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LABELING *IN VIVO* OF RAT LIVER PROTEINS BY TRITIUM-LABELED DIMETHYLNITROSAMINE

EFFECT OF PRIOR TREATMENT WITH 3-METHYLCHOLANTHRENE, PHENOBARBITONE, DIMETHYLFORMAMIDE, DIETHYLFORMAMIDE, AMINOACETONITRILE, ETHIONINE AND CARBON TETRACHLORIDE*

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Abstract—We studied the effect of seven agents on the radioactivity of the liver proteins, measured 2·5 hr after injecting a standard dose of tritium-labeled dimethylnitrosamine into rats. Protein labeling was slightly inhibited when the rats were pretreated with 3-methylcholanthrene and phenobarbitone. Pretreatment with dimethylformamide, diethylformamide and aminoacetonitrile produced a larger (76–92 per cent) inhibition of the labeling. The labeling was also inhibited by acute treatment with ethionine and carbon tetrachloride. The results are well correlated with known effects by these agents on dimethylnitrosamine metabolism and (for 3-methylcholanthrene and phenobarbitone) on liver carcinogenesis by nitrosamines.

THE LIVER carcinogen dimethylnitrosamine (DMN) is metabolized by an *N*-demethylating system in liver microsomes to give formaldehyde and a methylating agent, presumably derived from monomethylnitrosamine, which methylates cell macromolecules. Magee and Barnes[1] reviewed the metabolic studies and the evidence for the hypothesis that methylation of cell DNA, RNA or proteins is involved in DMN carcinogenesis. According to these observations and this hypothesis, agents that affect DMN metabolism should also affect the methylation of cell macromolecules by DMN and its carcinogenic activity. As an aid to establishing such correlations, we examined the action of seven drugs or toxic agents on the labeling *in vivo* of rat liver proteins by tritium-labeled DMN.

MATERIALS AND METHODS

Materials. Dimethylnitrosamine (DMN), dimethylformamide (DMF), diethylformamide (DEF) and 3-methylcholanthrene (3-MC) were obtained from Eastman Organic Chemicals, Rochester, N.Y.; aminoacetonitrile (AAN) bisulfate from Aldrich

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Chemical Company, Milwaukee, Wis.; DL-ethionine from Sigma Chemical Company, St. Louis, Mo.; CCl_4 from Fisher Scientific Company, Fair Lawn, N.J.; and phenobarbitone sodium from Merck & Co., Rahway, N.J.

Treatment of rats. Young adult male Wistar rats fed Purina laboratory chow were used, with female rats used in the ethionine experiments. Tritiated DMN was synthesized from [^{3}H]dimethylamine.[2] A standard intraperitoneal injection was given of 14·5 mg and 100 μc [^{3}H]DMN, dissolved in 20 ml of 0·025 M sodium phosphate buffer (pH 7), per kg of body weight. The injection was always given at 10 a.m.–12 noon. The rats were killed 2·5 hr later (except in experiment 4 of Table 1 where 5 hr elapsed) by bleeding under ether anesthesia, and weighed. The livers were removed, weighed and stored at −15°.

At various times before the [^{3}H]DMN injection, groups of rats were injected intraperitoneally with one of seven compounds. The doses and rat weights (at death) are given in Table 1. The 3-MC was injected as a solution of 4 mg/ml of olive oil 48 hr before the [^{3}H]DMN treatment. Control rats were not injected with olive oil, since similar experiments with [^{3}H]diethylnitrosamine had shown no differences between untreated rats and rats injected with oil.[3] An aqueous solution of phenobarbitone sodium was injected 24, 48 and 72 hr before the DMN. Freshly prepared aqueous solutions of AAN bisulfate (neutralized with NaOH), DMF and DEF were injected 30 min before the DMN, at concentrations corresponding to 10 ml solution/kg of body weight.

Ethionine (75 mg in 3 ml water) was injected at 9 a.m. and again at 4 p.m. into 130–150 g female rats, and [^{3}H]DMN was injected on the following morning. The rats were fasted from the night before ethionine treatment until they were killed. Control rats were fasted similarly and injected with 2 × 3 ml saline. A 50% (by volume) solution of CCl_4 in olive oil was injected at doses of 1·2, 2·4 and 3·6 ml/kg into 100–120 g male rats 24 hr before the DMN injection. Control rats were injected with 1·2 ml olive oil/kg. In the ethionine and CCl_4 experiments, portions of the liver were fixed in 10% formalin, and paraffin sections were stained with hemotoxylin and eosin for histological examination.

Isolation of liver proteins. The method was adapted from techniques described in the literature.[4–7] Control and experimental livers were always worked up at the same time. Each liver was homogenized with 8 ml of 5% trichloroacetic acid per g of liver in a Virtis homogenizer. The homogenate (40 ml) was centrifuged at 2°. The sediment was washed by centrifugation with 40 ml each of ice-cold 5% trichloroacetic acid (three times), ice-cold 10% potassium acetate in 95% ethanol (twice),[7] 3:1 ethanol–ether (twice; the second wash was incubated with the sediment at 40° for 20 min) and ether (twice). The resulting "protein–nucleic acid powder" was allowed to dry overnight in tubes tilted almost horizontal.

One ml (about 150 mg) of this powder was extracted with 8 ml of 10% NaCl at 100° for 1 hr to remove nucleic acids. The suspension was cooled in ice for 10 min and centrifuged. The sediment was re-extracted with hot 10% NaCl for 30 min, washed by centrifugation with 10 ml of 5% trichloroacetic acid (twice) and acetone (twice), and desiccated under vacuum at 4°. Two 20-mg samples were incubated overnight at 60° with 2·0 ml of NCS Solubilizer (Amersham/Searle Corp., Des Plaines, Ill.). Samples (0·5 ml) were assayed in a Packard scintillation counter (model 3003) after adding 15 ml toluene–PPO (0·5%)–POPOP (0·03%). The counting efficiency

was 20 per cent. The vials were counted for 10 min and those for the control rats showed 300–500 counts/min. The final protein preparation contained less than 2% nucleic acid, was obtained in 70–85 per cent yield by weight from the protein–nucleic acid powder, and showed about 25 per cent lower disintegrations per minute per milligram than the protein–nucleic acid powder.

RESULTS

In order to check the purity of the [^{3}H]DMN, samples were injected into rats, the urine was collected, and the urinary purines (excluding uric acid) were analyzed using two-dimensional paper chromatography.[8] Almost all (97 per cent) of the purine label appeared in 7-methylguanine, as shown by Craddock and Magee[9] [^{3}H]diethylnitrosamine was synthesized from [^{3}H]diethylamine by the method[2] used for ^{3}H-DMN and was injected into rats. When the urine was analyzed, labeled 7-ethylguanine was detected as a spot on the paper chromatogram close to adenine and coinciding with authentic material,* but the 7-ethylguanine contained only 40 per cent of the counts in 7-methylguanine. This suggests that the sample of [^{3}H]diethylnitrosamine contained small amounts of [^{3}H]DMN, which is much more active metabolically[10] and so would contribute disproportionately to the radioactivity of cell constituents and urinary purines. The presence of other labeled [^{3}H]methylalkylnitrosamines could be a contributory factor. Since this complication did not apply to [^{3}H]DMN (as shown above), this nitrosamine was chosen for the present study.

The effect of injecting various agents was then examined on the labeling of liver proteins after injection of [^{3}H]DMN (Table 1). In each experiment, the results were compared with those for control rats injected with [^{3}H]DMN alone. These control groups showed mean values of 300–473 dis./min/mg of protein. Pretreatment of male rats with 3-MC or phenobarbitone produced a small (15–19 per cent) inhibition of the labeling, which was statistically significant in experiment 2 but not in experiment 1. The results for the two experiments were expressed as per cent mean control value for the experiment, and combined. The 3-MC and phenobarbitone groups then showed values of 82 ± 12 per cent (8 rats) and 84 ± 12 per cent (8 rats), (mean and standard deviation) which are significantly different ($P < 0{\cdot}01$) from the value of 100 ± 7 per cent (8 rats) for the controls.

Much more marked effects, all highly significant, were found for DMF (76 per cent inhibition), DEF (91–92 per cent inhibition) and AAN (80–86 per cent inhibition). In these experiments, the total nucleic acids were counted after isolation from the hot NaCl extracts[5,6] and showed about three times the specific radioactivity (expressed as disintegrations per minute per milligram) of the proteins. DMF, DEF and AAN all strongly inhibited labeling of the nucleic acids, in addition to that of the proteins. However, nucleic acids were not assayed in the remaining experiments, because the preparations were found to be contaminated with about 20 per cent of protein.

Treatment with two doses of ethionine produced a significant inhibition of the protein labeling. The livers showed minimal to moderate fine vacuolation of the cytoplasm, predominantly in periportal areas. A single treatment with CCl_4 produced

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TABLE 1. LIVER PROTEIN RADIOACTIVITY AFTER INJECTING ^{3}H-DMN ALONE OR PRECEDED BY INJECTION OF VARIOUS COMPOUNDS*

Expt.	Compound	Dose	No. of rats	Mean rat wt. (g)	Mean liver wt. (g)	Protein radioactivity (dis./min/mg)†	Per cent inhibition	P <‡
1	—	—	4	146	7·9	382 ± 32	—	—
	3-MC	1·2 mg/rat	4	144	8·4	309 ± 57	19	NS§
	Phenobarb.	3 × 80 mg/kg	4	144	8·4	327 ± 57	15	NS
2	—	—	4	126	6·3	434 ± 14	—	—
	3-MC	1·2 mg/rat	4	119	5·8	362 ± 32	17	0·01
	Phenobarb.	3 × 80 mg/kg	4	123	7·4	365 ± 40	16	0·02
3	—	—	4	257	¶	358 ± 153	—	—
	DMF	144 mg/kg	4	215	¶	85 ± 56	76	0·02
	DEF	202 mg/kg	4	225	¶	31 ± 10	91	0·01
	AAN	200 mg/kg	3	240	¶	50 ± 19	86	0·02
4	—	—	3	118	¶	473 ± 75	—	—
	DEF	600 mg/kg	4	122	¶	37 ± 10	92	0·001
	AAN	300 mg/kg	4	127	¶	96 ± 40	80	0·001
5	—	—	5	142	5·4	413 ± 10	—	—
	Ethionine	2 × 75 mg/rat	5	141	6·3	242 ± 30	41	0·001
6	—	—	5	136	5·3	372 ± 30	—	—
	Ethionine	2 × 75 mg/rat	5	134	6·3	236 ± 50	37	0·001
7	—	—	4	127	5·6	306 ± 20	—	—
	CCl_4	0·6 ml/kg	3	117	5·9	211 ± 6	31	0·001
	CCl_4	1·2 ml/kg	4	113	6·2	190 ± 36	38	0·01
	CCl_4	1·8 ml/kg	3	113	6·2	160 ± 30	48	0·001
8	—	—	3	119	5·5	300 ± 10	—	—
	CCl_4	0·6 ml/kg	3	107	5·7	156 ± 36	48	0·01
	CCl_4	1·2 ml/kg	3	102	6·1	126 ± 6	58	0·001
	CCl_4	1·8 ml/kg	3	105	5·8	90 ± 6	70	0·001

* Experimental procedures are given in the text, which gives the abbreviations for the test compounds.
† Mean ± standard deviation.
‡ Significance of difference from the control group of the same experiment.
§ NS, not significant.
¶ Not measured.

significant inhibition of the labeling at all three dose levels used, and the inhibition increased with increasing dosage. The livers here showed mild to severe cellular degeneration in the central areas of the liver lobules, and in general the damage became more severe as the dose was raised. Clear correlations could not be demonstrated between the biochemical and histological results in individual animals, for either of these agents.

DISCUSSION

Tritium-labeled nitrosamines have been used in several metabolic studies.[11–13] Lee *et al.*[11] injected [^{14}C]DMN and [^{3}H]DMN into rats, compared the total radioactivity of RNA and the labeling of 7-methylguanine in several organs, and concluded that incorporation experiments may be carried out equally well with both kinds of label. All three hydrogen atoms of the DMN methyl group are known to be incorporated into 7-methylguanine.[14] The labeling of protein and nucleic acid by tritiated DMN, diethylnitrosamine, dimethylamine and diethylamine were compared by Mirvish,[3,15] who also briefly reported the effects of 3-MC and phenobarbitone on the labeling *in vivo* of protein and nucleic acid by [^{3}H]diethylnitrosamine. However, the present data on urinary metabolites of [^{3}H]diethylnitrosamine indicate that our results with this nitrosamine should be interpreted with caution.

The labeling of RNA and DNA at short times after injecting [^{14}C]- and [^{3}H]DMN is mostly due to the formation of 7-methylguanine, and not to the incorporation of DMN metabolites during biosynthesis of nucleic acids.[1] Similarly, the labeling of proteins is attributed to methylation of amino acids and to incorporation of DMN metabolites during protein biosynthesis. Magee and Hultin[5] found that histidine residues are methylated and presumably other amino acids are also methylated. The distribution of the label in proteins is more complex than in nucleic acids.[5,*] According to the current view of DMN metabolism,[1] both protein methylation and the production of DMN metabolites, e.g. formaldehyde, should depend on the microsome-mediated catabolism of DMN. Therefore, the observed variations in total protein labeling may mainly reflect effects on the DMN-catabolizing system. Furthermore, since short time intervals (mostly 2·5 hr) were used, the labeling may mainly reflect the extent of protein methylation. It should also be noted that the labeling of nucleic acids (where examined) appeared to vary similarly to that of the proteins.

The small inhibition produced by 3-MC and phenobarbitone may be compared with previous reports that treating rats with phenobarbitone did not affect the rate of DMN catabolism[16] and that phenobarbitone and 3-MC treatments suppressed the production of formaldehyde from DMN *in vitro* by rat liver microsomes.[17,18] For 3-MC, the effect is probably due to a decrease in the amount of *N*-demethylating enzyme,[19] and was also observed in starved rats.[20] With respect to carcinogenesis, treatment of rats with 3-MC did not increase the induction of liver tumors by DMN,[21] but treatment of mice with phenobarbitone inhibited the induction of liver tumors by diethylnitrosamine.[22] These results are unusual, since many microsomal drug-metabolizing enzymes are induced by 3-MC and phenobarbitone. We might therefore have predicted that DMN metabolism would be stimulated, leading to an increase in protein labeling.

* Unpublished observations by the author.

The strong inhibition of protein labeling by DMF and DEF is in agreement with Heath's finding[16] that these agents inhibited the catabolism of DMN, perhaps because of the analogy in structure [cf. DMN (Me_2N–N:O) and DMF (Me_2N–CH:O)]. The inhibition by AAN agrees with previous observations that this agent protects the liver against DMN-induced necrosis, suppresses the inhibition of protein synthesis by DMN, and inhibits both DMN catabolism and the methylation of tissue nucleic acids by DMN.[23]

A few hours after the injection of carbon tetrachloride[24,25] or ethionine,[26] alterations occur in the morphology of the endoplasmic reticulum, including an accumulation of triglycerides. Various functions of the endoplasmic reticulum are inhibited by carbon tetrachloride,[24] including the *N*-demethylation of dimethylaniline.[25] Thus the inhibition of protein labeling by these agents could be due mainly to effects on the metabolism of DMN by the endoplasmic reticulum. The mild degree of hepatic necrosis produced by carbon tetrachloride could be a contributory factor, since we should not expect the proteins of dead cells to become labeled. The effect of carbon tetrachloride on nitrosamine carcinogenesis has not been tested, but it may be relevant that the halogenated compounds, halothane and methoxyfluorane, did not affect the incidence of liver tumors (though they did affect the type of tumor) in mice treated with diethylnitrosamine.[22]

In conclusion, the observed inhibitory effects on the labeling of liver proteins are well correlated with known effects on the metabolism of DMN, as measured by formaldehyde production *in vitro* or the rate of DMN disappearance *in vivo*. The results probably reflect changes in the rate of DMN metabolism and (perhaps) in the degree of protein methylation by DMN. The results therefore support the view of Magee and Barnes[1] that DMN must be metabolized before it can react with (and especially methylate) cell macromolecules, and complement the recent report by Fiume *et al.*[23] on the action of AAN. Since alkylation of macromolecules may be involved in DMN carcinogenesis,[1] the results help to explain the results of carcinogenesis experiments in which 3-MC and phenobarbitone were administered to nitrosamine-treated animals,[21,22] and suggest that DMN carcinogenesis would be inhibited by concurrent administration of the other test agents.

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