The Interaction of 7,12-Dimethylbenz(a)anthracene with Cells Sensitive and Resistant to Toxicity Induced by This Carcinogen

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SUMMARY

The multiplication of monolayer cultures of transformed rodent cells and normal or transformed human cells was not inhibited by concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) 200–500 times greater than those that inhibited the multiplication of normal embryonic rodent cells. Fluorescence microscopy showed that DMBA localized to about the same degree within the cytoplasm of cells that were sensitive or resistant to the toxic effect of the carcinogen. Autoradiography with DMBA-3H and an aqueous cell fixative (formalin) revealed that the carcinogen was distributed throughout the cytoplasm and nucleus and that the concentrations were similar in sensitive and resistant cells. However, when the cells were treated with a fixative in which DMBA is soluble (Carnoy’s), most (90%) of the labeled compound was eliminated from the resistant cells whereas a considerable amount appeared to be bound within both the cytoplasm and the nucleus of sensitive cells. The difference in the binding of DMBA by the 2 types of cells was confirmed by isolating and assaying for radioactivity individual cellular constituents after cells had been exposed to DMBA-3H. Sensitive cells bound 10–50 times more DMBA to their nucleic acids and protein than did resistant cells. With mouse embryo cells, the amount of DMBA bound to DNA and protein was proportional to the growth-inhibitory effect of the carcinogen within the dose range 0.01–0.1 μg/ml.

INTRODUCTION

The multiplication of monolayer cultures of normal embryonic rodent cells is inhibited by the carcinogenic polycyclic hydrocarbons 3,4-benzpyrene and 3-methylcholanthrene (2, 4, 6). In contrast, transformed or malignant rodent and primate cells and normal primate cells are relatively resistant to the growth-inhibitory effect of these carcinogens (2, 4, 6).

To elucidate the mechanism of cytotoxicity and the possible relation of this effect to carcinogenicity, the binding of a carcinogenic polycyclic hydrocarbon, DMBA,4 to cells sensitive or resistant to the toxic effect of DMBA was compared. The difference in the response of sensitive and resistant rodent or primate cells was not related to uptake of the carcinogen by the cells but rather to differences in the amount of DMBA binding to cellular protein and nucleic acid.

MATERIALS AND METHODS

Carcinogen

Stock solutions of unlabeled DMBA (Eastman Kodak) were prepared by dissolving the carcinogen in DMSO (Matheson, Coleman and Bell) to a concentration of 1 mg DMBA per ml of solvent (6). Randomly labeled DMBA-3H, obtained from the Radiochemical Centre, Amersham, England, had a specific activity of 21 c/mmole. A stock solution containing 1 mc DMBA/ml was stored at —70°C. Immediately prior to use, aliquots of the stock solutions were diluted with culture medium to obtain the desired concentration of DMBA. The final concentration of DMSO in the cell cultures never exceeded 0.5%, a concentration that did not inhibit cell multiplication (6).

Cell Cultures

The cell cultures sensitive to the toxic effect of the carcinogen were secondary hamster and mouse embryo. The primary hamster and mouse embryo cultures were initiated by trypsinization of the embryos with a solution of 0.25% trypsin and 0.02% Versene in PBS (8) lacking calcium and magnesium ions. The resistant rodent cell cultures were L cells, a polyoma-transformed line of hamster embryo (Nil-2/Py) (7), and an SV40-transformed line of mouse embryo (SV-3T3 C12) (15). The resistant human cells were the 26th passage of a diploid cell strain (WI-38) (9) and an SV40-transformed line of human fibroblasts (W18VA2) (14). The cells were grown as monolayers in Eagle’s basal medium supplemented with 10% calf serum and containing 50 μg/ml Aureomycin.

Fluorescence Microscopy

Cells were grown on coverslips for 24 hr in medium containing 5 μg DMBA/ml and no antibiotics. The monolayers were washed

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2 Leukemia Society Scholar and Department of Pathology, University of Pennsylvania.


4 The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline.
twice with PBS, and the cells were examined without fixation in a dark-field fluorescence microscope (6). The excitation wavelength was 360 mµ, and the barrier filter excluded light with a wavelength below 460 mµ.

**Autoradiography**

Sixty-mm Petri dishes containing coverslips were seeded with 1–2 × 10⁶ cells. Twenty-four to 48 hr later the medium was replaced with medium containing 0.0075 µg/ml DMBA-H (0.62 µc/ml). At various time intervals thereafter, coverslips were washed twice in PBS. The cells were fixed by two different methods. In the 1st method, the cells were fixed in 10% formalin-saline solution, then washed in PBS and finally in distilled water. In the 2nd method, the cells were fixed in Carnoy’s solution (60% absolute ethanol, 30% chloroform, 10% glacial acetic acid) and rinsed in 70% ethanol. After air-drying, the coverslips were coated with liquid emulsion NTB 3 (Kodak), exposed in sealed boxes at 4°C for 18 days, developed in D-19, and fixed. The cells were stained with 0.05% toluidine blue at pH 4.

While focusing on the emulsion layer, random fields on each slide were photographed, using a 40 X objective. Grain counts in the cytoplasm and nucleus were made from prints (final magnification 520 X). Because the background varied considerably and was particularly high on formalin-fixed coverslips, the grains in cell-free regions were counted. The values obtained from areas equivalent and adjacent to those of individual cells were subtracted from the grain counts over the cells.

**Isolation of Cellular Constituents**

**Treatment of Cells with Labeled Hydrocarbon.** The cells were grown as monolayers in 4–6 one-liter Blake bottles. Labeled carcinogen was added when the cells were in the logarithmic phase of growth and the monolayers were about 50% confluent, usually 24–48 hr after seeding. With the WI-38 cells, however, each bottle was inoculated with 10⁴ cells and the cultures were used 7 days later, after 3–4 cell generations had occurred. The medium was removed from the monolayer cultures and replaced with 50 ml of fresh medium containing 1 µc/ml (0.012 µg/µl) DMBA-H. In experiments requiring higher concentrations of DMBA, unlabeled DMBA was added to give the desired concentration.

After 18–24 hr of incubation, the medium was decanted and the cell monolayers washed twice with 100 ml PBS. The cells were removed from the glass with a rubber policeman and suspended in PBS. They were washed twice in PBS by centrifugation and counted. The isolation of cellular constituents was begun immediately thereafter, or the cell pellet was frozen at −70°C until use. Cell death, evidenced by detachment from the glass, was not apparent in any of the cultures at the time of harvest. Individual experiments yielded 50–200 × 10⁶ cells for isolation of cellular constituents.

**Chemical Procedures.** Modifications of the procedures described by Kirby et al. (11–13) were used. The cells were suspended in 5% sodium p-aminomaleylate and lysed by the addition of 1/5 vol of 10% sodium deoxydisulfate. The resulting highly viscous solution was extracted with an equal volume of phenol:m-creosol:8-hydroxyquinoline:water mixture (500:70:0.5:55 by weight). After centrifugation, DNA was precipitated from the upper aqueous phase by the addition of an equal volume of 2-ethoxyethanol. The DNA was removed on a glass rod, and the addition of an equal volume of ethanol to the remaining solution caused precipitation of the RNA. The phenol layer was extracted once again with 5% p-aminomaleylate and then added to excess methanol, which precipitated the protein.

The DNA was freed of traces of RNA by treatment with crystalline ribonuclease (Worthington) at 37°C for 15 min, again extracted with the phenol reagent and precipitated as before with 2-ethoxyethanol. Carbohydrate contaminants were removed by extraction of the DNA from a 1.25 M potassium phosphate solution (pH 7.2) by 2-ethoxyethanol, as described by Kirby (12). To avoid prolonged dialysis, the DNA was precipitated from the high molarity phosphate solution by the addition of 1/4 volume of 1% cetyltrimethylammonium bromide, converted back to the sodium salt with 70% aqueous ethanol containing 2% sodium acetate, washed with ethanol, then with ether, and dried. The RNA was freed of carbohydrate by the same procedure used for DNA. Detailed analyses of nucleic acid isolated by these procedures have been reported (5).

The protein precipitated from the phenol layer was washed several times with methanol, then with ether, and dried.

**Radioactivity Assays.** The scintillation fluid for radioactivity assays was a solution of toluene, dioxane, and ethanol. The nucleic acids were assayed as aqueous solutions [in the case of DNA, after degradation with crystalline deoxyribonuclease (Worthington)]. The concentration of nucleic acid in the solution to be assayed was calculated from the optical density of the solution (pH 7.0) at 260 mµ, by assuming that at this wave length E₂₆₀ = 190 for RNA and 210 for DNA (after degradation with deoxyribonuclease). These were the values obtained with samples of nucleic acid in which the quantity was determined colorimetrically. Protein was assayed after dissolving in 10% aqueous tetraethylammonium hydroxide to a concentration of 5 mg/ml; 0.1 ml of this solution was used for radioactivity assay.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>The Effect of DMBA on Cell Multiplication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell</td>
<td>Day of exposure</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Hamster embryo</td>
<td>1</td>
</tr>
<tr>
<td>Mouse embryo</td>
<td>2</td>
</tr>
<tr>
<td>Nil:2/Py</td>
<td>4</td>
</tr>
<tr>
<td>L cells</td>
<td>3</td>
</tr>
<tr>
<td>SV-3T3C12</td>
<td>4</td>
</tr>
<tr>
<td>WI-38</td>
<td>7</td>
</tr>
<tr>
<td>WI8VA2</td>
<td>4</td>
</tr>
</tbody>
</table>

\( ^a \) DMBA, 7,12-dimethylbenz(a)anthracene.

\( ^b \) Unlabeled DMBA was added to replicate cultures at the time the cells were seeded. Cell counts were done on treated and control cultures at each time interval. The percent cell yield has been calculated as the number of cells in treated culture × 10⁶, divided by the number of cells in control culture.
The extent of binding of hydrocarbon was calculated from the observed specific activities of the isolated protein and nucleic acids. The concentrations of nucleic acid-\(P\) in the solutions assayed for radioactivity were derived from their optical densities by taking a value of \(\epsilon_p = 8750\) for DNA and \(\epsilon_p = 9000\) for RNA.

### RESULTS

**Effect of DMBA on Cell Multiplication**

The effect of DMBA on cell multiplication was determined by comparing, at various time intervals, the number of cells in cultures exposed to unlabeled DMBA with the number of cells in untreated control cultures (Table 1). The multiplication of the transformed rodent (Nil-2/PY, SV-3T3, and L cells) and the normal (WI-38) or transformed (W18VA2) human cells was not inhibited by concentrations of DMBA 200-500 times greater than those that inhibited the multiplication of embryonic rodent cells (hamster and mouse embryo). The relative sensitivity of the latter to DMBA varied with each primary culture, and the results with several different cultures have been averaged in Table 1. At a DMBA or DMBA-\(\text{-H}\) concentration of 0.01 \(\mu\text{g}/\text{ml}\), there was no significant inhibition of cell multiplication in the hamster embryo cultures after 24 hr. Therefore, this concentration and time of exposure were selected for the experiments measuring DMBA binding so that cell death would be minimal in sensitive cultures but incorporation of carcinogen might occur.

**Cellular Uptake of Carcinogen as Determined by Fluorescence Microscopy**

When cells grown in DMBA were examined in the fluorescence microscope, a diffuse blue-green fluorescence was observed throughout the cytoplasm. The majority of cells on a coverslip also contained distinct blue fluorescent granules in the cytoplasm. According to Allison and Mallucci (3), the distribution of these granules suggests that the carcinogen localizes within lysosomes. There was no fluorescence in the nucleus. No difference in the number of fluorescent granules or in the intensity of fluorescence was detected between cells sensitive and resistant to the toxic effect of the carcinogen. At the excitation and barrier wave lengths used, cells grown in the absence of carcinogen did not fluoresce.

**Uptake and Binding of Carcinogen as Determined by Autoradiography**

More quantitative evidence for cellular uptake of carcinogen by sensitive and resistant cells was obtained from autoradiography with DMBA-\(\text{-H}\) (Table 2). The grain counts after formalin fixation, an average of 13 cells and an additional 28 nuclei were counted; with Carnoys fixative, an average of 8 cells and an additional 21 nuclei were counted.

**TABLE 2**

<table>
<thead>
<tr>
<th>Fixative</th>
<th>Time</th>
<th>Mean No. of grains ± S.D.</th>
<th>Cell</th>
<th>Cytoplasm</th>
<th>Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 min</td>
<td>1 hr</td>
<td>4 hr</td>
</tr>
<tr>
<td>Formalin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Secondary hamster embryo</td>
<td>7.7 ± 4.8</td>
<td>17.9 ± 8.8</td>
<td>28.0 ± 9.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nil-2/PY</td>
<td>19.6 ± 11.8</td>
<td>32.4 ± 14.2</td>
<td>27.0 ± 18.0</td>
</tr>
<tr>
<td>Carnoys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Secondary hamster embryo</td>
<td>6.8 ± 4.0</td>
<td>14.7 ± 7.2</td>
<td>23.0 ± 8.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nil-2/PY</td>
<td>17.5 ± 11.1</td>
<td>26.1 ± 12.6</td>
<td>21.4 ± 13.2</td>
</tr>
</tbody>
</table>

\(a\) DMBA, 7,12-dimethylbenz(a)anthracene.

\(b\) Background grain counts of cell-free regions on the slides equivalent in area and adjacent to each cell have been subtracted from these values. With formalin fixation, an average of 13 cells and an additional 28 nuclei were counted; with Carnoys fixative, an average of 8 cells and an additional 21 nuclei were counted.

\(c\) N.C., not counted.

The extent of binding of hydrocarbon was calculated from the observed specific activities of the isolated protein and nucleic acids. The concentrations of nucleic acid-\(P\) in the solutions assayed for radioactivity were derived from their optical densities by taking a value of \(\epsilon_p = 8750\) for DNA and \(\epsilon_p = 9000\) for RNA.
Interaction of DMBA with Sensitive and Resistant Cells

CHART 1. Grain counts of autoradiographs of secondary hamster embryo (2° HE) and Nil-2/Py cells exposed to 7,12-dimethylbenz(a)anthracene-3H (0.0075 μg/ml) and fixed in formalin. The mean number of grains in the cell (○------○), cytoplasm (▲------▲), and nucleus (□------□) at each time interval has been plotted. Data are taken from Table 2.

Binding of Carcinogen to Cellular Constituents

The amount of DMBA-3H bound by normal or transformed rodent and primate cells was determined after the isolation of cellular constituents (Table 3). Normal rodent cells bound 10–20 times more DMBA to their nucleic acids and proteins than did transformed rodent cells. The amount of DMBA bound by the normal human diploid cell strain, WI-38, was similar to the amount bound by transformed cell lines.

With mouse embryo cells, the extent of binding was approximately proportional to dose within the range 0.01–0.1 μg/ml; no saturation of binding was observed. At these concentrations of DMBA, the amount bound was proportional to the growth-inhibitory effect of the carcinogen on the cells (Chart 3).

DISCUSSION

When compared with normal embryonic rodent cells, transformed rodent cells and normal or transformed primate cells are relatively resistant to toxicity induced by carcinogenic hydrocarbons (2, 4, 6, 16). One explanation of this difference would be that the hydrocarbon does not penetrate the resistant cells. This is not in accordance with the experimental evidence. Alfred (1) reported that mouse tumor cells grown in vitro took...
up more 3,4-benzpyrene from the medium than normal mouse embryo cells. Diamond (6) used fluorescence microscopy to show that 3,4-benzpyrene and 3-methylcholanthrene concentrate within the cytoplasm of both sensitive and resistant cells. In the present study, similar results were obtained by fluorescence microscopy with DMBA, and these observations were confirmed by autoradiography of formalin-fixed cells (Table 2).

Failure to detect fluorescence in the cell nucleus after exposure to carcinogenic hydrocarbons has been cited as evidence against their interaction with genetic material (10). However, the localization of DMBA within both the nucleus and the cytoplasm has now been demonstrated by autoradiography, a more sensitive technic.

The difference between sensitive and resistant cells in the response to carcinogen-induced toxicity was related to the amount of hydrocarbon firmly bound to the cells, as demonstrated by autoradiography and by radioactivity assays of isolated protein and nucleic acid. The autoradiographs of cells fixed with lipid solvents to remove unbound hydrocarbon showed that only the normal rodent cells retained any significant amount of labeled material. Bound hydrocarbon was detected in nucleus and cytoplasm. The isolation and radioactivity assays of cellular

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**CHART 2.** Grain counts of autoradiographs of secondary hamster embryo (2° HE) and Nil-2/Py cells exposed to 7,12-dimethylbenz(a)anthracene-3H (0.0075 µg/ml) and fixed in Carnoy’s solution. The mean number of grains in the cell (O-----O), cytoplasm (▲-----▲), and nucleus (□-----□) at each time interval has been plotted. Data are taken from Table 2.
DNA, RNA, and protein confirmed these observations. Normal rodent cells bound 10–50 times more DMBA-3H than did transformed rodent cells and normal or transformed primate cells (Table 3).

With normal rodent cells, the highest specific activity was obtained with DNA. The value for RNA was about one-half the DNA value, for protein about one-fourth. The amount of DMBA-3H binding to the DNA and protein of mouse embryo cells was approximately proportional to dose within the range 0.01–0.1 μg/ml; no saturation was reached. At the highest concentration of DMBA, the binding was equivalent to about one molecule of hydrocarbon per DNA molecule (molecular weight, 12.5 × 10^6) and one molecule of hydrocarbon per 1250 molecules of protein (molecular weight, 5 × 10^4). Since cell multiplication was markedly inhibited at these low levels of binding, the significant binding site for the cytotoxic effect may be DNA.

Resistance to DMBA-induced cytotoxicity appears to be due to a relative lack of intracellular binding of this carcinogen. Since binding probably requires metabolism of DMBA to a chemically reactive form, these results suggest that such a conversion does not occur in resistant cells. Resistant cells may be unable to metabolize the hydrocarbon or may metabolize it to a form that is chemically nonreactive and, therefore, nontoxic.

Brookes and Lawley (5) have shown that polycyclic hydrocarbons applied in vivo to mouse skin bind to cellular DNA in direct relation to the carcinogenic potency of the hydrocarbons. Thus, the binding of DMBA to cellular constituents seems to correlate with both the carcinogenic effect in vivo and the cytotoxic effect in vitro. It remains to be determined whether the 2 responses reflect quantitative differences in binding by individual cells or different modes of action of the carcinogen.

**ACKNOWLEDGMENTS**

The authors would like to thank Dr. H. Koprowski for his guidance and helpful suggestions made in the course of this study. They are indebted to Drs. V. Cristofalo, M. Roeller, and M. Umeda for providing some of the cells used and to Miss R. McFall for excellent technical assistance. Dr. Brookes also wishes to express his gratitude to Dr. C. Heidelberger for the hospitality.

<table>
<thead>
<tr>
<th>Type of cell</th>
<th>Cell</th>
<th>DMBA (μg/ml)</th>
<th>Time of exposure (hours)</th>
<th>Extent of binding to cellular constituents&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic rodent</td>
<td>Hamster embryo</td>
<td>0.012</td>
<td>24</td>
<td>DNA: 3.9, RNA: 2.17, Protein: 0.26</td>
</tr>
<tr>
<td></td>
<td>Mouse embryo</td>
<td>0.012</td>
<td>18</td>
<td>DNA: 2.75, RNA: 2.17, Protein: 0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.025</td>
<td>18</td>
<td>DNA: 4.45, RNA: 2.17, Protein: 0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>18</td>
<td>DNA: 14.0, RNA: 2.17, Protein: 0.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>18</td>
<td>DNA: 35.0, RNA: 2.17, Protein: 1.56</td>
</tr>
<tr>
<td>Transformed rodent</td>
<td>Nil-2/Py</td>
<td>0.012</td>
<td>24</td>
<td>DNA: 0.26, RNA: 0.11, Protein: 0.04</td>
</tr>
<tr>
<td></td>
<td>SV-3T3</td>
<td>0.012</td>
<td>24</td>
<td>DNA: 0.30, RNA: 0.11, Protein: 0.01</td>
</tr>
<tr>
<td></td>
<td>L-cells</td>
<td>0.012</td>
<td>24</td>
<td>DNA: 0.12, RNA: 0.11, Protein: 0.02</td>
</tr>
<tr>
<td>Diploid human</td>
<td>WI-38</td>
<td>0.012</td>
<td>24</td>
<td>DNA: 0.15, RNA: 0.11, Protein: 0.02</td>
</tr>
<tr>
<td>Transformed human</td>
<td>W18VA2</td>
<td>0.013</td>
<td>24</td>
<td>DNA: 0.20, RNA: 0.11, Protein: 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.023</td>
<td>24</td>
<td>DNA: 0.41, RNA: 0.11, Protein: 0.03</td>
</tr>
</tbody>
</table>

<sup>a</sup> DMBA, 7,12-dimethylbenz(a)anthracene.
<sup>b</sup> Values for nucleic acids are expressed as μmoles/mole P and for protein as μmoles/100 gm.

**TABLE 3**

*The Binding of DMBA-3H* by Cells in Tissue Culture

MAY 1967
shown during a year spent in his laboratory and for fruitful dis-
cussions concerning some aspects of this project.

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Interaction of DMBA with Sensitive and Resistant Cells

Fig. 1. Autoradiographs of cells exposed for 24 hr to tritiated 7,12-dimethylbenz(a)anthracene and fixed in Carnoy’s solution. A, Secondary hamster embryo cells; B, polyoma-transformed hamster embryo cells (Nil-2/Py). X 520.
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