ABSTRACT

Chronic exposure to the hepatocellular carcinogen diethylnitrosamine (DEN) causes a dose-dependent accumulation of the promutagenic DNA adduct O^-ethyldeoxythymidine in hepatocytes and increases in the number of initiated hepatocytes as indicated by γ-glutamyl transpeptidase positive foci. Initiation is thought to be dependent on the quantity of promutagenic DNA adducts, their efficiency for causing base pair mismatch, and the extent of replication in the target tissue. If the extent of replication is also dose dependent, then this dependence could alter the number of promutagenic DNA adducts that mispair prior to repair and enhance the clonal expansion of initiated cells.

We have examined the effect of DEN on hepatocellular proliferation over a wide range of doses. Six-week-old male F-344 rats were exposed to drinking water containing 0.4, 1, 4, 10, 40, or 100 ppm DEN for 1, 4, or 10 weeks. Following exposure to DEN, rats were injected i.p. with [3H]thymidine, sections from the left, right median and anterior right lobes of the liver were processed for autoradiography and the labeling index of hepatocytes determined. A progressive increase in hepatocyte replication was induced by exposure to 40 and 100 ppm DEN. This was especially marked in the left lobe where 40 and 100 ppm DEN induced increases of 800 and 1500%, respectively, over controls after 10 weeks of exposure. Exposure to 4 and 10 ppm DEN resulted in a 300 to 400% increase in hepatocyte replication in all lobes, whereas 1 and 0.4 ppm DEN did not significantly increase cell proliferation compared to unexposed controls.

INTRODUCTION

Cell replication is an essential step in the carcinogenic process (1–3). It is necessary for converting promutagenic lesions in DNA to mutations, for clonally expanding the number of initiated cells (4), and for fixing additional mutagenic events in initiated cells (5). While there have been numerous studies on dose and time relationships for chemical carcinogens and cell proliferation, there have been few reports examining dose responses covering orders of magnitude and using extended periods of carcinogen exposure (6). If carcinogen-induced cell proliferation is elevated with increasing dose, the quantitative relationships between DNA adducts, cell proliferation, and tumor induction. Therefore, we have examined differences in labeling indices of hepatocytes in the left, right median and anterior right lobes of rat livers during 10 weeks of continuous administration of drinking water containing DEN in concentrations ranging from 0.4 to 100 ppm.

MATERIALS AND METHODS

Male Fischer-344 rats (4 weeks old, 90–120 g) from Charles River Breeding Laboratories (Kingston, NY) were housed in polypropylene shoe box type cages on hardwood chip bedding. The animals were maintained on a 12-h light-dark cycle at a temperature of 22 ± 1°C and 55 ± 5% humidity in a room with a filtered air supply. Following a 2-week quarantine, rats were randomized into groups of seven (three or four per cage). Animals received NIH-07 rat chow and distilled deionized water containing 0, 0.4, 1, 4, 10, 40, or 100 ppm DEN (Eastman Kodak, Rochester, NY) for 1, 4, or 10 weeks ad libitum. DEN consumption was monitored at regular intervals throughout the experiment. At the end of the exposure period, animals were injected i.p. with [3H]thymidine (NEN, Boston, MA, specific activity, 6.7 Ci/mmol) at a concentration of 1 μCi/g body weight. Two h after injection, rats were killed by CO2 asphyxiation, livers were weighed and sections were taken from the left, right median and anterior right lobes of the liver. Liver sections were fixed in formalin, embedded in paraffin, sectioned at 5 μm and fixed to glass slides. The slides were then dipped in NBT2 emulsion (Eastman Kodak, Rochester, NY), exposed for 3 weeks, developed, and counterstained with hematoxylin & eosin. The percentage of labeled hepatocytes in each section was determined by dividing the number of labeled hepatocytes by the total number of hepatocytes in 10–20 random fields. A minimum of 1000 hepatocytes per liver lobe were counted to determine percentage labelling. A Neuman-Keuls multicomparison test (P < 0.05) was used to analyze differences between dose groups within each time point, and Student’s t test was used to analyze differences within dose groups.
RESULTS

The rate of water consumption per kilogram of body weight and thus DEN consumption, decreased with time for all dose groups (Fig. 1). Throughout the experiment the 100 ppm dose groups consumed proportionately less DEN than the lower dose groups. The 40 ppm dose groups also consumed proportionately less DEN during the final 3-4 weeks. Consumption curves were generated using the function $DEN(t) = ae^{-bt} + b$, where $t$ is time in days, $a$ is the difference between the initial and asymptotic rates of DEN consumption during long periods of exposure, and $k$ is the rate constant governing the decrease in the rate of DEN intake.

A dose-dependent increase in the labeling indices was evident after 1 week of administration in all three lobes examined (Table 1). In the 100-ppm group, labeling indices in the left and right median lobes continued to increase throughout the experiment. A progressive increase in labeling index was seen only in the left lobe of rats exposed to 40 ppm. Labeling indices in lower dose groups decreased from week 1 to week 4, after which they remained constant until termination of the experiment. Labeling indices in the 0.4- and 1-ppm dose groups were not significantly different from controls. The 4-ppm dose group showed significant increases compared to control, which varied with time and between lobes, whereas all lobes and times were significantly increased in animals exposed to 10 ppm (Table 1).

Labeling indices as a percentage of control in the left lobe were significantly greater than in the right median and anterior right lobes of animals after 1 week and 4 weeks of exposure, but by 10 weeks the right median lobe had relative indices significantly higher than the left or anterior right lobes (Fig. 2).

Histopathological changes were monitored on sections used to determine labeling indices and these revealed concentration-, time-, and lobe-related changes in the liver. This was seen as scattered clusters of hepatocytes with mild vacuolization at the earliest time point in rats exposed to 100 ppm DEN and progressed to random areas of nodular hyperplasia with cytomegaly, karyomegaly, and loss of normal arrangement of hepatic laminae at the latest time point at 100 ppm. Cytotoxic effects were localized predominantly in the midzonal region of the lobules examined and to a lesser extent in the centrilobular regions. The left lobe appeared to be most affected by DEN, while the right anterior lobe was least affected.

Labeled hepatocytes were randomly scattered and did not appear to be zonally organized in any of the lobes examined. There was a trend towards increased labeling in some of the areas of nodular hyperplasia in the left lobe after 10-week exposure to 100-ppm DEN, although this could not be clearly established due to the small number of nodules that were present.

DISCUSSION

Hepatocellular replication, as indicated by incorporation of tritiated thymidine during S phase, was examined over a DEN dose range that was previously used in tumor (8-10), DNA adduct (11), and hepatocyte initiation studies (14). Results indicate that rates of replication can be heterogeneous between lobes and are time- and concentration-dependent. Since relative liver weights did not increase during treatment, except for those of the exposures to 100 ppm DEN, for 10 weeks, it is assumed that the increase in the rate of replication relative to time and concentration was due to compensatory hyperplasia that occurs in response to the cytolethal effects of DEN (16-18). The increase in liver weight in rats exposed to 100 ppm DEN for 10 weeks may also represent proliferation associated with nodular hyperplasia and early neoplasia since this is a fully carcinogenic exposure regimen (18). Histopathological observations were comparable to previous studies employing necrogenic and subnecrogenic doses of DEN (19, 20).

Rates of hepatocyte replication were time- and concentration-dependent at the higher doses, reached a plateau in the intermediate doses and were not significantly greater than controls in the lower doses. Results suggest that a dose of 4 ppm is very

Table 1 Effect of DEN administration on labeling indices in the left, right median and anterior right liver lobes

<table>
<thead>
<tr>
<th>Lobes</th>
<th>Control</th>
<th>0.4</th>
<th>1.0</th>
<th>4.0</th>
<th>10.0</th>
<th>40.0</th>
<th>100.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>0.24 ± 0.07</td>
<td>0.62 ± 0.10</td>
<td>0.49 ± 0.11</td>
<td>1.14 ± 0.13*</td>
<td>1.44 ± 0.25*</td>
<td>1.17 ± 0.09*</td>
<td>1.47 ± 0.24*</td>
</tr>
<tr>
<td>Right median</td>
<td>0.33 ± 0.06</td>
<td>0.63 ± 0.11</td>
<td>0.50 ± 0.15</td>
<td>0.86 ± 0.07*</td>
<td>1.10 ± 0.22*</td>
<td>1.12 ± 0.10*</td>
<td>1.46 ± 0.18*</td>
</tr>
<tr>
<td>Anterior right</td>
<td>0.39 ± 0.14</td>
<td>0.75 ± 0.16</td>
<td>0.88 ± 0.19</td>
<td>0.90 ± 0.10</td>
<td>0.92 ± 0.22*</td>
<td>1.64 ± 0.13*</td>
<td>1.42 ± 0.21*</td>
</tr>
<tr>
<td>Week 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>0.30 ± 0.08</td>
<td>0.30 ± 0.02</td>
<td>0.38 ± 0.06</td>
<td>0.77 ± 0.07*</td>
<td>0.79 ± 0.11*</td>
<td>1.29 ± 0.14*</td>
<td>2.71 ± 0.26*</td>
</tr>
<tr>
<td>Right median</td>
<td>0.28 ± 0.08</td>
<td>0.23 ± 0.03</td>
<td>0.24 ± 0.06</td>
<td>0.47 ± 0.03</td>
<td>0.81 ± 0.07*</td>
<td>1.27 ± 0.11*</td>
<td>1.72 ± 0.16*</td>
</tr>
<tr>
<td>Anterior right</td>
<td>0.35 ± 0.06</td>
<td>0.36 ± 0.05</td>
<td>0.34 ± 0.06</td>
<td>0.37 ± 0.05</td>
<td>0.68 ± 0.07*</td>
<td>1.07 ± 0.07*</td>
<td>1.84 ± 0.20*</td>
</tr>
<tr>
<td>Week 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>0.25 ± 0.02</td>
<td>0.28 ± 0.04</td>
<td>0.33 ± 0.10</td>
<td>0.74 ± 0.05</td>
<td>0.80 ± 0.09*</td>
<td>2.00 ± 0.20*</td>
<td>3.87 ± 0.41*</td>
</tr>
<tr>
<td>Right median</td>
<td>0.12 ± 0.05</td>
<td>0.28 ± 0.05</td>
<td>0.26 ± 0.09</td>
<td>0.55 ± 0.07</td>
<td>0.64 ± 0.10*</td>
<td>1.38 ± 0.23*</td>
<td>2.37 ± 0.16*</td>
</tr>
<tr>
<td>Anterior right</td>
<td>0.18 ± 0.04</td>
<td>0.20 ± 0.05</td>
<td>0.27 ± 0.05</td>
<td>0.55 ± 0.11*</td>
<td>0.71 ± 0.09*</td>
<td>1.30 ± 0.09*</td>
<td>1.97 ± 0.23*</td>
</tr>
</tbody>
</table>

* Doses of DEN that produced labeling indices which were significantly higher than control indices at 1, 4, and 10 weeks in the left, right median and anterior right liver lobes, when analyzed by Neuman-Keuls multicomparison test ($P \leq 0.05$). Differences between lobes for each time was analyzed by Student's $t$ test ($P \leq 0.05$). Variation is expressed as the standard error of the mean.
near the lowest concentration of DEN which will induce a sustained increase in hepatocellular replication. Peto et al. (8) have shown that the concentration of DEN which results in a sharp increase in the slope of the Weibull curve for median time to hepatocellular tumor is between 1.5 and 4 ppm. We have previously shown that the dose response of O\textsuperscript{4}EtdT, the major promutagenic DNA adduct, is linear during chronic exposure to 0.4 to 40 ppm DEN (13). The significant increase in replication at the concentration of DEN that also results in a decrease in time to tumor suggests that the increase in hepatocyte replication is a major factor linking the nonlinear tumor response with the linear accumulation of DNA adducts. Although the groups exposed to intermediate doses of DEN have rates of replication significantly higher than controls, these rates do not increase progressively with time, but reach a plateau after the 4th week of the experiment. The liver's ability to dynamically compensate for cell loss as a result of toxic injury is well established (3, 16, 18, 21). The concentration-dependent increase in labeling in the absence of increased liver weight suggests that the liver is adapting to the cytotoxic effects of DEN at concentrations of 40 ppm and lower. The cytotoxic effects of DEN at the highest dose may be so severe that the liver can no longer adapt, and/or may represent proliferation associated with hyperplasia and early neoplasia. This increased cell death may explain why the concentrations of O\textsuperscript{4}EtdT were less than proportional to DEN concentrations during exposures to 100 ppm DEN (13). Death of initiated hepatocytes also represents a plausible explanation for the plateauing of hepatocyte initiation in rats exposed to 40 ppm DEN (14, 15). It has been noted in previous studies that hepatocytes in neoplastic nodules exhibit higher rates of DNA synthesis and mitotic activity than do normal hepatocytes (17, 22-24). These higher rates of replication are above the needs for homeostasis of cell number in the liver and may contribute to the progressive increase of labeling indices observed at the highest concentration in this study.

Interlobular differences of hepatocellular replication parallel observed interlobular differences in HCCA formation. Greater incidences of HCCA have been observed in the left lobe, followed by the right median and anterior right lobes during chronic administration of DEN (15). The greater labeling indices in the left lobe correlate with the preferential distribution to and/or metabolism of DEN in the left lobe, followed by the right median and anterior right lobes (14). Studies have also shown that O\textsuperscript{4}EtdT accumulation and GGT+ focus induction, relative to number and volume, show a similar lobe distribution (14).

This study indicates that DEN-induced changes in rates of replication are nonlinear with respect to administered dose and time, and correlate with incidence of hepatocellular carcinomas observed in previous studies. This nonlinearity has implications for the extrapolation of risks to low-dose exposure for environmental agents. Initiation, promotion, and progression are major factors involved in the carcinogenic process and cell replication is an essential determinant in each of these factors. We have demonstrated that the extent of cell proliferation can vary greatly between high and low exposures and over time. Risk assessments which use high- to low-dose extrapolations to predict tumor incidence depend on an agent's effect on rates of cell initiation and/or cell proliferation (7). Therefore, extrapolation from high to low exposure should include information on how agents affect rates of replication in order to make predictions of tumor incidence more accurate.

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Dose Response of Hepatocyte Replication in Rats following Continuous Exposure to Diethylnitrosamine

Frank H. Deal, Frank C. Richardson and James A. Swenberg


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