Carcinogenicity of Diallylnitrosamine Following Intragastric Administration to Syrian Hamsters [1,2,3]

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ABSTRACT—Single and multiple intragastric doses of diallylnitrosamine [(DAN) CAS: 16338-97-9] administered to Syrian golden hamsters induced tumors, primarily of the respiratory tract, in which the nasal cavity epithelium was the preferred site. When compared to the effect of DAN after subcutaneous administration at equal doses, the incidence of respiratory tract tumors was lower but that of hepatic tumors was higher, suggesting partial metabolism of DAN in the liver. Comparative metabolic and mutagenesis studies in BD IX rats (which reportedly are refractory to the carcinogenic effects of DAN), in Wistar rats, and in Syrian hamsters showed that a greater proportion of orally administered DAN was exhaled by both rat strains (12–19%) than by hamsters (2–4%). The activity of the microsomal fraction of the hamster liver for metabolizing DAN to allyl alcohol was about 10 times higher than that in rats, whereas no significant species differences were found with the cytosolic fraction. Pretreatment of animals with phenobarbital (PB) or pregnenolone-16α-carbonitrile (PCN) did not influence either microsomal or cytosolic enzyme activities in hamsters, whereas about a tenfold increase in enzyme activities was seen after pretreatment with PB in both rat strains and following PCN in Wistar rats. Moreover, in bacterial mutagenesis assays, hamster liver microsomes were twice as active as those in BD IX rats. The results are discussed in relation to the carcinogenicity of DAN in rats and hamsters.—JNCI 1985; 74:1043-1046.

About 300 nitrosamines have been studied for carcinogenicity in many species of animals and most have been found to be carcinogenic to various degrees of potency and tissue specificity, especially upon oral administration (*1*). One of these compounds, DAN, proved a potent respiratory tract carcinogen when given once or repeatedly to Syrian hamsters (*2*) but failed to induce tumors in BD IX rats (*1*). This inability may be due to DAN's species-related inactivation by the liver as a result of a "first pass effect." To clarify this issue and to elucidate possible strain differences in response, we tested the long-term effects of oral administration of DAN in Syrian hamsters and examined its metabolic activation in vivo and in vitro by livers from hamsters and from two strains of rats. BD IX rats were used, since they reportedly are unresponsive to DAN, and Wistar rats were examined for comparison.

MATERIALS AND METHODS

Chemicals.—DAN was synthesized and analyzed as previously described (*2*). A single contaminant, methyl-*N*-butylnitrosamine, at a level of 0.002%, was discovered by combined gas-liquid chromatography and mass spectrometry; however, because the impurity level was low, no further cleanup was attempted.

Carcinogenesis study.—Outbred 8-week-old Syrian golden hamsters from the Eppley colony were housed in plastic cages in groups of 5 separated by sex. They received Wayne pelleted diet (Allied Mills, Inc., Chicago, IL) and water ad libitum. The animals were kept under standard conditions (21±5°C, 55±5% humidity) in a fume hood, because of the volatility of DAN, and were weighed once weekly.

The LD_{50} of DAN was calculated by Weil's method (*3*), on the basis of mortality within an 8-day observation period. DAN, dissolved in olive oil, was instilled ig into groups of 10 hamsters (5 females, 5 males) as a single dose of either 500, 1,000, 2,000, or 4,000 mg/kg body weight. Survivors (group A) were observed for life. The chronic effect (group B) was examined by ig treatment with DAN of 3 groups of hamsters (each with 15 females and 15 males) once weekly for life with doses of either 87 (group 1B), 43.5 (group 2B), or 21.25 mg (group 3B) DAN/kg body weight, corresponding to 1/10, 1/20, and 1/40 of the LD_{50}, respectively. Controls (group 4B) received the same dose schedule of the solvent (10 ml/kg).

Hamsters were killed when moribund and complete necropsies were performed. Organs were fixed in 10% buffered Formalin. Bones were decalcified in Calcifier I (Surgipath Medical Industries, Northbrook, IL) and tissues were embedded in Paraplast. Step sections were prepared from the brain, nasal and paranasal cavities, larynx, trachea with attached stem bronchi, lungs,

ABBREVIATIONS USED: DAN=diallylnitrosamine; ig=intragastric, intragastrically; LD_{50}=median lethal dose(s); PB=phenobarbital; PCN=pregnenolone-16α-carbonitrile.

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pancreas, liver, kidneys, adrenal glands, stomach, urinary bladder, rectum, ovaries, vagina, uterus, Cowper's gland, prostate gland, and testes. Sections were stained with hematoxylin and eosin. Times stated were from the beginning of treatment. Sex differences are mentioned when found.

Metabolic studies.—DAN was administered orally to each of 5 male BD IX and 5 male Wistar rats (the respective LD_{50} were 1,270 and 1,140 mg/kg body weight) and to Syrian golden hamsters in amounts which corresponded to 1/10 of their respective oral LD_{50}. The animals were then placed in all-glass metabolism cages and urine and expired air were collected for 24 hours. All collected samples were analyzed for DAN by gas-liquid chromatography, as described (*2*).

For in vitro studies, each group of 5 male animals (BD IX rats, Wistar rats, and Syrian hamsters) were either untreated or pretreated with PB (20 mg/kg ip injections for 4 days) or PCN (5 mg/kg ip injections for 3 days). Pretreated animals were sacrificed 24 hours after their last injection. Upon sacrifice the livers were removed, homogenized in 0.05 *M* Tris buffer, pH 7.4, and the microsomal fraction was obtained by ultracentrifugation of the 10,000×*g* supernatant (S-10 fraction) at 106,000×*g* for 1 hour. Both the microsomal and S-10 fractions were incubated with DAN for 1 hour at 37°C. Final concentrations in the incubation media were DAN, 14 m*M*; Tris, 0.05 *M*, pH 7.4; $MgCl_2$, 5m*M*; NADPH, 0.16 m*M*; protein, 6 mg/ml. After incubation the reaction was stopped by adding $Ba(OH)_2$ and $ZnSO_4$ to the media. Allyl alcohol was distilled from the aqueous mixture with the use of a microcondenser apparatus and the distillate was then analyzed for allyl alcohol by gas-liquid chromatography on a 2-m column of 4% carbowax 20 *M* plus 0.8% KOH on Carbopack B. The column temperature was 100°C and carrier gas flow rate was 25 ml/minute. Retention time was 6.84 minutes. Allyl alcohol in a given distillate was identified by comparing the retention time of an unknown component peak with an authentic standard. The final product was confirmed by mass spectrometric analysis of portions from the eluted peak of interest.

Mutagenicity.—The method of Rao et al. (*4*) was used to assess the ability of the respective liver microsomes from the 2 rat strains and the hamsters to generate bacterial mutagens from DAN. The autotrophic strain of *Salmonella typhimurium* TA 1535 was kindly supplied by Dr. B. N. Ames, Department of Biochemistry, University of California, Berkeley, CA. Both the plate assay and liquid suspension tests were used. The dose range was 5-200 μg/plate, and the final microsomal protein concentration was 3 mg/plate.

The mammalian mutagen assay followed the methods outlined by Jones and Huberman (*5*), who used $1\text{-}2\times10^6$ Chinese hamster V79 cells over the DAN concentration range of 1-100 m*M*. Mutations were characterized by ouabain resistance.

For statistical evaluation the chi-square test was used.

TABLE 2.—*In vivo excretion of DAN (24-hr collection, ig administration)*[a]

Mode of excretion	Sex	Mean percent recovery of dose ± SEM in: Hamster	Wistar rat	BD IX rat
Urine	♂	0.15±0.03	0.4±0.03	0.6±0.06
	♀	0.3±0.04	0.6±0.03	1.1±0.12
Expired air	♂	2±0.14	12±0.09	16±1.1
	♀	4±0.19	15±0.11	19±1.2

[a] All values represent determinations from 10 animals, 5 of each sex.

RESULTS

Carcinogenicity study.—The LD_{50} of DAN was calculated to be 810 and 930 mg/kg body weight in females and males, respectively. All hamsters treated with 2,000 and 4,000 mg/kg of DAN (group A) died within 8 days, due to thoracic and abdominal hemorrhages and hepatocellular necrosis. A few hamsters that received 1,000 (group A1) and 500 mg/kg (group A2) of DAN died of acute toxicity. There were 16 survivors, 10 of which developed tumors in various tissues but predominantly in the respiratory tract and liver (table 1). The survival rates of hamsters treated weekly with DAN (group B) were lower in group B2 (43.5 mg/kg) and greatest in group B3 (21.75 mg/kg), when compared to controls, whereas body weight was not affected by treatment (weights ranged from 85 to 110 g). The main target

TABLE 1.—*Tumors found in Syrian hamsters treated ig with DAN and in untreated controls*

Group	Dose of DAN, mg/kg body weight	Effective No. of hamsters	Mean survival time, wk±SD	No. (%) of tumor-bearing animals	No. (%) of tumors in: Nasal cavities	Larynx	Trachea	Pharynx	Liver	Others, type (%)[a]
A1	1,000	7	61±8	3(43)	0	0	2(29)	0	1(14)	B(33)
A2	500	9	48±12	7(78)	2(22)	1(11)	2(22)	0	3(33)	C(11)
B1	87	27	38±12	24(89)	18(67)	14(52)	15(56)	1(4)	1(4)	B (20), D (7), E (15), F (7)
B2	43.5	27	35±15	16(59)	13(48)	1(4)	8(30)	3 (11)	1(4)	B(30), D(4)
B3	21.75	28	44±15	18(64)	1(4)	13(11)	11(39)	1 (4)	1(4)	B(22), E(7), F(11), G(4) H(7)
Control	0	30	45±12	7(23)	0	0	0	0	1(3)	B(40), D(7)

[a] B=adenoma, Cowper's gland; C=fibroma, intestine; D=adenoma, adrenal cortex; E=fibroleiomyoma, myometrium; F=polyp, gallbladder; G=adenoma, lung; H=papilloma, forestomach.

TABLE 3.—*In vitro metabolism of DAN*[a]

Pretreatment	Sex	Allyl alcohol production, mean nmol/mg protein/hr ± SEM in:					
		Microsomal fraction			S-10 fraction		
		BD IX rat	Wistar rat	Hamster	BD IX rat	Wistar rat	Hamster
None	♂	4.1±0.49	6.0±2.1	44.8±5.1	14.9±3.1	13.8±2.2	16.2±2.1
	♀	4.9±0.49	8.0±1.2	67.2±5.9	9.9±1.4	8.8±1.0	12.2±1.4
PB	♂	69.3±4.3	43.2±2.9	35.8±5.8	144.4±8.8	91.1±4.8	17.3±1.1
	♀	54.0±3.8	36.9±4.1	47.8±3.9	99.2±9.9	50.1±6.2	8.9±0.6
PCN	♂	9.6±0.81	52.7±6.3	31.6±2.9	22.4±3.0	124.1±14.1	12.1±2.4
	♀	9.6±0.91	44.8±5.9	39.4±4.3	26.1±3.1	72.6±9.1	9.4±1.3

[a] All values are the composites of three separate experiments. Five animals of each sex were used in each experiment.

tissues for DAN in these hamsters were the posterior nasal cavity, trachea, larynx, pharynx, and liver, respectively. All but 2 nasal cavity tumors were adenocarcinomas of the posterior region, as described (*6*), and 4 of these invaded surrounding tissues. The only 2 benign neoplasms in group A hamsters (both females) were papillomas of the anterior nasal region. The first nasal cavity adenocarcinoma was observed at 13 weeks in a female of group B1.

Tumors of the larynx and trachea were all papillary polyps and ranged in number between 1 and 4/hamster (an average of 2). Only 1 lung tumor (an adenoma) was seen in a female of group B3. Pharyngeal neoplasms were papillomas, which usually occurred as solitary growths. Liver neoplasms were all cholangiomas. The other tumors in table 1 were seen sporadically and their incidence and pattern were within the range of spontaneously occurring tumors in this hamster colony.

Metabolic studies.—The results comparing the in vivo excretion of orally administered DAN in Syrian hamsters and 2 strains of rats are shown in table 2. The recovery of intact DAN in the urine of both species was very low and was greater in rats than in hamsters. The amount of expired DAN in the two strains of rats was comparable and 4–5 times larger than that expired by the hamsters.

The in vitro production of allyl alcohol from DAN by different liver fractions from both species is shown in table 3. This product would be the end result arising from α-hydroxylation of DAN, followed by reaction of the unstable intermediate decomposition product with water. This metabolic pathway is purportedly one of activation for N-nitrosamines. As shown, there is a significant amount of allyl alcohol formed (i.e., α-hydroxylation) by enzymes in the cytosol (S-10 fraction), and for the rats it is generally larger than the microsomal activity. The microsomal metabolic activity of the untreated hamster is 3–15 times higher than any fraction from either strain of rat. There is much more metabolism of DAN by the hamster, and accordingly this factor may explain why less DAN is expired by this species (*see* table 2). Allyl alcohol production by hamster enzymes remains basically unchanged upon pretreatment; however, in both rat strains the enzyme activity was increased about 2–17 times after treatment with PB or PCN. As in the controls, the highest activity remained in the S-10 fraction after pretreatment.

Mutagenicity studies.—Because of the differences in metabolic activity between the two species, we sought to determine if such differences could also be demonstrated in a system with an endpoint more closely related to carcinogenicity. Therefore, we examined the relative mutagenicity of products derived from liver enzyme fractions from hamsters and BD IX rats. The results of studies with the cytosolic enzyme fractions from both species were unremarkable (data not shown); however, as can be seen in table 4, the ability of liver microsomes from untreated or PB-treated hamsters differed in the production of mutagens toward the tester strain of bacteria used. In essence, hamster activity was about twice ($P<.05$) that of the BD IX rat. For the untreated animals this difference in activity correlated with the relative activity seen in table 3; however, for the PB-treated animals the mutagenicity varied slightly with the metabolic activity of similarly treated animals.

DISCUSSION

Orally administered DAN, which was noncarcinogenic in BD IX rats (*1*), was found to induce tumors in Syrian hamsters; subcutaneous administration of DAN also induced tumors in this species (*2*). In both instances, the hamster respiratory tract was the main target tissue for DAN, regardless of the route of administration. However, when DAN was injected ig, a considerably lower tumor incidence (4–67% vs. 60–97%) was induced in the nasal and paranasal cavities and larynx and lungs, when compared with the tumor incidence after sc administration of the equivalent dose (*2*). This difference could be

TABLE 4.—*Bacterial mutagenicity of DAN (50 mM)*

Microsome source	Sex	Mean No. of *his* revertants/10^8 cells ± SEM	
		Untreated	PB pretreated
Hamster[a,b]	♂	344±32	373±21
	♀	222±11	325±20
BD IX rat[a,b]	♂	156±31	171±29
	♀	131±20	225±34

[a] Control (no microsomes): 39 revertants/10^8 cells.
[b] 5 animals of each sex.

due to partial detoxification of orally administered DAN on its first passage through the liver.

The present metabolism and mutagenicity studies demonstrated that in both strains of rats a large proportion of DAN is expired in the air and only a small fraction is metabolized in the liver, whereas in hamsters the opposite was the case. In addition, hamster microsomes were much more effective in mutagenesis assays than those of the rat liver. The results of our mutagenicity studies are in line with those of Andrews and Lijinsky (*7*), who demonstrated DAN to be mutagenic by both hamster and rat liver S-9.

Although our results could explain, in part, the species differences in response to DAN, the data cannot be considered conclusive, because the metabolic capacity of the main target tissues of DAN in hamsters, i.e., the respiratory epithelium, was not investigated. The importance of such studies is highlighted by our recent experiment, which showed selective induction of nasal and paranasal cavity cancer by DAN in BD IX rats (*8*). Since pretreatment with enzyme inducers significantly increased metabolism of DAN by rat liver microsomal and cytosolic fractions, the carcinogenic effect of the compound in pretreated rats would be of interest.

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