Environmental Health Perspectives
Vol. 50, pp. 131-138, 1983

Comparison of the Promoting Effects of Various Agents in Induction of Preneoplastic Lesions in Rat Liver

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The promoting activities of 29 compounds on induction of hyperplastic (neoplastic) liver nodules (HN) and the dose-dependent effects of tumor promoting agents were compared by using a short-term test system developed in this laboratory.

In tests on promoting activity, F344 rats were given a single dose (200 mg/kg) of *N*-nitrosodiethylamine (DEN), and from two weeks later, were treated with various test compounds for 6 or 10 weeks. They were subjected to partial hepatectomy 3 or 4 weeks after DEN treatment. The results showed that strong hepatocarcinogens, such as aflatoxin B_1, DEN, *N*-nitrosodimethylamine (DMN), 2-acetylaminofluorene (2-AAF), 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) and ethionine, induced many hyperplastic liver nodules, whereas dieldrin, 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), polychlorinated biphenyls (PCB) and α-hexachlorocyclohexane (α-HCH) induced few lesions. Nonhepatocarcinogens, such as N-nitrosoethylurea (ENU) and 3-methylcholanthrene (3-MC), only slightly induced hyperplastic nodules. Of the miscellaneous compounds tested, phenobarbital, deoxycholic acid and ethynyl estradiol also induced γ-glutamyl transpeptidase (γ-GT) positive foci.

In tests on the dose-dependent effects of promoting agents, DMN was given at different concentrations for 6 weeks from 2 weeks after DEN treatment. Results were quantitated by histochemical measurement of the number or area of γ-GT positive lesions induced. A long-term experiment on the effect of feeding DMN for 96 weeks was also done. Clear dose-dependent effects of DMN were seen in induction of γ-GT positive foci in the short-term experiment and neoplastic lesions in the long-term one.

Introduction

Since the proposal of a two-stage process of chemical carcinogenesis in mouse skin, extensive investigations have been carried out to understand the mechanism of the initiation-promotion process (*1*). Initiation is thought to introduce neoplastic information into the cells, possibly by some alteration of cellular DNA, whereas treatment with a promoter given continuously over a relatively long period is thought to allow phenotypic expression of the altered genome eventually leading to neoplastic growth. This two-stage process of carcinogenesis was also demonstrated more recently in liver carcinogenesis with 2-acetylaminofluorene (2-AAF) as an initiator and phenobarbital as a promoter (*2,3*). A similar two-stage mechanism has been proposed for carcinogenesis in urinary bladder (*4-6*), mammary gland (*7*), thyroid gland (*8*) and intestines (*9*).

The actual risk to humans of chemicals in the environment is usually the result of multiple exposures to a wide variety of agents, including carcinogens and promoters, and there is increasing evidence that these compound infact contribute to the induction of human cancer. However, it is difficult to test all possible factors in long-term *in vivo* experiments, and no suitable *in vitro* short-term test for promoting agents has yet been reported. Thus a short-term *in vivo* assay for promoting agents is urgently needed. Previous studies on this problem in this laboratory indicated that when rats were pretreated with *N*-nitrosodiethylamine (DEN) and then exposed to various promoting agents for 6 to 10 weeks, quantitatively detectable numbers of hyper-

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plastic (neoplastic) nodules (HN) were formed in their liver (*10*). The HN measured in this experiment included hyperplastic foci (areas of cellular alterations) and hyperplastic nodules (neoplastic nodules) of more than 0.2 mm diameter, as previously described (*10*). The aims of this study were to observe the sequence in which hepatocellular carcinomas develop from early preneoplastic lesions and to define HN; to study the dose-dependent effects in long-term experiments for carcinogenicity and in short-term tests for promoting activity on the incidence of neoplastic and preneoplastic lesions, and to compare the activities of various compounds as promoters of preneoplastic liver lesions in the short-term model system.

Hyperplastic (Neoplastic) Nodules in the Liver of Rats Treated with DEN followed by 2-AAF or α-HCH

The behavior and fate of hyperplastic nodules (HN) induced in an *in vivo* short-term screening test for hepatocarcinogens were studied (*11*). Fischer 344 rats were injected intraperitoneally with DEN (200 mg/kg body weight) and fed basal diet for first 2 weeks, followed by basal diet containing 2-AAF (200 ppm) or α-hexachlorocyclohexane (α-HCH) (1000 ppm) for 6 weeks and again placed on basal diet until week 50. Two-thirds partial hepatectomy was performed at the end of week 3. Animals were killed in week 4, 6, 8, 10, 20, 30, 40 or 50 of the experiment.

The numbers and areas of HN per unit area in microscopic sections of liver are shown in Figures 1 and 2, respectively. In the liver of rats treated with 2-AAF, the number and area of HN were maximal in week 10, and then gradually decreased to week 50 ($p<0.001$). However, the area remained almost constant from week 30 to 50. In the group given α-HCH, the number and area of HN gradually increased throughout the experiment. The decrease of HN in 2-AAF treated rats after week 10 was apparently due to their degeneration or disappearance. Therefore, the numbers and areas of degenerated or necrotic nodules which had a spongy or cystic appearance were also measured. The histological features of the lesions, which were diagnosed as degenerated hyperplastic nodules (DHN), were similar to those seen in spongiosis hepatis reported by Bannasch et al. (*12*). The number and area (not shown) of these degenerated hyperplastic nodules (DHN) increased with time until week 30 and then decreased or remained almost constant in rats given 2-AAF or α-HCH (Fig. 3). The maximal number of hepatocellular carcinoma was reached at week 40 (Fig. 4).

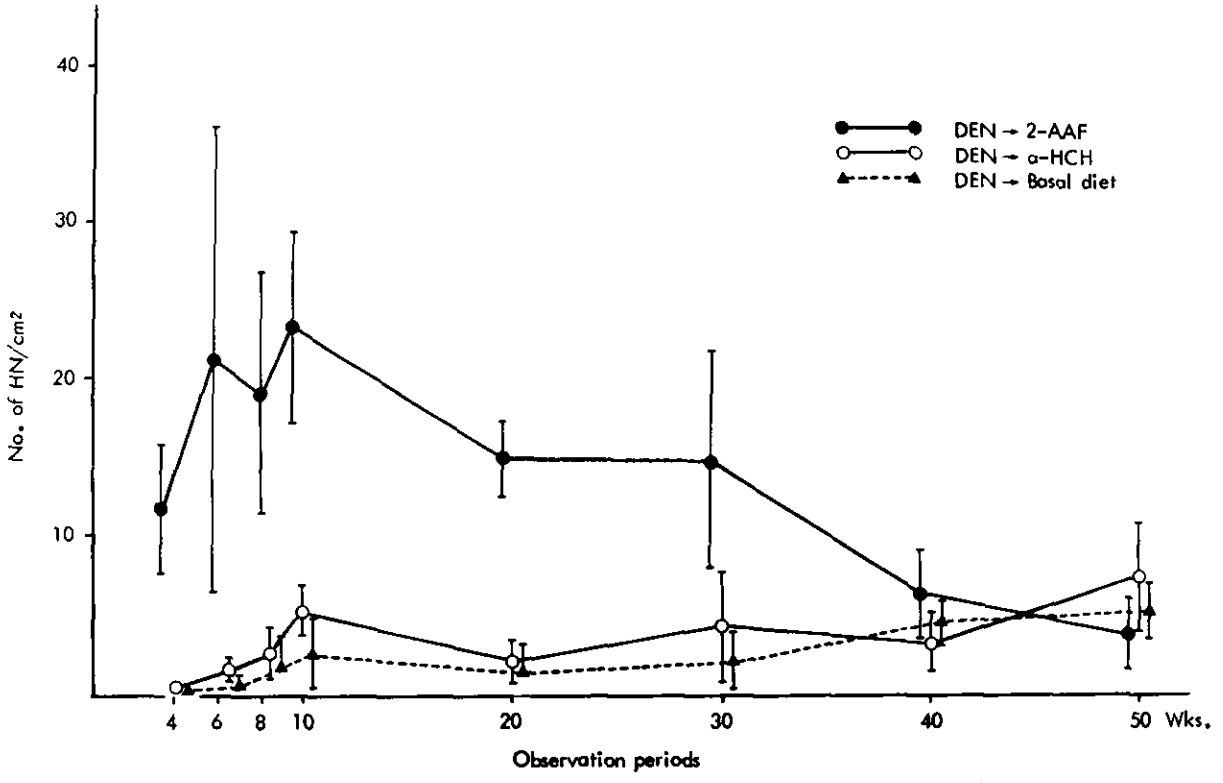

FIGURE 1. Sequential changes in the number of hyperplastic (neoplastic) nodules (HN).

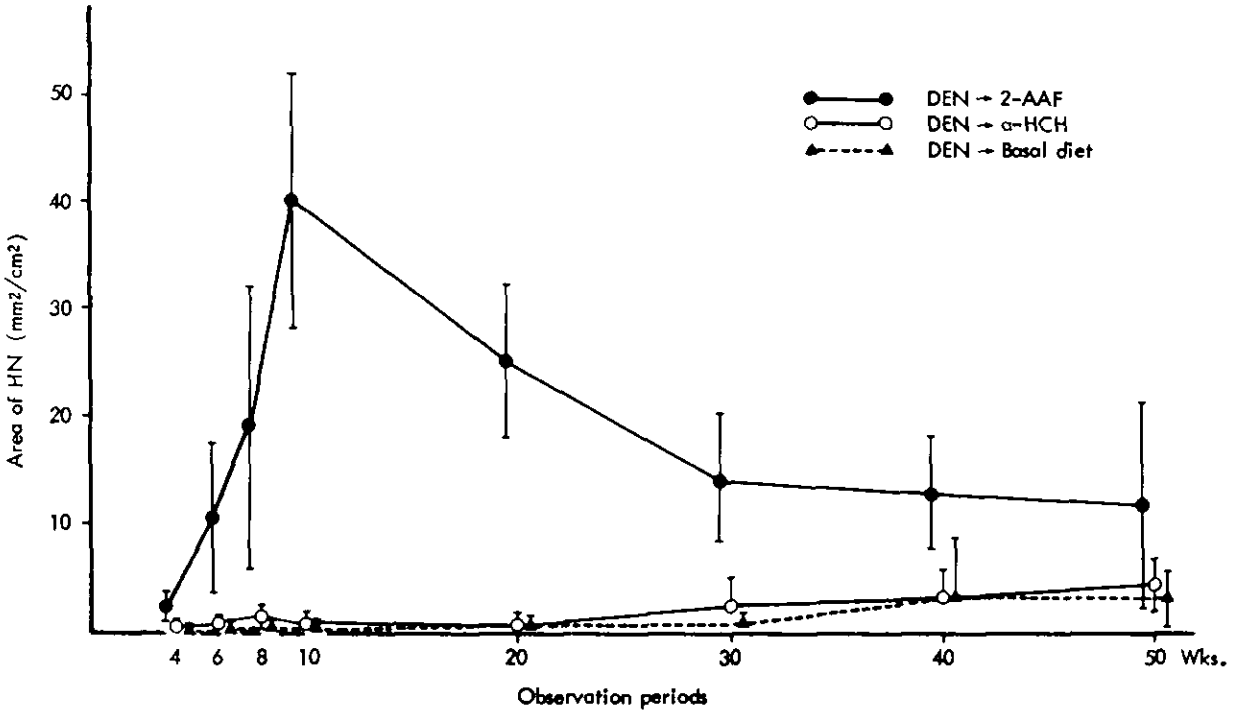

FIGURE 2. Sequential changes in the area of hyperplastic (neoplastic) nodules (HN).

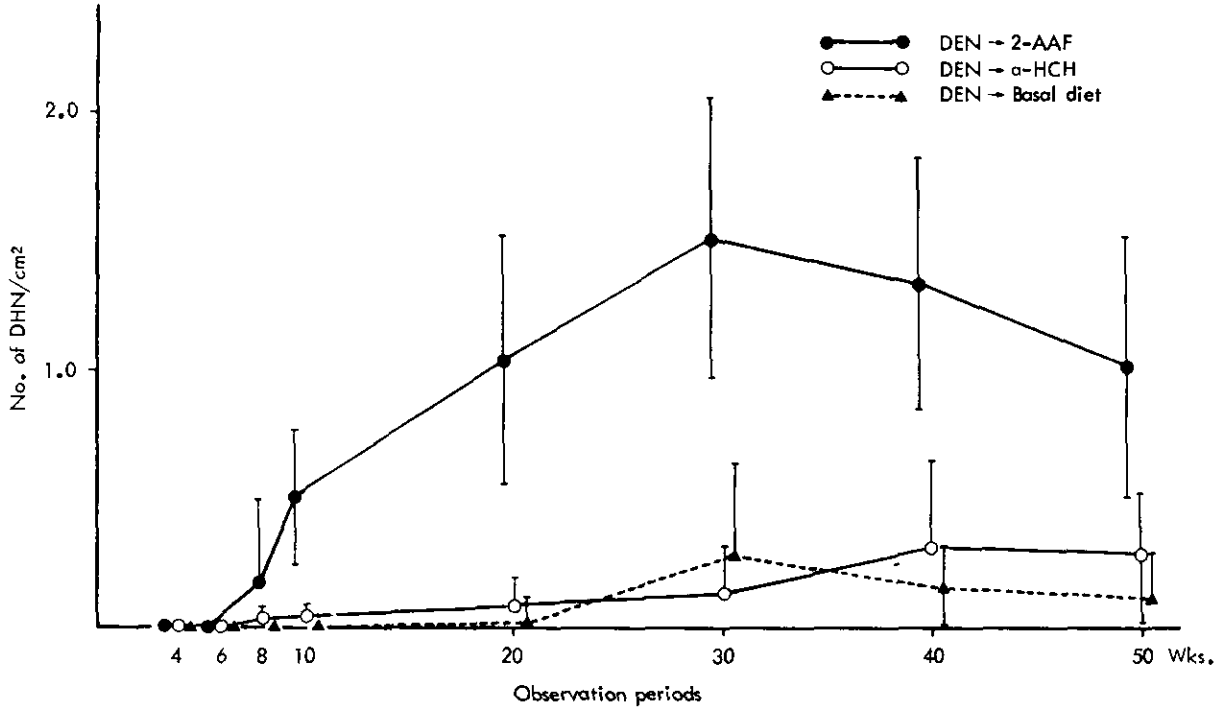

FIGURE 3. Sequential changes in the number of degenerated hyperplastic nodules (DHN).

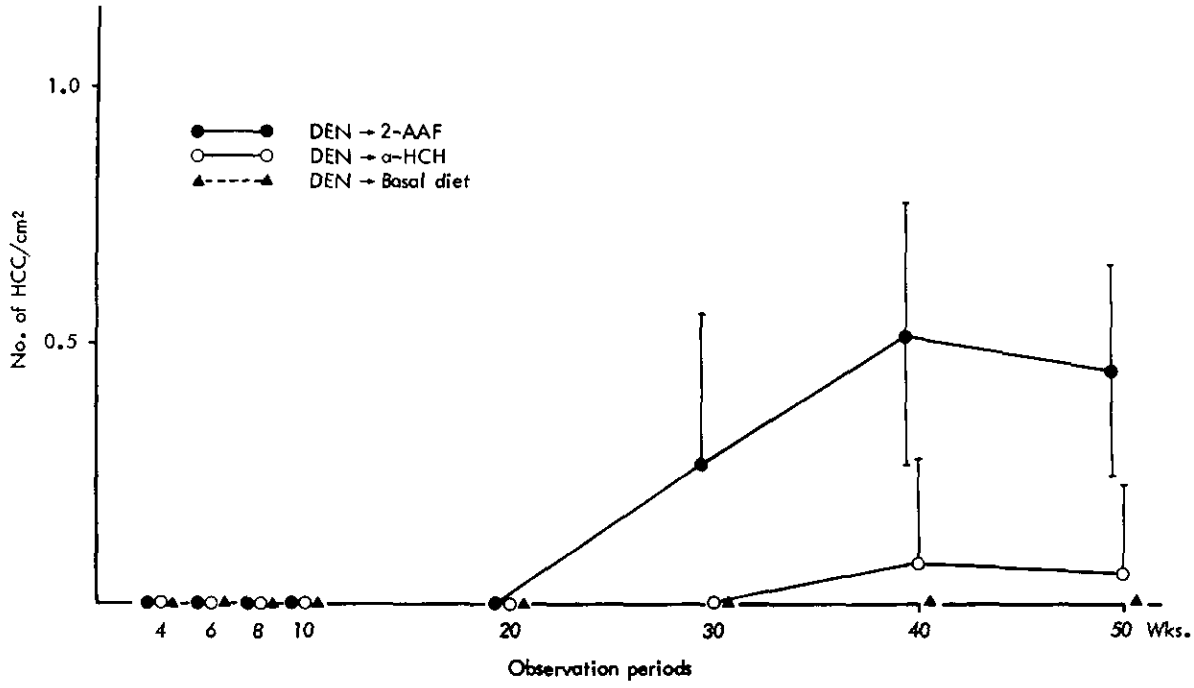

FIGURE 4. Sequential changes in the number of hepatocellular carcinomas (HCC).

This experiment shows that detectable preneoplastic lesions in the liver, measured as HN, were induced in DEN-treated rats by exposure to promoting agents for 6 weeks in combination with partial hepatectomy. Their number and area started to decrease 2 weeks after the end of exposure to promoting agents (10 weeks after DEN) due to degenerative change.

Dose-Dependent Effects of DEN in Induction of Preneoplastic Lesions of Rat Liver

N-Nitrosodimethylamine (DMN) was given at concentrations of 10.0, 1.0 and 0.1 ppm in the diet for 6 weeks after initiation with DEN to test its dose-dependent effect in promotion (Fig. 5). In this experiment, the number and area of preneoplastic liver lesions giving a positive reaction for γ-glutamyltranspeptidase (γ-GT) were measured (*13*). A long-term experiment in which DMN was given continuously in the diet for 96 weeks at the same concentrations as in the short-term test (10.0, 1.0 and 0.1 ppm) was also carried out to examine whether the incidence of γ-GT positive foci in the short-term experiment was comparable to that of HN in the long-term experiment. A significant dose-dependent response in

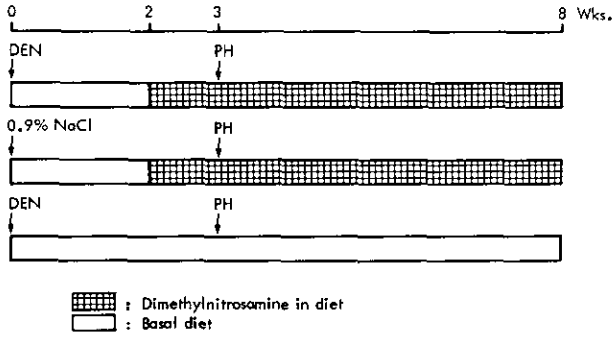

FIGURE 5. Experimental design of short-term test for promoting activity as determined by an enhancement of hyperplastic (neoplastic) nodules or γ-GT positive foci.

the induction of γ-GT positive foci in the short-term test and also HN or malignant neoplasias in the long-term test were shown among the higher range of doses (*14*). In these experiments the results of the short-term experiment clearly reflected those of the long-term one (Tables 1 and 2). Similarly, a clear dose-dependent effect on the enhancement of HN induction was seen with different doses of phenobarbital (*15,16*). Since most γ-GT positive cell foci develop into HN (*17*), this system for detecting preneoplastic changes seems suitable for analytical studies on promoters or factors that enhance chemical carcinogenesis.

Table 1. Dose-dependent effect of short-term treatment with *N*-nitrosodimethylamine (DMN) in induction of γ-glutamyl transpeptidase (γ-GT) positive foci in rats after initiation by *N*-nitrosodiethylamine (DEN).

Treatment with DEN	Dose of DMN, ppm	No. of animals	γ-GT positive foci in liver	
			No./cm²	Area, mm²/cm²
+	10.0	20	10.3 ± 2.9*	0.30 ± 0.1*
+	1.0	20	6.1 ± 1.6*	0.23 ± 0.1
+	0.1	19	4.1 ± 1.1	0.14 ± 0.1
+	0	21	4.0 ± 1.1	0.14 ± 0.1
−	10.0	17	<0.2	<0.01

* Significantly different from other groups.

Table 2. Dose-dependent effect in induction of liver tumors in rats treated with *N*-nitrosodimethylamine (DMN) for 96 weeks.

Dose of DMN, ppm	No. of rats	Changes in liver, %			
		Hyperplastic nodule	Hepatocellular carcinoma	Hemangio-endothelioma	Fibro-sarcoma
10.0	17	6 (35)*	1 (6)	3 (18)*	5 (29)*
1.0	15	1 (1)	1 (7)	0 —	1 (7)
0.1	9	0 —	0 —	0 —	0 —
0	7	0 —	0 —	0 —	0 —

* Significantly different from other groups.

Table 3. Quantitation of hyperplastic (neoplastic) nodules induced by test compounds in rat liver after initiation by *N*-nitrosodiethylamine (DEN).

Compound	Dose, ppm	Route[a]	Hyperplastic (neoplastic) nodules[b]			
			No./cm²		Area, mm²/cm²	
			DEN	Saline	DEN	Saline
Chlorobenzilate	3000	D	3.3 ± 1.2* (18)	0 (8)	1.0 ± 0.4*	0
Ethionine	2500	D	3.4 ± 1.3* (13)	0 (10)	1.0 ± 0.4*	<0.1
Quinoline	2500	D	1.2 ± 0.6 (14)	0 (9)	0.3 ± 0.2*	0
Thioacetamide	1000	D	10.8 ± 4.2* (12)	0.6 ± 0.4 (9)	6.6 ± 3.0*	0.1 ± 0.1
α-HCH	1000	D	3.3 ± 1.4* (12)	0 (8)	2.0 ± 0.8*	0
PCB	1000	D	1.4 ± 0.6 (12)	0 (8)	0.5 ± 0.3*	0
3′-Me-DAB	600	D	15.9 ± 4.8* (17)	1.9 ± 1.1 (9)	10.9 ± 5.5*	0.5 ± 0.3
DDT	500	D	3.6 ± 1.3* (13)	0 (8)	1.2 ± 0.5*	0
2-AAF	200	D	24.3 ± 7.6* (14)	10.0 ± 5.0 (9)	17.1 ± 5.4*	4.3 ± 3.6
Sterigmatocystin	120	D	7.8 ± 3.5* (13)	0.1 ± 0.2 (9)	3.7 ± 1.9*	<0.1
DEN	100	W	28.7 ± 16.1* (17)	16.7 ± 7.4 (10)	17.2 ± 9.6*	3.6 ± 2.9
DMN	100	D	9.5 ± 2.1* (17)	0.2 ± 0.3 (9)	5.7 ± 1.3*	<0.1
Dieldrin	100	D	2.7 ± 0.1* (13)	0 (8)	0.8 ± 0.4*	0
Hexachlorobenzene	50	D	3.2 ± 1.7* (21)	0 (10)	0.7 ± 0.4*	0
Aldrin	50	D	2.5 ± 1.3* (18)	0 (8)	0.5 ± 0.3*	0
Aflatoxin B_1	2	D	31.6 ± 7.4* (12)	3.5 ± 1.0 (9)	10.3 ± 5.5*	1.0 ± 0.3
Control	—	—	1.5 ± 1.2 (34)	—	0	—

[a] Route: D = in diet; W = in drinking water.
[b] Numbers in parentheses denote numbers of animals.
* Significantly different from control.

Effects of Exogenous and Endogenous Promoting Agents on Induction of Preneoplastic Lesions

In tests on the promoting activities of various compounds, rats were injected intraperitoneally with DEN or saline (control), given test compounds by an appropriate route for 6 weeks and then killed (Fig. 5). One week after the start of treatment with test compounds, a two-thirds partial hepatectomy was performed to stimulate cell proliferation (*18*). The doses and routes of administration of test compounds are shown in Tables 3-6. In tests on hormones and bile acids, a modified experimental schedule was used: test compounds were given for 10 weeks and partial hepatectomy was performed in week 4 (Fig. 5). Doses of chemicals were chosen on the basis of their LD_{50} values (*19*).

Results show that administration of strong hepatocarcinogens, such as aflatoxin B_1, DEN, DMN, 2-AAF, 3′-methyl-4-dimethylaminoazobenzene (3′-Me-

Table 4. Quantitation of hyperplastic (neoplastic) nodules induced by test compounds in rat liver after initiation by *N*-nitrosodiethylamine (DEN).

Compound[a]	Dose, ppm	Route[b]	Hyperplastic (neoplastic) nodules[c] No./cm² DEN	No./cm² Saline	Area, mm²/cm² DEN	Area, mm²/cm² Saline
BBN	1800	W	2.0 ± 1.4 (14)	0 (10)	0.2 ± 0.2	0
ENNG	20 mg × 7	P	1.1 ± 0.9 (15)	—	0.1 ± 0.1	0
MNNG	400	W	1.6 ± 0.7 (15)	9 (9)	0.2 ± 0.1	0
ENU	300	W	2.5 ± 1.1* (14)	0 (10)	0.4 ± 0.1*	0
BNU	20 mg × 7	P	1.2 ± 1.2 (12)	—	0.1 ± 0.1	0
3-MC	100	D	1.7 ± 1.0* (14)	0 (10)	0.7 ± 0.2*	0
7,12-DMBA	30 mg × 7	G	1.1 ± 1.1 (11)	—	0.1 ± 0.1	0
B(a)P	30 mg × 7	G	1.0 ± 0.6 (12)	—	0.1 ± 0.1	0
Amitrole	15000	D	3.6 ± 2.4* (15)	—	0.8 ± 0.7*	0
Control	—	—	1.5 ± 1.2 (34)	—	0	—

[a] Compounds: BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; ENNG, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; ENU, *N*-ethylnitrosourea; BNU, *N*-nitrosobutylurea, 7,12-DMBA, 7,12-dimethylbenz(a)anthracene; B(a)P, benzo(a)pyrene.
[b] Route: W = in drinking water; P = intraperitoneal injection; D = in diet; G = intragastric feeding.
[c] Numbers in parentheses denote numbers of animals.
* Significantly different from control.

Table 5. Quantitation of hyperplastic (neoplastic) nodules induced by test compounds in rat liver after initiation by *N*-nitrosodiethylamine (DEN).

Compound	Dose, ppm	Route[a]	Hyperplastic (neoplastic) nodules[b] No./cm² DEN	No./cm² Saline	Area, mm²/cm² DEN	Area, mm²/cm² Saline
Quercetin	50000	D	1.7 ± 0.8 (12)	0 (10)	0.3 ± 0.3	0
Caffeine	1000	W	1.6 ± 1.0 (20)	0 (10)	0.2 ± 0.1	0
BHT	1000	D	1.0 ± 1.2 (14)	0 (9)	0.1 ± 0.1	0
Endrin	25	D	1.8 ± 0.8 (16)	0 (8)	0.3 ± 0.2	0
Saccharin	50000	D	1.4 ± 1.3 (18)	0 (10)	0.2 ± 0.1	0
Phenobarbital	500	D	3.1 ± 2.3* (15)	0 (10)	1.0 ± 0.7	0
Control	—	—	1.5 ± 1.2 (34)	—	0	—

[a] Route: D = in diet; W = in drinking water.
[b] Numbers in parentheses denote numbers of animals.
* Significantly different from control.

Table 6. Quantitation of γ-glutamyl transpeptidase-positive foci induced by test compounds in the rat liver after initiation by *N*-nitrosodiethylamine (DEN).

Compound	Dose, ppm	Route[a]	γ-GT positive foci[b] No./cm² DEN	No./cm² Saline	Area, mm²/cm² DEN	Area, mm²/cm² Saline
Ethynyl estradiol	10	D	7.8 ± 2.1* (14)	0 (12)	1.5 ± 1.5*	0
Dexamethasone	0.5	D	5.6 ± 1.8* (11)	0 (13)	0.2 ± 0.1*	0
Testosterone	500	D	5.3 ± 1.3* (15)	0 (14)	0.2 ± 0.1*	0
Cortisone	10	D	3.8 ± 1.5* (15)	0 (12)	0.2 ± 0.1	0
Deoxycholic acid	5000	D	23.8 ± 9.8* (14)	0.5 ± 0.5 (7)	12.7 ± 11.2*	0.1 ± 0.1
Taurine	5000	D	3.9 ± 1.4* (12)	0 (15)	0.1 ± 0.0	0
Lithocholic acid	5000	D	3.5 ± 1.1* (14)	0 (15)	0.3 ± 1.1	0
Phenobarbital	500	D	14.4 ± 2.6* (11)	0 (10)	1.3 ± 0.5	0
Control	—	D	2.2 ± 0.9 (19)	— ( )	0.1 ± 0.1	—

[a] Route: D = in diet.
[b] Numbers in parentheses denote numbers of animals.
* Significantly different from control.

DAB), thioacetamide, sterigmatocystin and ethionine, resulted in large numbers of HN, whereas dieldrin, DDT, polychorinated biphenyls (PCB) and α-HCH had less promoting activity. Nonhepatocarcinogens, such as *N*-ethylnitroso urea (ENU), 3-methylcholanthrene (3-MO) and aminotriazole (amitrole) also caused slight induction of HN. Of the miscellaneous compounds tested, only phenobarbital caused appreciable HN induction (Tables 3-5).

Among the hormones and bile acids tested by the modified schedule, ethynyl estradiol, dexamethasone, testosterone and deoxycholic acid greatly increased both the number and area of γ-GT positive foci (Table 6). In this experiment, γ-GT positive foci were measured quantitatively becasue they appear earlier than HN and therefore their measurement is more sensitive than that of HN.

Hepatocarcinogens and chemicals with promoting activity in liver carcinogenesis gave positive results in this system. Liver carcinogens had strong promoting effects, and some nonliver carcinogens had weak promoting effects. Thus our system could be used for screening for hepatocarcinogens or promoters of hepatocarcinogenesis as epigenetic factors and also for estimating their potency.

Conclusions

The present work shows that detectable preneoplastic lesions are induced by short-term exposure (6-10 weeks) to promoting agents in rats initiated by DEN. Their promoting effect of DMN is dose-dependent in both short- and long-term tests. There was a good correlation between quantitative values for early preneoplastic lesions (γ-GT positive foci) and the incidences of the more advanced lesions, such as of hyperplastic (neoplastic) nodules and hepatocellular carcinomas.

Strong liver carcinogens were shown to have strong promoting effects. Thus various tumor promoting agents can be detected and their potencies can be measured with the system developed in this study. Figure 6 shows diagrammatically possible relationships between different neoplastic stages and their modifying factors. When the initiation is strong and proliferation of initiated cells is rapid, the patient will die of cancer before completing lifespan. However, when the proliferation is slow, the lifespan is completed before cancer develops; in other words, the patient does not die of cancer. The effect of promoter is thought to shorten the period of early or preneoplastic stage by increasing the angle of aslant lines in the diagram. If we could detect some compound with an effect to decrease this angle, the compound would be used as an antipromoter.

This investigation was supported in part by a grant-in-aid for cancer research from the Ministry of Education, Science and Culture of Japan, a grant-in-aid for cancer research from the Ministry of Health and Welfare of Japan, and grants-in-aid for medical research from Meihoku Labor Standard Association, Japan, the Japan Tobacco and Salt Public Corporation, Toyota Foundation, and Nissan Science Foundation.

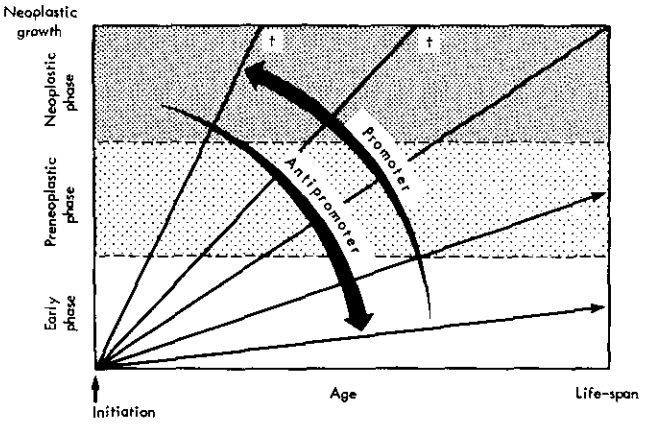

FIGURE 6. Diagrammatic representation of probable relationships between neoplastic growth and modifying factors. The vertical axis shows different stages of neoplastic development and the horizontal axis shows the time. The effect of promoter is thought to shorten the period of early or preneoplastic stage by increasing the angle of aslant lines, with eventual earlier development of cancer before completing life span.

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