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Lung

The Importance of Ultramicroscopic Emphysema in Cigarette Smoke-Induced Lung Disease

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Abstract. To determine the role of the alveolar pores in cigarette smoke-induced lung disease, we examined the alveolar pores of guinea pigs exposed to cigarette smoke for 12 months, and compared these data to those obtained from sham-smoked animals, correlating the data with airspace size and lung function. We found that the smoke-exposed animals had a larger mean number of pores per alveolus ($p < 0.001$), and the distributions of pore size and shape were significantly shifted to indicate a larger and more irregular pore configuration ($p < 0.001$, 01 respectively). In the smoke exposed group, there was a significant correlation of pore number with total lung capacity (TLC) (0.68 $p < 0.05$), RV (0.70, $p < 0.05$), and $FEV_{0.1}/FVC$ (−0.77, $p < 0.02$). No correlations were identified between pore size or shape and the lung function tests. We conclude that cigarette smoke exposure produces an increase in the number of alveolar pores, a process which we believe represents ultramicroscopic emphysema. These alterations appear to precede any increase in airspace size, and may help to explain abnormal lung function in cigarette smokers without macroscopic emphysema or small airways disease. This is the first study to clearly document an increased number of alveolar pores, with a significant number of either/or large and irregular pores, after chronic smoke exposure, but in the absence of gross emphysema.

Key words: Pores of Kohn—Emphysema—Scanning electron microscopy—Cigarette smoke.

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Introduction

Emphysema has been defined as "a condition of the lung characterized by abnormal, permanent enlargement of the airspaces distal of the terminal bronchioles, accompanied by destruction of their walls, and without obvious fibrosis" [14]. Although gross parenchymal destruction has become the hallmark in the recognition of emphysema, definition of the earlier phases of emphysema, prior to the formation of actual emphysematous spaces, continues to be difficult.

Alteration of the alveolar pores of Kohn have long been considered as a possible site for early lung destruction, but because they are normal anatomic structures, the differentiation between normality and abnormality has been difficult [7]; the terms "fenestrae" or "holes" have been used when abnormalities were thought to be present. Boren [1] examined thick sections of human lungs embedded in plastic, and concluded that holes in alveolar walls more than 20 μm in diameter were abnormal and were evidence of destruction. In a study using scanning electron microscopy, Nagai et al. [8] suggested that this value could be reduced to 10 μm. As might be expected, however, the size of pores is species dependent, and there may also be an aging effect [5, 11].

It has been difficult to attribute pulmonary function test alterations solely to either emphysematous lung destruction or to smoke-induced airway alterations. Loss of lung elastic recoil can occur both with and without the presence of grossly recognizable emphysema [9, 17]. In addition, Hogg et al. [4] demonstrated that macroscopic emphysematous holes were less compliant than the adjacent lung tissue. Thurlbeck [16] suggested that alteration of the scleroproteins in the lung and emphysema could be independent, with the changes in scleroprotein being primarily responsible for increasing the maximum distensibility of the lung, a suggestion supported by the theoretical work of Laros and Kuyper [6].

In his discussion of these issues, however, Thurlbeck [17] noted that the pulmonary function alterations could also be explained by subtle morphological changes. Cosio et al. [2] and Nagai et al. [8] examined the scanning ultrastructure of the alveolar pores in the lungs of human smokers. Although different sampling methodology was utilized, both groups demonstrated correlations between measurements of the pores and pulmonary function tests (see Discussion).

We have developed a guinea pig model of cigarette smoke-induced lung disease [19] in which chronic exposure to cigarette smoke produced increases in lung volumes, increased compliance, and decreased airflow. No gross emphysema could be identified in these lungs, although morphometric analysis demonstrated enlargement of in airspace size due to an increase in the chord length of both the alveoli and alveolar ducts. In the present study, we used scanning electron microscopy to examine the alveoli, and assessed whether chronic exposure to smoke would alter the alveolar pore number or size, and to determine whether these parameters would correlate with the measurements of lung function.

Methods

We utilized archival lung tissue and pulmonary function data from guinea pigs which had been exposed to cigarette smoke, or had a sham smoke exposure, for a period of 12 months [19].

Animals

The experimental animals consisted of Hartley strain female guinea pigs which weighed approximately 350 g at the onset of smoke or sham smoke exposure and weighed between 850 and 1000 g at the end of the exposure period.

Smoke Exposure

The smoke exposure was performed according to our usual protocol [19]; in brief, this consisted of the smoke of five nonfiltered cigarettes delivered into a nose-only chamber in aliquots of 20 ml per min. These conditions are sufficient to produce a carboxyhemoglobin of between 5 and 10%, values similar to those present in human cigarette smokers [10].

Pulmonary Function Testing

At the end of the 12-month exposure period, the animals were anesthetized and detailed pulmonary function tests were performed, including pressure volume curves with calculations of lung volumes and static compliance. A detailed description is provided in reference [19]. In brief, a 4.0 mm internal diameter polyethylene cannula was placed in the trachea, and a 10 cm long, water-filled catheter with multiple side holes was inserted into the esophagus to measure pleural pressure. Boyle's law was used to measure FRC, and lung volumes were obtained by inflation to +30 cm of H_2O pressure, designated as TLC, followed by slow deflation to –30 cm H_2O, designated as RV. Static compliance (Cst) was measured between 0 and 15 cm H_2O transpulmonary pressure. Flow measurements were performed by inflation of the lungs to +30 cm H_2O, followed by a rapid deflation to –30 cm H_2O. $FEV_{0.1}$ was calculated as the volume exhaled in the first 0.1 sec, and FVC was calculated from the flow volume curve.

Morphometric Analysis

After sacrifice, the lungs were fixation inflated to a standard pressure of 25 cms H_2O, followed by sectioning in the sagittal plane, paraffin embedding, and histological sectioning. The histological sections were examined morphometrically [19] to determine airspace size, calculating the mean linear intercept (Lm) [15].

Scanning electron microscopy samples were obtained from a parasagittal slice and were postfixed in buffered glutaraldehyde followed by buffered osmium. The lung tissue was then rinsed in distilled water, dehydrated with progressive alcohol rinses, followed by critical point drying. After placement on the tissue stub holders, the tissue was gold coated in a splutter coater. Specimens from 6 sham-smoked and 7 smoke-exposed animals were suitable for examination.

We attempted to obtain approximately 50 random alveoli, with selection based only on the presence of a relatively completed alveolus with a viewpoint perpendicular to the alveolar base. These were photographed at 1500× and printed on 8.5 × 11 inch paper, to a give a total magnification of 2830×. The photographs were measured in a blinded fashion. All alveolar pores that could be identified in an alveolus were counted, but detailed measurements were performed only on the alveolar pores on the base of the alveolus, thus eliminating errors due to projection angle. Alveolar pores that were obscured by macrophages were counted, but not measured unless a rim of pore could be identified around the macrophage. Each measurement included perimeter, area, and shape factor. The latter measurement

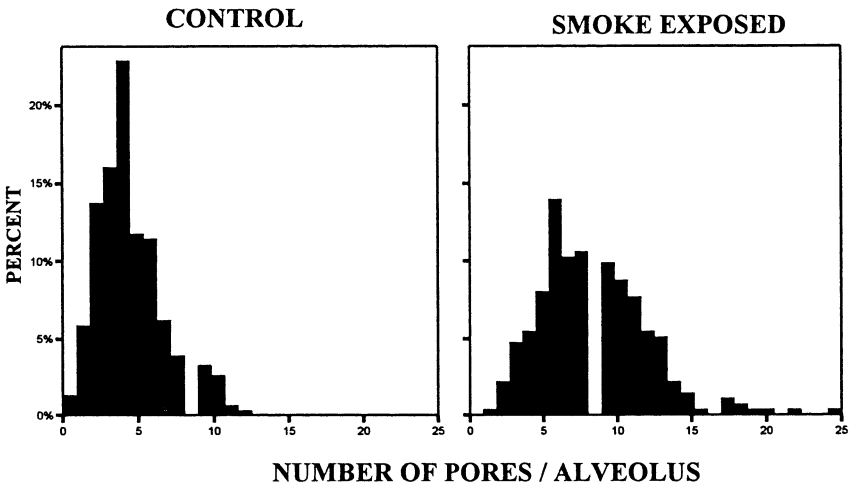

Fig. 1. Total distribution of the number of alveolar pores in each alveolus in control (sham smoke-exposed) and smoke-exposed animals. The smoke-exposed animals have a broader distribution with a marked shift to the right indicating an increased number of pores per alveolus ($p < 0.001$).

provides an indication of the shape of the pores and utilizes the formula 4 area/perimeter^2 (Bioquant^{TM} System IV, Nashville, Tennessee, USA). A shape factor of 1 would indicate a perfect circle, with fractional numbers indicating greater degrees of irregularity.

Statistical Analysis

Analyses were performed using the SYSTAT statistical analysis software [18]. The overall distribution of the number of holes in each alveolus, and the individual measurements of each hole were examined using a two-sample Kolmogorov-Smirnov test.

To determine correlations with airspace size or pulmonary function, we used the mean value for the numbers of holes, since these data were normally distributed. The areal data were not normally distributed, but a normal distribution could be obtained after logging the data; we therefore used a geometric mean. The shape data were not normally distributed as raw data, nor as logged or square root data; we therefore used the median value as being most representative. Statistical significance was assessed by linear regression with Pearson correlation.

Results

We counted alveolar pores in 306 alveoli from the six sham-exposed animals, and 273 alveoli from the seven smoke-exposed animals. The discrepancy in numbers is due to the necessity to discard photomicrographs in the smoke-exposed animals because macrophages obscured the field of vision. The control animals had 4.4 ± 2.3 pores per alveolus compared with 8.4 ± 3.7 pores in the alveoli from the smoke-exposed animals. Figure 1 illustrates the distribution of the numbers of pores in each alveolus, and shows the marked shift of the curve to the right in the smoke-exposed animals ($p < 0.001$), indicating the overall increase in pore number.

Measurements were made on 1072 pores from the sham-exposed animals, and on 1848 pores from the smoke-exposed animals. Figure 2 illustrates the distri-

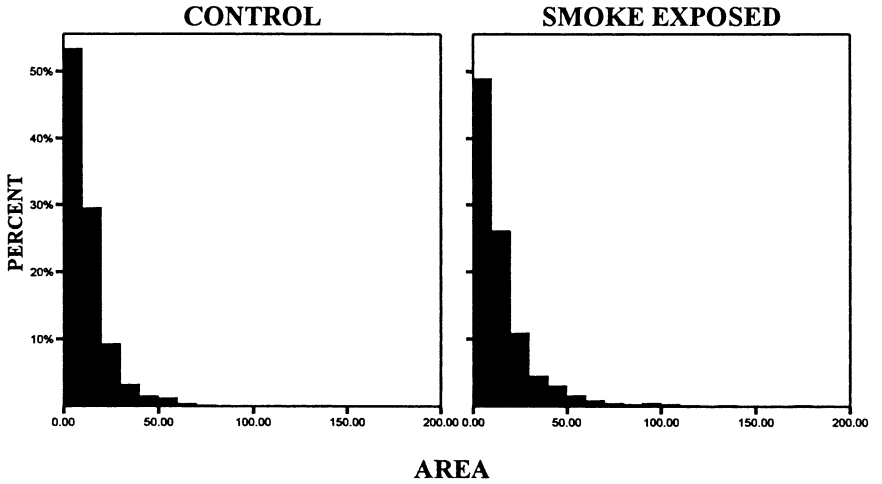

Fig. 2. Total distribution of the area of the individual pores in each alveolus (expressed in μ^2) in control (sham smoke-exposed) and smoke-exposed animals. The smoke-exposed animals have a distribution shifted to the right, accompanied by a long tail, indicating that many of the pores are larger than the range found in the control animals ($p < 0.001$).

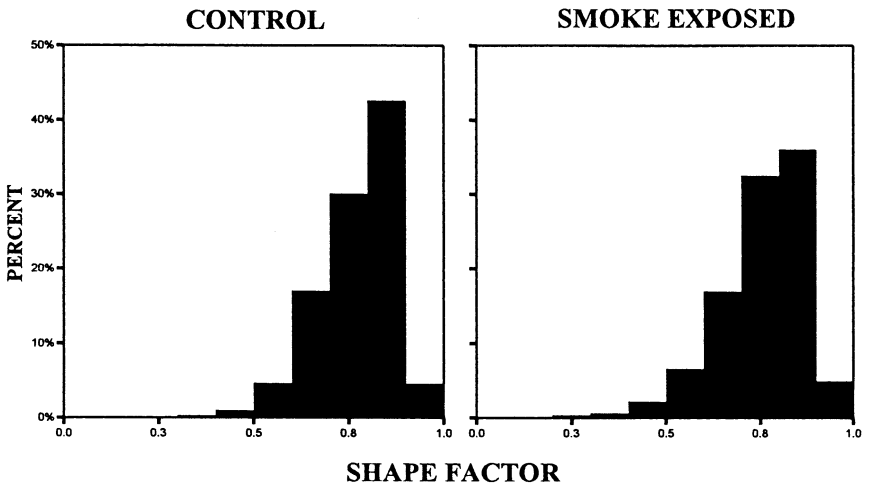

Fig. 3. The total distribution of the shape factors obtained from measurements of the individual pores in each alveolus in control (sham smoke-exposed) and smoke-exposed animals. The smoke-exposed animals have a distribution shifted to the left, indicating that there is an increased population of irregularly shaped pores in the smoke-exposed animals ($p < 0.01$).

bution of the pore area (expressed in microns^2), and demonstrates the shift of the curve to the right in the smoke-exposed animals ($p < 0.001$). The smoke-exposed animals had approximately 10% of pores with an area of greater than 40 μm^2, compared with only 4% of pores in the control animals. The distribution curves for the shape of the pores are shown in Figure 3. The curve from the

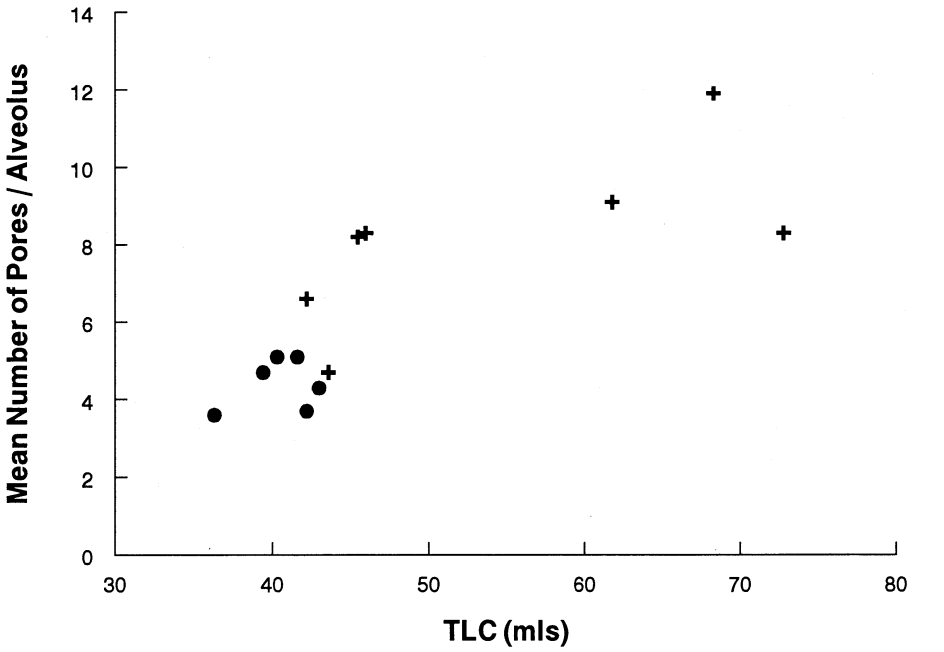

Fig. 4. Relationship between the mean number of pores per alveolus and the total lung capacity (expressed in ml). The overall correlation is significant ($r = 0.80$, $p < 0.001$). Correlation using only the smoke exposed animals is significant ($r = 0.68$, $p < 0.05$), although the wide confidence limits suggestion caution in interpretation. The control animals are represented by the filled circles, the smoke-exposed animals are represented by the crosses.

smoke-exposed animals is slightly shifted to the left ($p < 0.01$). The sham-exposed animals had approximately 65% of the pores with a shape factor of 0.75 or greater and approximately 6% of pores with a shape of factor of 0.60 or less. By contrast, the smoke-exposed animals had approximately 59% of pores with a shape factor of 0.75 or greater and approximately 10% with a factor of 0.60 or less. Smoke-exposed animals, therefore, had a greater degree of irregularity in their shape, whereas those from the control animals tended towards a more rounded configuration.

We did not find any correlations between the geometric mean area or the median shape of the pores and any of the pulmonary function test parameters. There were no correlations between the median shape and the geometric mean area of the pores, nor were there any correlations between either shape or area and numbers of pores. Neither the median shape nor the geometric mean area of the pores correlated with the airspace size (Lm). However, mean number of pores per alveolus correlated significantly (r, confidence interval, p value) with the TLC (0.80, 0.45–0.94, $p < 0.001$) (Fig. 4), FRC (0.81, 0.46–0.94, $p < 0.001$), RV (0.80, 0.43–0.94, $p < 0.001$), Cst (0.62, 0.10–0.87, $p < 0.05$), and $FEV_{0.1}/FVC$ (−0.71,

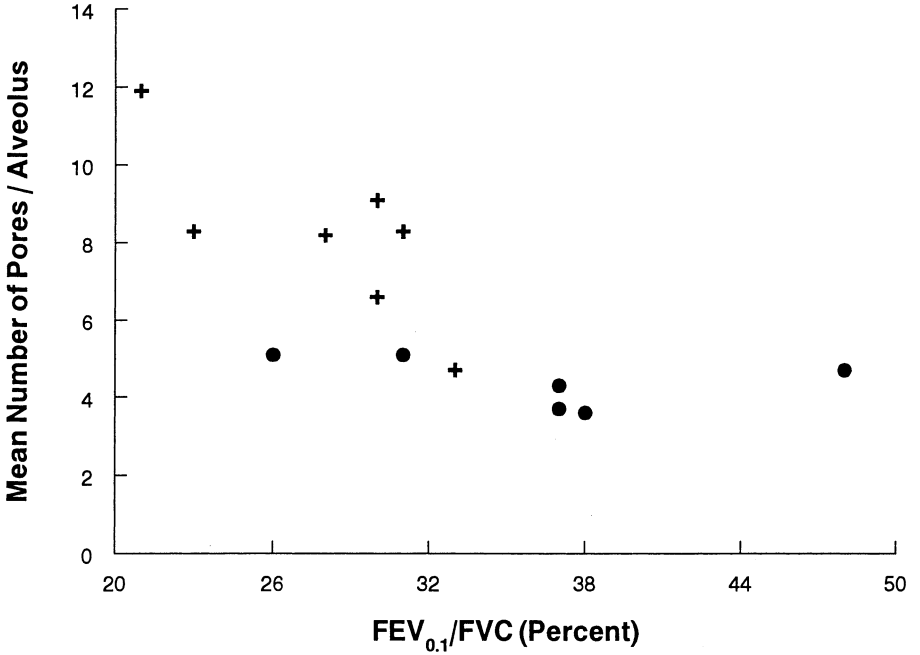

Fig. 5. Relationship between the mean number of pores per alveolus and the $FEV_{0.1}/FVC$ (expressed in percent). The overall correlation is significant ($r = -0.71$, $p < 0.001$). Correlation using only the smoke-exposed animals is significant ($r = -0.77$, $p = 0.02$), although the wide confidence limits suggest caution in interpretation. The control animals are represented by the filled circles, the smoke-exposed animals are represented by the crosses.

0.26–0.91, $p < 0.01$) (Fig. 5). In addition, the mean number of pores per alveolus correlated significantly with airspace size (0.74, 0.31–0.92, $p < 0.01$) (Fig. 6). Significant correlations between Lm and pulmonary function test were also observed [TLC (0.83, 0.52–0.95, $p < 0.001$), FRC (0.90, 0.70–0.97, $p < 0.001$), RV (0.83, 0.52–0.95, $p < 0.001$), Cst (0.70, 0.25–0.90, $p < 0.001$), and $FEV_{0.1}/FVC$–0.71, 0.26 −0.91, $p < 0.01$)].

When only the data from the smoke-exposed animals were examined, the mean number of pores per alveolus continued to significantly correlate with TLC (0.68, 0.15–0.95, $p < 0.05$), RV (0.70, 0–0.95, $p < 0.05$), and $FEV_{0.1}/FVC$ (−0.77, 0.05–0.96, $p = 0.02$), whereas those with FRC (0.62), and Cst (0.56) did not attain significance, p values 0.07, 0.19 respectively. Although statistically significant, the wide confidence limits of these correlation suggest that they be interpreted with caution. The correlation between mean numbers of pores per alveolus and Lm (0.57) was not significant ($p = 0.18$). Significant correlations between Lm and pulmonary function tests continued to be found [TLC (0.88, 0.36–0.98, $p < 0.01$), FRC (0.96, 0.72–0.99, $p < 0.001$), and RV (0.91, 0.51–0.99, $p < 0.01$)], whereas those with Cst (0.54), and $FEV_{0.1}/FVC$ (−0.55) did not reach significant p values 0.08, 0.20 respectively.

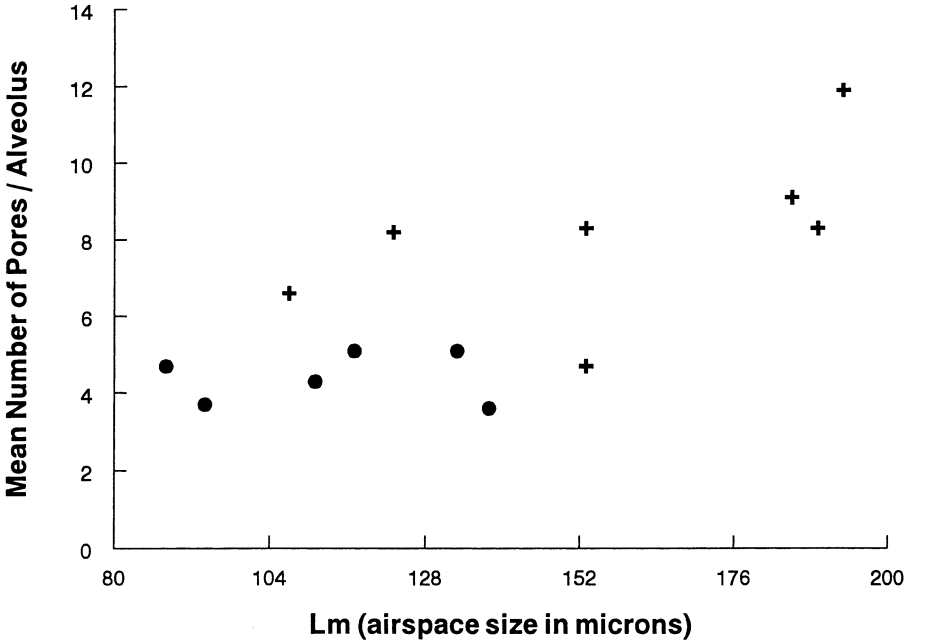

Fig. 6. Relationship between the mean number of pores per alveolus and the morphometric indicator of airspace size (Lm, expressed in microns). The overall correlation is significant ($r = 0.74$, $p < 0.01$). Correlation using only the smoke-exposed animals is not significant ($r = 0.57$, $p = 0.18$). The control animals are represented by the filled circles, the smoke-exposed animals are represented by the crosses.

Discussion

The present study demonstrates that chronic exposure to cigarette smoke is associated with an increase in the number of alveolar pores, and these pores are larger and more irregular then those found in an age-matched control guinea pig population. This is the first time that irregularity of pores has been clearly documented. The results suggest that pores do not increase in size from progressive dilatation of existing pores, but rather form from a destructive process.

The study further suggests that it is the number, rather than the size or shape, of the alveolar pores that appears to be an important correlate with lung function. Our data, therefore, are in general agreement with that of Nagai et al. [8], who examined human lungs that had gross evidence of emphysema, and non-emphysematous lungs, predominately from nonsmokers. These workers did not identify differences in the number of pores per alveolus when they compared the non-emphysematous lungs to areas in the emphysematous lungs which were distal to identifiable emphysema. Significant differences were found, however, when comparisons were made with areas between grossly emphysematous foci. Also in contrast to the present study, they identified correlations between area of pores, in addition to number of pores, with RV. However, similar to our study, they found that only number of pores correlated with FRC and Pl_{max}, and it did so in the

lung parenchyma both between and distant from grossly emphysematous lung lesions.

The study by Cosio et al. [2] examined the area rather than the number of pores. They found a correlation between the area of the pores near the terminal airway and airspace size as measured by the Lm; such a correlation did not exist with the peripheral airspaces. There was a greater area of holes in the center of the lobule, and they concluded that early lung destruction was concentrated in the proximal portion of the acinus. Since the mean hole area in the center of the lobule correlated with Pl_{90}, MMEF, and FEV_1, they suggested that alteration of the lung parenchyma in the center of the lobule might be important in the disturbances of pulmonary function found in smokers.

In comparing the present study in guinea pigs to those performed in humans, it is important to recognize that, as noted in the Introduction, this animal model of smoke-induced lung disease does not have grossly identifiable (macroscopic) emphysema. Furthermore, the increase in airspace size appears to be due to an increase in the mean chord length of both the alveolar ducts and alveoli [19]. This would suggest that in our model, the alveolar parenchymal alteration (destruction) is present both in the center and in the periphery of the lobule. Nevertheless, we do demonstrate that smoke exposure is associated with an increased overall number of pores, and a shift toward larger and more irregular pore configuration. Thus, although making no comment regarding the importance of serine versus metalloelastase, our data provide support to a destructive process such as that resulting from an imbalance between proteases and anti-proteases. Finally, we also demonstrate similar correlations between alterations in the alveolar pores and lung volumes, and airflow to those found in the human studies.

It is possible that the increase in alveolar pore number and size documented in this study is analogous to the "destructive index" described initially by Saetta et al. [12], and modified by Eidelman et al. [3]. Saetta et al. suggested that alveolar wall destruction occurred prior to the increase in airspace size, although this conclusion was disputed by the study of Saito et al. [13] who found that the two indices appeared to advance in parallel. In the present study, we did not find a correlation between pore area and Lm, and the relationship between pore number and Lm was only significant when both control and smoke animals were included in the analysis. However, examination of Figure 6 suggests that three of the four smoke-exposed animals, which have an Lm roughly in the range of the control animals, had an increase in the number of holes. Furthermore, only one of the three animals with the largest Lm had an obvious additional increase in number of holes. Although a definite conclusion would require analysis of a greater number of animals, this pattern would suggest that the increased numbers of pores preceded the increase in airspace size, rather than both occurring in concert. Thus, although it is likely that the alveolar breaks that constituting the DI_b portion of the destructive index in the two-dimensional microscopic sections are formed from the alveolar pores which we have identified using scanning ultramicroscopy, it appears that it is the number rather than the size of these abnormal pores that is the important feature.

In his Christie Lecture [17], Thurlbeck discussed the possible explanations for the loss of elasticity found in lungs with and without emphysema. Although macroscopic emphysema would seem an intuitive candidate, the data of Hogg et al. [4], and the fact that there was no gross emphysema present in the lungs, clearly eliminates the possibility of macroscopic destruction in the present study. It does not, however, eliminate the second possibility, which suggests that it is the alteration of the parenchyma because of the presence of multiple smaller holes in the alveoli that is important in the increase of lung compliance found in our model. We have titled the increased numbers of pores "ultramicroscopic emphysema" in recognition of this potential. However, the lack of significant correlation between the numbers of pores and airspace size in the smoke-exposed animals does not support this hypothesis, but rather suggests that the increase in airspace size is occurring independently, although it may be preceded by, and may or may not progress concurrently with an increase in alveolar pores. This pattern of progression would be in keeping with the third alternate hypothesis, namely, that airspace enlargement is due to alterations in the lung matrix [6] rather than its direct destruction.

Finally, it is important to reconcile the findings of the present study with other analyses of this lung model. In a study that examined the alveolar capillary network in the lungs of guinea pigs chronically exposed to cigarette smoke [20], we documented enlargement of the capillary ring areas, but could not find any evidence for capillary destruction. We considered that these findings were most consistent with a relatively homogenous alteration of the lung structure, with airspace enlargement rather than significant alveolar wall destruction. The increased numbers of alveolar pores must, therefore, be situated at the level of the capillary ring. This suggestion would be in accordance with the findings described in a very early study by Macklin [7] in which he noted that the pores appeared to occur as a result of atrophy of the ground substance near the center of the capillary mesh. This explanation would also be in keeping with the hypothesis that it is the lung matrix that is important, and the holes are only a visual clue to the disorganization within the matrix as a result of cigarette smoke-induced damage.

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