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Experimental Model of Bronchiolitis Obliterans : Pulmonary Response of Mice to High-Concentration Ozone Exposure

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ABSTRACT. In order to elucidate the pathogenesis of bronchiolitis obliterans, an experimental model was produced in mice using high-concentration ozone. A single exposure to 3.5 p.p.m. of ozone for 4 hours diffusely developed occlusion of the terminal bronchioles. These lesions consisted mainly of hyperplasia of bronchiolar and alveolar epithelial cells. Four exposures, at intervals of 3 days, resulted in more intense regeneration of the terminal airways with fibrous polypoid lesions protruding from the bronchiolar wall into the lumen. Although these lesions morphologically resembled bronchiolitis obliterans in human cases, they did not persist for a long period after the exposure. The explanation for this transient occurrence and the mechanism of the damage remains to be clarified.

Key words : bronchiolitis obliterans — ozone — terminal bronchiole — respiratory bronchiole — mouse

Bronchiolitis obliterans is an inflammatory and occlusive disease of the terminal bronchioles characterized by polypoid masses of fibrous tissue protruding into bronchiolar lumen.[1] The polypoid masses are the result of injury to the bronchiolar wall through the basement membranes with some derangement of elastic fibers. It has been suggested that bronchiolitis obliterans is merely a tissue reaction to any injury to distal bronchioles.[2] A wide variety of stimuli, such as infections, toxic-fumes, and collagen-vascular diseases, therefore, may produce the same tissue reaction pattern, although about one-third of such cases are clinically idiopathic. In addition, there are lesions called organizing pneumonia,[3] in which similar tissue reactions are localized in the alveolar ducts and alveoli.

In order to better understand the site and the mechanism of injury and its repair process, it is necessary to study the lesions in sequence from the early stage of insult to the repair stage with polypoid tissue. For these studies, good experimental models for such disorders should be sought first. Animals, toxic substances, and the manner of exposure should be properly chosen. At the beginning of this study, mice were exposed ozone, nitrogen dioxide, sulfur dioxide or formaldehyde of different concentrations and with varying frequency. In this communication, however, I describe the results in mice of 1 or 4 exposures to 3.5 ± 1.5 p.p.m. ozone, because these two groups were suitable for

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my purpose. Four exposures evoked severer lesions in the mice, and those lesions showed a very close resemblance to bronchiolitis obliterans in human cases.

MATERIALS AND METHODS

1. Single exposure group

Thirty female ICR mice (Clea Japan, Inc.), 6 to 8 weeks old and weighing 24 to 27 g each, were used in this study. All mice were exposed to 3.5 ± 1.5 p.p.m. of ozone for 4 hours. For exposure, ozone was generated from pure oxygen by a silent electrical discharge ozone generator (Clea Japan, Inc.) and mixed with room air in a chamber. The concentration of ozone was measured by Kitagawa precision gas detector tubes (Komei Rikagaku Kogyo Co.) every 30 minutes.

After exposure, all mice were removed to a normal ambient atmosphere. At 1, 6, 12 and 24 hours, and 2, 3, 4, 7, 14 and 28 days after exposure, three mice each were sacrificed by an intraperitoneally injected lethal dose of sodium pentobarbital, and a thoracotomy was performed. The trachea was cannulated, instilled with 10 per cent buffered formalin at 25 cm water pressure, and ligated. The thoracic organs were removed in toto. The nasal cavity, larynx, lymph nodes and intraperitoneal organs were also removed and fixed in 10 per cent formalin. All tissues were sliced after adequate fixation. Tissue slices were routinely processed, embedded in paraffin, and 4 μm thick sections were stained with Hematoxylin and Eosin. Silver impregnation for reticulin fibers or Weigert's elastic stain for elastic fibers was performed when necessary.

Thirty other mice, placed only in a normal ambient atmosphere, were sacrificed in similar manner as a negative control.

2. Four exposure group

Another 24 mice were exposed to the same dose of ozone 4 times at 3 day intervals. After the last exposure, the animals were treated in a similar manner to those in the single exposure group. The intervals between the last exposure and sacrifice of the animals were 1 and 24 hours, and 2, 3, 4, 7, 14 and 28 days. In addition, several mice were added at each exposure as positive controls to check the difference in severity from those receiving cumulative exposures.

RESULTS

1. Single exposure group

In general, lesions were confined mainly to the terminal bronchioles and their adjacent parenchyma, and distributed diffusely and bilaterally (Fig. 1). Morphologically, they varied to a slight degree among animals sacrificed at the same time sequence. The nasal cavity and non-respiratory organs were almost free of any pathologies at all times.

At 1 hour after the exposure, no apparent differences were observed between the ozone exposed mice and the controls. Between 6 and 12 hours, flattening of epithelial cells appeared in the terminal bronchioles. These epithelia showed cytoplasmic vacuolization and pyknotic nuclei, and some had desquamated into

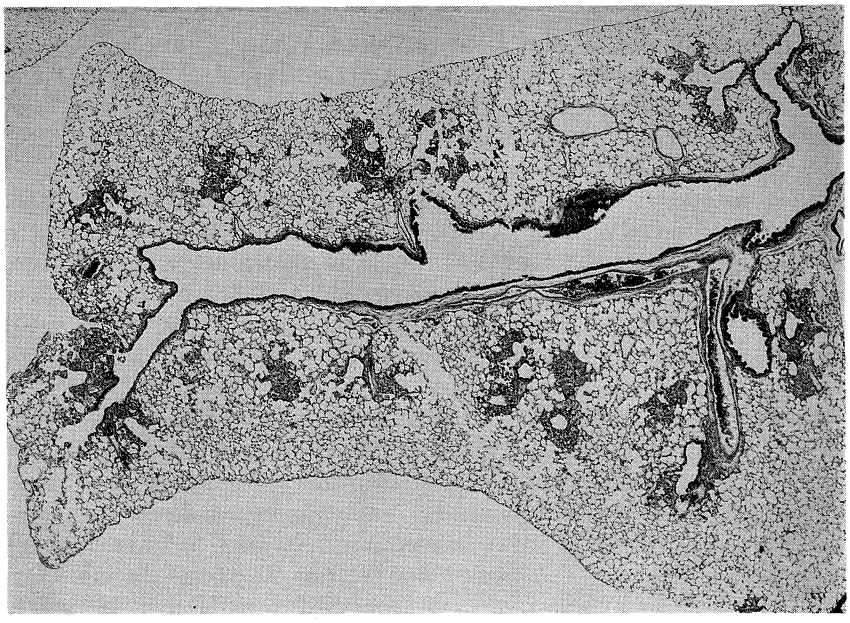

Fig. 1. Three days after a single exposure to ozone. Lesions are diffusely present and confined to the terminal bronchioles. HE, ×20.

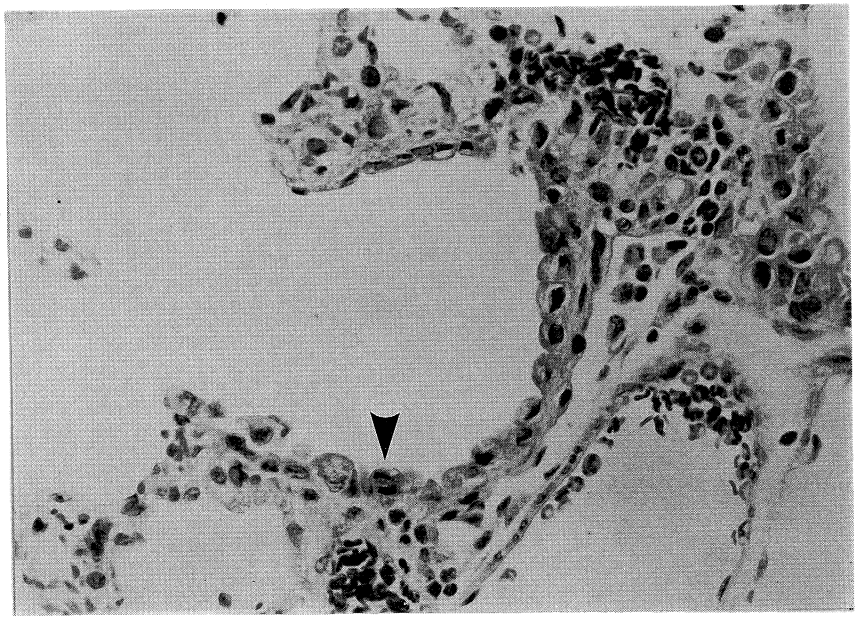

Fig. 2. Terminal bronchioles at 24 hours after a single exposure showing regeneration of epithelial cells. Note that a mitotic figure is present (arrowhead). HE, ×400.

the bronchiolar lumen. Cilia were observed vaguely. At 24 hours, flattening, desquamation, and loss of cilia were still present. Some terminal bronchioles, especially near the alveolar ducts, were replaced by single-layered, flattened epithelial cells, but some plump, polyhedral cells with bizarre nuclei were intermingled with them. Mitotic figures were occasionally seen (Fig. 2).

On the second day, these epithelial changes were more frequently noted and tended to spread into adjacent alveoli. Some terminal bronchioles had lost their lining epithelium, and others were covered with flattened and plump cells. They were thickened with infiltration of mononuclear cells, neutrophils and eosinophils, and their lumina were narrowed. Alveoli around the bronchioles were focally and partially lined with polyhedral cells which were similar to regenerating bronchiolar epithelium. These cells often occluded the alveolar lumen. There was also a scattering of foamy histiocytes (Fig. 3).

Regeneration was more prominent at days 3 and 4. Bizarre polyhedral cells with large hyperchromatic nuclei increased in number as well as in their atypism in the terminal bronchioles and their neighboring alveoli. The number of inflammatory cells in the bronchiolar wall was reduced, and spindle cells became prominent (Fig. 4). Silver impregnation revealed mild reticulin fibrosis in these bronchiolar walls. Reticulin and elastic fibers in the alveolar wall appeared increased in number and they encircled regenerating epithelial cells (Fig. 5). Alveoli adjacent to the bronchioles were filled with plump alveolar epithelial cells.

At day 7, the lesions became inconspicuous, with only mild alveolar epithelial swelling remaining. At days 14 and 28, lung histology showed no difference between the ozone exposed mice and the controls.

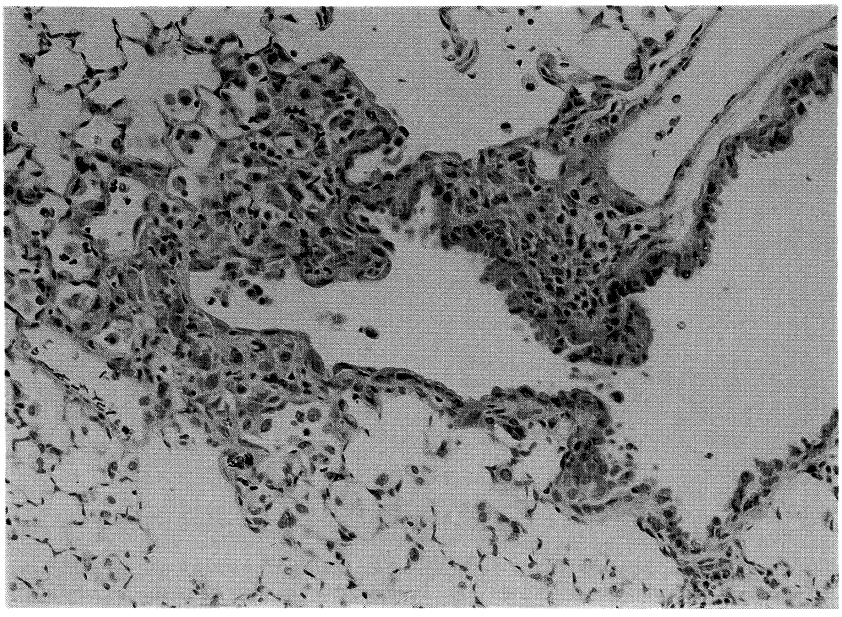

Fig. 3. Two days after a single exposure to ozone. The bronchiolo-alveolar junction is occluded by regenerating epithelial cells. HE, ×100.

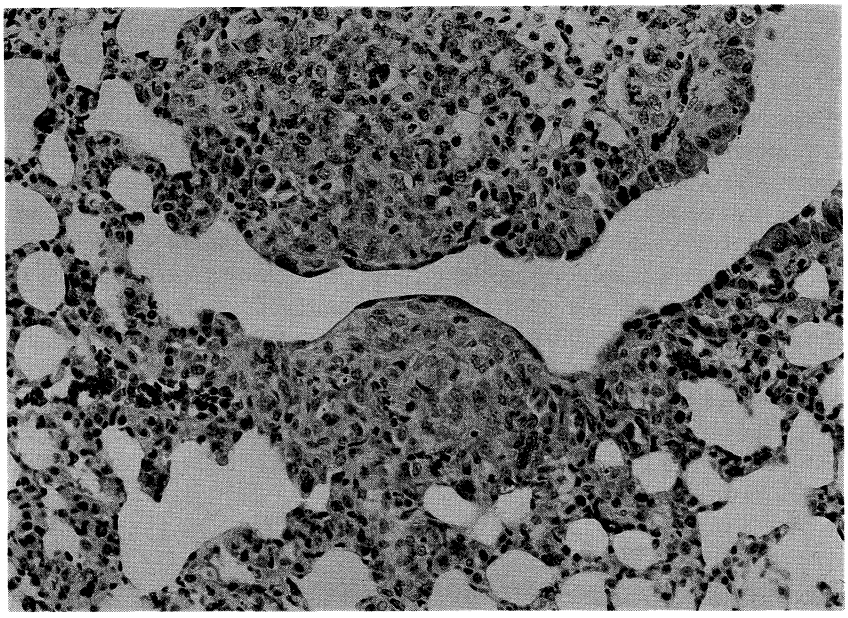

Fig. 4. Four days after a single exposure to ozone. The terminal bronchioles are still covered with flat regenerating epithelial cells but have been narrowed by plump spindle cells in their walls. HE, ×100.

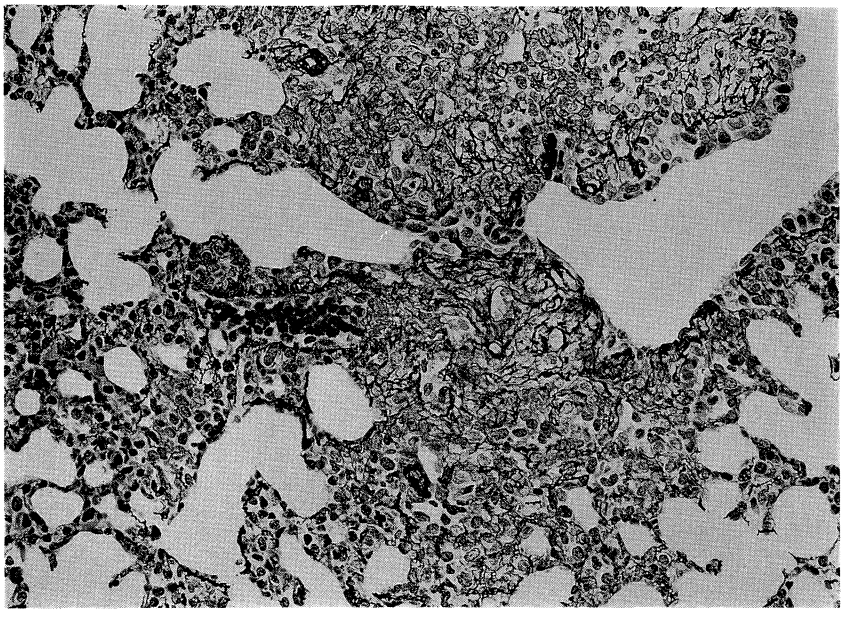

Fig. 5. Reticulin fibers at day 4. Mild reticulin fibrosis can be seen in the bronchiolar wall. Silver impregnation, ×100.

2. Four exposure group

At 1 hour after the last exposure, epithelial flattening was already obvious in all portions of the airway from the larynx and trachea to the terminal bronchioles, and epithelia also seemed to have lost their surface cilia. The epithelium had been desquamated to some degree from medium-sized bronchi to the terminal bronchioles. These epithelia had pyknotic nuclei and eosinophilic cytoplasm with vacuolization (Fig. 6). The nasal mucosa was unremarkable.

At 24 hours, epithelial damage was severer, especially at the distal terminal bronchioles. The central airways showed regenerated plump epithelial cells in places. Epithelial regeneration of the terminal bronchioles started on the second day after exposure, and the epithelial lining consisted of flattened and plump, cuboidal cells. The bronchiolar walls had accumulations of spindle-shaped or polyhedral cells. These cells made the bronchiolar walls thicker and their lumen narrower. Nevertheless, inflammatory cell infiltration was less conspicuous than that following a single exposure (Fig. 7). By this time, plump epithelial cells had piled up in the central airways. Some of these cells retained clear-cut ciliary structures.

Regeneration was maximum on the third day (Fig. 8). The terminal bronchioles, especially at their tail end, were sometimes nearly occluded by fibrous polypoid masses. These masses were composed of spindle or polyhedral plump cells, with some mononuclear cell infiltration. Sometimes they were lined with flattened epithelium. Reticulin fibrosis was evident in silver impregnated tissue sections (Fig. 9). In these polypoid lesions, fibers were partially continuous to those of the bronchiolar or alveolar walls. Elastic fibers, outlining the bronchiolar lumen, were destroyed in part by these polypoid tissue (Fig. 10). Minimal bronchopneumonias were present in association with obstruction of the terminal

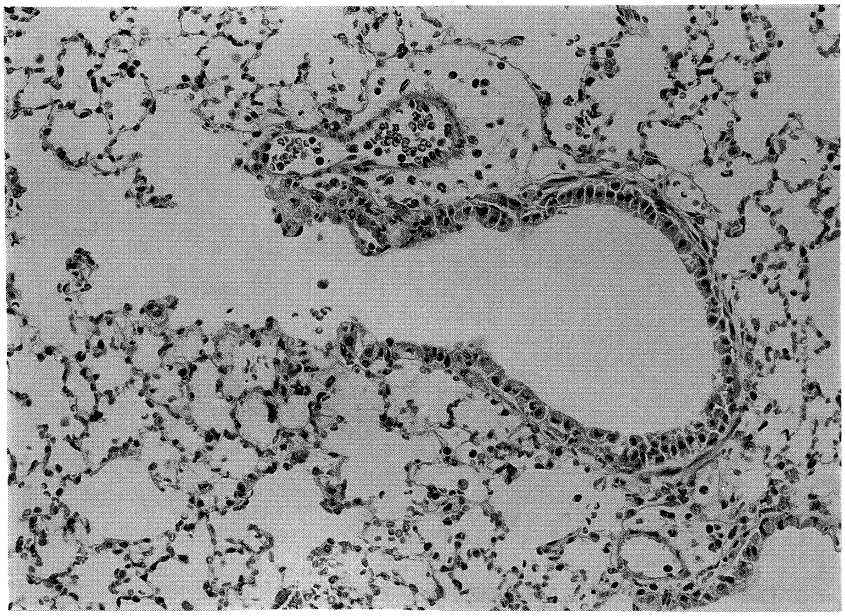

Fig. 6. Terminal bronchioles at 1 hour after the 4th exposure to ozone, showing marked epithelial desquamation. HE, ×100.

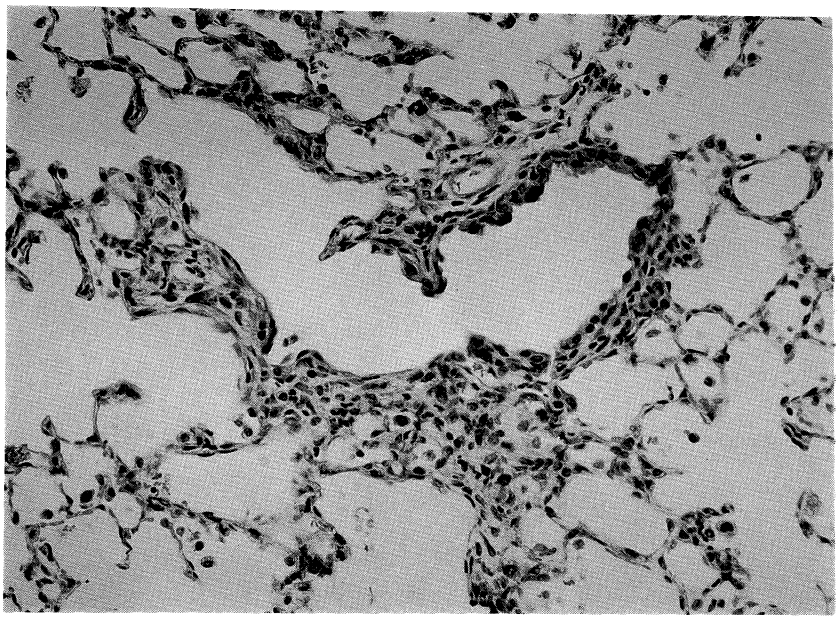

Fig. 7. Two days after a 4th exposure to ozone. Epithelial regeneration on the terminal bronchioles is severe, but inflammatory cell infiltration is minimal. HE, ×100.

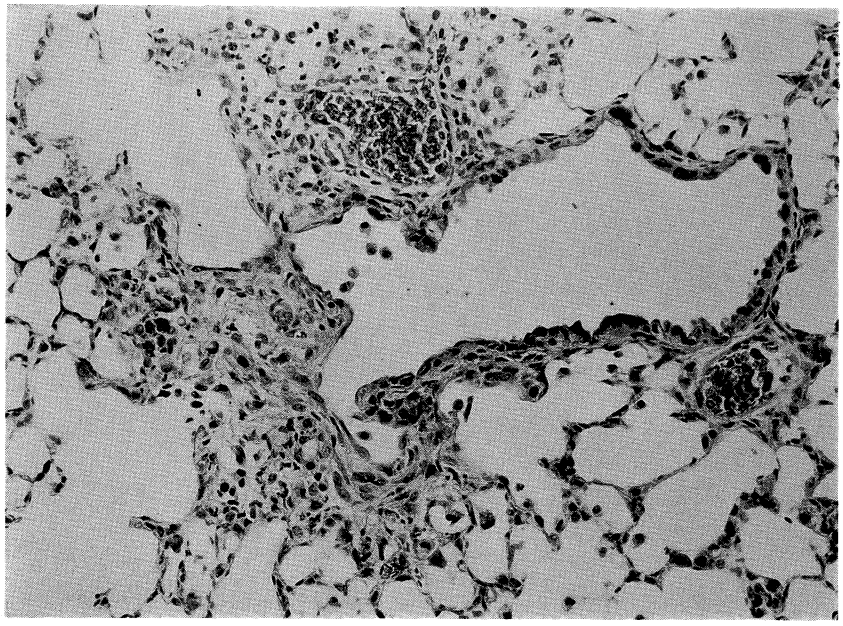

Fig. 8. Three days after a 4th exposure. Note the polypoid mass at the bronchiolo-alveolar junction. HE, ×100.

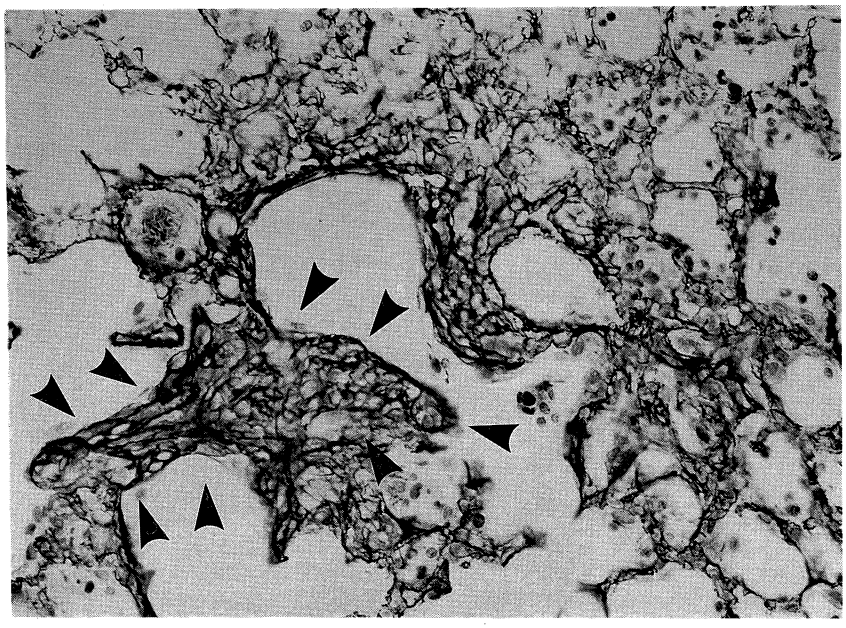

Fig. 9. Reticulin fibrosis is seen in the polypoid lesion (arrowhead) and the bronchiolar wall (The same specimen as in Fig. 8). Both fibers are partially continuous. Silver impregnation, ×100.

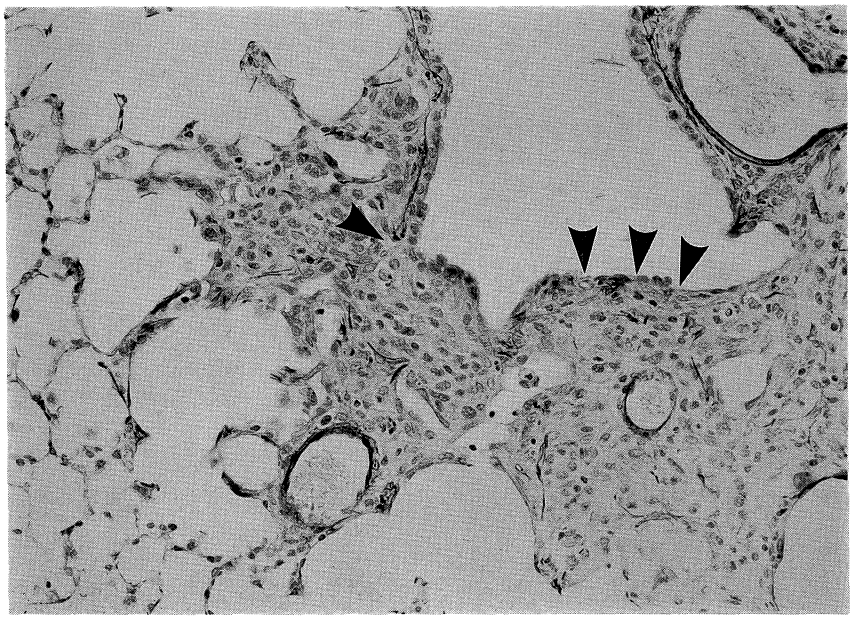

Fig. 10. Three days after a 4th exposure. The bronchiolar elastic fiber has been partially destroyed by the fibrous polypoid mass (arrowhead). Weigert's elastic stain, ×100.

bronchioles. The condition of the central airways was almost the same as in the controls.

At day 4, the luminal obstruction and epithelial regeneration decreased to greater extent. There were no appreciable lesions between days 7 and 28.

DISCUSSION

Ozone is a gas irritant to the human body. It is a principal oxidant gas of photochemical smog and highly ambient level of ozone may be detected in the welding industry. It is well known that ozone irritates respiratory systems and that it causes pulmonary edema and airway obstructive disease.[4,5] Many experimental studies have demonstrated, that an ambient level of ozone may produce small airway occlusion.[6–11] In most of these studies, the earliest change has been damage of the ciliated and type I alveolar epithelium.[9] Necrotic cell debris has also been seen in the bronchiolar lumen. Macrophages have been scattered in alveoli around the bronchioles.[12] In the later stage, epithelial hyperplasia have appeared in the junctional portion between the terminal bronchioles and alveoli.[6–8,11,12] Long-term exposure has resulted in metaplastic changes in the epithelium,[12,13] but has never produced fibrous obstruction of the small airways. In consideration of the fact that ozone has no delayed effect,[14] it is uncertain whether these pulmonary changes are derived from the direct effect of ozone or are secondary to the primary injury.

Experiments concerning acute high-concentration ozone exposure have been less frequently reported. Propper *et al.* exposed rats to 3 p.p.m. of ozone for 4 hours and found that ciliated and non-ciliated epithelia were equally damaged by such high-concentration ozone exposures.[15] Unfortunately, however, they described only the pathological findings immediately after exposure. Observations at the repair stage after high-concentration ozone exposure were made by Evans *et al.*[14] According to them, epithelial hyperplasia was prominent at the bronchiolo-alveolar junction. It seems generally accepted that there is a close correlation between the concentration of ozone and the intensity of the repair process.[6,14,16] I am not aware of experimental reports describing polypoid fibrous tissues in distal airways, similar to bronchiolitis obliterans in human cases.

In the current study, ozone was given in high-concentration and mice were kept in room air until the repair process had taken place. A single exposure resulted in a severer tissue reaction at the bronchiolo-alveolar junction, which was composed only of hyperplasia of the epithelium. Destruction of elastic fiber was indistinct. Reticulin fibers were slightly increased in the bronchiolar walls, seemingly in reaction to the epithelial hyperplasia. Obstructive change in the bronchioles as a result of epithelial hyperplasia is not essential to bronchiolitis obliterans. It is assumed that destruction of elastic fibers indicates damage to the bronchiolar and alveolar walls severe enough to cause the organizing tissue process to extend into the lumen, which is the histological hallmark of bronchiolitis obliterans. Therefore, I first intended to severely damage the bronchiolar wall by either increasing the concentration of ozone or prolonging the duration of exposure to it, and to observe a derangement of elastic fibers. Both of these procedures seemed to have a lethal effect on the mice, but did not produce the lesions I desired. Then, I planned repetition

of the same exposure. Good results were not achieved by the daily exposure reported by Freeman *et al.*[13] Therefore, the mice were exposed to ozone 4 times at the presumed time of epithelial regeneration. The interval of 3 days was based on the data of our experiments with a single exposure. By means of this experiment, I was able to successfully produce polypoid fibrous masses protruding from terminal bronchiolar wall into the lumen on the third day after the last exposure. The lesions were confined to distal bronchioles as with a single exposure. In addition to the presence of spindle and plump polyhedral cells and some mononuclear cell infiltration, tangles of reticulin fibers continuous to those of the bronchiolar and alveolar walls were seen to form polypoid masses. Elastic fibers were partially destroyed. These findings are consistent with bronchiolitis obliterans, and are equivalent to those seen in human cases. Therefore, I believe that the repeated exposures induced the intense fibrous repair process through damage to the basement membranes of this area.

Before concluding that this lesion is a good experimental model for bronchiolitis obliterans, it should be considered why the lesions persisted for a rather short period and whether or not such a quick change is suitable for studies to elucidate the pathogenetical mechanism of the disease. The fibrous lesions in this study were most conspicuous on the third day and mostly disappeared after the fourth day. This may be due to the tolerance of mice to the ozone. Several studies[17–19] have demonstrated that animals exposed to a short-term, ambient level of ozone were not sensitive to a following exposure to high concentration ozone. This phenomenon has been observed in several species, including mice, and cross-tolerance to some other irritant gases may develop.[19–21] Such tolerance has been reported to continue for one to three months. In those studies, however, pulmonary damage was determined by the presence of pulmonary edema alone. Autoradiographically, type I alveolar epithelial cells were already tolerant on the third day, and disappeared on the seventh day.[18] This may indicate that the tolerance may not last for a longer period. In my study, epithelial occlusion and inflammatory cell infiltration were minimal during the course of repeated exposures. This may indicate that some tolerance has developed. However, it is dubious that such tolerance took place during its recovery phase, to reduce and shorten the fibrosing process. Ozone has been implicated in fibrogenic responses.[17] Therefore, it is possible that the fibrotic process was exaggerated by the repeated exposures. If the ozone exposures had been repeated longer, the fibrous lesions might have existed longer.

The transiency of the lesions may be explained by species specificity. The reactivity to ozone and the anatomy of the airways or the lung may be different in mice and human beings. For example, it has been said that mice do not have the distinct zone of respiratory bronchioles that can be seen in human beings. Such structural difference may be responsible for the different degree of inflammatory response. In any case, though it was of short duration, the sequence of events seen in the present study was sufficiently long enough to gain some insights regarding the pathogenetic mechanism of bronchiolitis obliterans.

In conclusion, an experimental model for human bronchiolitis obliterans was successfully produced by repeated ozone exposure. Fibrous polypoid tissue masses were observed in the bronchioles on the third day after 4 exposures. Although the lesions were transient, it is believed that it is a good model for the study of bronchiolitis obliterans.

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