

Promotion by Dietary Phenobarbital of Hepatocarcinogenesis by 2-Methyl-*N,N*-dimethyl-4-aminoazobenzene in the Rat¹

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ABSTRACT

The hepatocarcinogenicity of 2-methyl-*N,N*-dimethyl-4-aminoazobenzene, previously shown to be noncarcinogenic in adult rats in the absence of further treatment, was observed by following a 1- to 6-week period of feeding this dye to weanling rats with the dietary administration of 0.05% phenobarbital for up to 70 weeks. Many large hepatocellular carcinomas developed in the phenobarbital-treated animals by 72 weeks, whereas a very small number of tiny neoplastic nodules, including one carcinoma, were seen in the rats not given this drug. This study suggests that the use of promoting agents, following the short-term administration of weak carcinogens for the liver, can be useful in demonstrating the initiating activity of such compounds. This system may be useful in the identification of such agents in the environment.

INTRODUCTION

Early studies by Miller and Baumann (9) showed that the azo dye 2-Me-DAB² was not carcinogenic when fed to adult rats for prolonged periods of time. Despite the lack of carcinogenicity of this compound, it yielded large amounts of protein-bound products in rat liver (8). In 1967, Warwick (21) confirmed that 2-Me-DAB was noncarcinogenic in adult rats, even in animals given several doses of carbon tetrachloride to stimulate cell division. However, partial hepatectomy of the rats either at the beginning of feeding of 2-Me-DAB or after 4 weeks of dye feeding resulted in the production of hepatomas in 8 of 10 animals.

Recent studies from our laboratory (12) have demonstrated that hepatocarcinogenesis by diethylnitrosamine given as a single dose within 24 hr after partial hepatectomy can be separated into 2 stages. When low doses of the carcinogen (5 to 20 mg/kg) were administered, no hepatomas occurred; this finding confirmed earlier studies by Scherer and Emmelot (16). Almost all of the animals fed phenobarbital for 6 months, commencing 2 months after the administration of the carcinogen, developed hepatocellular carcinomas. These investigations are consistent with the earlier studies of Peraino *et al.* (11) who demonstrated that the feeding of 0.02% 2-acetylaminofluorene to rats for 3 weeks produced only a very small number of hepatomas after 5 months, unless the animals were fed phenobarbital for several months after the cessation of carcinogen feed-

ing. Phenobarbital administration has likewise enhanced hepatocarcinogenesis by limited doses of 3'-methyl-*N,N*-dimethyl-4-aminoazobenzene (6).

The above studies indicated that the effects of potent hepatocarcinogens given at low doses could be "promoted" in a manner similar to the promotion of epidermal carcinogenesis (2, 3). In this paper, the promoting effect of phenobarbital is demonstrated for the weak carcinogen 2-Me-DAB.

MATERIALS AND METHODS

Materials. The basal diet (CE-2), which contained 24.5% protein, was obtained from Japan CLEA Co., Tokyo, Japan. Phenobarbital was a product of Iwaki Pharmaceutical Co., Tokyo, Japan. 2-Me-DAB was synthesized by the method of Miller and Baumann (9) and recrystallized from ethanol (m.p. 64-66°).

Rats and Feeding Protocol. Male Donryu rats 21 days of age were housed (5/cage) in an air-conditioned room in wire mesh cages. They were fed a diet containing 0.06% 2-Me-DAB for the time periods indicated in the tables and were then fed the basal diet for 2 weeks. The rats were then either fed the basal diet containing 0.05% phenobarbital or kept on the basal diet for the remainder of the experiment. Some rats were killed for histochemical studies at 12 and 24 weeks, and the remainder were killed at 72 weeks.

Histochemistry. Frozen sections of liver tissue were prepared in a cryostat and stained for ATPase by the method of Wachstein and Meisel (20). For each liver, the number and size of ATPase-deficient islands larger than 50 μ m in diameter were scored from several sections totaling about 5 sq cm. Routine histological examination of the tissues was carried out after fixation in 10% formalin, embedding in paraffin, sectioning, and staining with hematoxylin and eosin.

RESULTS

Previous studies by Pitot *et al.* (12) have demonstrated that the administration of 0.05% phenobarbital in the diet for periods up to 6 months did not produce islands of enzyme-altered cells in the livers of otherwise untreated rats. Inasmuch as these enzyme-altered islands have now been shown to be produced by the feeding of a variety of hepatocarcinogens, including diethylnitrosamine (16), 3'-methyl-*N,N*-dimethyl-4-aminoazobenzene (6), 2-acetylaminofluorene (13), and *N*-nitrosomorpholine (5), compounds producing similar effects may be considered as potentially hepatocarcinogenic. As seen from the data in Table 1, the administration of 2-Me-DAB in the diet of rats for either 3 or

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² The abbreviation used is: 2-Me-DAB, 2-methyl-*N,N*-dimethyl-4-aminoazobenzene.

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6 weeks resulted in the eventual appearance in the liver of significant numbers of foci that were distinguishable histochemically from the surrounding normal liver by a deficiency of ATPase (Fig. 1). However, in contrast to the 5- to 10-fold increase in the number of enzyme-altered islands observed on administration of phenobarbital for only 6 months to 3-month-old rats pretreated with a single, non-carcinogenic dose of diethylnitrosamine (12), phenobarbital administration did not increase significantly the number of these foci in the livers of weanling rats pretreated with 2-Me-DAB (Table 1). The number of these enzyme-altered foci was relatively low at 12 and 24 weeks but increased severalfold by 72 weeks.

In contrast to the above finding, the number of hepatocellular carcinomas in rats fed 2-Me-DAB for 1 to 6 weeks was far greater in those animals that subsequently received phenobarbital than in those not receiving this drug (Table 2). In the groups fed 2-Me-DAB for either 3 or 6 weeks prior to the administration of phenobarbital for up to 72 weeks, 10 of 17 animals had one or more carcinomas, many of which measured larger than 10 mm in diameter. In contrast, only 1 of 13 rats that received the dye for 3 or 6 weeks without subsequent administration of phenobarbital showed a histologically confirmed carcinoma. One of the animals in Group 3 died of leukemia at the 65th week.

Histology. Fig. 2 shows the histology of a tumor that was diagnosed as a nonmalignant neoplastic nodule. In Fig. 3 is

seen a gross photograph of a large hepatocellular carcinoma with a diameter of 55 mm and which developed in a rat fed 2-Me-DAB for 6 weeks and then maintained on the 0.05% phenobarbital diet until the end of the 72nd week. The high degree of differentiation of this neoplasm is seen in Fig. 4. Most hepatocellular carcinomas produced in the rats fed 2-Me-DAB and phenobarbital sequentially exhibited a high degree of differentiation which either was of the type seen in Fig. 4 or showed a glandular or pseudoacinar pattern (Fig. 5). The single small hepatocellular carcinoma that developed in a rat fed 2-Me-DAB for 6 weeks and then maintained on the basal diet until the end of the 72nd week is seen in Fig. 6. This neoplasm resembles that seen in Fig. 4, but it also exhibits some lipid accumulation within the tumor cells.

DISCUSSION

2-Me-DAB, like its structurally related carcinogenic aminoazo dyes, binds covalently to proteins, DNA, and RNA of rat liver (4, 8, 21). The levels of the DNA- and RNA-bound derivatives of 2-Me-DAB are similar to or somewhat lower than those observed for rats treated with *N,N*-dimethyl-4-aminoazobenzene, whereas the levels of the protein-bound derivatives are higher and take longer to reach the maximum level. The demonstration by Warwick (21) that this dye was hepatocarcinogenic when the rats were also subjected to partial hepatectomies suggested that proliferation of

Table 1
Number of enzyme-altered islands/sq cm in livers of rats fed 2-Me-DAB with or without subsequent administration of phenobarbital

Group	Administration of 2-ME-DAB (wk)	Phenobarbital ^a	No. of islands/sq cm liver section at		
			12 wk ^b	24 wk	72 wk
3	3	+	0.95 ± 0.9 ^c (7) ^d	1.1 ± 0.3 (7)	9.2 ± 3.9 (6)
4	3	-	0.70 ± 0.7 (7)	1.0 ± 0.9 (7)	4.0 ± 2.0 (5)
5	6	+			15.9 ± 10.0 (9)
6	6	-			8.0 ± 4.7 (6)

^a Groups denoted by + in this column were fed 0.05% phenobarbital as described in "Materials and Methods."

^b Number of weeks from start of experiment.

^c Mean ± S.D.

^d Numbers in parentheses, the number of animals studied.

Table 2
Number of tumors larger than 2 mm in diameter found at 72 weeks in rats fed 2-Me-DAB with or without subsequent administration of phenobarbital

Group	Administration of 2-Me-DAB (wk)	Phenobarbital ^a	Effective no. of rats ^b	No. of rats with tumors ^c (carcinomas)	No. of tumors ^c with the following diameters				Total no. of tumors ^c (carcinomas)	No. of tumors/rat
					2-4 (mm)	5-9 (mm)	10-20 (mm)	20+ (mm)		
1	1	+	4	0	0	0	0	0	0	
2	2	+	2	1 (0) ^d	6	0	0	0	6 (0) ^e	3.0
3	3	+	7	7 (3)	14	2	4	1	21 (7)	3.0
4	3	-	5	1 (0)	2	1	0	0	3 (0)	0.6
5	6	+	10	8 (7)	10	8	5	2	25 (9)	2.5
6	6	-	8	2 (1)	1	1	0	0	2 (1)	0.25

^a Groups denoted by + were fed 0.05% phenobarbital as described in "Materials and Methods."

^b Number of rats killed at 72 weeks.

^c The term "tumor" refers to all grossly identifiable masses in the liver, other than normal structures. Those tumors termed carcinomas were histologically compatible with that diagnosis. The tumors not classified as carcinomas exhibited the histopathology of neoplastic nodules (19).

^d Numbers in parentheses, number of rats with grossly overt carcinomas.

^e Numbers in parentheses, number of carcinomas.

hepatic cells might "fix" the transformation event (15) to yield initiated cells. In our experiments, the "fixation" of the 2-Me-DAB-induced damage to give initiated cells may also have been facilitated by the proliferation of the hepatocytes, inasmuch as the rats were started on the regimen at weaning. Other investigators (17) have demonstrated that weanling rats are more susceptible to carcinogenesis by chemicals than are older animals. The initiated cells are presumed to be precursors of the enzyme-altered foci (12).

In view of the mounting evidence for a 2-stage process of hepatocarcinogenesis in rats (7, 10-12, 16, 18), the production of hepatic tumors by a short-term feeding of 2-Me-DAB and the subsequent administration of phenobarbital demonstrates that initiating agents with little, if any, promoting capacity exist for liver as they do for skin carcinogenesis (1, 3). It should be noted, however, that it is not clear whether the enhancing effect of phenobarbital for hepatocarcinogenesis is strictly analogous to tumor promotion in mouse skin. Promoting agents for skin tumorigenesis (e.g., tetradecanoylphorbol acetate) induce extensive hyperplasia of the epidermis, whereas phenobarbital does not have a quantitatively similar effect on liver cells (10). Phenobarbital induces only a transient and relatively small increase in DNA synthesis in liver (10).

As with the enhancement of 3'-methyl-*N,N*-dimethyl-4-aminoazobenzene hepatocarcinogenesis by phenobarbital (6), the feeding of the latter compound after 2-Me-DAB administration appeared to cause an acceleration of cancer formation rather than an increase in the number of enzyme-altered foci, which are the putative precursors of hepatocellular carcinomas (12, 16). This was evident from the lack of a significant increase in the number of enzyme-altered foci in the livers of rats fed phenobarbital following the administration of 2-Me-DAB compared with the number in the livers of rats given only 2-Me-DAB (Table 1). Although administration of phenobarbital to otherwise untreated rats for 78 to 150 weeks has been reported to induce neoplastic nodules in the livers of Wistar rats (14), no neoplasms or enzyme-altered foci were found in livers of rats fed phenobarbital for up to 70 weeks under the conditions of the experiments reported here and in earlier studies from our laboratory (12).

These data suggest that the administration of promoting agents after the short-term administration of weak hepatocarcinogens can be useful in demonstrating the initiating activity of the latter agents. This phenomenon may be useful in the identification of such agents in the environment.

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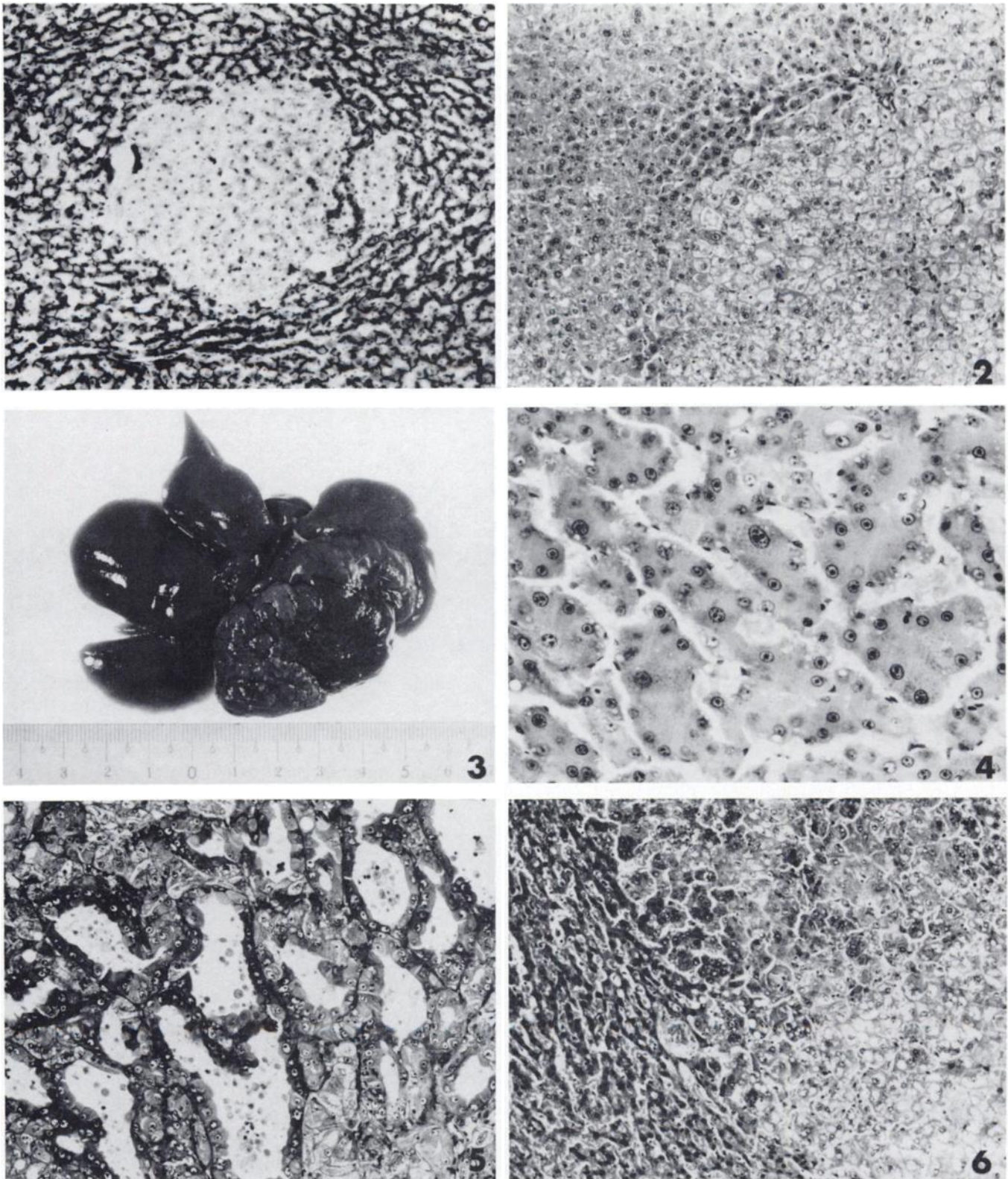


Fig. 1. An ATPase-deficient island in the liver of a rat in Group 5, which was killed at the 72nd week. Stained for ATPase activity, counterstained for nuclei with hematoxylin. $\times 120$

Fig. 2. A part of a neoplastic nodule that was considered nonmalignant from a rat of Group 5; it was killed at the 72nd week. H & E, $\times 120$.

Fig. 3. A gross photograph of a liver from a rat of Group 5 killed at the 72nd week. Note the large hepatocellular carcinoma 55 mm in diameter.

Fig. 4. Histology of the well-differentiated hepatocellular carcinoma shown in Fig. 3. H & E, $\times 240$.

Fig. 5. A well-differentiated hepatocellular carcinoma with a glandular pattern that developed in a rat of Group 5 by 72 weeks. H & E, $\times 120$.

Fig. 6. A well-differentiated hepatocellular carcinoma, 7 mm in diameter, from a rat of Group 6 that was killed at the 72nd week. H & E, $\times 120$.