**Morphometric Study on Age-Dependent Pulmonary Lesions in Rats Exposed to Nitrogen Dioxide**

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Abstract: Electronmicroscopic morphometry was performed on lung of 1, 3, 12 and 21 months-old rats exposed to 0.1, 0.5, 3 and 10 ppm nitrogen dioxide (NO₂) continuously for one month. The rats used in this experiment were all supplied at one time from one colony and kept under a barrier system until exposure.

Effects of aging on the responses of lungs to NO₂ were studied by comparing the dose-effect reaction patterns among the age groups.

A trend of dose-dependent increase of arithmetic mean thickness of air-blood barrier was found in all age groups examined. The responses of lung to NO₂ exposure showed age-related differences. Based on the morphometric index, the response declines from 1 to 12 months, but increases again in 21-months-old rats.

The compartmental components of alveolar wall tissue such as type I epithelial cells, type II epithelial cells, interstitial cells, interstitial matrix and capillary endothelium appeared to have various degrees of response due to both age at onset of exposure and NO₂ concentration, resulting in the appearance of varying stages in impairment or repair. Accordingly, the response of each compartmental component of lung to the concentrations of NO₂ did not always exhibit a simple dose-dependent increase or decrease but sometimes indicated a multiphasic reaction pattern.

**Keywords**: NO₂—Aging—Pulmonary lesion—Morphometry

**INTRODUCTION**

There are a number of reports including a series of works by Freeman and his colleagues concerning the morphological effects of the lungs to relatively low concentrations of NO₂ of one month or longer duration¹-⁶. The common fundamental lesions in these studies are recognized mainly at both the air-ways and bronchiopulmonary junction. They include: 1) uneven arrangement, shortening or loss of cilia in ciliated epithelium, hypertrophy, hyperplasia and hypersecretion of nonciliated epithelium, 2) fibrous thickening of connective tissue at the terminal end of air tracts, and 3) damage and desquamation of type I epithelial cells and subsequent proliferative response of type II epithelial cells.
On the other hand, except for slight enlargement of air spaces little change has been reported in the more peripheral region of the alveoli. However, it is sometimes very difficult to distinguish relatively weak morphological alterations appearing in the alveolar wall by lightmicroscopic observation.

Slight hypertrophy or atrophy of cells, microedema of interstitium and mild fibrosis recognized only by electronmicroscope are sometimes evaluated differently by different observers, and more objective, quantitative treatment of the materials is needed.

Age-related differences in sensitivity to NO₂ have been noted recently. It has been reported that lung impairment in neonatal mice and rats are slight while sensitivity increases in young adult animals and the onset of cell repair is slower in older rats. However, little is known about the relationship between the age of animals and effects on the lung of lower concentrations of NO₂ (<1 ppm) and NO₂ at ambient air pollution levels.

The present study was undertaken to detect quantitatively by morphometric methods differences in lung response by age of animals exposed to relatively low concentrations of NO₂. The quantitative analysis focuses particular attention on the alveolar wall at the peripheral region where lightmicroscopic observation is so far limited to detecting changes in each compartmental component such as alveolar epithelium, interstitium and capillary endothelium.

This work was done correlating biochemical examinations on the animal groups exposed to NO₂ performed at the same time, and the results were presented at an air pollution conference in 1980. The results of the biochemical examinations will be published in *Industrial Health*.

**MATERIALS AND METHOD**

1 **Experimental conditions**

Animals: Female rats of JCL-SD strain (SPF) were supplied 3 weeks after birth and maintained under a strict barrier-system until start of NO₂ exposure. The age of animals at onset of NO₂ exposure were 1, 3, 12 and 21 months. Thirty-five animals 1 to 12 months old were divided into five groups and exposed to 4 concentration levels of NO₂. One group served as box-control. Five animals selected randomly from the 7 rats of each group were used for biochemical analysis and the remaining 2 were used for morphometric evaluation. In the 21 month-old groups, 3 rats exposed to each dose level were used for morphological observation.

The four levels of NO₂ concentration were 0.1, 0.5, 3 and 10 ppm, and the mean NO₂ concentrations during the experiment were 0.11±0.06 (SD), 0.46±0.14, 2.8±0.63 and 8.8±1.2 ppm respectively. The exposure procedure has been reported previously. Feed (CE-2 of JCL) and sterilized water were given *ad libitum*.

Infectious quality of rat lungs: In order to detect very mild responses or minor
lesions caused by inhalation of low doses of pollutants it is very important to keep the animals free of any trace of lung infection. Before starting the present experiment, a preliminary check of lung quality was made by selecting 10 four-week-old rats from each of several colonies of animal breeders. Rats were chosen only from colonies which showed no small spots of bleeding or uneven contraction on the exterior of the lung to the naked eye nor signs of hypersecretion in the airways or infiltration of cells into interstitial space and perivascular connective tissue. Animals bred in the same colony at the same time were used in the present experiment. No infections including HVJ and M. pulmonis were observed by routine serological testing during the experimental period of 22 months.

2 Morphometry procedures

(1) Tissue preparation

All the rats were examined within 1 h after the cessation of one-month exposure to NO₂.

The thorax was opened under deep anesthesia with Na-pentobarbital. The trachea and the lower lobe of the right lung were ligated at the hilar region. Isotonic glutaraldehyde fixative (1.5% glutaraldehyde in 0.088 M cacodylate buffer at pH 7.4 with 1.5% dextran, osmolarity 330 mOsm) was dropped from a height of 25 cm and was instilled through a needle inserted in the trachea in situ, and at the same time depletion was done by cutting off the peritoneal artery. Within 2 min, the lung was filled with the fixative and fully re-enlarged inside the opened thorax. At this time the inserted needle was withdrawn and then 1 ml or more of the fixative was bubbled due to elasticity so that the lung was fixed at circa 50% volume level of total lung capacity.

Two vertical slices of about 2 mm thickness through the hilar region of the left lung were cut and fixed overnight in the same fixative at 0°C. After discarding the pleura, the upper slice was cut into 10 to 15 pieces and was kept for another 30 min in the fixative. Tissue blocks were washed thoroughly 3 times with ice-cold isotonic buffer (0.05 M cacodylate pH 7.4, +7.7% sucrose) for 2 h and postfixed with 1% OsO₄ solution for 1 h. Dehydration started from 70% alcohol, passed through propylene oxide and embedded in Epon 812 (Taab. Epikote 812).

The remaining portions of the left and the right lung tissue were prepared as routine paraffine embedded sections for lightmicroscopic observation.

(2) Sampling for electronmicroscopic morphometry

Sections of 1 μm thickness were cut from five blocks chosen randomly, stained with toluidine blue, screened under a lightmicroscope, and three blocks containing lung parenchyma wide enough for morphometric purposes were chosen. Thin sections were cut from these three blocks with a diamond knife, transferred onto the supporting grid (Maxtaform H₂), and stained with uranyl acetate and lead citrate.

As these sections covered 15 to 20 spaces of the supporting grid, 12 lung areas
were systematically recorded on electronmicrographs at initial magnification of \( \times 2000 \) from 12 consecutive fields of grid corners, and 10 of them were used for counting.

Alveoli adjacent to the ends of the terminal bronchioles and to the small blood vessels were excluded from the counts, and alveoli at the more peripheral regions were subjected to measurement.

(3) Morphometry

Arithmetic mean thickness of air-blood barrier (AMT)

Electronmicrographs were projected to a final magnification of \( \times 8000 \) on a screen by the coherent multipurpose test system described by Weibel\cite{15,16}, and AMT was measured.

Based on the results of a preliminary estimate of AMT using normal lung specimens of young adults, it was found that measuring a total of 60 micrographs per group would be sufficient to obtain the value for 95% confidence intervals within \( \pm 15\% \) of the mean\cite{17}. Measurement of AMT was performed on 60 micrographs per group consisting of 10 micrographs from 3 sections each of 2 experimental animals (90 micrographs for 21 M old groups, 10 micrographs \( \times 3 \) sections \( \times 3 \) animals).

AMT were expressed without any correction for shrinkage or elongation factors in the course of sample preparation because the procedures used were the same for all groups observed\cite{18}.

(4) Volume density of alveolar wall components

Volume density of each alveolar wall compartmental component was counted by the point counting method. Alveolar wall was defined as the tissue mass consisting of type I epithelial cells, type II epithelial cells, interstitial cells of all kinds, interstitial matrix (non-cellular interstitial space) and capillary endothelium. Alveolar macrophages and nucleated cells residing in the capillary lumen were excluded.

A test sheet containing 293 cross points was placed on each micrograph and the number of points distributed on each component counted. Student’s t-test for the mean of 60 micrographs and \( \chi^2 \)-test for the pooled sum of total points on each component of all 60 micrographs were applied to evaluate statistical significance.

(5) Mean number of alveolar cells and the volume density per cell

Differential cell counts of all cells of the alveolar wall with visible nuclei appearing in 12 grid spaces (127 \( \times \) 127 \( \mu \text{m}^2 \times 12 \)) were done directly on the electronmicroscopic field. The observation was made under magnification of \( \times 2000 \) and each cell was differentiated by the use of \( \times 10 \) ruper. Higher magnification was applied in the case of lesional changes since the precise differentiation of cell type was difficult under low magnification. Intermediate cells which lacked lamellar inclusion bodies and had only a few microvilli were counted as type I epithelial cells.
Comparison of cell numbers was made using the total sum of cell per group appearing in a unit area of section used. Volume density per cell was calculated as the number of points per mean number of cells with visible nuclei found in the measured area.

**RESULTS**

1 **Lightmicroscopic observations**

No loss of animals occurred during the exposure periods. The data of body weight and weight of lung per body weight presented by Hasegawa et al. showed that significant effects were noted only in the 10 ppm group of the 1-month-old (1 M) rats. No significant difference was noted in any other group.

Mild to moderate lung lesions were noted depending upon the NO₂ concentrations in all ages of rats under lightmicroscopic observations. The degree of the lesions differs by age, the strongest being found in the 1-M-old rats, and lighter lesions found in the 3 to 12-M-old rats. Age dependent differences in susceptibility appeared in the attacked foci at the 10 ppm level.

Hyperplastic foci with digital extrusion at the middle to terminal region of the bronchial tree were the most prominent feature of the lesions throughout all age groups. Moreover, the lesions extended to deeper airways in the 1-M-old groups and proliferative reaction accompanied by macrophage infiltration into the alveolar ducts and the bordering alveoli as well as enlargement of alveolar lumen were noted.

Impairment of the alveolar wall became too mild with age to permit precise evaluation under lightmicroscopic observation in the 12- and 21-M-old rats.

2 **Electronmicroscopic morphometry**

(1) **Changes of arithmetic mean thickness of air-blood barrier**

The values of AMT measured by Weibel's method and the increase of AMT presented by percentage against the control are shown in Figures 1a and 1b. NO₂ dose-dependent increase of thickness was indicated in rats of all ages. All the values of exposed groups at 0.1 and 0.5 ppm levels were higher than those of the controls of the same age, but no statistical significance was found except at the 0.1 ppm level of the 3-M-old group.

Significant increase of AMT started from the 3 ppm level except in the 12-M-old groups and a highly significant increase was seen in all the exposed groups at 10 ppm. The increase rate of AMT of the exposed groups compared to the control values was higher in the younger age groups and lowest in the 12-M-old groups but increased once again in the 21-M-old groups. The results indicate that age probably has a close relationship to susceptibility to NO₂ in rats.

(2) **Analysis of the factors related to changes of AMT value**

Volume density of alveolar wall tissue (VdAWT) and surface area of alveoles
Fig. 1. Changes of arithmetic mean thickness of air-blood barrier (AMT), volume density of alveolar wall tissue (VdAWT) and surface area (Sa). All the values are expressed as percentage of exposed groups to controls of the same age. This is also applied to Figs. 2, 3, 4, 6, 7 and 8.

1a; Changes of AMT of each age group. Bar indicates 95% confidence interval of the mean.

1b; Increase of AMT, percentage of exposed against the control value.

1c; Changes of VdAWT and Sa.

t-test; * p<0.05  ** p<0.01  *** p<0.001 p value control versus experimental.

z²-test; * p<0.05  ** p<0.01  *** p<0.001 ———/-, compared to indicated dose groups.

N.S.: non-significant.

All the values are expressed as percentage of exposed groups to controls of the same age. This is also applied to Figs. 2, 3, 4, 6, 7 and 8.
and capillary lumen (Sa) were first considered as the factors related to changes of AMT value.

AMT is defined as the mean thickness of alveolar wall tissue between the surfaces of the alveolar lumen and the capillary lumen. Thus increase of VdAWT and/or decrease of Sa will result in increase of AMT value.

Figure 1c shows changes in VdAWT and Sa, which appear to correspond to the changes of AMT in Figure 1a.

Table 1. Principal data of VdAWT and Sa.

A. Volume density of Alveolar Wall Tissue

<table>
<thead>
<tr>
<th>Age of rats §</th>
<th>Controls</th>
<th>0.1 ppm</th>
<th>0.5 ppm</th>
<th>3 ppm</th>
<th>10 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1→2M old</td>
<td>72±23</td>
<td>72±24</td>
<td>80±25</td>
<td>81±23</td>
<td>93±37***</td>
</tr>
<tr>
<td>4291b</td>
<td>4312</td>
<td>4828xxx</td>
<td>4380xxx</td>
<td></td>
<td>5552xxx</td>
</tr>
<tr>
<td></td>
<td></td>
<td>xxx v.s. 0.1 ppm</td>
<td></td>
<td>xxx v.s. 3 ppm</td>
<td></td>
</tr>
<tr>
<td>3→4M</td>
<td>72±24</td>
<td>82±29</td>
<td>68±24</td>
<td>77±24</td>
<td>80±28</td>
</tr>
<tr>
<td>4305</td>
<td>4928xxx</td>
<td>4063xxx</td>
<td>4594xxx</td>
<td>4829xxx</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>v.s. 0.1 ppm**</td>
<td>v.s. 0.5 ppm*</td>
<td></td>
<td>v.s. 3 ppmx</td>
</tr>
<tr>
<td>12→13M</td>
<td>79±33</td>
<td>77±33</td>
<td>76±26</td>
<td>72±29</td>
<td>80±26</td>
</tr>
<tr>
<td>4758</td>
<td>4604</td>
<td>4397xxx</td>
<td>4327xxx</td>
<td></td>
<td>4801</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v.s. 0.1 ppmx</td>
<td></td>
<td>v.s. 3 ppmxx</td>
<td></td>
</tr>
<tr>
<td>21→22M</td>
<td>79±22</td>
<td>78±29</td>
<td>88±28</td>
<td>86±32</td>
<td>95±31**</td>
</tr>
<tr>
<td>4718</td>
<td>6983</td>
<td>7894xxx</td>
<td>7741xxx</td>
<td></td>
<td>8558xxx</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v.s. 0.1 ppmxxx</td>
<td></td>
<td>v.s. 3 ppmxxx</td>
<td></td>
</tr>
</tbody>
</table>

B. Surface area: mean number of surface intercepts with 2.0 µ lines±SE/micrograph.

Total number of lines counted = 3540 (3310 for 21M old rats). §: period of exposure to NO₂.

<table>
<thead>
<tr>
<th>Age of rats §</th>
<th>Controls</th>
<th>0.1 ppm</th>
<th>0.5 ppm</th>
<th>3 ppm</th>
<th>10 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1→2M old</td>
<td>25±8</td>
<td>23±6*</td>
<td>23±6*</td>
<td>19±6*** v.s. 0.5 ppm**</td>
<td>20±6***</td>
</tr>
<tr>
<td>21±6</td>
<td></td>
<td></td>
<td></td>
<td>v.s. 3 ppm*</td>
<td></td>
</tr>
<tr>
<td>3→4M</td>
<td>25±7</td>
<td>24±7</td>
<td>21±5**  v.s. 0.1 ppm*</td>
<td>22±5**</td>
<td>19±6*** v.s. 3 ppm*</td>
</tr>
<tr>
<td>21±6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12→13M</td>
<td>25±7</td>
<td>24±8</td>
<td>23±6</td>
<td>23±6</td>
<td>21±6*** v.s. 3 ppm*</td>
</tr>
<tr>
<td>21±6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21→22M</td>
<td>23±6</td>
<td>20±6</td>
<td>23±6</td>
<td>21±5</td>
<td>21±6</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01, *** p<0.001 (one-sided test)
Comparisons are exposed groups to controls.
v.s. indicated dose groups.
χ²-test. x p<0.05, xx p<0.01, xxx p<0.001.
Table 1 includes the principal data of points counts and the results of statistical tests. The dose-effect patterns of VdAWT value and the concentrations of NO₂ appeared to be similar in both 1-M- and 21-M-old groups. In both age groups, AMT increased significantly at NO₂ levels beyond 0.5 ppm and markedly at 10 ppm.

Although the value of VdAWT in the 1-M- and 21-M-old groups increased with NO₂ concentration, the reaction pattern of the 3-M-old group differed from the other two groups. The values of exposed groups showed no significant difference from the control values by t-test except at the 0.1 ppm level, while the differences among the exposed groups themselves were significant. In the 3-M-old group, the value rose at 0.1 ppm, fell at 0.5 ppm and rose again dose-dependently at 3 and 10 ppm, resulting in a multiphasic reaction pattern of the concentrations of NO₂ (χ²-test).

In 12-M-old rats the values at each NO₂ level were observed to be insignificant to the control. Furthermore, the values among the exposed groups were also insignificant.

Using VdAWT as an index it was clearly shown that the pattern of dose-effect depended on the age of rats at exposure onset.

When Sa was used as an index of the reaction to NO₂, the results were very similar in the 1-, 3- and 12-M-old groups showing a trend of dose-dependent decrease. The degree of decrease became smaller with age, and in the 21-M-old groups no significant difference was found.

The above data seem to indicate that the dose-dependent increase of AMT was brought about by the two factors, the dose-dependent increase of VdAWT and the dose-dependent decrease of Sa. Analysis of the two factors showed clearly that the composition of alveolar wall tissue was not always the same among the different age groups and at various NO₂ levels even the calibrated AMT value were of the same order.

3 Changes of volume density of alveolar wall components

The contribution rate of each alveolar wall component, i.e., epithelial cells, interstitial cells, and matrix and capillary endothelial cells is shown in Figure 2. The value of VdAWT was expressed as the percentage to the control by the black bars. The contribution rate of each component to the total VdAWT is shown by the white bars.

The compartmental components did not always show linear responses to NO₂ dose. Some increased while others decreased with dose. The changes of VdAWT were the result of the sum of these componental changes.

In the 1-M- and 21-M-old groups, the reaction patterns of the VdAWT shown by the black bars were similar, as seen in Fig. 2, while the changes of the compartmental components were not. In 1-M-old groups, the contribution of interstitial (cell + matrix) component to the increase in total VdAWT was large at
The 0.5 ppm level. At 3 and 10 ppm, however, epithelial components contributed as well as the interstitium. On the other hand, in the 21-M-old group at the 0.1 ppm level, the increase of the volume density of the epithelium and interstitial cells seemed to counterbalance the decrease of the volume density of the endothelium and interstitial.

Fig. 2. Contribution rate of each component of alveolar wall to the VdAWT.

- t-test; * p<0.05 ** p<0.01 *** p<0.001 p value control versus experimental.
- $\chi^2$-test; $*$ p<0.05 $**$ p<0.01 $***$ p<0.001 compared to control.

*** / \, significant increase or decrease between indicated dose groups by $\chi^2$-test. These symbols also apply to Figs. 2-8.
Accordingly, no difference as a whole (black bar) was detected at this level but the absolute value of change shown by the white bar was almost the same as the 0.5 ppm level where the difference was significant. Increase of total matrix.

Fig. 3. Total volume density, number of cells and volume density/cell of type I epithelium.

3a: Total volume density  
3b: Number of cells with visible nuclei.  
3c: Volume density/cell.

NO$_2$ ppm: 0.1 | 0.5 | 3 | 10
Table 2. Principal data of alveolar wall components.

a. mean number of points on each component of alveolar wall±SE/micrograph.
b. total number of points on each component of alveolar wall/total points counted = 17580, (total points counted for the exposed groups of 21-M-old-rats = 26370). §: period of exposure to NO₂.

<table>
<thead>
<tr>
<th>Age of rats §</th>
<th>Controls</th>
<th>0.1 ppm</th>
<th>0.5 ppm</th>
<th>3 ppm</th>
<th>10 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type I epithelial cell</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1→2M</td>
<td>19±8*</td>
<td>20±8</td>
<td>21±8</td>
<td>21±11</td>
<td>22±11</td>
</tr>
<tr>
<td></td>
<td>1141b</td>
<td>1178</td>
<td>1238*</td>
<td>1261x</td>
<td>1314x³³</td>
</tr>
<tr>
<td>3→4M</td>
<td>20±10</td>
<td>19±7</td>
<td>17±6*</td>
<td>18±9</td>
<td>21±10</td>
</tr>
<tr>
<td></td>
<td>1217</td>
<td>1108x</td>
<td>1025xxx</td>
<td>1099x</td>
<td>1232 v.s. 0.3 ppm²²</td>
</tr>
<tr>
<td>12→13M</td>
<td>21±9</td>
<td>20±9</td>
<td>20±8</td>
<td>16±5***</td>
<td>20±10</td>
</tr>
<tr>
<td></td>
<td>1250</td>
<td>1168</td>
<td>1226</td>
<td>965 v.s. 0.5 ppm³³</td>
<td>1174 v.s. 3 ppm³³</td>
</tr>
<tr>
<td>21→22M</td>
<td>19±6</td>
<td>20±8</td>
<td>21±7</td>
<td>20±8</td>
<td>26±13***</td>
</tr>
<tr>
<td></td>
<td>1146</td>
<td>1764</td>
<td>1849</td>
<td>1786</td>
<td>2306 v.s. 0.3 ppm³³</td>
</tr>
</tbody>
</table>

| **Type II epithelial cell** | | | | | |
| 1→2M          | 6±14     | 6±12     | 7±10    | 11±17 | 15±21** |
|               | 369      | 383      | 441xxx  | 657xxx | 912xxx |
|               | v.s. 0.1 ppm³³ | v.s. 0.5 ppm³³ | v.s. 3 ppm³³ |
| 3→4M          | 6±9      | 7±13     | 4±12    | 6±12  | 10±15* |
|               | 329      | 436xxx   | 259     | 382   | 613 v.s. 3 ppm³³ |
|               | v.s. 0.1 ppm³³ | v.s. 0.5 ppm³³ | v.s. 3 ppm³³ |
| 12→13M        | 7±14     | 8±13     | 5±13    | 8±15  | 6±10  |
|               | 438      | 482      | 281xxx  | 493   | 343xxx v.s. 3 ppm³³ |
|               | v.s. 0.1 ppm³³ | v.s. 0.5 ppm³³ | v.s. 3 ppm³³ |
| 21→22M        | 4±10     | 6±13     | 5±11    | 7±15  | 9±18  |
|               | 238      | 578xxx   | 480     | 625   | 808 v.s. 3 ppm³³ |
|               | v.s. 0.1 ppm³³ | v.s. 0.5 ppm³³ | v.s. 3 ppm³³ |

| **Interstitial cell** | | | | | |
| 1→2M          | 12±9     | 12±10    | 13±10   | 14±14 | 16±11 |
|               | 712      | 713      | 783     | 847xxx | 928xxx |
| 3→4M          | 8±7      | 12±11*   | 9±9     | 11±9  | 13±13* |
|               | 492      | 745xxx   | 557     | 652   | 749 v.s. 3 ppm³³ |
|               | v.s. 0.1 ppm³³ | v.s. 0.5 ppm³³ | v.s. 3 ppm³³ |
| 12→13M        | 11±11    | 10±12    | 10±10   | 10±8  | 11±10 |
|               | 682      | 608      | 598     | 593   | 674 |
| 21→22M        | 11±8     | 12±10    | 13±10   | 15±11* | 12±10 |
|               | 630      | 1031     | 1123    | 1305 | 1077 |

| **Interstitial matrix** | | | | | |
| 1→2M          | 10±5     | 10±5     | 15±5*** | 13±5** | 16±7*** |
|               | 614      | 614      | 869 v.s. 0.1 ppm³³ | 778 v.s. 0.5 ppm³³ | 986 v.s. 3 ppm³³ |
VdAWT at the 10 ppm level was mainly due to the increase of volume density of type I and type II epithelial cells. As indicated above, each component of the alveolar wall showed fairly different responses to NO₂ though the reaction patterns of VdAWT were quite similar in 1-M- and 21-M-old rats.

In the multiphasic reaction pattern of the 3-M-old groups, the increase of the interstitial components contributed to significant increase of VdAWT even at 0.1 ppm, and the decrease in type I cell density resulted in the decrease of VdAWT at 0.5 ppm (t-test).

Among the 12-M-old groups no statistically significant change in VdAWT was detected by t-test; however, the componental difference was very clear. At 3 and 10 ppm levels of NO₂, the sum of the absolute values of change in each component was especially large; therefore, qualitative modification from the control was clear. Based on the χ²-test of the total number of points, significant decrease of VdAWT was noted at 0.5 and 3 ppm levels, even in the 12-M-old groups. The difference between 3 and 10 ppm was also found to be significant.

Table 2. (continued)

<table>
<thead>
<tr>
<th>Age of rats</th>
<th>Controls</th>
<th>0.1 ppm</th>
<th>0.5 ppm</th>
<th>3 ppm</th>
<th>10 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>3→4M</td>
<td>17±7</td>
<td>21±8*</td>
<td>16±6</td>
<td>18±8</td>
<td>17±8</td>
</tr>
<tr>
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<td>1034</td>
<td>1256***</td>
<td>957</td>
<td>1073</td>
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<td></td>
<td>v.s. 0.1 ppm***</td>
<td></td>
<td>v.s. 0.5 ppm*</td>
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</tr>
<tr>
<td>12→13M</td>
<td>17±9</td>
<td>17±9</td>
<td>16±6</td>
<td>19±8</td>
<td>24±11***</td>
</tr>
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<td></td>
<td>1013</td>
<td>1020</td>
<td>940</td>
<td>1115</td>
<td>1453</td>
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<td></td>
<td></td>
<td>v.s. 0.5 ppm***</td>
<td></td>
<td>v.s. 0.5 ppm***</td>
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<tr>
<td>21→22M</td>
<td>23±8</td>
<td>21±8</td>
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<td>Capillary endothelial cell</td>
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<td>1→2M</td>
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<td>24±9</td>
<td>25±10</td>
<td>22±9</td>
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<td></td>
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<td>v.s. 0.5 ppm***</td>
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<td>v.s. 3 ppm*</td>
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<tr>
<td>3→4M</td>
<td>21±7</td>
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<td>20±8</td>
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<td></td>
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<td>1388</td>
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<td></td>
<td>v.s. 0.1 ppm*</td>
<td></td>
<td>v.s. 0.5 ppm*</td>
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</tr>
<tr>
<td>12→13M</td>
<td>23±11</td>
<td>22±9</td>
<td>23±9</td>
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<td>19±7*</td>
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<td>1161***</td>
<td>1157***</td>
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<td>v.s. 0.5 ppm***</td>
<td></td>
<td>v.s. 0.5 ppm***</td>
<td></td>
</tr>
<tr>
<td>21→22M</td>
<td>23±12</td>
<td>20±8</td>
<td>24±11</td>
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<td>24±9</td>
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<td>1351</td>
<td>1757***</td>
<td>2132***</td>
<td>1981</td>
<td>2116</td>
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<tr>
<td></td>
<td></td>
<td>v.s. 0.1 ppm***</td>
<td></td>
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</tr>
</tbody>
</table>

* p>0.05, ** p<0.01, *** p<0.001 (one-sided test)
Comparisons are exposed groups to controls.
v.s. * indicated dose groups.
χ²-test. x p>0.05, xx p<0.01, *** p<0.001.
4 Changes of compartmental components of alveolar wall

(1) Type I and Type II epithelial cells

Total volume density, the number of cells with visible nuclei under electron-
microscope and volume density per cell (total volume density/cell number) of
type I epithelial cells are shown in Figures 3a, 3b and 3c. All the values are
expressed as percentage of exposed groups to controls of the same age. All prin-
cipal data are shown in Table 2.

Figure 3a shows that the total volume density of type I cells increases in the
1-M- and 21-M-old exposed groups while it decreases in the 3 and 12-M-old
groups. The number of type I cells showed a trend to decrease at lower concen-
trations and increase at NO₂ levels higher than 3 ppm in 1 and 3-M-old rats. In
the 12-M-old groups number of cells decreased only at 3 ppm and in the 21-M-old
groups, increase at 0.5 and 10 ppm levels was seen (Fig. 3b).

Volume density per cell of type I cells, as shown in Figure 3c, generally indi-
cated only small changes in the exposed groups, except the 0.5 ppm group of
1-M-old rats which showed cell hypertrophy. The cell size of all age groups at the
10 ppm level was small; in 3-M-old rats, particularly, the volume density per cell
at 3 and 10 ppm of NO₂ was reduced to about half the control volume.

The various values of the three indexes regarding type II epithelium are shown
in Figures 4a, 4b and 4c. Although the values were the highest for the compart-
mental components of the alveolar wall, however, type II cells appeared sporadi-
cally in the electronmicroscopic fields differed from those of the other components.
It probably comprises a rather large deviation from the mean value derived from
the 60 micrographs observed. For this reason, the significant difference of volume
density of the type II cells, by t-test was observed only at the 10 ppm level in the
1- and 3-M-old groups (Fig. 4a*). χ²-test using the total sum of the points
(17,580 test points) distributed on the type II cells of 60 micrographs revealed
that all values obtained for the exposed groups, except 0.1 ppm level of 1-M- and
12-M-old rats, were significant to the control value (Fig. 4a□). The differences
among the levels of NO₂ were also significant (Fig. 4a□□). Volume density
of the type II cells increased dose-dependently in 1-M-old groups, however, “up-
down-up” patterns with NO₂ dose were observed in the older groups.

The number of type II cells generally tended to increase with exposure, and
it was particularly obvious in the 3-M-old groups. Similar patterns of reaction
regarding volume density per cell were noted in the 1-M- and 21-M-old rats and
in the 3-M- and 12-M-old rats. Cell hypertrophy was noted with increasing dose
of NO₂ in 1-M- and 21-M-old rats. On the contrary, volume density per cell of
the type II cells decreased in all exposed groups of 3-M- and 12-M-old rats
according to increase in the number of cells. This was especially noticeable at
the 0.5 ppm level.

(2) Numerical ratio of type II cells to type I cells

The numerical ratios of type II/type I cells are shown in Figure 5. The ratio of
the controls was approximately 1.0 in all age groups. The exposed groups in
which ratio of type II/type I was larger than 20% compared to the control were
the 0.5 ppm 1-M-old group, 0.1 and 0.5 ppm 3-M-old groups, 0.1 to 3 ppm
12-M-old groups, and the 3 ppm 21-M-old group. In all the above groups, the type II cells exceeded the type I cells in number. The high ratio of type II/type I found at low concentrations of NO₂ may be considered useful indicator of the susceptibility of the epithelial cells to NO₂. This will be discussed below.

(3) Response of interstitial cells

The interstitial cells were identified from the non-cellular interstitial matrix and separate counts of volume density were performed. The various types of cells residing in the interstitial spaces were not differentiated and all were included as interstitial cells. Even fractions of damaged fibroblasts derived from the tissue impaired by exposure to higher concentrations of NO₂ was counted as the cell component if such was recognized.

The indexes concerning the interstitial cell components are demonstrated in Figs. 6a, 6b and 6c. The total volume density of the interstitial cells increased in the exposure groups of 1, 3 and 21-M-old rats (significant by $\chi^2$-test) and decreased in the 12-M-old groups (significant by $\chi^2$-test). The increments detected in the 0.1 and 10 ppm groups of 3-M-old rats and in the 3 ppm groups of 21-M-old rats were also found to be significant by t-test. The changes in the numbers of interstitial cells did not always coincide with the changes in the total volume density. Therefore, no dependency of volume density per cell of the interstitial cells to NO₂ dose was demonstrated.

In 3-M-old rats the number of interstitial cells remained constant in all the exposed groups, and the increase in total volume density was presumed to be due to cell hypertrophy.

(4) The interstitial matrix component (non-cellular component)

The change in volume density of the interstitial matrix compared to the control
was marked in the 1-M-old groups (Fig. 7a). Significant increase (t-test) was shown in all groups exposed to over 0.5 ppm NO₂. Significant increase (t-test) was also noted in the other age groups: at 0.1 ppm in 3-M-old rats, at 10 ppm in 12-M-old rats and at 0.5 ppm in 21-M-old rats. The differences among dose levels were significant for all age groups (χ²-test). Therefore, no consistent relationship between volume density of interstitial matrix and NO₂ concentrations could be detected.

It is clear in Table 2 that the volume density of the interstitial matrix of control...
Fig. 7. Total volume density of interstitial matrix, total points of interstitial matrix and total volume density of interstitium (interstitial cells + interstitial matrix).

7a: Total volume density  7b: Total points  7c: Total volume density of interstitium.

△ significant to control value of 1-M-old rats p<0.001 (7b).
animals increased with age. The number of total points of interstitial matrix against NO$_2$ concentrations are shown in Fig. 7b. Compared to the value of 1-M-old control rats, all the values of the older control groups were significantly high (p<0.001, t-test). The control values for the 3 and 12-M-old groups were about 1.6 times larger than that of 1-M-old rats, and that of the 21-M-old rats

Fig. 8. Total volume density, number of cells and volume density/cell of capillary endothelium.

8a: Total volume density  8b: Number of cells with visible nuclei
8c: Volume density/cell.
even higher, being 2.2 times that of 1-M-old rats and 1.35 times larger than that of 12-M-old rats.

The ratio of interstitial matrix to the total volume density of interstitium was elevated in the groups over 3 M (Fig. 7c). Increase in the total volume density of interstitium was recognized at NO₂ levels over 3 ppm irrespective of animal age. The volume density of both interstitial cell and matrix of 3-M-old rats at 0.1 ppm showed significant increase (t-test).

(5) The capillary endothelium

The data for the capillary endothelium are shown in Figures 8a, 8b and 8c. The capillary endothelium showed slight responses to NO₂ according to the volumic or numerical indexes used here, but they were not dependent on NO₂ dose or age.

Slight hypertrophy of the endothelium was seen in 1-M-old rats exposed to 0.5 ppm. Diminution of cell size was noted in rats older than 3-M at NO₂ levels higher than 3 ppm, and it was marked in the 21-M groups where diminution was recognized even at the low concentration of 0.1 ppm.

DISCUSSION

Changes in cell population and volume density of the alveolar components have been described as lung responses to inhalation of hazardous gases such as NO₂ and O₃.²⁰-²⁴ In order to compare the results of inhalation experiments performed by various investigators, common substratum data on the normal cell population of rat lung are required as well as a common fixative procedure (fundamental to morphometric estimation). Recently quantitative data on cell population of normal rat lung were reported by Crapo et al.²¹ and Haies and coworkers.²⁵ Because the procedure for tissue preparation in the present study is comparable to that of Crapo, the number of cells and the volume density of the lung cell components of normal rats from 4 to 22 M are shown in Table 3 as relative percentage to the total, together with the data of Crapo et al.²¹.

The relative percentages of the number of cells with visible nuclei which express the normal cell ecology of rat lung at the respective ages agreed well with each other. The relative percentages of the number of nuclei/unit area (Na) of lung cells of the control rats of 4 M in this paper agreed well with the data of 3-M-old rats cited from Crapo. The relative volume density of our study and that of Crapo's also showed fairly good accordance although the volume density of interstitial matrix of the controls was slightly lower. The interstitial matrix of the 21-M control groups increased more than twice that of the 1-M-old controls, causing decrease in the relative ratio of the other components of the lung.

It has been reported that the osmolarity of both glutaraldehyde and buffer solution in the first fixative strongly affect cell dimensions. For morphometry of the lung, therefore, the procedure for the first fixation should be carried out very carefully.²⁶ According to the fixation method applied in this study no change
such as edema or shrinkage of alveolar tissue of control groups was observed under high magnification by electron microscope. The cell dimension in the present study was considered to be in a comparable range with that of Mathieu’s and Crapo’s data. Based on these control data, it seemed reasonable to compare the data with those of exposed groups.

AMT is a parameter related to the mass of tissue constructing the barriers and consuming oxygen in the lung. Hence increase of the AMT value means the lowering of gas exchange efficiency which may be disadvantageous to the host. The AMT of the control groups in this study were found to be 1.22±0.11, 1.05±0.12 in the 1, 3, 12 and 21-M-old age groups respectively. These values are in the normal range, similar to the values described by Weibel et al., although the value for the 3-M-old group is somewhat lower than those for the other age groups.

After exposure to NO₂ gases, AMTs of all age groups were found to show dose-dependent increments. Regarding AMT and increase rates, differences of response of rat lung to NO₂ were found by animal age at exposure onset. The reactivity was highest in the 1-M-old groups and decreased in order in the 3-M-old and 12-M-old groups, then increased again in the 21-M-old groups. It was demonstrated that the increase of AMT resulted from the NO₂ dose-dependent

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Table 3. Relative number and Relative volume density for various cell types of rat lung by age (Control groups).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Relative % of cell/unit area, 1.16×10⁻² cm²</th>
<th>Age at sacrifice, counts from 2 rats</th>
<th>Crapo et al.²¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2M %</td>
<td>4M %</td>
<td>13M %</td>
</tr>
<tr>
<td>Type I Epithelial cells</td>
<td>12.0</td>
<td>12.2</td>
<td>46</td>
</tr>
<tr>
<td>Type II Epithelial cells</td>
<td>13.9</td>
<td>12.5</td>
<td>47</td>
</tr>
<tr>
<td>Interstitial cells</td>
<td>22.1</td>
<td>27.4</td>
<td>103</td>
</tr>
<tr>
<td>Endothelium</td>
<td>49.3</td>
<td>44.7</td>
<td>168</td>
</tr>
<tr>
<td>Macrophages</td>
<td>2.6</td>
<td>3.2</td>
<td>12</td>
</tr>
</tbody>
</table>

| Total No. of cells | 714 | 376 | 508 | 432 | 205 |

Relative volume density for various cell types %

| Type I Epithelial cells | 26.6 | 28.3 | 26.3 | 24.3 | 25.6 |
| Type II Epithelial cells| 8.6  | 7.6  | 9.2  | 5.0  | 6.2  |
| Interstitial cells      | 16.6 | 11.4 | 14.3 | 13.4 | 9.7  |
| Interstitial matrix     | 14.3 | 24.0 | 21.3 | 28.7 | 33.6 |
| Endothelium             | 33.9 | 28.6 | 28.9 | 28.6 | 24.9 |

| Total % | 100 | 99.9 | 100 | 100 | 100 |

* the value for D is cited from Crapo et al.²¹

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such as edema or shrinkage of alveolar tissue of control groups was observed under high magnification by electron microscope. The cell dimension in the present study was considered to be in a comparable range with that of Mathieu’s and Crapo’s data. Based on these control data, it seemed reasonable to compare the data with those of exposed groups.

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increase of the total volume density of alveolar wall tissue and the dose-dependent decrease of the surface area of alveolar wall.

No attempt was made in the present experiment to detect the effects of elevation of AMT by physiological respiration methods. However, persistent tachypnea was observed in rats under continuous exposure to 0.8 or 2 ppm NO₂ for long periods. Regarding the changes of blood gas content after NO₂ exposure, lowering of arterial blood oxygen tension (PaO₂) under relatively high concentrations of NO₂ for long periods has been reported.

Recently, Suzuki et al. in a study of chronic NO₂ exposure performed at the National Institute for Environmental Studies confirmed significant decrease of PaO₂ without accompanying lowered PaCO₂ in rats after continuous exposure to NO₂ at concentrations of 0.4 and 4 ppm for 9 months. The results of this study indicate that suppression of gas exchange ability is characteristic of hypoxemic respiratory failure. Morphological findings of marked alteration and constriction at terminal airways support the above, but the importance of the changes at the peripheral region of the alveolar wall where gas exchange takes place should be kept in mind when explaining the hypoxemic nature. No significant increase of AMT was found after 9 months exposure to NO₂ at 4 ppm although a trend of increase was observed. The effects of not only lesions of the terminal airways, but also of alterations of the alveolar wall components on the failure of gas exchange ability should be further investigated.

It was recognized by the present experiment that the increase of AMT as a whole effect of continuous exposure to NO₂ for one month and compartmental difference of alveolar wall corresponded to age at exposure onset. If the concentration of NO₂ is high enough to induce clear injury to the alveolar epithelium, cell division of type II epithelium will take place as the result of necrosis and desquamation of type I cells. It is recognized that the divided cells are cuboidal in shape, resistant to NO₂, and transformed into the more squamous type I cell within a few days, thus completing the repair of the epithelial system.

Evans et al. showed that under sublethal dose of NO₂ impairment of the alveolar epithelium was stronger in older rats than in young rats, indicating delay of the repair process. The peak of proliferative response lasted one week and the lung gained resistance to NO₂. However, the mitotic indexes did not return to control levels, indicating a somewhat incomplete adaptation of the lung tissue to the NO₂.

In the present study, the number of type I epithelial cells after continuous inhalation of NO₂ at 10 ppm for one month showed increase of 28% in the 1 M, 98% in the 3 M, 5% in the 12 M, and 50% in the 21 M groups compared to the control groups of the same age. What is the explanation for the presence of an excess number of type I epithelial cells per unit surface area of lung? There are two possibilities regarding the NO₂ resistance of the type I epithelial cell newly differentiated from the divided type II cell. First, the newly differentiated
type I epithelial cells are still preserving NO₂ resistance after returning to its original size; second, the renewed cell is tolerant to NO₂ during the process transforming into the type I cell, but loses its microvilli and lamellar inclusion bodies characteristic of the type II cell. The resistance gradually diminishes as differentiation proceeds toward its original size. If the latter case is true, both the incomplete adaptation to the NO₂ and the increase in the number of type I cells would be explained well.

In the course of cellular repair of type I cells by cell division of type II cells, the differences in cell size and surface area covered by one of these two cell types should be noted. Type I epithelium has the largest surface area among the lung cell types, and the luminal surface it covers is 57\textsuperscript{21)} to 73\textsuperscript{25)} times larger than that of type II epithelium. The volume density of the type I cells is estimated to be 3 to 5 times (present study), 2.5 times\textsuperscript{25)} or 6.4 times\textsuperscript{21)} larger than that of the type II cells. These facts imply that a young renewal cell transformed from a type II into a type I cell has smaller volume density and surface area than the lost original type I cell. In fact, Cabral-Anderson \textit{et al.} observed increase of cellularity under heavy epithelial injury\textsuperscript{11}). If resistance to NO₂ decreases gradually with cell differentiation of the renewal epithelium, then after the peak period of cleavage under relatively higher concentration of NO₂ the resistance will be maintained with less frequent cleavage corresponding to the speed of differentiation of the renewal epithelium. If this is the case, then the fact that in Evans' study the mitotic indexes did not return to control level under continuous inhalation can be well understood. The above is supported by the present study in which the presence of an excess number of type I epithelial cells and the decrease of volume density per cell were confirmed in all age groups of rats at the 10 ppm level (Fig. 3).

The proliferation of type II epithelial cells should occur prior to proliferation of type I epithelial cells. The number of type II epithelial cells at the 10 ppm level increased markedly in the 1, 3 and 21-M-old groups and decreased in the 12-M-old group. Although the number of type I and type II cells of all age groups except the 12-M-old groups increased, the ratio of type II/type I cells remained at control level, as shown in Figure 5. It is reasonable to presume that the increased number of epithelial cells and reduced volume density per cell indicate the proliferation of type II cells and transformation into type I cells occurring or having occurred recently. The responses of the 12-M-old groups are different showing little change in the number of cells from the other age groups. Nevertheless, the occurrence of impairment and repair of the cells was suggested from the fact that the ratio of type II/type I varied with the concentrations of NO₂ and the volume density per cell of the type II epithelium decreased.

The responses of each age groups to low levels (0.1 and 0.5 ppm) of NO₂ are difficult to explain consistently. For example, the relatively high ratio values of type II/type I cells at 0.1 and 0.5 ppm levels of 3-M-old rats resulted from a
slight decrease of the number of type I cells and the proliferation of type II cells. It is likely that the accumulation of very mild lesions under continuous exposure for one month caused type II cells to proliferate and a small peak of cell division to take place. On the contrary, with 1-M-old rats at 0.5 ppm, the striking decrease of the number of type I cells with accompanying cell hypertrophy pushed the ratio of type II/type I higher despite a slight decrease in the number of type II cells. These facts may be explained by assuming that impairment of the type I cells has already proceeded while the division of the type II epithelium has not yet occurred. A similar trend of cell response was found at the 0.1 ppm level.

The responses of the alveolar epithelial system to NO$_2$ exposure are concluded to be as follows: the lesions of type I cells and their repair caused by cell division and transformation of type II cells are common fundamental processes, but the degree of impairment and the speed of repair differ with age of animal. Exposure to NO$_2$ at 10 ppm for one month causes definite lesions to the lung of all age groups. However, the peak stage of cell proliferation of the type II epithelium is already passed, and a small degree of turnover or repair of the epithelium maintaining adaptive response to NO$_2$ remains. On the other hand, at lower levels of exposure the lung lesions may accumulate gradually with prolonged exposure, and it is probable that the speed of development or repair of the lesion is age dependent.

It has been reported that the effects of NO$_2$ on the other cell populations of alveolar wall are not always as clear as they are in the epithelium. Evans et al. observed the incorporation of H$^3$-tymidine into mononuclear cells in the capillaries, capillary endothelial cells, cuboidal epithelial cells, interstitial cells, and alveolar macrophages, but the main labeling was found in circulating mononuclear cells in capillaries and "intermediate" epithelial cells. Crapo et al. demonstrated that in rats exposed to 25 ppm NO$_2$ for 6 h/day for 5 consecutive days the number of type II epithelial cells, interstitial cells and alveolar macrophages increased significantly. In the present study, the number of interstitial cells increased markedly by NO$_2$ exposure in the 1 and 21-M-old groups and it was about 1.5 times as high at the 10 ppm level with accompanying reduction of volume density per cell. Exposed groups of 3-M-old rats showed cell hyperplasia without any changes in the number of cells. These observations suggest that the interstitial cells also undergo a slight degree of cell proliferation after NO$_2$ exposure, and that the reaction is more active particularly in young and aged rats. One of the reasons for the fact that the magnitude of reaction does not always correspond to NO$_2$ dose may be due to the heterogeneous cell population of the interstitial cells.

The changes in volume density of interstitial matrix are derived from edema and addition of fibrous components. Widening of the air-blood barrier in the aged human lung due to edema and fibrous change is known. The increase of alveolar interstitial matrix with aging in normal control rats was measured quanti-
tatively for the first time in this study (Fig. 7b). The same type of change in normal rats related to aging has been reported on the basement membrane of the renal tubules, where the thickness of the membrane of 14-M-old rats was measured as being twice as that of 4-M-old rats\(^{35}\). It was shown clearly in the present study that increase of interstitial matrix occurred in the case of NO\(_2\) dose higher than 0.5 ppm in 1-M-old rats and that the increment was also high. On the contrary, little change was noted in the other age groups except at the 0.1 ppm level in 3-M-old groups and at the 10 ppm level in 12-M-old groups which showed fairly large increases.

As indicated in Figure 7c, volume density of the total interstitium of all age groups increases with NO\(_2\) dose over 3 ppm. The large contribution of the total interstitium to the increase of VdAWT is important whether or not the rates of increase of the total interstitium are so large compared to the other components. Under continuous exposure to 4 ppm NO\(_2\) for 27 months, lower rates of increase of the total volume density of interstitium than in younger rats and qualitative alteration of the interstitial lesions were observed\(^{31}\). The results obtained with 21-M-old rats in the present study are in agreement with this result.

There is controversy over whether or not exposure to NO\(_2\) causes some damage to the capillary endothelium\(^{21,36,37}\). Guidotti reported impairments of capillary endothelium showing increased redundancy and frequency of vesicles after short exposure to rather high concentrations of NO\(_2\) as well as interstitial edema resulting from higher permeability of the capillary wall\(^{38}\). Kohno \textit{et al.}\(^{39}\) also observed a remarkable change in pinocytotic vesicles of rats exposed to 0.5 ppm NO\(_2\) for 2 months, and they noted a resemblance to effects caused 3 days after exposure to 20 ppm NO\(_2\) for 20 hours.

Correlation between NO\(_2\) dose and changes of the capillary endothelium was not so clear in all age groups in the present investigation. Slight hyperplasia and decrease in the number of endothelial cells observed in 1-M-old rats at NO\(_2\) doses over 0.5 ppm was considered to imply a mild lesion of the endothelium. With rats older than 3 M, no consistent correspondence between the number of endothelial cells and the volume density per cell was observed. However, the volume density per cell of the groups exposed to NO\(_2\) over 3 ppm was small: the trend was particularly evident in the 21-M-old groups. The findings that the magnitude of change of the capillary endothelium is less than that of the other cell types and that the absence of correlation with NO\(_2\) dose may suggest a shorter circuit for endothelial renewal.

Summarizing the above results, it is clear that the dose of NO\(_2\) and the increase of AMT are closely related. Using AMT as an index, it was concluded that there exist age dependent differences in response to the same concentration of NO\(_2\) and that the degree of response falls with aging from 1 M to 12 M while it rises at 21 M. Those results, however, are derived as the sum total of the increase or decrease of each compartmental component of the alveolar wall; thus little or no
change in AMT does not necessarily mean low sensitivity to NO₂.

The cellular responses to NO₂ were measured as either numerical changes or changes of volume density. The 12-M-old rats differ somewhat from the other age groups because the responsiveness of the alveolar cells is generally low, and except for a slight increase of the number of the type II cells at low levels of NO₂, most of the cellular components remain steady or show a trend of slight decrease at high NO₂ levels. The significant increase of AMT found at the 10 ppm level was caused mainly by the increase of the interstitial matrix.

The cellular reactions were high in 1 and 21-M-old groups with similar patterns of reaction. The response of alveolar cells seems to be high in young rats, decreasing gradually in middle age rats and rising again in old rats. The rise of cell reactivity and the increase of interstitial cells in 21-M-old rats should be particularly noted because of the qualitative alteration of lung lesions in aged rats. Accelerated alteration of the elastic system in the alveolar wall of aged rats is presumed to cause interstitial impairment in addition to the increment of interstitial matrix with aging.

Incipient emphysema induced by relatively low concentrations of NO₂ has been reported with beagle dogs⁷. In lifetime exposure to NO₂ at polluted or ambient air concentrations, however, typical emphysema did not appear in rodents which had only 2 to 3 years life spans, but tachypnea and enlargement of lung were observed³. In a preliminary estimation by lightmicroscope, the mean alveolar size of rats exposed to NO₂ at 1 M of age for one month did not change at NO₂ levels lower than 3 ppm. However, 45% increase at 5 ppm and 78% increase at 10 ppm were observed, resulting in marked reduction of the number of alveoles counted per unit area of lung. With the lung tissue fixed through tracheal instillation, one electronmicrograph recorded with direct magnification of ×2000 contains parts of alveoles but not two complete alveoles. Accordingly, much more tissue per unit area of electronmicroscopic morphometry were not observed with higher concentration groups compared to control groups. Nevertheless, increase of volume density and number of cells with visible nuclei per unit area were observed with exposure groups. As various types of alveolar cells divide by NO₂ inhalation¹², the comparison of cell population of alveolar wall components should be done using the total number of cells per unit area excluding mononuclear cells in capillary lumen and alveolar macrophages in air space.

Both Yuen²⁰ and Crapo et al.²¹ have reported increase in the number of the type II cells by cell differentiation under electronmicroscope. Their data are based on the count of alveolar wall tissue excluding alveolar macrophages in the lumen and nucleated cells in the capillary. It is widely accepted that proliferative changes develop at the broncho-pulmonary junction in continuous exposure to a relatively low concentration of NO₂. However, detailed information on the larger area of the peripheral alveoles was insufficient and only slight enlargement or varying size of alveoles was noted²,³¹.
The present investigation was done on the alveolar wall excluding the alveolar ducts, and showed clearly the occurrence of NO₂ dose-dependent response at the peripheral alveolar walls and age-dependent differences in reactivity. The re-elevation of cellular responses at old age and numerical increase of interstitial cells strongly suggest effects on the elastic system of lung parenchyma particularly in aged animals. The possibility of a gradual accumulation of lung lesions in a larger area of alveolar wall under continuous exposure to low concentration of NO₂ as a causative factor of emphysema cannot be excluded in animals with long life spans.

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