Effect of Exposure of Guinea Pigs to Cigarette Smoke on Elastolytic Activity of Pulmonary Macrophages*

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Study objective: To determine the effect of exposure to cigarette smoke on the elastolytic activity of guinea pigs' alveolar macrophages (AMs), and to compare elastolytic activity of AMs obtained by BAL with that of lung macrophages (LMs) obtained from minced lung tissue.

Methods: AMs were obtained by BAL from seven adult guinea pigs exposed to cigarette smoke for 5 d/wk during 6 weeks, as well as from age-matched control guinea pigs. From each animal, one lung was used to obtain LMs by mincing and teasing the lung, followed by enzymatic digestion and isolation of mononuclear cells by Hypaque-Ficoll separation. The other lung was inflated and fixed to quantitate emphysema by the destructive index (DI). Elastolytic activity (microgram of elastin degraded by 10^6 macrophages) was determined at 24, 48, and 72 h, by culturing AMs and LMs (1×10^6 cells in 1 mL of medium) in 3H-elastin-coated wells.

Results: In animals exposed to cigarette smoke, the total number of BAL cells (8.6±2.1×10^6) and DI (21.8±8.1) were significantly higher than in nonexposed animals (6.4±1.8×10^6, p<0.05 for cells, and 12.1±4.1, p<0.01 for DI). Elastolytic activity of AMs from smoke-exposed guinea pigs was significantly higher at 24, 48, and 72 h than elastolytic activity of AMs from control animals (19.0±9.4 vs 10.0±5.3, p<0.05 at 72 h). Likewise, elastolytic activity of LMs was significantly higher in exposed than nonexposed guinea pigs (11.8±7.7 vs 7.4±5.0 at 72 h, p<0.05). Elastolytic activity of LMs was not significantly different from elastolytic activity of AMs, both in exposed guinea pigs (11.8±7.7 vs 19.0±9.4 at 72 h) and nonexposed animals (7.4±5.0 vs 10.0±5.3 at 72 h).

Conclusions: These results indicate that elastolytic activity of both AMs and LMs of guinea pigs increases significantly after exposure to cigarette smoke and that AMs and LMs have similar elastolytic activities.

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Key words: cigarette smoke exposure; elastase; elastolytic activity; emphysema; lung macrophages

Abbreviations: AM=alveolar macrophage; COHb=carboxyhemoglobin; DI=destructive index; EA=elastolytic activity; LM=lung macrophage; NS=not significant; PMSF=phenylmethyl-sulfonyl-fluoride

The current hypothesis of the pathogenesis of emphysema postulates that an imbalance between proteases and antiproteases leads to the destructive changes in the lung parenchyma.1 Neutrophils have been considered to be the main cells involved in this elastolytic injury.2 However, there is more recent evidence suggesting that alveolar macrophages (AMs) may play a prominent role in the degradation of lung elastin.3-5 Studies of the elastolytic properties of lung macrophages (LMs) as well as their possible role in mechanisms leading to emphysema have been based on AMs retrieved from BAL.3-7 However, because these AMs are not directly in contact with the interstitial matrix, the macrophages resident in the lung interstitium may be the more relevant cell in relation to potential elastolytic damage of lung parenchyma.

In this context, our study had two aims; first to evaluate the effect of cigarette smoke exposure on...
AM elastolytic activity (EA) in guinea pigs, and second to compare the EA of AMs with that of macrophages extracted from lung tissue (predominantly interstitial macrophages) both in guinea pigs exposed and not exposed to cigarette smoke.

**MATERIALS AND METHODS**

**Preparation of Elastin-Coated Culture Plates**

\(^{3}\)H-elastin was prepared by reductive alkylation of bovine ligamentum mchaei elastin (Elastin Products Company, Owensville, Mo) using sodium borohydride\(^{6}\) as previously reported.\(^8\) Culture plates were prepared by spreading evenly 250 μg of the \(^{3}\)H-elastin suspension (specific activity = 1.584 cpm/μg) on the inside bottom of 16-mm wells of 24-well tissue culture plates (Costar; Cambridge, Mass). The elastin was dried at 45°C and the \(^{3}\)H-elastin-coated plates were then stored at 4°C until used.

**Experimental Exposure to Cigarette Smoke**

Approval from the Institutional Animal Research Committee was obtained. Seven adult guinea pigs weighing 600 to 630 g were exposed to the smoke of 20 commercial nonfilter cigarettes per day, for 5 days, during 6 weeks. The smoke exposure was accomplished by enclosing the animals (three or four at a time) in a plastic chamber 100 cm long, 60 cm wide, and 50 cm high, which had two holes on one lower edge that held two cigarettes used for smoke exposure. The animals were exposed to the smoke by lighting the cigarettes and inhaling the smoke through the chamber using a suction vacuum attached to the opposite upper corner of the chamber; in this manner, the smoke was dispersed throughout the chamber. The cigarettes were lit and "smoked" over a period of 10 min and followed by a period of 10 to 20 min without cigarette smoking. The cycle was repeated until a total of 20 cigarettes were "smoked" over a period of about 5 h.

To confirm that this system led to significant smoke inhalation, we obtained blood measurement of carboxyhemoglobin (COHb) by co-oximetry in another group of animals exposed to cigarette smoke under identical conditions. As control group for the effects of cigarette smoke exposure, we studied seven guinea pigs placed in a similar chamber for a similar period of time for 5 days during 6 weeks under the same conditions but without using any cigarette, so that only room air was being aspirated into the chamber.

**Bronchoalveolar Lavage**

After 6 weeks of experimental or sham exposure, guinea pigs were anesthetized by intraperitoneal injection of pentobarbital (28 mg/kg). A tracheostomy was performed under sterile conditions and a 0.21-cm catheter was introduced into the trachea. Guinea pig lungs were washed very gently by instilling five aliquots of 10 mL of saline solution each. The fluid recovered from the lavage was kept on ice until analysis. Cells in the BAL were separated from the lavage fluid by centrifugation at 4°C. The sedimented cells were washed twice in Hank’s balanced salt solution and then resuspended in serum-free RPMI 1640 medium containing 25 mmol/L Hepes buffer, 2 mmol/L glutamine, 100 U/mL penicillin, and 10 mg/mL streptomycin (GIBCO; Grand Island, NY). The number of macrophages per milliliter was determined by counting the cells in a hemocytometer and cell viability was analyzed by trypan blue exclusion. Slide preparations were prepared to determine the BAL cell differential count. Cell counting was performed on a total of 300 cells by a pathologist unaware of the origin of the fluid. The cell suspension was adjusted to a count of 1×10⁶ macrophages per milliliter for assay of elastolytic activity.

**Preparation of LMs**

To further remove AMs from the lung, two additional BALs with 10 mL of saline solution each were performed. After the lavage, the animals were killed with an intraperitoneal overdose of sodium pentobarbital. The left lung was used for the histologic study and the right lung for obtaining LMs. The right lung was minced into pieces of 2 to 4 mm with scissors and excess blood was removed by rinsing the lung pieces with saline solution. The tissue was then incubated with RPMI-1640 medium and trypsin (166 μg/mL) EDTA (66 μg/mL) for 30 min at 37°C. To remove particulate matter, the medium was filtered through gauze and then centrifuged for 10 min at 1,500 g at 4°C. The cell pellet was resuspended in medium and the LMs were separated by using a Hypaque-Ficoll gradient.\(^{10}\)

The LMs were washed twice in medium and then centrifuged. The LMs in the pellet were considered to be predominantly interstitial macrophages; they were resuspended in RPMI-1640 medium, counted, and adjusted to a concentration of 1×10⁸ LM per milliliter. Cell viability was determined by trypan blue exclusion.

**Measurements of AM and LM EA**

EA in AMs and LMs was measured by the method originally described by Chapman and Stone\(^8\) and later used by other investigators.\(^{11,12}\) Prior to use, the \(^{3}\)H-elastin-coated wells of culture plates were washed three times with phosphate-buffered saline solution and then loaded in duplicate (or in triplicate depending on the total amount of available cells) with either 1 (or 0.5) mL of either AMs or LMs in RPMI-1640 at 1×10⁶ cells per milliliter. For every assay, blanks (RPMI-1640 without cells) in duplicate were routinely included and used to correct for nonspecific release of radioactive elastin. The cell cultures were then incubated at 37°C, 100% humidity, and 95% air-5% CO₂ for 2 h for allowing macrophages to adhere to the \(^{3}\)H-elastin-coated wells, and then nonadherent cells were removed. The wells with \(^{3}\)H-elastin and the adherent macrophages were then filled with 1 (or 0.5) mL of RPMI-1640 supplemented with 1% (vol/vol) nonessential amino acids and 10% fetal (vol/vol) bovine serum, and the culture plates were returned to the incubator for culture periods up to 72 h. Every 24 h, the medium was removed and stored at 4°C until analysis, and replaced by fresh medium. At the end of the 72-h incubation period, the samples of each 24-h incubation were spun in a microcentrifuge (13,000×g at 4°C for 5 min) and 100 μL of the supernatants was assayed for solubilized \(^{3}\)H-elastin by scintillation counting in 10 mL of Aquasol-2 (New England Nuclear; Dupont, UK). Results of the EA in micromgrams of elastin degraded per 100 μL of medium were calculated as follows:

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\text{cpm macrophage sample–cpm blank)/specific activity of \(^{3}\)H-elastin.}
\]

Results were then expressed as microgram of elastin degraded by 10⁶ cells by dividing the total amount of elastin degraded in each well by the number of macrophages in that well.

**Experiments to Control for Possible Differences Due to Method**

**Effect of Hypaque-Ficoll:** Since LMs were isolated by using the Hypaque-Ficoll gradient method, and AMs were not, we deter-
mined if AMs had similar EA whether or not they were obtained by using Hyapque-Ficoll. Therefore, a separate experiment was made with three additional guinea pigs to compare EA of AMs prepared from BAL without and with the use of Hyapque-Ficoll.

**Trypsin Treatment:** To determine the influence of the enzymatic digestion with trypsin used during recovery of LMs on EA, AMs from three guinea pigs exposed to cigarette smoke and obtained as described previously were assayed for EA without and after incubation with trypsin for 30 min. The EA from AMs subjected to enzymatic treatment was then compared with EA of the AMs without trypsin exposure.

**Effect of Neutrophil-Derived Elastase:** To determine the possible contribution of EA derived from neutrophils on the determined macrophage EA, AMs from three guinea pigs exposed to cigarette smoke and obtained as described previously were assayed for EA without and with the addition of 100 mmol/L of phenylmethyl-sulfonyl fluoride (PMSF), a potent inhibitor of neutrophil elastase.

**Histologic Assessment**

After fixation with 10% buffered formalin at a constant pressure of 25 cm H₂O, the left lung was processed for morphologic evaluation. Five-micrometer sections were stained with hematoxylin-eosin and examined on light microscopy by a pathologist who did not know whether the animal had been exposed to cigarette smoke. To assess the degree of parenchymal destruction, lung samples were further evaluated by using the destructive index (DI) described by Saetta et al. Light microscopy at 10X magnification was used to grade the stained sections of the lung. For each tissue, 20 nonoverlapping fields randomly chosen from one slide were examined with an ocular with cross hair and 36 points. Any field containing structures, like vessels or airways, whose maximal diameter was larger than 0.60 mm was not used for the analysis. Alveoli or alveolar ducts lying under these points were classified as normal or destroyed. They were considered to be normal when they were surrounded by intact or continuous walls or when these walls were disrupted only in one place. Alveolar spaces were considered to be destroyed when the wall of an alveolus was disrupted in two or more places or there were three or more disruptions of contiguous alveoli. DI was calculated according to the following formula: DI=100×D/(D+N), where D indicates "destroyed" and N indicates "normal."

**Statistical Analysis**

The unpaired Student’s t test was used for comparing results in nonexposed and smoke-exposed animals, while the paired t test was used when comparing the two types of macrophages from the same animal. The p values <0.05 were considered to indicate a statistically significant difference.

**Table 1—BAL Cell Profile (Mean±SD)**

<table>
<thead>
<tr>
<th></th>
<th>Total Cells, ×10⁶</th>
<th>Macrophages, %</th>
<th>Neutrophils, %</th>
<th>Lymphocytes, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoke exposed</td>
<td>8.6±2.1</td>
<td>65.85±15</td>
<td>11±7.4</td>
<td>22.2±8.4</td>
</tr>
<tr>
<td>Control animals</td>
<td>6.4±1.8</td>
<td>63.85±15</td>
<td>7±3.1</td>
<td>27.8±5.9</td>
</tr>
<tr>
<td>p Value</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Results**

After 6 weeks of tobacco smoke or air exposure, there were no significant differences in body weight between the two groups of guinea pigs. A significant increase in the mean level of COHb was observed in the cigarette smoke-exposed animals (19.3±5.4% vs 6.5±3.2% in the control group; p<0.05). Table 1 shows the cell profile from BAL. The total amount of cells was significantly higher in smoke-exposed guinea pigs compared with control animals (8.6±2.1 vs 6.4±1.8, p<0.05). Macrophages were the most prevalent cell population in both groups of animals, but no significant differences were found in any type of inflammatory cells. The cell viability was always higher than 90%.

**Histologic Assessment**

Morphologic changes are illustrated in Figure 1. The lungs of all cigarette smoke-exposed guinea pigs showed inflammatory changes as foci of interstitial

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**Figure 1.** Top (a): normal lung. Bottom (b): smoke-exposed lung exhibiting peribronchiolar, interstitial, and intra-alveolar inflammation, with areas of rupture of the alveolar septa can be seen (hematoxylin-eosin, original magnification ×4).
and intra-alveolar infiltration by lymphocytes and macrophages and in some cases by polymorphonuclear cells. In most animals, lesions compatible with bronchiolitis were found. Areas of disruption of alveolar septa were noticed mainly under the pleura, and this observation was corroborated by the DI that was significantly higher in guinea pigs exposed to cigarette smoke (21.76±8.11) than in the control animals (12.17±4.11; p=0.016).

**Elastolytic Activity**

Results of EA of AMs and LMs derived from control and smoke-exposed animals are shown in Table 2. In general, mean values of EA obtained from AMs tended to be higher than those obtained from LMs in both tobacco smoke-exposed animals and control animals. However, no significant differences between the two cell preparations were found by the paired t test. EA of both types of macrophages increased with duration of culture. EA of AMs was significantly higher in smoke-exposed guinea pigs compared with the control animals at all time intervals of culture. Similar results were observed with LMs, although no significant difference was observed at 72-h culture.

**Effect of Hypaque-Ficoll Separation, Trypsin Treatment, and PMSF on EA**

We first determined if the total number of cells recovered was affected by the use of Hypaque-Ficoll. The mean number of cells obtained from the BAL of three guinea pigs exposed to cigarette smoke was 10.93±5.8×10⁵. Half of the BAL was processed to obtain mononuclear cells by using the Hypaque-Ficoll method and a total of 5.06±4.69/10⁵ cells were recovered. This number was not significantly different from the 5.41±5.3/10⁵ cells in the remaining half of the BAL not subjected to Hypaque-Ficoll. In addition, EA (microgram of degraded elastin ×10⁶ cells) of AMs purified by the Hypaque-Ficoll separation was not different from AMs not treated with Hypaque-Ficoll (24±7 vs 22±8 at 72 h, p=not significant [NS]).

Since enzymatic digestion with trypsin was used to obtain LMs, we additionally evaluated EA from AMs after incubation with this enzyme. Our results show that EA from AMs treated with trypsin exhibited a consistently lower EA by a factor of 17±3% (n=6 assays from three guinea pigs). When EA of LMs from all the experiments (shown in Table 2) was adjusted by using this factor to correct for the trypsin effect, EA of LMs still remained slightly lower by 15 to 20% than the corresponding AM values. PMSF treatment did not significantly affect EA; the EA of AMs with PMSF at 72 h was 22±4, while that of AMs without PMSF was 23±6 (p=NS).

**DISCUSSION**

The evaluation of the EA of AMs or LMs raises two issues: first, whether the observed EA is really due to the macrophages and not to neutrophils, and second, if the so-called LMs are indeed coming from the interstitium of the lung. It was assumed in this article that the cells responsible for the EA were macrophages based on stained cytospin preparations of the obtained cells. Contaminating neutrophils were removed after a 2-h period of incubation to allow adherence of macrophages prior to determination of EA. This adherence step indicates that the EA determined was due to macrophages and not to nonadherent cells such as neutrophils. Furthermore, in a separate experiment (with three guinea pigs), EA of AMs obtained with the Hypaque-Ficoll method to remove contaminating neutrophils was the same as that obtained without Hypaque-Ficoll, supporting the notion that in our assay, there was no EA due to neutrophils. Moreover, the routine use of fetal bovine serum rich in serine proteinases inhibitors such as α1-antitrypsin in all cultures would inhibit any elastase derived from neutrophils but not macrophages. The addition of PMSF, a potent inhibitor of neutrophil elastase, did not affect cultured AM EA, indicating that there was no EA derived from neutrophils.

The best method to determine the origin of the macrophages from lung tissue is by cell markers.

### Table 2—EA of AMs and LMs of Control and Cigarette Smoke-Exposed Guinea Pigs

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Nonexposed</th>
<th>Exposed</th>
<th>p</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM</td>
<td>LM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>4.72±2.10</td>
<td>2.97±2.37</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>7.67±3.71</td>
<td>5.13±3.49</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>9.99±5.26</td>
<td>7.36±4.99</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.94±2.65*</td>
<td>6.41±1.95</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.21±6.07*</td>
<td>10.12±4.54</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.01±9.42*</td>
<td>11.82±7.68</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

*AM exposed vs nonexposed AM, p<0.05.
'Exposed LM vs nonexposed LM, p<0.05.
'Exposed LM vs nonexposed LM, p=NS.
specific for alveolar but not for interstitial macrophages, but we were unable to use these techniques. We attempted to decrease contamination of LMs by AMs by washing out the lung with an excess of saline solution after the BAL, and by rinsing and eliminating the remaining blood from the tissue while processing the tissue cells. These maneuvers would not completely eliminate the resident macrophages in the alveoli and the blood. The studies by Dethloff and Lehnert indicate that such preparations considered to be of interstitial macrophages would have significant contamination of AMs. However, Weissler and coworkers observed that macrophages obtained from minced lung tissue (as we did) are functionally different from those obtained by BAL, in terms that they induce significantly more T-cell proliferation in a mixed leukocyte reaction, suggesting macrophages different from AMs. Despite this possible contamination of LMs by AMs, our results clearly suggest that EA of LMs increases significantly after exposure to cigarette smoke.

Significant exposure to cigarette smoke was clearly demonstrated by the levels of COHb (19%) in smoke-exposed guinea pigs. However, the nonexposed animals also had high levels of COHb (about 6%) in comparison to human levels. The reason for this finding is not clear; a possible explanation is that the levels were tested in a co-oximeter standardized for determining human blood COHb and not guinea pig COHb.

Significant inflammatory and parenchymal changes compatible with early emphysema were observed in guinea pigs exposed to cigarette smoke, indicating that even the degree of exposure to tobacco smoke that we used (5 h/d, 5 d/wk for 6 weeks) was deleterious. This is not surprising since Wright and Churg have previously observed changes of emphysema in guinea pigs exposed to the smoke of 10 cigarettes during 5 d/wk for 1 to 12 months. Furthermore, we have previously demonstrated that foci of moderate peribronchiolar, interstitial, and intra-alveolar inflammation are observed in the same model at 4 weeks exposure.

Our findings of increased EA in both AMs and LMs from guinea pigs exposed to cigarette smoke suggest that increased EA by LMs may contribute to the observed destructive changes in the lung parenchyma. Supporting this point of view are the findings of increased EA in smokers as reported by Chapman and Stone and reviewed by Shapiro and associates. Actually, it is important to point out that macrophages are the most abundant inflammatory cells found in BAL of cigarette smokers, as well as in respiratory bronchioles where emphysematous changes are first manifested. Moreover, Finkelstein et al. have recently demonstrated that the extent of lung destruction in human emphysema is directly related to the number of AMs and T lymphocytes, but not with the number of neutrophils.

Despite the numerous studies on pathogenetic mechanisms in emphysema using BAL, to our knowledge, our study is the first one to attempt to compare EA of both AMs and LMs. The importance of attempting to evaluate interstitial macrophages’ enzymatic activity through the use of minced lung tissue is that the interstitial macrophages, in contrast to AMs, are in closer contact with the elastic fibers that may be more susceptible to elastolysis by interstitial than AMs. Nevertheless, our results showed that the EA of these two types of macrophages is similar both in smoke-exposed and in nonexposed guinea pigs.

There was a nonsignificant trend for the LMs to show a lower EA than the AMs. This tendency was not modified after correcting for the putative enzymatic effect of trypsin, to which the tissue cells were submitted during their isolation.

In general, our results support the notion that AMs and LMs are similar in a variety of activities, as has been demonstrated in other different studies. For example, Adamson and coworkers compared rat alveolar and interstitial macrophage activity to stimulate fibroblasts after exposure to silica and asbestos. They found that the two macrophage populations responded equally to particles with respect to secretion of fibroblast growth factors.

However, the time course of EA of guinea pig AMs and LMs is comparable to that previously reported by Senior et al. and Chapman and Stone, and Ofulue et al. regarding EA of human AMs; the slight differences in the amount of degraded elastin and in the time course of elastin degradation may be due to the species difference.

In summary, our study indicates that cigarette smoke exposure in guinea pigs not only induces changes compatible with emphysema, as has been reported previously, but also results in a significant increase of EA by macrophages that may contribute in part to the destructive changes observed in emphysema. The enzyme(s) responsible for this activity deserve further study, but the recently described metalloelastase, and the 92-kd gelatinase B, both secreted by macrophages, are strong candidates. Actually, an increase in 92-kd type IV collagenase expression by macrophages has been found previously in this experimental model. Our study also suggests that the EA of macrophages from minced lung tissue is similar to that observed in macrophages obtained from BAL.
REFERENCES

21. Adamson IYR, Letourneau HL, Bowden DH. Comparison of alveolar and interstitial macrophages in fibroblast stimulation after silica and long or short asbestos. Lab Invest 1991; 64:339-44