of malignant, miscellaneous, and transudative pleural samples. There were no differences among the diagnostic groups based on age or sex. The characteristics of pleural effusion samples according to each diagnostic group are summarized in Table 1. The experimental protocol was approved by the Human Ethics Review Committee and a signed consent was obtained from each individual participating in the study.

**Defensins Assay**

The concentration of defensins was measured by RIA established in our laboratory.9 We synthesized full-length defensin using a peptide synthesizer (Model 430; Applied Biosystem; Foster City, Calif), which was then purified by reverse-phase high-performance liquid chromatography (RP-HPLC). In RP-HPLC, synthetic defensin was eluted at a position identical to that of native defensin isolated from human leukocytes. Synthetic defensin (5 mg) was used to immunize New Zealand white rabbits by multiple intracutaneous and subcutaneous injections. Defensin was radioiodinated and the 125I-labeled peptide was purified by RP-HPLC on a column (TSK ODS 120A; Tosoh Co Ltd; Tokyo, Japan). The diluted sample or standard peptide solution (100 μL) was incubated with 100 μL of antiserum diluent (final dilution of 1/21,000) for 24 h. The 125I-labeled defensins solution (16,000 counts/min in 100 μL) was added, and the mixture was incubated again for 24 h. In the next step, normal rabbit serum and antirabbit IgG gout serum were added and stored for 16 h. Bound and free ligands were separated by centrifugation. All procedures were performed at 4°C and the samples were assayed in duplicate. In this assay, the minimum detectable level was 22 pg and half-minimum inhibition by the peptide was observed at 130 pg. We used 0.5 μL of pleural fluid to determine the concentrations of defensins. The intra-assay and interassay coefficients of variation were 3.5% and 5% at 50% binding, respectively. The RIA system specifically detected mature defensins and prodefensins, which was confirmed by RP-HPLC coupled with the RIA.8

**In Vitro Release of Defensins From Neutrophils**

Heparinized venous blood samples were collected from healthy subjects. Erythrocytes were separated with dextran, then

**Table 1—Sixty-One Pleural Effusion Samples**

<table>
<thead>
<tr>
<th>Effusion Type</th>
<th>Leukocyte Count, ×10⁶/L</th>
<th>PMN Count, ×10⁶/L</th>
<th>Protein, g/dL</th>
<th>LDH, IU/L</th>
<th>Glucose, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transudates (n=11)</td>
<td>180 (10-400)</td>
<td>29 (0-106)</td>
<td>2.10 (1.3-3.9)</td>
<td>218 (94-414)</td>
<td>144 (104-166)</td>
</tr>
<tr>
<td>Empyema (n=11)</td>
<td>18,100 (6,300-38,000)</td>
<td>16,652 (6,000-34,700)</td>
<td>4.35 (4.4-7)</td>
<td>2,963 (808-8,630)</td>
<td>10 (0-40)</td>
</tr>
<tr>
<td>Parapneumonic (n=3)</td>
<td>3,000 (70-8,000)</td>
<td>1,500 (0-4,000)</td>
<td>4.05 (3.3-4.8)</td>
<td>403 (197-609)</td>
<td>101 (75-124)</td>
</tr>
<tr>
<td>Tuberculous (n=15)</td>
<td>2,300 (600-18,000)</td>
<td>340 (30-16,400)</td>
<td>3.73 (2.5-4.2)</td>
<td>773 (292-1,549)</td>
<td>64 (3-128)</td>
</tr>
<tr>
<td>Malignant (n=18)</td>
<td>70 (40-2,000)</td>
<td>110 (0-1,800)</td>
<td>5.01 (3.4-6.7)</td>
<td>892 (197-3,149)</td>
<td>127 (110-156)</td>
</tr>
<tr>
<td>Miscellaneous (n=3)</td>
<td>60 (10-140)</td>
<td>12 (0-30)</td>
<td>3.29 (2-4.1)</td>
<td>150 (85-345)</td>
<td>122 (80-160)</td>
</tr>
</tbody>
</table>

*All parameters are expressed as median (range). PMN=polymeronuclear leukocytes.*
Figure 1. The plots on a logarithmic scale indicate (top [A]) defensins, (center [B]) IL-8, (bottom [C]) G-CSF concentrations in 61 pleural effusion samples. The horizontal dashed line indicates the lowest level in empyema, and horizontal solid lines represent the mean value for each group.
cells at that stage was ≥95%, as confirmed by the trypan blue exclusion. To stimulate the production of defensins, the cultured neutrophils were incubated with phorbol myristate acetate (PMA), recombinant human endothelial IL-8 (Pepro Tech Inc; London, UK), and recombinant human G-CSF at 37°C for 30 min. After culture, the supernatant fluid was examined for the presence of defensins.

Chromatographic Characterization of Defensins

Samples of transudate and exudate pleural effusions were prepared from two separate patients. RP-HPLC was performed using 5 to 10 μL pleural fluid on a column (TSK ODS SIL 120A; Tosoh Co Ltd.). A linear gradient of acetonitrile (CH3CN) from 10 to 60% in 0.1% trifluoroacetic acid (pH 2.0) was used at a flow rate of 0.5 mL/min for 40 min. All fractions of defensins were measured by RIA.

IL-8 and G-CSF Assay

IL-8 was measured with a double antibody (sandwich) enzyme-linked immunosorbent assay (Tore Fuji Bionis; Tokyo, Japan) using murine monoclonal antibodies. The primary antibody was used for capturing the antigen while the secondary antibody was used for recognition of the antigen, as described previously.12 G-CSF was measured by enzyme-linked immunosorbent assay (Amersham, Tokyo, Japan) using horseradish peroxidase-labeled antihuman G-CSF antibody. Levels below the lower limit of detection (IL-8, 3 pg/mL; G-CSF, 30 pg/mL, respectively) were considered to be equivalent to zero.

Statistical Analysis

Data are expressed as mean±SEM unless stated. Pleural fluid values from different diagnostic groups were compared by analysis of variance with Tukey's test. Correlation between variables was determined by a simple linear regression analysis. A p value <0.05 denoted the presence of a statistically significant difference.

RESULTS

All pleural fluid samples analyzed in the present study contained defensins. Although the concentration of defensins varied from one type of effusion to another, it was significantly higher in empyema (13,265.8±1,895.2 ng/mL) than in other groups. Furthermore, the concentration of defensins was >5,100 ng/mL in all the samples of empyema, which was true for none of the other 50 effusion samples tested (Fig 1, top [A]). The concentration of defensins in each exudative effusion of other groups was similar, as was the level in the transudative group. The concentration of IL-8 in pleural effusions was markedly higher in empyema (27,977.6±10,276.9 pg/mL) compared with that in other groups (Fig 1, center [B]). IL-8 concentrations in empyema were >1,400 ng/mL in each sample, which was also true for only 3 of the other 50 effusion samples. The latter three samples were from tuberculous effusion. G-CSF levels in pleural effusion samples were also the highest in empyema (9,242.5±2,984.2

![Figure 2](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21761/)
pg/mL), and were >770 pg/mL in all these samples, which was true for none of the other 50 effusion samples (Fig 1, bottom [C]).

The release of defensins from neutrophils was studied in vitro by stimulating isolated neutrophils with PMA, IL-8, or G-CSF. Stimulation of neutrophils with PMA caused the release of defensins in a dose-dependent manner (Fig 2, top [A]). Defensins released by stimulation with IL-8 were lower than those with PMA, but a significant increase in defensins release was noted at 500 ng/mL of IL-8 (Fig 2, center [B]). In contrast, G-CSF failed to stimulate neutrophils to release defensins (Fig 2, bottom [C]).

Mature defensins and prodefensins were present in normal human plasma in a 3:1 ratio as detected by RP-HPLC (Fig 3, top left [A]). Prodefensin was not detected in the culture medium of neutrophils stimulated with PMA (Fig 3, top right [B]). Prodefensin accounted for 90% of defensin molecules in transudative pleural fluid (Fig 3, bottom left [C]). In contrast, mature defensin accounted for 95% of defensin molecules in the empyema fluid (Fig 3, bottom right [D]). There was a significant correlation between the concentration of IL-8 and defensins in pleural effusion when all samples were pooled together (p<0.0001; Fig 4, top [A]). A similar correlation was present in empyema alone (r=0.623, p<0.05). There was also a significant correlation between G-CSF concentration and that of defensins in all pleural effusion samples (Fig 4, center [B]) and also empyema alone (r=0.745, p<0.01). The concentration of IL-8 correlated significantly with that of G-CSF when all samples were pooled together (Fig 4, bottom [C]) but such correlation was not present in empyema.

**DISCUSSION**

The major findings of the present study were the presence of high concentrations of defensins in pleural effusion of patients with empyema. Because the localization of defensins is restricted to cells of neutrophil lineage,1,2,13 such high levels must be due to neutrophil accumulation in the pleural space in these patients. Furthermore, the level of defensins in pleural effusion was >5,100 ng/mL in patients with empyema but not in any of the other patients. This finding suggests that these peptides may serve as a diagnostic marker for empyema.

High plasma and blood concentrations of defensins are present during infections.8,14,15 The elevated levels of the peptides in the pleural spaces of patients with empyema may beneficially reflect the host immune response to infection of the pleural linings. Defensins are also cytotoxic to human con-

![Diagram of RP-HPLC profiles](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21761/ on 06/26/2017)
Figure 4. Top (A): correlation between pleural defensins and IL-8 concentrations. Center (B): pleural defensins concentrations in ng/mL vs G-CSF concentrations. Bottom (C): pleural concentration of IL-8 and G-CSF.

In this study, we examined the correlation between pleural defensins and IL-8 concentrations. The correlation coefficient between defensins and IL-8 was 0.762 with a p-value of <0.0001 (Fig. 4A). The correlation between defensins and G-CSF was also observed, with a coefficient of 0.827 and a p-value of <0.0001 (Fig. 4B). The correlation between IL-8 and G-CSF was 0.629 and significant at a p-value of <0.002 (Fig. 4C).

We found that the release of defensins is stimulated by IL-8, and this stimulation results in an increase in the release of defensins. This increase is more pronounced in cases with high levels of IL-8, and the effect is more significant in cases with high levels of G-CSF.

Our results also showed a high correlation between IL-8 and G-CSF in pleural effusion of patients with empyema. This is consistent with previous studies that have reported the presence of high levels of IL-8 and G-CSF in pleural effusion of patients with empyema.

IL-8 is a major neutrophil chemotactic factor in pleural effusion of patients with empyema. It is secreted by pleural mesothelial cells, alveolar macrophages, lymphocytes, and lung fibroblasts. Neutrophil transendothelial migration into the pleural space is enhanced by IL-8. Examination of IL-8-stimulated neutrophils shows that IL-8 may induce not only neutrophil migration to inflammatory lesions but also the release of defensins at the site. Previous studies have also reported the presence of a large number of neutrophils and high concentrations of IL-8 in pleural fluids of some patients with tuberculosis, as was the case in three of our tuberculosis patients. Although these three patients had many neutrophils and high levels of IL-8 equivalent to those of empyema patients (Fig 1, center [B]), their defensin levels were lower than those with empyema.

The present study also showed high concentrations of G-CSF in pleural effusion of patients with empyema compared with those of other conditions. In addition, there was a significant correlation between G-CSF and defensins in these samples. G-CSF is a primary cytokine that promotes the formation of granulocytic colonies from committed precursor cells, stimulates the proliferation of neutrophils, and enhances phagocytosis, killing, and production of reactive oxygen intermediates. Serum levels of G-CSF are known to increase during infections, but the underlying mechanism is not yet known. We have also reported that administration of human recombinant G-CSF increases plasma and blood concentrations of defensins. We also found that the stimulation by administration of G-CSF resulted in gene expression of defensins and increased neutrophil defensins content in peripheral blood (unpublished data). Thus, neutrophil migration and defensins release induced by IL-8, the stimulatory effects of G-CSF on defensins content.
may account altogether for the elevated concentrations of defensins in pleural effusion of patients with empyema.

We also demonstrated in the present study that the major molecular form of defensins in transudative fluid was prodefensin while that in empyema fluid was a mature form of protein. Because mature defensins bind in plasma to macromolecular proteins, they cannot transfer from the blood into transudative fluids. In contrast, prodefensins with molecular weights of approximately 7.5 kd could flow into the pleural space. In empyema, many neutrophils containing mature defensins transmigrate into the pleural space through inflammatory-damaged pleura. Because mature defensins are present only in mature neutrophils, the elevated levels of defensins in empyema probably result from damage of neutrophils, which release the mature protein into the pleural space.

In conclusion, our results showed a marked elevation of defensins concentrations in empyema compared with pleural effusion in other diseases. These high concentrations may play an important role in the host defense or have a cytotoxic function in empyema.

REFERENCES