Modulation of the Cell Cycle of Cultured Mouse Liver Cells by Benzo(a)pyrene and Its Derivatives

James C. Bartholomew, Andrew L. Pearlman, Joseph R. Landolph, and Kenneth Straub

Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720

ABSTRACT

We have used flow cytometry to monitor the alterations in cell cycle distributions caused by chemical carcinogens. Two closely derived mouse liver cell strains growing in culture have been studied with regard to the effect of benzo(a)pyrene and derivatives of benzo(a)pyrene on DNA synthesis. The carcinogenicity, mutagenicity, and cytotoxicity of this hydrocarbon to cells of mammalian origin has been extensively studied (1, 2, 3, 4, 5, 6, 7, 8, 9, 10). Stock cultures of a highly inducible AHM activity (27) were derived from NMuLi (36) mouse liver epithelial cells by the cloning technique of Puck et al. (39). The sensitivity of these clones to BaP cytotoxicity was first demonstrated by using a clonal assay and shown to correlate with the presence of a highly inducible AHM activity (27). All cells were cultured in plastic dishes (Falcon Plastics, Oxnard, Calif.) and incubated at 37°C in a 5% CO₂, 95% air incubator. The medium used to grow the cells was Eagle's minimal medium (12) (Grand Island Biological Co., Grand Island, N. Y.) containing 10% donor calf serum (Flow Laboratories, Rockville, Md.) and 10 μg insulin per ml (Schwarz/Mann, Orangeburg, N. Y.). The cells were judged free of Mycoplasma by incorporation of [3H]TdTh (20.1 Ci/mmol; New England Nuclear, Boston, Mass.) into the nucleus of the cell and not the cytoplasm (18). Stock cultures were maintained by subculturing the cells twice weekly at a cell density of 1 x 10⁶/sq cm. BaP and the derivatives were dissolved in DMSO (Matheson, Coleman, & Bell, Los Angeles, Calif.) immediately before addition to the cultures. The final DMSO concentration in all experiments was 0.1%. Cell counts were determined by using a Model ZBI Coulter counter (Coulter Electronics Inc., Hialeah, Fla.).

INTRODUCTION

BaP is a common environmental contaminant produced by the combustion, liquifaction, or gasification of fossil fuels (5, 6, 45). The carcinogenicity, mutagenicity, and cytotoxicity of this hydrocarbon to cells of mammalian origin has been extensively studied (1, 11, 15, 23, 26, 28, 29, 32, 33). All of these biological effects have been shown to involve metabolism of BaP by the AHM of the cell (15, 23). This enzyme system converts BaP into many products (8, 40), but recent studies have implicated the conversion into the (--)t-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene via the combined enzymatic action of AHM and epoxide hydrase followed by a second epoxidation in the 9,10-positions to give r-t-7,8-dihydroxy-t-9,10-oxyl-7,8,9,10-tetrahydrobenzo(a)pyrene (diol-epoxide 1) as the important pathway in carcinogenesis and mutagenesis (4, 9, 25, 38, 42).

Alterations in cellular physiology caused by BaP and/or the above metabolites have been described in the literature. Much of this research has been concerned with the inhibition of DNA synthesis (22, 37, 43). This focus on DNA synthesis has resulted from the observation that many, if not all, chemical carcinogens bind to DNA and interfere with DNA replication, leading to transformation, mutation, or cell death (14, 19, 24, 31). The mechanism of DNA synthesis in eukaryotic cells is only beginning to be understood (17, 41), and little information exists as to how chemical carcinogens interfere with this process. A precise understanding of how these compounds alter the biochemistry of DNA synthesis coupled with an understanding of the chemistry of carcinogen DNA interaction would aid greatly in our ability to predict the carcinogenicity of individual compounds. As a prelude to studying the biochemistry of carcinogen inhibition of DNA synthesis, we studied the effect of BaP and some of its carcinogenic derivatives on the kinetics of cell movement through the DNA synthetic period of the cell cycle.

MATERIALS AND METHODS

Cells and Culture Techniques. The cells used in this study were derived from NMuLi (36) mouse liver epithelial cells by the cloning technique of Puck et al. (39). The sensitivity of these clones to BaP cytotoxicity was first demonstrated by using a clonal assay and shown to correlate with the presence of a highly inducible AHM activity (27). All cells were cultured in plastic dishes (Falcon Plastics, Oxnard, Calif.) and incubated at 37°C in a 5% CO₂, 95% air incubator. The medium used to grow the cells was Eagle’s minimal medium (12) (Grand Island Biological Co., Grand Island, N. Y.) containing 10% donor calf serum (Flow Laboratories, Rockville, Md.) and 10 μg insulin per ml (Schwarz/Mann, Orangeburg, N. Y.). The cells were judged free of Mycoplasma by incorporation of [3H]TdTh (20.1 Ci/mmol; New England Nuclear, Boston, Mass.) into the nucleus of the cell and not the cytoplasm (18). Stock cultures were maintained by subculturing the cells twice weekly at a cell density of 1 x 10⁶/sq cm. BaP and the derivatives were dissolved in DMSO (Matheson, Coleman, & Bell, Los Angeles, Calif.) immediately before addition to the cultures. The final DMSO concentration in all experiments was 0.1%. Cell counts were determined by using a Model ZBI Coulter counter (Coulter Electronics Inc., Hialeah, Fla.).
**[3H]dThd Incorporation.** At each time point, cultures were pulsed with 10 μCi [3H]dThd per ml for 15 min. Autoradiography was carried out as previously described (2). For determination of [3H]dThd incorporated during the pulse, the cells on the dish were washed 2 times with Saline GM (1.5 mM Na₂HPO₄, 1.1 mM KH₂PO₄, 1.1 mM glucose, 0.14 mM NaCl, and 5 mM KCl at pH 7.5) and removed from the dish with DISPO [Saline GM containing 0.5 mM EDTA and 0.1 mg crystalline trypsin per ml (Grand Island Biological)]. The suspended cells were filtered onto Millipore HAWP filters with 0.45-μm pore size (Millipore Corp., Bedford, Mass.). The cells on the filter were washed 2 times with cold 5% trichoroacetic acid, 2 times with cold H₂O, and 1 time with 90% ethanol. The filters were air dried, placed in scintillation vials, and counted by using a Packard Instrument Co., Inc., Downers Grove, III.

**FCM.** FCM has been described in detail elsewhere (7, 20, 21), and in these experiments a fluorescent DNA probe was used to identify where individual cells were located in the cell cycle. FCM has many advantages over [3H]dThd incorporation for measuring cell cycle parameters (10, 13, 16), but most importantly for these studies, FCM measures total DNA content per cell and is therefore not affected by carcinogen-induced DNA repair.

Cells were stained with propidium iodide by using the technique described by Crissman and Steinkamp (7). The DNA content of the stained cells was analyzed by using a flow cytometer as described previously (2). Analysis of the resulting histograms was carried out by using a program developed by Pearlman et al. In general, the program is based on the approach described by Fried et al. (13) and allows for interactive processing of data with a CDC6600 computer (Digital Equipment Corp., Maynard, Mass.) after transforming the data to log space, allowing for variation in spacing between G₁ and G₂ + M, and extracting a representative G₁ spread function from samples having a low contribution due to cells in S. In test cases with or without carcinogens, this data analysis technique was within experimental error of standard autoradiography techniques for estimating the proportion of a population in the S phase of the cell cycle. Kinetic modeling studies were carried out as described by Gray (16).

**BaP Derivatives.** The BaP derivatives used in these studies were synthesized as described by Meehan et al. (30).

**AHM Induction and Assay.** AHM induction and assay was as described previously (26, 27). The assay is essentially that described by Gray (16). AHM induction was measured in NMuLi clone 7 and clone 8.

**RESULTS**

**Growth Studies.** The 2 clones used for these studies were isolated from a parent culture of NMuLi cells which had previously been shown to have an inducible AHM activity and be highly sensitive to the cytotoxic action of BaP (7). Neither clone has been exposed to BaP during isolation; however, NMuLi clone 7 cells in comparison to NMuLi clone 8 cells had considerably lower levels of basal and induced AHM activity (Table 1). The relative sensitivity of the 2 clones to growth inhibition by BaP and BaP derivatives reflected this difference in AHM activity. The 37% lethal dose values listed in Table 2 indicate that clone 8 was equally sensitive to BaP and 7,8-diol toxicity, whereas diol-epoxide was approximately 10 times more cytotoxic than BaP to this clone. Clone 7 was as sensitive as clone 8 to diol-epoxide toxicity and was 2.5 times less sensitive to BaP toxicity. The 7,8-diol did not reduce clone 7 growth below 37% of the control, and the tetrob was not toxic to either clone. DMSO, the solvent in which the compounds were added, was only slightly toxic at the 0.1% level used in these experiments.

The growth curves of cells in medium containing approximately 8 μM of the derivatives were determined as shown in Chart 1. This concentration of each derivative was chosen because it gave the maximum cell cycle effect in the studies reported below. The growth parameters of the cells from these 2 clones in the absence of test compounds differed slightly as shown in Chart 1 and Table 3. The primary difference was that clone 8 had a reduced G₁ residence time relative to clone 7 and an increased saturation density. Growth of NMuLi clone 7 and clone 8 was completely arrested by the diol-epoxide. The other compounds did not affect significantly the doubling time of clone 7 cells; however, they did have an effect on the saturation density of this clone. The tetrob increased the saturation density relative to the control to 127%. BaP and 7,8-diol decreased the saturation density to 31% and 36% of control, respectively. The growth of cells with an inducible AHM was not affected by this concentration of tetrob, but BaP and the 7,8-diol both greatly increased the doubling time of clone 8 cells and reduced the saturation density.

**Perturbation of Actively Growing Cells.** Perturbations in the cell cycle distributions caused by a compound were studied by FCM analysis of the DNA content per cell and [3H]dThd incor-

---

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>pmol 3-hydroxybenzo(a)pyrene/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 7</td>
<td>0.65</td>
</tr>
<tr>
<td>Clone 8</td>
<td>14.80</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>37% lethal dose (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaP</td>
<td>3.33</td>
</tr>
<tr>
<td>7,8-Diol</td>
<td>1.86</td>
</tr>
<tr>
<td>Diol-epoxide</td>
<td>0.02</td>
</tr>
<tr>
<td>Tetrob</td>
<td>—</td>
</tr>
</tbody>
</table>

---

Chart 1. Growth of NMuLi clone 7 (Cl 7) and clone 8 (Cl 8) in the presence of BaP derivatives. The compounds were added to the culture medium from a solution in DMSO (1 mg/ml) 24 hr after seeding the cells. The final concentrations were: BaP, 8.0 µM; 7,8-diol, 7.0 µM; diol-epoxide, 6.7 µM; and tetrol, 6.4 µM.

Table 3

<table>
<thead>
<tr>
<th>NMuLi clone</th>
<th>Doubling time (hr)</th>
<th>Length of cell cycle phase (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(x 10^6 cells/sq cm)</td>
<td>G1</td>
</tr>
<tr>
<td>Cl7</td>
<td>16.50 ± 0.21</td>
<td>1.1</td>
</tr>
<tr>
<td>Cl8</td>
<td>14.98 ± 0.52</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* Computed from the cell counts by using a least-squares program. The values reported had correlation coefficients greater than 0.995.

** Calculated from the FCM DNA histograms and the doubling times (2) and assumes that all cells are cycling.

*p Mean ± S.D.

The alterations in the cell cycle distributions for all cell cycle phases are shown in Chart 3. Diol-epoxide caused an increase in the proportion of the cells in S in both clone 7 and clone 8 cultures beginning at about 4 hr after adding the compounds. NMuLi clone 8 cells accumulated in S faster than clone 7 cells presumably due to the shorter residence time in G1 (Table 3). BaP caused similar alterations in cell cycle distributions in NMuLi clone 8 cultures, but the increase in fraction of the population in S began between 6 and 8 hr after BaP addition instead of at 4 hr as for diol-epoxide. This time lag corresponds to the time necessary to induce AHM in these cells (3). BaP had essentially no effect on the cell cycle distribution of clone 7 cells. 7,8-Diol had similar effects on both cell types as BaP, both in the extent of perturbation and the time of appearance.
of cells in S. The tetrol did not alter the cell cycle distribution of either clone.

Chart 4 presents the DNA histograms from clone 8 cells prepared for analysis daily, beginning 24 hr after treatment with BaP, diol-epoxide, or DMSO (solvent controls). In the controls, the cell cycle distributions progressed toward a higher proportion of the population in G1 as the cultures approached saturation density. In these cells that have an inducible AHM, BaP [as well as 7,8-diol (data not presented)] prevented this accumulation of cells in G1. Diol-epoxide also prevented this G1 accumulation and maintained even more cells in S and G2 + M than did BaP. [3H]dThd incorporation (15-min pulse) on Day 3 or 4 of the experiment followed by autoradiography of the cells indicated that, even though cells were in S as indicated by FCM analysis and by counting the number of cells incorporating [3H]dThd in the BaP- and diol-epoxide-treated cultures, their DNA synthesis rate during the pulse determined by counting grains per labeled nucleus was less than 1% that of controls. These observations suggest that, after the initial wave of cells moves from G1 through S and into G2 + M in the diol-epoxide-treated cultures, movement around the cycle becomes extremely slow or arrested. The effects are not just on a single cell cycle phase since at no time after exposure do all the cells accumulate in one particular cell cycle phase.

Cell Cycle Perturbations of Serum-stimulated Cells. The alterations in cell cycle distribution caused by diol-epoxide and diol-epoxide-generating compounds described in Chart 3 could be due to a carcinogen-induced stimulation of cells from G1, an inhibition of DNA synthesis, or some complex combination of these 2 phenomena. To obtain information on these alternate descriptions of carcinogen effects on the cell cycle, NMuLi clone 7 and clone 8 cells were stimulated from saturation density cultures with fresh medium and serum in the presence or absence of BaP. NMuLi clone 7 and clone 8 cells become distributed in G1 upon reaching saturation densities in culture. As seen in Chart 5, cells from both clones can be stimulated by subculturing to move from G1 into S and G2 + M. The period between subculturings and the appearance of cells in S is composed of the time necessary to recover from being at saturation density as well as the normal G1 period (44, 46). Clone 7 cells were approximately 2 hr slower than clone 8 cells in moving into and through S, again reflecting the 2 hr difference in the G1 period of these clones. BaP added to these stimulated cells did not affect the time it takes for cells from either clone to begin appearing in S. However, NMuLi clone 8 cells but not clone 7 cells traversed S slower in the presence of BaP than in its absence. Also, with clone 8 the number of cells moving through S at any particular analysis time in the presence of BaP is greater than that in the controls. This increased number of cells in S was not due to BaP increasing the number of cells stimulated by serum since, in an experiment in which colchicine (0.2 μg/ml) was added to trap in mitosis cells stimulated by serum, the proportion of the population building up behind the colchicine block was not affected by BaP. This experiment suggests that BaP does not alter the ability of serum to promote the G0 to G1 transition.

Kinetic cell cycle modeling (16) of the NMuLi data presented in Chart 5 revealed that the apparent increase in cells in S can be explained by a greater effect of the carcinogen on S relative to G1 (Table 4). Also, these modeling studies indicated that the dispersion of the cell cycle times is not affected by BaP but
that the rate of DNA synthesis slows down as the cells exposed to BaP move through S. The maximum rate of DNA synthesis in the cultures treated with BaP occurred when the serum-stimulated cells had traversed 0.2 of the S period. The cultures without BaP had a maximum DNA synthetic rate as the stimulated population traversed 0.5 of the S period (Table 4).

**DISCUSSION**

These experiments demonstrated that BaP or the 7,8-diol, when they could not be metabolized to the diol-epoxide, had very little effect on cell cycle progression. When these compounds could be metabolized, they gave a cell cycle response identical to the diol-epoxide. At the concentrations tested, they primarily slowed the progress of cells through S. They had no effect on the G2 to G transition probability (46), nor on the lag time from serum stimulation to the appearance of cells in S. All of these processes, however, are inhibited at concentrations above 40 μM (data not presented). The wave of cells moving through S beginning 4 hr after treatment of a randomly growing population and the increased proportion of the population in S at individual analysis times after serum stimulation of BaP- (or diol-epoxide-) treated cultures were probably both due to the effect of diol-epoxide on slowing progress through S. Kinetic modeling studies of logarithmically growing populations indicates that, when progress through a compartment in the cell cycle is inhibited and the dispersion in the transit times is not changed, the proportion of the population in the inhibited cell cycle phase will increase (16). Modeling of the population distribution as a function of time after serum stimulation in the presence of BaP, as well as the results from the incorporation of [3H]dThd in diol-epoxide-treated logarithmetic cells, indicated that the rate of DNA synthesis was continually decreasing as cells progressed through S.

The events described here take place in the first generation after adding the toxic compounds. Under the conditions of these experiments, there does not appear to be a second generation. The wave of cells passing through S does not return and the population dies, with cells distributed in all phases of the cell cycle.

The simplest interpretation of the relationship between the cell cycle perturbations reported here and the initiation of carcinogenesis is that some cells survive the toxic effects of these chemicals with alterations in their genome that reflect changes in growth control. Painter and Howard (37) have shown that there is a good positive correlation between the ability of a chemical to inhibit DNA synthesis and its carcinogenicity. They have suggested that all chemical carcinogens would inhibit DNA synthesis and that inhibition of DNA synthesis could be used as an assay for chemical carcinogens. The results of our study point to the importance of looking at the detailed effect of test compounds on the population dynamics rather than by measuring DNA synthesis at some arbitrary point after application of a compound.

**ACKNOWLEDGMENTS**

We thank Jean Lawson and Maria Costin for technical assistance and Marlyn Amban, Rosette Ajemian, and Beth Klingel for help in preparing the manuscript. In addition, we would like to thank Jan Curtis for analyzing the FCM samples. A very special thanks goes to Joe Gray of the Lawrence Livermore Laboratory for allowing us to use his cell cycle modeling program and working with us to model our data.

**REFERENCES**


**Table 4**

**Kinetic modeling of BaP perturbations in cell cycle parameters of NMuLi clone 8 cells**

<table>
<thead>
<tr>
<th>Length of cell cycle phase (hr)</th>
<th>Dispersion in Cycle Times (Coefficient of Variation)</th>
<th>DNA-synthetic rate maximum*</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>0.35</td>
<td>0.50</td>
</tr>
<tr>
<td>S</td>
<td>4.4</td>
<td>18.8</td>
</tr>
<tr>
<td>G2 + M</td>
<td>2.5</td>
<td>2.8</td>
</tr>
</tbody>
</table>

* The point in the S period at which DNA is being made most rapidly (expressed as a fraction of S with 0 at the G1-S boundary and 1.0 at the S-G2 boundary).
Modulation of the Cell Cycle of Cultured Mouse Liver Cells by Benzo( a)pyrene and Its Derivatives


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/39/7_Part_1/2538

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.