

## Pulmonary Tumors Induced in Mice by Vinyl Chloride Monomer<sup>1</sup>

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Neoplastic effects of vinyl chloride were studied in lungs of 27 mice exposed to vinyl chloride monomer at 2500 and 6000 ppm for 5 and 6 months. Pulmonary tumors were observed in 26 of 27 experimental animals. By light microscopy, the tumors were multiple and arranged in either tubulo-papillary or adenomatous formations. Although occasional mitotic divisions and invaginations into the bronchiolar lumen were observed, no metastases were found. By electron microscopy, short microvilli, tight junctions between two adjacent cells, appearance of osmiophilic lamellar bodies, large mitochondria of irregular shape, well-developed Golgi complexes, continuous or discontinuous basement membranes, occasional appearance of "sequestration" and of crystalloids, and lack of both cilia and mucous secretory granules were observed as characteristic features of the neoplastic cells. Some of the cells were poorly differentiated and were equipped with poorly developed organoids, without formation of osmiophilic lamellar bodies. The pulmonary tumors corresponded to "alveologenic tumors or alveologenic cancer." It is suggested that the neoplastic cells were transformed from type II alveolar epithelium via its hyperplastic form. It is concluded that mouse lung is an extremely sensitive indicator of the oncogenicity of vinyl chloride.

### INTRODUCTION

Hepatic hemangiosarcoma has been accepted as a serious health hazard associated with vinyl chloride exposure among workers in vinyl chloride polymerization plants (1-7). A risk of lung cancer has also been reported among the workers on the basis of epidemiological studies (8, 9).

Experimental studies in rats, mice, and hamsters have shown that, in addition to liver, various organs such as lung, brain, breast, and skin, including sebaceous glands, were involved in induction of primary neoplasia by vinyl chloride. Although a number of studies have demonstrated that pulmonary tumors can be induced by vinyl chloride in mice, the significance of such occurrence and the nature of the tumors have not yet been appropriately explored (10-18).

We have undertaken a preliminary study of pulmonary oncogenetic effects of vinyl chloride in mice to provide information on these questions. A number of significant observations have been made: frequent occurrence of pulmonary tumors, unique ultrastructure of the neoplastic cells, findings concerning the precursor of these cells, as well as data regarding the submicroscopic aspects

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FIG. 1. Two pulmonary tumors induced by vinyl chloride are seen in the peripheral part of a mouse lung (2500 ppm in group III). Hematoxylin and eosin:  $\times 64$ .

of the neoplastic transformation from the precursor cells. Details of these findings are reported here.

#### MATERIALS AND METHODS

Twenty-seven CD1 Charles River white strain male mice, 4 to 5 weeks old at first exposure, were used. These animals were divided into three groups. In group I, six animals were exposed to vinyl chloride at 2500 (three mice) and 6000 (three mice) ppm/hour, 5 hours/day, 5 days/week, for 5 months. They were then kept for 6 days without exposure before sacrifice. In group II, 13 mice were exposed at 2500 (seven mice) and 6000 (six mice) ppm for 6 months and were kept for an additional 2 days for recovery before sacrifice. In group III, eight animals (seven at 2500 ppm and one at 6000 ppm) were exposed to vinyl chloride monomer for 6 months followed by a 37-day recovery period. The inhalation exposures were accomplished at the Industrial Bio-Test Laboratory, Northbrook, Ill.

In addition to the experimental animals, 16 mice (four for group I, four for group II, three for group III, and five which were 12 months old) were simultaneously maintained as controls. The control mice were primarily of interest for providing data concerning the spontaneous occurrence of pulmonary tumors.

Under anesthesia, the mice of both experimental and control groups were sacrificed by decapitation. Lungs were examined under a dissecting microscope after the organs were taken out of the bodies to determine whether tumors had occurred.

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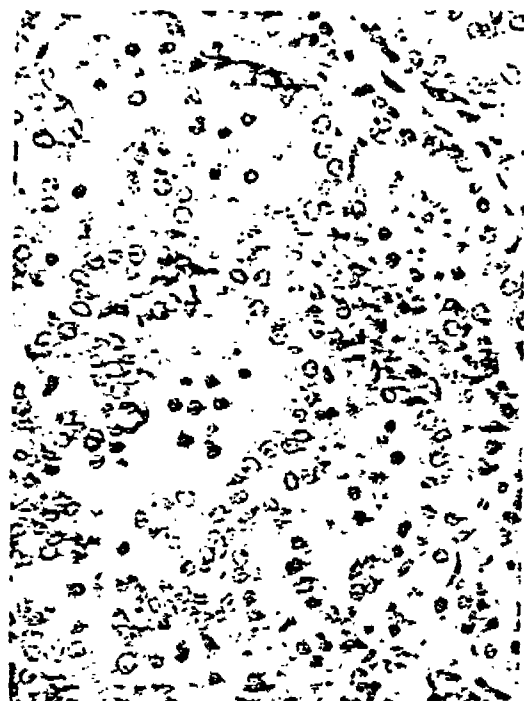


FIG. 2. A part of the lower tumor seen in Fig. 1. A tubulo-papillary pattern is observed. The neoplastic cells are basophilic. Hematoxylin and eosin:  $\times 400$ .

For light microscopy, the organs were fixed in 10% neutral buffered formalin and embedded in paraffin after dehydration in alcohol. Five- to six-micrometer sections were made and stained with hematoxylin-eosin, Weigert's silver, periodic acid-Schiff's (PAS) with and without digestion by diastase, elastin, and Van Gieson's picrofuchsin technique. For electron microscopy small pieces, smaller than  $1 \text{ mm}^3$ , were taken from both pulmonary tumors and non-neoplastic pulmonary tissues and were fixed in 1% phosphate buffered osmic acid at pH 7.2-7.4 for 2 hours. After alcohol dehydration, the blocks were embedded in epoxy resin. Ultrathin sections were obtained with an LKB microtome. The sections were stained with uranyl acetate and lead. A Siemens 101 electron microscope was used for ultrastructural observations. In addition to the lungs, other major organs, including the liver, were examined. To the present, three hepatic hemangiosarcomas were observed in the 2500-ppm series of group III. Details of the hepatic effects of vinyl chloride will be reported elsewhere (19).

## OBSERVATIONS

### A. Gross Anatomical Findings

Pulmonary tumors were observed in all experimental mice except one from the 6000-ppm series of group II (26 of 27). None were found in 16 controls. These tumors were round, whitish in color, multiple in number, and variable in size from

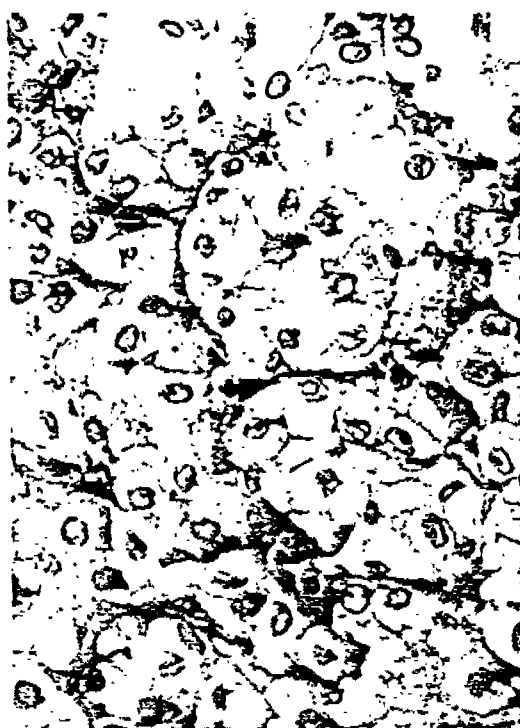


FIG. 3. A part of the upper tumor seen in Fig. 1. An adenomatous pattern is seen. Hematoxylin and eosin;  $\times 560$ .

1 to to 5 mm in diameter. No metastases to regional lymph nodes or other organs were observed. Neither parenchymal fibrosis nor fibrotic adhesions of the pleura were detected.

#### B. Light Microscopy

As shown in Fig. 1, the tumors were usually seen in the peripheral part of lung parenchyma, although occasionally tumors were found in more proximal parts of the lung. No direct connections of the tumors with bronchi or bronchioles were observed. The neoplastic cells were arranged in various ways, such as tubulo-papillary (Fig. 2) and adenomatous forms (Fig. 3). Polymorphism and atypical structures were not striking. However, sometimes abnormal mitoses were observed, as shown in Fig. 4 (arrow labeled M). The nuclei were round in shape and small, and chromatin was generally finely distributed. Nucleoli were generally poor in development. Two different types, eosinophilic and basophilic, were distinguished in the neoplastic cells (Figs. 2 and 3). Some of the cells stained with PAS (Fig. 4; arrow), and the substance so stained was digested by diastase, suggesting that it was glycogen. The neoplastic tissue was not encapsulated by connective tissue. Often, air spaces separated neoplastic tissue from normal tissue. Although malignant invasion, such as destruction of preexisting tissue, was not observed in the animal lungs, invagination of the neoplastic tissue into bron-

FIG. 4. Neoplastic cells stained by diastase. The arrow labeled M indicates an abnormal mitosis.

chiolar air spaces and reticular connective tissue changes of the lung parenchyma. In Fig. 5, the lining cells may be alveolar cells. Hyperplasia is shown in Fig. 6. The lobule of the lung is not always clearly identifiable. Identification is not feasible.

#### C. Electron Microscopy

Figure 7 (Figs. 1 and 2). Ultrastructure: round- or oval-shaped, electron-lucent neoplastic cells.



FIG. 4. Neoplastic cells stained with PAS. An arrow indicates fine PAS-positive material digested by diastase. The arrow labeled M shows an abnormal mitosis (2500 ppm in group III).  $\times 560$ .

cholar air spaces was detected in instances of extremely large tumors. Collagen and reticular fibers showed little development in the neoplastic tissues. In addition to neoplastic changes, as shown in Figs. 5 and 6, focal and multiple hyperplastic changes of the alveolar lining cells were noted in lungs exposed to vinyl chloride. In Fig. 5, the hyperplastic cells are seen just beneath the visceral pleura. Since the lining cells beneath the thin connective tissue of the visceral pleura are known to be alveolar epithelium, the hyperplastic cells are assumed to be alveolar epithelial cells. Hyperplastic cells are also found in the deeper part of lung parenchyma, as shown in Fig. 6. Occasionally, neoplasia and hyperplasia coexisted in the same lobe of the lung, and the distinction between neoplastic and hyperplastic cells was not always clear, as some cellular similarities were found between the two. Confident identification of the cell types of both neoplastic and hyperplastic cells was not feasible at the level of light microscopy.

#### *Electron Microscopy*

Figure 7 is derived from neoplastic tissue of the tubulo-papillary form (see Fig. 2). Ultrastructural characteristics of the neoplastic cell included microvilli, large, round- or rod-shaped mitochondria, well-developed Golgi complexes, and osmiophilic lamellar bodies. Junctional structures (arrows in Fig. 7) between adjacent neoplastic cells and a basement membrane (B) were usually observed. Figure



FIG. 5. Hyperplastic pulmonary cells are seen beneath the visceral pleura of a mouse lung (2500 ppm in group III). Hematoxylin and eosin;  $\times 430$ .



FIG. 6. Proliferated alveolar lining cells in the deeper part of lung parenchyma. (2500 ppm in group III). Hematoxylin and eosin;  $\times 170$ .



FIG. 7. A low-power junctional structure.  $\times 7900$ .

8 was derived poorly formed : Fig. 7. However Golgi complex two. Irregular mitochondria v mic reticulum partsments for compartments scopic observ



FIG. 7. A low-power electron micrograph of a well-differentiated pulmonary tumor. Arrows indicate junctional structures between neoplastic cells. B. Basement membrane (2500 ppm in group III).  $\times 7900$ .

8 was derived from an adenomatous area shown in Fig. 3. Neoplastic cells had poorly formed tubular lumens and microvilli and were smaller than those shown in Fig. 7. However, other ultrastructural characteristics, such as mitochondria, Golgi complexes, and osmiophilic lamellar bodies, were almost identical in the two. Irregular arrangements of cristae mitochondriales were fairly common, and mitochondria were often wrapped by well-developed smooth-surfaced endoplasmic reticulum (Fig. 9). Some of the neoplastic cells contained cytoplasmic compartments formed by membrane structures (Fig. 10). The occurrence of such compartments has been reported by Svoboda (20), who made electron microscopic observations on the neoplastic cells in mouse pulmonary tumors induced

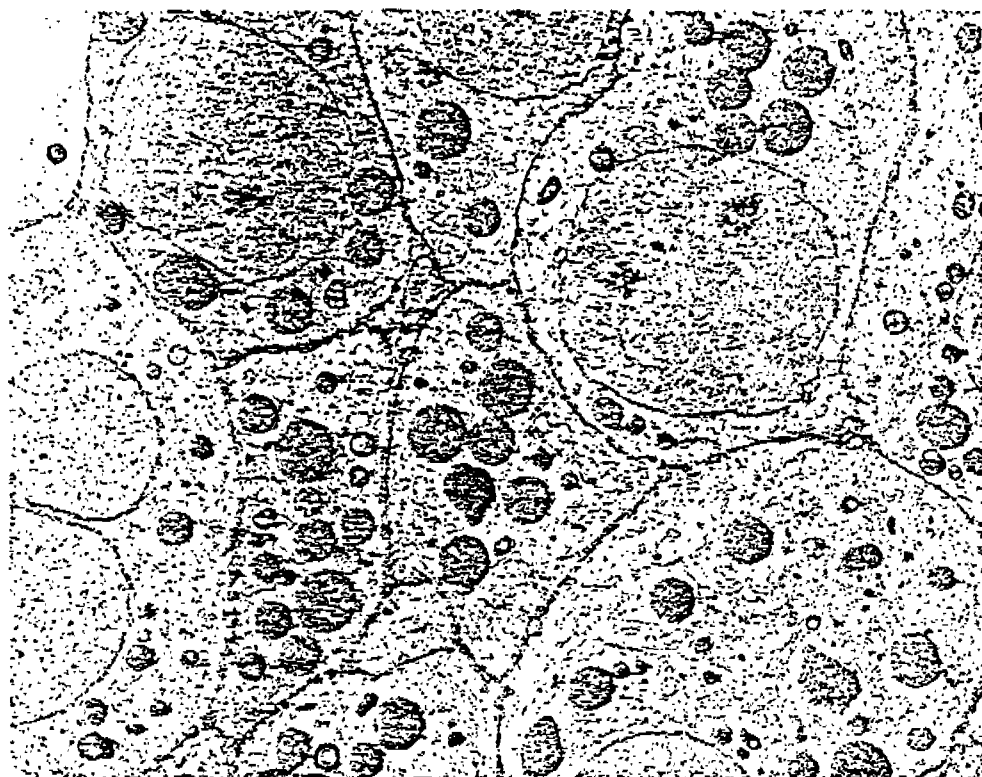


FIG. 8. A differentiated neoplasm seen in the animal lung shown in Fig. 3. Formation of the tubular lumen and microvilli is poor.  $\times 6500$ .

spontaneously or by urethane. Intracytoplasmic vacuoles containing electron-dense granules (diameter: approximately 400 Å) and amorphous material were frequently observed in the cell cytoplasm (Fig. 11). Since the limiting membrane was attached to ribosomes, the vacuole was identified as a cisterna of endoplasmic reticulum. The intracisternal granules did not stain with uranyl acetate alone and therefore were assumed to be glycogen (Fig. 12). Occasionally, crystalloid structures were observed in the cytoplasm of the neoplastic cells (Fig. 13), though their significance has not been clarified. The above-described neoplastic cells were quite similar in ultrastructure to type II alveolar epithelium. Capillaries in the neoplastic tissue consisted of single layers of nonfenestrated endothelium as seen in the normal alveolar capillary. In addition to well-differentiated neoplastic cells, poorly differentiated ones were also recognized (Fig. 14). As seen in Fig. 14, the cells were cuboidal or cylindrical in shape and lacked formation of osmiophilic lamellar bodies. Mitochondria were small in size, although the cells were relatively rich in rough-surfaced endoplasmic reticulum. These cells seemed to correspond to the basophilic ones observed by light microscopy. Except for the lack of a large amount of glycogen, these cells resembled immature alveolar epithelium, as observed in fetal lung in late gestation. Neither cilia nor mucinous

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FIG. 9. A part of the cell cytoplasm of a neoplastic cell. The arrow indicates irregular cristae mitochondriales (2500 ppm in group III).  $\times 29,400$ .

secretory granules were observed in the neoplastic cells. Based on these findings, it was strongly suggested that the neoplastic cells were derived from the alveolar epithelium, particularly from type II cells. A hyperplastic pulmonary alveolus is illustrated in Fig. 15. Electron microscopically, aspects of the hyperplastic cells were evidently those of type II alveolar cells, although they showed some differences in ultrastructure from the normal type II cell. Mitochondria were large in size and irregular in shape (Fig. 16), and, occasionally, retention of huge osmiophilic lamellar bodies was noted in the cell cytoplasm (Fig. 16). Cristae mitochondriales were arranged irregularly (Fig. 16), and well-developed endoplasmic reticulum was frequently seen in the cytoplasm (Fig. 17). Early stages of the membrane formation responsible for "cytoplasmic compartments" were observed in the hyperplastic cell (arrow in Fig. 17). It is suggested that the membrane was derived from the endoplasmic reticulum. In many respects, the hyperplastic type II cell is assumed to be the precursor of the neoplastic cell. An intermediate form between type I and II cells was frequently observed in the hyperplastic pulmonary alveoli. The arrow in Fig. 15 indicates a part of the cell cytoplasm which may represent a transitional form between type I and II cells. Though the



FIG. 10. Intracytoplasmic compartments formed by membrane structures (2500 ppm in group III).  $\times 20,000$ .

attenuated cytoplasm corresponded to that of a type I cell, the presence of microvilli on the cell surface was characteristic of a type II cell.

#### DISCUSSION

Experimental studies (10-18) on the oncogenicity of vinyl chloride have revealed that the monomer can induce various neoplasms including hepatic hemangiosarcoma (rat, mice, hamsters), Zymbal gland carcinoma in the external auditory meatus (rats), breast cancer (mice), nephroblastoma (rats), "lung adenoma" (mice), skin trichoepithelioma (hamsters), lymphoma (hamsters), and forestomach papilloma (hamsters).

Evidence of the pulmonary oncogenicity of vinyl chloride in animals has been obtained: (i) Viola *et al.* (10, 11) have reported that rats exposed to vinyl chloride exhibited lung cancer (32%). Histological features of the cancers were stated to be those of adenocarcinoma, with the exception of a single epidermoid tumor. Maltoni and Lefemine (12, 13), however, reviewed the histological slides of Viola *et al.* and stated that the lung cancers reported by the latter were *not* primary tumors of the lungs, but metastatic cancers from Zymbal glands. (ii) Maltoni and Lefemine (12, 13) also have reported on the pulmonary oncogenicity of vinyl

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FIG. 11. Glycogen granules in a cisterna of the endoplasmic reticulum (2500 ppm in group III).  $\times 60,000$ .

chloride on the basis of their own data. Though they could not find bronchogenic carcinoma in rats, rare pulmonary hemangiosarcomas and fibrosarcomas were induced in these animals. Unlike the case in rats, pulmonary tumors ("adenomas") were found in mice (89 of 471). Maltoni and Lefemine (12, 13)



FIG. 12. Single staining by uranyl acetate. Glycogen granules are not detected in the cisterna. A step section in the specimen of Fig. 11.  $\times 60,000$ .



FIG. 13. Crystalloid structure seen in the cytoplasm of a neoplastic cell (2500 ppm in group III).  $\times 30,000$ .

noted that some of the adenomas underwent malignant transformation. (iii) Kep-linger and associates (14) have found "alveologenic adenomas" in the lungs of mice exposed to vinyl chloride (44 of 49). (iv) Lee and associates (15) have stated that "bronchiolar adenoma" developed in mice 2 months after exposure to vinyl chloride at 50–1000 ppm. (v) Holmberg and associates (16) found "alveologenic adenoma" in 13 of 24 mice exposed to vinyl chloride at 50 ppm for 24–52 weeks. Our present study has also confirmed that pulmonary tumors are frequently induced by vinyl chloride (26 of 27).

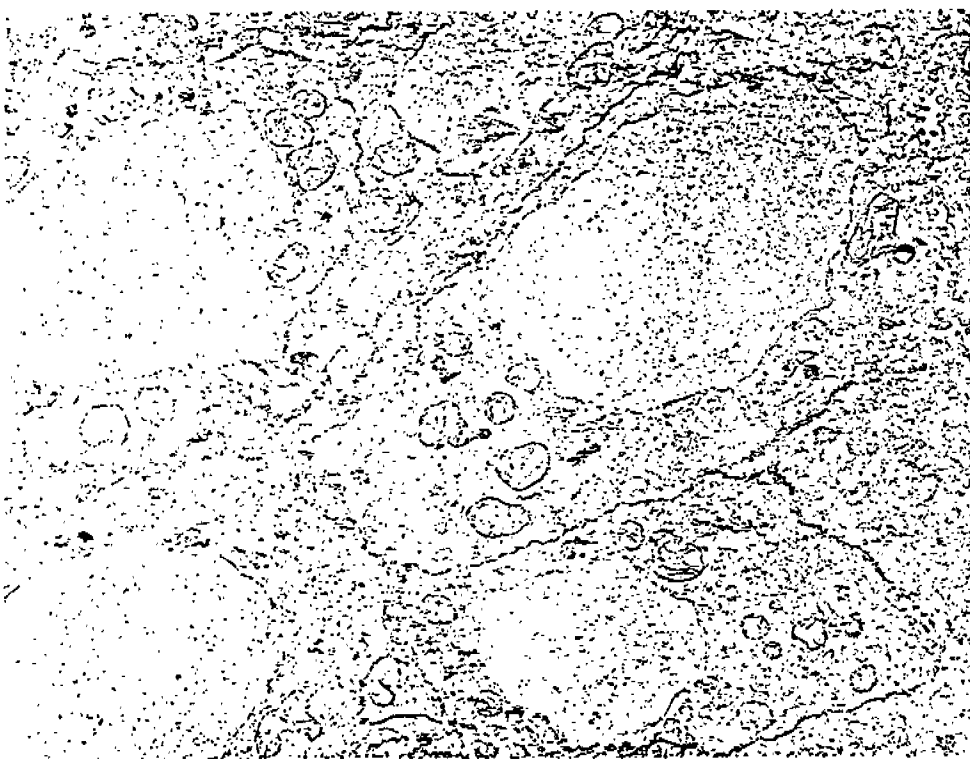


FIG. 14. Poorly differentiated neoplastic cells. No osmiophilic lamellar bodies are observed (6000 ppm in group III).  $\times 6200$ .

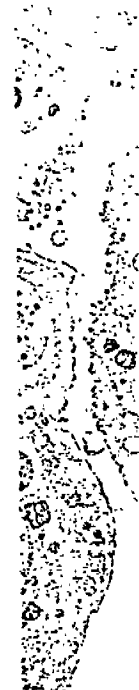


FIG. 15. Epithelium.  $\times 4200$ .



FIG. 16. Mitochondria.

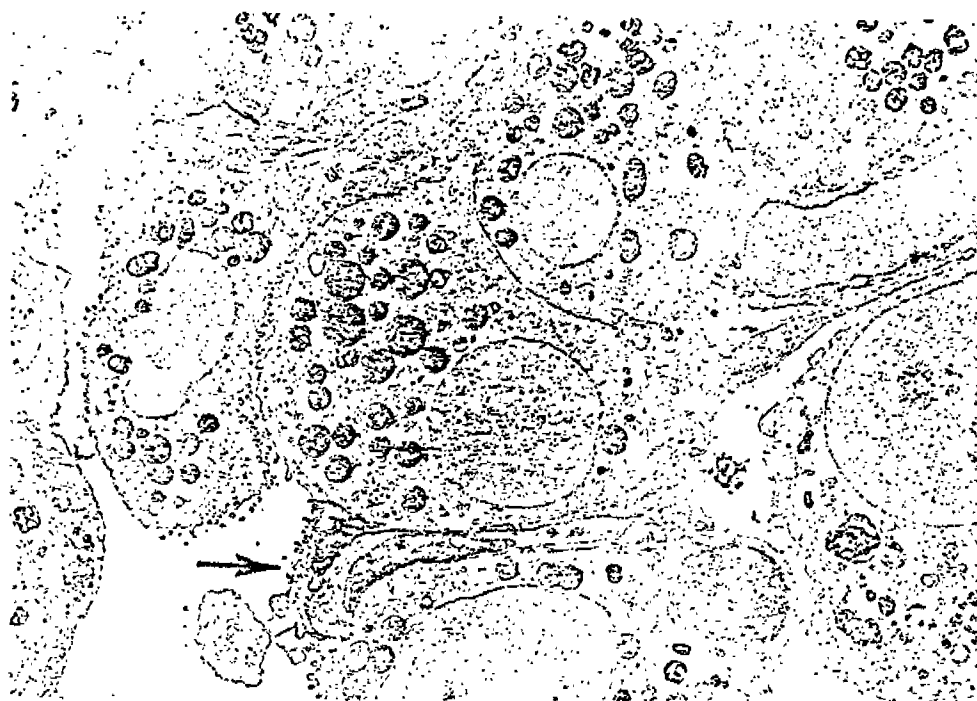


FIG. 15. Part of a hyperplastic alveolus. Hyperplastic cells are identified as type II alveolar epithelium. An arrow indicates a transitional form between type I and II cells (2500 ppm in group I).  $\times 4200$ .

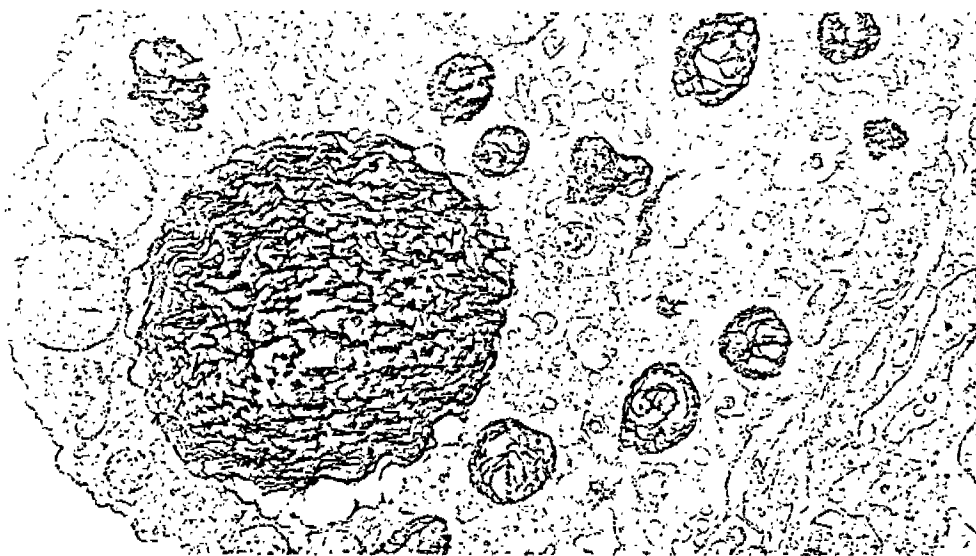


FIG. 16. A hyperplastic type II cell. A huge osmiophilic lamellar body and irregular cristae mitochondria are seen (6000 ppm in group III).  $\times 11,400$ .



FIG. 17. Cytoplasm of a hyperplastic type II cell. Arrows indicate membrane formation. Endoplasmic reticulum is well developed (2500 ppm in group III).  $\times 26,600$ .

The reports by Lee *et al.* (15) and Holmberg *et al.* (16) are noteworthy since the pulmonary tumors are induced by low doses and relatively long exposure and also because these reports demonstrated that the oncogenicity of vinyl chloride is dose related. Based on all the data available, it can be concluded that, of all the tumors induced by vinyl chloride in all species, those in the lungs of mice appear earliest, and that mouse lung is an extremely sensitive organ for demonstrating the oncogenicity of vinyl chloride.

Gross anatomical and histological aspects of the tumors in our investigation corresponded to the "alveologenic tumor or cancer" of Stewart *et al.* (21, 22), though other designations such as pulmonary adenoma, bronchiolar adenoma, and adenoma becoming malignant have also been used. The alveologenic tumor has been induced by various carcinogens (21-26) such as polycyclic hydrocarbons, urethane, nitrogen mustard, methylcholanthrene, and nitrofur derivatives and is known to occur spontaneously with aging (27-29). The cancer induced has been distinguished from that occurring spontaneously by multiple primary foci, occasional formation of huge tumors, and occurrence without any relation to aging. It is also known that in certain strains, such as A and DD, spontaneous tumors are quite common after 10 to 12 months of age. Spontaneous pulmonary tumors could be excluded in our experimental animals; in addition to the above-mentioned points, neoplastic changes in the lungs were absent in the controls.

Electron microscopically, alveolar epithelium, particularly the type II cell, was assumed to be the precursor of vinyl chloride-induced tumor in the mouse lung. This assumption was derived from ultrastructural similarities between the normal type II cell and the neoplastic cell. Similar suggestions have been made by other investigators (20, 26, 30) after studying pulmonary tumors induced by agents other than vinyl chloride.

It was noteworthy that the processes of transformation of the normal alveolar epithelium into the neoplastic cell could be followed morphologically on the level of ultrastructure; the appearance of an intermediate form between type I and II cells in the alveolar lining, the disappearance of type II cell in the lining due to replacement by the hyperplastic type II cells, which were transformed from the intermediate form, and the neoplastic change of the type II cell were assumed to

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be a sequence of the process. The intermediate form appears in certain pathological conditions, such as pulmonary asbestosis (31) and radiation pneumonitis (32) prior to cuboidal metaplasia of the attenuated alveolar epithelium. Embryologically, both type I and II cells are of the same origin, the entodermal epithelium (33, 34). It has been accepted that, at a late stage of gestation, rapid attenuation of the immature alveolar epithelium, similar to the type II cell, occurs in mammals (33, 34). Some of the neoplastic cells, distinguished as poorly differentiated, were similar in ultrastructure to the immature alveolar epithelium of fetal lung. From these perspectives, the process of neoplastic alteration observed in the epithelium may be interpreted as a retrograde process of the normal differentiations of the alveolar epithelium.

Recently, Waxweiler and associates reported an increased number of deaths due to lung cancer among vinyl chloride workers (8). They observed 12 cases compared to the 7.7 cases expected. Eight of the twelve were examined histologically and were classified as undifferentiated large-cell carcinoma (five cases) and adenocarcinoma (three cases). Since these human lung cancers are of bronchogenic origin, it may be that target pulmonary cells in vinyl chloride carcinogenesis are different in human and mouse lung.

Neoplastic invasion and metastases were not found in our material. However, it is known (21, 35-38) that sometimes both induced and spontaneous alveologenic tumors of mice show such changes and that the malignant transformation occurs with some delay after initiation of the tumor. In addition, transplantation of this tumor has been accomplished (39). Stewart and associates (21) therefore dubbed it an "alveologenic tumor" or "alveologenic cancer." Maltoni and Lafemine (13) have found that some vinyl chloride-induced pulmonary tumors undergo transformation. During the short period of observation in our experiments, we did not observe this.

Alveologenic tumors may be considered unique in some ways, since it is possible to observe the process of malignant transformation sequentially from the precursor to the malignant cell via hyperplastic and benign neoplastic states.

Hepatic hemangiosarcoma is recognized as a specific malignant tumor related to vinyl chloride exposure. The tumor can be induced in a variety of experimental animals (mice, rats, and hamsters) (12, 13), and histological features of the tumor are almost identical in humans and animals. In contrast, the intrapulmonary target cells of vinyl chloride oncogenesis may be different in humans and mice. Beyond these differences, moreover, the induction of alveologenic tumors in mice by vinyl chloride may be predictive of a risk of human bronchogenic cancer from the chemical. Consistent with this is the fact that various carcinogens such as polycyclic aromatic hydrocarbons, nitrogen mustard, and chromate compounds are known to induce alveologenic tumors in mice, on one hand, and, on the other, to be associated with excess bronchogenic carcinoma among workers exposed to the carcinogens (40). It is noteworthy that a similar relation has been suggested for vinyl chloride.

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