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# Lung deformation and macrophage displacement in smoke-exposed and normal mice (*Mus musculus*) following different fixation procedures

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**Summary.** Lung deformation (shrinkage or inflation) and displacement of pulmonary parenchymal macrophages were evaluated after immersion fixation, intratracheal instillation of fixative and lung lavage followed by intratracheal fixative instillation in cigarette smoke-exposed, sham-treated and control pallid male mice. Lung volume displacement and lung section and alveolar area analysis revealed that degree of deformation was uniform in lungs from all treatment groups fixed by immersion but not by instillation of fixative and fixative instillation following lavage. In situ pulmonary parenchymal macrophage number per lung section area of fixative-instilled lungs and lavaged lungs followed by fixative instillation was significantly greater than in those following immersion fixation in all corresponding treatment groups. A paucity of macrophages was noted in airways of fixative-instilled and lavaged followed by instillation of fixative lungs. Pulmonary macrophages were uniformly distributed throughout lung parenchyma following immersion fixation, while in fixative-instilled and lavaged prior to instillation of fixative lungs these cells tended to be concentrated in alveoli near terminal bronchioles. Lavage procedures removed an unknown portion of lung macrophages and appeared to ineffectively sample the pulmonary parenchymal macrophage population. Intratracheal instillation of fixative with or without prior lavage apparently alters the distribution of pulmonary macrophages by displacing airway phagocytes into the alveoli. Data reported suggest that fractional estimates of in situ lung parenchymal macrophage population can be obtained by counting the number of these cells per area of tissue from lungs fixed by immersion.

**Key words:** Tissue fixation – Lung – Lung deformation – Macrophage displacement – *Mus musculus*

## Introduction

Evaluating the role of pulmonary parenchymal macrophages (septal and alveolar) in development of pulmonary fibrosis following cigarette smoke inhalation (Matulionis 1984; Matulionis and Simmerman 1985; Matulionis et al. 1985) requires assessment of in situ lung phagocyte numbers and macrophage ultrastructure from the same lung. Macrophages can be easily quantified at the light microscopic level by counting these cells per unit area of lung tissue fixed by immersion adjacent to regions sampled for electron microscopy. However, during this type of tissue preparation differential shrinkage and/or collapse (deformation) of lungs could occur as a result of conditions induced by different experimental treatments (i.e., smoke exposure vs. sham manipulation and control). This in turn would influence macrophage number per lung area in animals of the different treatments groups. An alternative fixation method is intratracheal fixative instillation. Under strictly controlled conditions (Hayatdavoudi et al. 1980; Mazzone et al. 1980) such procedures give well-fixed, uniformly inflated lungs. However, intratracheal instillation of fixative may cause displacement of lung macrophages (Brain et al. 1984). Pulmonary macrophage numbers are often evaluated in fluids obtained by lung or bronchoalveolar lavage (Daniele et al. 1985). This procedure, however, does not account for interstitial macrophages and contribution of cells from airways (Rossman et al. 1981), especially during airway inflammatory reactions caused by smoking or infection (Daniele et al. 1985).

In view of the above, this study was designed to determine in smoke-exposed, sham-treated and control animals: 1) whether differential deformation (inflation or shrinkage) of lungs occurs following immersion fixation as a result of abnormal con-

ditions induced by cigarette smoke inhalation, 2) the degree of pulmonary macrophage displacement by intratracheal instillation of fixative, and 3) whether lung lavage procedures can be used to obtained cells for estimating the pulmonary parenchymal macrophage population size.

## Materials and methods

Fifty-four 5- to 6-month-old pallid (pa) male mice (*Mus musculus*) were used in this study. The animals were housed, maintained and monitored for disease in environmentally controlled quarters at the University of Kentucky Tobacco and Health Research Institute as described earlier (Matulionis 1984). Eighteen mice were exposed to cigarette smoke from 2R1 Reference cigarettes (32.9 mg tar; 2.19 mg nicotine per cigarette) (University of Kentucky Tobacco and Health Research Institute) for five months, once in the a.m. and once in the p.m. seven days per week (Matulionis 1979, 1982). Smoke exposure was carried out in the Kentucky smoking machine (Matulionis et al. 1985) and consisted of eight to ten 35-ml puffs of 100% smoke diluted to 20% with air. The "puffs" of smoke lasted for 2 s and were delivered at the rate of one/min. The diluted smoke from each puff remained in the smoke inhalation chamber for 15 s after which the chamber was flushed with fresh air. The fresh air was left in the chamber for 45 s before the next puff exposure. A similar number of animals were sham-treated (Matulionis 1979, 1982), and 18 additional mice served as absolute controls. Sham treatment was carried out in machines never before used for smoke exposures and, except for the substitution of fresh air for smoke, the sham-treated mice were manipulated exactly as the smoke-treated animals. Lungs of six mice from each of these groups were fixed by immersion (I), by intratracheal instillation of fixative (FI) and by intratracheal instillation of fixative following lung lavage (LFI). The mice were anesthetized by intraperitoneal injection of sodium pentobarbital (The Butler Co., Columbus, Ohio, USA) 50 mg/kg body weight), weighed, exsanguinated by severing the abdominal aorta and the lungs autopsied. Lungs designated for immersion fixation were obtained as follows. The thoracic cavity was opened by removing the anterior aspect of the rib cage and the left-lung root was ligated with a silk suture. Lungs were removed from the chest via the suture after cutting the lung root medial to the ligature and placed in a 3.5% cacodylate buffered (pH 7.4) glutaraldehyde fixative. Care was taken not to touch the lungs during these procedures. Lungs were divided into portions for light and electron microscopy and displacement volume determined in the glutaraldehyde fixative as described below.

Lavage of lungs was accomplished as follows. The trachea was cannulated with an Intramedic R Luer-End Catheter, No. 7532, (Clay Adams; Parsippany, NJ, USA) secured to the trachea and attached to a 10-ml syringe containing 7 ml of calcium- and magnesium-free phosphate-buffered saline. A small cut was made in the diaphragm. One milliliter of saline was slowly infused manually and allowed to remain in the lungs for 30 s. The catheter was then disconnected from the syringe and the saline was allowed to drain by gravity into a centrifuge tube positioned 20 cm below the animal. This procedure was repeated seven times. Tubes containing lavage fluid were then placed in an ice bath. Approximately 85% of the instilled fluid was recovered. Total number of cells in pulmonary lavage fluid was determined using standard hemocytometer blood cell procedures. Cell differentials were obtained on a basis of identification of 500 cells at a magnification of 1,000 × using a Honeywell ACS 1000 cell counter (Honeywell, Hopkins, MA, USA). The total number of cells was converted arithmetically to the number of neutrophils, lymphocytes, and macrophages present in the lavage fluid (total cell number × differential %).

Intratracheal instillation of fixative (3.5% cacodylate buffered pH 7.4 glutaraldehyde) was achieved via the catheter used for lavage or via one installed as above into the trachea of animals whose lungs were infused with fixative but not lavaged. The fixative was placed into a 10-ml syringe and positioned 20 cm above the animal's chest. The diaphragm was opened and the fixative allowed to fill the lungs within the chest cavity by gravity. Flow rate of the fixative was not measured. Fixative instillation was maintained until the lungs stopped expanding. At this point the rib cage was removed, the root of the left lung ligated with a silk suture and the lung removed by way of the ligature after cutting the root medial to the suture.

Left-lung displacement volume was determined by immersing the lungs into a 5-ml plastic syringe filled with 3.5% cacodylate-buffered (pH 7.4) glutaraldehyde fixative. The fluid was adjusted to the 5-ml mark prior to immersion of tissue. A 1-ml syringe (calibrated to 0.01 ml) was attached to the larger syringe with a 3-way stopcock. Fluid displaced by the lungs was drawn into the 1-ml syringe and adjusted exactly to the 5-ml mark of the larger syringe. Fluid displaced by the lungs could be measured at 0.005-ml increments. All lungs were weighed down by identical lead spheres attached to the silk suture tied around the lung root. The volume of the suture and lead spheres was subtracted from lung plus suture and lead sphere values. Following determination of the whole left-lung volume, lungs were immersed into the glutaraldehyde fixative and transected into a cephalic 2/3 and caudal 1/3 portions. Subsequently the volume of the cephalic 2/3 piece was determined. Glutaraldehyde fixative was used to determine lung volume and to dissect the lungs in order that fixation of tissue for electron microscopy not be delayed.

Fixation for light microscopy of all the cephalic 2/3 lung pieces, (i.e., those designated for immersion fixative, those instilled with glutaraldehyde and those lavaged first followed by fixative instillation) was continued for 72 h by immersion in 10% neutral buffered formalin, a fixative better suited for large tissue pieces. These portions of lung were in the glutaraldehyde fixative for 2–3 min. Lung volume was again determined following formalin fixation and after dehydration and clearing (just prior to tissue embedment into paraffin). All lung displacement volume values were normalized by body weight to eliminate inherent lung volume differences resulting from different sizes of animals.

The cephalic lung segments, following paraffin embedment, were serially sectioned at 4-μm thickness through approximately 360 μm of tissue starting at the inferior border of the cephalic 2/3 of the lung. One slide containing six to nine sections was prepared from the beginning, middle and end of the serial sections and stained with a periodic acid-Schiff (PAS) reagent, hematoxylin (Matulionis 1984). PAS-hematoxylin stained macrophages magenta which clearly distinguished these cells from all other cells. All lung sections were cut from identical locations in relation to the external pulmonary surface.

Tissue samples for ultrastructural examination were consistantly obtained from the same location, i.e., from two 1-mm slices of the caudal 1/3 of the left lung. Slices were subsequently dissected into 18 to 22 1-mm cubes. Fixation of 14 randomly selected cubes was continued by immersion in the glutaraldehyde fixative and processed for electron microscopy according to procedures described elsewhere (Matulionis 1975).

Cross-sectional areas of four or five lung sections at the beginning, middle and end of sampling region, prepared for light microscopy, were measured and calculated using area-

**Table 1.** Pulmonary lavage cell population

| Treatment | Total cell counts[a] | Differentials (%)[b] | | | Number[a] | | |
|---|---|---|---|---|---|---|---|
| | | PMN[c] | Lym[d] | Mac[e] | PMN[c] | Lym[d] | Mac[e] |
| Smoke | 28.43±1.63 | 55.7±5.4 | 3.7±0.8 | 40.54±5.2 | 16.1±2.2 | 1.1±0.25 | 11.3±1.0 |
| Sham | 11.06±1.31 | 16.7±4.3 | 5.2±2.8 | 76.0±4.9 | 2.0±0.7 | 0.5±0.3 | 8.3±0.9 |
| Control | 8.89±1.78 | 6.1±0.7 | 6.2±1.1 | 87.6±1.3 | 0.5±0.07 | 0.6±0.2 | 7.8±1.5 |

[a] Mean±one standard error of the mean $\times 10^4$
[b] Mean±one standard error of the mean
[c] neutrophils; [d] lymphocytes; [e] lavagable macrophages

perimeter analysis on the Zeiss Video Plan Image Analysis System (Carl Zeiss Inc., Thornwood, NY, USA). Alveolar area and maximum diameter were determined in five microscopic fields (at 250×) that covered approximately 60% of the lung section by the same procedure and instrument as above. Ninety to 120 alveoli were assessed per each animal. Tissue used for these determinations was also used to quantify the number of pulmonary macrophages (alveolar and septal) per area of lung tissue (Matulionis and Simmerman 1985).

To negate the influence of inflation resulting from instillation of fluids on *in situ* macrophage numbers per area of lung tissue sections, the numbers of phagocytes in intratracheally fixative-instilled (FI) and lavaged followed by fixative instillation (LFI) lungs were normalized by sectional areas of lungs fixed by immersion (I). Since lung macrophage numbers were determined per unit area of lung tissue (Matulionis and Simmerman 1985), such normalization was necessary because otherwise the density of parenchymal macrophages in fluid-instilled lungs would be artificially decreased since they would be distributed over a larger area. Thus, macrophage numbers (Mn) for each animal of a particular treatment group (smoke-exposed, sham-treated, controls) were adjusted by calculating $\frac{A_{FI} \text{ or } A_{LFI} \times M_n}{A_I}$ where $A_{FI}$ and $A_{LFI}$ is the sectional area of fixative-instilled and lavaged followed by fixative instillation lungs, respectively, of individual animals from a particular treatment group and $A_I$ is the mean sectional area of lungs fixed by immersion from animals subjected to similar treatments.

Numerical data were evaluated statistically using the $F$ test (analysis of variance) and Fisher's multiple comparison procedures. A $P$ value of $\leq 0.05$ was required for significance.

## Results

The general health of animals during the 5-month study was good. Mice were free of Sendai virus and *Mycoplasma pulmonis* infections at the onset and duration of the experiment. Mortality rate was minimal and attributed to natural or explainable causes. Body weights of smoke-exposed (25.8±0.5 gm) and sham-treated (25.9±0.15 gm) animals were similar though significantly ($P=0.004$) lower than those of absolute controls (29.2±1.28 gm).

Total number of lung lavagable cells was significantly larger ($P=0.0001$) in smoke-exposed mice than in sham-treated and control animals (Table 1). Neutrophils comprised the largest portion of lavagable cells in the smoke-exposed group of animals while macrophages were most abundant in sham and control mice (Table 1). However, macrophage numbers were significantly larger ($P=0.05$) in lung lavage fluid of smoke-exposed mice than in those of the control but not ($P=0.08$) the sham group (Table 1). Similar numbers of neutrophils were present in lavage fluid of sham and control animals ($P=0.4$). Lymphocytes were more numerous, though not significantly, in lavages of smoke-treated mice than in those of sham and control mice (Table 1).

Displacement volume of the entire left lung prior to formalin fixation was largest from animals subjected to pulmonary lavage followed by intratracheal instillation of glutaraldehyde (LFI) and smallest in lungs designated for immersion fixation (I) (Table 2). Lung volume following instillation of glutaraldehyde (FI) was between values of the two above groups (Table 2). Cephalic (superior) 2/3 portion lung volume was influenced by LFI, FI and I procedures similarly to the entire lung (Table 2). Following formalin fixation, shrinkage of LFI lungs occurred while FI and I lung volume increased (Table 2). After tissue processing, the superior 2/3 lung volume of LFI and FI animal lungs was variable over treatment while that of I lungs was uniform, although the latter was significantly ($P=0.0001$) smaller than the former lungs (Table 2). Considerable shrinkage (43 to 55%) occurred in all lungs during dehydration and clearing of tissue.

Lung section area of paraffin-embedded tissues fixed by only immersion was similar ($P\geq 0.21$) in smoke-exposed, sham-treated, and control mice (Fig. 1). Sectional area of lungs subjected to FI and LFI in all animals was significantly larger ($P=0.0001$) than that of lungs fixed by immersion alone (Fig. 1). Lung section areas of smoke-exposed FI, sham-treated FI and control LFI animals were similar ($P\geq 0.39$) but significantly ($P\leq 0.03$) smaller than those of smoke-exposed LFI, sham-treated LFI and control FI mice

**Table 2.** Lung Displacement Volume (mean ± one standard error of the mean) of smoke-exposed, sham-treated, and control animals after immersion fixation (I), intratracheal fixative instillation (FI) and lavage followed by intratracheal fixative instillation (LFI)

| Treatment | Whole left lung | Lung displacement volume[a] (CC) | | |
|---|---|---|---|---|
| | | Superior 2/3 left lung | Post fixation | Post processing |
| Smoke-exposed | | | | |
| I-lung | 0.1269 ± 0.004 | 0.0814 ± 0.004 | 0.1145 ± 0.007 | 0.0489 ± 0.002 |
| FI-lungs | 0.2974 ± 0.014 | 0.2182 ± 0.012 | 0.2521 ± 0.009 | 0.1294 ± 0.003 |
| LFI lungs | 0.5120 ± 0.021 | 0.3570 ± 0.010 | 0.3257 ± 0.022 | 0.1620 ± 0.010 |
| Sham treated | | | | |
| I-lungs | 0.1215 ± 0.006 | 0.0786 ± 0.005 | 0.1082 ± 0.004 | 0.0499 ± 0.007 |
| FI-lung | 0.2915 ± 0.018 | 0.1954 ± 0.016 | 0.2423 ± 0.013 | 0.1345 ± 0.005 |
| LFI-lungs | 0.4811 ± 0.028 | 0.3262 ± 0.016 | 0.2811 ± 0.013 | 0.1457 ± 0.005 |
| Control | | | | |
| I-lung | 0.1372 ± 0.007 | 0.0917 ± 0.008 | 0.1131 ± 0.006 | 0.0594 ± 0.005 |
| FI-lungs | 0.2534 ± 0.012 | 0.1906 ± 0.010 | 0.2073 ± 0.007 | 0.1078 ± 0.006 |
| LFI-lungs | 0.3738 ± 0.021 | 0.2667 ± 0.020 | 0.2156 ± 0.012 | 0.1060 ± 0.005 |

[a] Normalized for body weight to eliminate inherent differences in lung volume resulting from different sizes of animal

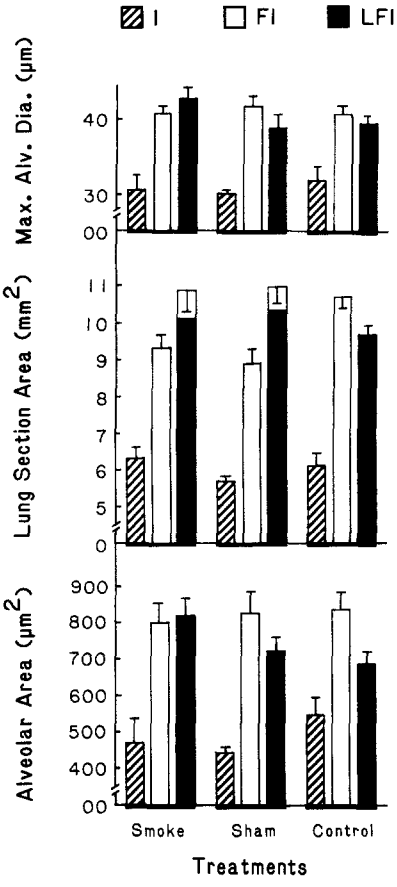


**Fig. 1.** Alveolar area ($\mu m^2$), lung section area ($\mu m^2$) and maximum (Max.) alveolar (Alv.) diameter (Dia.) (μm) of smoke-exposed (smoke) sham-treated (sham) and control animal lungs fixed by immersion (*I*), intratracheal fixative instillation (*FI*) and lavaged followed by intratracheal fixative instillation (*LFI*)

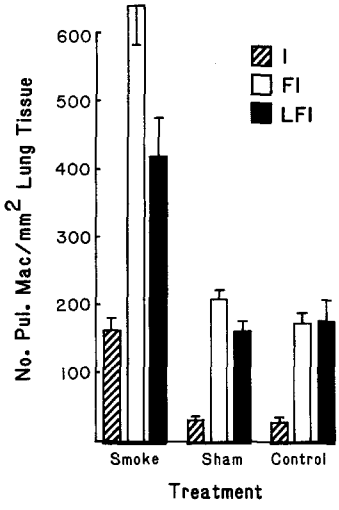


**Fig. 2.** Number (No.) of pulmonary (Pul.) macrophages (Mac.) per $mm^2$ of lung tissue in lungs of smoke-exposed, sham-treated and control animals fixed by immersion, intratrachial fixative instillation and lavaged followed by intratracheal instillation of fixative. Abbreviations as in Fig. 1

(Fig. 1). Area of alveolar spaces in lungs subjected to immersion fixation (I) was likewise similar ($P \geq 0.12$) but significantly ($P = 0.0001$) smaller than values obtained from FI and LFI lungs (Fig. 1). Alveolar area in control and sham LFI lungs was similar but smaller than that recorded from smoke-exposed LFI lungs ($P = 0.04$) (Fig. 2). Area of air spaces was similar in FI lungs ($P \geq 0.6$) of all treatment groups (Fig. 1). Maximum diameter of alveoli in I lungs of smoke-exposed, sham-treated, and control mice was uniform (Fig. 1). Likewise, this parameter was similar in FI and LFI lungs of all treatment groups, although it was sig-

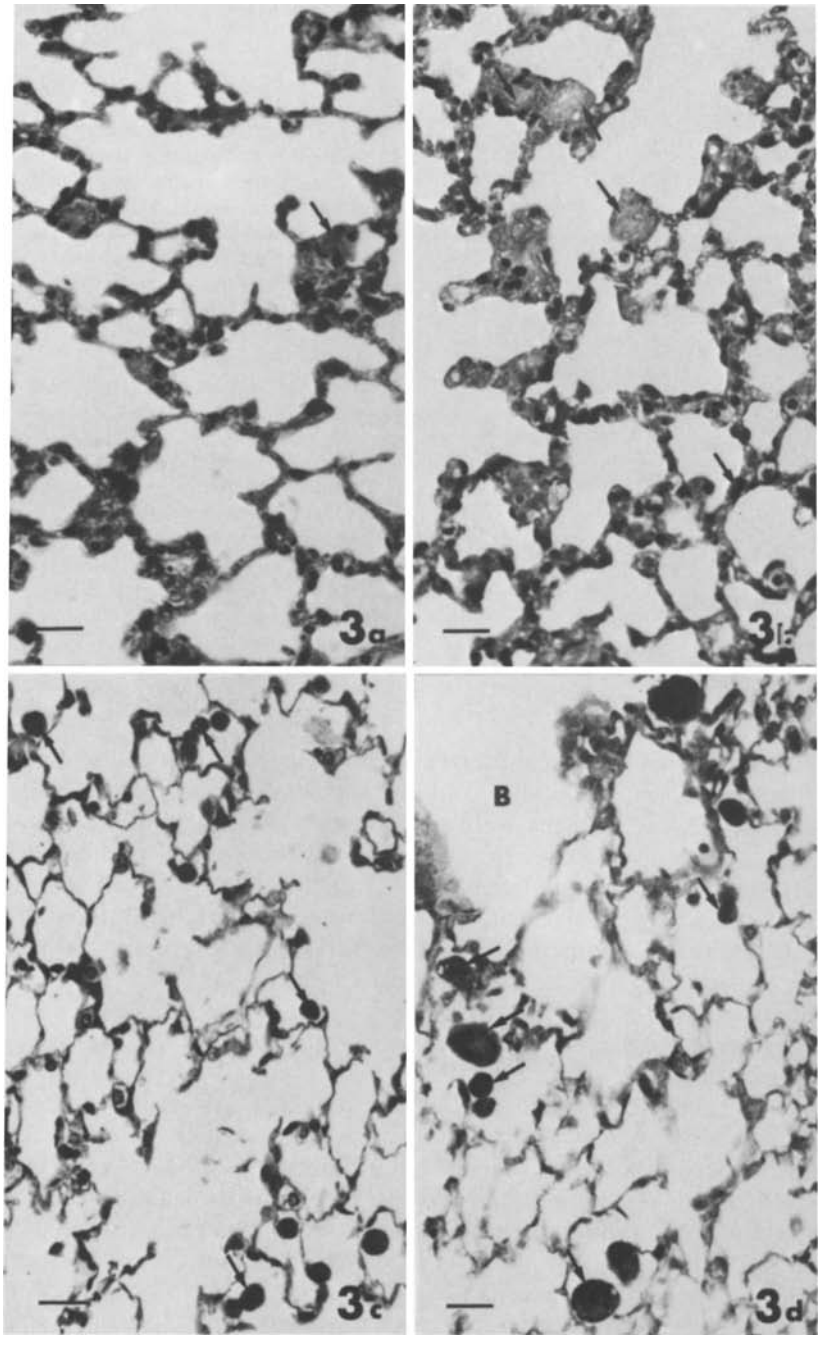


**Fig. 3.** Light micrographs of lungs fixed by immersion (3a, 3b) and lavaged followed by intratracheal instillation of fixative (3c, 3d) from sham-treated (3a, 3c) and smoke-exposed (3b, 3d) mice. Macrophages (*arrows*) in slide preparations stained magenta and were clearly visible. Terminal airways (B). ×350. *Scale bar*=20 μm

nificantly larger ($P \leq 0.027$) (range, $38.9 \pm 1.3$–$43.3 \pm 1.7$ μ) than alveolar diameter values in I lungs (Fig. 1).

The number of pulmonary macrophages (septal and alveolar) per $mm^2$ of FI and LFI lung tissue from smoke-exposed animals was dramatically ($P=0.0001$) higher than all other values (Fig. 2). Phagocyte population in lungs of smoke-exposed mice, fixed by immersion, was similar to that noted in intratracheally fixative-instilled ($P \geq 0.84$) and lavaged followed by instillation of fixative lungs ($P \geq 0.29$) of sham and control animals (Fig. 2). These values were, however, approximately six times larger ($P=0.0001$) than those recorded from immersion-fixed of sham-treated and control mice (Fig. 2). The paucity of macrophages in the latter lungs compared to all others was clearly visible at the light microscopic level (Fig. 3).

Morphology and distribution of macrophages differed in lungs fixed by immersion from those of fixative-instilled and lavaged followed by fixative instillation. In the former, macrophages

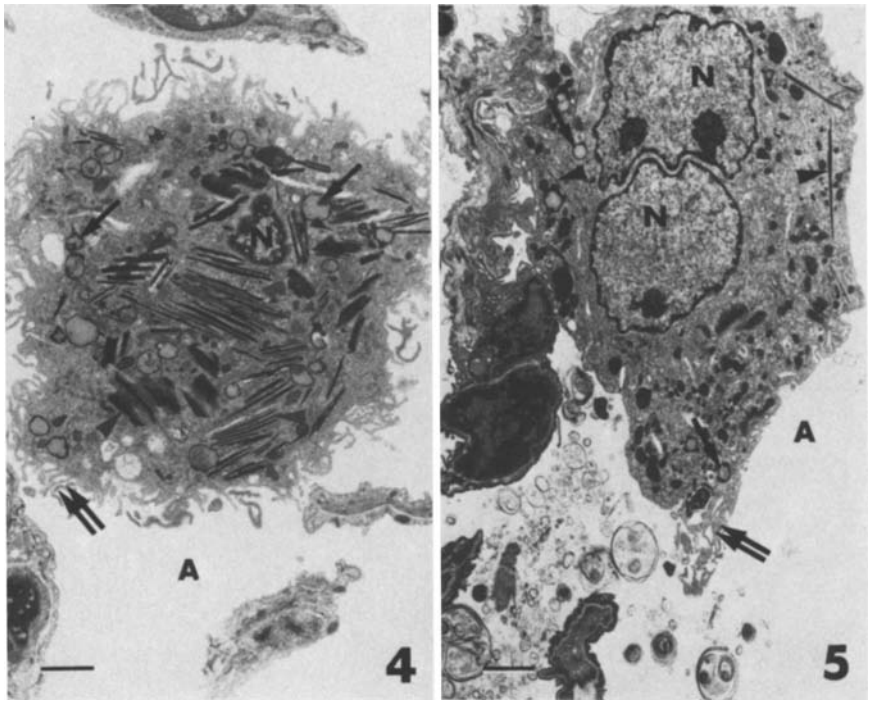


**Fig. 4.** Electron micrograph of a pulmonary macrophage from smoke-exposed animal lung which was lavaged first followed by intratracheal instillation of fixative. Note the lysosome-like bodies (*arrows*), numerous crystal-like inclusions (*arrow heads*) and cytoplasmic cell processes (*double arrows*). Alveolar space, *A*; tangential section of nucleus, *N*. ×3630 *Scale bar* = 2 μm

**Fig. 5.** Electron micrograph of a macrophage from a smoke-exposed mouse lung which has been fixed by immersion alone. Lysosome-like bodies, *arrows;* crysalline inclusions, *arrow-heads;* cytoplasmic processes, *double arrows*; alveolar space, *A*; nucleus, *N*. ×3630. *Scale bar* = 2 μm

stained light magenta, were most often elongate and were attached to or embedded in the alveolar wall (Fig. 3a, b). In the latter 2 groups these cells stained dark magenta, were rounded and were usually free in the alveolar spaces (Fig. 3c, d). Identification of macrophages in lung sections was not problematic since they were stained clearly and differentially from all other cells. Solitary macrophages, uniformly distributed, were observed in lungs fixed by immersion (Fig. 3a, b). However, in FI and LFI lungs clusters of 2 to 4 phagocytes were noted (Fig. 3c, d). Such clusters and, in general, more macrophages were seen in close proximity to terminal airways (Fig. 3d). Airways macrophages were seldom observed in fixative-instilled lungs and in those lavaged prior to instillation of fixative. However, they were common in lungs fixed by immersions alone.

Ultrastructural assessment of lung tissue substantiated the light microscopic morphology and location of macrophages described above. Further, at this level of resolution, clearly fewer phagocytes were encountered in lungs fixed by immersion than in lungs fixed via intratrachial instillation of fixative with or without prior lavage. In addition, it was noted that rounded macrophages in FI and LFI lungs of smoke-exposed (compare Figs. 4 and 5), sham-treated and control animals were characterized by many slender cytoplasmic processes which emanated from the surface with relative uniformity. Also, many macrophages in these lungs were characterized by numerous dense lysosome-like bodies, and in smoke-exposed animals the phagocytes were often replete with crystalline inclusions (Fig. 4). Fewer lysosome-like bodies and especially crystal-like inclusions were noted in phagocytes of smoke-exposed animal lungs fixed by immersion (Fig. 5) than in pulmonary macrophages of FI and LFI lungs.

## Discussion

The effects of smoke inhalation on pulmonary tissue and macrophages were similar to those described earlier (Matulionis 1984; Matulionis and Simmerman 1985; Matulionis and Traurig 1975) and will not be discussed. However, it should be noted that, as in earlier observations, inhalation of cigarette smoke causes a marked increase of in situ pulmonary macrophages and structural tissue changes which resemble lung fibrosis (Matulionis 1984; Matulionis and Simmerman 1985; Matulionis et al. 1985). Germane to the present study are: 1) influence of differential lung deformation on quantification of in situ pulmonary macrophages per unit area of lung sections; 2) importance of sampling similar lung regions during quantitation of macrophage numbers per lung section area; 3) effect of fluid instillation into the lungs on macrophage displacement, and 4) evaluation of possible use of lung lavage to obtain macrophages for estimating their population.

Manifestation of lung fibrosis or other conditions subsequent to cigarette smoke inhalation (Matulionis and Simmerman 1985) could affect shrinkage or inflation of lungs differentially during fixation and subsequent processing of tissue for microscopic analysis. In this event interpretation of quantitative data regarding macrophage numbers per unit area of lung sections would be difficult if not impossible since they may reflect volumetric lung change rather than altered phagocyte population size. The superior 2/3 lung portion initially subjected to lavage followed by intratracheal instillation of fixative (LFI) shrank after 72 h of formalin fixation. Suprisingly, the volume of the superior 2/3 segments of the fixative-instilled and immersion-fixed lungs increased. The latter observation does not agree with that of Mazzone et al. (1980) who reported that dog lungs shrank consistantly during a 24-h fixation period. However, data obtained in this study cannot be validly compared with those reported by the above investigators since different species of animals and technical procedures were used. Pronounced shrinkage of mouse lungs, regardless of experimental treatment (i.e., smoke, sham, control) or fixation procedure, occurs during dehydration and clearing of tissues. It is significant to note that deformation (shrinkage or inflation). of I lung preparation was uniform over treatments while that of FI and LFI lungs was not. This differential deformation of lungs fixed via intratracheal instillation of fixative with or without prior lung lavage must be accounted for, if in situ number of cells per area of lung tissue is to be compared over treatment.

Degree of lung shrinkage or inflation (i.e., deformation) evaluated by assessing sectional area of lungs, area of alveoli and alveolar diameter was similar in lungs of smoke-exposed, sham-treated and control animals fixed by immersion. Lung section and alveolar areas were not equal in the LFI and FI lungs of animals subjected to different treatments. However, maximum diameter of alveoli was similar in FI and LFI lungs over treatment. The cause for variability of lung section and alveolar area in FI and LFI lungs is unknown at present. But it is suspected that instillation of fluids into the lungs was influential in this respect because all factors affecting the degree of lung inflation (Hayadovoudi et al. 1980) as a result of intratracheal fixative instillation were not totally controlled. Nevertheless, the present study demonstrates that lung section area, alveolar area and alveolar diameter are not influenced differentially by smoke exposure or sham treatment following immersion fixation. Therefore, such preparations can be used to quantitate in situ macrophage number per unit area of lung tissue even without adjustment for degree of inflation or shrinkage, whereas instillation of fixative into the lungs via the trachea affects lung section and alveolar area and must be accounted for before the number of lung macrophages per area of lung tissue in animals subjected to different treatments can be meaningfully interpreted.

When quantifying pulmonary macrophages per unit area of tissue, the region of lungs sampled should be the same in all animals since numbers of phagocytes could vary differentially in different parts of the organ. Volume displacement of lungs in all animals after transection into superior 2/3 and inferior 1/3 revealed that lungs were divided accurately linearly since volume of the former lung portions was uniform. This observation indicates that all lung sections for light microscopy were obtained from the same location of the lungs because they were always cut from the same lung region, i.e., starting at the cut end of the superior 2/3 lung segment.

Displacement of phagocytes from the airways as a result of intratracheal instillation of fixatives or fluids into the alveoli could significantly alter the number of these cells in the parenchymal compartment of the lungs (Tyler et al. 1985). The present study substantiates this possibility since in all animals groups following intratrachial instillation of fixative with or without prior lavage the number of macrophages were markedly higher in parenchymal regions of lungs when compared to that noted in lungs fixed by immersion. This observation is not in accord with that of Brain et al. (Brain et al. 1984) who reported a decrease in parenchymal macrophage numbers in hamsters following intratracheal instillation of fixative. The discrepancy in data might be related to the different species of animals or procedures used to quantify the cells. Origin of the displaced macrophages in FI and LFI lungs appears to be the airways, since few were observed in these passageways after instillation of fluids intratracheally. Similar observations were noted in hamsters where the number of airway macrophages was reduced significantly following fixative instillation when compared to lungs fixed by vascular perfusion (Brain et al. 1984). Possible contribution of cells from airways to lavage fluid in humans, previously acknowledged (Davis et al. 1982; Rossman 1981) indicates that cells in these passageways can be displaced. In addition, clusters of free-floating macrophages observed near the terminal airways in lungs subjected to FI and LFI procedures suggest that cell displacement is not

uniform. Subcellular assessment of the round, unattached macrophages in fixative-instilled and lavaged followed by fixitive instillation lungs revealed them to be laden with considerably more phagocytosed material than most phagocytes noted in lungs fixed by immersion. This would suggest that these macrophages are older and perhaps represent a certain portion of airway macrophages which were being cleared from the lungs. Intratracheal instillation of fluid into alveoli appears to displace these cells from the trachea into the lungs altering the in situ lung parenchymal macrophage population quantitatively and qualitatively.

Lung lavage removed a relatively small portion of pulmonary macrophages from lungs of smoke-exposed and none from sham-treated and control animals when compared with the phagocytes quantified in situ following intratracheal instillation of fixative alone. This observation suggests that lavage procedures inappropriately sample the lung macrophage population. Although bronchopulmonary lavage is a useful technique to obtain lung cells and fluids and has provided significant diagnostic information (Daniele et al. 1985) the kinetics of cell removal from the lungs is multifactorial, complex and not totally understood (Davis et al. 1985). It appears that if in situ quantification of parenchymal lung macrophages is desired, counting these cells per area of tissue fixed by immersion yields more accurate fractional estimates of the phagocyte population size than does quantification of these cells obtained by lavage procedures.

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