Inhibition of DNA Synthesis by Chemical Carcinogens in Cultures of Initiated and Normal Proliferating Rat Hepatocytes

Deborah L. Novicki, Mark R. Rosenberg, and George Michalopoulos

Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710

ABSTRACT

Rat hepatocytes in primary culture can be stimulated to replicate under the influence of rat serum and sparse plating conditions. Higher replication rates are induced by serum from two-thirds partially hepatectomized rats (Michalopoulos, G., Cianciulli, H. D., Novotny, A. R., Kligerman, A. D., Strom, S. C., and Jirtle, R. L. Cancer Res., 42: 4673-4682, 1982). The effects of carcinogens and noncarcinogens on the ability of hepatocytes to synthesize DNA were examined by measuring the incorporation of [3H]thymidine by liquid scintillation counting and autoradiography. Hepatocyte DNA synthesis was not decreased by ethanol or dimethyl sulfoxide at concentrations less than 0.5%. No effect was observed when 0.1 mM ketamine, Nembutal, hypoxanthine, sucrose, ascorbic acid, or benzo(e)pyrene was added to cultures of replicating hepatocytes. Estrogen, testosterone, tryptophan, and vitamin E inhibited DNA synthesis by approximately 50% at 0.1 mM, a concentration at which toxicity was noticeable.

Several carcinogens requiring metabolic activation as well as the direct-acting carcinogen N-methyl-N'-nitro-N-nitrosoguanidine interfered with DNA synthesis. Aflatoxin B1 inhibited DNA synthesis by 50% (ID50) at concentrations between 1 x 10^-8 and 0.1 mM, a concentration at which toxicity was noticeable.

In a recent report, we have shown that hepatocytes can be stimulated to replicate in primary culture. We have used this system of replicating hepatocytes in order to precisely quantitate the effect of hepatocarcinogens on hepatocyte replication and compare the relative response of initiated versus normal hepatocytes under identical culture conditions. Previous studies have demonstrated the relative resistance of cells from neoplastic (5, 16) or regenerating (44) liver to the cytocidal toxicity induced by chemical compounds. This study focuses on the effect of chemicals on hepatocyte replication. This type of study has not been possible until now due to lack of systems that allow replication of hepatocytes in primary culture.

INTRODUCTION

Liver is one of the sites primarily involved in the biotransformation of xenobiotic compounds. Metabolic activation is an obligatory step for the interaction of a number of carcinogens with the presumed cellular target, DNA (29, 34). Cultures of isolated hepatocytes have been used extensively in studies of drug and carcinogen metabolism (52). Hepatocytes or enzyme extracts derived from liver cells have been combined with a variety of replicating cell types: bacteria (2); human diploid fibroblasts (32); or V79 cells (24) to provide bioactivation of promutagens in mutagenicity assays or in conjunction with BHK (38, 41) and C3H/10T1/2, SHE, and BALB/c 3T3 (7) cells in transformation assays. UDS (50) or carcinogen binding (51) can be measured in hepatocytes as a direct indication of carcinogen interaction with DNA. To date, direct measurement of mutagenesis and neoplastic transformation of parenchymal hepatocytes in primary culture have not been feasible end points for the determination of genotoxicity. Although cell division can be stimulated in primary cultures of hepatocytes (26), optimization of conditions that would allow cloning of hepatocytes in primary culture has not yet been achieved.

The importance of cell replication to the development of neoplasia has been well documented (6). An early consequence of the interaction of hepatocarcinogens with liver in vivo is the production of "preneoplastic hepatocytes" which exhibit altered morphological and histochemical characteristics (15). A dose of a hepatocarcinogen administered 24 hr after partial hepatectomy results in the production of hepatocyte populations that are positive for GGT, an oncostatic marker not generally found in adult parenchymal hepatocytes (17). The cells comprising these enzyme-altered foci have been found to be resistant to the toxic effects of a number of carcinogens (18) as well as antineoplastic (5) and hepatotoxic compounds (21). The number and size of nodules can be increased by the use of liver tumor promoters (8). Depending on the tumor production protocol used, many of these hyperplastic nodules can progress to hepatocellular carcinoma (37, 39, 49).

In a recent report, we have shown that hepatocytes can be stimulated to replicate in primary culture. We have used this system of replicating hepatocytes in order to precisely quantitate the effect of hepatocarcinogens on hepatocyte replication and compare the relative response of initiated versus normal hepatocytes under identical culture conditions. Previous studies have demonstrated the relative resistance of cells from neoplastic (5, 16) or regenerating (44) liver to the cytocidal toxicity induced by chemical compounds. This study focuses on the effect of chemicals on hepatocyte replication. This type of study has not been possible until now due to lack of systems that allow replication of hepatocytes in primary culture.

MATERIALS AND METHODS

Materials. Male Fischer 344 rats were purchased from Harlan Sprague-Dawley, Madison, WI. Tissue culture medium, Eagle's MEM with Earle's salts, was a product of Grand Island Biological Co., Grand Island, NY. FBS was obtained from Sterile Systems, Logan, UT. EGF was purchased from Collaborative Research, Danvers, MA. FBS was obtained from Flow Laboratories, McLean, VA. Other reagents were purchased from Sigma Chemical Co., St. Louis, MO.

Received December 14, 1983; accepted September 26, 1984.

1 The abbreviations used are: UDS, unscheduled DNA synthesis; GGT, y-glutamyltranspeptidase; MEM, minimal essential medium; MNG, N-methyl-N'-nitro-N-nitrosoguanidine; DMSO, dimethyl sulfoxide; ABi, aflatoxin B1; AAF, 2-acetylaminoazobenzene; 3'-MeDAB, 3'-methyl-4-dimethylaminoazobenzene; 1-NA and 2-NA, 1- and 2-naphthylamine; DEN, diethylnitrosamine; DMN, dimethylnitrosamine; EGF, epidermal growth factor; FBS, fetal bovine serum; HPRS, hepatocarcinometized rat serum; TCA, trichloroacetic acid; BejP, benzo(e)pyrene.

2 To whom requests for reprints should be addressed.

3 This study was supported by Grant CA R01 35373 from the NIH.
of 0.33 N NaOH was added to each culture to solubilize the cells. The third partial hepatectomy performed as by Higgins and Anderson (20).as described (26) from male retired Fischer breeder rats 48 hr after two-
jCi/ml (specific activity, 40 to 60 Ci/mmol). Serum (HPRS) was prepared (50 fig/ml), EGF (10 ng/ml), and 

Hepatocyte Isolation and Tissue Culture Conditions. Hepatocytes were isolated from 125- to 200-g male Fischer rats by collagenase perfusion using a modification (9) of the method of Seglen (46). Isolated hepatocytes were inoculated in 1-ml aliquots into collagen-coated (24) 35-mm-diameter tissue culture plates (Falcon Plastics) at a density of 100,000/ml in MEM with Earle’s salts, supplemented with 0.2 mM serine, 0.2 mM aspartate, 1 mM pyruvate (11), 5% FBS, insulin (10^{-7} M), and gentamicin (50 g/ml). Four hr later, the plating medium was removed and replaced by MEM supplemented with serine, aspartate, and pyruvate as above with the addition of 25% HPRS, insulin (10^{-7} M), gentamicin (50 g/ml), EGF (10 ng/ml), and [H]-thymidine at a concentration of 10 Ci/ml (specific activity, 40 to 60 Ci/mmol). Serum (HPRS) was prepared as described (26) from male retired Fischer breeder rats 48 hr after two-thirds partial hepatectomy performed as by Higgins and Anderson (20). Chemicals added to the cultures were dissolved in DMSO and added in 10-uL aliquots so that the concentration of DMSO did not exceed 0.1%. The cell cultures were incubated for 48 hr in a 90% humidity (7% CO2) atmosphere at 37°.

Quantitation of Uptake of [H]-Thymidine. Control and treated cultures were harvested at the end of the 48-hr incubation period by removing the medium and passing the tissue culture plates through 6 successive washes in large volumes of 0.85% NaCl solution. The plates were inverted to drain for approximately 5 min, after which time 1 ml of 0.33 n NaOH was added to each culture to solubilize the cells. The plates were incubated for 30 min at 37°. The contents of the plates were transferred to tubes and placed on ice. The NaOH was neutralized with HCl, and TCA was added to a final concentration of 10%. Samples were centrifuged for 15 min at 4° at 4000 x g. The cell pellets were resolubilized in 0.5 ml of 0.33 n NaOH; 0.3-ml aliquots were taken from each sample for scintillation counting in a Searle Analytic 81 using Aquasol (New England Nuclear, Boston, MA) acidified with 0.1 ml of 40% TCA.

Autoradiography. Hepatocyte cultures were washed as described above to remove unbound [H]-thymidine and dead cells. The cultures were fixed in 10% buffered formalin for 24 hr, rinsed with distilled water, rinsed with 95% ethanol, and dried. NTB3 photographic emulsion was diluted 1:1 with distilled water and used to coat the tissue culture plates. The emulsion was exposed for a total of 10 days. Autoradiographed culture dishes were developed using D-19 developer (diluted 1:1 with H2O) and fixed. Plates were allowed to dry and were stained with hematoxylin and eosin. The labeling index (percentage) was calculated by counting the number of labeled nuclei from a total of at least 200 nuclei at a magnification of X 400. The counted fields were chosen at random, and all cells in a field were counted.

DNA Isolation and Binding of [H]-IAAF. Experiments were done with either FBS or HPRS present in the medium as specified in “Results.” The cell density was increased to 200,000 hepatocytes/35-mm-diameter tissue culture plate. Titrated AAF was added to the cultures 4, 24, or 48 hr postinoculation at a concentration of 10^{-4} M (21.9 mCi/mmol). Cultures were harvested 24, 48, or 72 hr after addition of the carcinogen, respectively. Cells from 35 dishes/segment were harvested to 0.85% NaCl solution by scraping with a rubber policeman, pooled, and centrifuged at 4000 X g. The cell pellets were solubilized in 3 ml of 6 M NaSCN. Samples were carefully layered onto a preformed step gradient of CsCl (47) and centrifuged for 17 hr at 25° in a Beckman SW 41 rotor at 28,000 rpm.

The gradients were fractionated, and the DNA-containing fractions were dialyzed twice against 4-liter volumes of 0.85% NaCl solution (saline). A Cary 219 scanning UV-visible spectrophotometer was used to quantify the absorption of the dialysate at 260 and 280 nm. Contamination of the DNA by protein was not observed. A standard curve was generated by using known quantities of purified calf thymus DNA. After quantitation of DNA content, duplicate 1.5-ml samples were mixed with 10 ml of Aquasol for scintillation counting.

In order to concurrently assess the effect of unlabeled AAF on DNA synthesis in hepatocytes isolated from the same perfusion as the ones used with the labeled AAF experiments, cultures of hepatocytes made from the same preparations as the ones used with the labeled AAF were treated with unlabeled AAF at a concentration of 1 X 10^{-5} M and [H]-thymidine for the same time periods as described for radio labeled AAF and processed for autoradiography.

Cytotoxicity of Carcinogens. Hepatocytes with [H]-thymidine-precabeled DNA were produced by performing two-thirds partial hepatectomy upon adult male Fischer 344 rats followed by i.p. injection of 1 ml of [H]-thymidine per 100 g of body weight at 22, 24, and 26 hr posthepatectomy. One month later, isolated hepatocytes were prepared as described above. The highest carcinogen doses used in this study were applied to the liver cell cultures under conditions identical to those used for measuring the effect of the chemicals on the hepatocyte replication.

The radioactivity of cell cultures was assessed by scintillation counting after TCA precipitation as described previously. Toxicity was determined as the loss of label from hepatocyte cultures by comparing the radioactivity associated with the DNA of treated versus control cultures. Viability of attached cells was determined by trypan blue dye exclusion (7).

Production of GGT-positive Hepatocytes. GGT-positive and GGT-negative hepatocytes were compared with respect to survival and ability to synthesize DNA in the presence of toxic levels of a number of carcinogens. To obtain populations of GGT-positive cells, rats were partially hepatectomized and given injections of DEN (100 mg/kg) 24 hr later. The rats were maintained on food and water ad libitum for 2 months. Hepatocytes were prepared, cultured, treated with carcinogens, and processed for autoradiography as described. Fixation of the cultures was with ice-cold 95% ethanol rather than formalin. The cultures were histochemically assayed for the presence of GGT activity using the procedure of Wachstein and Meizel (54). Labeling indices were determined for GGT-positive and GGT-negative hepatocytes. Cultures prepared from hepatocyte rats which had not received DEN were stained for GGT to reveal the proportion of GGT-positive cells not due to carcinogen initiation.

RESULTS

Effect of HPRS and Solvent Concentration on the Incorporation of [H]-Thymidine. The uptake of [H]-thymidine (measured as radioactivity associated with TCA-precipitable material) with increasing concentrations of serum from hepatopoenized animals is shown in Chart 1 (left abscissa). A statistically significant increase in dpm/culture occurred between control (0% serum) and all other serum doses (p < 0.01). HPRS concentrations greater than 10% decreased the mean incorporation of radio labeled thymidine. In view of the similar labeling indices at 25 and 50% serum concentration (right abscissa), the decrease in counts of thymidine is probably due to dilution of radiolabeled thymidine by thymidine of the serum. Fewer grains per nucleus were seen in the labeled nuclei at higher serum concentrations (data not shown). We did not dialyze serum for removal of thymidine because, in other studies (27, 28), we have found that dialyzed serum is much less effective than undialyzed serum for stimulation of DNA synthesis. Dialysis reduces the stimulatory capacity of serum by 80%. Chart 1 (right abscissa) indicates that the labeling index increases with increasing serum concentration.
INHIBITION OF HEPATOCYTE DNA SYNTHESIS BY CARCINOGENS

Chart 1. Dose response of incorporation of [3H]thymidine (left abscissa) or labeling index (right abscissa) to increasing concentrations of HPRS. Cells were cultured for 48 hr with medium containing [3H]thymidine (10 μCi/ml) and the indicated concentrations of HPRS. The dpm/culture were determined by liquid scintillation counting after TCA precipitation. Parallel cultures were processed for autoradiography and determination of labeling indices as described in "Materials and Methods." Points, mean of pooled data (5 samples) from 2 experiments; bars, S.E. •, dpm; • labeling index.

Table 1

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Dose (%)</th>
<th>dpm/culture</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>242,724 ± 28,065a</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>95% ethanol</td>
<td>1</td>
<td>134,831 ± 21,450b</td>
<td>56b</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>195,440 ± 25,579</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>248,325 ± 5,531</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>275,742 ± 8,592</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>201,493 ± 26,930</td>
<td>84</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.5</td>
<td>150,591 ± 16,177</td>
<td>62b</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>235,483 ± 19,606</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>213,593 ± 21,273</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>254,634 ± 37,439</td>
<td>105</td>
</tr>
</tbody>
</table>

*Mean ± S.E. of 3 cultures.

Statistically significant differences between control and solvent-treated cultures (p < 0.05).

Fig. 1 is a photomicrograph of one field from an hepatocyte culture autoradiograph. HPRS was used in subsequent experiments at 25%; the labeling index in cultures with 25% HPRS was sufficiently high (54%) to observe inhibition, if present.

Ethanol and DMSO were added to hepatocyte cultures at the indicated concentrations to assess solvent effects on the incorporation of [3H]thymidine. Table 1 shows that ethanol or DMSO concentrations less than 0.5% did not significantly decrease the incorporation of thymidine.

Cytotoxicity of Carcinogens. Carcinogens were applied to hepatocyte cultures in which the DNA had been prelabeled in vivo with tritiated thymidine by injection after partial hepatectomy 1 month prior to cell isolation. In this study, the death and loss of cells are measured as loss of DNA-associated radioactivity. The data in Charts 2A and 3A demonstrate that mM concentrations of nitrosamines resulted in significant toxicity (p < 0.01). AB, concentrations less than 1 × 10⁻³ M did not result in statistically significant decreases in DNA per culture. 3'-MeDAB, 1-NA, and 2-NA all resulted in significant cell death at 5 × 10⁻⁴ M concentrations (p < 0.02); lower concentrations were not toxic. B(a)P did not cause significant toxicity at any of the concentrations tested. Significant toxicity due to AAF was not observed until the concentration reached 1 × 10⁻⁸ M (p < 0.05). The viability of carcinogen-treated cells from each group was assessed by trypan blue dye exclusion after the cultures were washed in preparation for NaOH solubilization and TCA precipitation. The hepatocytes that remained attached to the collagen-coated dishes at the end of the 48-hr incubation period were >95% viable in all treatment groups (data not shown), indicating that viable cells remained attached to collagen-coated dishes during carcinogen treatment, whereas the nonviable cells were removed by the washing procedure.

Inhibition of DNA Synthesis by Carcinogens. Several hepatocarcinogens, B(a)P, 1-NA, and 2-NA, were tested across a wide range of concentrations as shown in Charts 2B and 3B. AAF treatment resulted in significant inhibition of DNA synthesis measured by both labeling index and [3H]thymidine counts per culture at concentrations higher than 1 × 10⁻⁸ M (p < 0.01). 3'-MeDAB was an effective inhibitor at concentrations greater than 1 × 10⁻⁸ M (p < 0.01). B(a)P inhibited DNA synthesis at a concentration as low as 1 × 10⁻⁸ M (p < 0.05). The nitrosamines
INHIBITION OF HEPATOCYTE DNA SYNTHESIS BY CARCINOGENS

Binding of [³H]AAF to Hepatocellular DNA. Changes in microsomal functions of hepatocytes in primary culture have been described by several investigators (3, 18, 31). In order to examine the capability of replicating hepatocytes to activate the procarcinogen AAF, and to compare it to that of nonreplicating cells, separate cultures of replicating or nonreplicating hepatocytes were exposed to $1 \times 10^{-6}$ M tritiated AAF for three 24-hr periods as described. Chart 5 demonstrates that similar activation and DNA binding occurred in cultures of replicating hepatocytes (corresponds to 25% HPRS; labeling index, 54%) or nonreplicating hepatocytes (corresponds to 10% FBS; labeling index 11%). Concurrent experiments were conducted using equimolar unlabeled AAF to investigate the effect of $1 \times 10^{-6}$ M AAF on the labeling index of HPRS-stimulated cells when AAF is added at time points later than 4 hr. AAF treatment within the first 24-hr culture period resulted in a reduction in the labeling index from 7% (control) to <1%. When AAF was added in the second 24-hr culture period, the labeling index of AAF-treated cells was 9% less than control cells (49%). When AAF was added in the third 24-hr culture period, the labeling index in treated cells was 7% less than control, 23 and 30%, respectively. A comparison of these findings (not shown) to the data from AAF-treated cultures in which the carcinogen was added 4 hr after isolation and incubated for 48 hr (Charts 2C and 3C) yields the following information. Most of the untreated hepatocytes underwent DNA synthesis in the second 24-hr period in culture as described previously (26). After 24-hr in culture, $1 \times 10^{-5}$ M AAF did not inhibit DNA synthesis to the extent seen in cells treated for 48 hr from 4 hr after isolation (9 versus 82%). The results of this study and those of Chart 5 show that most of the activation of AAF takes place in the first 24 hr. Thus, in the experiments in which AAF was continuously present for the first 48 hr (Chart 3, B and C), the accrued effect in the first 24 hr substantially inhibits replication in the second 24-hr period.

Differential Sensitivities of GGT-positive and GGT-negative Hepatocytes to Carcinogen Inhibition of DNA Synthesis. Hepatocyte cultures prepared from rats treated with DEN followed by partial hepatectomy (2 months prior to use for the preparation of hepatocytes) were treated with the carcinogens shown in Table 2. Marked resistance of GGT-positive hepatocytes to toxicity or inhibition of DNA synthesis exerted by carcinogens was observed in all cases. In the presence of carcinogen doses known to be toxic to uninitiated hepatocytes, GGT-positive cells were often the only cells attached to the tissue culture dishes at the end of the 48-hr incubation period (data not shown). The percentage of GGT-positive cells in the cultures was approximately 10% (the percentage of GGT-positive cells in cultures of hepatocytes from animals given only partial hepatectomy without DEN was less than 1%).

DISCUSSION

A wide variety of chemical carcinogens has been shown to be metabolized to reactive electrophilic intermediates that are capable of binding to nucleophilic sites in DNA, RNA, proteins, and other cellular molecules (4, 19, 45). It is generally felt that the interaction of carcinogens with DNA is a critical step in carcinogenesis (43). Suppression of cell replication and DNA synthesis is frequently caused by chemical carcinogens, and it is considered to be the result of either genotoxicity due to the formation of toxic metabolites or the interference with macromolecular synthesis (44).
INHIBITION OF HEPATOCYTE DNA SYNTHESIS BY CARCINOGENS

CHART 4. Effect of several noncarcinogenic compounds on the DNA synthesis of replicating hepatocytes. Columns, mean of duplicate samples from 2 experiments; bars, S.E.

CHART 5. Binding of $[^3H]$AAF to hepatocellular DNA in cells cultured with 25% HPRS (A) or 10% FBS (B). Hepatocytes were incubated with AAF for 4 to 24 hr, 24 to 48 hr, or 48 to 72 hr and harvested. DNA from each incubation period was purified on CsCl gradients, and the radioactivity was determined by liquid scintillation counting. DNA was quantitated by A$_{260}$ nm.

TABLE 2
Differential effect of carcinogens on DNA synthesis in hepatocytes positive or negative for GGT

<table>
<thead>
<tr>
<th>Additive</th>
<th>Dose</th>
<th>GGT positive</th>
<th>GGT negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>51 ± 7%</td>
<td>53 ± 4</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.1%</td>
<td>54 ± 3</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>$AB_1$</td>
<td>1 μM</td>
<td>16 ± 14</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>$AB_1$</td>
<td>0.1 μM</td>
<td>21 ± 7</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>$AB_1$</td>
<td>10 μM</td>
<td>42 ± 7</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>DEN</td>
<td>10 μM</td>
<td>51 ± 3</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>DEN</td>
<td>5 μM</td>
<td>46 ± 6</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>3'-MeDAB</td>
<td>0.1 μM</td>
<td>59 ± 4</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>3'-MeDAB</td>
<td>10 μM</td>
<td>54 ± 3</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>AAF</td>
<td>0.1 μM</td>
<td>30 ± 7</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>AAF</td>
<td>10 μM</td>
<td>46 ± 3</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>AAF</td>
<td>1 μM</td>
<td>51 ± 5</td>
<td>20 ± 5</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of 3 experiments.

DNA adducts or nonspecific cytotoxicity. As mentioned in "Materials and Methods," the measurement of thymidine uptake by liquid scintillation was expressed as dpm per plate. This allows for the possibility that some of the observed decline in thymidine uptake seen by this method can be due to lower numbers of cells per plate. To allow evaluation of this parameter, the results shown in terms of dpm per plate in Charts 2 and 3 are shown in parallel with the measurement of the percentage of cells that remained in the plate, so that a direct comparison between the cell killing induced by the carcinogen and the observed DNA synthesis inhibition can be made. We have measured cell death and inhibition of DNA synthesis at equimolar carcinogen concentrations in order to distinguish, as much as is practically possible, between these 2 possibilities. It is likely that the effects observed with carcinogens that inhibited DNA synthesis at very low concentrations (AAF, $AB_1$) were due primarily to genotoxicity, since no cell death was seen at those concentrations. Higher concentrations of all the carcinogens tested could result in subtle cytotoxicity that did not result in measurable cell death. Cytotoxicity with or without genotoxicity was probably an important factor at the high carcinogen concentrations, especially at the concentrations that were shown in Charts 2A and 3A to result in statistically significant death of hepatocytes.

The dose-response curves of carcinogen inhibition of hepatocyte DNA synthesis (Charts 2B and 3B) did not always parallel the carcinogenic potency observed in vivo. The hepatocarcinogenic potency in vivo can, theoretically, be affected by several parameters. These are: (a) the capability of the carcinogen to interact with hepatocellular DNA (as determined by pathways of activation and detoxification and metabolite diffusion resulting in final concentrations of ultimate carcinogenic metabolites in the proximity of hepatic DNA); (b) the cytotoxicity of the carcinogen toward parenchymal hepatocytes. Carcinogens that are more cytotoxic can cause death of hepatocytes followed by hepatocyte replication due to stimulation of hepatocyte regeneration. Hepatocyte regeneration enhances the carcinogenic response (37, 39); (c) the degree of inhibition of the cell replication of
The naphthylamines only caused inhibition of hepatocyte DNA synthesis (42). Liver is capable of bioactivation of 1-NA and 2-NA but alkylation (1), the former resulting in termination of DNA synthesis in vitro was shown to be differentially affected by AAF (36) and ducts caused by DEN and DMN are quite small and would not be expected to cause the same degree of helical distortion in DNA. The in vitro systems with other cell types, DNA synthesis was quantitated by observing the decrease in incorporation of tritiated thymidine into newly synthesized DNA by scintillation counting and autoradiography. The differential sensitivities of normal and DEN-initiated hepatocytes to inhibition of DNA synthesis by carcinogens were examined by autoradiography.

At equimolar concentrations, AAF was a stronger inhibitor of DNA synthesis of normal hepatocytes than B(a)P. AAF was also more hepatocytotoxic than B(a)P. AAF is known to be a stronger hepatocarcinogen in vivo than B(a)P (22, 33).

AAF and 3’-MeDAB are considered strong hepatocarcinogens (55). AAF is much stronger than 3’-MeDAB in suppression of DNA synthesis at low concentrations, and thus, it is presumably more capable of generating genotoxic DNA adducts. At relatively high concentrations (10^{-5} M), 3’-MeDAB is well tolerated, whereas AAF is only slightly cytotoxic. The relative advantage of 3’-MeDAB that makes it a strong hepatocarcinogen may lie in the fact that the initiated cells appear quite resistant to 3’-MeDAB even at high concentrations (10^{-4} M).

Aflatoxin is a potent hepatocarcinogen for the rat (12). Of the carcinogens tried in this study, aflatoxin is considered to be the most potent. In our study, it induced the strongest inhibition of DNA synthesis even at very low concentrations, and it also caused the strongest cytotoxicity as compared to equimolar concentrations of all the carcinogens used in this study. At higher concentrations of AB, however, it inhibited the DNA synthesis of initiated hepatocytes quite strongly.

The nitrosamines caused suppression of DNA synthesis only at concentrations that were clearly cytotoxic. DMN and DEN are known hepatocarcinogens (25). The difference between the nitrosamines and the other carcinogens as inhibitors of DNA synthesis may lie in the nature of the DNA adducts formed. In comparison to AAF, B(a)P, AB, and 3’-MeDAB, the DNA adducts caused by DEN and DMN are quite small and would not be expected to cause the same degree of helical distortion in DNA. The in vitro systems with other cell types, DNA synthesis in vitro was shown to be differentially affected by AAF (36) and UV irradiation (35, 53) as opposed to depurination (48) or methylation (1), the former resulting in termination of DNA synthesis and the latter resulting only in miscoding.

The naphthylamines have been shown to be bladder carcinogens (42). Liver is capable of bioactivation of 1-NA and 2-NA but is also very efficient at detoxification of these compounds (23). The naphthylamines only caused inhibition of hepatocyte DNA synthesis at concentrations that produced cytotoxicity to cultured hepatocytes.

DNA synthesis estimated by liquid scintillation was, with the exception of AB, a more sensitive indicator of a decrease in DNA synthesis than was autoradiographic quantitation of labeled indices. This could be due to the fact that, even with reduced DNA synthesis (e.g., 50% decrease in the number of participating replicons), a nucleolus could accumulate enough radioactive thymidine to be scored as positive for DNA synthesis in autoradiographic measurements.

It has been shown previously (30) for most of the carcinogens used in this study that they can induce DNA repair that can be monitored by uptake of [3H]thymidine, measured by autoradiography or liquid scintillation techniques. Thus, it is possible that the estimation of the suppression of DNA synthesis may have been affected by thymidine uptake due to DNA repair. We do not think, however, that this interference was of serious consequence for either the measurements by liquid scintillation or the labeling index determination. In studies of UDS in nonproliferating hepatocytes done in our laboratory, we have found that the uptake of thymidine due to the UDS is usually less than 10% of the uptake due to hepatocyte replication. Thus, the measurements of thymidine uptake by liquid scintillation should not have been interfered by more than 10%. In addition, this interference should only be considered for the concentrations of carcinogens that allow measurement of UDS. As we mentioned further in the discussion, inhibition of DNA synthesis can be seen at concentrations which are 100 to 1000 times lower than the lowest concentrations that allow UDS to be measured. The measurement of inhibition of DNA synthesis at the concentrations of carcinogens that do not induce UDS should not be affected by UDS. The measurements at concentrations that also induce UDS should be corrected for the contribution of approximately 10% of counts due to UDS. It should also be pointed out that any contribution due to UDS would only give a false result by showing less inhibition of DNA synthesis. The main point of this report is to show that hepatocarcinogens inhibit DNA synthesis in replicating hepatocytes. If it is considered that a percentage of the observed thymidine uptake is due to UDS, then this consideration should strengthen the main conclusion of this report. The measurements of labeling index by autoradiography should be affected even less by UDS. The exposure of the emulsion-coated plates for the purpose of these studies was 10 days. In that period of time, the small amount of thymidine uptake due to UDS does not result in significant numbers of grains per nucleus above background. In studies where UDS is measured by autoradiography (40), grains of thymidine are seen over most of the cells. This is not seen in our studies. Even with the higher concentrations of carcinogens used in this study, it was clear in all autoradiographs that the hepatocytes could be separated in 2 populations of cells. One population of cells had only background counts (less than 5 grains per nucleus), whereas the other population had, even at the highest carcinogen concentrations used, more than 40 grains per nucleus. It was impossible to distinguish and count the grains in the nucleus in the low carcinogen concentrations because, due to the high number of grains, the nuclei appeared homogeneously black. In the higher concentrations of carcinogens, a lower number of grains was seen, as manifested by the fact that the individual grains could be clearly distinguished. In all instances, however, the labeled...
cells did not have less than 40 grains per nucleus, whereas only background numbers could be seen in the unlabeled cells. Thus, UDS should not have been a factor to be considered in the measurement of the labeling index.

In a study published previously (26), we reported that optimal hepatocyte DNA synthesis measured by autoradiography occurred in the presence of 50% serum from partially hepatectomized rats. Labeling indices as high as 80% were observed. In this study, the highest labeling index (50%) was observed with the presence of 50% serum from partially hepatecto-

Several studies have shown that hepatocytes in primary culture have a decreased capability to activate chemical carcinogens. In the system described in this report, the results obtained must also have been affected by alterations induced in cell culture. The AAF-induced inhibition of DNA synthesis decreased as the time in culture increased. The binding of radiolabeled AAF to hepatic DNA also decreased as a function of time. This result may be related to the fact that cytochrome P-450 concentrations decline in culture (3, 18, 31), as well as to the decreased sensitivity of hepatocytes to carcinogens or toxins during regeneration (45), or both. These alterations can result in different rates of metabolic activation of the carcinogens by the cells in vitro as compared to in vivo. Future studies focusing on specific carcinogens can make correlations between indices used in this study and indices of DNA damage derived from direct assessment of DNA adduct formation. In such studies, the potential errors due to altered metabolic activation can be theoretically overcome.

For some of the carcinogens used in this study, inhibition of DNA synthesis is affected at concentrations that are much lower than the concentrations that are effective in other genotoxicity assays utilizing hepatocytes. This is true for AAF, B(a)P, 3'-MeDAB, and AB1, in comparison with assays using UDS (30), cell-mediated mutagenesis (32), DNA binding (51), etc. In the presence of DNA adducts that prevent the DNA synthesis, the whole length of the DNA chain undergoing DNA synthesis is prevented from participating in the process for a long distance downstream from the point of blockage of DNA polymerase. This can result in easily measurable decrease of thymidine incorporation. Other systems mentioned above are not as sensitive, because they rely on, e.g., measurable thymidine incorporation above background (UDS) or diffusion of metabolites to adjacent cells (cell-mediated mutagenesis) or adequate binding of radioactive carcinogen to DNA (DNA binding, usually hampered by the lack of labeled carcinogen of adequately high specific activity).

Another consideration in using this assay for establishing genotoxicity is the fact that inhibition of DNA synthesis can result from a variety of reasons that are not necessarily related to genotoxicity (e.g., cytotoxicity, etc.). This is shown in Chart 4, where high concentrations of known nongenotoxic compounds inhibited DNA synthesis at concentrations that resulted in grossly visible cell death. Thus, inhibition of DNA synthesis cannot be itself be used as proof of genotoxicity of a compound of unknown genotoxic potential. The results of this study, however, suggest that for some compounds of already proven genotoxic potential, inhibition of hepatocyte DNA synthesis may be the most sensitive in vitro assay that can be used to determine the lowest level of a known chemical carcinogen that can be shown to have a genotoxic effect. Thus, this assay may be of value for measurements required for policy decisions in which the lowest measurable genotoxic concentration of a known carcinogen needs to be empirically assessed.

REFERENCES

INHIBITION OF HEPATOCYTE DNA SYNTHESIS BY CARCINOGENS


Inhibition of DNA Synthesis by Chemical Carcinogens in Cultures of Initiated and Normal Proliferating Rat Hepatocytes

Deborah L. Novicki, Mark R. Rosenberg and George Michalopoulos


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/45/1/337

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.