Biochemical Basis for Cytotoxicity of 7,12-Dimethylbenz(a)anthracene in Rat Liver Epithelial Cells[1]

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ABSTRACT

When the effects of 7,12-dimethylbenz(a)anthracene (DMBA) on normal and malignant rat liver epithelial cells were compared in a colony inhibition assay, this carcinogen showed a preferential cytotoxic action on the normal cells. In investigations of the biochemical basis of this selective toxicity, it was found that both cell lines were similarly effective in binding DMBA to DNA and that both cell lines had the capacity to metabolize this carcinogen. However, the hepatoma cells were more efficient than were the normal cells in generating very polar metabolites (not organic solvent extractable). These studies suggest that the basis of the resistance of the hepatoma cells to the toxicity induced by DMBA lies in their ability to detoxify biologically active metabolites. Several phenols were examined as possible toxic metabolites of DMBA, but these were not toxic at dose levels at which DMBA kills most of the normal cells.

INTRODUCTION

To improve the sensitivity of detection of transformed epithelial cells *in vitro*, we have been investigating conditions (21, 22) which would increase the percentage of transformed cells in a given population of rat liver epithelial cells in culture (20). Since the polycyclic hydrocarbon DMBA[3] has been found to exhibit a selective cytotoxicity towards normal rodent cells in culture (11, 12, 19), we examined the effect of this carcinogen on normal rat liver epithelial cells and on hepatocellular carcinoma cells in culture. In this paper, we report the selective toxicity of DMBA for normal liver cells *in vitro* and the relationship between cytotoxicity, hydrocarbon metabolism, and the binding of DMBA to cellular DNA.

MATERIALS AND METHODS

Cell Lines. The previously described (20) control epithelial cell line NRL 11 derived from the liver of a 10-day-old male Wistar rat and the hepatocellular carcinoma cells, HL 5, isolated from a histologically confirmed tumor developed in the same strain of rat by feeding 4-dimethylaminoazobenzene (30) were used. These cell lines were maintained routinely as monolayer cultures in Ham's F10 medium (18) supplemented with 10% fetal bovine serum. The cells were grown on plastic Petri dishes and incubated at 37° in humidity cabinets with a gas

phase of 5% CO_2 in air. The monolayers were subcultured weekly with 0.05% trypsin. These cell lines had been in culture for 120 to 150 days (subculture, 17 to 22) and stored frozen before the experiments.

Chemicals. DMBA and BF were purchased from Sigma Chemical Co., St. Louis, Mo. [G-^{3}H]DMBA (specific radioactivity, 9 Ci/mmol) was purchased from Amersham/Searle Corp., Arlington Heights, Ill. Labeled and unlabeled DMBA were chromatographically purified as previously described (14). [G-^{3}H]-DMBA isolated by this procedure was 99% radiochemically pure as determined by thin-layer chromatography on Eastman 6060 silica gel sheets in hexane.

Markers prepared for HPLC studies were 7-hydroxymethyl-12-methylbenz(a)anthracene, 12-hydroxymethyl-7-methylbenz(a)anthracene, 7,12-dihydroxymethylbenz(a)anthracene (2, 7), cis-5,6-dihydro-5,6-dihydroxydimethylbenz(a)anthracene (9), the 5,6- and 8,9-trans dihydrodiols of DMBA (31), and 2-, 3-, and 4-hydroxy-DMBA (26). Dimethylbenz(a)-anthracene-5,6-oxide was prepared according to the method of Dansette and Jerina (10), and nucleoside adducts were prepared from this, as previously described (6).

Cytotoxicity Studies. Monolayers of the cell lines were detached with trypsin, and the resultant cell suspensions were plated at a density of 20 cells/sq cm (500 cells in 5 ml medium in 60-mm dishes). After 1 day, the medium was removed, and an equal volume of either the control medium or media with different concentrations of DMBA (0.001, 0.01, 0.1, 1.0 μg/ml) was added to the cultures. DMBA was dissolved in DMSO, and the final concentration of this solvent in culture media was 0.25%. The cells were refed on the sixth day with appropriate media, fixed in methanol, and stained with Giemsa on the 11th day. The colonies were counted with an automatic colony counter (a minimum of 3 dishes/dose). The above protocol was also used for experiments with 2-, 3-, and 4-hydroxy-DMBA; with BF; and with a simultaneous treatment with BF and DMBA. The possible recovery from the toxic effects of DMBA was assessed by feeding the cells with regular culture medium after 5 days of DMBA treatment.

DNA Binding Studies. Confluent monolayers of either cell line in 150-sq cm plastic flasks were treated with a solution of [^{3}H]DMBA in DMSO (20 μg/ml) such that the final concentration of DMBA was 0.05 μg/ml of medium, and the medium contained 0.25% DMSO. After 24 hr, cells were harvested by trypsinization, and DNA was isolated from the cell pellets by lysis of the cells in Triton X-100 and lysis of the nuclei in 0.5% sodium dodecyl sulfate, followed by isopyknic centrifugation in cesium chloride, all as previously described (24). Binding to DNA was determined from the specific radioactivity of the DNA after deoxyribonuclease I treatment, with the value of εP for DNA being taken as 8750. DNA solutions were degraded enzymically to deoxyribonucleoside mixtures with deoxyribonuclease I, snake venom phosphodiesterase, and bacterial

alkaline phosphatase, as described earlier (27), and chromatography on Sephadex LH-20 in a methanol-water gradient was as described by Baird and Brookes (3).

Metabolism Studies. Cells were plated at 4 × 10³/sq cm (10⁵ cells in 5 ml medium in 60-mm dishes) and maintained in culture for 7 days. The cells were treated with [³H]DMBA on the seventh day when the cells formed a confluent monolayer. [³H]DMBA was dissolved in DMSO and diluted with culture medium to give final concentrations of 0.001 µg DMBA per ml (0.037 µCi/ml) or 0.1 µg DMBA per ml (3.7 µCi/ml). At the time of DMBA treatment, the culture medium was removed, and 5 ml of medium containing [³H]DMBA were added to each dish. After further incubation at 37° for 24 hr, the culture media were aspirated and saved. The cells were detached with a rubber policeman, transferred into vials using a total of 5 ml Dulbecco's phosphate-buffered saline (Grand Island Biological Co., Grand Island, N. Y.), frozen, and then thawed. The culture media and the thawed cell suspension were separately extracted with ethyl acetate (3 × 5 ml). After drying the extracts over MgSO₄, aliquots were examined by HPLC using a Spectra Physics Modular HPLC equipped with Model 740B pumps. The metabolic mixture was separated on a 5 µm Spherisorb octadecyl silane column (3.2 × 250 mm; Phase Separations, Ltd., Hartwell, United Kingdom) using a No. 4 concave gradient of 10 to 70% acetonitrile in water for 100 min at a flow rate of 0.8 ml/min. The UV-absorbing markers added to the mixture were detected by continuously monitoring the column effluent at 254 nm. Radioactive metabolites were detected by liquid scintillation counting of each fraction (0.8 ml) using PCS (Amersham/Searle Corp.).

In some experiments, the medium after extraction with ethyl acetate was treated with β-glucuronidase (4) from either *Helix pomatia* (type H-1 containing sulfatase activity) or bovine liver (type B-10 containing almost no sulfatase activity). Ethyl acetate extracts of β-glucuronidase-treated medium were analyzed by HPLC as above.

RESULTS AND DISCUSSION

To determine the growth-inhibitory effect of DMBA on normal epithelial rat liver cells and rat hepatoma cells in culture, the effect of various concentrations of this carcinogen on relative plating efficiencies for these cells was determined. At concentrations of greater than 0.01 µg/ml, DMBA dramatically reduced survival of normal cells (NRL 11), whereas, even at 1.0 µg/ml, it only marginally inhibited growth of the hepatoma cell line (HL 5) (Table 1). The inhibitory effect seemed to be cytotoxic rather than cytostatic because recovery of the normal cells after removal of DMBA from the culture medium was not observed. However, the cytotoxicity was abrogated by coincubation of NRL 11 cells with BF (Table 1). Since this compound inhibits the metabolism of DMBA (13), it is clear that the cytotoxic action of DMBA was mediated by its metabolism in the NRL 11 cultures as it is in other cells (13). This suggests that the resistant HL 5 cultures may differ substantially from the NRL 11 cultures with respect to their capacity to metabolize DMBA. Indeed, there are many reports on the metabolism of hydrocarbons by normal liver cells (5, 8, 12, 22, 23, 28, 29), but HTC hepatoma cells do not efficiently metabolize hydrocarbons (5).

Since the mechanism of binding of DMBA to DNA is becoming increasingly clear (see also Refs. 6, 14, and 25), the conversion of DMBA to reactive derivatives in the 2 cell lines was examined by measuring the binding of DMBA (0.05 µg/ml) to DNA. After a 24-hr incubation period, the binding in each cell line was similarly low, *i.e.*, 4.1 and 5.6 µmol/mol of DNA phosphorus for HL 5 and NRL 11, respectively. Therefore, these cells do not behave like the sensitive and resistant cells studied by Diamond *et al.* (12) where 10- to 50-fold greater binding to the DNA of sensitive cells was observed. Moreover, chromatographic analysis of the DMBA-nucleoside adducts formed in these liver cells (Chart 1) revealed no qualitative differences between the 2 cell lines, each yielding DMBA-nucleoside adducts similar to those previously found in mouse and hamster fibroblasts which arise from the 1,2,3,4-ring diol-epoxide of DMBA (25). It does not seem likely, that the basis for the selective toxicity of DMBA for these 2 cell lines lies in the metabolism involved in DNA binding.

Therefore, a more detailed study of the metabolic products formed in these cells was undertaken. The general approach followed was that ethyl acetate extracts of medium from confluent cultures which had been incubated for 24 hr with [³H]-DMBA were examined by HPLC, as illustrated in Chart 2 for DMBA (0.001 µg/ml dose). Markers which cochromatographed with radioactive peaks were DMBA itself (*I*), the *trans*-8,9-dihydrodiol of DMBA (*B*), 7-hydroxymethyl-12-methyl-benz(a)anthracene (*E*), and the mixture of 2-hydroxy- and 3-hydroxy-DMBA (*G*). Other radioactive metabolites, such as those eluting in Fractions 49 and 57 in Chart 2, remain unidentified. The aqueous phases from the ethyl acetate extractions were then treated with β-glucuronidase. This treatment was followed by further extraction with ethyl acetate. Chromatographic examination of these extracts (illustrated in Chart 3 for NRL 11 cultures exposed to DMBA, 0.1 µg/ml) show that β-glucuronidase treatment of the aqueous phases released substantial amounts of radioactivity which coeluted with the mixture of 2-hydroxy- and 3-hydroxy-DMBA as found by Baird *et al.* (4) in studies with hamster embryo cells. These findings did not vary substantially with the sulfatase content of the β-glu-

Table 1

DMBA-induced cytotoxicity in rat liver cell lines

Determined as described in "Materials and Methods."

Treatment	Concentration (µg/ml)	NRL 11 (normal)		HL 5 (hepatoma)	
		No. of colonies/dish	Relative plating efficiency	No. of colonies/dish	Relative plating efficiency
Control		148	100	99	100
DMBA[a]	0.001	156	105	112	113
	0.01	118	80	110	111
	0.1	14	9	86	87
	1.0	0	0	85	86
Control		157	100	108	100
DMBA[b]	0.001	156	99	104	96
	0.01	152	97	86	80
	0.1	28	18	86	80
Control		187	100	122	100
BF[a]	2.0	161	86	112	92
DMBA[a]	0.1	2	1	130	107
DMBA[a] BF[a]	0.1 2.0	148	79	110	90

[a] Medium containing appropriate chemical(s) was used to refeed cultures on Day 6.

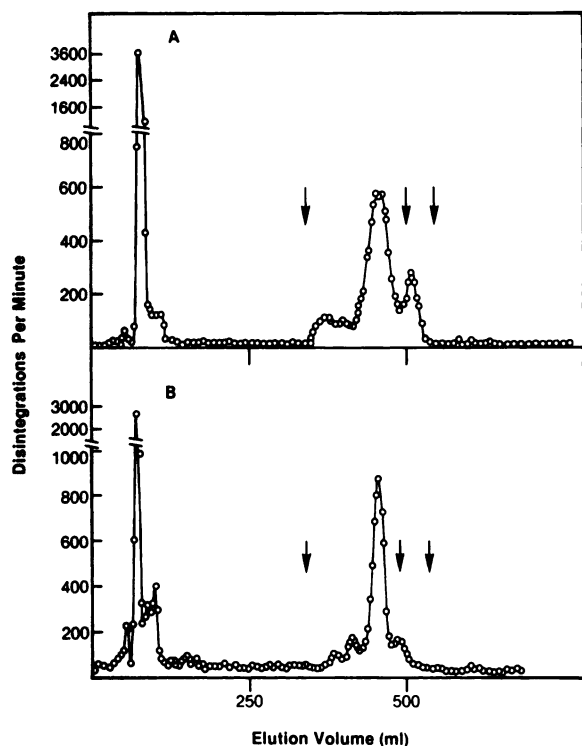[b] On Day 6, cultures were refed with regular medium.

Chart 1. Chromatography on Sephadex LH-20 eluted with a methanol-water gradient of deoxyribonucleoside mixtures from DNA (3) of normal rat liver hepatocytes, NRL 11 (A), and of rat hepatoma cells, HL 5 (B), each exposed to [³H]DMBA (0.05 μg/ml) for 24 hr. *Arrow at 340 ml*, position of elution of an added marker of 4-(p-nitrobenzyl)pyridine, *arrows at 495 and 540 ml*, position of elution of the UV-absorbing nucleoside adducts from the K-region oxide (6).
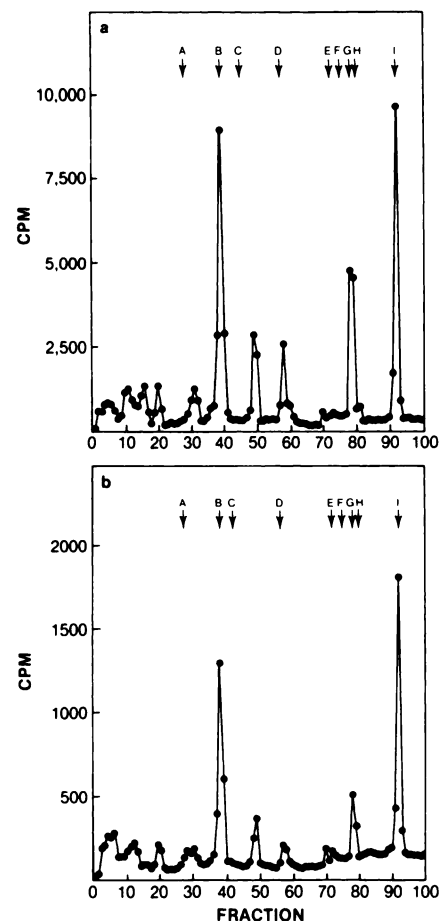

Chart 2. HPLC of ethyl acetate-extractable DMBA metabolites released into the media after treatment of normal (a) and malignant (b) rat liver epithelial cells with [³H]DMBA (0.001 μg/ml) for 24 hr. Samples were chromatographed as described in "Materials and Methods." *Lettered arrow*, UV-absorbing standards added to the radioactive metabolite mixture: A, trans-5,6-dihydro-5,6-dihydroxy dimethylbenz(a)anthracene; B, trans-8,9-dihydro-8,9-dihydroxydimethylbenz-(a)anthracene; C, 7,12-dihydroxymethylbenz(a)anthracene; D; cis-5,6-dihy-dro-5,6-dihydroxydimethylbenz(a)anthracene; E, 7-hydroxymethyl-12-methyl-benz(a)anthracene; F, 12-hydroxymethyl-7-methylbenz(a)anthracene; G, 2- and 3-hydroxy-DMBA; H, 4-hydroxy-DMBA; I, DMBA.
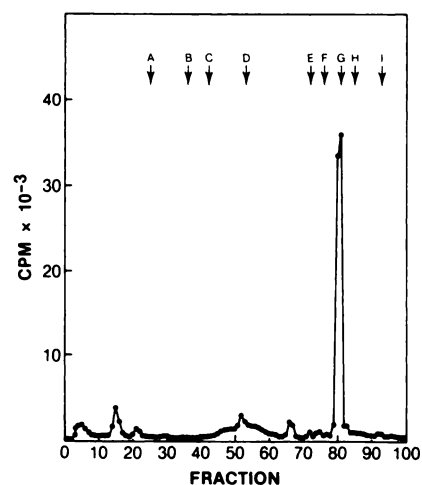
curonidase preparations, indicating that little conjugation with sulfate occurs in either cell line. (The radioactivity remaining in the aqueous phase after this enzymic treatment could represent very polar metabolites or other conjugates such as glutathione conjugates). Similar extractions and analyses of the radioactivity left within the cells were also undertaken, and a summary of the quantitative findings from these experiments for cells exposed to [³H]DMBA (0.1 μg/ml) are summarized in Table 2. Similar studies at the lower dose of DMBA showed that less DMBA was left unmetabolized. Nevertheless, the overall differences between the 2 cell lines were similar to those summarized in Table 2.

In the sensitive NRL 11 cultures, more DMBA is recovered unmetabolized than in HL 5 cultures, and there are correspondingly greater total amounts of metabolites recovered from the HL 5 cultures. Although the initial extraction of the media with ethyl acetate yields greater amounts of organic-soluble metabolites from the NRL 11 cultures, there are considerably greater amounts of nonextractable metabolites in the HL 5 cultures. Indeed, this latter represents the most notable difference in metabolism between the 2 cell lines. For example, although similar total yields of phenols are obtained in both cases, a greater fraction of these are conjugated in the HL 5 cultures (*i.e.*, released into the organic phase only after β-glucuronidase treatment).

These observations suggested a possible basis for the selective toxicity observed, *i.e.*, that toxic metabolites are more efficiently conjugated or otherwise detoxified in the resistant HL 5 cultures. This possibility was investigated by examining

Chart 3. HPLC of β-glucuronidase-released [³H]DMBA metabolites. The ethyl acetate-extracted medium from normal rat liver epithelial cells treated with [³H]DMBA (0.1 μg/ml) was treated with β-glucuronidase as described in "Materials and Methods" and reextracted with ethyl acetate. *Lettered arrows*, same standards as in Chart 2.

Table 2

Percentage of total radioactivity found in various fractions after incubation for 24 hr with [³H]DMBA (0.1 µg/ml)

Cells and media were separately extracted with ethyl acetate (Extraction 1), the aqueous phase was treated with β-glucuronidase and extracted with ethyl acetate again (Extraction 2), and the extracts were examined by HPLC, as described under "Material and Methods." The percentage of total radioactivity found under various peaks in chromatograms is given.

Cell type	Procedure	Cells						Medium					
		H₂O phase	Ethyl acetate phase					H₂O phase	Ethyl acetate phase				
			8,9-Diol[a]	7-CH₂OH	Phenols	DMBA	Total unidentified[b]		8,9-Diol	7-CH₂OH	Phenols	DMBA	Total unidentified
NRL 11	Extraction 1		0.6	0.2	0.8	2.5	1.8		9.9	1.7	5.3	26.3	19.0
	Extraction 2	0.9	0.0	0.2	0.3	0.0	0.4	14.8	0.0	0.2	9.3	0.0	5.8
HL 5	Extraction 1		0.6	0.1	0.7	1.2	1.4		5.8	0.6	1.9	10.0	12.7
	Extraction 2	1.7	0.0	0.2	0.6	0.0	0.7	31.7	0.0	1.2	15.5	0.0	13.4

[a] 8,9-Diol, trans-8,9-dihydro-8,9-dihydroxy-7,12-dimethylbenz(a)anthracene; 7-CH₂OH, 7-hydroxymethyl-12-methylbenz(a)anthracene.
[b] Total unidentified, radioactivity throughout the chromatograms not eluting with markers available (see charts).

Table 3

Effect of DMBA phenols on growth of normal hepatic and hepatoma cell lines determined as described in "Materials and Methods"

Treatment	Concentration (µg/ml)	Relative plating efficiency	
		NRL 11 (normal)	HL 5 (hepatoma)
4-Hydroxy-DMBA	0.01	111	100
	0.1	107	100
3-Hydroxy-DMBA	0.01	94	100
	0.1	79	97
2-Hydroxy-DMBA	0.1	100	100

the toxicity of the phenols available to us (*i.e.*, 2-, 3-, and 4-hydroxy-DMBA) in both cell lines (Table 3). Although the NRL 11 cultures showed some slight indication of greater sensitivity to the toxic effects of 3-hydroxydimethylbenz(a)anthracene, none of the phenols approached the toxicity exhibited by DMBA itself (Table 1). It could be argued that the effect of metabolic generation of these metabolites within the cell cannot be accurately reproduced by their addition to culture media, but these findings (Table 3) do not indicate that the toxicity of DMBA is directly mediated by these phenolic metabolites.

These studies demonstrate that DMBA exhibits a selective cytotoxic action towards normal rat liver epithelial cells and could therefore be used as a selective pressure immediately prior to the assay of the hepatoma cells. However, the hope that the metabolite(s) responsible for this biological effect might be identified was not realized. The rather limited cytotoxicity exhibited by the DMBA phenols was somewhat surprising since 3-hydroxybenzo(a)pyrene is fairly toxic in several cell lines (15), and Diamond (11), citing unpublished observations, has reported that 4-hydroxy-DMBA is similarly cytotoxic. However, the benzo(a)pyrene studies involved doses of an order of magnitude greater than those used in the present study, and even so, at the low end of the dose range the phenol was less toxic than benzo(a)pyrene in normal hamster cells.

It is of interest to compare the findings herein with earlier studies of hydrocarbon toxicity in cultured mammalian cells. Andrianov *et al.* (1) initially reported that sensitivity to benzo(a)pyrene increased with capacity for metabolism of this carcinogen, and Diamond *et al.* (12) found that sensitivity of various cells to DMBA was related to their capacities to bind DMBA to cellular macromolecules. Binding to cellular constituents, which requires metabolism of the hydrocarbon, is seem-ingly a better indicator of sensitivity to toxicity than is the ability to metabolize the hydrocarbon because Diamond (11) noted 2 cell lines which were resistant to both DMBA and benzo(a)-pyrene but were able to efficiently metabolize these hydrocarbons to water-soluble derivatives. Since we found comparable levels of binding to DNA in sensitive and resistant cells, these results are not consistent with a generalization relating toxicity to binding to DNA unless there are subtle quantitative differences in product distributions or large differences in repair capacity in the 2 cell lines studied.

At present, the cellular target for toxic action of DMBA has yet to be defined and could well be one of the cellular macromolecules not examined in this study such as RNA or protein. It does seem likely, however, that some toxic metabolite of DMBA is produced in these cells, and that the hepatoma cells which are more efficient in converting DMBA to water-soluble derivatives are simply detoxifying this metabolite through further metabolism or conjugation more effectively than do the normal cells. This is in concert with recent studies by Gentil *et al.* (16) who report that in hamster, rat, and rabbit embryonic cells DMBA-induced cytotoxicity is inversely related to the conversion of metabolized DMBA to water-soluble derivatives.

ACKNOWLEDGMENTS

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