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Effect of 1,2-Dimethylhydrazine and Diethylnitrosamine on Cell Replication and Unscheduled DNA Synthesis in Target and Nontarget Cell Populations in Rat Liver Following Chronic Administration

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ABSTRACT

Marked differences in the rates of *de novo* DNA synthesis and unscheduled DNA synthesis were observed in hepatocytes and nonparenchymal cells (NPC) isolated from Fischer 344 rats following *in vivo* exposure to 1,2-dimethylhydrazine (SDMH) or diethylnitrosamine (DEN) for 1.5, 4, 8, 16, and 28 days. Liver cells were labeled *in vivo* with bromodeoxyuridine and [^{3}H]thymidine, following which, hepatocytes and NPC were separated by elutriation centrifugation. Purified DNA was then separated into fully replicated and nonreplicated DNA by ultracentrifugation in CsCl gradients. Following exposure to SDMH, the rate of *de novo* DNA replication in NPC was greatly increased over both control NPC and the corresponding hepatocytes for the entire time course. Unscheduled DNA synthesis was only observed in hepatocytes after exposure to SDMH. Following exposure to DEN, both cell populations exhibited changes in *de novo* DNA synthesis that were directly proportional to one another except at 1.5 days. Increases occurred first in hepatocytes at 1.5 days but were delayed in NPC until 4 days. Hepatocyte replication returned to control values by 28 days. Unscheduled DNA synthesis was much lower following exposure to DEN than SDMH. Therefore, SDMH, which induces hemangioendotheliomas of the liver at the dose given, induces a marked increase in cell replication in the target cell population and unscheduled DNA synthesis in the resistant cell population. DEN, which induces hepatocellular carcinoma, moderately stimulates cell replication in both liver cell populations and induces little unscheduled DNA synthesis in either population.

INTRODUCTION

The replication of DNA containing promutagenic damage is thought to be necessary for the initiation of carcinogenesis (4). Evidence supporting this theory includes the fact that DEN[2] is a more potent hepatocarcinogen in young rats, and a single dose of dimethylnitrosamine rarely induces liver tumors in rats unless a potent stimulus for cell replication, such as partial hepatectomy, is also given (4, 16). It is postulated that the mitogenic stimulus is required because the rate of normal liver cell replication is so low that promutagenic DNA damage is repaired before the DNA replicates. Following partial hepatectomy, a wave of cell replication occurs causing the damaged DNA to replicate. Abanobi *et al.* have demonstrated that, following partial hepatectomy, the actual DNA containing the promutagenic adduct-O6MG is replicated, and the majority of the adducts are in a stable, S_1 nuclease-resistant conformation (1). Since the rate of cell replication in normal rat liver is low and liver possesses efficient mechanisms for removal of O^6-alkylguanine, it is unclear why chronic administration of low doses of DEN, dimethylnitrosamine, or SDMH induce liver tumors (6, 7, 13, 15, 19).

Previous studies on the effect of chemical carcinogens on cell replication *in vivo* have utilized either autoradiography or scintillation counting following the incorporation of [^{3}H]thymidine (17). Autoradiography allows identification of the labeled cell type but is relatively insensitive at distinguishing *de novo* from repair synthesis. Biochemical methods which separate *de novo* synthesis from repair synthesis using hydroxyurea or BrdUrd usually use whole organ DNA and do not distinguish which cell type is replicating. This latter point is particularly important when studying liver carcinogenesis. SDMH and DEN are both hepatocarcinogens when administered chronically at low doses in the drinking water to rats (3 mg/kg and 40 ppm, respectively), but DEN induces hepatocellular carcinomas (16, 19) and SDMH induces malignant hemangioendotheliomas (6, 7). We have shown previously that, following administration of [^{14}C]SDMH, O6MG was rapidly removed from the DNA of hepatocytes but remained in the DNA of the NPC (11). Furthermore, O6MG selectively accumulated in the NPC following a second dose of SDMH. Studies utilizing chronic administration of SDMH demonstrated that levels of O6MG in the NPC increased during the first 8 days of administration and declined over the next 20 days (10). In contrast, the amount of O6MG in hepatocytes decreased rapidly during the first 3 days of carcinogen administration and remained at low levels thereafter.

Preliminary evidence for an increased rate of cell turnover in the target cell population was suggested in the [^{14}C]SDMH experiment by the selective metabolic incorporation of ^{14}C into normal purines of NPC (11). Previous studies using whole-liver DNA or autoradiography have shown increased incorporation of [^{3}H]thymidine following exposure to DEN and SDMH (5, 12, 18). However, none of these investigations quantified *de novo* and repair synthesis in specific hepatic cell populations. We report here that SDMH and DEN have markedly different effects on replication of target and nontarget cell populations in the rat liver. Thus, under exposure conditions that selectively induce tumors of hepatocytes or NPC, the combination of cell

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[2] The abbreviations used are: DEN, diethylnitrosamine; O6MG, O^6-methylguanine; SDMH, 1,2-dimethylhydrazine; BrdUrd, bromodeoxyuridine; NPC, nonparenchymal cells.

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replication and persistant promutagenic DNA damage correlate with carcinogenesis in the target cell population.

MATERIALS AND METHODS

Animals

All animals were male COBS® CDF® (F344)/CrlBR rats that weighed 150 to 250 g at the beginning of the experiments. They were maintained on a 12-hr light-dark cycle throughout a 2-week acclimation period and during carcinogen exposures. Laboratory chow (Wayne Certified Lab Blox, Allied Mills, Chicago, Ill.) was provided *ad libitum*, and all animals were housed 3/cage throughout the experiments.

Materials

BrdUrd, SDMH dihydrochloride, and type 1 collagenase were obtained from Sigma Chemical Co., St. Louis, Mo. DEN was obtained from Eastman Kodak Co., Rochester, N. Y. [^{3}H]Thymidine (specific activity, 21 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. Hanks' balanced salt solutions with and without Ca^{2+} and Mg^{2+}, and phenol red were obtained from Grand Island Biological Co., Grand Island, N. Y. CsCl was obtained from Varicoid Chemical Co., Elizabeth, N. J.

Carcinogen Exposures

SDMH. Rats were weighed and housed 3/cage, and water consumption was measured for 7 days. Then SDMH was added to deionized water such that the dose of SDMH would be approximately 3 mg/kg/day based on average weight and water consumption per cage. Water and carcinogen were kept in amber bottles and changed each 5 days.

DEN. Rats were housed 3/cage and exposed to DEN in drinking water at 40 ppm. Water containing DEN was kept in amber water bottles and changed every 5 days.

Experimental Design

Groups of 3 animals were exposed to SDMH or DEN in the drinking water continuously for 1.5, 4, 8, 16, and 28 days. On the morning of sacrifice, a 300-mg pellet of BrdUrd prepared in a pellet press (Parr Instrument Co., Moline, Ill.) at 250 inch-lb of torque was inserted s.c. After 1 hr, 5 hourly injections of [^{3}H]thymidine were given i.p. to a final dose of 0.5 μCi/g of body weight. One hr following the last injection, the rats were anesthetized with sodium pentobarbital (Abbott Laboratories, North Chicago, Ill.), the livers were perfused with collagenase (3), and the resultant mixed liver cell suspension was separated into hepatocytes and NPC by elutriation centrifugation as described previously (11). DNA was purified by hydroxyapatite chromatography (2), and the replicated fully-BrdUrd-substituted DNA was separated on neutral CsCl gradients (density, 1.72 g/ml). The density was determined by reading the refractive index (1.4012) on a refractometer (Bausch & Lomb, Rochester, N. Y.). Samples were centrifuged in a Sorvall Tv850 vertical rotor at 40,000 rpm (129,000 rcf) in a Sorvall OTD65 ultracentrifuge for at least 40 hr. Gradients were harvested from the top by pumping Fluoinent (Instrument Specialities Co., Lincoln, Nebr.) into the bottom of the tubes (1.5 ml/min) in an ISCO Model 185 gradient fractionater. UV absorbance was monitored with a LKB 2138 Unicord S detector and traced on a Linear Instruments recorder. Fractions of 1.5 ml were collected, 0.5 ml was taken for scintillation counting, the remainder of the samples containing the DNA were combined, and the amount of DNA was quantified by UV absorbance. Data were calculated as dpm in either the normal density (unscheduled synthesis) or BrdUrd-labeled DNA (*de novo* synthesis) per mg of total DNA. Means were compared using Student's *t* test (2-tailed). The control animals were treated in the same manner to determine normal levels of replication in hepatocytes and NPC.

RESULTS

Representative UV and radioactive profiles of gradients containing DNA from hepatocytes or NPC of a control animal and a rat exposed to SDMH for 8 days are presented in Chart 1. In control animals, all of the radioactivity sedimented to a more dense position in the gradient than did the unlabeled DNA. Activity was low in hepatocytes and 2.2-fold higher in NPC. After 8 days of exposure to SDMH, there was a slight increase in radioactivity in the density-labeled DNA of hepatocytes and a small peak of activity in the normal density DNA. The corresponding NPC exhibited a striking increase in radioactivity in the density-labeled DNA and only background levels of radioactivity in the normal density of DNA.

Following administration of SDMH for 1.5, 4, 8, 16, and 28 days, there was a large increase in *de novo* DNA synthesis by NPC over both control NPC and the corresponding hepatocytes (Chart 2). Maximal DNA synthesis was reached by 16 days and remained at that level through 28 days. *De novo* DNA synthesis in hepatocytes was slightly increased over control levels following 1.5 days of administration of SDMH, returned to control levels after 8 days of exposure, and increased again at 16 and 28 days. The NPC/hepatocyte ratio of *de novo* DNA synthesis increased sharply through 8 days and declined thereafter because of increased activity in hepatocytes (Chart 4).

After 1.5 days of exposure to DEN, *de novo* DNA synthesis in hepatocytes increased significantly. No increase in NPC *de novo* synthesis was observed until 4 days (Chart 3). Following exposure for 4, 8, and 16 days, both cell populations had significant increases in *de novo* DNA synthesis (Chart 3). These increases were similar in proportion so that the NPC/hepatocyte ratio of *de novo* synthesis was the same as controls (Chart 4). Following 28 days of exposure, the rate of *de novo* synthesis in hepatocytes had returned to control levels, and the rate in NPC had also decreased significantly (Chart 3).

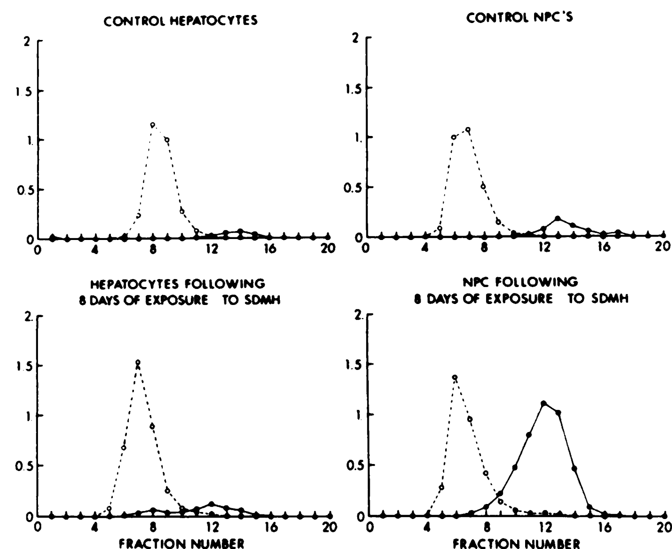

Chart 1. Representative UV (○) and radioactive (●) profiles of CsCl gradients harvested from the top containing DNA from hepatocytes and NPC. Presented are profiles of a control animal and one exposed to SDMH (3 mg/kg/day) in the drinking water for 8 days. On the morning of sacrifice, liver cell DNA was labeled with BrdUrd and [^{3}H]thymidine *in vivo* as described in "Materials and Methods." Hepatocytes and NPC were separated by centrifugal elutriation, and the replicated (BrdUrd-labeled) DNA was separated in CsCl gradients. Data are normalized to 1 mg of DNA for comparison.

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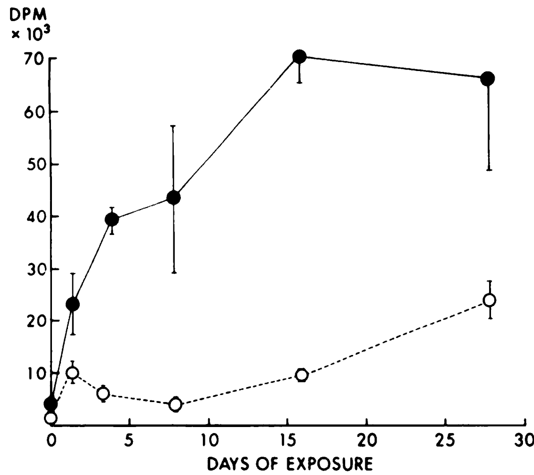

Chart 2. Measurement of dpm/mg DNA in the replicated (BrdUrd-labeled) DNA of hepatocytes (○) and NPC (●) from animals exposed to SDMH in the drinking water (3 mg/kg/day) for various times. On the morning of sacrifice, liver cell DNA was labeled with BrdUrd and [^{3}H]thymidine *in vivo* as described in "Materials and Methods." Hepatocytes and NPC were separated by centrifugal elutriation, and the replicated (BrdUrd-labeled) DNA was separated in CsCl gradients as described in "Materials and Methods." Activity is expressed as dpm/mg total DNA in the more dense DNA. Zero time represents control values. *Time points,* means for 3 animals; *bars,* S.E.

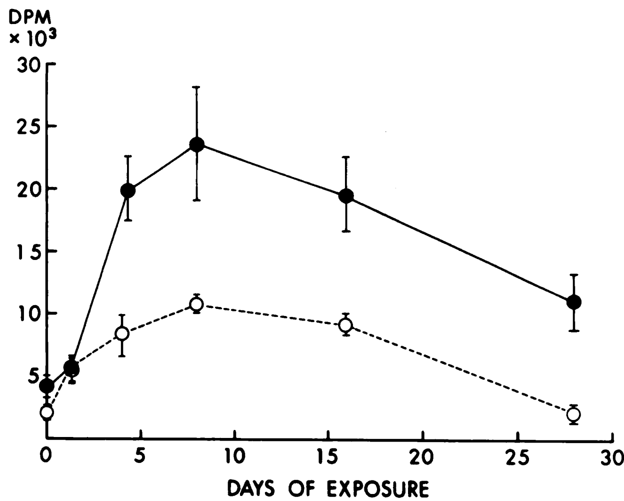

Chart 3. Measurement of dpm/mg DNA in the replicated (BrdUrd-labeled) DNA of hepatocytes (○) and NPC (●) from animals exposed to DEN in the drinking water (40 ppm) for various times. On the morning of sacrifice, liver cell DNA was labeled with BrdUrd and [^{3}H]thymidine *in vivo* as described in "Materials and Methods." Hepatocytes and NPC were separated by centrifugal elutriation, and the replicated (BrdUrd-labeled) DNA was separated in CsCl gradients as described in "Materials and Methods." Activity is expressed as dpm/mg total DNA in the more dense DNA. Zero time represents control values. *Time points,* means for 3 animals; *bars,* S.E.

Significant levels of radioactivity found in the normal density DNA, which represent unscheduled DNA synthesis, were observed almost exclusively in hepatocytes (Table 1). The highest levels of unscheduled synthesis occurred in hepatocytes following administration of SDMH for 1.5, 4, 8, and 16 days. Lower levels of unscheduled DNA synthesis were observed in hepatocytes following exposure to DEN for 4 and 16 days. A small but significant increase over controls was also observed in the NPC following exposure to DEN for 8 days.

DISCUSSION

Chronic administration of DEN or SDMH at doses which induce hepatocellular carcinomas or malignant hemangioendotheliomas of the liver, respectively, have strikingly different effects on cell replication in the different cell populations. SDMH causes a selective increase in cell replication in the target cell population (Chart 2). Furthermore, this increase occurs during the first 2 weeks of SDMH administration, a period in which O6MG formation exceeds removal (10). In contrast, much smaller elevations in hepatocyte replication were observed, with greatest increases after 16 and 28 days of exposure. This is during the period that O6MG levels in hepatocytes are extremely low (10). Whether this increase in hepatocyte replication is due to SDMH toxicity or to the increased turnover of vascular lining cells is unclear.

During exposure to DEN, both hepatocytes and NPC exhibited comparable increases in cell replication at 4 and 8 days and similar decreases at 16 and 28 days. Replication of hepatocytes was significantly elevated after 1.5 days of exposure, but increased NPC was not detected until 4 days. The increase

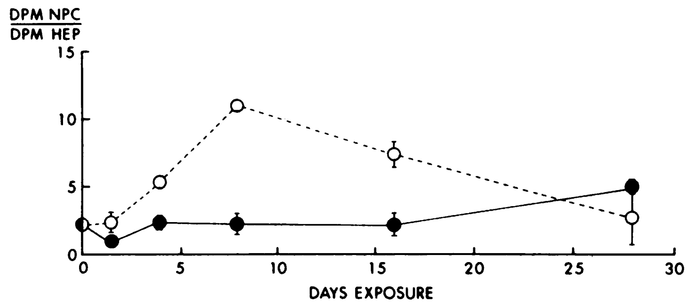

Chart 4. Ratio of NPC/hepatocyte *de novo* DNA synthesis after exposure to SDMH (○) or DEN (●) for various periods of time. Animals were exposed to SDMH (3 mg/kg/day) and DEN (40 ppm) in the drinking water for various periods of time. On the morning of sacrifice, liver cell DNA was labeled with BrdUrd and [^{3}H]thymidine as described in "Materials and Methods." Hepatocytes and NPC were separated by centrifugal elutriation, and the replicated (BrdUrd-labeled) DNA was separated in CsCl gradients. Activity is expressed as dpm/mg total DNA in the more dense DNA. Zero time represents control values. *Time points,* means for 3 animals; *bars,* S.E.

Table 1

Effect of SDMH or DEN administration on unscheduled DNA synthesis in hepatocytes and NPC

Rats were exposed to DEN (40 ppm) or SDMH (3 mg/kg/day) in the drinking water for various periods of time. The DNA was labeled with BrdUrd and [^{3}H] thymidine *in vivo* as described in "Materials and Methods." The cells were separated by centrifugal elutriation, and the replicated more-dense DNA was separated in CsCl gradients as described in "Materials and Methods." Radioactivity contained in the normal density DNA was defined as unscheduled DNA synthesis.

Carcinogen	Time of exposure (days)	NPC (dpm/mg DNA)	Hepatocyte (dpm/mg DNA)
Control	0	95 ± 26[a]	71 ± 31
SDMH (3 mg/kg/day in the drinking water)	1.5	87 ± 68	2027 ± 147[b, c]
	4	0	718 ± 218[b, c]
	8	47 ± 22	1277 ± 256[b, c]
	16	0	1307 ± 215[b, c]
	28	267 ± 90	191 ± 64
DEN (40 ppm in the drinking water)	1.5	61 ± 53	416 ± 174
	4	240 ± 83	372 ± 92[b]
	8	381 ± 91[b]	242 ± 164
	16	186 ± 108	239 ± 32[b]
	28	0	706 ± 492

[a] Mean ± S.E.
[b] Significantly different than control ($p < 0.05$).
[c] Significantly different than corresponding treated cell type ($p < 0.05$).

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in NPC at 4 days was such that the normal ratio replication of NPC to hepatocyte returned to the control value (Chart 4). The delayed response in NPC leading to a restoration of the control NPC/hepatocyte ratio suggests that the NPC may be dividing in response to the loss of underlying hepatocytes. Conversely, the selective toxic effects of SDMH on NPC indicate that large losses of vascular lining cells can be tolerated with only small increases in hepatocyte replication.

Another significant aspect of the hepatocyte response to DEN exposure was that, by 28 days, the rate of replication had returned to that of controls. This suggests that, even with continuous administration of DEN, there may be distinct initiation and promotional stages to the action of DEN. It has been demonstrated that administration of DEN in a similar manner for just 4 weeks induces a 13% incidence of hepatocellular carcinomas at 20 weeks (19). If, however, animals are treated with phenobarbital for an additional 6 weeks, there is an 89% incidence of hepatocellular carcinoma. This is similar to the 100% incidence achieved when rats are treated for 10 weeks with DEN. Since the rate of hepatocyte replication returned to control values at 4 weeks, the response to an additional 6 weeks of exposure to DEN is not simply due to increased DNA replication. Selective replication of initiated cells cannot be ruled out, however.

In contrast, the rate of NPC replication during exposure to SDMH remained elevated at the highest level during the third and fourth week. This may reflect the inability of NPC to adapt to SDMH cytotoxicity. Levels of O6MG at 28 days are approximately one-third that present at 8 days (10). The potential for initiation, *i.e.,* replication of DNA containing O6MG, is greatest during the first month of SDMH administration even though the carcinogen is administered for the life span of the rat. Promotion effects of SDMH may reside in its selective mitogenic potential.

Following administration of SDMH, unscheduled DNA synthesis was mainly detected in hepatocytes. The highest activity was observed at 1.5 days and had returned to control values by 28 days. This correlates with the fact that hepatocytes are resistant to carcinogenesis with SDMH and have a high capacity for DNA repair (13, 14). The type of lesion repaired and the specific mechanism of repair cannot be determined from these data. Since hepatocytes continually remove O6MG during the 28-day exposure (11) and detectable unscheduled DNA synthesis decreases over 28 days, these data suggest that unscheduled DNA synthesis does not reflect repair of O6MG. This observation is consistent with repair of O^6-alkylguanine by specific glycosylases or demethylases since [^{3}H]thymidine would not be incorporated (8, 9, 14). Much lower levels of unscheduled DNA synthesis were observed after exposure to DEN. This may reflect the lower extent of alkylation achieved when ethylating agents *versus* methylating agents are administered at nearly equamolar doses.

In summary, we have demonstrated that SDMH induces high levels of replication in NPC during the period of maximal O6MG and induces unscheduled DNA synthesis selectively in hepatocytes. Except for an initial selective increase in hepatocyte replication after 1.5 days of administration, DEN induced similar changes in cell replication in both cell populations.

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