ENVIRONMENTAL RESEARCH 32, 91–103 (1983)

Neoplastic Effect of Vinyl Chloride in Mouse Lung—Lower Doses and Short-Term Exposure

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Received September 13, 1982

Neoplastic pulmonary effects of lower doses of vinyl chloride (0 = control, 1, 10, 100, 300, and 600 ppm) and short-term exposure (4 weeks) by inhalation have been studied by light and electron microscopy in 220 mice. Except for dead or seriously sick animals, a large majority of the animals were sacrificed at three different stages: immediately after exposure, 12 weeks later, and 40 or 41 weeks after exposure. Six mice (4: 600 ppm, 2: 0 ppm) were kept longer than 41 weeks to examine the effects of the chemical after a long-term recovery period. Alveologenic tumors were first observed 10 weeks after exposure to 600 ppm. In the subgroups exposed to higher concentrations (600 and 300 ppm) the incidence of tumors was higher and their appearance was earlier than in the subgroups exposed to lower concentrations (100, 10, and 1 ppm). These findings indicated a dose–response relationship for incidence of alveologenic tumors, and the latency period was inversely related to dose. By light and electron microscopy, there was no obvious evidence that tumor cells were derived from Clara cells of the terminal bronchioles. Rather, neoplastic cells in both the tubulopapillary and adenomatous forms of the pulmonary tumors possessed all or some of the ultrastructural characteristics of type II alveolar cells, based on observations of mitochondria, microvilli, osmiophilic lamellar bodies, and other criteria. Type II alveolar cells are therefore considered to be the most sensitive in mice to the neoplastic effect of vinyl chloride.

I. INTRODUCTION

In previous studies, a high incidence of pulmonary tumors as well as non-neoplastic alterations of bronchioles and alveoli was observed in mouse lung exposed to vinyl chloride at heavy doses (2500 and 6000 ppm) for a long duration (5 and 6 months) (17, 18). The induced pulmonary tumors corresponded to those reported by Stewart *et al.* (15, 16) as alveologenic tumors originating in type II alveolar cells. It has been suggested that the mouse lung is an extremely sensitive indicator for the oncogenicity of vinyl chloride. A dose–response relationship has been demonstrated in the production of alveologenic tumors with vinyl chloride (3, 6, 8). In addition, a delayed appearance of the neoplastic effect after periods of recovery, following exposure to vinyl chloride, has been postulated (3, 10, 17).

In this study, the neoplastic effect of exposure to lower doses of vinyl chloride (0 = control, 1, 10, 100, 300, and 600 ppm), and short-term exposure (4 weeks), has been studied using light and electron microscopy in 220 mice. Details of the findings are reported here.

0013-9351/83 $3.00

II. MATERIAL AND METHODS

(*a*) *Test material.* Vinyl chloride was supplied by the Dow Chemical Company, Midland, Michigan. The material was greater than 99% pure. Prior to use in this study, the purity was confirmed by gas chromatography.

(*b*) *Test animals.* Two hundred and twenty CD1 Charles River male mice, 5–6 weeks old at first exposure, were used. Exposure was for 6 hr per day, 5 days per week, for a total of 4 weeks in all animals, but six different doses of vinyl chloride were administered as follows: 600 ppm (40 mice), 300 ppm (30), 100 ppm (30), 10 ppm (30), 1 ppm (30), and 0 ppm (60 = controls).

(*c*) *Chambers, vapor generation, and analyses.* The chambers used for this study were stainless steel and Rochester-type glass, approximately 1 m^3 in volume. The chambers were operated under dynamic airflow conditions with temperature and humidity controlled to approximately 70°F and 50% rh. Vinyl chloride gas was metered at a controlled rate into the airstream where it was further diluted to the desired concentration. The analytical concentration of vinyl chloride in each exposure chamber was continuously monitored by infrared spectroscopy at a wavelength of 10.9 μm. Standards for analysis were made by injecting a known volume of vinyl chloride gas into a 100-liter Saran gas sampling bag filled with a known volume of air. The nominal concentration of vinyl chloride in the chamber was the ratio of the rate at which the test material was dispensed to the ratio of total airflow through the chamber. These inhalation experiments were performed under the supervision of Dr. M. J. McKenna of the Toxicology Research Laboratory, Health and Environmental Science, U.S.A. Dow Chemical, Midland, Michigan.

(*d*) *Procedure of animal sacrifice.* A large majority of the animals were sacrificed at three different stages: (a) immediately after exposure (a total of 70 mice, 10 each of the 600, 300, 100, 10, and 1 ppm groups, and 20 of the 0 ppm group); (b) 12 weeks after exposure (a total of 61 mice, 9 of the 600, 9 of the 300, 6 of the 100, 9 of the 10, 10 of the 1, and 18 of the 0 ppm groups); and (c) 40 or 41 weeks after exposure (a total of 58 mice, 7 of the 600, 7 of the 300, 9 of the 100, 9 of the 10, 9 of the 1, and 17 of the 0 ppm groups). Between the first and second sacrifices, 14 mice (6 of the 600, 1 of the 300, 4 of the 100, 1 of the 10, and 2 of the 0 ppm groups) were found dead or were killed. Eleven mice (4 of the 600, 3 of the 300, 1 of the 100, 1 of the 10, 1 of the 1, and 1 of the 0 ppm groups) were found dead or were killed between the second and third sacrifices. A small number of animals (4 of the 600 and 2 of the 0 ppm groups) were allowed a long-term postexposure recovery period (42–65 weeks) prior to sacrifice or death.

(*e*) *Histopathological and electron microscopic procedures.* All animals were systematically autopsied. Sacrifice was performed by ether anesthesia. Lungs were inflated with paraformaldahyde (for sacrificed mice) or with 10% neutral buffered Formalin (for mice found dead). Then, to confirm the presence of pulmonary tumors, and to count the number of the tumors, all lobes of the lungs were thoroughly examined under a dissecting microscope. The lungs, together with a majority of organs, including liver, brain, kidney, adrenal, stomach, intestine, esophagus, testis, and seminal vesicle, were fixed in 10% neutral buffered for-

malin and embedded in paraffin after dehydration in alcohol. Five- to six-micrometer sections were made and stained with hematoxylin–eosin, Gomori's silver, periodic acid–Schiff's (PAS), Masson's trichrome, and Van Gieson's picrofuchsin technique. For electron microscopy, small pieces (smaller than 1 mm^3) were taken from the tissues that were first fixed in paraformaldehyde and then fixed in 1% phosphate-buffered osmic acid. 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. Siemens 101 and Hitachi 11DS electron microscope were used for ultrastructural observation.

III. RESULTS

(A) *Induction of Pulmonary Tumors*

Table 1 summarizes the results of induction of pulmonary tumors in mice exposed to low doses of vinyl chloride for 4 weeks and sacrificed or found dead at various times after exposure.

The first pulmonary tumor was observed in 1 mouse of the 600 ppm group that was found dead 10 weeks after exposure. The tumor was a single alveologenic tumor. At the time of the second sacrifice (12 weeks after exposure), a high incidence of alveologenic tumors was seen in the subgroups exposed to heavier doses [600 and 300 ppm, 88.8% (8 of 9) and 66.6% (6 of 9), respectively] although the tumor was not induced in the subgroups exposed to lower doses (100, 10, and 1 ppm). Alveologenic tumors induced in the 300 ppm group were all single tumors; however, the tumors of the 600 ppm group consisted of multiple tumors in 6 of 8 (75%) while the remaining 2 of 8 (25%) were single tumors. Between the second and third sacrifices (40 or 41 weeks after exposure), again, animals of the lower doses (100, 10, and 1 ppm) did not develop any pulmonary tumors, but those of the heavier doses—subgroups (600 and 300 ppm)—showed a high incidence of tumors (4 of 4 [100%] and 2 of 3 [66.6%], respectively), all of which were multiple neoplasms in the lungs. At the third sacrifice, the pulmonary tumors were induced in all subgroups (600, 300, 100, 10, and 1 ppm) and the incidence of tumor production was 6 of 7 (85.7%) in the 600 ppm, 5 of 7 (71.4%) in the 300 ppm, 6 of 9 (66.7%) in the 100 ppm, 3 of 9 (33.3%) in the 10 ppm, 1 of 9 (11.1%) in the 1 ppm, and 0 of 17 (0%) in the 0 ppm (control) groups. The percentage of induced tumors which were multiple was 6 of 6 (100%) in the 600 ppm, 3 of 5 (60%) in the 300 ppm, 3 of 6 (50%) in the 100 ppm, 0 of 3 (0%) in the 10 ppm, and 0 of 1 (0%) in the 1 ppm groups. In the 600 ppm group, sacrificed over 41 weeks after exposure, a multiple alveologenic tumor was found in 4 of 4 (100%), although 2 controls did not develop any pulmonary tumor.

These findings demonstrate a dose–response relationship for incidence of alveologenic tumors, and the latency period inversely related to dose. Further, the incidence of multiple alveologenic tumors seems to be related to two factors, dose and the recovery period after exposure.

It is not possible to compare accurately the size of the induced tumors. However, in general one can say that larger tumors are seen in the heavier-dose group

TABLE 1
INDUCTION OF MOUSE PULMONARY TUMORS WITH LOW DOSES OF VINYL CHLORIDE (VC) EXPOSED FOR 4 WEEKS

Sacrificed or found dead time	No. of animals	VC-induced pulmonary tumors		Muliple foci of VC-induced pulmonary tumors		Spontaneous pulmonary tumors	
		No.	%	No.	%	No.	%
Immediately after exposure							
600 ppm	10 (9 sac. 1 f.d.)	0	0	0	0	0	0
300 ppm	10 (sac)	0	0	0	0	0	0
100 ppm	10 (sac)	0	0	0	0	0	0
10 ppm	10 (sac)	0	0	0	0	0	0
1 ppm	10 (sac)	0	0	0	0	0	0
0 ppm	20 (sac)	0	0	0	0	0	0
Between first and second sacrifices							
600 ppm	6 (2 sac, 4 f.d.)	1 (f.d.,s)[a]	16.6	0 (of 1)	0	0	0
300 ppm	1 (f.d.)	0	0	0	0	0	0
100 ppm	4 (1 sac, 3 f.d.)	0	0	0	0	0	0
10 ppm	1 (sac)	0	0	0	0	0	0
0 ppm	2 (f.d.)	0	0	0	0	0	0
12 Weeks after exposure (second sacrifice)							
600 ppm	9 (sac)	8 (2 s, 6 m)	88.8	6 (of 8)	75	0	0
300 ppm	9 (sac)	6 (s)	66.6	0 (of 6)	0	0	0
100 ppm	6 (sac)	0	0	0	0	0	0
10 ppm	9 (sac)	0	0	0	0	0	0
1 ppm	10 (sac)	0	0	0	0	0	0
0 ppm	18 (sac)	0	0	0	0	0	0
Between the second and third sacrifices							
600 ppm	4 (1 sac, 3 f.d.)	4 (m)	100	4 (of 4)	100	0	0
300 ppm	3 (1 sac, 2 f.d.)	2 (m)	66.6	2 (of 2)	100	0	0
100 ppm	1 (sac)	0	0	0	0	0	0
1 ppm	1 (f.d.)	0	0	0	0	0	0
0 ppm	1 (f.d.)	0	0	0	0	0	0
40 or 41 Weeks after exposure (third sacrifice)							
600 ppm	7 (sac)	6 (m)	85.7	6 (of 6)	100	1[b]	14.3
300 ppm	7 (sac)	5 (3 m, 2 s)	71.4	3 (of 5)	60	0	0
100 ppm	9 (sac)	6 (3 m, 3 s)	66.7	3 (of 6)	50	1[b]	11.1
10 ppm	9 (sac)	3 (s)	33.3	0 (of 3)	0	0	0
1 ppm	9 (sac)	1 (s)	11.1	0 (of 1)	0	1[b]	11.1
0 ppm	17 (sac)	0	0	0	0	3[b]	17.6
Over 41 weeks after the exposure							
600 ppm	4 (sac)	4 (m)	100	4 (of 4)	100	0	0
0 ppm	2 (1 sac, 1 f.d.)	0	0	0	0	0	0

[a] The first mouse bearing a pulmonary tumor, found dead 10 weeks after exposure. sac. = sacrificed; f.d. found dead; s = a single pulmonary tumor; m = multiple pulmonary tumors.

[b] Spontaneous pulmonary tumor; a single, small (not identified by either naked eye and a stereoscope, but barely identified by light microscopy of lung section slides) tumor appeared in and after the period of third sacrifice (49 weeks old in age or older).

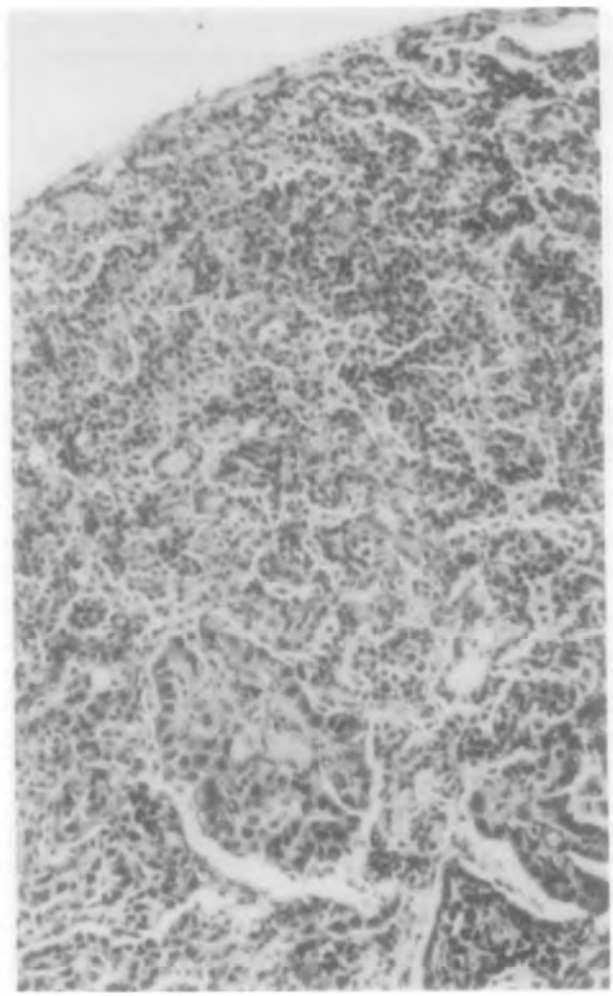
and also when the recovery period was longer. Huge alveologenic tumors were observed in only two mice of the 600 ppm group, found dead 40 weeks after and sacrificed 55 weeks after exposure, and the tumors seen in the groups exposed to smaller doses (100, 10, and 1 ppm) were generally smaller.

In addition to the induced pulmonary tumors, spontaneous alveologenic tumors presented in six animals (3 of 0 ppm, 1 of 10 ppm, 1 of 100 ppm, and 1 of 600 ppm) sacrificed 40 and 41 weeks after exposure. These tumors were excluded from the calculations of incidence of vinyl chloride-induced alveologenic tumors. Details of this will be described in the Discussion.

(B) Light and Electron Microscopic Observations of the Alveologenic Tumors

The histological aspects of the pulmonary tumors were similar to those induced by heavy doses of vinyl chloride with a long exposure. No metastases to other organs were observed. The tumors were usually seen in the peripheral part of the lung; however, occasionally, the tumors were found in more centrally located areas. The tumors were not encapsulated by connective tissue. The neoplastic cells were arranged in the tubular, papillary, and adenomatous fashions. When adenomatous, the cells were small in size, cuboidal in shape, and similar to hyperplastic type II cells which have been postulated as the presursor of the neoplastic cell. When tubular or papillary, the cells were usually large in size and cuboidal or cylindrical in shape, but small, cuboidal cells were also identified. Pleomorphism and anaplastic features were not striking and mitoses were uncommonly observed. In rare cases a huge tumor almost completely occupied a whole lobe of lung. Figure 1 indicates a part of such a huge pulmonary tumor (600 ppm, sacrificed 55 weeks after exposure). Figures 2–4 are light microscopic pictures of higher magnification, taken from either the peripheral (Fig. 2) or the deeper (Figs. 3 and 4) parts of the tumor. In addition to the alveolar (Fig. 2) and tubulo-papillary (Fig. 4) patterns, an intermediate form (Fig. 3) was also observed in the huge tumor.

Electron microscopically, the neoplastic cells of these patterns possessed all or some of the ultrastructural characteristics of type II cells, such as microvilli, large round- or rod-shaped mitochondria, osmiophilic lamellar bodies, junctional structures, multivesicular bodies, and a basement membrane. Less differentiated cells possessed only a few of those characteristics. Interestingly, the neoplastic cells of the tubulo-papillary pattern, seen in the huge tumor (600 ppm, 55 weeks after exposure) still showed some of the ultrastructural characteristics, such as microvilli, osmophilic lamellar bodies, and multivesicular bodies. Size and shape of the mitochondria of the neoplastic cells varied; however, cristae of mitochondria were usually well developed. Figure 5 is a low-power electron micrograph of the tubulo-papillary pattern seen in the huge tumor for which light microscopy illustrations are shown in Figs. 1–4. A part of Fig. 5 is enlarged in Fig. 6. The electron micrographs clearly indicate that the neoplastic cells forming the tubulo-papillary pattern still show some similarities to type II cells: well-developed microvilli (Figs. 5 and 6), lamellar bodies (Fig. 5), and cristae-rich mitochondria with a clear matrix (Fig. 6) are characteristic features seen in type II cells. Mitochondria of Clara cells in terminal bronchioles of the mouse are known to be



FIGS. 1–4. Taken from a huge pulmonary tumor seen in a mouse exposed to 600 ppm vinyl chloride for 4 weeks and sacrificed 55 weeks after exposure.

FIG. 1. A low-power view. ×78.

round in shape, dark in the tone of the mitochondrial matrix, and poor in the development of mitochondrial cristae. Such mitochondria were not present in the neoplastic cells of any patterns seen by us. In addition to the above findings, intracytoplasmic compartments formed by a membrane structure and glycogen granules in a cisterna of the endoplasmic reticulum were frequently observed in the neoplastic cells, as stated in our previous report (17).

Hypercellularity of the alveolar lining cells was frequently observed around the induced pulmonary tumors. In the previous study (17), it had been strongly suggested that such a change was the precondition for the alveologenic tumor production because electron microscopically, the proliferated cells were quite similar to the normal type II cell on the one hand, and also to the well-differentiated cell type

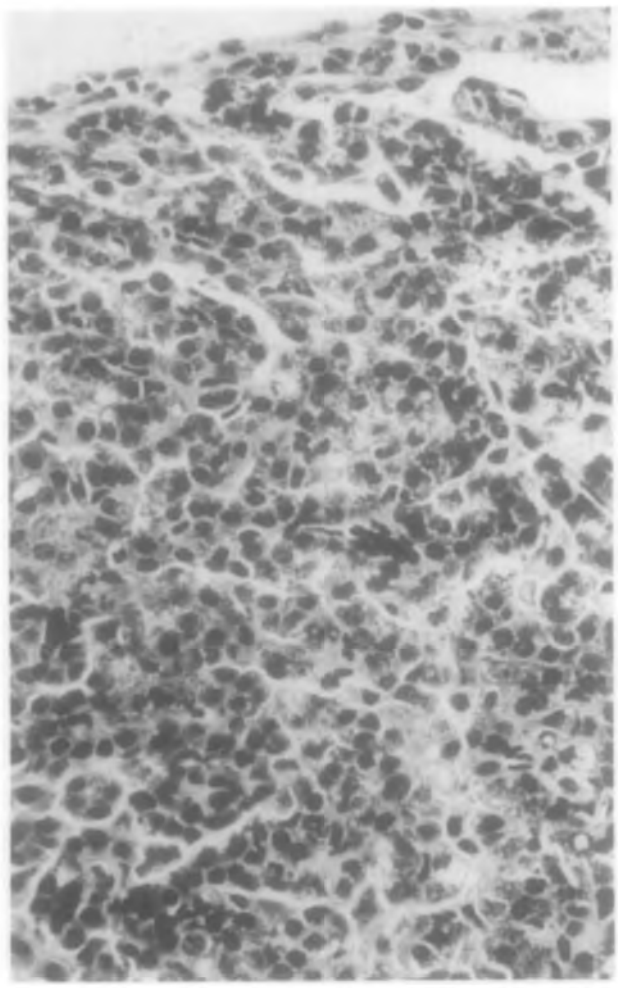


FIG. 2. Alveolar fashion seen in the peripheral part of the tumor. × 196.

of the alveologenic tumor on the other hand. A small proportion of the subgroups exposed to heavier doses (2 of 20 in the 600 ppm and 1 of 10 in the 300 ppm groups) already showed such an alteration immediately after exposure to vinyl chloride.

(C) *Tumors Other than Pulmonary Tumors*

Hemangiosarcomas were induced in both the subcutaneous connective tissue of the ear (10 ppm; 29 weeks after exposure) and liver (600 ppm; 65 weeks after). Details of those tumors have been reported elsewhere (19).

IV. DISCUSSION

Hepatic hemangiosarcoma induced by vinyl chloride, Thorotrast, or arsenics (1, 2, 7, 9, 11, 12, 21) has been known to be a "signal" [I. J. Selikoff (13)] of human

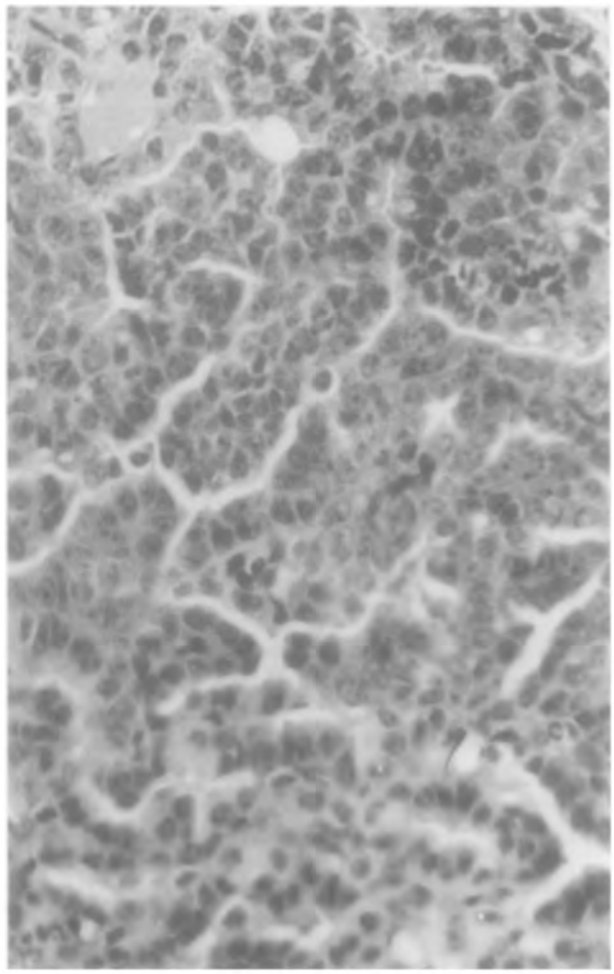


FIG. 3. An intermediate pattern between the alveolar and tubulo-papillary. ×196.

cancer. Maltoni and Lefemine (10) have clearly documented that hepatic hemangiosarcomas can be induced in mouse, rat, and hamster exposed to vinyl chloride at various doses and different exposure times.

Among vinyl chloride workers, in addition to hepatic hemangiosarcoma, an increased number of deaths due to bronchogenic cancer has been postulated (20, 22). To the present, however, lung tumors have not been induced by vinyl chloride in laboratory animals except the mouse. Although alveologenic tumors of the mouse have been frequently produced by vinyl chloride (3, 6, 8, 10, 16, 17), the cell origin and malignancy of the alveologenic tumors are still in controversy (4, 5, 15, 16). Regardless of this controversy, however, the induction of alveologenic tumors by vinyl chloride may be indirectly predictive of risk of human bronchogenic carcinoma from the chemical, since a number of chemical carcinogens, such as polycyclic aromatic hydrocarbons, nitrogen mustard, and chromate com-

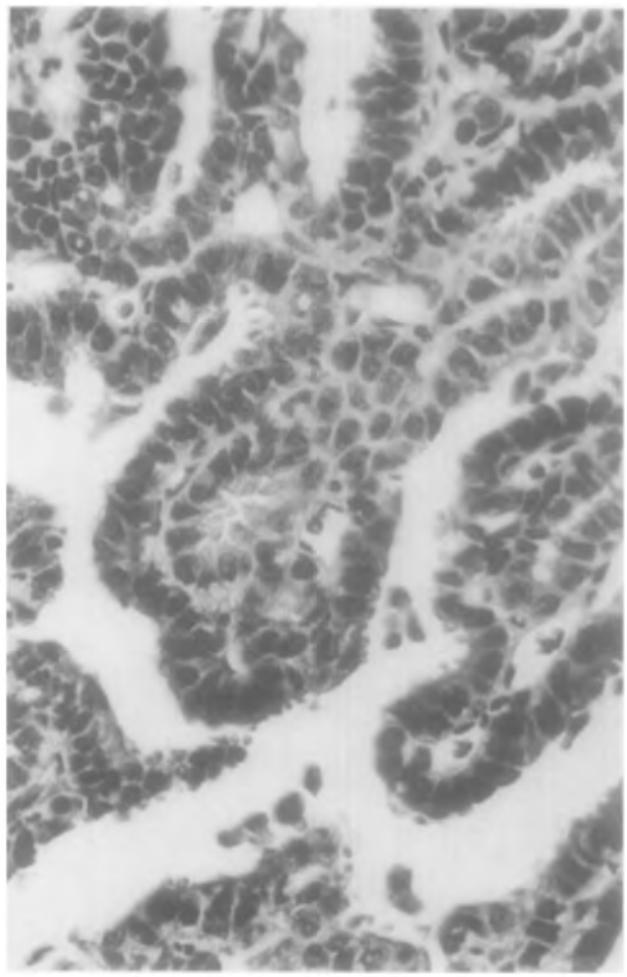


FIG. 4. Tubulo-papillary pattern seen in the deeper part of the tumor. × 196.

pounds have also been known to induce alveologenic tumors in the mouse, and to be associated with excess bronchogenic carcinoma among workers exposed to the carcinogens.

The cell origin of alveologenic neoplasms has been disputed. Type II alveolar cells, or terminal bronchiolar cells, or either alveolar and bronchiolar cells have been reported as the progenitor cell of the tumors.

Recently Kauffman *et al.* (4, 5) have divided mouse pulmonary tumors induced by transplacental administration of ethyl nitrosourea into two groups, benign and malignant. The two were histologically characterized by alveolar and tubulo-papillary patterns, respectively. According to these investigators, the type II cell is the progenitor of the benign form and the Clara cell of the terminal bronchioles the progenitor of the malignant form. In the present study, however, we have failed to detect Clara cell tumors among the mouse pulmonary tumors induced by

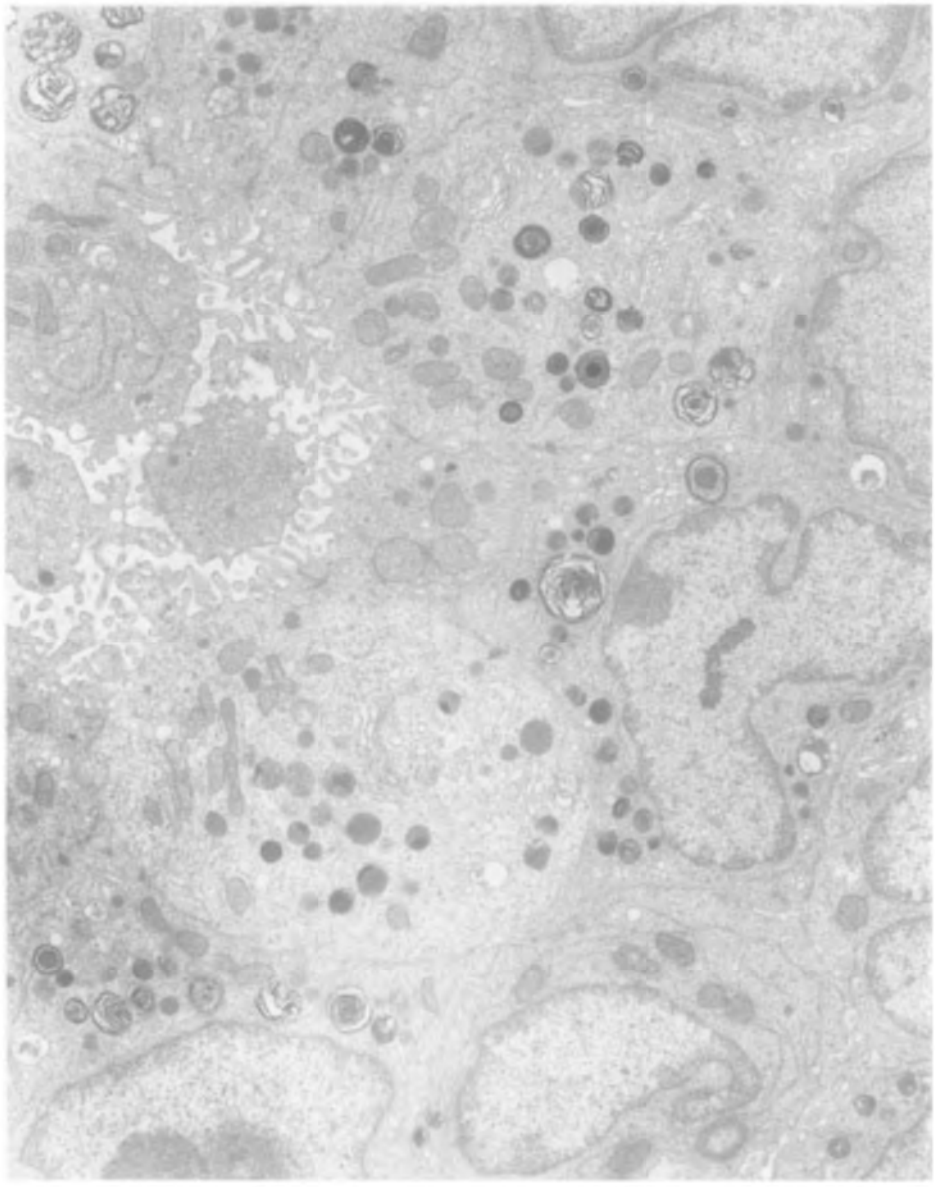


FIG. 5. A low-power electron micrograph showing neoplastic cells, seen in the tubulo-papillary pattern of the huge tumor shown in Fig. 1. ×10,500. Some ultrastructural characteristics of the type II cells, such as microvilli, osmiophilic lamellar bodies, and cristae-rich mitochondria, are shown in the neoplastic cells illustrated in this figure and figure 6.

vinyl chloride, although the tumors induced included various patterns; alveolar, tubulo-papillary, and mixed forms. It is known that the Clara cell of the mouse contains unique mitochondria which are round or ovoid in shape, large in size, dense in the mitochondrial matrix, and very poor in mitochondrial cristae.

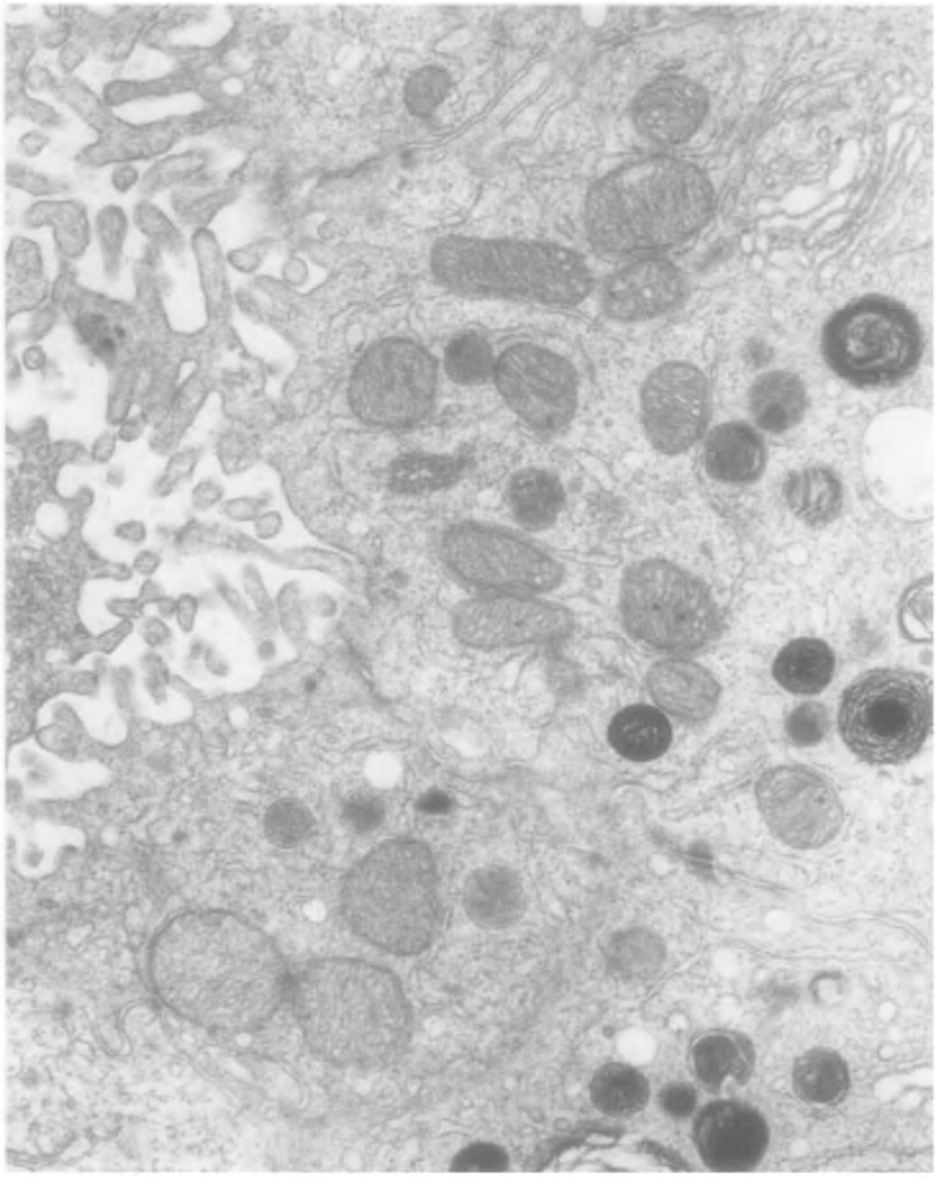


FIG. 6. A part of Fig. 5 ×21,900.

Unlike the type II cell, the Clara cell lacks osmiophilic lamellar bodies and microvilli (14). Such unique mitochondria were not detected in the neoplastic cells of any of the induced tumors, while microvilli, osmiophilic lamellar bodies, cristae-rich mitochondria with clear matrix, and multivesicular bodies, which are commonly seen in the type II cell, were often observed in the tumor cells of the tumors we observed. From this, it is suggested that the type II cell is the sole progenitor

cell of the tumor. If this suggestion is accepted, the term of "alveologenic tumor" (Stewart) would be accepted as adequate.

It is known that alveologenic tumors spontaneously occur in the mouse lung. Stewart *et al.* reported that the incidence of spontaneous pulmonary tumors was quite high in certain strains, such as A and DD after 10 to 12 months of age, and that these spontaneous tumors could be differentiated from those produced by carcinogens by their small size and their single number, as well as the older age of the individual mouse (15, 16). Taking this into consideration, alveologenic tumors seen in six mice (3 of the 0 ppm, 1 of the 1 ppm, 1 of the 100 ppm, and 1 of the 600 ppm groups) sacrificed 40 and 41 weeks after exposure have been considered spontaneous. They were single in number and were barely identified under a light microscope. In addition, the age of the mice (over 49 weeks old) was old enough to expect production of spontaneous tumors.

It has been suggested that the latency of alveologenic tumors after exposure to vinyl chloride is dose related (3, 6, 8, 10). Indeed, in this study, the latency was shorter in the groups exposed to heavier doses. The shortest latent period for tumor induction was 10 weeks after exposure in the 600 ppm group, 12 weeks after exposure in the 300 ppm, and 40 weeks after exposure in the 100, 10, and 1 ppm groups. No multiple tumors were induced in the groups of 10 and 1 ppm. As stated earlier, the size of the induced tumor appears to be related to both the dose and to the recovery time.

Previous findings of a number of investigators support the idea that alveologenic tumors induced by vinyl chloride (a) seem to appear earliest among all neoplasms induced by the chemical in all species of laboratory animals (3, 6, 8, 10, 17, 18); (b) the incidence of the tumors produced is dose related (3, 6, 8, 10); and (c) the latency of the tumor is also dose-related (3, 6, 8, 10). In this study, evidence to support all three of these suggestions has been presented. In addition, electron microscopic observations have focused on the type II alveolar cell as the progenitor cell of the tumor. Thus, it is considered to be the cell type most sensitive to the oncogenicity of vinyl chloride.

ACKNOWLEDGMENTS

The author would like to express his appreciation to Dr. I. J. Selikoff for his review and Dr. S. Frank for her editorial assistance. The author also thanks Mr. R. Ashley, Mrs. A. Calderaro, Mr. S. Yuen, and Mr. R. Fuller for their technical assistance, and Mrs. J. Roberts for secretarial assistance. This work was supported by Research Grant OH 00681 from the National Institute for Occupational Safety and Health, U.S. Department of Health and Human Services.

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