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Dose-dependent DNA Ruptures Induced by the Procarcinogen Dimethylnitrosamine on Primary Rat Liver Cultures[1]

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ABSTRACT

The effect of certain procarcinogens, among which dimethylnitrosamine (DMN) is included, has been difficult to detect in several short-term assays. An alternative system, in which DMN effects could be easily quantitated, might be useful in studies of chemical carcinogenesis and enviromental contamination. To develop such a system, we tested the possibility of measuring the amount of breakage produced by DMN on radiolabeled DNA of primary liver cultures. Rat liver cells were isolated 20 to 24 hr after partial hepatectomy, cultured, and pulse labeled *in vitro* with [^{3}H]thymidine. Radioactively labeled cultures were treated with DMN or with the direct carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and then lysed directly onto alkaline sucrose gradients. DMN and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine caused a dose-dependent reduction in the molecular weight of DNA, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine being approximately 1000 times more potent than DMN. DNA breaks appeared to be carcinogen specific and not due to cell death since treatment with high doses of cycloheximide, a noncarcinogenic hepatotoxic, was without significant effect. Our data indicate that detection of DNA breaks constitutes a more sensitive assay of DMN effects than does unscheduled DNA synthesis in primary liver cultures. Therefore, it could be useful to extend our work to determine the general applicability of quantitation of DNA breaks in liver cells as a short-term assay for the identification of possible carcinogens and procarcinogens.

INTRODUCTION

Most of the chemical carcinogens interact covalently with DNA (15). Furthermore, they induce specific DNA repair mechanisms including ruptures, removal of DNA-carcinogen adducts, and UDS (15).[4]

Procarcinogens exert their effect only if they are converted to the ultimate carcinogen (19) and are usually assayed in systems in which an activating fraction from liver is added to the target organisms (1, 8, 12, 16, 17). Different classes of procarcinogens, requiring specific enzymatic systems to become activated, have been shown to elicit UDS in primary cell cultures of rat hepatocytes (10, 21). Such cultures have been proposed for the *in vitro* detection of procarcinogens as a system not requiring an exogenous fraction for procarcinogen activation (11, 20).

Several carcinogens have been shown qualitatively to induce ruptures in rat liver DNA (5, 14). The procarcinogen DMN induced DNA breaks in cultured fibroblasts only if these cells were mixed with a liver microsomal fraction (8) and also caused barely detectable UDS in primary rat liver cultures (10, 21).

In this paper, we show that both DMN and the direct carcinogen MNNG cause dose-dependent breaks in radioactively labeled DNA from rat liver cultures and that their effect can be easily quantitated.

MATERIALS AND METHODS

Isolation of Liver Cells. Male Wistar rats (190 to 210 g) were subjected to 67% partial hepatectomy (6) and then starved for 20 to 24 hr. The remaining liver was then perfused *in situ* via the portal vein by the method of Berry and Friend (2) with the Salt Solution TD for 3 min at 37° and a flow rate of 15 ml/min by means of a Buchler peristaltic pump. Following this, the liver was perfused with type I collagenase (100 units/ml; Sigma Chemical Co., St. Louis, Mo.) in TD at 37° for 10 min at a rate of 10 ml/min. The cells were dispersed by gently combing the excised liver with a stainless steel pin brush in a 37° TD solution containing type I collagenase (100 units/ml), DNase I (1 μg/ml; Sigma Chemical Co.), 5 mM $CaCl_2$, and 5 mM $MgCl_2$. The cell suspension was then filtered through a nylon mesh of 200 μm/side, allowed to sediment for 10 min, resuspended in TD solution, and allowed to sediment again. The supernatant was discarded, and the cells were resuspended in 10 ml of Eagle's MEM supplemented with 7% newborn calf serum (Biocel, Mexico City, D.F., Mexico) and 1 μM insulin (Laboratorios Lilly, Mexico City, D.F., Mexico). The yields averaged 50 million cells per hepatectomized rat with 90% viability as measured by trypan blue exclusion.

Plating and [^{3}H]dThd Incorporation into DNA. One million cells were inoculated into 35- × 10-mm tissue culture dishes (Falcon Plastics Co., Oxnard, Calif.) in 1.5 ml of supplemented MEM containing [^{3}H]dThd (5 μCi/ml; 47 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.) and placed in a 37° humidified incubator gassed with 95% air:5% CO_2. After 2 hr, detached cells were washed out, and the dishes were refed with supplemented MEM and incubated for an additional 2 hr.

Pulse Labeling of Rat Hepatocyte DNA. The cells were plated as above, except that [^{3}H]dThd (1 μCi/ml) was added to each dish. After 2 hr labeling, the cells were lysed with 0.5 ml 5% sodium dodecyl sulfate. The lysate was precipitated in cold 10% trichloroacetic acid, collected on 24-mm Whatman GF/A glass fiber filters, washed twice with 3 ml of cold 5% trichloroacetic acid, and twice with cold absolute ethanol. Each filter was then placed in a glass vial with 2 ml of Instagel (Packard Instrument Co., Inc., Downers Grove, Ill.). Tritium radioactivity

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[4] The abbreviations used are: UDS, unscheduled DNA synthesis; DMN, dimethylnitrosamine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; salt solution TD, 137 mM NaCl: 50 mM KCl: 0.4 mM Na_2HPO_4: 25 mM Tris: 0.001% phenol red (pH 7.5); MEM, Eagle's minimal essential medium; dThd, thymidine; M_w, weight-average molecular weight; M_r, relative weight-average molecular weight; D_{50}, mean effective dose.

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was measured in a Packard liquid scintilllation spectrophotometer.

Treatment with Chemicals. MNNG (ICN K & K Laboratories, Inc., Plainview, N. Y.), DMN (Eastman Organic Chemicals, Rochester, N. Y.), and cycloheximide (Sigma Chemical Co.) were dissolved immediately before use in serum-free MEM (pH 7.2) and added to the dishes 4 hr after plating the cells, while controls were exposed to carcinogen-free medium. Following treatment, the dishes were washed twice, and the cells were scraped off with a rubber policeman in 0.2 ml TD.

Centrifugation in Alkaline Sucrose. Samples of 100 μl, containing approximately 5×10^4 cells, were layered onto a 100 μl layer of lysing solution (1 M NaOH, 0.1 M EDTA, and 1% sodium dodecyl sulfate) previously placed on top of a 3.7 ml alkaline 5 to 20% (w/v) sucrose gradient (0.7 M NaCl, 0.3 M NaOH, and 0.01 M EDTA). One tenth ml of lysing solution was added on top of the cell suspension; the centrifuge tubes were then incubated for 1 hr at 37° and run in a Beckman model L4 ultracentrifuge with a SW-56 rotor at 50,000 rpm for 30 min at 20°. After centrifugation, approximately 25 fractions of 6 drops each were collected in glass vials from the bottom of pierced tubes. Each fraction was neutralized with 0.2 ml of 0.2 M HCl, and then 0.8 ml of Instagel was added to determine the radioactivity.

Calculation of Molecular Weight of DNA. The average molecular weight of DNA in a given fraction was calculated with the formula (9):

$$M = \left(\frac{K}{L\omega^2 t}\right) 1/k_D 1/k$$

where D is the distance in cm traveled by molecules in a run where the rotor was spun for ω rpm for t hr, and K, L, and k are constants. K resulted in 7.08×10^{10}, assuming a value of $s_{20,w} = 71$ for the T4-phage DNA used as standard for calibration. For L and k, the values used were 0.0528 and 0.400, respectively, determined previously by Studier (18). The M_w was calculated from the distribution of radioactivity through each gradient (13) using a programmable calculator.

RESULTS

Incorporation of [^{3}H]dThd into DNA. The maximum rate of [^{3}H]dThd incorporation into TCA-precipitable material (Chart 1) was achieved 2 to 4 hr after plating, and it was kept at around 50% of the maximum from 4 to 12 hr. The incorporation rate was negligible 23 to 25 hr after plating (4% of the maximum). Therefore, the first 2 hr after plating were chosen for tritium labeling. This interval was followed by a 2-hr incubation in complete medium without radioactive dThd to allow maturation of the recently synthesized radiolabeled DNA.

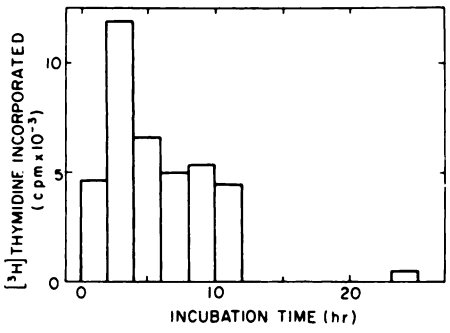

Chart 1. [^{3}H]dThd incorporation by cultured hepatocytes. [^{3}H]dThd (1 μCi/ml) was added to each dish at the beginning of 2-hr intervals after which the cells were lysed with sodium dodecyl sulfate. The lysates were precipitated with cold trichloroacetic acid, and the precipitates were collected onto glass fiber filters and counted. *Bars*, mean of 2 determinations.

M_w of Control Hepatocyte DNA. The M_w of DNA from control cultures in different experiments (mean of 2 determinations) ranged between 2.14 and 3.71×10^8, with an average of 2.98×10^8. Since the S.E. was ±5%, M_w variations smaller than 10% of the M_w of control DNA were not considered statistically significant ($p < 0.05$).

Quantitation of the Decrease in the Size of DNA from Treated Hepatocytes. Treatment of cultured hepatocytes for 1 hr with MNNG (1 to 50 μg/ml) (Chart 2*A*) or for 2 hr with DMN (30 to 1200 μg/ml) (Chart 2*B*) reproducibly decreased the sedimentation velocity of DNA. The M_r of DNA from treated cultures was calculated by comparison with that of DNA from untreated cultures and plotted as a function of carcinogen concentration (Chart 3). MNNG decreased M_r proportionally to the dose (Chart 3*A*); the D_{50} of MNNG, *i.e.*, that producing one average break per DNA molecule and thus decreasing M_r to one-half the original, was 11.2 μM (calculated by linear regression of the experimental values). The highest dose of MNNG

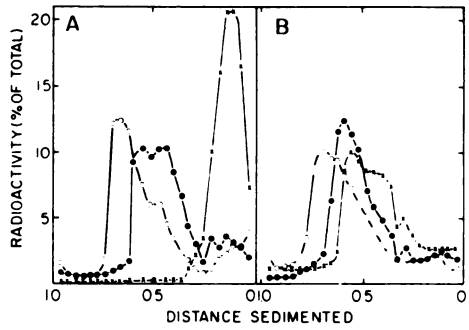

Chart 2. Effect of MNNG and DMN on the sedimentation velocity of hepatocyte DNA. The cultures were incubated for 2 hr in medium containing [^{3}H]dThd (5 μCi/ml) and for an additional 2 hr in fresh medium without [^{3}H]dThd and then treated with different concentrations of MNNG or DMN. After treatment, the cells were detached, lysed, and centrifuged in alkaline sucrose gradients. The sedimentation is depicted from *right* to *left*. *A*, sedimentation profiles of control culture DNA (○) and of cultures treated for 1 hr with 6.8 μM (●) or 0.34 mM (×) MNNG; *B*, sedimentation profiles of control culture DNA (○) and of cultures treated for 2 hr with 0.45 mM (●) or 16.2 mM DMN (×).

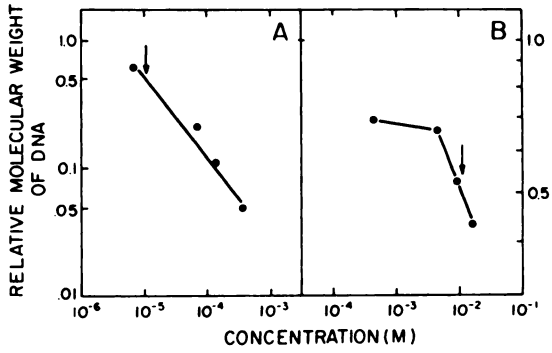

Chart 3. Effect of MNNG and DMN on the relative molecular weight of hepatocyte DNA. The M_w's of DNA from 2 control cultures and 4 cultures treated with different concentrations of MNNG (*A*) or DMN (*B*) were determined and expressed as a fraction of the controls. *Points*, mean of 2 experiments done with cultures obtained from different rats. ——, drawn to fit the corresponding equations obtained by linear regression. ↓, mean effective dose of each chemical. *Scales* of the *ordinates* are different.

tested (340 μM) caused a 20-fold decrease in the original size of DNA. Treatment with DMN in the range 4.46 to 16.2 mM was also followed by a proportional decrease in the size of DNA (Chart 3*B*). The lowest dose of DMN tested (0.45 mM) significantly diminished the DNA M_r (30% below the control value). The D_{50} of DMN was 10.8 mM, approximately 1000-fold larger than that of MNNG.

Cycloheximide Effect on Molecular Weight of Hepatocyte DNA. Liver cell cultures were also treated with different cycloheximide concentrations. Their DNA sedimentation profiles were obtained, and their M_r was calculated as before. The highest dose of cycloheximide tested (10 mM) was similar to the highest dose of DMN and was the only one causing a slight but significant decrease in the size of DNA (Table 1).

DISCUSSION

Since most chemical carcinogens injure DNA, many short-term tests for their assay are in fact systems for the detection of agents causing DNA damage (3). Such damage can be measured by determining UDS or DNA strand breaks in alkaline sucrose gradients (17). Both approaches have been used for detection of chemical carcinogens on cultured fibroblasts (17). Only a limited number of carcinogens can be detected on fibroblasts since these cells lack enzymatic activities that are neccesary to metabolize and activate procarcinogens to their ultimate carcinogenic forms (11).

It has been suggested that detection of DNA breaks is closer to the actual event of DNA alteration than is the repair end point (17). A target system in which DNA breaks produced by carcinogens and procarcinogens were measured would have this advantage over UDS detection. Primary rat hepatocyte cultures appear to constitute an adequate system for procarcinogen detection, since the same cells might activate and act as the target of chemicals. These facts led us to try to quantitate ruptures induced in hepatocyte DNA by the procarcinogen DMN, previously shown to be difficult to detect in several short-term *in vitro* assays (11, 21), and to compare DMN effects with those of a direct carcinogen (positive control) and a hepatotoxic noncarcinogen (negative control).

DNA was radiolabeled in hepatocytes isolated 20 to 24 hr after partial hepatectomy when about 30% of all parenchymal cells have been reported to incorporate [^{3}H]dThd (7). The cultured hepatocytes incorporated [^{3}H]dThd efficiently, and the label was autoradiographically located in the nuclei (data not shown). Before carcinogen treatment, the cells were pulse labeled and then incubated in fresh medium for a period long enough to allow maturation of the recently synthesized DNA.

The molecular weights of DNA from untreated cultures were in agreement with those reported previously for mammalian DNA by other workers using alkaline sucrose centrifugation (4), a method that can be used to quantitate single-stranded DNA breaks but cannot distinguish between preexisting breaks and hydrolysis of alkali labile sites. Quantitation of DNA breaks induced by carcinogens was performed by comparing the relative molecular weight of DNA from control cultures with that of treated ones, and it allowed us to obtain dose-response curves and D_{50}, a parameter that might be used to compare the potency of different chemical carcinogens.

Table 1

Effect of cycloheximide on the molecular weight of DNA

Cycloheximide concentration (mM)	Relative molecular wt[a]
0.0	1.00 (0.08)[b]
0.1	0.99 (0.08)
0.5	0.97 (0.03)
1.0	1.03 (0.04)
10.0	0.85 (0.06)

[a] Mean of 2 determinations on different cultures. The relative molecular weight of DNA from untreated cultures was defined as 1.00.
[b] Numbers in parentheses, relative dispersion.

High concentrations of DMN (10 to 100 mM) have to be used to produce detectable UDS in primary rat hepatocyte cultures (21) or in freshly isolated hepatocytes (11). The D_{50} for DMN in our system was 10.8 mM, and even 1/24 such concentration produced a highly significant diminution in DNA size (see Chart 3*B*). Lower DMN concentrations than those shown here might have even greater effects if exposure times were also modified. Our data, however, clearly show that measurement of breaks in labeled DNA is a much more sensitive assay than is detection of UDS in rat liver cells.

Production of DNA breaks appear to be carcinogen specific and not due to the endonucleolytic degradation of DNA accompanying cell death (22), since only the highest tested dose of the hepatotoxic cycloheximide caused a slight decrease in the size of DNA. This agrees with the finding that cycloheximide and several other hepatotoxic noncarcinogenic agents, when tested *in vivo*, induced no apparent strand breaks in liver DNA except after cell death (15).

The finding that the administration of a wide variety of carcinogens (methylating and ethylating nitrosamines and nitrosamides, aromatic amines, cyclic nitroso compounds, azo dyes, and intercalating agents) induces hepatic DNA strand breaks *in vivo* (15) and the results obtained by us with the alkylating carcinogens MNNG and DMN appear to support the idea that detection of DNA breaks in cultured liver cells could constitute an adequate system to screen suspected chemical carcinogens.

Our work also suggests that determination of DNA ruptures could be advantageous over UDS, especially for the detection of procarcinogens, since it allows dose-response analysis and is more sensitive. Further work with different classes of chemical carcinogens is needed to evaluate the applicability of quantitation of DNA breaks in cultured liver cells as a screening test for suspected chemical (pro)carcinogens.

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