Protection of Cultured Hamster Embryonic Cells from 7,12-Dimethylbenz(a)anthracene Cytotoxicity and the Induced Synthesis of Aryl Hydroxylase

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SUMMARY

Treatment of secondary cultures of hamster embryonic cells with an excess of thymidine (5mM) caused an inhibition of DNA synthesis which was reversible. This treatment permitted a study of the effects of polycyclic hydrocarbons on cells inhibited at the G1-S boundary and on cells entering the S phase of the cell cycle. Cytotoxic damage caused by 7,12-dimethylbenz(a)anthracene (DMBA) treatment of cells after their release from thymidine inhibition was prevented by the simultaneous exposure to an equimolar concentration of benz(a)anthracene (BA) (4μM). The DMBA depression of the rate of DNA synthesis and the inhibition of cell division were also prevented by simultaneous BA treatment. These results suggest that protection from DMBA cytotoxicity may be due to a BA induction of aryl hydroxylase which may have degraded the DMBA to less toxic metabolites. DNA synthesis was shown not to be required for induction of aryl hydroxylase; in fact, an equal amount of the enzyme activity was induced during either synthesis or inhibition of DNA. Cultures induced simultaneously with BA + DMBA synthesized a higher level of aryl hydroxylase than cultures with either BA or DMBA.

INTRODUCTION

The simultaneous treatment of Syrian hamster cells with equimolar concentrations of benz(a)anthracene (BA) and 7,12-dimethylbenz(a)anthracene (DMBA) resulted in a reduction of binding of DMBA-3H to DNA and less inhibition of DNA synthesis and mitotic activity compared to that usually produced with DMBA alone (2). This protection from the cytotoxic action of DMBA may have been due to the increased synthesis of aryl hydroxylase induced by BA (3). Recent studies of the effects of actinomycin D on the induction of aryl hydroxylase (benzpyrene hydroxylase) suggests that this process is mediated by DNA-dependent RNA synthesis (3).

In the present studies, attempts were made to determine whether the protection from overt DMBA cytotoxicity was associated with the simultaneous BA induction of aryl hydroxylase in these cells. Protection was defined as the prevention of the DMBA initiated inhibition of DNA synthesis and cell division.

MATERIALS AND METHODS

Preparation of Cultures. Primary cultures derived from 12- to 14-day-old golden Syrian hamster embryonic tissues were grown in Eagle's minimum essential medium + 10% calf serum (CM) in an atmosphere of 5% CO2 and air at 37°C. Secondary cultures prepared from 5-day-old primary cultures were grown in CM at an initial population density of 2 X 10^6/60 mm plastic Petri dish (Falcon Plastics, Inc.), 4.0 X 10^6/100 mm dish or 20 X 10^6/150 mm dish.

Preparation of Polycyclic Hydrocarbons (PCH) in Solution. A suspension of 5-8 mg of BA or DMBA/100 ml CM was shaken and filtered through a Seitz filter as previously described (1). The concentration of PCH in the filtrate was determined as follows: 1 volume of filtrate plus 1 volume of redistilled benzene were shaken for 1 minute, and the PCH in the organic phase was measured in the Aminco Bowman spectrophotofluorometer. BA was measured at an excitation of 340 m/μ and an emission of 398 m/μ, DMBA was measured at an excitation of 300 m/μ and an emission of 430 m/μ. More than 95% of the PCH in the aqueous filtrate was benzene extractable as shown by comparison to a standard curve obtained by extracting BA-3H or DMBA-3H dissolved in CM as above. Stock filtrates of BA (40 μM) and DMBA (56 μM) were stored in amber bottles at 4°C.

Inhibition of DNA Synthesis with Excess Thymidine and PCH Treatment. The general procedure for inhibiting cells in

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1Presented in part at the 59th annual meeting of the American Association for Cancer Research, April 13, 1968.
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their completion of DNA synthesis is presented in Chart 1. Cells which had been doubly blocked with 5 mM thymidine (10) were released by washing the monolayers three times with phosphate-buffered saline (PBS, pH 7) and refed with CM containing PCH (4 μM) and thymidine (5 mM or deoxycytidine (10^{-4} M). After PCH treatment the medium was removed, the monolayers were washed twice with PBS, and the cells were labeled for 20 minutes with 0.2 μc/ml thymidine-2-14C (30 mc/mimole) + 0.2 μc/ml deoxycytidine-3H (500 mc/mimole) [or with 1 μc/ml thymidine-methyl-3H (6.7 c/mimole)]. Incorporation was stopped by decanting the medium and washing the monolayers 4-5 times with cold PBS containing a 1000-fold excess of unlabeled thymidine or deoxycytidine. Cells were harvested by scraping, and RNA was separated from DNA by alkaline hydrolysis according to the procedure of Fleck and Munro (9).

**Aryl Hydroxylase Assay.** The enzyme assay using 3,4-benz(a)pyrene as a substrate was a modification (11) of the method described by Wattenberg and Leong (20), except that 3 μmoles MgCl2 were added to the assay flask (18). The amount of product was determined in the Aminco-Microfluorometer by comparison with a standard solution of 3-hydroxybenzo(a)pyrene. Protein analysis was made on cell homogenates by the Lowry method (16).

**RESULTS**

**Rate of Incorporation of Deoxycytidine-3H (CdR-3H) and Thymidine-2,14C (TdR-14C) into DNA during PCH treatment.** Secondary cultures were released from thymidine block and labeled simultaneously with CdR-3H and TdR-14C, and the radioactivity of the DNA fraction was measured. PCH treatment had only a slight effect on the rate of CdR-3H incorporation into DNA (Chart 2, lower). The maximum rate of incorporation of this precursor occurred at one hour, which was prior to the initiation of TdR-14C incorporation into DNA (Chart 2, upper). The maximum value of DMBA-treated cells for TdR-14C incorporation was approximately 50% of control or BA values, but the slope of the curve for DMBA cells was the same as for other treatments. Cells treated simultaneously with BA + DMBA were more effective in DNA synthesis than those treated with DMBA alone.

**Effect of PCH Treatment on Cell Division.** The inhibition of cell division by DMBA at a concentration of 4 μM was prevented by simultaneous treatment with an equimolar concentration of BA (Table 1). Pretreatment with DMBA alone for 4 hours caused a delay in population doubling, and cells only divided once. Cultures pretreated under DMBA conditions for 24 hours resulted in an initial toxicity so that the populations were unable to double. Duplicate cultures pretreated with BA, DMBA + BA, or under control conditions all became confluent monolayers, and no cytotoxicity was observed during subsequent growth in CM alone.
An increased synthesis of aryl hydroxylase during PCH treatment was expected, and the activity of this enzyme system was measured. The BA (4 \mu M)-induced synthesis of aryl hydroxylase (per cell basis) reached a peak within 24 hours and declined to a baseline level during the following 48 hours (Chart 3). It was possible to reinduce the enzyme to maximum levels of synthesis within 24 hours in these confluent cultures after the original enzyme activity had declined. The fact that it was possible to reinduce the enzyme in confluent cultures at a time when the rate of DNA synthesis is minimal suggested that actively replicating DNA is not required for this induction phenomenon. To investigate this, enzyme induction by BA and TdR-^3H incorporation were measured (a) in cells in which DNA synthesis was blocked by excess thymidine and (b) in cells in which this block was reversed with Cdr. At various times thereafter the cells were washed with cold PBS and harvested; a sample of the cells was used for enzyme assay and the remainder for the extraction of DNA (9). Under blocked culture conditions, DNA synthesis was inhibited by 99% at time zero of the experiment (Table 2). The rates of enzyme induction by BA and the amounts of enzyme induced were similar in cultures continued in the presence of excess thymidine and in cultures released from the thymidine block with Cdr (Table 2).

**Enzyme Induction during Protection from DMBA Toxicity.** Exposure to BA + DMBA of cells that had just been released from thymidine inhibition with Cdr resulted not only in the prevention of DMBA-initiated cytotoxicity, but also in an

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### Table 1

<table>
<thead>
<tr>
<th></th>
<th>No. of cells/100 mm dish in CM (X 10^6) posttreatment at:</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>4 hours pretreatment in:</td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>2</td>
</tr>
<tr>
<td>BA</td>
<td>2</td>
</tr>
<tr>
<td>DMBA</td>
<td>2</td>
</tr>
<tr>
<td>BA + DMBA</td>
<td>2</td>
</tr>
<tr>
<td>24 hours pretreatment in:</td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>3</td>
</tr>
<tr>
<td>BA</td>
<td>4</td>
</tr>
<tr>
<td>DMBA</td>
<td>2</td>
</tr>
<tr>
<td>BA + DMBA</td>
<td>2</td>
</tr>
</tbody>
</table>

Number of cells in cultures growing in CM following pretreatment with PCH. PCH concentrations: BA = 4 \mu M, DMBA = 4 \mu M, BA + DMBA = 4 \mu M of each. Time zero refers to the starting point of these experiments, following PCH pretreatment. BA, benz(a)anthracene; DMBA, 7,12-dimethylbenz(a)anthracene; PCH, polycyclic hydrocarbons; CM, Eagle's minimum essential medium + 10% calf serum.

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### Table 2

<table>
<thead>
<tr>
<th>Added to CM</th>
<th>cpm TdR-^3H/mg DNA, X 10^{-3} at:</th>
<th>( \mu )moles enzyme product^a at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1 hr</td>
</tr>
<tr>
<td>Thymidine</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Deoxycytidine</td>
<td>2</td>
<td>212</td>
</tr>
<tr>
<td>BA + thymidine</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>BA + deoxycytidine</td>
<td>2</td>
<td>56</td>
</tr>
</tbody>
</table>

Rates of BA (2 \mu g/ml)-induced aryl hydroxylase synthesis and incorporation of thymidine (TdR)-^3H into DNA of cells maintained in an excess of thymidine (5 mM), or released from thymidine inhibition with deoxycytidine (10^{-4}M). See Table 1 for abbreviations.

^a \mu moles hydroxylated benz(a)pyrene, compared to a 3-hydroxy-benz(a)pyrene standard.
increase of the enzyme activity to a level slightly higher than the sum of the activities obtained from each of the PCH inducers considered separately. This effect, particularly when expressed on a basis of μmoles of product per mg protein, was higher for the two PCH’s in combination than for each PCH expressed on a basis of a moles of product per mg protein, inducers considered separately. This effect, particularly when the sum of the activities obtained from each of the PCHs was higher for the two PCH's in combination than for each PCH.

### Table 3

<table>
<thead>
<tr>
<th>Treatment for 24 hours</th>
<th>Concentration of inducer (μM)</th>
<th>μmoles of enzyme product per mg cell protein</th>
<th>per 10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM</td>
<td>0</td>
<td>31</td>
<td>6</td>
</tr>
<tr>
<td>BA</td>
<td>4</td>
<td>318</td>
<td>62</td>
</tr>
<tr>
<td>DMBA</td>
<td>4</td>
<td>76</td>
<td>36</td>
</tr>
<tr>
<td>BA + DMBA</td>
<td>4 + 4</td>
<td>549</td>
<td>58</td>
</tr>
</tbody>
</table>

Induction of aryl hydroxylase during BA protection from DMBA cytotoxicity in cultures released from thymidine block. See Table 1 for abbreviations.

## DISCUSSION

Present experiments utilized secondary cultures doubly blocked with thymidine (less than 1% of the cells in DNA synthesis) in studying the rates of incorporation of CdR-3H and TdR-14C into DNA following the reversal of thymidine inhibition. There was an early and rapid incorporation of CdR-3H into DNA which reached a maximum rate 1 hour prior to the initiation of TdR-14C incorporation. The addition of exogenous CdR-3H is responsible for the immediate restoration of the normal rate of DNA synthesis. DMBA exerted only a slight effect on the incorporation of CdR-3H while the rate of incorporation of TdR-14C was significantly decreased in the presence of this carcinogen during the DNA synthetic period. The suppression of TdR-14C incorporation was associated with cytotoxicity and a subsequent inhibition of cell division (2, 14).

The shape of the curves (Chart 2) suggests that, while DMBA did not inhibit the incorporation of CdR-3H into DNA after TdR-14C incorporation was initiated, DMBA may have depressed either the rate of phosphorylation of TdR-14C or the DNA polymerase reaction. On the other hand, cells pretreated with BA or BA + DMBA for 4 to 24 hours, and then grown in CM alone for 5–6 days, had a growth rate similar to the control cultures. Protection from DMBA toxicity was attributed to the BA induction of aryl hydroxylase which probably degraded the DMBA to nontoxic metabolites. Aryl hydroxylase was inducible in confluent nondividing cultures; this suggested that the induction of this enzyme does not require active DNA synthesis. The high rate of enzyme formation during thymidine-inhibited DNA synthesis suggests that cells in the G1-S boundary of the mitotic cycle are as efficient in synthesizing this inducible enzyme system as are cells in progress through other periods of the cycle.

This cytotoxic DMBA activity on hamster cells results in a permanent damage which can be attributed to the persistance of DMBA or its metabolites within the cells (8). Protection from DMBA cytotoxicity with simultaneous BA + DMBA treatment, which resulted in a higher aryl hydroxylase activity than with either inducer alone, was probably related to the additive effect of the two PCH’s. These results support the findings of Steiner and Falk (19) on the protection from the tumorigenicity of a potent carcinogenic PCH (1,2,5,6-dibenzanthracene) applied to mouse skin simultaneously with 1,2-benzanthracene. Such protection may also be related to the reduced carcinogenic activity of hepatocarcinogens when fed with certain PCH’s (17), or to the inhibition of DMBA-induced adrenal necrosis by other PCH’s (15). It would be important to determine whether this transitory resistance to DMBA toxicity (protection) observed during aryl hydroxylase synthesis is related to susceptibility to PCH-induced neoplastic transformation in cultured cells (4, 8). Long-term cultures of hamster cells and certain neoplastic cells are noninducible for aryl hydroxylase. Yet some of these cell lines can take up and tolerate 100 times more toxic PCH than can normal rodent cells in culture (6). The increased resistance of abnormal cell lines to DMBA toxicity appears to be related to a decreased capacity for DNA binding of PCH (7), and their inability to be induced for aryl hydroxylase may suggest the absence of molecular binding sites for PCH inducers. In vitro neoplastic transformation of normal cells growing in the presence of PCH (4, 8, 12, 13) strongly suggest the involvement of specific binding sites for PCH. The probable molecular sites involved in PCH induction of aryl hydroxylase may be different from those involved in chemical carcinogenesis since no relationship has been established between the inducibility of this enzyme system and the carcinogenic index of a variety of chemicals (18).

The present observations on the phenomenon of protection from DMBA cytotoxicity may be explained by one or both of the following mechanisms: (a) simultaneous BA + DMBA treatment induces high levels of aryl hydroxylase which catabolize both inducers, and (b) competition between BA and DMBA for DNA binding sites resulting in a reduction in DMBA binding (2) and the subsequent expression of its toxic action.

## ACKNOWLEDGMENTS

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## REFERENCES


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