Human Alveolar Macrophage Proteolytic Enzyme Activities in Chronic Obstructive Pulmonary Disease*

Lack of Correlation with Functional Abnormalities

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The occurrence of emphysema in people deficient in alpha1-antitrypsin and the production of emphysema in experimental animals with elastolytic enzymes suggest proteolysis as a mechanism for the development of emphysema. To investigate the possible role of pulmonary alveolar macrophages in the pathogenesis of emphysema, we measured elastase, acid protease, and elastase-like esterase activities in macrophages from patients with chronic obstructive lung disease and attempted to correlate the level of enzyme activity with the severity of pulmonary function abnormality measured in these patients. Compared to values for cigarette smokers with normal pulmonary function, these macrophage enzyme activities were not increased in patients with chronic obstructive lung disease, and there was no correlation of high elastase activity with more severe degrees of pulmonary function abnormality. These findings lead us to believe that the absolute level of proteolytic enzymes in pulmonary alveolar macrophages is not in itself a determinant of emphysema.

Laurell and Eriksson's4 report of the association of serum alpha1-antitrypsin deficiency and the premature onset of emphysema, aroused much interest in proteolysis as a mechanism in the production of emphysema.2-4 The proteolytic theory has been further supported in the laboratory with the production of emphysema in animal models by instilling various enzyme preparations into the lung.2-4 This experimental emphysema is morphologically similar to the emphysema seen in humans and appears to be best correlated with the elastolytic activity of the various enzymes used.5 With this as a background, the proteolytic theory has been applied to human emphysema with particular emphasis on the emphysema seen in cigarette smokers. The two most likely sources of endogenous proteolytic enzymes in cigarette smokers are the alveolar macrophage and the peripheral blood leukocyte. The alveolar macrophage is a potential source of proteolytic enzymes in the cigarette smoker because it is increased in number and has increased levels of elastase-like esterase and acid protease when compared with alveolar macrophages of nonsmokers and with the peripheral blood leukocyte.7

Because measurements of obstructive airway disease and emphysema are correlated,8,9 a logical extension to the proteolytic hypothesis would be to see if alveolar macrophage enzyme levels are abnormally increased in patients with emphysema and if they correlate with measurements of obstructive airway disease. To investigate this, we have determined the activities of several alveolar macrophage enzymes in cells from a group of cigarette smokers whose pulmonary function tests ranged from normal to severe obstructive airway disease and have compared the level of enzyme activities with severity of pulmonary function abnormalities.

Materials and Methods

We studied patients scheduled for thoracotomy and lung resection at either the University of Florida Shands Teaching Hospital or the Gainesville Veterans Administration Hospital. Before surgery, their evaluation included history, physical examination, routine laboratory studies, chest roentgenogram, bronchoscopy, and routine pulmonary function studies which consisted of forced vital capacity (FVC), forced expiratory volume in one second (FEV1), FEV1/FVC ratio, CO diffusing capacity (Dco)10, and lung volumes.11 Predicted values for FVC, Dco, and lung volumes were derived from previously reported formulas.10,13,14 Patients were not included in the study if extensive tumor or pleural effusion caused restriction of lung volumes.

Alveolar macrophages were obtained for study by lavage of lungs surgically resected for carcinoma. We did not think that in vitro lavage was justified, since the procedure is not without hazard in patients with obstructive airways dis-

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From the outset, the possible influence of carcinoma was taken into consideration in this study. It was not known if the presence of carcinoma would change the cell population in these lungs or possibly affect the enzyme activity of alveolar macrophages: therefore, great care was taken not to lavage the cancer-containing part of the lung, and very careful differential counts were made.

Immediately after removal of an entire lobe or lung, the specimen was lavaged with cooled normal saline solution until approximately 500 ml of fluid was obtained. The lavage fluid from each lobe was centrifuged at 250 g at 4°C for 10 minutes. The supernatant fluid was decanted and the cell sediment subjected to hypotonic shock to lyse the red blood cells present. This was done by adding 1 ml of sterile distilled water for every milliliter of cellular material present. Tonicity was restored after 30 seconds by adding 3.5 percent NaCl to the solution. The solution was centrifuged at 250 g at 4°C for 10 minutes. The supernatant was decanted and discarded.

Cell viability was determined at this time by using the dye exclusion technique.15 The cells were then washed twice with Hanks' balanced salt solution, and total and differential cell counts were done with a standard hemocytometer. Because of the large size and brownish intracellular inclusions of smokers' alveolar macrophages these cells are very easily distinguished from leukocytes in unstained preparations. Only specimens with greater than 80 percent viability and containing fewer than 10 percent leukocytes were used in this study. The final cell mixture was stored at -70°C until the enzyme analysis could be performed at a later date. Previously, it had been determined that this method of storage did not affect macrophage elastase-like esterase or acid protease activities.7

At the time of enzyme determination, the frozen cell specimens were allowed to thaw and the cells were disrupted with a Bronwell Biosonic III homogenizer as previously described.7 The alveolar macrophage elastase-like esterase activity was determined in cell homogenates from 34 patients, using p-nitrophenyl N-tert-butyloxycarbonyl-L-alanate (NBA) as substrate.16 Activity was expressed as ΔA447,5 per minute per 10⁸ cells. The alveolar macrophage–acid protease

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was determined in 34 subjects by the method of Anson as modified by Fress and associates, using denatured hemoglobin as substrate at pH 3.2. Activity was expressed as units per 10^8 cells. Elastase activity was measured in macrophage homogenates from 24 subjects according to Takahashi and coworkers using tritiated elastin as substrate. Incubation of cell homogenate and substrate was carried out for 24 hours and activity was expressed as mg of elastin degraded per 10^8 cells. This assay is highly sensitive and specific for elastolytic enzymes in that it will accurately detect activities equivalent to 0.1 µg of porcine pancreatic elastase and tritium is not solubilized by trypsin, chymotrypsin, or collagenase.

The nonpaired t test and coefficient of correlation were used in the statistical analysis of our data.

**Results**

Alveolar macrophages from resected lungs of 39 patients met the criteria of 80 percent viability and presence of less than 10 percent leukocytes. All patients were chronic smokers with a mean pack-year history of 51 and a range of 20 to 80 pack years. Thirty-seven patients were men and two were women. The distribution of ages and pulmonary function values for the group are illustrated in Figure 1. The mean age of these patients was 57 years, and results of routine pulmonary function studies ranged from normal to severe obstructive ventilatory abnormalities. Approximately one-half of the subjects had evidence of hyperinflation of lung volumes and a decreased diffusing capacity for carbon monoxide. All subjects did not receive the complete battery of pulmonary function tests; therefore, the total number of subjects represented by each histogram in Figure 1 is not the same.

Figure 2 compares the macrophage elastase-like esterase and acid protease activities observed in this group of patients with those previously reported for alveolar macrophages obtained by in vivo lavage from normal cigarette smokers. The elastase-like esterase level in this study is 5.60 ± 0.40 ΔM_47.5 per min per 10^8 cells (mean ± SEM) and the acid protease activity is 30.60 ± 2.70 units per 10^8 cells (mean ± SEM). These data indicate that patients with bronchogenic carcinoma associated with cigarette smoking have the same macrophage elastase-like esterase and acid protease activities as do normal cigarette smokers. We have also had the opportunity to study macrophage enzyme activities in resected lungs containing carcinoma from two nonsmokers and two ex-smokers, and the enzyme activities were as would be expected for normal nonsmokers and ex-smokers without carcinoma. Thus, we have been able to compare two macrophage enzyme activities in nonsmokers, ex-smokers, and cigarette smokers with and without bronchogenic carcinoma. It is unlikely that these three groups of subjects with carcinoma would have, respectively, maintained low, intermediate, and high enzyme activities if carcinoma had had a significant influence on the activity of the two enzyme levels measured.

The subjects in this study were divided into those with normal pulmonary function test and those with evidence of chronic obstructive pulmonary disease (COPD). The criteria for inclusion in the normal group was an FEV1/FVC ratio of .75 or greater with lung volumes and diffusing capacity within ± 20 percent of the predicted values for subjects 50 years of age or younger. For subjects older than 50, an FEV1/FVC ratio of .70 was accepted as normal if the other criteria were met. Fourteen subjects were considered to have normal pulmonary function tests and 25 were placed in the COPD group. In Table 1 the mean values for alveolar macrophage enzyme activities for these two groups are compared with each other, as well as mean values for normal cigarette smokers. There are no significant differences in these groups and these data indicate that patients with COPD have the same elastase-like esterase, acid protease, and elastase activity as cigarette smokers without COPD.

An attempt was made to correlate absolute values for elastase, elastase-like esterase and acid protease with FEV1/FVC, total lung capacity (TLC), functional residual capacity (FRC), RV/TLC, and Dco to see whether the level of these enzymes was related to the degree of physiologic abnormality observed. These physiologic parameters were chosen because airway obstruction with hyperinflation of lung volumes and decreased diffusing capacity would be most suggestive of emphysema. As illustrated in Figures 3, 4, and 5, there were no significant correlations between the macrophage enzyme

**Table 1—Comparison of Proteolytic Enzyme Activities of Alveolar Macrophages from Three Different Groups of Cigarette Smokers**

<table>
<thead>
<tr>
<th>Enzymes*</th>
<th>Elastase mg elastin degraded 10^8 cells</th>
<th>Acid Protease units/10^8 cells</th>
<th>Elastase-like Esterase ΔM_47.5/min/10^8 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal cigarette smokers</td>
<td>6.9 ± 0.1</td>
<td>30.5 ± 2.9</td>
<td>5.60 ± 0.40</td>
</tr>
<tr>
<td>Cigarette smokers with carcinoma of lung and normal pulmonary function</td>
<td>5.7 ± 0.1</td>
<td>29.8 ± 3.0</td>
<td>4.7 ± 1.5</td>
</tr>
<tr>
<td>Cigarette smokers with carcinoma of lung and COPD</td>
<td>5.6 ± 0.1</td>
<td>32.3 ± 4.2</td>
<td>5.8 ± 1.4</td>
</tr>
</tbody>
</table>

*Values given are mean ± standard error.
ELASTASE

mg Elastin Degraded / 10^6 Cells

activities and any of these physiologic parameters. Although the data are not plotted, there was no correlation of macrophage enzyme activities with age of the subjects or the quantity of cigarettes smoked.

DISCUSSION

At present there are multiple factors, both environmental and genetic, associated with the development of pulmonary emphysema. However, the specific mechanisms by which emphysema develops are not known. On an environmental basis, cigarette smoking stands out as the most significant factor with pack-years of smoking being directly related to anatomic severity of emphysema. Of the genetic factors, homozygous alpha1-antitrypsin deficiency has been well documented as being associated with emphysema.1 Galdston and co-workers thought that the level of leukocyte proteolytic enzyme activity was a factor that was important in expression of emphysema in patients who are partially or totally deficient in serum alpha1-antitrypsin. Alveolar macrophage enzyme levels could also be a determinant of emphysema either on a genetic basis, such as the one suggested by Galdston for leukocytes, or in response to environmental factors, such as cigarette smoking. Cigarette smoking has an established association with increased lysosomal enzyme activity in alveolar macrophages; however, there is considerable variation among individual smokers. If genetic or environmental factors influence the level of proteolytic enzymes in macrophages, people with the highest proteolytic enzyme activities might be at greater risk of developing lung disease. In the present study, the finding that the activities for alveolar macrophage elastase, elastase-like esterase, and acid protease in patients with COPD are the same as in normal cigarette smokers is evidence against high macrophage enzyme response to cigarette smoking being the primary determinant for development of lung disease. The lack of correlation of enzyme activities and measurements of pulmonary functional abnormalities, however, does not rule out the participation of alveolar macrophages in tissue damage.

In this study we have assumed that a neutral protease with elastolytic activity would be the most important enzyme to consider as a participant in the production of emphysema. This was based on the physiologic abnormality of decreased elastic recoil.
Figure 4. Alveolar macrophage elastase-like esterase activity is plotted against various pulmonary function parameters measured in these subjects. Correlation coefficient \( r \) is given for each comparison.

Figure 5. Alveolar macrophage acid protease activity is plotted against various pulmonary function parameters measured in these subjects. Correlation coefficient \( r \) is given for each comparison.
in the emphysematous lungs\textsuperscript{34,35} and on the data suggesting that elastic tissue is responsible for elastic properties of lung.\textsuperscript{5} Also, only elastolytic enzymes have been shown to produce emphysema in experimental animals.\textsuperscript{5,34,37} Initially the synthetic substrate, NBA, used to measure the elastase-like esterase activity was thought to be specific for elastase;\textsuperscript{16,18} however, recent studies suggest that macrophages have esterases that hydrolyze this substrate but do not have true elastolytic activity.\textsuperscript{29} When this was realized, we began using tritiated elastin as a substrate which is specific for true elastolytic enzymes and we found there was no correlation of NBA activity to tritiated elastin activity in these cells. Therefore, the elastase-like esterase activity should not be considered to be a quantitation of human alveolar macrophage elastase. Nevertheless, the activity observed with the tritium-labeled elastin provides firm evidence that cigarette smokers' alveolar macrophages possess a true elastase in amounts comparable to polymorphonuclear leukocytes (J. O. Harris, unpublished observations).

The data from this study do not establish a definite relationship between alveolar macrophage proteolytic enzymes and emphysema, but do suggest that if a relationship exists, it is not simply one of highest enzyme activities causing the more severe physiologic abnormalities. Possibly, alveolar macrophage enzymes have no role in the pathogenesis of emphysema or they may participate in a secondary fashion in conjunction with deficient antiprotease activity or factors that promote excessive release of lysosomal enzymes from alveolar macrophages. Werb and Gordon\textsuperscript{30} demonstrated that thioglycolate-stimulated mouse peritoneal macrophages secrete elastase into culture media when maintained in vitro and similar findings have been reported for human alveolar macrophages from cigarette smokers.\textsuperscript{31} Although it is not known if these in vitro observations reflect what occurs in vivo, it does raise the interesting possibility that longterm secretion of elastase may be more important than the absolute level of elastase activity in smokers' alveolar macrophages. In assessing the data presented in this report, it should be pointed out that elastase activity was measured at one point in time in alveolar macrophages from these patients and we do not know if this truly reflects the rate of elastase synthesis and absolute elastase activity over the long period of time during which emphysema developed in these subjects.

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