Chemical Quantification of Unscheduled DNA Synthesis in Cultured Hepatocytes as an Assay for the Rapid Screening of Potential Chemical Carcinogens

Felix R. Althaus,2 Susan D. Lawrence, Gerald L. Sattler, David G. Longfellow, and Henry C. Pitot3

ABSTRACT

Technical modifications of the quantitative determination of unscheduled DNA synthesis in cultured hepatocytes are described which allow for the rapid identification of potentially carcinogenic chemicals on a large-scale screening basis. The test is based on the biochemical quantification of [methyl-3H]thymidine incorporation into DNA in the presence of hydroxyurea following isolation of nuclei from hepatocytes treated with the agent under study. This procedure ("nuclei procedure") eliminates most of the background radioactivity which otherwise obscures the stimulation of DNA repair synthesis by agents that induce a relatively weak response. By combining the nuclei procedure with a double-labeling technique, test results can be obtained within a few hr after exposure of hepatocytes to the test agents. A test series involving 41 agents confirmed the reliability of the nuclei procedure for the assay of DNA repair synthesis. In addition, chemicals which had yielded conflicting results previously in the autoradiographic hepatocyte DNA repair test, such as 4-acetylaminofluorene, or which had passed unrecognized in previous in vitro tests, such as the potent liver carcinogen methapyrine hydrochloride, scored clearly positive in our test protocol.

INTRODUCTION

In the hierarchy of test systems for chemical carcinogenesis, cultured hepatocytes derived from various species have become increasingly important because of their differentiated procarcinogen-metabolizing capacity. This capacity makes liver cells also a probable site for the early interaction with carcinogens, since it is well established that the carcinogenic properties of a large number of chemicals arise from the metabolic activation of their precursors, procarcinogens (17). Thus, hepatic cells also offer a realistic end point for carcinogen testing, i.e., DNA damage by ultimate carcinogens. The occurrence of DNA damage can be measured either directly (12) or indirectly by determining DNA repair synthesis which follows damage to DNA (12). DNA repair synthesis is more amenable to rapid analysis than the identification of DNA damage by carcinogens. Therefore, for practical purposes, analysis of DNA repair synthesis is more suitable for large-scale screening of potentially carcinogenic agents.

The majority of published carcinogen testing using a DNA repair system has been performed using autoradiographic analysis of the incorporation of [3H]thymidine into DNA during repair synthesis (unscheduled DNA synthesis). This technique was originally used by Rasmussen and Painter (20) and others (10), and it was later adapted by Williams (25) for use in hepatocyte cultures. In this technique, unscheduled DNA synthesis is quantified by counting the number of activated silver grains in the radiographic emulsion over the nuclei which contain [methyl-3H]thymidine radioactivity incorporated into DNA. Due to the large differences in [3H]thymidine incorporation into DNA during replicative synthesis and repair synthesis of DNA, these 2 processes can be readily distinguished by nuclear grain counting. However, this analysis of DNA repair synthesis is relatively time consuming and therefore does not adequately meet the requirements of a procedure suitable for the rapid screening of thousands of chemicals for their carcinogenic potential. In view of the enormous costs of carcinogen testing, efficiency often becomes a major determinant in the choice of a test system. We therefore decided to evaluate other procedures for large-scale screening of carcinogens in the hepatocyte DNA repair test. The present report demonstrates that, using biochemical procedures to quantify DNA repair synthesis in cultured hepatocytes, test results can be obtained within a few hr after carcinogen exposure of cells.

MATERIALS AND METHODS

Materials. Leibovitz (L-15) medium, penicillin-streptomycin, and newborn calf serum were purchased from KC Biologic, Inc., Lenexa, Kan. Nicotinamide, isonicotinamide, insulin, bovine serum albumin, collagenase type I, hydroxyurea, and deoxynucleotide triphosphates were obtained from Sigma Chemical Co., St. Louis, Mo. Bisbenzimidazole trihydrochloride pentahydrate was purchased from Aldrich Chemical Co., Milwaukee, Wis. Digitonin was obtained from Fisher Scientific Co., Pittsburgh, Pa. Cyclohexanediaminetetraacetate was purchased from Fluka AG Chemische Fabrik, Buchs, Switzerland. [methyl-3H]Thymidine (42 to 58 Ci/mmol), [14C]thymidine, and [methyl-3H]TTCP (50 Ci/mmol) were purchased from Amersham/Searle Corp., Arlington Heights, Ill. The carcinogens listed in Table 2 were obtained from the following sources: methyl methanesulfonate, urethan (ethyl carbamate), safrole, and 4-aminobiphenyl from Aldrich; 3-methylcholanthrene from Calbiochem-Behring Corp.; methapyrine hydrochloride, 17β-estradiol, 4-nitroquinoline 1-oxide, and 7,12-dimethylbenz(a)anthracene from Sigma; thiourea from Matheson, Coleman, & Bell, East Rutherford, N. J.; thiocetamide from Fisher; and benzidine from J. T. Baker Chemical Co., Phillipsburg, N. J. Profloxine was obtained from Dr. Steve Graves, Midwest Research Institute, Kansas City, Mo. α-Hexachlorocyclohexane and its metabolite β-pentachlorocyclohexene were a gift from Dr. W. Parzefall, Phillips Universität, Marburg, Germany. N-Acetoxy-2-acetylaminofluorene and 2-methyl-4-dimethylaminoazobenzene were a gift...
from Dr. E. Miller and Dr. J. Miller, and Aroclor 1254 was generously supplied by Dr. A. Poland from McArdle Laboratory. All the other compounds mentioned in Table 2 were obtained from the National Cancer Institute Repository, IIT Research Institute, Chicago, Ill.

**Culture of Adult Rat Hepatocytes.** Hepatocytes were isolated from the livers of adult male albino rats (The Holtzman Co.; 220 to 280 g, fed ad libitum) by the collagenase perfusion method of Berry and Friend (4) as modified by Bonney et al. (6). The hepatocytes were then plated at a cell density of 10 to 13 x 10^6 cells in 10 ml of L-15 medium [supplemented with 18 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, albumin (2 mg/ml), 5% newborn calf serum, penicillin (100 µg/ml), streptomycin (100 µg/ml), glucose (1.5 mg/ml), and insulin (0.5 µg/ml)] onto 100-mm Falcon tissue culture dishes. L-15 medium contains 8.2 µM nicotinamide. A medium change was routinely performed 4 hr after plating to remove unattached cells, and culture was continued for the times indicated. For treatment of cultures with test agents, stock solutions of chemicals in H2O, medium, or dimethyl sulfoxide were added to the cultures. The concentration of dimethyl sulfoxide in the medium never exceeded 1%. For treatment with UV, the culture medium was removed, and the monolayers were irradiated with a germicidal lamp (254 nm) at an incident dose rate of 6 J/sq m/sec.

**Measurement of DNA Repair Synthesis (Nuclei Procedure).** DNA repair synthesis following treatment of hepatocyte cultures with UV or chemical carcinogens was measured by determining the amount of [methyl-3H]thymidine (10 µCi/plate) incorporated into nuclear DNA in the presence of hydroxyurea (10 mM). [methyl-3H]Thymidine was added to the medium immediately following the treatment of cultures by the agent under study which was continuously present in the medium during the labeling period. At the end of these experiments, the monolayers were washed with ice-cold phosphate-buffered saline (136.9 mM NaCl-2.7 mM KCl-4.3 mM Na2HPO4-1.5 mM KH2PO4, pH 7.4) containing 2 mM thymidine, and the cells were scraped off the plates by means of a rubber policeman. All the following procedures were carried out at 4°C. Hepatocellular nuclei were isolated as described previously (1). Briefly, hepatocytes were pelleted, and approximately 20 x 10^6 cells were resuspended in 900 µl of hypotonic buffer (10 mM Tris-HCl-5 mM MgCl2, pH 8.0). The cells were homogenized, incubated in 1% Triton X-100, and homogenized again, and nuclei were separated from other cellular material. Alternatively, this separation could be conveniently carried out using a Sorvall desk top centrifuge. In this procedure, the homogenate was centrifuged for 10 min at 800 rpm in a Sorvall Model GMC-2 centrifuge at 4°C. The supernatant was discarded, and the pellet was resuspended in 1 ml of 0.25 M sucrose-50 mM Tris-HCl-25 mM KCl-15 mM MgCl2 (pH 8.0) and carefully layered over 1 ml of 0.8 M sucrose-Tris-HCl-KCl-MgCl2 (4°C, concentrations as above) in 15-ml Corex tubes. Centrifugation was carried out at 2500 rpm for 10 min in a Sorvall Model GMC-1 instrument. The pellet contained the nuclear fraction. The amount of radioactive DNA incorporated into nuclear DNA was then determined as described previously (1). Using these procedures, [methyl-3H]thymidine incorporation into nuclear DNA of untreated control cultures amounted to less than 100 dpm/µg DNA during an 18-hr incubation period, which reflects the very low replicative DNA synthesis of these nonproliferating hepatocytes.

**Purification of Hepatocellular DNA on Cesium Chloride Gradients.** Hepatocytes were solubilized at room temperature in 500 µl 0.01 M Tris-Cl (pH 8.0) containing 1% sodium dodecyl sulfate and 10 mM EDTA. CsCl was then added in Tris-Cl-EDTA buffer to a final density of approximately 1.64 g/ml, and the suspension was centrifuged at 10,000 x g for 30 min at 10,000 x g in a fixed-angle rotor. The supernatant was layered on 1.5 ml CsCl-Tris-Cl-EDTA (density, 1.79 g/ml) and centrifuged for 2 hr at 34,000 rpm in a Beckman Model SW 56 rotor. Fractions of 300 µl were collected from the bottom of the gradient tubes, and the amount of radioactive DNA and the density of each fraction were determined. The DNA was contained in 2 consecutive fractions, and these 2 fractions had almost exactly the same amount of radioactivity per µg DNA (Table 1) as reported previously by Sirica et al. (23) using the same procedure. However, the DNA banded at a higher density (1.739 g/ml) than the one reported by Sirica et al. (23). The reason for this discrepancy of results is unclear at this time.

**DNA Repair Synthesis in Permeabilized Cells.** Hepatocytes were permeabilized with 0.005% digitonin as described by Fiskum et al. (11). For determination of DNA repair synthesis in permeabilized cells, the procedures of Ciarrocchi and Linn (6) was modified as follows. The reaction mixture contained in 55 µl consisted of 2 x 10^6 permeabilized hepatocytes in hypotonic NaHCO3 as above; 27 mM K2HPO4 (pH adjusted to 6.1 with citric acid); 2 mM dithiothreitol; 9 mM MgCl2; 4.5 mM ATP; 0.09 mM concentrations each of dATP, dGTP, and dCTP; and 0.5 µCi of [3H]dTPP (50 Ci/mmol). The final pH of the reaction mixture is 6.9.

After incubation at 37°C for 15 min, the reaction was stopped by addition of 200 µl of ice-cold 20% trichloroacetic acid, and the radioactivity of the trichloroacetic acid-insoluble fraction was determined on Whatman GF/C glass filters by scintillation counting. Incorporation of [3H]dTPP into DNA was linear for at least 15 min but continued to increase at a linear, although lower, rate for more than 60 min.

**Formation of DNA Strand Breaks.** The procedures by Birnboim and Jevcak (5) were adapted for use in hepatocyte culture in order to determine the formation and removal of DNA strand breaks in response to carcinogens. All the following procedures were carried out under subdued light. Hepatocytes were washed with an ice-cold solution of 75 mM NaCl-25 mM EDTA, and the cells were scraped off the plates at 0.25 M cesinomositol-10 mM sodium phosphate-1 mM MgCl2 (pH 7.2). The cells were pelleted and resuspended in this same buffer, and 200 µl of cell suspension containing 4 to 6 x 10^6 cells were aliquoted into sets of triplicate glass tubes (10 x 75-mm dial tubes; American Hospital Supply Corp., McGraw Park, III.). Cells lyzed and chromatin disruption were achieved by addition of 200 µl of a solution containing 9 mM urea-10 mM NaOH-2.5 mM cyclohexaneiminediacarboxylic acid-0.1% sodium dodecyl sulfate followed by incubation at 6°C for 10 min. The rate of DNA unwinding after addition of alkalai was then measured using ethidium bromide as a probe for DNA structure (5). A minimum of 3.6 x 10^6 cells (4 x 10^6 cells/tube) was used for a single determination in triplicate. The fluorescence ratio of the total amount of double-stranded DNA (T-value) versus the basal value (B-value, no double-stranded DNA remaining) ranged between 1.9 and 2.2 under these conditions (5).

**Other Procedures.** The DNA content of samples was determined according to the method by Burton (7). For very small amounts of sample, the method of Labarca and Paigen (13) was used.

**RESULTS**

We have measured DNA repair synthesis by determining [methyl-3H]thymidine incorporation into nuclear DNA of carcinogen-treated hepatocytes. This incorporation was shown to reflect DNA repair synthesis of these nonproliferating hepatocytes by a number of laboratories including our own (1, 16, 23, 27). However, the sensitivity of this technique, i.e., the stimulation of DNA repair synthesis that can be measured in response to a given carcinogen, is dependent on the degree to which unspecific background radioactivity can be eliminated (18). Therefore, 3 different methods were evaluated to quantify [methyl-3H]thymidine incorporated into hepatocellular DNA. These data are summarized in Table 1. DNA repair synthesis in response to the same dose of UV (60 J/sq m) was analyzed in 3 different ways. The highest stimulation of DNA repair synthesis (19-fold) was measured when nuclei were isolated prior to DNA extraction (nuclei procedure). This procedure yielded markedly low control values, i.e., 93 dpm/µg DNA. In

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**Hepatocyte DNA Repair Test for Chemical Carcinogens**

AUGUST 1982
Comparison of analytical techniques for the quantitation of DNA repair synthesis in cultured hepatocytes

Hepatocytes were irradiated with UV (60 J/sq m) and were subsequently maintained in the presence of [methyl-3H]thymidine and hydroxyurea (10 mM) in the culture medium. Eighteen hr after UV treatment, the hepatocytes were harvested, and DNA repair synthesis was measured by 3 different methods as described under “Materials and Methods.”

Table 1

<table>
<thead>
<tr>
<th>Analytical procedure</th>
<th>% of recovery of DNA</th>
<th>UV (60 J/sq m)</th>
<th>Stimulation of DNA repair synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct extraction of DNA</td>
<td>100</td>
<td>1700 ± 81*</td>
<td>6400 ± 63</td>
</tr>
<tr>
<td>Solubilization of cells, purification of DNA on cesium chloride gradients</td>
<td>58</td>
<td>519 ± 21</td>
<td>2023 ± 25</td>
</tr>
<tr>
<td>Nuclei procedure</td>
<td>84</td>
<td>93 ± 4</td>
<td>1788 ± 19</td>
</tr>
</tbody>
</table>

* Mean ± S.E.

Chart 1. Dose response (A) and time course (B) of the effect of UV light (254 nm) on DNA repair synthesis of cultured hepatocytes. In A, hepatocytes were irradiated with the indicated doses of UV 18 hr prior to harvesting cells. In B, hepatocytes were irradiated with UV at the times indicated prior to harvesting of cells. Hydroxyurea (10 mM) was added to the culture medium 1 hr prior to UV irradiation. DNA repair synthesis was measured by the nuclei procedure as described under “Materials and Methods.” The values reflect [methyl-3H]thymidine incorporation into nuclear DNA above the levels of untreated controls, m², sq m.

Chart 2. Effect of methyl methanesulfonate (MMS; 5 × 10−4 M) on the rate of incorporation of radioactivity from [3H]dTTP into DNA (A) and the formation and removal of DNA strand breaks (B) in cultured hepatocytes. Experimental procedures were as described under “Materials and Methods.” The data in B represent the percentage of double-stranded DNA remaining after a 60-min DNA unwinding period at 15°C as described by Birnboim and Jevcak (5). Values are averaged from 2 determinations.
resulted in a maximum rate of DNA repair synthesis 1.5 hr after hepatocytes had been exposed to this ultimate carcinogen, and after 4 and 6 hr, the rate of DNA repair synthesis had declined to low levels slightly above the incorporation rate of untreated control cultures. On the basis of these combined data, we selected an 18-hr treatment period for the testing of carcinogens in the hepatocyte DNA repair test, and we used the nuclei procedure described under “Materials and Methods” to analyze repair incorporation of [methyl-3H]thymidine into nuclear DNA.

Table 2 summarizes our test results with 41 compounds. Nineteen of these agents were furnished by the National Cancer Institute and tested in a double-blind manner. In order to allow rapid comparison with literature data, the test results are listed in the alphabetical order of the test compounds. For each test compound, a broad dose range was tested, and the maximally effective dose was retested at least one more time in a separate culture of hepatocytes. If no clear dose-response relationship was found, a higher dose range, including cytotoxic doses, was selected for the second testing.

In a recent study (3), we have demonstrated that inhibitors of nuclear ADP ribose biosynthesis, such as nicotinamide or...
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isonicotinamide, enhance the stimulation of DNA repair synthesis elicited by carcinogens or by UV. We therefore also tested most of the agents under study in hepatocytes that had been maintained in the presence of nicotinamide (25 mm) or isonicotinamide (10 mm). These results are also summarized in Table 2.

Using the nuclei procedure to analyze DNA repair synthesis in cultured hepatocytes, we were able to obtain quantitative test results within 1 or 2 days after carcinogen exposure of cells. Most of the time for analytical procedures was spent on the extraction and measurement of hepatocellular DNA. Therefore, we evaluated a procedure which did not require quantification of DNA and therefore would reduce the time for analytical procedures. Chart 3 shows the results of an experiment in which a double-labeling procedure was used to quantify DNA repair synthesis. In this procedure, carcinogen-treated hepatocyte cultures were maintained in the presence of [\(^{3}H\)thymidine, while untreated control cultures simultaneously received \([^{14}C\)thymidine. At the end of this exposure, hepatocytes were harvested and homogenized. \(^{3}H\)- and \(^{14}C\)-labeled homogenates prepared from equal numbers of cells were pooled, and the ratio of \(^{3}H\) over \(^{14}C\) dpm was determined in the acid-insoluble fraction of isolated nuclei. The ratio of \(^{3}H\) over \(^{14}C\) radioactivity from 2 control cultures receiving the 2 differently labeled forms of thymidine served as a control. This double-labeling technique for the determination of DNA repair synthesis yielded accurate quantitative results within a few hr after carcinogen exposure. The dose-response curve shown in Chart 3 also indicates that this improvement in testing efficiency apparently is not associated with a loss in sensitivity.

DISCUSSION

The hepatocyte DNA repair test designed by Williams (25) has demonstrated clearly the validity of the approach to use liver epithelial cells as a system to provide procarcinogen activation as well as an end point to determine DNA damage by carcinogens in a eukaryotic system. However, the use of autoradiography in the determination of DNA repair synthesis has some technical shortcomings which could seriously limit the use of this test system on a large-scale basis. A major shortcoming of this procedure is that it is relatively time consuming, which is due in part to the time required for exposure of the radiometric emulsion as well as for the grain counts. In addition, the autoradiographic response itself is somewhat variable due to lot differences in emulsion sensitivity as well as emulsion thickness (19). Thus, for example, up to 5-fold differences in the number of grains in untreated control cultures have been reported (19). These analytical problems might have contributed to the sometimes conflicting results obtained with this technique. For example, Williams (26) and Tong et al. (24) presented positive as well as negative test results for benz(a)anthracene and N-4-fluorethylacetamide in separate reports using autoradiographic analysis of DNA repair synthesis. However, using a biochemical procedure to quantify DNA repair synthesis, the same authors found both compounds to be positive in the hepatocyte DNA repair test (24). This illustrates clearly the problems with autoradiographic methods and demonstrates the potential of biochemical procedures for analysis of DNA repair synthesis.

The present report illustrates that DNA repair synthesis measured by biochemical determination of [methyl-\(^{3}H\)]thymidine incorporation into DNA requires rigorous elimination of contaminating radioactivity. In previous studies (16, 23), this was attempted by purification of total cellular DNA on cesium chloride gradients. However, this relatively tedious method still yields fairly high background counts in untreated control cultures (Table 1). In liver cells, the high level of radioactivity in the cytoplasmic fraction (18) due to thymidine catabolism (27) might be the cause of this contamination, since isolation of nuclei prior to DNA extraction eliminates this radioactivity from the acid-insoluble fraction. The nuclei procedure outlined in this report therefore provides a very simple procedure to determine DNA repair synthesis in liver cells in an accurate and sensitive manner. This is also illustrated by the fact that we found a much higher stimulation of DNA repair synthesis in response to UV than observed by others (27). Moreover, we were able to measure a stimulation of repair synthesis by agents that give a weak response, which otherwise would be obscured by contaminating radioactivity in the acid-insoluble fraction of these cells (Table 1). An important consideration in the design of in vitro test systems for screening potentially carcinogenic chemicals is the cost-benefit relationship. Only simple and efficient test systems are likely to find approval on a large scale by industry and regulating agencies. It is desirable that time-consuming analytical procedures can be reduced to a minimum. The double-labeling procedure shown in Chart 3 is very promising in this regard, since it yields quantitative results within a few hr after treatment of hepatocytes with the agent under study. This apparently was not associated with a loss of sensitivity of the test. The 41 agents we tested in the hepatocyte DNA repair test (Table 2) comprise a broad spectrum of chemical classes of known carcinogens, procarcinogens as well as direct-acting carcinogens, liver tumor promoters, and noncarcinogenic compounds. All the compounds that have been recognized as carcinogens were positive in the test with the exception of diethyldithiobistrol, ethylenethiourea, and thioacetamide. Diethylstilbestrol was also reported negative in the autoradiographic hepatocyte DNA repair test by Probst et al. (19). However, this same compound stimulated DNA repair synthesis in HeLa cells (14). The reasons for this discrepancy of results are not ob-
REFERENCES


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REFERENCES


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