PULMONARY EMPHYSEMA is a chronic disease, characterized morphologically by abnormal permanent enlargement of the respiratory air spaces, that results from destruction of the alveolar walls (37). Progressive degradation of elastin in the alveolar walls is a key feature in the pathogenesis of emphysema (17, 24, 36). One known cause of emphysema is a genetic deficiency of \( \alpha_1 \)-proteinase inhibitor (\( \alpha_1 \)-PI), an inhibitor of neutrophil elastase (6). However, the major known cause of emphysema is cigarette smoking (14). Lung elastin degradation in emphysema associated with \( \alpha_1 \)-PI deficiency is triggered by the unopposed action of neutrophil elastase because of the insufficient levels of its inhibitor (17, 24, 36). The initiating elastinolytic factor in cigarette smoking-induced emphysema is still not clear.

Emphysema in chronic smokers is believed to be the result of increased neutrophil and/or macrophage elastinolytic activity within the alveolar structures (17, 24, 36) that is due to smoking-induced accumulation of neutrophils and macrophages in the lower respiratory tract (7, 10, 16, 22). Because neutrophils exert 10-fold more elastinolytic activity per cell than do macrophages (4), neutrophils were considered to be the principal pathogenic cells in emphysema in chronic smokers (16, 17, 22, 24). However, attention has now focused on macrophages as a potential principal source of elastinolytic activity within the lung of smokers (17, 24, 36) because macrophages accumulate precisely in the centriacinar zones of the lung where emphysema in smokers occurs, unlike neutrophils that are uniformly distributed in the lung (25). Morphological data from surgically resected lungs of smokers have shown a direct relationship between the extent of smoking-induced destruction of lung parenchyma and the number of macrophages, not neutrophils, in the lung parenchyma (5, 8). However, because these studies (5, 8, 16, 22) were conducted at a single time point in smokers with long smoking history, the earliest neutrophil or macrophage responses in relation to the onset of lung parenchyma destruction in smokers are not known.

The aim of present study was to determine the relative elastinolytic potentials of neutrophils and macrophages in both the onset and progression of cigarette smoke-induced emphysema, using rats chronically exposed to cigarette smoke inhalation. This model of cigarette smoke-induced emphysema has been well characterized (7, 15, 39). Our first specific objective was to examine the time course of cigarette smoke-induced accumulation of neutrophils and macrophages in the lung and the relationships to the onset and progression of emphysema lesions in the lung. Although analysis of bronchoalveolar lavage fluid cells has been useful in studies of the inflammatory response in the lungs of human smokers (16, 22) and smoke-exposed animals (7, 10), we chose to examine both bronchoalveolar lavage fluid and lung interstitial inflammatory cells because it is not known whether responses of the bronchoalveolar lavage fluid inflammatory cells are truly representative of the lung interstitial pool. We reasoned that the interstitial neutrophils and/or macrophages, by virtue of their close proximity to the connective tissue matrix, would have the greatest potential to play a role in the breakdown of elastin in the lung parenchyma. Then, our second specific objective was to
examine the time course of accompanying changes in elastinolytic activity exerted in vitro by lung neutrophils and macrophages of cigarette smoke-exposed rats and the relationships with indexes of in vivo lung elastinolytic damage.

MATERIALS AND METHODS

Animals and cigarette smoke exposure. Sixty specific pathogen-free male Sprague-Dawley rats (littermates from 10 litters; 289 ± 15 g initial body weight; Charles River Breeding Laboratories, Guelph, ON) were divided into two equal groups. One group was exposed daily to smoke from commercial nonfiltered cigarettes (10 cigarettes·rat⁻¹·day⁻¹), 5 days/ wk, with smoke-generating and head-only exposure chambers (1 rat/chamber) similar to those described by Simani et al. (34). The other group of rats was exposed to normal room air delivered similarly and served as control animals. Blood samples were obtained periodically within 2 h after smoke or air exposure from the tail vein and analyzed for blood carboxyhemoglobin levels (12). Average carboxyhemoglobin values over the entire exposure duration was 10.1 ± 1.5% (SD) in the smoke-exposed rats compared with 0.9 ± 0.3% in the air-exposed rats (P < 0.001), indicating successful smoke inhalation by the smoke-exposed rats. After each exposure session, the rats were housed in individual metabolite cages in pathogen-free quarters maintained at constant humidity and temperature. Smoke-exposed rats exhibited reduced food intake and somatic growth rate (12, 15, 21), factors known to affect lung elastin breakdown and alveolar structure (26, 28).

We observed that the food consumption by our smoke-exposed rats was ~8 g food pellets·100 g body wt⁻¹·day⁻¹, which was ~75% of the amount consumed by normal air-exposed rats. On this basis, the smoke-exposed and control air-exposed rats received and consumed the same amount of food, resulting in an increase of 0.8 ± 0.2 (SD) g body wt⁻¹·day⁻¹ for the smoke-exposed rats compared with an increase of 0.9 ± 0.1 (SD) g body wt⁻¹·day⁻¹ for the control air-exposed rats (no significant difference between the two groups). All rats were provided with water for ad libitum consumption.

Animal killing and lung perfusion. The rats were examined at 0, 1, 2, 3, and 6 mo of smoke or air exposure. At each time point, six rats per group were euthanized with sodium pentobarbital sodium (50 mg/kg body wt ip), usually ~18 h after the last cigarette smoke or air exposure, and killed by exsanguination from the abdominal aorta. The lung vascular beds were perfused in situ via the pulmonary artery with 100 ml of Ca²⁺⁻ and Mg²⁺⁻ free phosphate-buffered saline (PBS) at a rate of 17 ml/min. This protocol consistently rendered the lung vasculature free of blood cells and protein components.

Bronchoalveolar lavage and quantitation of lavage fluid neutrophils and macrophages. Immediately after lung perfusion of each rat, bronchoalveolar lavage was performed by instilling 5 ml of PBS into the lung via a tracheal cannula and carefully withdrawing the fluid; this was repeated seven times. About 89% of the total instilled volume per rat was recovered, without a significant difference between the smoke-exposed and control air-exposed rats. The lavage fluid was centrifuged at 500 g for 10 min at 4°C. The resultant clear supernatant (cell-free lavage fluid) was decanted into polystyrene tubes and stored at −70°C for subsequent analysis described in Analysis of neutrophil or macrophage elastinolytic activities in vitro and Estimation of in vivo lung elastin breakdown. The pelletted lavage fluid cells were washed three times in Dulbecco’s modified Eagle’s medium containing 2 mmol glutamine, 100 μg penicillin/ml, and 100 U streptomycin/ml (DMEM; GIBCO BRL Laboratories, Grand Island, NY) and resuspended in 1.5 ml of DMEM. Viability of the cells (judged by exclusion of trypan blue solution) averaged 91 ± 2% (SD) and was consistent between the two experimental groups per time point examined. The total number of cells per rat lavage fluid volume was counted in triplicate in a hemocytometer chamber. Differential cell counts were determined from triplicate slides of each sample (with Cytospin 2, Shandon Instruments, Pittsburgh, PA) stained with Diff-Quik (Baxter, McGaw Park, IL) according to standard morphological criteria; 400 cells/slide were counted. The number of lavage fluid neutrophils or macrophages per rat lung was calculated from the total and differential cell counts.

Quantitation of lung interstitial neutrophils and macrophages. Immediately after bronchoalveolar lavage of each rat, the right main bronchus was ligated. The right lung was removed, and the interstitial cells were isolated as described by Lavnikova et al. (20). Briefly, the right lung was trimmed of nonparenchymal tissue, weighed, and cut into 500-μm slices. Duplicate 350-μg slices/rat right lung were dissociated by a 90-min treatment in 10 ml of DMEM containing collagenase (175 U/ml, type A; Boehringer Mannheim, Laval, PQ), DNase (50 U/ml, Sigma type III), and 10% fetal bovine serum (GIBCO BRL). The dissociated tissue was filtered through nylon gauze, the filtrate was centrifuged, and the pelletted lung interstitial cells were washed three times in DMEM and resuspended in 3.5 ml of DMEM. Viability of the interstitial cells (judged by exclusion of trypan blue solution) averaged 89 ± 3% (SD) and was consistent between the smoke-exposed and air-exposed rats per time point examined. Total and differential cell counts were determined in triplicate as described in Bronchoalveolar lavage and quantitation of lavage fluid neutrophils and macrophages and used to calculate the number of interstitial neutrophils or macrophages per rat lung. Data from the right lung were projected to both lungs on the basis of lung weight.

Analysis of neutrophil or macrophage elastinolytic activities in vitro. Immediately after procurement of the lavage fluid or interstitial cell populations, neutrophils and macrophages in each cell population were isolated by Ficoll-Hypaque fractionation (3). Purity of the isolated cells was ascertained cytochemically (40), and their viabilities were found to be >90%, without a significant difference between smoke-exposed and air-exposed rats. The isolated neutrophils or macrophages were then examined for their elastinolytic activity (4, 33) as previously described (27). Briefly, intact neutrophils or macrophages per rat sample were loaded in triplicate into [3H]elastin-coated wells (10⁶ cells·well⁻¹·ml DEMEM⁻¹) and cultured in a 37°C, 5% CO₂ incubator for 24 h (neutrophil or macrophage cultures) or 72 h (macrophage cultures only). At the end of the incubation, the culture medium was removed and centrifuged (10,000 g, 5 min, 4°C), and the supernatant was analyzed by β-scintillation for degraded [3H]elastin. Specific elastinolytic activity by neutrophils or macrophages is expressed as micrograms of elastin degraded per 10⁶ neutrophils or macrophages. Total elastinolytic potential by neutrophils or macrophages per rat lung was estimated as specific elastinolytic activity multiplied by the total number of 10⁶ neutrophils or macrophages recovered per rat lung.

Elastinolytic activity of the cell-free lavage fluid was also determined. Ten- and twenty-milliliter aliquots of cell-free lavage fluid from each rat were dialyzed against distilled water at 4°C, lyophilized, and resuspended in 1 ml of DMEM. These lavage fluid preparations were first preincubated at 37°C for 30 min without and with 0.1 mM methoxy succinyl-L-alanyl-L-alanyl-L-prolyl-L-valyl-chloromethyl ketone (MSAAPVCMK; a neutrophil elastase inhibitor; 29) Enzyme Systems, Liver-
more, CA) or 10 mM EDTA (an inhibitor of macrophage metalloelastase (1, 4)) and then added to [3H]elastin-coated wells. After 72 h of incubation at 37°C, the medium was quantitated by β-scintillation for degraded [3H]elastin. The elastinolytic activity exerted by each cell-free lavage fluid was expressed as micrograms of elastin degraded per rat lavage fluid volume. The relative contribution of neutrophils or macrophages to the lavage fluid elastinolytic activity was estimated from the percent inhibition of the lavage fluid elastinolytic activity by MSAAPVCMK or EDTA, calculated by comparison with the activity in the absence of these inhibitors.

Estimation of in vivo lung elastin breakdown. Lavage fluid levels of elastin-derived peptides and desmosine, an amino acid unique to mature elastin, were measured as biochemical indexes of in vivo lung elastin breakdown. Elastin-derived peptides in 0.2-ml aliquots of cell-free lavage fluid were quantitated by an enzyme-linked immunosorbent assay (11) as described by Schriver et al. (32), with rat lung α-desmosine and goat anti-rat lung α-elastin (Elastin Products, Owensville, MO) as the standard and primary antibodies, respectively, and peroxidase-conjugated rabbit anti-goat IgG (Calbiochem, San Diego, CA) as the secondary antibody. Each rat sample was assayed in triplicate and gave reproducible results (coefficient of variation ranged from 1.9 to 2.7%). Results are expressed as nanograms of elastin-derived peptide per milligram of albumin. Albumin content was determined by reaction with bromcresol green (30), with rat albumin (both from Sigma, St. Louis, MO) as the standard.

For desmosine quantitation, 1- and 2-ml aliquots of cell-free lavage fluid were first dialyzed against distilled water with 500-Da molecular-mass exclusion limit membranes to remove salts that can interfere with the desmosine assay. Greater than 98% of elastin peptides in each sample (as judged by assay described above) were retained in the dialysis tubing. Subsequently, the postdialysis samples were lyophilized, hydrolyzed in 1 ml of 6 N HCl at 110°C for 48 h, and loaded onto Whatman CF11 cellulose minicolumns, followed by a wash with an n-butanol-acetic acid-water mixture, and desmosine was eluted out with distilled water essentially as described by Skinner (35). The column recovery of various amounts of [3H]desmosine that were routinely added to some samples before or after hydrolysis was found to be 95.4 ± 3.7% (SD), without a significant difference between columns. Thereafter, each eluant of test sample was lyophilized and redissolved in 1 ml of distilled water, and the amount of desmosine in 0.2-ml aliquots was determined by an enzyme-linked immunosorbent assay as described by Laurent et al. (19), with rabbit anti-desmosine (Elastin Products) as the primary antibody and biotinylated anti-rabbit IgG (Calbiochem) as the secondary antibody. Each rat sample was processed and assayed in triplicate and gave reproducible results (coefficient of variation ranged from 2.1 to 3.7%). Results are expressed as nanomoles of desmosine per rat lung total lavage fluid volume.

Morphometric determination of emphysema in lungs. Immediately after bronchoalveolar lavage and removal of the right lung from each rat, the left lung was fixed with 10% phosphate-buffered Formalin (pH 7.4) by inflation via the attached tracheal cannula at a constant transpulmonary pressure of 25 cmH₂O. Two midsagittal blocks of each fixed lung were obtained and embedded in paraffin. From each block, three 5-μm serial sections were equidistantly cut, picked up on a slide, and stained with hematoxylin and eosin. The histological slides were coded by external personnel. With the standard morphometric technique according to Thurlbeck (38) with modifications previously described (18, 26), each slide was analyzed by projecting a microscopic image of the lung section on a screen with a square reference lattice containing 2 diagonally placed test lines and 42 equidistantly distributed points. Twenty randomly selected fields per slide were sampled. The number of intersections of alveolar walls with the test lines were counted and used to quantitate mean linear intercept (Lₜ; the average distance between alveolar walls). Increasing Lₜ was taken as evidence of air space enlargement (7, 38, 39). The number of test points falling on alveolar wall tissue (Pₚ) in relation to the reference lattice (Pᵣ) was also counted and used to determine alveolar wall tissue volume density (Vₚ; expressed in percent). Decreasing Vₚ was taken as evidence of lung parenchyma destruction (7, 8, 15). All quantitations were corrected for tissue shrinkage during processing.

Statistical analysis. All data are expressed as means ± SD. Statistical analysis of data was performed with GraphPad Prism-StatMate (GraphPad Software, San Diego, CA). Student’s t-test or analysis of variance and Student-Newman-Keuls test were used for comparison of the experimental groups. Linear regression analysis was used to determine correlations between measured parameters. In all analyses, P values < 0.05 were considered significant.

RESULTS

Figure 1 shows the time-course data of morphometrically determined changes in lung structural parameters of alveolar Lₜ (Fig. 1A) and Vₚ (Fig. 1B) in the smoke-exposed rats compared with control air-exposed rats. At month 1 of exposure, Lₜ and Vₚ in smoke-exposed and control rats were not significantly different. However, at month 2 of exposure, Lₜ in smoke-exposed rats increased to 123% of the control value (P < 0.05) and Vₚ decreased to 71% of the control value (P < 0.05). These changes continued with exposure duration such that, by month 6 of exposure, Lₜ in smoke-exposed rats had increased to 130% of the control value (P < 0.02) and Vₚ decreased to 51% of the control value (P < 0.01). Figure 2 shows representative histological appearances of the lungs of control air-exposed and smoke-exposed rats. Air space enlargements were clearly evident in the lungs of the smoke-exposed rats.

Figure 3 shows the time course of accompanying changes in the number of neutrophils in the lavage fluid (Fig. 3A) versus that in the lung interstitium (Fig. 3B). At month 1 of exposure, the number of lavage fluid and interstitial neutrophils in the lungs of smoke-exposed rats was increased to 175 and 123%, respectively, of control values (P < 0.01 and 123%, respectively, of control values (P < 0.05, respectively). At months 2–6 of exposure, although the number of lavage fluid neutrophils in smoke-exposed rats remained increased (P < 0.01 vs. the control value), the number of interstitial neutrophils declined to the value in the control rats.

Figure 4 shows the time course of accompanying changes in the number of macrophages in the lavage fluid (Fig. 4A) versus that in the lung interstitium (Fig. 4B). At month 1 of exposure, the number of lavage fluid or interstitial macrophages in the lungs of smoke-exposed rats did not significantly differ from that in control air-exposed rats. However, at month 2 of exposure, the number of lavage fluid and interstitial macro-
phages in the lungs of smoke-exposed rats was increased to 135 and 155%, respectively, of control values (P < 0.05 and P < 0.02, respectively). This increase in the number of lavage fluid and interstitial macrophages in the lungs of smoke-exposed rats remained to month 3 (125 and 171%, respectively, of control values; P < 0.05 and P < 0.01, respectively) and month 6 of exposure (114 and 166%, respectively, of control values; P > 0.05 and P < 0.01, respectively).

Analysis of lung neutrophil-specific in vitro 24-h elastinolytic activity (that is, µg elastin degraded·24 h⁻¹·10⁶ live neutrophils⁻¹) showed that specific elastinolytic activity by lavage fluid neutrophils was identical to that by interstitial neutrophils for each experimental group at the various exposure periods (P = 0.42–0.76). Therefore, the data for the lavage fluid and interstitial neutrophils were combined for comparison between smoke-exposed and air-exposed groups, and these combined data are presented in Fig. 5A. According to the data, at months 1–6 of exposure, specific 24-h elastinolytic activity by lung neutrophils of smoke-exposed rats was not significantly different from that of the control air-exposed rats. However, at month 2 of smoke exposure, the total 24-h elastinolytic potential of the total macrophages in lung was increased to 148% of the control value (P < 0.01); at month 3 of smoke exposure, to 198% of the control value (P < 0.02); and at month 6 of smoke exposure, to 222% of the control value (P < 0.03). Analysis of lung macrophage elastinolytic activity in 72-h cultures (data not shown) also indicated a similar time-course change in the macrophage-specific in vitro 72-h elastinolytic activity with duration of smoke exposure of the rats. As shown in Fig. 6B, the total 24-h elastinolytic potential of total macrophages in the lung (that is, macrophage-specific elastinolytic activity multiplied by the total number of 10⁶ macrophages per rat lung) was unchanged at month 1 of smoke exposure. However, at month 2 of smoke exposure, the total 24-h elastinolytic potential of the total macrophages in lung was increased to 179% of the control value (P < 0.01); at month 3 of smoke exposure, to 198% of the control value (P < 0.005); and at month 6 of smoke exposure, to 222% of the control value (P < 0.005). Note that, at months 2–6 of smoke exposure, total elastinolytic potentials of lung macrophages were significantly higher than those of lung neutrophils (P < 0.02).

Data of elastinolytic activity exerted in vitro by cell-free lavage fluids from the smoke-exposed rats compared with that from the control air-exposed rats
are shown in Fig. 7. According to the data, cell-free
lavage fluid elastinolytic activity of smoke-exposed rats
was 4.5-fold higher than that of control air-exposed rats
at month 1 of exposure (P < 0.001) and remained
increased at months 2–6 of exposure (P < 0.001).
Additional data revealed that smoke-exposed rat cell-
free lavage fluid elastinolytic activity at month 1 of
exposure was 85 ± 5% inhibited by MSAAPVCMK (a
neutrophil elastase inhibitor) compared with 29 ± 6%
inhibition by EDTA (a macrophage metalloelastase
inhibitor), whereas the activity at months 2–6 of expo-
sure was 34 ± 7% inhibited by MSAAPVCMK com-
pared with 69 ± 5% inhibition by EDTA. In contrast,
control air-exposed rat lavage fluid elastinolytic activ-
ity at month 1 of exposure was 49 ± 4% inhibited by
MSAAPVCMK compared with 48 ± 3% inhibition by
EDTA; the activity at months 2–6 of exposure was 47 ±
9% inhibited by MSAAPVCMK compared with 50 ± 3%
inhibition by EDTA.

Figure 8 shows the data of in vivo lung elastin
breakdown in the rats, depicted by changes in the levels
of elastin-derived peptides (Fig. 8A) and desmosine,
a mature elastin unique amino acid, found in the lavage
fluid (Fig. 8B). According to the data, at month 1 of
exposure, the levels of elastin-derived peptides and
desmosine in the lavage fluid of smoke-exposed rats
were similar to the levels in control air-exposed rats.
However, at month 2 of exposure, the smoke-exposed
rat lavage fluid elastin-derived peptide and desmosine
levels increased to 178 and 145%, respectively, of
control values (P < 0.001) and remained increased at
month 3 (176 and 162%, respectively, of control values;
P < 0.001) and month 6 of exposure (159 and 160%,
respectively, of control values; P < 0.001).

Relationships between measured variables in the
smoke-exposed rats were evaluated. The levels of elas-	in-derived peptides or desmosine in the lavage fluid
did not correlate with cell-free lavage fluid elastinolytic
activity [r = 0.11, not significant (NS) and r = 0.13, NS,
respectively] or with lung neutrophil elastinolytic poten-
tial (r = 0.22, NS and r = 0.14, NS, respectively) but
correlated with lung macrophage elastinolytic potential
(r = 0.69, P < 0.01 and r = 0.74, P < 0.01, respectively).
Lm (the morphometric index of air space
enlargement in emphysema) correlated with the levels
of elastin-derived peptides or desmosine in the lavage
fluid (r = 0.40, P < 0.02 and r = 0.49, P < 0.01,
respectively), did not correlate with cell-free lavage
fluid elastinolytic activity (r = 0.09, NS) or lung neutro-
phil elastinolytic potential (r = 0.19, NS) but correlated
with lung macrophage elastinolytic potential (r = 0.67;
P < 0.01), and did not correlate with the number of
lavage fluid (r = 0.09; NS) or lung interstitial neutro-
phils (r = 0.08; NS) or the number of lavage fluid
macrophages (r = 0.29; NS) but correlated with num-
ber of lung interstitial macrophages (r = 0.62; P <
0.01). VVpt (the morphometric index of lung paren-
thyma destruction in emphysema; note that VVpt de-
creases with increasing emphysema) correlated nega-
tively with the levels of elastin-derived peptides or
desmosine in the lavage fluid (r = 0.53, P < 0.01 and
r = 0.67, P < 0.01, respectively) and did not correlate
with cell-free lavage fluid elastinolytic activity (r =
0.12; NS) or lung neutrophil elastinolytic potential (r =
0.33; NS) but correlated negatively with lung macro-
phage elastinolytic potential (r = 0.73; P < 0.01). There
was no correlation between VVpt and the number of
lavage fluid neutrophils (r = 0.10; NS) or lavage fluid
macrophages (r = 0.10; NS), but there was a direct
correlation with the number of lung interstitial neutro-
phils (r = 0.72; P < 0.05) and a negative correlation
with the number of lung interstitial macrophages (r =
-0.68; P < 0.01).
DISCUSSION

Our study in cigarette smoke-exposed rats provides the first known description of the time course of the chronic smoking-induced accumulation of neutrophils versus macrophages in both lung lavage fluid and lung interstitium and describes how this inflammatory response is related to the time course of onset and progression of emphysema lesions in the lungs. Cigarette smoking is the major known cause of emphysema (14). Data from our study clearly show that the onset of neutrophil accumulation in lung lavage fluid and lung interstitium of smoke-exposed rats (month 1 of exposure) preceded the time of onset of an increase in \( L_m \) and a decrease in \( V_{V_{pt}} \) in the lungs of these rats (month 2 of exposure; Fig. 1). Increased \( L_m \) and decreased \( V_{V_{pt}} \) values are established morphometric indexes of air space enlargement and destruction of alveolar walls, respectively (7, 8, 15, 38, 39), the hallmark lesions of emphysema (37). Also, our data show that neutrophil accumulation in the lung interstitium declined during progression of the lung damage (months 3 and 6 of smoke exposure). In contrast, our data clearly show that the onset of macrophage accumulation in lung lavage fluid and lung interstitium of the smoke-exposed rats (month 2 of exposure) coincided with the onset of lung damage, and this macrophage accumulation in the lungs continued during the progression of lung damage.

Fig. 3. Time course of accumulation of neutrophils in lavage fluid (A) and lung interstitium (B) of smoke-exposed (■) and control air-exposed (□) rats. Data are means ± SD; \( n = 6 \) rats/group. *Significant difference compared with corresponding control air-exposed rats, \( P < 0.05 \).

Fig. 4. Time course of accumulation of macrophages in lavage fluid (A) and lung interstitium (B) of smoke-exposed (■) and control air-exposed (□) rats. Data are means ± SD; \( n = 6 \) rats/group. *Significant difference compared with corresponding control air-exposed rats, \( P < 0.05 \).
The degree of accumulation of either neutrophils or macrophages in lung interstitium versus lung lavage fluid is particularly interesting because the interstitial cells, by virtue of their closer proximity to the interstitial connective tissue matrix, would have the greatest potential to play a role in destruction of the alveolar walls. Our data indicated that the accumulation of lung interstitial neutrophils declined at months 2–6 of smoke exposure, whereas the accumulation of lavage fluid neutrophils remained increased. Perhaps neutrophils migrated from the lung vascular beds through the interstitium and into the alveolar spaces without a significant increase in the number of interstitial neutrophils. However, this mechanism does not seem to completely account for the sustained increase in lavage fluid neutrophils in our smoke-exposed rats because comparison of the number of lung interstitial neutrophils with the number of lavage fluid neutrophils does not suggest that significant neutrophils migrated from the interstitium into the lavageable airway spaces. Moreover, it should be noted that bronchoalveolar lavage fluid includes airway neutrophils, not only alveolar neutrophils. Despite these quantitative limitations, we believe that our data of an apparent decline in neutrophil accumulation in the lung interstitium during the onset and progression of smoke-induced lung damage is a real phenomenon. Eidelman et al. (5) and Finkelstein et al. (8) have shown, from morphological data derived at a single time point in surgically resected human lungs of long-time cigarette smokers, that the number of neutrophils in the alveolar septum was inversely related to the extent of parenchymal...
destruction present in the lungs of these smokers. Our time-course data of the accumulation of lung interstitial neutrophils and the relationships to the morphometric indexes of emphysema in our smoke-exposed rats agree with this observation. Interestingly, the data of Finkelstein et al. (8) also showed that the extent of lung parenchymal destruction in the human smokers was directly related to the number of macrophages in the alveolar septum. Our data clearly show that the time course of the accumulation of macrophages in the lung, particularly that of lung interstitial macrophages rather than that of lavage fluid macrophages, coincided with the temporal sequence of the indexes of lung damage in our smoke-exposed rats. Moreover, the morphometric indexes of lung damage in our smoke-exposed rats correlated strongly with the accumulation of lung interstitial macrophages but did not correlate significantly with the accumulation of alveolar macrophages. In essence, data in the present study (Figs. 1–4) and from the other investigators (5, 8) suggest that accumulated macrophages in the lung interstitium (not alveolar macrophages) may be more relevant than neutrophils in the pathogenesis of cigarette smoke-induced emphysema.

Because neutrophils exert a 10-fold higher in vitro elastinolytic activity per cell than the macrophages (4) and individuals with a genetic deficiency of α1-PI, the major inhibitor of neutrophil elastase, also develop emphysema (6), accumulated neutrophils in the lungs of smokers have long been portrayed as the critical pathogenic factor in smoking-induced emphysema (17, 24, 36). However, our data indicated that although rat lung neutrophils exert a 10-fold higher in vitro elastinolytic activity per cell than macrophages, the smoke-exposed rat lung neutrophil-specific in vitro elastinolytic activity (that is, per 10^6 intact neutrophils) was identical to that exerted by control air-exposed rat lung neutrophils throughout the 6-mo exposure duration (Fig. 5A). In addition, the total neutrophil elastinolytic potential in the lung (that is, specific elastinolytic activity multiplied by the total number of 10^6 lavage fluid and interstitial neutrophils per rat lung) of the smoke-exposed rats declined during the onset and progression of lung damage in these rats (Fig. 5B compared with Fig. 1). These data indicate that intact neutrophil-directed elastinolytic activity in the lung

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Fig. 7. Time course of changes in elastinolytic activity exerted in vitro by cell-free lavage fluid of smoke-exposed (■) and control air-exposed (□) rats. Data are means ± SD; n = 6 rats/group. *Significant difference compared with corresponding control air-exposed rats, P < 0.05.

Fig. 8. Time course of changes in levels of elastin-derived peptides (A) and desmosine in lavage fluid (B) of smoke-exposed (■) and control air-exposed (□) rats. Data are means ± SD; n = 6 rats/group. *Significant difference compared with corresponding control air-exposed rats, P < 0.05.
does not seem to be temporally associated with lung damage in our smoke-exposed rats.

Interestingly, lung intact macrophages of our smoke-exposed rats exerted significantly increased in vitro specific elastinolytic activity (that is, per $10^6$ intact macrophages) starting at month 2 of exposure and continuing to month 6 of exposure (Fig. 6A), a time course change similar to that of the onset and progression of lung damage in these rats (Fig. 1). Although Sansores et al. (31) have shown that specific elastinolytic activity of lung macrophages of guinea pigs was significantly increased after a 1.5-mo exposure to cigarette smoke, our study is the first to describe the time course of upregulation of lung macrophage elastinolytic activity in an experimental model of cigarette smoke-induced emphysema. Human studies conducted at a single time point showed that intact alveolar macrophages of long-time chronic smokers with emphysema exert greater specific elastinolytic activity in vitro than those of healthy nonsmokers (23). In our study, rat lung alveolar and interstitial macrophages exhibited similar degrees of in vitro specific elastinolytic activity. This is not surprising because Sansores et al. (31) have shown that guinea pig alveolar and interstitial macrophages have similar specific elastinolytic activities. These observations are important, particularly considering that macrophage elastinolytic activity requires direct contact of the macrophages with the elastin substrate (that is, it is contact dependent), unlike neutrophil elastinolytic activity, which is due to secreted proteinases (4, 33). Because interstitial macrophages are in close proximity to the interstitial connective tissue matrix, the greatly increased number of interstitial macrophages in the lungs of our smoke-exposed rats perhaps created a more relevant total elastinolytic burden on the lung interstitial elastin of these rats. This concept is supported by our observation that the time course of the morphometric indexes of lung damage in our smoke-exposed rats correlated better with the time course of lung interstitial macrophage accumulation than with that of the lavage fluid macrophage accumulation. More interestingly, our smoke-exposed rat lung macrophage total elastinolytic potential was significantly greater than the lung neutrophil total elastinolytic potential (Fig. 5B compared with Fig. 6B), which suggests that the total elastinolytic activity of accumulated macrophages in the lung interstitium may be the more relevant pathogenic factor than the elastinolytic activity of the accumulated neutrophils in lung interstitium in cigarette smoke-induced emphysema.

We considered the possibility that elastinolytic proteinases secreted by neutrophils into the lung extracellular milieu may be more relevant than intact macrophage-mediated elastinolytic activity in the elastinolytic lung damage in our smoke-exposed rats. This is important because Fujita et al. (9) have shown that there is a significant moderate correlation between levels of neutrophil elastase activity in lung lavage fluid and the degree of emphysema in chronic smokers. According to our data (Fig. 7), although the elastinolytic activity exerted by cell-free lavage fluid of the smoke-exposed rats was greatly increased at month 1 of exposure (a time without any lung damage), the increase in cell-free lavage fluid elastinolytic activity remained consistent at months 2–6 of smoke exposure (the period associated with steadily increased lung damage). Moreover, there was no significant correlation between cell-free lavage fluid elastinolytic activity (which is due to secreted elastinolytic activity) of our smoke-exposed rats and the indexes of lung damage in these rats. However, the more noteworthy data were derived by incorporation of inhibitors into our assay of cell-free lavage fluid elastinolytic activity, which revealed that the lavage fluid elastinolytic activity at months 2–6 of smoke exposure was 34% inhibited by MSAAPVCMK [a neutrophil elastase inhibitor (29)] compared with 69% inhibition by EDTA [a macrophage metalloelastase inhibitor (1, 4)]. These data suggested that secreted neutrophil elastase in the lung could not be the critical pathogenic factor for the emphysema in our smoke-exposed rats but that macrophage metalloelastase may be the more prominent elastinolytic factor. This observation agrees with the recent observation by Hautamaki et al. (13), which showed that macrophage elastase-deficient [MME$^{-/-}$] mice subjected to cigarette smoke inhalation did not develop emphysema.

Interestingly, the time course of the increase in in vivo lung elastin breakdown in our study, increased levels of elastin-derived peptides and desmosine in the rat lavage fluid were used as indexes of in vivo lung elastin breakdown. Unlike studies (2, 32) in human smoking-related emphysema, which used levels of elastin-derived peptides in lavage fluid for demonstrating in vivo lung elastin breakdown, we recognized that an increase in lavage fluid elastin peptides could be due to either a breakdown of mature, cross-linked elastin or a newly synthesized immature tropoelastin produced as part of the repair process of damaged elastin. Thus we measured the lavage fluid levels of desmosine because this amino acid is unique to mature elastin. However, we found that the time courses of both indexes of in vivo lung elastin breakdown were similar in our smoke-exposed rats and coincided with the time course of the onset and progression of emphysema lesions in the lungs (Fig. 8 compared with Fig. 1). In addition, the observed time course profiles of the biochemical indexes of in vivo lung elastin breakdown in our smoke-exposed rats (Fig. 8) are in agreement with the time course of a histologically determined decrease in lung elastic fiber length in smoke-exposed guinea pigs observed by Wright and Churg (39). Thus we are convinced that our data of increased levels of elastin-derived peptides and desmosine in the lavage fluid truly reflect the breakdown of lung interstitial elastin in our smoke-exposed rats.

Interestingly, the time course of the increase in in vivo lung elastin breakdown in our smoke-exposed rats was similar to the time course of the increase in the lung macrophage in vitro elastinolytic activity, not the neutrophil elastinolytic activity, of these rats (Fig. 8 compared with Figs. 5 and 6). Although the elastinolytic activity as measured in vitro may not necessarily indicate perfectly what is going on in vivo in the lung, our data suggest that the increase in macrophage
elastinolytic activity has a more likely potential to play a prominent role than neutrophil elastinolytic activity in the onset of lung interstitial elastin breakdown in our smoke-exposed rats. Perhaps neutrophils are important early, setting the elastinolytic process in motion, but the elastinolytic damage does not become measurable until later on in the process when the macrophages become increased and activated. However, it is important to note that the indexes of in vivo lung elastin breakdown correlated more strongly with lung macrophage in vitro elastinolytic activity than with neutrophil elastinolytic activity. Although this relationship may not necessarily be that of cause and effect, it does suggest that lung macrophage elastinolytic activity rather than lung neutrophil elastinolytic activity may be the major contributing elastinolytic factor in our smoke-exposed rats and perhaps in chronic human smokers.

In conclusion, our study has provided the first available data that describe the temporal sequence of cigarette smoke-induced accumulation of neutrophils and macrophages in both lung lavage fluid and lung interstitial and how these are related to the onset and progression of emphysema lesions in the lung. Our data suggest that lung interstitial accumulation of macrophages and their enhanced elastinolytic activity rather than that of neutrophils is more closely associated with the onset and progression of cigarette smoke-induced emphysema.

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