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Induction of Squamous Cell Carcinomas and Adenocarcinomas in Mouse Lung by Intratracheal Instillation of Benzo(a)pyrene and Urethan

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Benzo(a)pyrene 및 Urethan의 마우스 氣管內 注入에 의한 扁平上皮癌과 腺癌의 發生

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초록 : 성숙한 A/J, C_{57}BL/6N, DBA/2 및 NIH-GP系 마우스에 benzo(a)pyrene과 charcoal powder를, C_{57}/6N 및 NIH-GP系 마우스에 Urethan을 氣管內 注入하여 肺臟의 腫瘍發生과 組織變化를 觀察한 바 다음과 같은 結果를 얻었다.

Benzo(a)pyrene을 注入한 群에서는 扁平上皮癌 및 腺癌, 그리고 扁平上皮癌과 腺癌의 혼합 발생例를 볼 수 있었다. 이와 같은 病變은 A/J 및 C_{57}BL/6N系 마우스에서 發生率이 높았으며 A/J系 마우스에서 扁平上皮癌의 發生 및 分化가 현저하였다. 한편 DBA/2 및 NIH-GP系 마우스에서는 腫瘍 發生率이 극히 낮았다.

Benzo(a)pyrene의 注入時 동일 계통의 마우스에서도 대량을 注入할 때 腺癌보다 扁平上皮癌의 發生이 많았으며 A/J系 마우스에서는 扁平上皮癌 單獨 發生例가 다수 觀察되었고 C_{57}BL/6N系 마우스에서는 腺癌 및 腺癌과 扁平上皮癌의 혼합 形態가 主로 觀察되었다. 그리고 氣管支上皮의 扁平上皮化生 및 扁平上皮癌의 發生時 alcian blue-PAS 양성반응세포가 관여함을 알 수 있었고 腺腫樣組織 發生例에서도 扁平上皮化生 및 扁平上皮癌으로 分化되는 경우도 觀察되었다.

Urethan 注入群은 극히 낮은 腫瘍發生率을 나타내어 發癌物質의 種類, 用量 및 마우스의 系統에 따라 腫瘍의 發生率 및 形態의 차이가 인정되었다.

Introduction

To complement and increase the significance of the study of human lung carcinomas (McDowell *et al.*, 1978a; Trump *et al.*, 1978) we saw the necessity of studying the morphologic and histochemical characteristics of lung tumor in an experimental animal model.

There have been many studies on induction of experimental lung tumors in mice by chemical carcinogens and results have been reviewed (Shimkin and Stoner, 1975; Shimkin, 1955). Most of the pulmonary tumors induced by chemical carcinogens in mice have been pulmonary adenomas. Because of the morphological and biological characteristics, the site of origin and the spontaneous occurrence of this type of tumor, the pulmonary adenoma system is generally not

considered to be an adequate model for studies designed to elucidate the pathogenesis of human bronchogenic carcinoma. Recently, in the studies on carcinogenesis of respiratory tracts in experimental animals, other animals such as hamsters (Takayama *et al.*, 1985; Yarita *et al.*, 1978; Saffiotti *et al.*, 1972a; Saffiotti *et al.*, 1972b), rats (Deutsch-Wenzel *et al.*, 1983; Schreiber *et al.*, 1972), rabbits (Hirao *et al.*, 1980; 1978) and dogs (Matsumura *et al.*, 1978) replaced mice.

Several procedures applicable to experimental animals have been well documented; these include the insertion of material through the trachea by surgical means (Deutsch-Wenzel *et al.*, 1983; Mossman and Craighead, 1978; Kobayashi *et al.*, 1976), application in the form of inhalants (International Agency for Research in Cancer, 1973) and intratacheal instillation (Ho and Frust, 1973; Nettesheim and Hammons, 1971; Saffiotti *et al.*, 1968). Each of these methods has its inherent advantages and limitations. The surgical technique permits only a single treatment, while the inhalation of aerosol does not allow for accurate quantitation of the material that penetrated into the lungs. Although the intratracheal instillation is an artificial process, precise amount of the substance can be administered repeatedly.

The mouse is an ideal experimental animal because more is known about its viral and genetic profilies, as well as types and frequency of spontaneous tumor incidence, than any other members of the rodent family (Staat, 1980; 1976; 1972). The numerous strains and mutations available facilitate the selection of specific characteristics required by the study. Due to the small size of the animal, intratracheal instillation of material into mouse lungs is difficult. The technique reported for the hamster or rat was found to be unsatisfactory because of two major problems; the material can easily be injected erroneously down the esophagus and the internal injuries suffered during treatment are too numerous for serial instillations. For these reasons, intratracheal instillation treatment of mice was seldom used.

Successful induction of squamous cell carcinomas by repeated intratracheal administration of a carcinogen to mice was reported by Nettesheim and Hammons (1971) and by Ho and Frust (1973). Yoshimoto *et al.* (1980; 1977) showed that squamous cell carcinomas could be induced rapidly and in high incidence by repeated intratracheal instillation of benzo(a)pyrene and charcoal powder into the lungs of C_{57}BL/6 mice.

This report describes studies on the effect of benzo(a)pyrene or urethan instillation on the induction of squamous cell carcinomas, adenocarcinomas and adenomas in the lungs of 4 mouse strains, A/J, C_{57}BL/6N, DBA/2 and NIH-GP.

Materials and Methods

Animals: Ten-week-old female inbred A/J, C_{57}BL/6N, DBA/2 and noninbred NIH-GP mice of our institute colony (strains originally derived from the National Institute of Health in U.S.A.) were used. They were fed on standard animal diet (NIH-7-open formula ration) and given water *ad libitum*.

Preparations of carcinogens: (3,4-benzo(a)pyrene, BP, Sigma Chemical Co.) was solved in small amount of aceton. Charcoal powder(Wako Pure Chemical Ind., Ltd., Osaka, Japan) was ground in a mullite mortar thoroughly for about 24hr. BP solution and charcoal powder were mixed and sterile saline solution was added to the mixture to give a final concentration of 50mg BP and 25mg charcoal powder or 25mg BP and 25mg charcoal powder per ml saline solution. For the purpose of removing aceton and attaching BP to charcoal powder, mixture of BP solution, charcoal powder and saline was bubble by nitrogen gas. This suspension was kept homogeneously by continuous stirring during the procedure of intratracheal instillation.

BP coated charcoal powder particles were in the 0.5 to 15μm size range.

Urethan (Ethyl Urethan, Fisher Chemical Co.) 1mg or 10mg in 0.02ml sterile saline solution was used.

Intratracheal instillation: Intratracheal instillation of the carcinogen was essentially the same as that described for hamster (Saffiotti *et al.*, 1972 a) and mouse (Ho and Frust, 1973).

Each was aneshetized with ether and fixed in a supine position. For the purpose of mouth opening and confirming epiglottis location, otoscope (Heine,

Table 1. Experimental Groups and Treatment

Treatment	Strains	Numbers of animals
BP(1mg)+charcoal powder(0.5mg)/ 0.02ml saline/head, 8 instillations*	A/J	28
	C_{57}BL/6N	26
	DBA/2	28
	NIH-GP	26
BP(0.5mg)+charcoal powder(0.5mg)/ 0.02ml saline/head, 11 instillations*	A/J	25
Charcoal powder (0.5mg)/0.02ml saline/head, 8 instillations*	A/J	15
	C_{57}BL/6N	13
	DBA/2	12
	NIH-GP	13
Urethan(1mg)/0.02ml saline/head, 11 instillations*	C_{57}BL/6N	21
	NIH-GP	18
Urethan(10mg)/0.02ml saline/head, 1 instillation	C_{57}BL/6N	18
	NIH-GP	19
Saline 0.02ml/head, 11 instillations*	C_{57}BL/6N	14
	NIH-GP	17

* 1 intratracheal instillation/week.
BP : 3, 4-benzo(a)pyrene

milex, West Germany) was used. After the tongue of the mouse was pulled outward and laterally with a small forceps, the salivary secretion in the oral cavity was wiped away with a sterile paper string. The 22-gauge blunt needle of a tuberculin syringe was inserted slowly between the vocal cords into the trachea and pushed forward almost to the bifurcation of the trachea. Carcinogens were injected slowly and the needle was withdrawn.

Mouse strains, kinds of carcinogens and treatments are summarized in Table 1.

Histopathological examination: The mice were examined daily and weighed weekly throughout the observation period of 13 weeks. Animals were either allowed to die naturally or killed at the end of the experiment and they were all necropsied. The lungs were excised *en bloc* with trachea, and liver, spleen, kidneys, heart and stomach were removed. They were fixed in Lillie's alcoholic lead nitrate formalin solution, embedded in paraffin, and sectioned at 5μm thickness. The specimens were stained with hematoxylin and eosin(H-E), and alcian blue-periodic acid Schiff(AB-PAS).

Results

Changes in survival rate and mean body weight

Changes in survival rates and mean body weight of experimental and control animals are shown in Fig. 1 and 2. The mean body weights of the mice

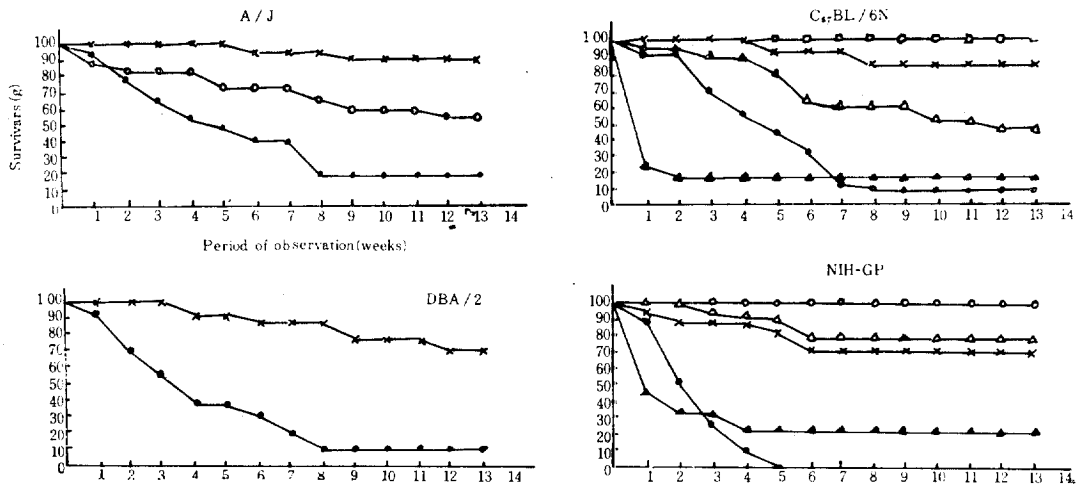

Fig. 1. Changes in cumulative survival rate ×——× Charcoal powder □——□ Saline ●——● 1mg B(a)P △——△ 1mg Urethan ○——○ 0.5mg B(a)P ▲——▲ 10mg Urethan

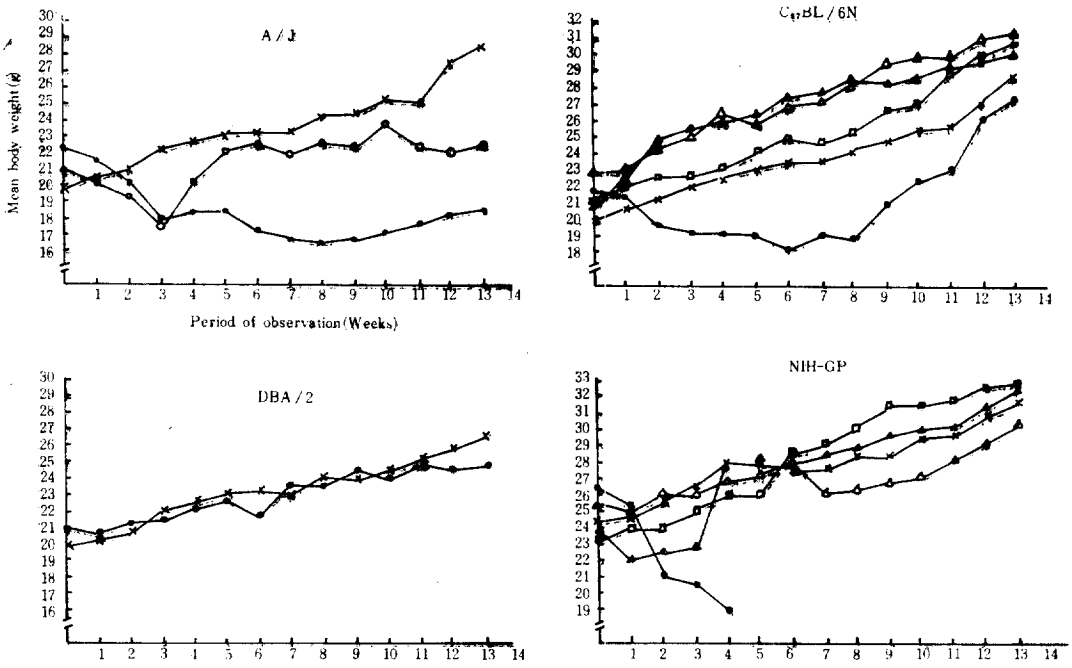

Fig. 2. Changes in mean body weight ×——× Charcoal powder □——□ Saline ●——● 1mg B(a)P △——△ 1mg Urethan ○——○ 0.5mg B(a)P ▲——▲ 10mg Urethan

given a high dose of BP instillation once a week for 8 weeks decreased greatly during treatment but increased gradually after cessation of treatment. On the other hand, the mean body weight of mice treated with 11 low doses of BP increased steadily during treatment. In the control group and urethan instillation group, mean body weights of the mice increased steadily in spite of treatment.

The experimental group had a considerably shortened life span. Especially, the mean body weight of NIH-GP mice given BP decreased greatly during treatmen. NIH-GP mice given BP, C_{57}BL/6N and NIH-GP mice given 10mg urethan died in the early period of treatment.

Macroscopic observations

The mixture of BP and charcoal powder distributed more densely in the right lobes of the lung than in the left lobes. In both A/J and C_{57}BL/6N mice treated 8 times with a high does of BP, nodular lesions were observed on the surface of the lung and on the cut surface of the lung from 7 weeks after the first instillation (Fig. 3). These nodular lesions were always surrounded by charcoal deposits. Small tumors were about 5mm in diameter.

In low dose BP treated A/J mice, nodular lesion was not found on the surface of the lung. Occasionally, tumor nodules were seen as round, white swellings on the surface of the lung in mice treated with urethan.

Microscopic findings

Incidence and histological types of tumors in the lung of the mice instillated with BP or urethan are summarized in Table 2 and 3.

1) **1mg BP-0.5mg charcoal powder treated A/J mice**: One week after third instillation, hyperplastic and squamous metaplastic changes of the epithelium in bronchi and bronchiole were usually observed(Fig. 4, 5). The hyperplastic cells in the bronchi were stained purple or sky blue with AB-PAS and squamous metaplastic cells were also stained sky blue(Fig. 5). At the same time, focal neoplastic cells were observed in on case.

In week 7, moderately to highly keratinized microtumors were observed in the lung(Fig. 6, 7, 8).

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In week 13, squamous cell carcinomas and adenocarcinomas were seen in all examined animals(Fig. 13, 14). The changes of surrounding tissue, such as mononuclear cell infiltration and hyperemia in this treatment group, was less prominent than in 1mg BP instillation group(Fig. 12). Tumor induction time was also delayed.

3) 1mg BP-0.5mg charcoal powder treated C_{57}BL/6N mice: One week after third instillation, AB-PAS positive cell hyperplasia and focal adenocarcinomas were ovserved in bronchi and bronchiole.

After sixth instillation, adenocarcinomas and squamous cell carcinomas were seen in each of the examined animals. But squamous cell carcinoma lesion was rare in this group(Fig. 15, 16).

4) 1mg BP-0.5mg charcoal powder treated DBA/2 and NIH-GP mice: In the DBA/2 mice, hyperemia, edema and mononuclear cell infiltration were not observed only after week 5. In one case, focal adenocarcinoma was seen at week 13.

In the NIH-GP Mice, severe hyperemia and hemorrhage caused death of animal at first and second week after instillation(Fig. 17). In week 4, adenocarcinoma was observed in one case.

The changes of lung tissue in these strains of mice by the BP instillation, such as tumor induction, hyperplasia and metaplasia of bronchial epithelium, were less pronounced than in A/J and C_{57}BL/6N.

In BP treated groups, the cells of squamous mataplasia and of squamous cell carcinoma had alcianophility in early period. On the other hand, In case of highly differentiated squamous cell carcinoma and massively keratinized squamous metaplasia, tumor cells and metaplastic cells were not stained with alcian blue.

5) 1mg and 10mg urethan treated C_{57}BL/6N and NIH-GP mice: In the mice treated with 1mg of urethan, pyperemia, hemorrhage, edema and necrosis of bronchial epithelium were seen in the lung at 6th week. Metaplastic and hyperplastic changes of bronchial epithelium as well as tumor occurrence were rare. Adenoma was seen in only one NIH-GP mouse (Fig. 18, 19) and adenocarcinoma was observed in one C_{57}BL/9N mouse at week 13(Fig. 20).

In the mice treated with 10mg of urethan, severe hyperemia and hemorrhage were observed. Thereafter, animal died in the early period of experiment. Adenoma was seen in only one case of NIH-GP mouse (Fig. 21).

Discussion

Squamous cell carcinomas and adenocarcinomas were readily induced in the lungs of A/J and C_{57}BL/6N mice by repeated intratracheal instillation of BP with charcoal powder suspened in saline solution.

Many chemical and environmental agents have been examined for carcinogenic activity using the tumorigenic response of the mouse lung as a measure. The mouse lung has been shown to be sensitive to all major classes of chemical carcinogens and the induction of lung tumors in mice appears to be a sensitive measure of the carcinogenic potential of agents found in the environment. The mouse lung tumor system is an excellent tool in carcinogenesis research. (Mc Dowell *et al.*, 1979).

In a wide spectrum of susceptibility to spontaneous lung tumors, there is a general agreement that the A strain has the highest incidence of lung tumors. Strains BALB/C, CR, O_{20} and DD are of intermediate susceptibility. Among the relatively resistant strains are the CBA and C3H. The most resistent strains are the DBA, C_{57}BL and C_{57}L. Many investigations on pulmonary anenoma induction in mice reiterate that the susceptibility to carcinogens is in direct relation to the spontaneous occurrence of the tumor. Thus, strain A mice are the most susceptible to a wide variety of carcinogens, in terms of earlier appearance and greater multiplicity of tumors. Strain C_{57}BL is among the most resistant to all chemical classes of carcinogens(Shimkin and Stoner, 1975; Shimkin, 1955). But, there were obvious differences between induction of squamous cell carcinoma or adenocarcinoma by intratracheal instillation of BP and induction of adenoma by intravenous, intraperitoneal or subcutaneous injection of BP in strain spectrum of susceptibility to induction of tumor. Thus, although there was difference in the type of induced tumor, C_{57}BL/6N, which had the lowest susceptibility of all the strains to spontaneous lung tumor, produced

malignant tumor of the lung at the same time with A/J. On the other hand, DBA/2 and NIH-GP mice did not produce pulmony tumor.

When a high dose of BP(1mg BP and 0.5mg charcoal powder) was instillated to A/J mice, squamous cell carcinomas were induced at high incidence. On the other hand, when a low dose of BP(0.5mg BP and 0.5mg charcoal powder) was instillated, adenocarcinomas were induced at high incidence. These results are in agreement with the suggestions(Yoshimoto *et al.*, 1980, 1977) that a larger quantity of intratracheal BP instillation was needed for induction of squamous cell carcinoma than for induction of adenocarcinoma in the lung of mice.

Original site of these tumors could not be determined. Serial killing of the mice and serial sections of the lung will be needed to answer the question of whether these tumors are bronchogenic, as in hamster (Saffiotti *et al.*, 1968), or bronchiolo-alveolar in origin, as in rats(Schreiber *et al.*, 1972).

Many investigator presented data suggesting the hypothesis that, in the bronchial epithelium, epidermoid metaplasia can result from direct conversion of columnar mucous cells to keratinizing epidermoid cells(Mc Dowell *et al.*, 1979; Trump *et al.*, 1978). Morphological and histochemical studies of regeneration in hamster trachea and of epidermoid metaplasia and carcinoma *in situ* in human bronchus and hamster trachea indicate clearly that mucous cells are involved in all of these processes(McDowell *et al.*, 1978 a, b). Areas of epidermoid metaplasia often lay adjacent to areas showing AB-PAS reactive cell hyperplasia. The cells of epidermoid metaplasia and of squamous cell carcinoma had alcianophility in early period. The results obtained in this study suggest that AB-PAS reactive cells have a significant role in epidermoid metaplasia and squamous cell carcinoma formation.

Although the induced neoplasms are very similar morphologically to those observed in the human lung (Becci *et al.*, 1978), differences in carcinogen distribution and activity due to varying of the physical properties of the carrier particle(Henry *et al.*, 1975) make the effective dose as well as the localization and incidences of tumors difficult to predict and control. To overcome these and other problems, many investigators used water soluble carcinogens (Stinson and Lilga, 1980; Becci *et al.*, 1980). In this study, urethan was instillated, as water soluble carcinogen, but the tumor incidence was very low.

Conclusions

The present study was carried out to observe the histopathological changes in the lung of A/J, C_{57}BL/6N, DBA/2 and NIH-GP mice intratracheally instillated with benzo(a)pyrene or urethan.

In the group of intratracheal instillation with benzo(a)pyrene and charcoal powder, squamous cell carcinomas and adenocarcinomas were induced. Tumors were induced in high incidence in the lung of A/J and C_{57}BL/6N mice. Especially, squamous cell carcinomas were induced in high incidence and were well-differentiated in the lung of A/J mice.

A large quantity of benzo(a)pyrene instillated intratracheally was needed for induction of squamous cell carcinoma than for induction of adenocacinoma in the lung of mice.

In the C_{57}BL/6N mice, it is generally considered that a lower spontaneous lung tumor incidence strain than the A/J mice, adenocarcinomas were induced with high frequency, whereas squamous cell carcinoma were induced in the lung of the A/J mice.

The alcianblue-PAS positive cells might play an important role in the occurrence of squamous metaplasia and squamous cell carcinoma.

Tumors were induced with low frequency in the DBA/2 and NIH-GP mice.

In the group of intratracheal instillation with urethan, the tumor incidence was very low.

Thus, the histologic types and incidence of tumors varied depending on mouse strain, kinds and amounts of carcinogens employed.

These findings suggest that mice appear adequate for studies on the pathogenesis of adenocarcinoma and squamous cell carcinoma.

Legends for Figures

Fig. 3. Gross appearance of pulmonary tumor nodules in the right lobe and functional hypertrophy in the left lobe of A/J mouse intratracheally instillated with 1mg benzo(a)pyrene(BP). Experimental period, 13 weeks.

Fig. 4. Alcian blue-periodic acid Schiff(AB-PAS) positive cell hyperplasia of terminal bronchiolar epithelium of A/J mouse intratracheally instillated with 1mg of BP. Experimental period, 7weeks. AB-PAS stain. ×100.

Fig. 5. Squamous metaplasia of bronchiolar epithelium of A/J mouse intratracheally instillated with 1mg of BP. The bronchiolar lumen is stenotic with keratinized squamous cells. The metaplastic cells have alcianophility. Experimental period, 5weeks. AB-PAS stain. ×100.

Fig. 6. Bronchogenic tumor and stenosis of bronchi with keratinized squamous cell in the A/J mouse intratracheally instillated with 1mg of BP. The tumor expands into the surrounding alveolar spaces. Experimental period, 7 weeks. Hematoxylin and eosin(H-E) stain. ×20.

Fig. 7. Moderately-keratinized squamous cell carcinoma in the A/J mouse intratracheally instillated with 1mg of BP. Note the irregular growth proliferating activities. Experimental period, 7 weeks. H-E stain. ×50.

Fig. 8. Moderately-keratinized focal squamous cell carcinoma of A/J mouse intratracheally instillated with 1mg BP. The tumor cells have alcianophility. Experimental period, 13 weeks. AB-PAS stain. ×50.

Fig. 9. Highly-keratinized squamous cell carcinoma with epithelial pearls of A/J mouse intratracheally instillated with 1mg BP. Experimental period, 13 weeks. H-E stain. ×50.

Fig. 10. Adenomatosis of A/J mouse intratracheally instillated with 1mg BP. Alcian blue positive tall columnar cells line the wall of alveolus. Experimental period, 13 weeks. AB-PAS stain. ×50.

Fig. 11. Adenomatosis, squamous metaplasia and squamous cell carcinoma in the A/J mouse intratracheally instillated with 1mg BP. Squamous metaplastic cells and tumor cells have alcianophility. Experimental period, 13 weeks. AB-PAS stain. ×50.

Fig. 12. Hyperplasia of bronchial epithelium and focal squamous cell carcinoma of A/J mouse intratracheally instillated with 0.5mg BP. Experimental period, 13 weeks. H-E stain. ×50.

Fig. 13. Adenocarcinoma in the A/J mice intratracheally instillated with 0.5mg of BP. The tumor has a glandular acinar-like pattern. Experimental period, 13 weeks. H-E stain. ×50.

Fig. 14. Moderately-keratinized focal squamous cell carcinoma in the A/J mouse intratracheally instillated with 0.5mg BP. The tumor cells have slight alcianophility. Experimental period, 13 weeks. AB-PAS stain. ×50.

Fig. 15. Adenocarcinoma in the C_{57}BL/6N mouse intratracheally instillated with 1mg BP. AB-PAS positive tumor cells are seen. Experimental period, 6 weesk. AB-PAS stain. ×50.

Fig. 16. Poorly differentiated adenocarcinoma in the C_{57}BL/6N mouse intratracheally instillated with 1mg BP. Note the glandular structure and polymorphic tumor cells. Experimental period, 13 weeks. H-E stain. ×50.

Fig. 17. The lung lesion of the NIH-GP mice intratracheally instillated with 1mg of BP. Severe hyperemia and hemorrhage are seen. Experimental period, 3 weeks. H-E stain. ×50.

Fig. 18. Peripheral pulmonary adenomatous cell arrangement in the NIH-GP mice intratracheally instillated with 1mg of urethan. Experimental period, 13 weeks. H-E stain. ×50.

Fig. 19. Pulmonary adenoma in the NIH-GP mice intratracheally instillated with 1mg of urethan. Experimental period, 13 weeks. H-E stain. ×100.

Fig. 20. Adenocarcinoma in the C_{57}BL/6N mice instillated with 1mg of urethan intratracheally. The tumor cells have alcianophility. Experimental period, 13 weeks. AB-PAS stain. ×50.

Fig. 21. Papillary type pulmonary adenoma in the NIH-GP mice instillated with 10mg of urethan intratracheally. Experimental period, 13 weeks. H-E stain. ×50.

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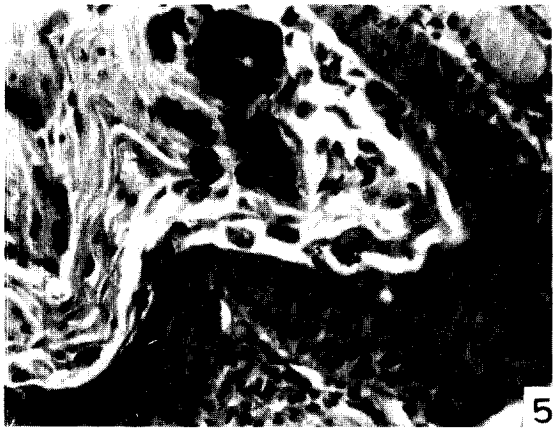

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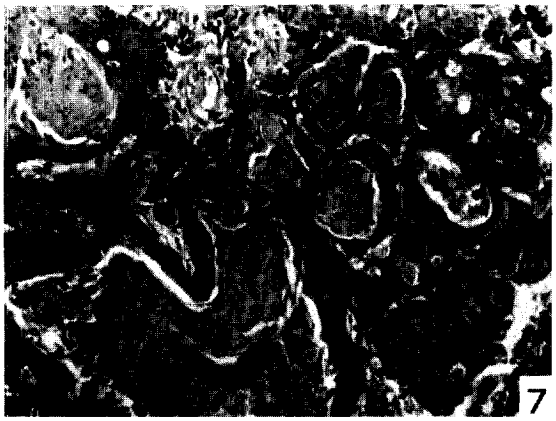

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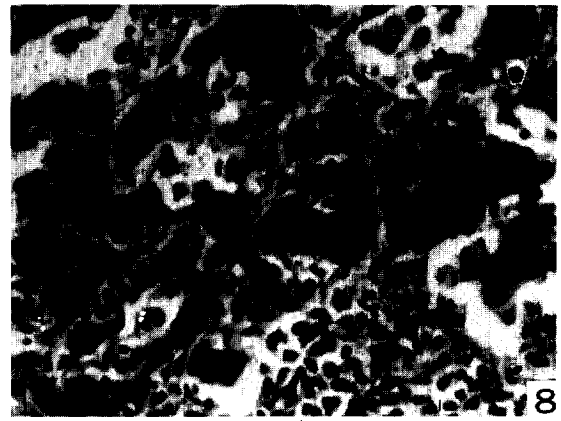

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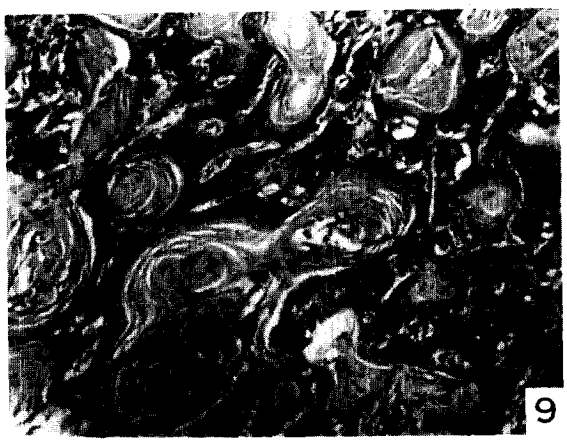
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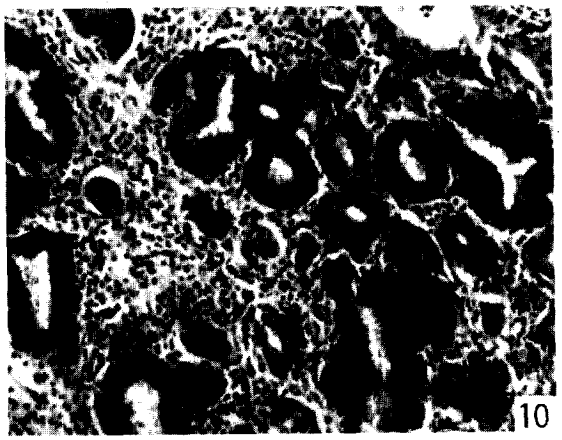
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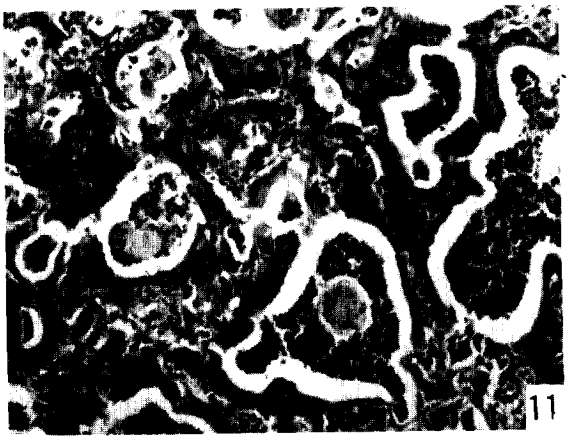
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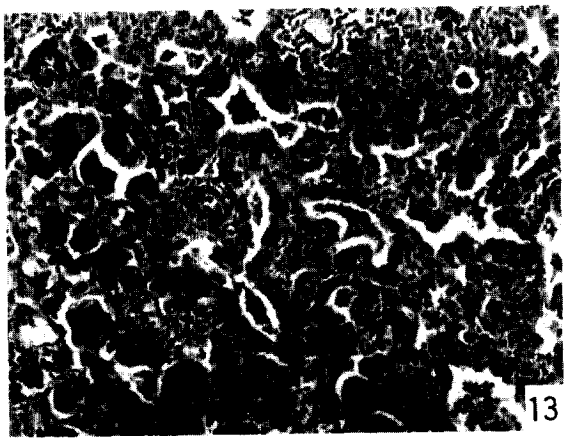
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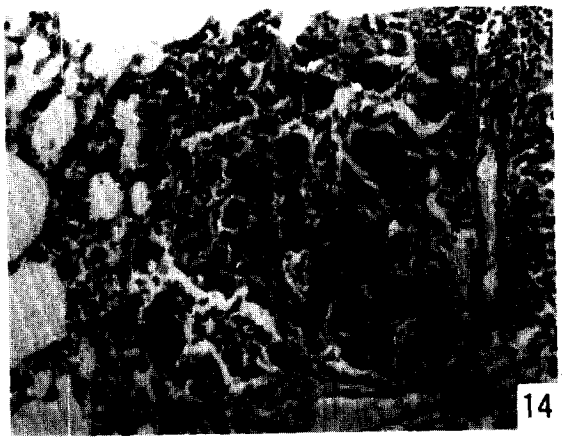
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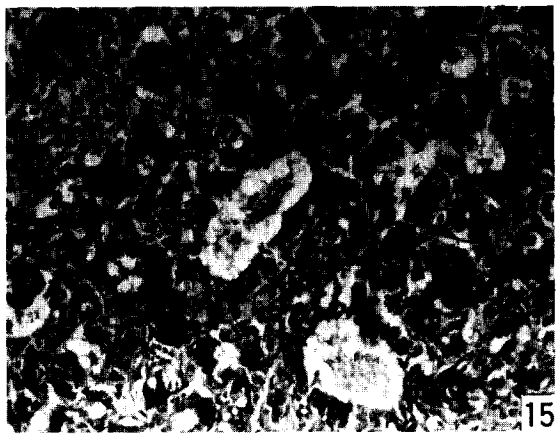
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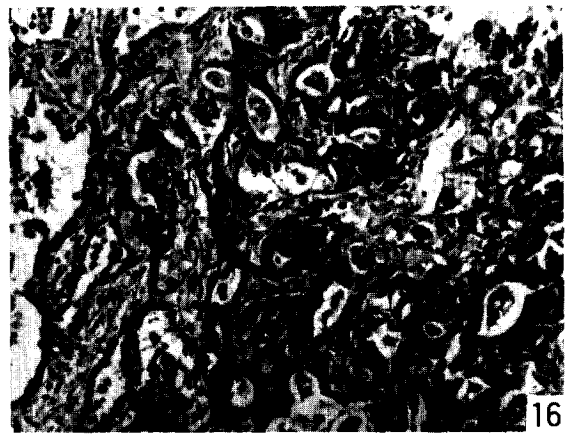
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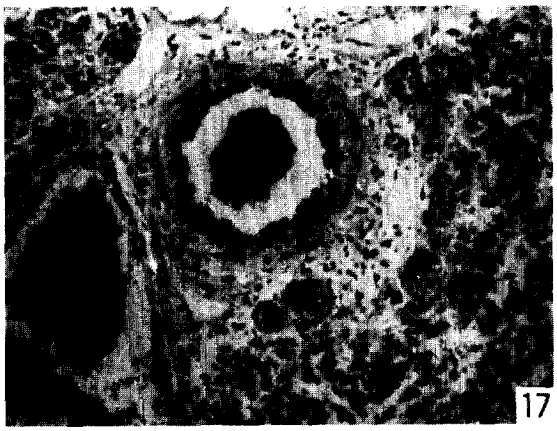
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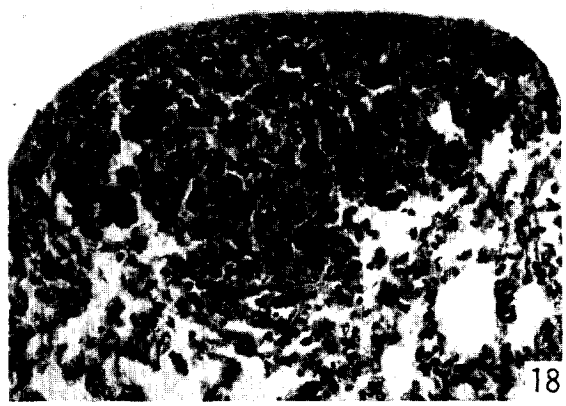
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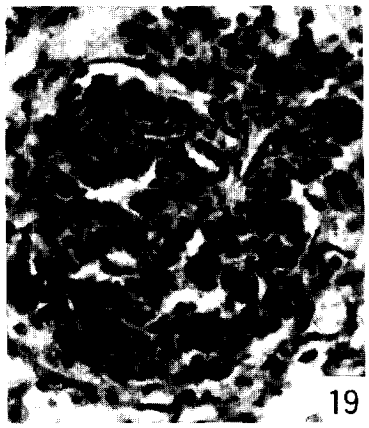
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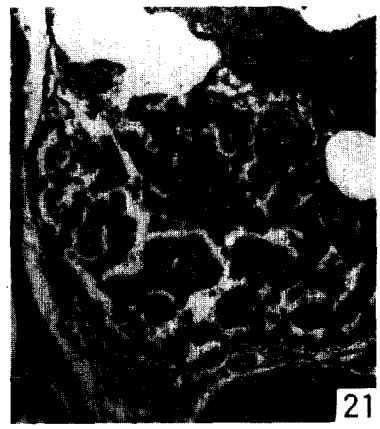
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