Effects of depletion of neutrophils or macrophages on development of cigarette smoke-induced emphysema

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Ofulue, A. Felix, and Mary Ko. Effects of depletion of neutrophils or macrophages on development of cigarette smoke-induced emphysema. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L97–L105, 1999.—The aim of this study was to ascertain the putative roles of neutrophils or macrophages in the pathogenesis of cigarette smoking-induced emphysema on the basis of effects of anti-neutrophil (anti-PMN) antibody or anti-monocyte/macrophage (anti-MoMac) antibody on the development of emphysema in cigarette smoke-exposed rats. Rats were treated with rabbit anti-PMN or anti-MoMac antibody and exposed 7 days/wk for 2 mo to cigarette smoke inhalation; rats treated with nonimmunized rabbit IgG (control antibody) and exposed to cigarette smoke or normal room air served as controls. Antibody treatments began 24 h before the start of smoke or air exposure and was continued with 1 treatment/wk. Total and differential cell counts in bronchoalveolar lavage fluid and collagenase-dissociated lung and determinations of the elastinolytic activity of lung neutrophils or macrophages in [3H]elastin-coated wells indicated specific suppression of neutrophil accumulation and neutrophil-related elastinolytic burden in the lungs of the anti-PMN antibody-treated smoke-exposed rats, in contrast to specific suppression of macrophage accumulation and macrophage-related elastinolytic burden in the lungs of the anti-MoMac antibody-treated smoke-exposed rats. Cigarette smoke exposure-induced lung elastin breakdown (quantitated by immunologic assay of levels of elastin-derived peptides and desmosine in lavage fluid) and emphysema in the lungs (based on morphometric analysis of alveolar mean linear intercepts and alveolar tissue density in fixed lungs) were not prevented in the lungs of anti-PMN antibody-treated smoke-exposed rats but was clearly prevented in lungs of the anti-MoMac antibody-treated smoke-exposed rats. These findings implicate macrophages rather than neutrophils as the critical pathogenic factor in cigarette smoke-induced emphysema.

anti-neutrophil antibody; anti-monocyte/macrophage antibody; lung elastin breakdown

PULMONARY EMPHYSMA is a chronic disease characterized by destruction of the alveolar walls, with subsequent abnormally permanent enlargement of the respiratory air spaces (33). Progressive breakdown of elastin in the lung parenchyma is a key feature in the pathogenesis of emphysema (3). The major known cause of emphysema is cigarette smoking (11), but the initiating elastinolytic factor in smoking-induced emphysema is still not clear. The other known cause of emphysema is a genetic deficiency of α1-proteinase inhibitor, an inhibitor of neutrophil elastase (5). In α1-proteinase inhibitor deficiency-associated emphysema, lung elastin breakdown is undoubtedly triggered by the unopposed action of neutrophil elastase due to the insufficient levels of its inhibitor in the bronchoalveolar epithelial lining (15, 23, 32). In cigarette smoking-associated emphysema, there is debate whether increased macrophage and/or neutrophil elastinolytic activity within the alveolar matrix, resulting from a smoking-induced accumulation of macrophages and neutrophils in the lung, may be responsible for the elastinolytic damage (15, 23, 32). A direct relationship between the extent of smoking-induced emphysema and the number of macrophages, but not of neutrophils, in the lung parenchyma has been shown by morphological data derived at a single time point from surgically resected lungs of human smokers (4, 8), which suggests that macrophages may be more important than neutrophils in the pathogenesis of cigarette smoking-induced emphysema.

In earlier study, Ofulue et al. (24) have shown that the time course of lung elastin breakdown and development of emphysema in cigarette smoke-exposed rats correlated with an increase in macrophage-related elastinolytic burden in the lung but not with the neutrophil-related elastinolytic burden. The aim of the present study was to undertake a direct evaluation of the roles of neutrophils and/or macrophages in cigarette smoke-induced emphysema. The approach was to use an anti-neutrophil (anti-PMN) or an anti-monocyte/macrophage (anti-MoMac) antibody to specifically suppress accumulation of neutrophils or macrophages in the lungs of cigarette smoke-exposed rats and investigate the effects on cigarette smoke-induced lung elastin breakdown and the development of emphysema.

MATERIALS AND METHODS

Anti-PMN and anti-MoMac antibodies. In preliminary studies, rats were treated with a rabbit anti-rat PMN or anti-rat macrophage (anti-Mac; both from Accurate Chemical & Scientific, Westbury, NY) antibody. We discovered that the anti-PMN antibody was effective in reducing the number of neutrophils in the lungs but that the anti-Mac antibody did not result in a sustained reduction of macrophages in the lung. Apparently, the anti-Mac antibody reacts only with mature macrophages at sites of tissue damage that are then rapidly removed and replaced by bloodborne monocytes that do not react with the anti-Mac antibody (7). Thus we produced an anti-MoMac antibody.

Monocytes from peripheral blood and macrophages from bronchoalveolar lavage (BAL) fluid and peritoneal fluid, obtained from adult Sprague-Dawley rats, were isolated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia Chemicals, Dorval, PQ) according to the method of Boyum (1). The mononuclear cells (monocytes or macrophages and
lymphocytes) were collected from the interface, washed twice in phosphate-buffered saline (PBS), resuspended in PBS, and added to Sepacel-MN (a colloidal silica-based medium from Sepratech, Oklahoma City, OK), and the separation of monocytes or macrophages from lymphocytes was achieved by centrifugation (upper band was monocytes or macrophages; lower band was lymphocytes) essentially as described by Vissers et al. (35). The final monocyte or macrophage preparations (≥97% purity) contained ≤3% contaminating lymphocytes. Neutrophils were isolated from the bottom layer of the Ficoll-Hypaque centrifugation after removal of the mononuclear cells and were separated from the erythrocytes by hypotonic lysis of the erythrocytes as described by Boyum (1). This method yielded neutrophils of ≥98% purity with no contaminating monocytes, macrophages, or lymphocytes. The purity of all isolated cells was ascertained by standard morphological criteria in Diff-Quik-stained cytospin preparations. The isolated rat monocytes/macrophages or neutrophils (≥99% viability) were then used to immunize New Zealand White rabbits for the production of polyclonal anti-rat MoMac or anti-rat PMN antibody. The rabbits were immunized with four weekly subcutaneous injections of a 1:1 monocyte/macrophage mixture (1 × 10^6 total cells) or 1 × 10^6 neutrophils in Freund's complete adjuvant (Difco, Detroit, MI); booster injections in Freund's incomplete adjuvant (Difco) were repeated at monthly intervals. Blood was obtained from the immunized rabbits at 4 mo, and the serum was separated and decompiments by heating at 56°C for 40 min. Each antiserum was absorbed with rat erythrocytes and lymphocytes as described by Rehm et al. (26). Specificity of the anti-MoMac sera was enhanced by further absorption with rat neutrophils, whereas specificity of the anti-PMN sera was enhanced by further absorption with rat monocytes and macrophages. All cells used for the absorption procedure were ≥97% pure. Each antiserum IgG fraction was isolated by protein A-Sepharose (Pharmacia) affinity chromatography, concentrated by ultrafiltration (Amicon, Beverly, MA), and sterilized by passage through 0.45- and 0.02-µm membrane filters (Millipore, Bedford, MA). The final anti-MoMac IgG or anti-PMN IgG was divided into 2-ml aliquots and stored at −20°C. Control IgG antibody (control Ab) was obtained from serum of nonimmunized same-stock rabbits and processed identically.

The anti-MoMac and anti-PMN IgGs were judged for cytotoxicity against monocytes, macrophages, neutrophils, and lymphocytes (based on uptake of a 0.1% trypan blue solution). Briefly, 2.5 × 10^5 cells (each cell type ≥97% purity) were incubated at 37°C for 30 min with various dilutions of anti-MoMac antibody, anti-PMN antibody, or control Ab in Hank's solution and 10% inactivated autologous rat serum and 10% fresh rabbit serum as complement source, and the cells were examined for uptake of trypan blue in a hemocytometer. Anti-MoMac antibody dilutions of 1:32 to 1:512 resulted in the death of >48% of the monocytes or macrophages but of ≤0.5% of the neutrophils or lymphocytes. Anti-PMN antibody dilutions resulted in the death of ≥55% of the neutrophils but of ≤1% of the monocytes, macrophages, or lymphocytes. Control Ab dilutions did not result in the death of >1% of any of the cells examined.

Experimental protocol. Twenty-four specific pathogen-free male Sprague-Dawley rats (littermates; 286 ± 13 g initial body wt; Charles River Breeding Laboratories, Guelph, ON) were divided into four equal groups: 1) rats exposed to normal room air and treated with control Ab; 2) rats exposed to cigarette smoke and treated with control Ab; 3) rats exposed to cigarette smoke and treated with anti-PMN antibody; and 4) rats exposed to cigarette smoke and treated with anti-MoMac antibody. Treatment with control Ab, anti-PMN antibody, or anti-MoMac antibody (1.0 mg·0.2 ml⁻¹·rat⁻¹ ip; saturating concentration as determined by preliminary experiments) was started 24 h before the initiation of cigarette smoke or air exposure and was continued every Monday at 0800. Exposure to cigarette smoke (10 nonfiltered cigarettes·rat⁻¹·day⁻¹) or normal room air was 7 days/wk with smoke- or air-generating delivery machines and head-only exposure chambers (1 rat/chamber) as previously described (24). Blood was taken from the tail vein every Wednesday and Saturday at 0800 and 1600 for total and differential counts of peripheral blood leukocytes. Blood carboxyhemoglobin levels within 2 h after smoke- or air exposure were 11.9 ± 1.4% (SD) in the control Ab smoke-exposed rats, 11.7 ± 1.5% in the anti-PMN antibody smoke-exposed rats, 12.0 ± 1.7% in the anti-MoMac antibody smoke-exposed rats (no significant difference between smoke-exposed groups), and 0.9 ± 0.3% in the air-exposed control rats (P < 0.001 compared with the smoke-exposed groups), confirming successful smoke inhalation by the smoke-exposed rats. After each exposure session, the rats were housed in individual metabolic cages in pathogen-free quarters maintained at constant humidity and temperature and were provided with water for ad libitum consumption. All rats received and consumed the same amount of food daily (8 g food pellet·100 g body wt⁻¹·day⁻¹·rat⁻¹). Body weight of the rats was measured daily. The gram body weight increases per rat per day were 1.1 ± 0.1 (SD) for air-exposed control rats, 1.0 ± 0.2 for the control Ab smoke-exposed rats, 0.8 ± 0.3 for the anti-PMN antibody smoke-exposed rats, and 0.9 ± 0.3 for the anti-MoMac antibody smoke-exposed rats (no significant difference between the groups). At 2 mo of smoke or air exposure, ~15 h after the last exposure session, the rats were killed by anesthetization with pentobarbital sodium (50 mg/kg body wt ip) and exsanguination from the abdominal aorta.

Quantitation of neutrophils and macrophages in lungs. BAL fluid neutrophils or macrophages were quantitated essentially as previously described (24). Briefly, BAL was performed immediately after each rat was killed, and recovery of the instilled fluid was >91%, without a significant difference between the four rat groups. Each recovered BAL fluid was centrifuged (500 g for 10 min at 4°C), the resultant supernatant (cell-free BAL fluid) was decanted into polystyrene tubes and stored at −70°C for subsequent analysis, and the pelleted cells were washed three times in Dulbecco's modified Eagle's medium containing 2 mmol glutamine/l, 100 µg penicillin/ml, and 100 U streptomycin/ml (DMEM; GIBCO BRL, Grand Island, NY) and resuspended in 1.5 ml of DMEM. Total and differential cell counts per rat BAL fluid were determined in triplicate and used to calculate the number of BAL fluid neutrophils or macrophages per rat lung.

Lung interstitium neutrophils or macrophages were quantitated from collagenase-DNase-digested right lung tissues according to Lavnikova et al. (19) essentially as described in the previous study by Ofuole et al. (24). Briefly, duplicate 350 mg lung slices/rat were dissociated by treatment in DMEM containing 175 U collagenase/ml, 50 U DNase/ml, and 10% fetal bovine serum, and the interstitium cells were obtained by filtration and centrifugation. These are optimal conditions needed to obtain a consistent maximal yield of cells (13, 19) and were confirmed by preliminary studies. The isolated lung interstitium cells were washed three times in DMEM and resuspended in 3.5 ml of DMEM. Total and differential cell counts were determined in triplicate and used to calculate the number of interstitium neutrophils or macrophages per rat.
lung. Data were projected to both lungs on the basis of lung weight.

Analysis of neutrophil or macrophage elastinolytic activity in lungs. Neutrophils and macrophages in BAL fluid or lung interstitium populations were immediately fractionated with Ficol-Hypaque (1). The isolated neutrophils or macrophages (≥89% viable as judged by exclusion of a 0.1% trypan blue solution) were washed, resuspended in DMEM, added to [3H]elastin-coated wells, incubated at 37°C for 24 h, and examined for elastinolytic activity essentially as previously described (24, 25). Elastinolytic activity is reflected by the micrograms of [3H]elastin degraded and is expressed as described (24, 25). Elastinolytic activity per 10^6 neutrophils or macrophages = (elastinolytic activity/10^6 neutrophils or macrophages) × (total millions of neutrophils or macrophages in BAL fluid + lung interstitium/rat lung).

Evaluation of cell-free enzyme activities in BAL fluid. Elastinolytic activity exerted by cell-free BAL fluid was determined in [3H]elastin-coated wells as previously described (24). Briefly, aliquots of cell-free BAL fluid were dialyzed against distilled water at 4°C, lyophilized, resuspended in DMEM, and added to [3H]elastin-coated wells. After 72 h of incubation at 37°C, the medium was assayed by β-scintillation for degraded [3H]elastin, and the results are expressed in units per rat total BAL fluid volume, where 1 unit of activity is equivalent to 1 μg of [3H]elastin degraded per 72 h.

Myeloperoxidase (MPO) activity in cell-free BAL fluid was quantitated by spectrophotometry (2). Briefly, aliquots of cell-free BAL fluid were diluted with an equal volume of 1.0% hexadecyltrimethylammonium bromide and combined with 0.5 mM o-dianisidine and 0.3 mM hydrogen peroxide in 50 mM phosphate buffer (pH 6.0) at 25°C, and the change in absorbance of the reaction mixture at 460 nm was determined. MPO activity is expressed in units per rat total BAL fluid volume, where 1 unit of activity is defined as that degrading 1 micromole of hydrogen peroxide per minute.

Estimation of in vivo lung elastin breakdown. Levels of elastin-derived peptides and desmosine (an amino acid unique to mature elastin) in BAL fluid were measured as indexes of in vivo lung elastin breakdown essentially as previously described (24). Briefly, elastin-derived peptides were estimated by an enzyme-linked immunosorbent assay (29), and the results are expressed in nanograms of elastin-derived peptides per milligram of albumin. Albumin content was determined by reaction with bromocresol green (28), with rat albumin (both from Sigma, St. Louis, MO) as the standard. Desmosine in BAL fluid was extracted through Whatman CF11 cellulose (31) and quantitated by an enzyme-linked immunosorbent assay (18), and the results are expressed in nanomoles of desmosine per rat total BAL fluid volume.

Determination of emphysema in lungs. Standard morphometric technique (17, 34) was used to determine the presence of emphysema in the lungs as previously described (24). Briefly, 5-μm sections were cut from midsagittal blocks of paraffin-embedded fixed left lungs and stained with hematoxylin and eosin, and 20 randomly selected fields were sampled 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. The number of intersections of alveolar walls by the test lines was counted and used to quantitate alveolar mean linear intercept (Lm, the average distance between alveolar walls); increasing Lm was taken as evidence of air space enlargement (6, 34). The number of test points falling on alveolar tissue (Pm) in relation to the reference lattice (Pt) was also counted and used to determine alveolar tissue density (Vv, expressed in percent); decreasing Vv was taken as evidence of lung parenchyma destruction (6, 34). All quantitations were corrected for tissue shrinkage during processing.

Statistical analysis. All data are expressed as means ± SD. Data were analyzed by analysis of variance (ANOVA) and Student-Newman-Keuls test with Prism-StatMate software (GraphPad Software, San Diego, CA). P values ≤ 0.05 were considered significant.

RESULTS

Table 1 shows the effects of the antibodies on peripheral blood leukocytes. The cigarette smoke exposure-induced increase in the number of neutrophils in the peripheral blood was reduced in the anti-PMN antibody smoke-exposed rats (= 53% of the value in the air-exposed control rats), whereas the number of monocytes was similar to that in the control Ab smoke-exposed rats. In contrast, the cigarette smoke exposure-induced increase in the number of monocytes in the peripheral blood was reduced in the anti-MoMac antibody smoke-exposed rats (= 50% of the value in the air-exposed control rats), whereas the number of neutrophils was similar to that in the control Ab smoke-exposed rats. The anti-PMN and anti-MoMac antibodies also reduced the number of lymphocytes in the peripheral blood, but the degree of the effects was identical.

Table 2 shows the effects of the antibodies on lung accumulation of neutrophils and macrophages in the rats. The cigarette smoke exposure-induced increase in the number of neutrophils in BAL fluid and lung interstitium was reduced in the anti-PMN antibody smoke-exposed rats (less than or equal to the value in the air-exposed control rats), whereas the number of macrophages was similar to that in the control Ab smoke-exposed rats. In contrast, the smoke exposure-induced increase in the number of macrophages in BAL fluid and lung interstitium was reduced in the anti-MoMac antibody smoke-exposed rats (less than or equal to the value in the air-exposed control rats), whereas the number of neutrophils was similar to that in the control Ab smoke-exposed rats.
in the control Ab smoke-exposed rats. The anti-PMN and anti-MoMac antibodies did not alter the number of lymphocytes in the BAL fluid or lung interstitium in the smoke-exposed rats.

Table 3 shows the accompanying changes in elastolytic activity of the lung neutrophils or macrophages. Elastolytic activity per 10^6 neutrophils was not altered by cigarette smoke exposure, but the elastolytic activity per 10^6 macrophages was increased, and these conditions remained in the anti-PMN and anti-MoMac antibody smoke-exposed rats. Total elastolytic activity per lung by the neutrophils or macrophages (that is, elastolytic activity per 10^6 neutrophils or macrophages × total millions of neutrophils or macrophages per rat lung) was affected. The cigarette smoke exposure-induced increase in neutrophil total elastolytic potential per lung was reduced in the anti-PMN antibody smoke-exposed rats (less than or equal to the value in the air-exposed control rats), whereas macrophage total elastolytic potential per lung was similar to that in the control Ab smoke-exposed rats. In contrast, the cigarette smoke exposure-induced increase in macrophage total elastolytic potential per lung was reduced in the anti-MoMac antibody smoke-exposed rats.

Table 3. Elastolytic activities of lung neutrophils versus macrophages

<table>
<thead>
<tr>
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<th>Elastolytic Activity/10^6 cells</th>
<th>Elastolytic Activity/Lung</th>
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<tbody>
<tr>
<td></td>
<td>Neutrophils</td>
<td>Macrophages</td>
</tr>
<tr>
<td>Control</td>
<td>49.3 ± 6.8*</td>
<td>4.8 ± 0.9*</td>
</tr>
<tr>
<td>CigSmoke</td>
<td>52.2 ± 8.8*</td>
<td>6.1 ± 1.0†</td>
</tr>
<tr>
<td>CigSmoke + Anti-PMN</td>
<td>51.7 ± 9.1*</td>
<td>5.9 ± 1.2†</td>
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<tr>
<td>CigSmoke + Anti-MoMac</td>
<td>50.9 ± 9.3*</td>
<td>6.5 ± 0.9</td>
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Values are means ± SD; n = 6 rats/group. One unit of elastolytic activity is 1 µg [3H]elastin degraded/24 h in vitro by live cells; n = 6 rats/group. For each parameter measured, values of groups with different symbols are significantly different (P < 0.05) by ANOVA and SNK test.
treatment of cigarette smoke-exposed rats with anti-PMN antibody reduced the cigarette smoke-induced increase in the number of peripheral blood neutrophils and the number of neutrophils in BAL fluid and lung interstitium without affecting blood monocytes or lung macrophages (Tables 1 and 2). Because of the cytotoxic nature of the anti-PMN antibody (see Materials and Methods), we suspect that the anti-PMN antibody must have destroyed a greater part of the excess peripheral blood neutrophils induced by smoke exposure and thus limited the number of circulating neutrophils available for recruitment into the lungs of anti-PMN antibody smoke-exposed rats. The reduced number of lung neutrophils in these rats could not be due to destruction of neutrophils within the lung as judged by MPO activity in cell-free BAL fluid. MPO, an enzyme abundant in azurophil granules of neutrophils but relatively absent in macrophages, is released extracellularly by activated neutrophils during phagocytosis or after lysis of neutrophils as a result of necrosis (2). Thus the presence of MPO in cell-free BAL fluid reflects the presence of active phagocytosis and degranulation by neutrophils and/or active destruction of neutrophils within the lung (2, 36). In this context, we reasoned that if the anti-PMN antibody treatment was causing neutrophil destruction within the lungs of anti-PMN...
antibody smoke-exposed rats, their cell-free BAL fluid MPO activity should increase more than that in the control Ab smoke-exposed rats. However, our data showed that MPO activity in cell-free BAL fluid of the anti-PMN antibody smoke-exposed rats was significantly lower than that in the control Ab smoke-exposed rats and comparable to that in air-exposed control rats (Table 4). Thus we are convinced that the reduced number of lung neutrophils in the anti-PMN antibody smoke-exposed rats could not be due to destruction of neutrophils within the lung.

An anti-rat MoMac antibody was developed to investigate the role of macrophages in our rat model of smoke-induced lung damage. It is well known that the accumulated macrophages in the lungs of smokers originate from circulating blood monocytes that migrate into the lung (14). Moreover, monocytes and macrophages seem to differ in the nature and degree of cell-surface receptors (7, 14). These characteristics may account for the success of our anti-MoMac antibody to reduce the smoke-induced increase in the number of peripheral blood monocytes and lung macrophages without affecting blood neutrophils or lung neutrophils (Tables 1 and 2). A conventional anti-Mac antibody was not a reliable tool for our study because it did not result in a sustained reduction of macrophages in the lung. Another study (7) has shown that an anti-Mac antibody reacts only with mature tissue macrophages that are often removed at the sites of tissue damage and rapidly replaced by circulating monocytes from peripheral blood. Because of the cytotoxic nature of the anti-MoMac antibody (see MATERIALS AND METHODS), it is very likely that the anti-MoMac antibody treatment must have destroyed a large part of the smoke-induced excess monocytes in the peripheral blood and thus limited the number of circulating monocytes available for recruitment into the lungs of anti-MoMac antibody smoke-exposed rats. Because the cell-free BAL fluid MPO activity in the anti-MoMac antibody smoke-exposed rats was not increased more than that in the control Ab smoke-exposed rats (Table 4), it is reasonable to assume that the anti-MoMac antibody treatment was not causing destruction of neutrophils within the lungs of the anti-MoMac antibody smoke-exposed rats.

The anti-PMN or anti-MoMac antibody did not affect the number of lung lymphocytes (Table 2), although they did suppress the number of peripheral blood lymphocytes (Table 1). These findings are consistent with data from another study (22) that has shown a lack of correlation between alterations in blood lymphocytes and those in BAL fluid. It is known that blood lymphocytes do not necessarily reflect lymphocyte changes in organs and/or tissues (37). Systemic lymphopenia after administration of anti-PMN or anti-Mac antibodies has been described in various animal studies (16, 30). This lymphopenia cannot likely be due to destruction of circulating lymphocytes because our anti-PMN or anti-MoMac antibody was not cytotoxic to lymphocytes (see MATERIALS AND METHODS). The anti-PMN and anti-MoMac antibody-induced systemic lymphopenia seem to be an immunologically insignificant response to cell-specific antibodies (21, 30). Relevant to our study is the fact that the anti-PMN or anti-MoMac antibody did not affect the number of lung lymphocytes because there is speculation that lung lymphocytes may play a role in cigarette smoking-related emphysema (8). In this regard, our data demonstrate that the anti-PMN or anti-MoMac antibody is a reliable tool for investigating the role of neutrophils or macrophages in our model of cigarette smoke-induced lung damage.

The suppression of neutrophil accumulation in the lungs of anti-PMN antibody smoke-exposed rats was accompanied by a significant reduction in the cigarette smoke-induced increases in the intact neutrophi-
mediated elastinolytic burden in the lung (Table 3). But our data showed that the anti-PMN antibody smoke-exposed rats and control Ab smoke-exposed rats had similar increased levels of elastin-derived peptides and desmosine in BAL fluid (Fig. 1) and similar increased $L_m$ and decreased $V_{V Pt}$ values (Fig. 3). Increases in the levels of elastin-derived peptides and desmosine in BAL fluid are indexes of increased in vivo lung elastin breakdown (24, 29), a critical component in the development of emphysema (15, 23, 32). Increased $L_m$ and decreased $V_{V Pt}$ are morphometric indexes of air space enlargement and destruction of the alveolar wall, respectively (6, 8, 34), criteria accepted as indicating emphysema (33). On this basis, our data provide direct evidence that intact neutrophil-mediated elastinolytic mechanisms could not be the critical pathogenic factor for emphysema in our cigarette smoke-exposed rats. Perhaps it is important to note that the anti-PMN antibody used to define the role of neutrophils did not prevent an accumulation of macrophages in the lungs (Table 2). These macrophages with their resultant elastinolytic burden in the lung were fully operative in the anti-PMN antibody smoke-exposed rats (Table 3) and thus could be responsible for lung elastin breakdown in our smoke-exposed rats even in the presence of lung neutrophil depletion.

The suppression of macrophage accumulation in lungs of anti-MoMac antibody smoke-exposed rats was accompanied by a significant reduction in cigarette smoke-induced increases in intact macrophage-mediated elastinolytic burden in the lung (Table 3). Interestingly, our data showed that the anti-MoMac antibody smoke-exposed rats had levels of elastin-derived peptides and desmosine in BAL fluid and values of $L_m$ and $V_{V Pt}$ similar to those in the air-exposed control rats (Figs. 1 and 3), which indicate the prevention of smoke-induced lung damage in the anti-MoMac antibody smoke-exposed rats despite the fact that the intact neutrophil-mediated elastinolytic burden was still increased in the lungs of these rats (Table 3). These findings indicate that the intact macrophage-mediated elastinolytic burden in the lung could be responsible for lung elastin breakdown and emphysema in our smoke-exposed rats even in the presence of excess neutrophils. Our study did not attempt to identify the enzyme(s) responsible for the intact macrophage-mediated elastinolytic activity, but macrophage metalloelastase and 92-kDa gelatinase B are strong candidates. Hautamaki et al. (10) have shown that macrophage metalloelastase-deficient [MME(-/-)] mice subjected to cigarette smoke inhalation did not develop emphysema. Finlay et al. (9) have found an increase in the 92-kDa gelatinase B expression by alveolar macrophages from emphysema patients. Cysteine proteinases may also be involved in the intact macrophage-mediated elastinolytic activity because increased expression of cathepsins L and S have been shown in alveolar macrophages of cigarette smoke-exposed rats and human smokers (20, 27).

It is not clear whether cell-free BAL fluid elastinolytic activity participates in cigarette smoke-induced elastinolytic lung damage. The preceding report by Ofulue et al. (24) showed that the time course of increase in cell-free BAL fluid elastinolytic activity in cigarette smoke-exposed rats did not correlate with the time course of elastinolytic lung damage in these rats. In the present study, the smoke-induced increase in cell-free BAL fluid elastinolytic activity was reduced in both the anti-PMN and anti-MoMac antibody smoke-exposed rats (Table 4), yet only the anti-PMN antibody smoke-exposed rats showed evidence of elastinolytic lung damage and emphysema (Figs. 1–3). This observation supports the contention that the lung damage in the anti-PMN antibody smoke-exposed rats was not due to neutrophil lysis and the resultant release of a potent elastinolytic factor(s). A complete reduction in the smoke-induced increase in cell-free BAL fluid elastinolytic activity in the anti-PMN antibody smoke-exposed rats indicated that neutrophils were the major source of an elastinolytic factor(s) in the alveolar spaces of our smoke-exposed rats. The partial reduction in the cigarette smoke-induced increase in cell-free BAL fluid elastinolytic activity in the anti-MoMac antibody smoke-exposed rats indicated that macrophages contribute a released elastinolytic factor(s) in the alveolar spaces of our smoke-exposed rats. The macrophage-related elastinolytic factor(s) accounts for the cell-free BAL fluid elastinolytic activity in the anti-PMN antibody smoke-exposed rats and these rats developed emphysema, which suggests that macrophage-related cell-free BAL fluid elastinolytic activity may possibly be a relevant factor in cigarette smoke-induced elastinolytic lung damage and emphysema.

Data from present study clearly implicate intact macrophage-mediated elastinolytic activity as the likely critical pathogenic factor in cigarette smoke-induced elastinolytic lung damage. This finding strengthens the preceding report by Ofulue et al. (24) that showed that the time course of lung elastin breakdown and development of emphysema in cigarette smoke-exposed rats correlated significantly with an influx of macrophages and an increase in macrophage-mediated elastinolytic burden in the lung but did not correlate with an influx of neutrophils and an increase in neutrophil-mediated elastinolytic burden in the lung. Although a smoking-induced influx of neutrophils and an increased neutrophil-dependent elastinolytic burden in the lung have long been viewed as the critical pathogenic factor for the breakdown of lung parenchymal elastin and consequent development of emphysema in chronic smokers (15, 23, 32), data from present study now clearly raise awareness that macrophage-mediated elastinolytic activity may be more important than that of neutrophils in the pathogenesis of smoking-induced emphysema. Our findings (24; present study) have relevance to emphysema in human smokers because the extent of emphysema in surgical lungs of human smokers has been shown to be unrelated to the number of neutrophils in the alveolar septum but directly related to the
number of macrophages in the alveolar septum (4, 8). We do recognize that reconciling data in cigarette smoke-exposed rats with what happens in human smokers has to be done with caution because not every smoker develops emphysema. Perhaps what we are observing in smoke-exposed rats is the initial response in human smokers that may be taken over by an unsuccessful repair process and sustained lung damage over longer periods of time in some smokers.

In conclusion, data from the present study show that suppression of neutrophil influx and neutrophil-mediated elastinolytic burden in the lungs of cigarette smoke-exposed rats failed to prevent lung elastin breakdown and emphysema in these rats. In contrast, suppression of macrophage influx and macrophage-mediated elastinolytic burden in the lungs prevented lung elastin breakdown and emphysema in smoke-exposed rats. These findings provide direct evidence that cigarette smoke-induced lung elastin breakdown and consequent emphysema may not be neutrophil dependent but is more likely macrophage dependent.

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