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Influence of Age on Hepatocarcinogenesis in Rats Ingesting Diethylnitrosamine

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SUMMARY

The effect of age on hepatocarcinogenesis was investigated on Fischer male rats of 3, 6, 9, and 12 months old utilizing diethylnitrosanime at 40 ppm in drinking water for 10 weeks. Tumor incidence larger than 5 mm in diameter was significantly higher in 3-month-old rats than in other groups. Tumor incidence was not significantly different among 6-, 9-, and 12-month-old rats. The resistance to hepatocarcinogens in aged animals was disscussed on the basis of hormonal status, mixed function oxidases and cell replication.

INTRODUCTION

It is well known that carcinogenesis is modified by many host factors such as age, sex, genetics, and hormonal and nutritional status (11). Among these factors, age might be very important because in human and experimental animals, the incidence of spontaneous neoplasms generally increases with age. Many investigations on chemichal carcinogenesis used newborn, infant, or younger animals, partly because using these animals results in the use of a smaller amount of chemicals and a considerable reduction in time and upkeep costs, and partly because the induction of tumors in many tissues was readily achieved in these animals. Toth (27) reviewed twenty-nine reports of chemical carcinogenesis in newborn animals, Della Porta and Terracini (6) reviewed infant mice, and Scoental (25) reviewed transplacental carcinogenesis. The available experimental evidences have demonstrated that younger animals are more susceptible to chemical carcinogens than adult animals in general. However, as pointed out by Toth (27), some tissues provided more tumors when the chemicals were given at older ages than at birth. In fact, Franks and Carbonell (10) reported that, when a single subcutaneous dose of 3-methylcholanthrene was given to weanling (21 days), adult (6 months), and old (20 months) C57 Bl mice, all aged mice, 96% of adult mice, but only 75% of weanling mice, developed tumors 140 days after the carcinogen injection. Ebbesen (7-9) found that aging increased the susceptibility of mouse skin to

DMBA-induced carcinogenesis in which skin grafting was used in syngeneic hosts of various ages. Berry and Wagner (1) studied the incidence of mesothelioma with injection of asbestos in the thorax of 2-, and 10-month-old rats, and found early development of tumors in old rats. However, attempting to distinguish experimentally between the environmental and aging mechanisms for the increased incidence of neoplasms, Peto *et al.* (14) found that using skin-painted mice with 3,4-benzpyrene starting from ages 10, 25, 40, or 55 weeks, the incidence rate increased approximately as a power of the duration of exposure to the carcinogen, and was independent of any intrinsic effects of aging such as failing immunologic surveillance or age-related hormone changes.

Thus it seems that the effect of host ages on carcinogenesis differed from organ to organ. In the present experiment, we investigated the effect of age on the incidence of hepatomas induced by diethylnitrosamine, using 3-, 6-, 9-, and 12-months old Fischer male rats.

MATERIALS AND METHODS

Fischer male rats of one month age were purchased from Charles River Co. Japan. They were maintained on a diet of commercial chow and water *ad libitum* until the administration of the carcinogen. DEN was disolved in drinking water at 40 ppm and given to rats for 10 weeks *ad libitum*. DEN administration was started when rats became 3-, 6-, 9-, and 12-month old. Daily intake of drinking water was measured in order to calculate the amount of the carcinogen administered.

All rats were killed 10 weeks after termination of the carcinogen treatment. The livers were fixed in buffered formalin for 2-3 days and sliced into sections 2 mm thick, then the number and size of tumors larger than 5 mm in diameter were measured. Representative tumors were blocked in paraffine, and stained with hematoxylin and eosin for histological examinations.

RESULTS

Intake of DEN

Fig. 1 shows cumulative intake of DEN per rat. Rats of all groups ingested DEN constantly. However, differences of amount ingested were noted among the groups. The average amounts of daily intake per rat in groups 1 (3 months), 2 (6 months), 3 (9 months), and 4 (12 months) were 0.77, 1.16, 0.83, and 1.03 mg, and total amounts were 53.6, 81.0, 57.8, and 72.2 mg, respectively. Rats in group 1 ingested the smallest amount of DEN, and the rats in group 2 ingested the largest amount.

Incidence of liver tumors

The number of rats with tumors and the average number of tumors per rat

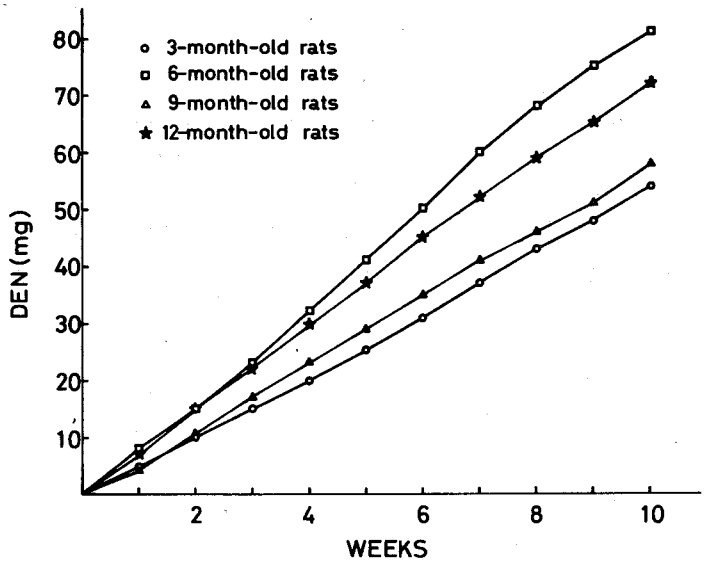

Fig. 1. Cumulative intake of DEN per rat. Ingested amount of drinking water per cage was measured daily, divided by the number of rats in each cage, and the amount of DEN per rat was calculated.

were shown in Table 1. The data in Table I deal with only tumors larger than 5 mm in diameter, because tumors less than 2 mm in diameter were so numerous in group 1 that accurate estimation of number could not be obtained. All rats in group 1 had liver tumors larger than 5 mm in diameter. The average number of tumors larger than 5 mm in diameter in group 1 was 8.30. Sixteen tumors were larger than 10 mm in diameter and found in ten rats, the largest tumors being 25 mm in diameter.

In group 2 where the rats were administered DEN at the age of 6 months, 75%

Table 1. *Incidence of Tumors Larger Than 5 mm in Diameter*

Group	Age at treatment (months)	No. of rats Initial	Effective	No. of rats with tumors (%)	Average No. of tumors per rat
I	3	16	14	14 (100)	8.30 ± 3.81^{a}
II	6	14	8	6 (75)	2.25 ± 2.05^{b}
III	9	14	10	6 (60)	1.80 ± 1.98^{c}
IV	12	16	11	7 (63)	2.00 ± 1.84^{d}

a; mean±SD.

Significantly different from group I at: b: $P<0.05$, c: $P<0.005$, d: $P<0.001$.

of rats had tumors larger than 5 mm, the average number of which was 2.25 per rat. Only eight tumors were larger than 10 mm in diameter and found in four rats, the largest tumors being 16 mm in diameter.

In group 3 where rats were ingested DEN at age of 9 months, 60% of rats had tumors larger than 5 mm. Average number of tumors per rat was 1.80. Six rats possessed tumors larger than 10 mm in diameter. The largest ones were 18 mm in diameter. In group 4 where rats were ingested DEN at the age of 12 months, 63% of rats had tumors larger than 5 mm, the average number of which was 2.00 per rat. Three rats had a tumor larger than 10 mm in diameter each. The largest one was 16 mm in diameter.

The statistically significant difference of the average number of tumors larger than 5 mm per rat was obvious between group 1 and groups 2–4, but a significant difference was not obtained among groups 2–4.

Histology of tumors

The majority of tumors larger than 5 mm in diameter induced by DEN were trabecular hepatocellular carcinomas, which can be subdivided into well-, moderately, and poorly-differentiated trabecular types. Well-differentiated trabecular hepatocellular carcinomas consisted of cells with larger and eosinophilic cytoplasms, arranged in plates as one or more cells thick (Figs. 2, 3). Poorlydifferentiated ones consisted of rather small cells with more basophilic cytoplasms and arranged more compactly than well-and moderately-differentiated ones (Figs. 4, 5). Rarely, anaplastic hepatocellular carcinomas were observed in all groups (Figs. 6, 7). Histological differences were not observed among the groups.

DISCUSSION

Cancer increases with age in humans as well as in animals. A human will develop cancer during the next 5 years is only 1 in 700 if he is aged 25 but is 1 in 14 if he is 65 years old (14). Although there are many literatures concerning experimental carcinogenesis using younger animals, experiments concerning age-related carcinogenesis are limited.

With respect to hepatocarcinogenesis, Della Porta and Terracini (6) reviewed the incidences of thymic lymphomas, lung adenomas, and hepatomas induced by chemical carcinogenesis. Among the newborn, infant and younger adult mice, the younger animals were more susceptible to the action of some chemical carcinogens, and they developed more hepatomas, lung adenomas and thymic lymphomas than adult mice similarly treated. Decloitre *et al* (5) demonstrated also using azo-dye that the hapatoma incidence was higher in 4- to 6-week-old rats than in 10-week -old rats.

In the present experiment, we examined the effect of age on hepatocarci-

nogenesis using 3-, 6-, 9-, and 12-month-old rats. Our results showed clearly that liver tumors were more numerous in 3-month-old rats than in 6-, 9-, and 12-month -old rats, in spite of the increased intake of DEN in the latter groups. However, there is no statistically significant difference in the incidence of tumors among 6-, 9-, and 12-month-old rats.

Reuber and his associates compared the tumor incidence of liver in both male and female rats at age of 4, 12, 24, and 52 weeks old induced by DEN (17), N-4-(4' -fluorobiphenyl)acetamide (26), 3'-methyldimethylaminoazobenzene(20), and N-2 -fluorenyldiacetamide (22). These investigations all showed that more numerous and larger tumors of the liver were found in younger rats than in older rats. Thus, from above mentioned reports and our own, it is clear in respect of hepatocarcinogenesis that younger animals were more susceptible than old animals.

The mechanisms by which older animals are more refractory to carcinogenic stimuli may be very complicated, because both the aging process and carcinogenesis are influenced by many additional factors. Reuber and his associates placed great importance on hormonal status. Castration (16, 21), thyroidectomy (15, 16), hypophysectomy (18) and alloxan diabetes (20) all decreased the development of hepatocellular carcinoma. However, although exogenous testosterone restored hepatic carcinogenesis in castrated male rats, the same treatment failed to restore significantly in castrated old male rats (23).

Although hormonal factors act an important role in aging and carcinogenesis, other factors must be considered. Most carcinogens must be metabolically converted to highly reactive derivatives which react with important macromolecules such as DNA, RNA and proteins by mixed function oxidases. These enzymes are known to change during aging (12, 13, 24). These changes might be influence on hepatocarcinogenesis. In fact, Decloitre *et al* (5) showed that metabolites of p -dimethylaminoazobenzen binding to DNA and protein in rat liver were significantly decreased by 70% between 4 and 12 weeks of age. They presumed that the high ability for carcinogen activation and normal detoxication would make young rats especially susceptible to the carcinogenic effect, and by contrast, the low degree of activation and normal detoxication in older rats would make them more resistant. Thus, the changes of microsomal drug metabolizing enzymes with aging reflect also on chemical carcinogenesis.

On the other hand, cell replication plays an essential role in carcinogenesis (4) The mitotic activity of many tissues is higher in young animals than in older animals. Many experiments of hepatocarcinogenesis used young animals or partially hepatectomized animals. A single treatment of an adult animal with a hepatocarcinogen rarely induces hepatomas. However, a single treatment does induce hepatomas in newborn or in partially hepatectomized adult animals. To produce

hepatomas in the adult animals, chronic administration of the carcinogens is essential. This chronic administration can lead to progressive liver cell damage and hence to restorative hyperplasia, so that the rate of cell replication is increased. Morever, it is known that regeneration of rat liver is retarded with age. Bucker *et al* (2, 3) clearly demonstrated that compared with weanling, young adult and old rats, incorporation of thymidin into the DNA and cell proliferation rate after partial hepatectomy were less in older rats than in younger rats. It might be conclded that all these factors act on hepatocarcinogenesis, resulting in fewer hepatomas in old animals.

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REFERENCES

1. BERRY, G. and WAGNER, J. C.: Int. J. Cancer **17,** 477–483 (1976).
2. BUCHER, N. L. and GLINOS, A. D.: Cancer Res. **10,** 324–332 (1950).
3. BUCHER, N. L., SWAFFIELD, M. N. and DITROIA, J. F.: Cancer Res. **24,** 509–512 (1964).
4. CRADDOCK, V. M.: Cell proliferation and experimental liver cancer. *In*: Liver Cell Cancer eds. by CAMERON, H. M., LINSELL, D. A. and WARWICK, G. P. pp 153–201 Elsevier, Amsterdam (1976).
5. DECLOITRE, F., CHAUVEAU, J. and MARTIN, M.: Int. J. Cancer **11,** 676–680 (1973).
6. DELLA PORTA, G. and TERRACINI, B.: Progr. Exp. Tumor Res. **11,** 334–363 (1969).
7. EBBESEN, P.: Nature **241,** 280–281 (1973).
8. EBBESEN, P.: Science **183,** 217–218 (1974).
9. EBBESEN, P.: J. Natl. Cancer Inst. **58.** 1057–1060 (1977).
10. FRANKS, L. M. and CARBONELL, A. W.: J. Natl. Cancer Inst. **52,** 565–566 (1974).
11. HOMBURGER, F.: Modifiers of carcinogenesis. *In*: The physiopathology of cancer ed. by HOMBURGER, F. vol. I, pp 110–154. S. Karger, Basel (1974).
12. KATO, R. and TAKANAKA, A.: J. Biochem. **63,** 406–408 (1968).
13. MATHUR, D. P.., BOREN, J. A., SMYTH, R. D. and REAVEY-CANTWELL, N. H.: Res. Comm. Chem. Path. Pharm. **11,** 39–44 (1975).
14. PETO, R., ROE, F. J. C., LEE, P. N., LEVY, L. and CLACK, J.: Br. J. Cancer **32,** 411–426 (1975).
15. REUBER, M. D.: J. Natl. Cancer Inst. **35,** 959–966 (1965).
16. REUBER, M. D.: J. Natl. Cancer Inst. **36,** 775–781 (1966).
17. REUBER, M. D. and LEE, C. W.: J. Natl. Cancer Inst. **41,** 1133–1140 (1968).
18. REUBER, M. D.: J. Natl. Cancer Inst. **43.** 445–452 (1969).

19. REUBER, M. D.: Gann **62,** 157–161 (1971).
20. REUBER, M. D., STROMBERG, K. and GLOVER, E. D.: J. Natl. Cancer Inst. **48,** 675–683 (1972).
21. REUBER, M. D.: J. Natl. Cancer Inst. **53,** 883–886 (1974).
22. REUBER, M. D.: Path. Microbiol. **43,** 31–37 (1975).
23. REUBER, M. D.: Europ. J. Cancer **12,** 137–141 (1976).
24. SCHLEDE, E.: Naunyn Schmiedebergs Arch. Pharmacol. **282,** 311–315 (1974).
25. SCOENTAL, R.: Ann. Rev. Pharmacol. **14,** 185–204 (1974).
26. STROMBERG, K. and REUBER, M. D.: J. Natl. Cancer Inst. **44,** 1047–1054 (1970).
27. TOTH, B.: Cancer Res. **28,** 727–738 (1968).

Figure Legends

Fig. 2. Well-differentiated trabecular hepatocellular carcinoma in 3-month-old group. Trabecular pattern in one or more cell thickness was clearly visible (X144).

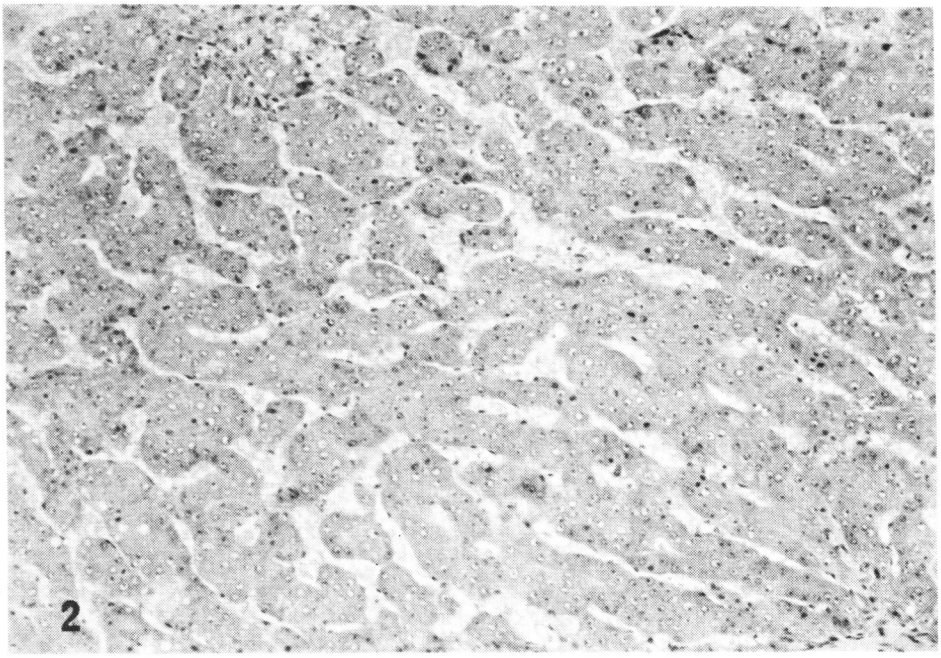
Fig. 3. Well-differentiated trabecular hepatocellular carcinoma in 6-month-old group. The histological characteristics were the same as those of Fig. 2. (X144).

Fig. 4. Poorly-differentiated trabecular hepatocellular carcinoma in 3-month-old group. The cells are smaller and more basophilic than those of Figs. 2 and 3, and are compactly arranged, but the trabecular pattern is visible. (X360).

Fig. 5. Poorly-differentiated trabecular hepatocellular carcinoma in 12-month-old group. The histological characteristics were the same as those of Fig. 4. (X360).

Fig. 6. Anaplastic hepatocellular carcinoma in 3-month-old group. The cells are rather large and compactly arranged, but occasionaly adenomatous patterns are noted. (X144).

Fig. 7. Anaplastic hepatocellular carcinoma in 12-momth-old group. Irregular shaped cells of various size are compactly arranged. Mitoses are numerous and an abnormal mitotic figure is seen in right upper corner. (X144).



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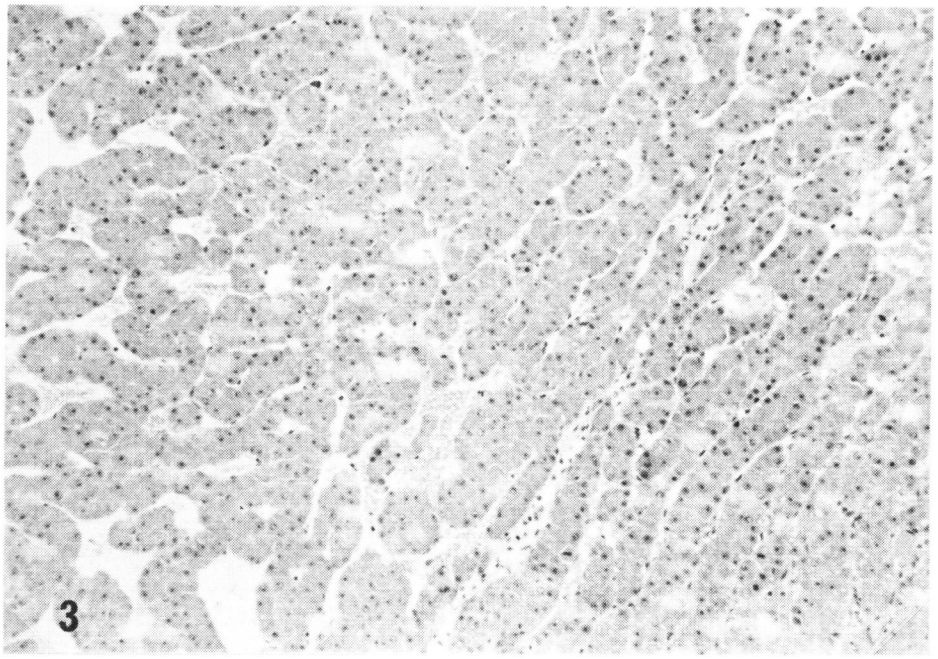

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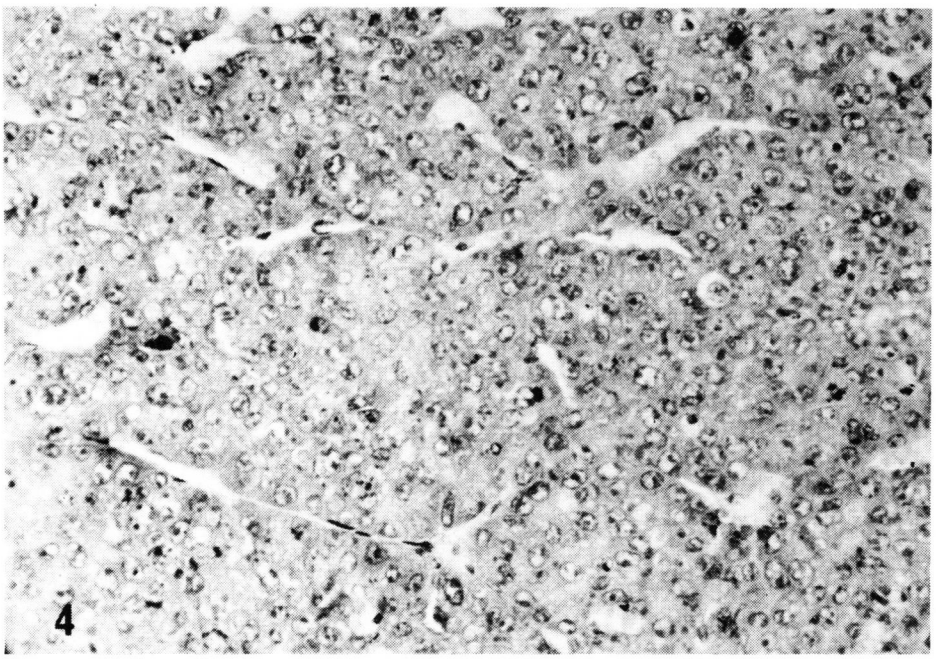
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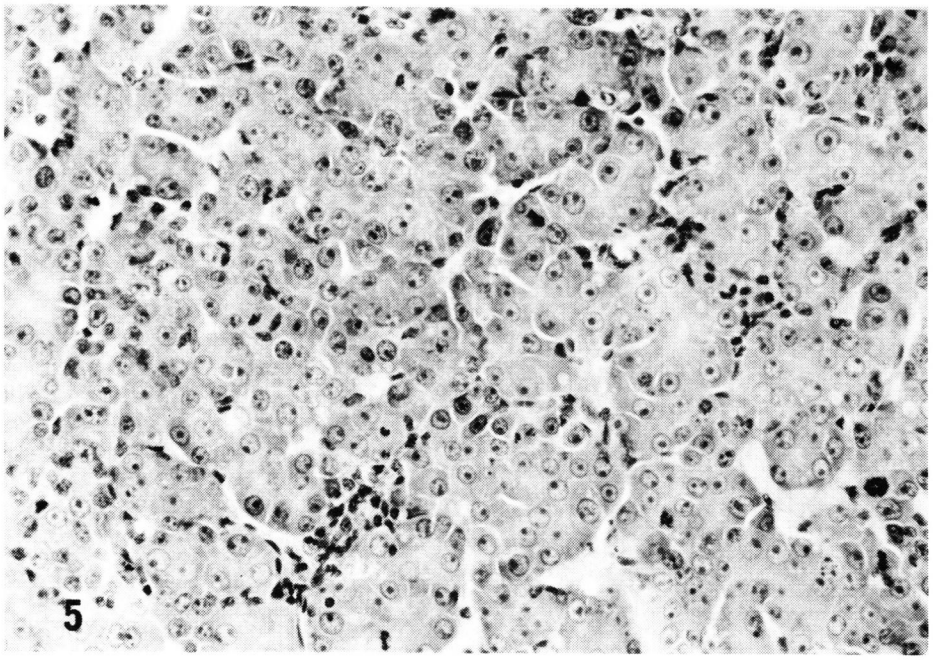
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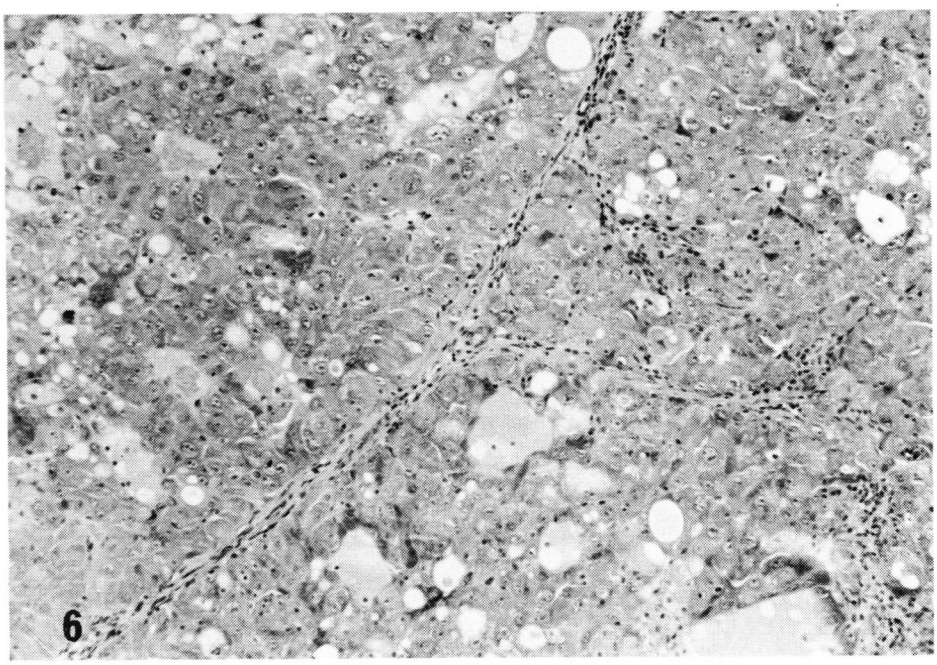
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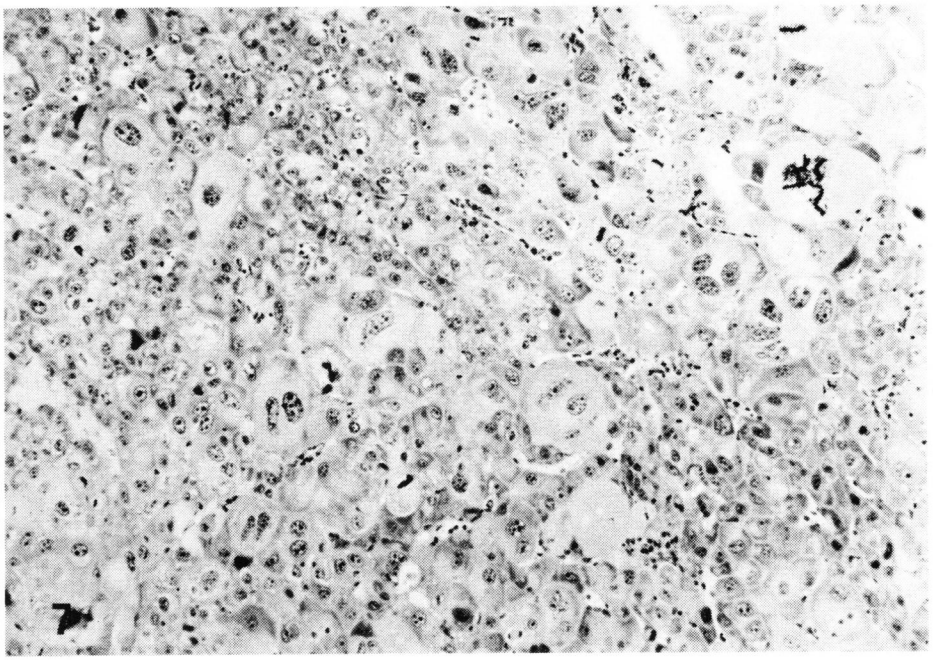
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