Binding of 7,12-Dimethylbenz[a]anthracene to BALB/c Mouse Mammary Gland DNA in Organ Culture 1, 2

Amit B. Kundu, 3 Nityanand T. Telang, 3 and Mihir R. Banerjee 3, 4, 5

ABSTRACT—The second thoracic mammary glands of immature female BALB/c mice were cultivated to alveolar development in a chemically defined medium with insulin plus prolactin plus aldosterone plus hydrocortisone (IPAH medium). The glands were treated with labeled (H or 14C) or unlabeled 7,12-dimethylbenz[a]anthracene (DMBA) in dimethyl sulfoxide (DMSO) for 24 hours between days 3 and 4 of culture. For binding studies, isolated mammary gland DNA was treated with RNase and pronase and the radioactivity in DNA was measured. The DNA of glands treated with [14C]DMBA was also analyzed in a CsCl density gradient. The DNA of glands treated with labeled DMBA was found to contain bound radioactive. UV absorption spectral analysis also showed the presence of the DMBA nucleus at the 310-nm region in the DNA of DMBA-treated glands. Nearly 100% of the initial [3H]DMBA-DNA radioactivity was retained up to 5 months, and at 8 months 72% of the radioactivity was present in the DNA. For morphologic studies, observation of the glands after treatment with equimolar concentrations (0.004 μM) of DMBA, dibenz[a,h]anthracene, benzo[a]anthracene, or anthracene in IPAH medium revealed that DMBA was most potent in inducing nodulalike alveolar lesions (NLAL) in the mammary glands in culture. Thus the binding of DMBA to DNA corresponds to its high noduligenic action in the mammary glands in the present organ culture model. The results appear to resemble DMBA-DNA interaction associated with the carcinogenic action of DMBA. The biologic characteristics of the NLAL are being assessed by mammary fat pad transplantation.—J Natl Cancer Inst 61: 465-469, 1978.

Hyperplastic alveolar nodules constitute an essential precursor stage in murine mammary tumor virus- and chemical carcinogen-induced mouse mammary carcinogenesis (1, 2). However, recent reports (3, 4) indicate that in the rat a hyperplastic alveolar nodulalike discrete precursor step may not always be associated with chemical carcinogenesis in the mammary gland. With the exception of a single report (5), induction of malignant transformation of cells in tissue fragments or in an organ culture still remains a challenge. With the use of some modifications of the methods of organ culture of the whole mammary gland developed by Ichinose and Nandi (6), studies in our laboratory have previously shown (7) that DMBA or MCA treatment of cells in organ culture of the whole mammary gland in IPAH medium produces a high incidence of NLAL. The biologic characteristics, particularly with regard to tumorigenic potential of the cells in NLAL-containing glands, are being assessed by various modes of mammary fat pad transplantation in vivo. The ability of the hydrocarbon to produce the mammary lesions in culture led to the present studies concerning biochemical interactions between the hydrocarbon and the macromolecules of the mammary glands cultivated in this unique whole-organ culture model containing only the hormones. In addition, the action of several other less potent carcinogenic and noncarcinogenic hydrocarbon compounds to induce NLAL was also assessed, and the results are presented in this report.

MATERIALS AND METHODS

Animals and organ culture.—As a prerequisite for the culture procedure (8-10), BALB/c female mice (3-4 wk old) were primed for 9 days by daily sc injections of 1 μg estradiol-17β and 1 mg progesterone in a 0.1-ml saline (0.9%) suspension. On day 10, mice were killed. The entire second thoracic mammary gland was excised on a sterile Dacron raft and then placed in a Falcon plastic culture dish containing chemically defined Waymouth's MB 752/1 medium (1 ml medium/gland), supplemented with 35 μg t-glutamine/ml, 3.5 μg penicillin/ml, and appropriate combinations of polypeptide and steroid hormones. The cultivation was done at 37° C in a 95% O2 and 5% CO2 atmosphere, as previously described (7, 11). In the present studies the mammary glands were cultivated in IPAH medium (5 μg insulin/ml, 5 μg prolactin/ml, 1 μg aldosterone/ml, and 5 μg hydrocortisone/ml) for 9 days with change of medium at 2-day intervals (7).

Treatment with [3H]DMBA and [14C]DMBA.—For biochemical studies [3H]DMBA (12 Ci/mmmole; purity, 98%) was dissolved in 911 μl of DMSO, and to this solution 89 μl of 0.04 M unlabeled DMBA in DMSO was added to obtain 1.0 ml of a stock solution of the labeled carcinogen (5,000 μCi/ml). For treatment of the glands with [3H]DMBA, 2 μl of this solution (10 μCi) was added per ml of the culture medium. The final concentration of [3H]DMBA in medium was 7.8 μM. [14C]DMBA (10.55 mCi/mmmole; purity, 99%) was dissolved in 0.5 ml of DMSO, 136 μl of this stock solution

ABBREVIATIONS USED: BA = benz[a]anthracene; DBA = dibenz[a]anthracene; DMBA = 7,12-dimethylbenz[a]anthracene; DMSO = dimethyl sulfoxide; IPAH medium = chemically defined medium containing insulin, prolactin, aldosterone, and hydrocortisone; IPH medium = chemically defined medium containing insulin, prolactin, and hydrocortisone; MCA = 3-methylcholanthrene; NLAL = nodulalike alveolar lesion(s); 0.01 X SSC = 0.01 X standard saline citrate (0.0015 M sodium chloride plus 0.00015 M sodium citrate).

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was made 100 ml with the use of the culture medium, and this [14C]DMBA stock medium (0.68 μCi/ml, 16.5 μg/ml) was used as culture medium during treatment. Because of the lower specific activity of [14C]DMBA, this higher concentration of the carcinogen was used and the final concentration of [14C]DMBA in the medium was 65 μM.

For DMBA-DNA binding studies, the glands were cultivated in the labeled DMBA-containing medium for 24 hours between days 3 and 4 of culture in IPAH medium or IPH medium; the concentration of each hormone was the same as indicated earlier. At the end of the treatment period, the glands were repeatedly washed in cold 0.9% saline, frozen in liquid nitrogen, and stored at -80°C.

**DNA isolation and determination of DMBA binding.**

[3H]DMBA- or [14C]DMBA-treated glands (0.6-0.7 g, 40-50 glands pooled) were homogenized in a buffer (0.1 M Tris, 0.1 M NaCl, and 5 mM EDTA at pH 8.0; 10 ml/g tissue), a 0.1 volume of 10% sodium dodecyl sulfate was added to the homogenate, and the mixture was shaken at room temperature for 5 minutes. Subsequently, isolation of DNA was done according to Marmur's method (12) of chloroform isooamyl alcohol extraction with some modifications (13). The DNA preparation was treated with RNase and pronase as described (13, 14). After RNase and pronase digestion, DNA was precipitated with cold ethanol, and the pellet was repeatedly washed with ethanol and ether. The DNA samples after dilution with 0.01 X SSC were measured for absorbance (260 nm), and radioactivity in the DNA was monitored in Bray's solution with the use of a Beckman LS 350 liquid scintillation counting system. CsCl density gradient analysis of purified DNA of glands cultivated with [14C]DMBA was done as previously described (13). To assess the possible alteration in the DNA molecule after DMBA treatment, an absorbance profile of purified DNA samples dissolved in 0.01 X SSC in water was measured between the 220- and 340-nm range with the use of a Beckman (DB-GT) spectrophotometer according to standard procedures (15). DNA was determined by both UV absorbance (260 nm) and by diphenylamine color reaction.

**Treatment with different polycyclic aromatic hydrocarbons.**—For morphologic studies, the glands were cultivated in IPAH medium containing the hormone combination found to be most conducive to DMBA induction of NLAL (7). Between days 3 and 4 of culture, the glands were treated for 24 hours with equimolar (0.004 μM) concentrations of DMBA, DBA, BA, and anthracene dissolved in DMSO. After hydrocarbon treatment the medium was removed by suction, glands were rinsed with fresh medium, and the cultivation was continued for another 6 days (total, 9 days) in polycyclic aromatic hydrocarbon-free medium, containing the same combination of hormones. Subsequently, regression of normal alveoli was accomplished after 15 days' cultivation in medium with insulin and aldosterone as previously described (9, 11). Throughout the 24-day culture period, medium was changed at 2-day intervals. DMSO-treated glands served as controls.

### RESULTS

The results (table 1) show that a high level of radioactivity was present in the DNA of glands cultivated in the presence of IPAH medium or IPH medium. Although the specific activity of [3H]DMBA-DNA of glands cultivated with IPAH medium was

<table>
<thead>
<tr>
<th>Medium</th>
<th>Total radioactivity, counts/min in DNA</th>
<th>Specific activity, pmoles DMBA bound/mg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPAH</td>
<td>11,447.0</td>
<td>1.06</td>
</tr>
<tr>
<td>IPH</td>
<td>8,231.0</td>
<td>0.91</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>undigested</th>
<th>digested</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPAH</td>
<td>4,463.0</td>
</tr>
<tr>
<td>IPH</td>
<td>3,440.0</td>
</tr>
</tbody>
</table>

* Glands were treated with [3H]DMBA (0.004 μM; 10 μCi/ml of medium) between days 3 and 4 of culture in appropriate medium. After 24 hr of treatment, the glands were repeatedly washed with cold 0.9% saline and frozen. DNA was isolated (see Materials and Methods), and the radioactivity of the DNA before (undigested) and after (digested) RNase and pronase digestion was determined.
TEXT-Figure 2.—UV absorbance spectra of DNA in 0.01 X SSC in water. DNA isolated from [3H]DMBA-treated mammary glands cultured in IPAH medium (●—●), calf thymus DNA (Δ—Δ), and DNA from glands not treated with DMBA (●—●).

TEXT-Figure 3.—Radioactivity of [3H]DMBA-DNA complex measured over an 8-mo period. Between days 3 and 4 of culture in IPAH medium, the glands were treated with [3H]DMBA. DNA isolated from frozen glands (see "Materials and Methods") was dissolved in 0.01 X SSC and stored in aliquots at −20°C. These DNA samples were used for [3H]DMBA-DNA complex stability studies at the different time points shown.

TABLE 2.—Incidence of NLAL induced by polycyclic aromatic hydrocarbons in cultured mammary glands

<table>
<thead>
<tr>
<th>Polycyclic aromatic hydrocarbons</th>
<th>No. of glands/</th>
<th>Percent glands with</th>
<th>Total No. of</th>
<th>NLAL per gland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>glands with NLAL</td>
<td>with NLAL</td>
<td>NLAL</td>
<td>per gland</td>
</tr>
<tr>
<td>DMSO (control)</td>
<td>22/0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DMBA</td>
<td>33/27</td>
<td>82</td>
<td>168</td>
<td>5</td>
</tr>
<tr>
<td>DBA</td>
<td>29/13</td>
<td>45</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>BA</td>
<td>33/10</td>
<td>30</td>
<td>20</td>
<td>0.6</td>
</tr>
<tr>
<td>Anthracene</td>
<td>34/7</td>
<td>20</td>
<td>15</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* NLAL were scored from stained wholemount preparations of the regressed glands as described (7).

* Data represent average number of NLAL/gland scored from number of glands treated with DMSO or the hydrocarbons.

slightly greater (14%) than that of [3H]DMBA-DNA of glands cultivated with IPH medium, the difference was too little to attach a significance at this time. Inasmuch as the [3H]DMBA radioactivity in the DNA may have been caused by tritium exchange, the isolated DNA from cultured glands treated with [14C]DMBA was also examined. A correspondence between the buoyant density banding pattern and radioactivity of the [14C]-DMBA—DNA was also observed in the purified DNA after equilibrium density gradient analysis in CsCl (text-fig. 1).

The UV absorption spectra (text-fig. 2) of the [3H]-DMBA—DNA complex were analyzed to see whether the hydrocarbon moiety was detectable at the appropriate wavelength. The profile (text-fig. 2) shows the presence of a shoulder at the 308-310 nm region in the DNA from glands treated with [3H]DMBA. Analysis of mammary gland DNA not treated with DMBA failed to show a similar shoulder at the same region, and the spectrum was virtually identical to that of calf thymus DNA used as a control.

To determine the stability of the [3H]DMBA—DNA complex, samples of DNA of [3H]DMBA-treated glands were measured for radioactivity at different intervals during an 8-month period. The results (text-fig. 3) showed virtually no loss of radioactivity at 5 months after the initial DMBA—DNA complex formation, and at 8 months 72% of the radioactivity was sustained in the DNA. Table 2 shows the results of the morphologic studies. Consistent with our previous findings (7), over 80% of the glands treated with DMBA contained NLAL. The incidence of NLAL induced by DBA was about half that induced by DMBA. The NLAL-inducing capabilities of BA were even lower and anthracene showed only a little effect.

DISCUSSION

The high level of radioactivity in purified DNA of glands cultivated in the presence of [3H]DMBA suggests that a measurable amount of the hydrocarbon or its metabolites binds to DNA of the mammary cells in the culture medium. We have previously reported (9) that the mammary fat pad after 4 days' cultivation in IPAH medium is virtually filled with the lobuloalveolar parenchyma. Therefore, the [3H]DMBA—DNA...
binding should represent results of interactions of the hydrocarbon mostly with the mammary parenchymal cells. Inasmuch as the results show that a substantial level of $^{14}$C-radioactivity is also associated with the purified DNA, conceivably the [H]$^{14}$DMBA radioactivity is mostly due to its association with the DNA rather than to tritium exchange (15, 16). Thus one may reasonably conclude that the radioactivity in the DNA is due to a DNA-hydrocarbon interaction and that the hydrocarbon appears to bind to the DNA of the mammary glands cultivated in the chemically defined medium. Moreover, the results of CsCl density gradient analysis of the [H]$^{14}$DMBA-labeled DNA provide additional evidence that the interaction of the hydrocarbon is with the DNA itself.

The observation that DMBA-treated mammary cell DNA also shows a measurable level of absorbance at 310 nm is consistent with a recent report (15) that DMBA or its derivatives covalently bound to DNA exhibit a significant absorbance at the same region of the spectrum (310 nm), whereas unmodified nucleic acid fails to do so. Thus, possibly the hydrocarbon is in a covalent bond with the nucleic acid of the mammary cells observed in the present study. The radioactivity in the DNA months after treatment of the cells with [H]$^{14}$DMBA further indicates a high degree of stability of the nucleic acid-DMBA complex. Inasmuch as the affinity of the metabolic derivative(s) of the hydrocarbons to bind to cellular DNA is much greater than that of the parent compound (17-20), the present results strongly indicate that the mammary cells in this unique organ culture model do have the capability to metabolize DMBA in a manner similar to that of other mammalian cells in vivo and in culture. Studies in our laboratory have also shown a high level of aryl hydrocarbon hydroxylase activity in glands treated with DMBA in the present organ culture model, and these results will be presented in a separate report.

The results of morphologic studies demonstrate the nodulogenic potential of various polycyclic aromatic hydrocarbons in the mammary glands in culture (table 2). The potent oncogenic DMBA gives the highest incidence of NLAL, whereas less oncogenic DBA, BA, and anthracene show a much lower ability in the order cited to induce the mammary alveolar lesions. The pronounced difference between DMBA and the three low-potency hydrocarbons to induce NLAL in the mammary gland is in agreement with similar patterns of ‘transforming’ abilities of these compounds in other cell types (21-23).

Binding of oncogenic DMBA to mammary cell DNA has been reported to be associated with DMBA-induced rat mammary carcinogenesis (3, 24). A similar pattern of MCA binding also has been observed (14) in mouse mammary hyperplastic alveolar nodules, which are highly susceptible to both MCA (14, 25) and DMBA (26) carcinogenesis. Thus our previous findings (7, 27) and the present results that DMBA induction of NLAL corresponds to binding of DMBA to mammary cell DNA in organ culture appear to be of interest because similar biochemical interactions between DMBA and cellular DNA are believed to be characteristic of DMBA oncogenesis in other cell types (21, 22, 28-31). However, the carcinogenic potential of the NLAL yet remains to be assessed.

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