Effects of Delays in the Cell Cycle on the Induction of Preneoplastic and Neoplastic Lesions in Rat Liver by 1,2-Dimethylhydrazine¹

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ABSTRACT

This study was designed to explore further the relationship between cell proliferation and the induction of early putative preneoplastic lesions by carcinogens. Rats were given a non-necrogenic dose of 1,2-dimethylhydrazine 24 hr before being subjected to partial hepatectomy. Beginning 4 hr later, hydrocortisone was injected 10 times at 4-hr intervals to delay progression through the cell cycle, including inhibition of DNA synthesis by at least 85% for about 40 hr. At the appropriate time thereafter, the putative preneoplastic hepatocytes were selectively stimulated to grow in vivo into γ-glutamyltransferase-positive focal lesions. Animals given hydrocortisone showed a large decrease (71%) in the number of γ-glutamyltransferase-positive foci. In contrast, when hydrocortisone was given at 6 days after partial hepatectomy, no inhibition in the induction of hepatic lesions was observed. In the next experiments, rats were treated with 1,2-dimethylhydrazine and were subjected to partial hepatectomy at 12, 24, or 48 hr or 1 week thereafter. A significant number of γ-glutamyltransferase-positive foci was found when partial hepatectomy was performed at 12 or 24 hr but far fewer were found when the operative partial hepatectomy was delayed to 48 hr or 1 week later. Similarly, in long-term experiments, six of 14 animals developed primary hepatocellular carcinoma 13 months after the time of injection of 1,2-dimethylhydrazine when partial hepatectomy was performed at 12 hr, while none of the animals developed liver cancer when the operation was performed at 48 hr. These results imply that the majority of biochemical lesions induced by 1,2-dimethylhydrazine that are relevant to the induction of liver preneoplasia and neoplasia are short-lived and that their persistence is associated with some cellular activity closely related to the cell cycle.

INTRODUCTION

Accumulating evidence indicates the importance of an early episode of cell proliferation in the development of cancer in many experimental systems (4, 24, 26, 37). For the liver, the most convincing older data came from the many studies showing that carcinogens, ordinarily not carcinogenic for the liver, may become so if administered even as a single dose in close association with PH (see Ref. 4 for other references). More recently, it has been shown that cell proliferation is essential for the induction of very early focal lesions in the liver during initiation (2, 3, 35, 38, 39). However, the precise role of cell proliferation in the initiation process is not clear. A common hypothesis visualizes its predominant role as stimulating DNA replication which somehow "fixes" permanently one or more biochemical lesions in DNA in the daughter strands (4, 25).

Utilizing one biological model for the sequential analysis of liver carcinogenesis (2, 31), we showed previously that 1,2-DMH, a colon carcinogen, could induce presumptive preneoplastic foci of altered cells in rat liver only when coupled with a cell-proliferative stimulus such as PH (35, 38). To further elucidate the role of cell proliferation in hepatocarcinogenesis, the present study was conducted to investigate whether 2 different types of delays in the cell cycle would influence the induction of hepatic preneoplasia and neoplasia by 1,2-DMH in a similar fashion.

In designing the initial experiments, use was made of classical inhibitors of DNA synthesis, such as hydroxyurea. Attempts to use this approach were unsuccessful, because of the early death of the animals. Resort was then made to the use of HC. This compound, an effective inhibitor of DNA synthesis (27), was successfully used recently in a study using a single dose of MNU as the carcinogen and liver cancer appearance as the end point (16). With this approach, the progression through the cell cycle was delayed by administration of HC during G1 after the injection of 1,2-DMH in association with PH, and the effect of this treatment on the genesis of very early presumptive preneoplastic foci of resistant hepatocytes was measured. In addition, the effect of delaying the time of performance of PH after the administration of the carcinogen on the induction of presumptive preneoplastic and neoplastic lesions was observed. The results of these experiments are the subject of this paper.

MATERIALS AND METHODS

Chemicals

1,2-DMH (Aldrich Chemical Co., Milwaukee, Wis.) was recrystallized with ether before use. Hydrocortisone-21-sodium succinate and other biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo. [methyl-3H]Thymidine (specific activity, 40 Ci/mmol) was purchased from New England Nuclear, Montreal, Quebec.

Animals

Male Fischer 344 rats (Charles River Breeding Laboratories, Wilmington, Mass.) were maintained on a semisynthetic diet containing 24% protein (Bio-Serv, Inc., Frenchtown, N. J.) and a daily cycle of alternating 12-hr periods of light and dark. The animals were given food and water ad libitum and were acclimatized to their environment until they weighed in the range of 175 to 200 g.
Experimental Regimen

Regimen 1 (Chart 1). Freshly dissolved 1,2-DMH in 0.9% NaCl solution containing 1.5 mg/100 ml EDTA (pH 6 to 7) was administered i.p. at a dose of 100 mg/kg body weight. In a previous study, this dose was found to be nonnecrogenic to male Fischer 344 rat liver cells. At 24 hr, a time by which 1,2-DMH has been reported to be completely metabolized (8, 9, 23), the rats were subjected to a standard two-thirds PH (12). Commencing at either 4 hr or 6 days after PH, HC was given at a dose of 50 mg/kg i.p. 10 times at 4-hr intervals with control animals receiving 0.9% NaCl solution in its place. After a 2-week recovery period, 3 rats from each of the experimental and control groups were killed, and their liver weights were compared. There was no difference between the 2 groups. The remaining animals were then placed on a "selection" regimen as described previously (2, 35, 36) in order to stimulate the rapid growth of resistant hepatocytes. The control groups C1 and C2 were given 0.9% NaCl solution in place of HC and 1,2-DMH, respectively.

Regimen 2 (Chart 2). Rats were given 1,2-DMH (100 mg/kg) i.p. and were subjected to PH at various time periods thereafter, i.e., at 12, 24, or 48 hr or 1 week. The experimental groups were designated as E3, E4, E5, and E6, respectively. After a recovery period, the animals were submitted to the "selection" protocol as described in Regimen 1. The control groups were subjected to sham hepatectomy at 12 hr after the administration of 1,2-DMH (C1) or given 0.9% NaCl solution in place of 1,2-DMH (C2).

Regimen 3 (Long-Term Study). Groups of rats from the experimental groups (E3 and E5) and the control groups (C1) were kept on the basal diet for 12 months following selection, at which time the experiments were terminated. At the time of death or of sacrifice at the termination time, the liver and other organs were carefully examined, and each liver lobe was sectioned, fixed in 10% formalin or ice-cold acetone, and processed for histological or histochemical examination, respectively. Various organs such as the lung, colon, and kidney were also analyzed for any possible changes including metastasis. The presence and number of nonmalignant hepatocyte nodules ("persistent hyperplastic nodules") and of hepatocellular carcinomas were noted.

Measurement of DNA Synthesis

Incorporation of [methyl-3H]thymidine into Liver DNA. Groups of 3 rats were treated with 1,2-DMH (100 mg/kg) and were subjected to PH at either 12 or 24 hr later. At indicated time intervals after the operation (i.e., 12, 16, 20, 28, 32, 36, 42, 48 hr), [methyl-3H]thymidine (50 μCi/100 g body weight) was administered i.p., and rats were killed 1 hr later. The excised livers from each group were weighed and homogenized in ice-cold 0.15 M NaCl-0.1 M EDTA, pH 8.0. Extraction of liver DNA from each homogenate was performed according to the method of Munro and Fleck (20). The radioactivity of the aliquots of acid-soluble supernatant was determined in Aquasol scintillation fluid (Amersham/Searle Corporation) in an Intertechnique liquid scintillation spectrometer. The concentration of DNA of the samples was measured by absorbance at 260 nm in a Beckman spectrophotometer.

Autoradiography. Groups of 4 rats were given 1,2-DMH (100 mg/kg) 24 hr before being subjected to PH. At 4 hr after PH, HC or 0.9% NaCl solution was administered 10 times i.p. at 4-hr intervals. In order to determine the cumulative labeling indices of hepatocytes, an injection of [methyl-3H]thymidine (50 μCi/100 g body weight) was given 1 hr after each injection of HC. The animals were subsequently killed at 1 hr after the tenth administration of radioactive thymidine, i.e., 42 hr after PH. Sections of liver were processed for autoradiography, while the remaining rats were utilized to determine the incorporation of [methyl-3H]thymidine into DNA.

Autoradiography was carried out on deparaffinized sections by coating with Kodak NTB-2 emulsion, exposing the emulsion for at least 2 weeks, and then developing and counterstaining with hematoxylin and eosin or periodic acid-Schiff for microscopic examination.

For the determination of hepatocyte labeling indices, the percentage of hepatocyte nuclei with more than 5 grains was counted, using a magnification of ×400. Labeling indices for individual livers were taken as the average value determined from counts of 10 random fields, each field consisting of about 150 to 200 cells.

Histochemical Staining for γ-GT. Liver harvested on the final day of each regimen was examined histochimically for the localization of γ-GT using the method described previously (22, 29). For quantitation of foci, the number of γ-GT-positive lesions was counted by low-power microscopy and expressed as foci per sq cm of sectional area. The area of liver section was measured with a planimeter.

RESULTS

Effects of 1,2-DMH on DNA Synthesis following PH. Since chemical carcinogens are known to inhibit cell proliferation and/or DNA synthesis following PH, initial experiments were designed to study the effect of 1,2-DMH on DNA synthesis triggered by PH. As shown in Chart 3, administration of 1,2-DMH 12 hr before PH did not alter the position of the peaks of [methyl-3H]thymidine incorporation but decreased the extent of labeling by about 30 to 35%. However, when PH was performed 24 hr after 1,2-DMH, essentially the same specific activity was found in the experimental and control animals (Chart 4).

Inhibition of DNA Synthesis following 1,2-DMH plus PH by Multiple Injections of HC. The next series of experiments was
The results presented in Table 1 show that the administration of 1,2-DMH at 24 hr prior to PH decreased the labeling index and the labeling of DNA by about 75% and 89%, respectively. The inhibition of DNA synthesis lasted for 4 to 6 hr after the last injection of HC, and rapid recovery thereafter was seen (data not presented).

The labeling index and the labeling of DNA were determined according to the method of Munro and Fleck (20). Results are obtained from duplicate samples of individual livers. Points, mean from the results on 3 rats; bars, S.E.

The amount of radioactive label in liver DNA was determined according to the method of Munro and Fleck (20). Results are obtained from duplicate samples of individual livers. Points, mean from the results on 3 rats; bars, S.E.

Conducted to analyze the inhibitory effects of HC on DNA synthesis after the administration of 1,2-DMH 24 hr before PH. The results presented in Table 1 show that the administration of 1,2-DMH at 24 hr prior to PH decreased the labeling index by 10% and the extent of DNA labeling by 23%. Multiple injections of HC beginning at 4 hr after PH drastically reduced the labeling index and the labeling of DNA by about 75% and 89%, respectively. The inhibition of DNA synthesis lasted for 4 to 6 hr after the last injection of HC, and rapid recovery thereafter was seen (data not presented).

Effects of HC Treatment on the Induction of γ-GT-positive Foci by 1,2-DMH with PH. Having established that 1,2-DMH does not inhibit liver DNA synthesis to a major degree following PH under these experimental conditions and having shown that HC could inhibit to a major extent DNA synthesis after 1,2-DMH and PH, it became of interest to study the effects of HC on the induction of foci with enzyme-altered hepatocytes by 1,2-DMH. Examination of the results in Table 2 clearly shows that, when treatment with HC was begun at 4 hr after PH (Group E1), a large decrease (71%) in the number of γ-GT-positive foci was seen. This was in contrast to the animals that received the initial injection of HC at 154 hr (E2) in which no significant decrease in the number of foci was seen compared to the appropriate controls (C1). The large majority of hepatocytes have traversed the S phase by this time. It is important to point out that the dose of 1,2-DMH used, 100 mg/kg, is on the part of the dose-response curve that is essentially linear with dose (38). Therefore, the conditions used in this study with γ-GT-positive foci as end point seem to be appropriate for a valid assay. In control rats that received PH plus HC (Group C2) but no 1,2-DMH, few if any foci were observed.

Effects of Time of Performance of PH on the Induction of Presumptive Preneoplastic and Neoplastic Lesions in Rat Liver by 1,2-DMH. As reported in an earlier publication (38), 1,2-DMH induced foci of altered, γ-GT-positive liver cells when PH was performed at 12 hr after the administration of the carcinogen. The results presented in Table 3 show that a significant number of γ-GT-positive foci were obtained in 1,2-DMH-treated animals when PH was delayed until 24 hr. However, with longer delays between the time of injection of the 1,2-DMH and the time of performance of PH (48 hr or 1 week), there was a large reduction in the number of foci seen.

In the long-term study, the majority of the animals in both experimental and control groups survived throughout the 13-month period following the initial administration of 1,2-DMH. When PH was performed at 12 hr after 1,2-DMH, about 43% (6 of 14) of the animals developed frank primary hepatocellular carcinoma with one animal showing multiple pulmonary metastases (Table 3). One animal in this group also developed...
carcinoma of the colon. The main histological pattern of the liver cancers was the trabecular type without acinar structure. The remaining 57% (8 of 14) of the animals all had one or more persistent hyperplastic nodules in their liver. When PH was delayed until 48 hr after 1,2-DMH (E5), neither persistent hyperplastic nodules nor liver cell cancer was found. The predominant histological lesions detected in this group were the remodeling type of hyperplastic nodules with characteristic patchy \( \gamma \)-GT-staining appearance (6, 22). Similarly, in the sham-operated group (C1) and control group (C2), a few \( \gamma \)-GT-positive hyperplastic lesions were seen in some animals. It should be pointed out that all the animals included in Table 3 were exposed to the 2-week period of dietary 2-acetylaminofluorene plus \( \mathrm{CCL}_4 \) in order to select the resistant hepatocytes. Yet, only those in Group E3 developed hepatocellular carcinoma by 13 months.

**DISCUSSION**

The results of this study clearly show that a delay in the cell cycle, with or without temporary interruption, significantly decreased the initiation of liver carcinogenesis and the induction of early presumptive preneoplastic changes with 1,2-DMH. These findings in cell proliferation agree in principle with the results of other studies using (a) early events induced by several other indirect- or direct-acting carcinogens (2, 3, 35), (b) carcinogenesis in some other organs or tissues (1, 21), and (c) some cells in vitro (14, 15, 19). The results of this study also confirm in principle those of Kaufman et al. (16) with the use of \( \mathrm{MNU} \) as the carcinogen, HC as the inhibitor, and hepatocellular carcinoma as the end point.

This study also shows that the persistence of some one or more biochemical effects or lesions induced by 1,2-DMH is closely associated with a tissue or cellular activity related to the cell cycle. Although DNA replication is a cellular activity that is very special to cell proliferation, there are a variety of other known activities such as formation of chromosomes, cytokinesis, and reorganization of DNA and chromatin associated with the resting cell, to name but 3, that are also uniquely correlated with cell proliferation and the cell cycle. Although it is easier to formulate a possible mechanism using DNA replication to "fix" a lesion (4, 25), the evidence that can be mustered to support this hypothesis is entirely circumstantial and suggestive.

HC is a potent inhibitor of DNA synthesis in the liver. However, the mechanism of this inhibitory effect and other possible biochemical effects of this compound during the cell cycle are unknown. Until a much better understanding of these effects is acquired, it is impossible to relate the influences of HC on carcinogenesis with any particular biochemical alteration. Also, since the progression of cells through the cell cycle seems to be a highly coordinated complex series of closely interdependent events, any attempt to inhibit one step or another without introducing a delay in the movement through the cell cycle as additional variables seems not to be possible with the known inhibitors at this time.

The present findings also confirm previous data on the need for properly timed cell proliferation in the induction of early hepatocyte alterations by several carcinogens (2, 3, 13, 35, 38, 39). As with \( \mathrm{MNU} \) (2), diethylnitrosamine (13, 30), and a wide spectrum of other chemical carcinogens (35, 36) including 1,2-DMH (35, 38), the induction of early focal hepatocyte changes in the liver decreases significantly when cell proliferation is delayed for periods longer than 24 hr after the administration of the initiating dose of the carcinogen.

In this context, it is important to emphasize that 1,2-DMH is predominantly a carcinogen for the colon (5, 10, 32, 33) and to a lesser degree for the vascular endothelium of the liver (5, 33, 34) but not for hepatocytes, even though it extensively methylates the DNA of liver (11, 17, 18, 23, 28). The results of the present study suggest that the probable reason for the ineffectiveness of this carcinogen in inducing liver cell cancer may be the lack of a measurable degree of necrosis and subsequent cell proliferation with the doses used (39). Clearly, a single dose of 100 mg 1,2-DMH per kg can initiate the development of a significant incidence of hepatocellular carcinoma if cell proliferation is provided at an appropriate time.

Finally, an important question is the nature of the time dependence for cell proliferation in its role in the induction of early lesions with 1,2-DMH and with other carcinogens. The data are most easily interpreted in terms of time for repair or removal of some carcinogen-induced lesions. The time de-

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**Table 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of ( \gamma )-GT-positive foci/sq cm</th>
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<tbody>
<tr>
<td>C1</td>
<td>1,2-DMH + PH + 0.9% NaCl solution</td>
<td>15.4 ± 1.5 ( ^{(26)} ) (26)</td>
</tr>
<tr>
<td>E1</td>
<td>1,2-DMH + PH + HC (4 hr)</td>
<td>4.4 ± 1.0 (12)</td>
</tr>
<tr>
<td>E2</td>
<td>1,2-DMH + PH + HC (154 hr)</td>
<td>14.7 ± 1.9 (5)</td>
</tr>
<tr>
<td>C2</td>
<td>0.9% NaCl solution + PH + HC (4 hr)</td>
<td>0.6 ± 0.3 (8)</td>
</tr>
</tbody>
</table>

\( ^{(a)} \) E1 is significantly different from C1 and E2 \( (p < 0.001) \) and is not significantly different from C2 \( (p = 0.12) \). E2 is not significantly different from C1 \( (p = 0.80) \).

\( ^{(b)} \) Experimental details are given in "Materials and Methods" and in the legend to Chart 1. Group E1 was given HC commencing at 4 hr after PH, whereas Group E2 was given the same doses of HC but commencing at 154 hr after PH.

\( ^{(c)} \) Mean ± S.E. of 2 separate experiments.

\( ^{(d)} \) Numbers in parentheses, effective number of rats.

**Table 3**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of ( \gamma )-GT-positive foci/sq cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>E3</td>
<td>1,2-DMH + PH (12 hr)</td>
<td>19.3 ± 2.6 ( ^{(16)} ) (16)</td>
</tr>
<tr>
<td>E4</td>
<td>1,2-DMH + PH (24 hr)</td>
<td>15.4 ± 1.5 (28)</td>
</tr>
<tr>
<td>E5</td>
<td>1,2-DMH + PH (48 hr)</td>
<td>6.2 ± 1.2 (10)</td>
</tr>
<tr>
<td>E6</td>
<td>1,2-DMH + PH (156 hr)</td>
<td>5.4 ± 1.1 (11)</td>
</tr>
<tr>
<td>C1</td>
<td>1,2-DMH + sham hepatoectomy (12 hr)</td>
<td>3.6 ± 0.8 (14)</td>
</tr>
<tr>
<td>C2</td>
<td>0.9% NaCl solution + PH (12 hr)</td>
<td>0.8 ± 0.2 (12)</td>
</tr>
</tbody>
</table>

\( ^{(a)} \) E3 and E4 are both significantly different from either E5, E6, C1, or C2 \( (p < 0.001) \). E3 is not significantly different from E4 \( (p = 0.27) \). No significant difference between E5 versus C1 and C2; E6 versus C1 and C2 \( (p > 0.3) \) for each determination.

\( ^{(b)} \) Experimental details are given in "Materials and Methods."

\( ^{(c)} \) Mean ± S.E.

\( ^{(d)} \) Numbers in parentheses, effective number of rats.

\( ^{(e)} \) Only animals without hepatocellular carcinoma are included in this group.

\( ^{(f)} \) Those with nodules and hepatocellular carcinoma are included in the next column.

\( ^{(g)} \) One animal in Group E3 had pulmonary metastases, and one animal in the same group developed carcinoma of the colon.

\( ^{(h)} \) ND, these groups not included in the long-term studies.

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pendence curve and the availability of assays for resistant hepatocytes or other possible types of early liver cell changes (7) make it possible to critically analyze possible correlations between specific biochemical lesions and the appearance of early biological changes in liver cells that may relate to the ultimate development of cancer.

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