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Modulation of Diethylnitrosamine Carcinogenesis in Rat Liver and Oesophagus

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Abstract A series of 16 experiments, using a total of 2,000 BD_6 rats, was designed in order to assess the ability of 8 individual agents or their combinations to modulate the liver and oesophageal carcinogenesis induced by multiple doses of diethylnitrosamine (DEN). Of the antioxidants tested, sodium selenite, ascorbic acid, and butylated hydroxytoluene generally exhibited protective effects on both types of tumors. In contrast, retinoic acid behaved as a promoter of DEN hepatocarcinogenesis, but this effect could be eliminated by its combination with either selenite or butylated hydroxytoluene. Caffeine and theophylline, when individually assayed, were devoid of significant protective effects, and the latter methylxanthine stimulated oesophageal tumorigenesis when administered after exposure to the carcinogen. Caffeine tended to decrease the multiplicity of liver tumors and potentiated the inhibitory effect of selenite in the liver. Irrespective of combination with caffeine, treatment with phenobarbital before each DEN injection tended to reduce the multiplicity of both liver and oesophageal tumors. On the other hand, the metabolic inhibitor diethyldithiocarbamate, given after each DEN injection, dramatically enhanced the incidence and multiplicity of oesophageal tumors. Thus, on the whole, modulation of DEN carcinogenesis varied depending on test agents, their combinations, dosages, treatment schedules, and target organ.

Key words: antioxidants, diethylnitrosamine, liver tumors, methylxanthines, modulation of carcinogenesis, modifiers of metabolism, oesophageal tumors.

The primary prevention of cancer is based both on the avoidance of exposure to recognized risk factors and on the protection of the host organism by means of chemopreventive agents. Experimental models in laboratory animals provide valuable tools for identifying protective factors and evaluating benefits and risks resulting from treatment with chemopreventive agents.

We report here the results of a series of experiments aimed at assessing the modulation of diethylnitrosamine (DEN) carcinogenicity in two rat organs (i.e., liver and oesophagus) in different stages of the carcinogenesis process. Another goal of the present study was to investigate the effects resulting from combined exposures to some test agents, thus mimicking the real life situation. The modulators under study included eight agents to which humans are quite extensively exposed, either because they are naturally occurring substances introduced with the diet and beverages or because of their use as drugs or food and cosmetic additives. Some test agents, such as sodium selenite, retinoic acid, ascorbic acid, and butylated hydroxytoluene (BHT), are well-known antioxidants; others, such as phenobarbital, diethyldithiocarbamate, and the methylxanthines caffeine and theophylline, are effective modulators of metabolism and/or DNA repair. It should be emphasized, however, that, like most inhibitors of mutagenesis and carcinogenesis, all test agents are known to work via multiple mechanisms [1]. With the exception of partial reports on selenite [2] and diethyldithiocarbamate [3], all data presented in this article are original.

METHODS

Table I shows a general outline of the 16 separate experiments performed, which involved the use of a total of 2,000 adult female BD_6 rats treated with DEN and 8 agents. All chemicals were commercially available (Sigma Chemical Co., St. Louis, MO). DEN was injected i.p., at a dose of 80 mg/kg b.w., once a week for 5–10 weeks, except in experiment 2, in which the

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carcinogen was added to drinking water, at a concentration of 100 mg/l, for 3.5 months. Of the modulators tested, sodium selenite, ascorbic acid, caffeine, and theophylline were added to drinking water according to various treatment schedules (see Table I); BHT and retinoic acid were dissolved in corn oil and given by gavage; phenobarbital and diethyldithiocarbamate were dissolved in 0.15 M NaCl and administered i.p.

All rats, either killed 6 months after the first injection of carcinogen or dead before that time, were analyzed at autopsy for the presence of macroscopically visible tumors. All liver lesions appearing as single or multiple nodules larger than 1 mm were recorded [4]. Oesophageal lesions exhibiting a three-dimensional structure with a height of at least 1 mm were classified as tumors [5]. Representative samples of pathological lesions were processed by standard histological techniques.

RESULTS

Table I summarizes the results of the 16 experiments performed in terms of survival at the time of registration of the earliest tumor, incidence (i.e., percent of tumor-bearing rats), and multiplicity (i.e., mean number of tumors per rat). The data reported refer to the total of macroscopically visible tumors. At the end of all experiments, only liver and oesophageal tumors were detected. The incidence of tumors in DEN-treated rats varied between 44.1% and 100% in the liver and between 5.9% and 50% in the oesophagus, depending on the total dose of carcinogen administered. A wide spectrum of benign and malignant epithelial and mesenchymal tumors occurred in the liver, whereas oesophageal tumors were almost exclusively squamous cell papillomas. The spectrum of histological types of liver tumors was not appreciably influenced by the modulators tested.

Sodium selenite significantly inhibited the multiplicity of liver tumors, in a dose-dependent fashion, when added to drinking water throughout the duration of the experiments, starting 7 days before the first DEN administration (experiments 1, 2, and 7–9). In experiment 7 selenite, at a concentration of 1 mg/l, significantly decreased the incidence and multiplicity of oesophageal tumors. When administration of selenite started 4 days after the last DEN injection (experiment 10), no effect was observed on liver and oesophageal carcinogenesis.

Ascorbic acid significantly decreased the multiplicity of liver tumors, albeit in the absence of a dose-related response, when added to drinking water starting 1 day before the first DEN injection and lasting until either the end of the experiment (experiments 3 and 6) or 2 days after the last DEN injection (experiment 4). Conversely, liver tumors were not significantly affected when treatment with ascorbic acid started 2 days after the last DEN injection (experiment 3). Oesophageal tumors were significantly inhibited only at the lowest dose tested (3 g/l water), when administration lasted throughout the experiment (experiment 6) or started 2 days after the last DEN injection (experiment 3).

BHT significantly inhibited the multiplicity of both liver and oesophageal tumors, with a similar protective effect when the carcinogen was injected for 6 weeks (experiment 6) or 8 weeks (experiment 5).

Retinoic acid (experiments 5 and 7), when tested at 20,000 IU/kg, produced a significant enhancement of liver tumor multiplicity, whereas oesophageal tumors tended to be decreased, but not to a significant extent.

Caffeine consistently yet not significantly decreased the multiplicity of liver tumors in all three experiments in which treatment started 3 days before the first DEN injection and lasted until 14 days after the last DEN injection (experiments 8, 9, and 14), but not when treatment continued until the end of the experiment (experiment 15) or started 4 days after the last DEN injection (experiment 10). No significant effect was produced by caffeine on oesophageal carcinogenesis, irrespective of the treatment schedules used.

The only significant effect produced by theophilline (experiments 12 and 13) was an enhancement of the multiplicity of oesophageal tumors when this methylxanthine was administered after the last DEN injection.

Phenobarbital (experiment 14) appreciably decreased the multiplicity of both liver and oesophageal tumors. However, the recorded differences were not statistically significant. Similar figures were recorded when phenobarbital was combined with caffeine.

When administered 4 h after each DEN injection, diethyldithiocarbamate (experiments 15 and 16) significantly enhanced the multiplicity of liver tumors, but in one experiment only. A dramatic increase of both tumor incidence and multiplicity (twentyfold) was induced in the oe-

TABLE I. Modulation of DEN Carcinogenesis in BD_6 Rats

Experiment no.	Treatment[a]	Initial/ surviving rats (No.)	Liver		Oesophagus	
			Rats with tumors (%)	No. of tumors/rat (mean ± SE)	Rats with tumors (%)	No. of tumors/rat (mean ± SE)
1	DEN (i.p., 80 mg/kg × 8 weeks)	20/16	93.8	4.3 ± 0.96	31.2	0.63 ± 0.30
	+Se, p.o., 5 mg/l[b]	20/18	94.4	2.2 ± 0.58	50.0	0.67 ± 0.30
	+Se, p.o., 10 mg/l[b]	20/13	69.2	1.2 ± 0.27**	38.5	0.38 ± 0.14
2	DEN (p.o., 100 mg/l × 3.5 mo.)	25/19	94.7	10.3 ± 2.05	36.8	0.58 ± 0.22
	+Se, p.o., 10 mg/l[b]	25/16	93.7	6.3 ± 1.55	25.0	0.31 ± 0.15
3	DEN (i.p., 80 mg/kg × 10 weeks)	30/26	100	8.1 ± 1.22	34.6	0.54 ± 0.17
	+AsA, p.o., 3 g/l[c]	30/27	96.3	4.3 ± 1.02*	18.5	0.19 ± 0.08
	+AsA, p.o., 10 g/l[c]	30/25	88.0	5.2 ± 0.96	28.0	0.40 ± 0.17
	+AsA, p.o., 15 g/l[c]	30/25	92.0	3.9 ± 0.84*	24.0	0.24 ± 0.09
	+AsA, p.o., 3 g/l[d]	30/20	100	6.9 ± 1.23	10.0	0.15 ± 0.11*
	+AsA, p.o., 10 g/l[d]	30/24	100	6.5 ± 1.15	33.3	0.46 ± 0.16
	+AsA, p.o., 15 g/l[d]	30/18	100	7.3 ± 1.5	27.8	0.50 ± 0.28
4	DEN (i.p., 80 mg/kg × 8 weeks)	35/35	94.3	4.5 ± 0.75	31.4	0.37 ± 0.10
	+AsA, p.o., 3 g/l[e]	40/39	89.7	2.2 ± 0.28*	28.2	0.41 ± 0.14
	+AsA, p.o., 10 g/l[e]	40/37	94.6	7.0 ± 1.08	32.4	0.41 ± 0.21
	+AsA, p.o., 15 g/l[e]	40/31	90.3	4.0 ± 0.84	25.8	0.42 ± 0.15
5	DEN (i.p., 80 mg/kg × 8 weeks)	39/30	83.3	2.9 ± 0.56	20.0	0.20 ± 0.07
	+BHT, p.o., 50 mg/kg[b]	38/29	72.1	1.5 ± 0.35*	3.5	0.07 ± 0.07
	+RA, p.o., 10,000 IU/kg[b]	37/23	91.3	3.5 ± 0.71	13.0	0.13 ± 0.07
	+BHT[b] + RA[b]	40/28	92.9	3.0 ± 0.51	3.6	0.04 ± 0.04
6	DEN (i.p., 80 mg/kg × 6 weeks)	30/29	51.7	0.66 ± 0.14	13.7	0.14 ± 0.07
	+BHT, p.o., 50 mg/kg[b]	30/26	23.1	0.27 ± 0.11*	0	0*
	+AsA, p.o., 3 g/l[c]	30/18	25.0	0.25 ± 0.12*	0	0*
7	DEN (i.p., 80 mg/kg × 8 weeks)	30/20	85.0	2.5 ± 0.42	30.0	0.51 ± 0.20
	+Se, p.o., 1 mg/l[b]	30/20	75.0	1.7 ± 0.35	0*	0*
	+RA, p.o., 20,000 IU/kg[b]	30/15	93.3	4.6 ± 0.78*	13.3	0.22 ± 0.15
	+RA[b] + Se[b]	30/18	78.5	3.6 ± 1.00	0*	0*
8	DEN (i.p., 80 mg/kg × 9 weeks)	42/36	100	5.3 ± 0.56	50.0	0.65 ± 0.13
	+Se, p.o., 5 mg/l[b]	42/42	95.2	3.4 ± 0.40**	51.2	0.83 ± 0.16
	+Caf, p.o., 300 mg/l[f]	42/38	94.7	3.8 ± 0.55	54.3	1.11 ± 0.22
	+Se[b] + Caf[f]	42/38	92.1	2.4 ± 0.31***	43.2	0.68 ± 0.15
9	DEN (i.p., 80 mg/kg × 7 weeks)	33/28	64.3	1.4 ± 0.26	25.0	0.32 ± 0.12
	+Se, p.o., 10 mg/l[b]	35/35	31.4*	0.4 ± 0.10***	14.3	0.14 ± 0.06
	+Caf, p.o., 600 mg/l[f]	34/27	55.6	0.8 ± 0.32	11.1	0.11 ± 0.11
	+Se[b] + Caf[f]	54/48	12.5***	0.2 ± 0.07***	8.3	0.08 ± 0.04
10	DEN (i.p., 80 mg/kg × 9 weeks)	36/28	100	6.5 ± 0.77	28.6	0.39 ± 0.13
	+Se, p.o., 10 mg/l[g]	36/22	95.5	6.4 ± 0.10	18.2	0.23 ± 0.11
	+Caf, p.o., 600 mg/l[g]	36/29	100	7.6 ± 1.35	20.7	0.30 ± 0.12
	+Se[g] + Caf[g]	36/24	100	8.5 ± 0.94	41.7	0.50 ± 0.15
11	DEN (i.p., 80 mg/kg × 9 weeks) +Se, p.o., 5 mg/l[g]	41/35	100	5.1 ± 0.56	48.2	0.55 ± 0.13
	+Caf, p.o., 300 mg/l[g]	41/41	97.6	4.5 ± 0.50	55.3	0.87 ± 0.19
12	DEN (i.p., 80 mg/kg × 8 weeks)	40/40	90.0	3.2 ± 0.49	15.0	0.15 ± 0.06
	+Theo, p.o., 600 mg/l[f]	40/34	97.1	3.6 ± 0.50	8.8	0.12 ± 0.07
	+Theo, p.o., 600 mg/l[g]	40/38	97.4	3.7 ± 0.38	28.9	0.40 ± 0.11*
13	DEN (i.p., 80 mg/kg × 8 weeks)	40/28	100	5.1 ± 0.62	14.3	0.18 ± 0.09
	+Theo, p.o., 600 mg/l[b]	40/29	89.7	3.6 ± 0.54	17.2	0.17 ± 0.07
14	DEN (i.p., 80 mg/kg × 8 weeks)	30/22	81.8	3.2 ± 0.67	18.2	0.27 ± 0.15
	+PB, i.p., 80 mg/kg[h]	30/15	100	1.9 ± 0.43	0	0
	+Caf, p.o., 600 mg/l[f]	30/18	72.2	1.2 ± 0.23	22.2	0.28 ± 0.14
	+PB[h] + Caf[f]	30/17	94.1	2.2 ± 0.43	0	0

Table I continued on next page

TABLE I. Modulation of DEN Carcinogenesis in BD_6 Rats (continued)

Experiment no.	Treatment[a]	Initial/ surviving rats (No.)	Liver		Oesophagus	
			Rats with tumors (%)	No. of tumors/rat (mean ± SE)	Rats with tumors (%)	No. of tumors/rat (mean ± SE)
15	DEN (i.p., 80 mg/kg × 5 weeks)	40/34	44.1	0.8 ± 0.22	5.9	0.06 ± 0.04
	+DDTC, i.p., 50 mg/kg[i]	40/31	77.4	2.5 ± 0.60**	77.4***	1.26 ± 0.18***
	+Caf, p.o., 600 mg/l[b]	40/27	40.7	1.1 ± 0.34	11.1	0.11 ± 0.06
16	DEN (i.p., 80 mg/kg × 5 weeks)	30/29	51.7	0.66 ± 0.14	13.7	0.14 ± 0.07
	+DDTC, i.p., 50 mg/kg[i]	30/18	44.4	0.50 ± 0.15	94.4***	2.83 ± 0.44***
	+DDTC, i.p., 50 mg/kg[j]	30/28	32.1	0.54 ± 0.19	3.6	0.04 ± 0.04

[a]Abbreviations: Se, sodium selenite; AsA, ascobic acid; BHT, butylated hydroxytoluene; RA, retinoic acid; Caf, caffeine; Theo, Theophylline; PB, phenobarbital; DDTC, dietyldithiocarbamate.
[b]Treatment started 7 days before the first DEN administration and lasted until the end of the experiment.
[c]Treatment started 1 day before the first DEN injection and lasted until the end of the experiment.
[d]Treatment started 2 days after the last DEN injection and lasted until the end of the experiment.
[e]Treatment started 1 day before the first DEN injection and lasted until 2 days after the last DEN injection.
[f]Treatment started 3 days before the first DEN injection and lasted until 14 days after the last DEN injection.
[g]Treatment started 4 days after the last DEN injection and lasted until the end of the experiment.
[h]Three daily PB injections before each DEN injection.
[i]Four hours after each DEN injection.
[j]Twenty-four hours after each DEN injection.
*Statistical significance, as assessed by χ^2 analysis (incidence) or Student's t-test (multiplicity): $P < 0.05$ as compared to rats treated with DEN only.
**Statistical significance, as assessed by χ^2 analysis (incidence) or Student's t-test (multiplicity): $P < 0.01$ as compared to rats treated with DEN only.
***Statistical significance, as assessed by χ^2 analysis (incidence) or Student's t-test (multiplicity): $P < 0.001$ as compared to rats treated with DEN only.

sophagus under the same experimental conditions. No significant effect was observed when diethyldithiocarbamate was administered 24 h after each DEN injection.

The combinations of retinoic acid with either BHT (experiment 5) or sodium selenite (experiment 7) were devoid of significant effects on liver tumors. Combination of selenite with retinoic acid (experiment 7) did not affect the ability of this microelement, at 1 mg/l water, to inhibit oesophageal carcinogenesis.

Combination of caffeine with selenite (experiments 8 and 9) did not affect the oesophageal tumor frequency but further potentiated the inhibition of liver carcinogenesis produced by each one of these two agents, when applied individually. In experiment 8, such a combination gave a significantly higher protection ($P < 0.05$) than caffeine alone. However, the decrease of liver tumor multiplicity produced by cotreatment with these two agents was less than additive. When both caffeine and selenite were administered after the last DEN injection (experiments 10 and 11), no effect was observed on liver or oesophageal carcinogenesis.

DISCUSSION

Treatment of rats with DEN resulted in the formation of tumors, which after 6 months were only detected in the liver and the oesophagus, thus confirming the typical organotropism of this carcinogen in BD_6 rats [6].

Three of the four antioxidants tested (i.e., selenite, ascorbic acid, and BHT) exerted protective effects on the liver carcinogenicity of DEN when these chemopreventive agents were administered throughout the whole experiment. Additionally, under the same conditions, BHT and ascorbic acid, at least at the lowest tested dose of 3 g/l, inhibited oesophageal carcinogenesis. Selenite and ascorbic acid were also administered after withdrawal of exposure to the carcinogen without any effect on liver tumor frequency but with a significant decrease of the multiplicity of oesophageal tumors by ascorbic acid. This finding suggests that vitamin C is capable of inhibiting the promotion of this type of tumors, which is consistent with the notion that, due to the importance of free radicals in tumor promotion in the oesophagus, antioxidants play a promi-

nent protective role during this carcinogenesis step [1]. The inhibition of DEN-induced carcinogenesis by ascorbic acid is also in accordance with the epidemiological evidence showing a decreased cancer risk in humans receiving a diet rich in vitamin C, which, however, is generally ascribed to the ability of this vitamin to prevent the endogenous synthesis of *N*-nitrosamines [7].

In contrast, retinoic acid tended to reduce the multiplicity of oesophageal tumors but at the same time significantly enhanced DEN-induced hepatocarcinogenesis, the latter effect being substantially attenuated or even eliminated by its combination with other antioxidants. Conflicting data are available in the literature concerning modulation of oesophageal carcinogenesis by retinoids [8], and limited data are available on modulation of liver carcinogenesis by this category of compounds [9]. Our findings are in agreement with the reported enhancement of murine hepatocarcinogenesis by retinoic acid itself and two synthetic retinamides at doses that inhibited cancer induction in other tissues [9]. As mentioned above, such an adverse effect was abolished when retinoic acid was administered in combination with either BHT or selenite, thus indicating that the combined application of more than one modulator can result in a neutralization of their adverse effects on carcinogenesis. On the whole, the results obtained in this study with antioxidants confirm that these agents can affect in a variable mode tumor initiation and especially tumor promotion, depending on their doses and redox potential [1].

Humans are extensively exposed to the methylxanthines caffeine and theophylline. The former compound is known to modulate DNA repair, thereby affecting toxic and mutagenic activities of alkylating agents [10]. Controversial results are, however, available on the ability of caffeine to inhibit chemically induced tumors [10,11], and specific information is scanty in the case of theophylline. For instance, neither caffeine nor theophylline significantly influenced the intestinal carcinogenesis induced by 1,2-dimethylhydrazine in rats [12]. The results obtained in the present study show that administration of caffeine during the period of treatment with DEN tends to reduce the number of liver tumors and to potentiate the liver protection afforded by selenite. On the other hand, the only effect observed in theophylline-treated rats consisted in a stimulation of the advanced stages of oesophageal tumorigenesis. The distinctive influence of these two methylxanthines in the experimental model used suggests the involvement of different mechanisms, possibly related to the differential efficiency of caffeine and theophylline in modulating DNA repair and the metabolism of cyclic nucleotides [11], which may be involved in tumor initiation and promotion, respectively [1].

Pretreatment of rats with phenobarbital tended to decrease the multiplicity of liver tumors and completely prevented those in the oesophagus, although these differences did not attain the threshold of statistical significance. This potent enzyme inducer has been previously reported to inhibit chemical hepatocarcinogenesis in rodents, when applied prior to the carcinogen [13]. These effects were not further modified by the combined treatment of rats with phenobarbital and caffeine.

Dithiocarbamates were proposed as suitable chemopreventive agents in the case of *N*-nitrosamine–induced tumors, because of their ability to inhibit the metabolic activation of these carcinogens [14]. However, our results indicate that the block of metabolism produced by diethyldithiocarbamate, when injected 4 h after administration of DEN as suggested in rat hepatocarcinogenesis models [15], increased the multiplicity of liver tumors in one out of two experiments and especially caused a dramatic enhancing effect on the number of oesophageal tumors. A similar potentiation of oesophageal carcinogenesis had been observed in rats treated with DEN and disulfiram [16], whose molecule is formed of two diethyldithiocarbamate moieties. Thus, inhibitors of metabolism appear to behave as double-edged swords, and their possible use as chemopreventive agents should be considered with caution.

In conclusion, modulation of DEN carcinogenesis varied depending on several factors, including 1) the type of agent and its putative mechanism(s), 2) their combinations, 3) the treatment schedules, with special reference to dosage and administration times along the carcinogenesis process, and 4) the target organ. As a consequence, besides protective effects, under certain conditions even well-known chemopreventive agents produced adverse effects.

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