Role of Acute Hepatic Necrosis in the Induction of Early Steps in Liver Carcinogenesis by Diethylnitrosamine¹

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ABSTRACT

Experiments were performed to assess the role of liver cell necrosis in the induction of early steps in liver carcinogenesis with diethylnitrosamine, as measured by the appearance of foci of resistant hepatocytes that stain for γ-glutamyl transpeptidase and that are presumptive preneoplastic lesions in the rat. With the use of a necrogenic dose of diethylnitrosamine and an assay for the carcinogen-induced early stages or resistant hepatocytes, the number of enzyme-altered foci was decreased to a major extent (up to 62%) by posttreatment with diethyldithiocarbamate, a derivative of disulfiram. Such posttreatment decreased to a large degree (78%) the cumulative labeling index of hepatocytes following an initial exposure to diethylnitrosamine. The performance of partial hepatectomy up to 68 hr after such posttreatment restored the level of the induction of the resistant hepatocytes. Nonnecrogenic doses of diethylnitrosamine or dimethylnitrosamine induced virtually no foci of resistant hepatocytes but did so when coupled with cell proliferation. These results establish clearly an important role for liver cell necrosis in the production of early steps in liver carcinogenesis in one model. The mechanism for this effect appears to be by the induction of compensatory liver cell proliferation.

INTRODUCTION

The possible relationship of acute and chronic liver injury to the pathogenesis of liver cancer in humans and in experimental animals has been discussed for many years (1, 7–9, 11, 12, 20, 22, 26, 42, 44, 47). An association between cirrhosis of the liver and liver cancer has been known for a long time. More recently, hepatitis and chronic liver injury related to hepatitis B virus have been receiving increasing attention as factors that are probably important in the genesis of human liver cancer (1, 2, 24, 33, 48). Also, many of the chemical carcinogens that induce liver cancer in experimental animals are acutely hepatotoxic.

Despite the general interest in this area, little of a definitive nature concerning the possible role of liver cell injury in one or more steps in the carcinogenic process in the liver has been documented. A commonly associated cell response, cell proliferation, has been implicated in the overall process of liver cancer development with several chemicals, especially ones that are not ordinarily considered to be hepatocarcinogens (6, 7, 25, 42). Also, cell proliferation has been shown to be important or essential in the genesis of 2 types of very early lesions in the liver: (a) enzyme-deficient islands (28–30) and (b) carcinogen-resistant hepatocytes (3, 37–39, 45), each induced by many carcinogens. However, what role, if any, is played by acute liver cell injury in the early steps or in the subsequent development of liver cancer has not been clarified.

Recently, 2 developments in our laboratory have permitted us to formulate an approach to the question, does liver cell death play any role in the initial steps of liver carcinogenesis, and if so, what role?

One development is a new model for liver carcinogenesis that enables an analysis of the first few steps in the process, including an assay for one type of carcinogen-altered cell, a resistant hepatocyte (34, 35). This is based on the hypothesis, for which there is increasing evidence (3, 12, 23, 34, 35, 37–39, 45), that one form of early change may consist of the induction of altered hepatocytes resistant to the inhibitory effects of 2-AAF³ on cell proliferation. Such resistant hepatocytes can be selectively stimulated to proliferate rapidly to produce nodules by the use of an appropriate selection pressure, such as a short exposure to 2-AAF in combination with a stimulus for hepatocyte proliferation such as PH or CCl₄ administration (3, 23, 35, 37–39, 45). In this model, with DENA as the initiating carcinogen, a linear continuity (12, 13) between early resistant hepatocytes, nodules, and metastasizing hepatocellular carcinoma has been established (35), thus suggesting that the foci of resistant hepatocytes, as a group, are early precursor lesions in liver carcinogenesis. Since histochemical γ-GT activity appears in 90 to 95% of the early hyperplastic foci and nodules under these circumstances (23), this is a convenient marker for their quantitation.

The second development is the dissociation of some biochemical and metabolic effects of DENA and DMN, including alkylation of DNA and RNA, from their cytocidal effects by the use of posttreatment with DEDTC (46). By administering DEDTC at 4 or 8 hr after the administration of a usually necrogenic dose of DENA (or of DMN), liver cell necrosis can be prevented almost completely without inhibiting at least some of the known interactions of these nitrosamines with cellular nucleic acids. Thus, if such interactions with DNA or other cellular components are important in the initiation of carcinogenesis with chemicals, it might be possible to study the role of liver cell necrosis in the beginning of liver cancer development.

Using these combined approaches, it has been shown that liver cell necrosis can play an important role in the induction of early changes in liver carcinogenesis with DENA and DMN.

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³ The abbreviations used are: 2-AAF, 2-acetylaminofluorene; PH, partial hepatectomy; DENA, diethylnitrosamine; γ-GT, γ-glutamyl transpeptidase; DMN, dimethylnitrosamine; DEDTC, diethyldithiocarbamate; i.g., intragastric.
The experimental evidence in support of this conclusion and its possible mechanism are the subjects of this paper.

MATERIALS AND METHODS

**Chemicals.** DENA was obtained from Eastman Kodak Co., Rochester, N. Y., and DMN was obtained from Merck-Schuchardt, Munich, West Germany. Their chemical purities were routinely checked by UV spectrophotometry and gas chromatography prior to use. Sodium salt of DEDTC and all 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, Canada.

**Animals.** Male Fischer 344 rats (Microbiological Associates, Walkersville, Md.), weighing 175 to 200 g, were maintained on a semisynthetic diet containing 24% protein (Bio Serv, Frenchtown, N. J) and a daily cycle of alternating 12-hr periods of light and darkness. The animals were given food and water ad libitum and were acclimatized to their environment for 1 week before the start of the experiment.

**Experimental Regimen.** In Regimen 1 (Chart 1), freshly dissolved DENA in 0.9% NaCl solution was injected i.p. at a dose of 100 mg/kg body weight. DEDTC was dissolved in 0.9% aqueous NaCl solution and was administered i.p. at a dose of 50 mg/kg 3 times at 4-hr intervals, beginning at various time periods from 4 hr to 10 days after DENA administration. In control groups, rats received equivalent volumes of 0.9% NaCl solution instead of DENA (Group C2) or DEDTC (Group C1). After a 2-week recovery period, the animals were assayed for resistant cells in the liver (34, 35, 39) by being placed on a basal diet containing 0.02% 2-AAF in order to create an inhibitory effect on cell proliferation (34) and 1 week later being subjected to a standard two-third PH procedure (16). After a total period of 14 days on the 2-AAF diet, the animals were sacrificed, and liver samples were processed for histological examination and for quantitative histochemical analysis.

In Regimen 2 (Chart 2), PH was performed 3 days following the administration of DENA and DEDTC. Because the dose of DENA might inhibit the normal regenerating potential after PH (34, 35), the animals were given no further treatment for 4 weeks. At that time, the animals were assayed for resistant hepatocytes by placing them on a diet containing 0.02% 2-AAF in order to create an inhibitory effect on cell proliferation (34) and 1 week later being subjected to a standard two-third PH procedure (16). After a total period of 14 days on the 2-AAF diet, the animals were sacrificed, and liver samples were processed for histological examination and for quantitative histochemical analysis.

In Regimen 3 (Chart 3), a single nonnecrogenic dose of either DMN (10 mg/kg body weight) or DENA (15 mg/kg body weight) was administered i.p., and at 4 or 24 hr later, respectively, the rats were subjected to PH or sham hepatectomy (Group C1). Resistant hepatocytes were stimulated selectively to grow according to the procedure described previously in Regimen 2. Control Group C2 received vehicle plus PH.

All animals were fasted overnight before sacrifice.

**Incorporation of [³H]Thymidine into DNA.** Groups of 4 rats were given DENA (100 mg/kg i.p.), and 4 hr later they were given injections of either DEDTC (50 mg/kg) 3 times at 4-hr intervals or 0.9% NaCl solutions. From 1 to 14 days, [methyl-³H]thymidine was given i.p. at 8 a.m., 12 noon, 4 p.m., 8 p.m., and 12 midnight at a dose of 0.2 µCi/g body weight to label proliferating hepatocytes. At the time of sacrifice, part of the liver was processed for the determination of the specific activity of DNA. DNA was isolated and purified by the procedure of Kirby and Cook (18). Appropriate aliquots from the remainder of the liver were processed for autoradiographic analysis of labeled cells.

For autoradiographic analysis, multiple sections of the liver approximately 1 mm thick were obtained and placed in 4% formalin fixative for 24 hr. They were then processed and stained with hematoxylin and eosin. Additional contiguous paraffin sections were cut for autoradiographic analysis. These were deparaffinized, stained with eosin or periodic acid-Schiff, air dried, coated with Kodak NTB3 emulsion, and stored desiccated at 4°C for at least 2 weeks. They were then developed and counterstained with hematoxylin for microscopic examination.

**Histochemical Localization of γ-GT.** Livers harvested on the final day of each regimen were studied histochemically for the localization of γ-GT using the method described by Rutenberg et al. (27) as modified by Ogawa et al. (23). The number of γ-GT-positive foci was counted by low-power microscopy and expressed as foci per sq cm of section area. The long and short axes of the foci were measured with the use of a microm-
The results presented in Table 1 show that DEDTC, when given at 4 or 8 hr after DENA, decreased to a considerable degree (62 and 30%, respectively) the number of y-GT-positive foci induced by a necrogenic dose (100 mg/kg) of DENA (Fig. 1). It was shown previously that a 4-hr posttreatment with DEDTC effectively prevented almost all necrosis and that a 8-hr posttreatment prevented a considerable amount of necrosis with the same dose of DENA (46). The prevention of necrosis was not complete but almost so, as indicated by the degrees of elevation in serum sorbitol dehydrogenase and glutamate-pyruvate transaminase and by histological examination (46). The inhibitory effect on the genesis of foci of y-GT-positive and -resistant hepatocytes does not appear to be related to any effect of DEDTC per se, since this compound had no effect when given at 24 hr or at 10 days, times well before the beginning of the assay procedure for resistant foci. DEDTC had no effect on liver cell necrosis when given at 24 hr (46). The range in the diameters of all the y-GT-positive foci in representative sections (110 to 1380 μm) and the mean diameters under each condition were essentially the same (Table 1). The findings validate the use of the number of foci per area as a quantitative measure.

Since cell proliferation is required for the induction of resistant hepatocytes during initiation by many carcinogens (3, 37-39, 45), it is possible that the role of liver cell necrosis in initiation is in the stimulation of cell proliferation as a regenerative response. This was tested by determining whether PH performed after the administration of DEDTC would reverse the inhibitory effect on the development of DENA-induced y-GT-positive foci of resistant hepatocytes. The experimental design is illustrated in Chart 2.

As is evident from the results in Table 2, PH reversed completely the decrease in the number of foci seen with posttreatment with DEDTC. The 67% decrease seen in the group with DENA plus DEDTC became a 41% increase when PH followed the DEDTC. This “overshooting” with PH was also seen in the group treated with DENA only, which showed a 74% increase in the level of induction of foci of resistant hepatocytes.

If the role of necrosis in the induction of resistant hepatocytes with DENA is mainly to stimulate cell proliferation, posttreatment with DEDTC should decrease, if it does not prevent completely, the postnecrotic proliferation of hepatocytes. In order to test for this possibility, the cumulative labeling index of hepatocytes was measured over a 2-week period after the administration of DENA with and without posttreatment with DEDTC. As may be seen in Table 3, the cumulative labeling index with DENA alone was 13%, and this was decreased to a large degree (78%) by the 4-hr posttreatment with DEDTC. These observations are consistent with the suggestion that the DEDTC might be inhibiting the induction of early changes in liver carcinogenesis by decreasing secondarily the regenerative hepatocyte proliferation as a response to its effect in decreasing the degree of cell necrosis. It should be pointed out that theoretically some of the decrease in labeling index seen in the DENA-DEDTC-treated animals could be due to an inhibition of thymidine incorporation by the DEDTC. This possibility is very unlikely, since DEDTC had no effect on liver regeneration following DENA-induced necrosis (46) when given 24 hr after the DENA. Also, the 14-day period of accumulation of labeled hepatocytes makes any such inhibitory effect of DEDTC very improbable, since DEDTC must be given repeatedly every few hr if measurable effects studied are to be sustained even for a few hr (46).

Effect of PH on the Induction of Foci of Resistant Hepa-
Liver Necrosis and Early Steps in Liver Carcinogenesis

Effect of DEDTC on the hepatic hyperplasia induced by DENA

<table>
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<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Index of labeled nuclei (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>DENA + 0.9% NaCl solution</td>
<td>13.0 ± 1.85 (4)</td>
</tr>
<tr>
<td>2</td>
<td>DENA + DEDTC</td>
<td>2.9 ± 1.4 (4)</td>
</tr>
<tr>
<td>3</td>
<td>0.9% NaCl solution + DEDTC</td>
<td>&lt;0.1 (4)</td>
</tr>
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* Group 1 is significantly different from Group 2 (p < 0.01). Groups 1 and 2 are significantly different from Group 3 (p < 0.001) (Student’s t test).

After the appropriate treatment, animals were given [3H]thymidine injections (0.2 μCi/g body weight) i.p. beginning at Day 1 and continuing to Day 14. Each animal received 5 doses daily (8 a.m., 12 noon, 4 and 8 p.m., and midnight) for 13 days and was killed on the 14th day. The livers were processed for autoradiography. Only the hepatocytes with heavily labeled nuclei were counted using a magnification of 400-fold. The heavily labeled cells represent proliferating cells. In contrast, cells with sparsely labeled nuclei (<30 grains), probably representing repair replication, were not counted. This type of cutoff may eliminate some bona fide hepatocytes in S phase, but this was deliberate. Labeling indices for individual livers were taken as the average value determined from counts of 10 random high-power fields, each consisting of 150 to 200 cells.

Mean ± S.E.

Numbers in parentheses, effective number of rats.

tocytes by Nonnecrogenic Doses of DENA or DMN. If necrosis is playing a role in the initiation with DENA or with DMN by associated regenerative cell proliferation, nonnecrogenic doses of each nitrosamine should induce few if any foci unless coupled with a stimulus for cell proliferation. As seen in Table 4, PH performed at 4 hr after DMN or at 24 hr after DENA increased considerably the number of foci induced with the compounds. It should be pointed out that the number of foci induced by carcinogens coupled with PH varies considerably with the timing of each procedure (3, 39, 45) and that the time sequence as used here does not lead to a maximum induction of foci. This is why the number of foci in these experiments is considerably less than in other types of experiments, such as in those included in Tables 1 and 2. In these experiments, times for PH were selected at which no detectable levels (less than 5 to 10 ng/ml) of DMN (4 hr) or of DENA (24 hr) were found in the blood and liver (46).

It should be noted that in Experimental Regimens 2 and 3, CCl₄ was used instead of PH as a liver cell proliferative stimulus in the assay for resistant hepatocytes (3, 38, 39, 45). The foci obtained under these conditions were indistinguishable from those seen when 2-AAF plus PH is used as the selecting environment (Fig. 2). This similarity was evident with respect to all parameters including vesicular nuclei with prominent nucleoli, pseudoacinor arrangement of the hepatocytes, and tendency to accumulate glycogen (Fig. 3). However, with the 2-AAF plus CCl₄, an extra week on stock diet after the 2-week exposure to dietary 2-AAF was allowed in order to ensure the production of grossly visible grayish-white elevated nodules (38, 39). It should be emphasized that the control rats exposed to 2-AAF plus PH or 2-AAF plus CCl₄ without prior treatment with DENA developed very few if any resistant foci (Tables 1, 2, and 4).

DISCUSSION

The results of this study established for the first time an essential role of liver cell necrosis in the development of early putative preneoplastic liver lesions induced by a necrogenic dose of DENA.

The observation that cell proliferation induced by an exogenous stimulus such as PH could reverse the inhibitory effect of DEDTC suggests that those interactions of DENA with the target molecule(s) essential for the development of preneoplastic foci were not affected by such treatment. This supposition is further strengthened by the results of a previous study in which it was shown that DEDTC posttreatment did not affect the total extent of ethylation of liver nucleic acids although the rates of alklylation were different (46). The results presented also suggest that the key role played by necrosis in the induction of early events in liver carcinogenesis may be by stimulating compensatory cell proliferation. However, it is not yet unequivocally established whether compensatory cell replication is the only mechanism through which necrosis may exert its effect on the induction of preneoplastic lesions. Suffice it to say that DENA-induced liver cell necrosis is important but not optimum for the induction of preneoplastic lesions, since supplementation with PH resulted in a further increased appearance of γ-GT-positive foci (Table 2, compare Group C2 with C3). Even though the mechanism responsible for stimulation of cell proliferation by the toxic effects of the carcinogen may or may not be identical to that operating after PH, it has been shown that the proliferative response resulting from acute exposure to DENA is less effective in the induction of putative preneoplastic foci than is two-thirds surgical removal of liver.

The importance of cell proliferation in the induction of preneoplasia is further substantiated by the observation that a nonnecrogenic dose of DMN or DENA induced preneoplastic lesions only in rats that were subjected to PH but not in those that received SH following the administration of the carcinogen.

The mechanism whereby cell proliferation participates in the early events in the carcinogenic process is not known. However, an attractive possibility is the replication of carcinogen-damaged DNA, prior to its repair, with “fixation” of the damage in the daughter strands (7, 25, 42).

Several studies have shown that DEDTC or disulfiram, its parent compound, inhibited the development of tumors in various organs induced by a variety of carcinogens (31, 32, 43). However, in most of these instances, the protective agent was given either along with or prior to the administration of the carcinogen. Under these experimental conditions, it is possible that DEDTC or disulfiram may inhibit the activation of the carcinogen and thus may reduce the tumor incidence by decreasing the generation of the ultimate form(s) of the carcinogen.

The mechanism whereby posttreatment with DEDTC at 4

Table 4

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of γ-GT-positive foci/sq cm</th>
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<tr>
<td>E</td>
<td>DMN + PH</td>
<td>12.4 ± 1.85 (15)</td>
</tr>
<tr>
<td></td>
<td>DENA + PH</td>
<td>8.0 ± 1.0 (18)</td>
</tr>
<tr>
<td>C1</td>
<td>DMN + SH</td>
<td>1.8 ± 0.5 (8)</td>
</tr>
<tr>
<td></td>
<td>DENA + SH</td>
<td>1.1 ± 0.3 (19)</td>
</tr>
<tr>
<td>C2</td>
<td>0.9% NaCl solution + PH</td>
<td>0.8 ± 0.2 (12)</td>
</tr>
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</table>

* Experimental Groups E are significantly different from Control Groups C1 and C2 (p < 0.001). Groups C1 are not significantly different from C2 (p = 0.48 for DMN and p = 0.68 for DENA) (Student’s t test).

* Mean ± S.E. for 2 separate experiments.

* Numbers in parentheses, effective number of rats.

* SH, sham hepatectomy.
or 8 hr largely prevents hepatocyte necrosis is not known. Since later treatment with DEDTC does not appear to inhibit cell autolysis (necrosis) that characterizes cells following their death. Rather, it would appear that DEDTC somehow reverses some reversible step in the multistep sequence that seems to be characteristic of cell death in many cell types including the liver (46). Conceivably, its role as a free radical "scavenger" or as a chelating agent for some ions may be important in this context.

The results of this study offer a possible explanation for the effects of liver injury with cell death in various infections in the genesis of liver cancer. For example, hycanthone, an antischistosomal drug and a nonliver carcinogen, becomes carcinogenic in mice infected with schistosomes (14, 15) or in noninfected mice if coupled with PH (40). Infection with schistosomes or other trematodes is associated with liver cell death and liver cell proliferation (4, 10, 14, 15, 19, 36, 40). Thamavit et al. (36) reported that an otherwise noncarcinogenic dose of DMN induced cholangiocarcinoma in Syrian golden hamsters when they were infected with the parasite Opisthorchis viverrini. Similarly, Domingo et al. (10) observed that low doses of 2-amino-5-azotoluene produced a much higher incidence of hepatomas in infected mice with Schistosoma mansoni than in uninfected mice. Equally convincing is the fact that several carcinogens that normally do not induce liver cancer in adult animals, especially with a single dose, induce preneoplastic and neoplastic liver lesions when given after PH (5–7, 17, 21, 38, 41).

Finally, and most importantly, the results of the present study implicate liver cell injury as a possible factor in the genesis of human liver cancer. The increasing suspicion of a relationship between hepatitis B-virus and liver cancer (1, 2, 24, 33, 48) and the long-known association between nonneoplastic liver disease, including cirrhosis, and liver cancer could be in part explained by a role for liver cell death in the induction of very early changes in liver carcinogenesis by chemicals.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. Histochemical staining for γ-GT of a liver section from a rat showing γ-GT-positive foci of resistant proliferating hepatocytes distributed randomly throughout the lobe. The animal received DENA (100 mg/kg), followed by a selection procedure as shown in Chart 1. × 10.

Fig. 2. Photomicrograph of a liver section showing a γ-GT-positive focus of proliferating hepatocytes. The rat was treated with DENA (100 mg/kg) and DEDTC 4 hr later with PH 72 hr later, followed by a selection procedure as shown in Chart 2. H & E. × 40.

Fig. 3. Higher magnification of Fig. 2 showing portions of the hyperplastic focus with one arrow of at least 3 mitotic figures seen in the whole focus. The architecture of the hepatocytes is not evident in this particular focus since the sinusoids are collapsed. The liver cells show vacuolization largely because of periodic acid-Schiff-positive material (glycogen) removed by amylase. Note the sharp surrounding border containing oval cells. H & E. × 200.
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