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# Genotoxic Effects of Five Polycyclic Aromatic Hydrocarbons in Human and Rat Mammary Epithelial Cells

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Five polycyclic aromatic hydrocarbons (PAHs) of different carcinogenic activities were evaluated for their effects on DNA synthesis ($^3$HTdR labeling index (L.I.)) of rat and human mammary epithelial cells (MEC) and for their effects on chromosomes in MEC-mediated sister chromatid exchange (SCE) assays. When compared with DMSO-treated cells, exposures of rat MEC to the two most potent carcinogens (5 μg/ml for 24 hr), i.e., 7,12-dimethylbenz(a)anthracene (DMBA) and benzo(a)pyrene (B[a]P), resulted in a 45–62% reduction in the L.I. of rat MEC. Another carcinogen, 20-methylcholanthrene (MCA), produced a 35–48% reduction in L.I., while the noncarcinogenic PAHs, 1,2-benzanthracene (BA) and benzo(e)pyrene (B[e]P), showed no effect. Similarly, exposures of human MEC to DMBA and B[a]P resulted in a 50–90% depression in L.I. while BA was significantly less effective (30% reduction). When cocultivated with Chinese hamster V-79 cells in the presence of PAH,both rat and human MEC can activate and release the active metabolites to induce SCE in V-79 cells. In the rat MEC-mediated assay for all 5 PAHs, the frequencies of SCE per chromosome in DMBA-, B[a]P-, MCA-, BA-, B[e]P-, and DMSO (solvent control)-treated groups were 6, 3, 1.4, 0.7, 0.4, and 0.3, respectively. DMBA was most effective in increasing SCE, while B[e]P was ineffective. In the human MEC-mediated assay, B[a]P was more effective than DMBA in inducing SCE, and the frequencies of SCE per chromosome were 4.5 and 3.6 in B[a]P- and DMBA-treated groups, respectively. Comparing depression of L.I., SCE, and in vivo carcinogenecity for the 5 PAHs, SCE mediated by rat MEC is better correlated with carcinogenecity in rat than L.I. depression.

**Key words: sister chromatid exchanges, labeling index depression**

## INTRODUCTION

Many of the polycyclic aromatic hydrocarbons (PAHs) are widespread in our environment and are carcinogenic in experimental animals. The potent carcinogen benzo[a]pyrene (B[a]P) in particular is ubiquitous in the soil, water, and air of industrialized regions and seems to be an indicator of PAH pollution in general [Shabad, 1980]. Because of the extent of environmental pollution by PAHs and the carcinogenic potency of many PAHs, these contaminants constitute a potentially significant human health hazard. Certain of these PAHs, e.g., 7,12-dimethylbenz(a)anthracene (DMBA), are powerful oncogenic agents for the mammary glands of rodents [Foulds, 1975]. However, organ specificity and species difference in chemical carcinogenesis have complicated extrapolation from animal cancer data to human. In spite of the possible involvement of environmental chemicals in the etiology of human cancer, in vivo tests are unlikely to be performed in humans. Much effort has been directed toward the development of short-term bioassay systems using human cells and/or tissues for testing hazardous chemicals.

In cell-mediated mutation assays, Gould et al. [1986] demonstrated that human mammary epithelial cells (MECs) were able to activate B[a]P as well as DMBA and release the active metabolites that induced mutation in the indicator V-79 cells. They compared human and rat MEC-mediated mutations and found that B[a]P was more mutagenic than DMBA in human MEC-mediated assays whereas a reverse effect was observed in rat MEC-mediated mutation. The results suggested that B[a]P may be a stronger carcinogen than DMBA in human mammary glands. Nevertheless, there is no formula to equate the in vitro results to in vivo toxicity and/or carcinogenicity in humans.

In an attempt to use the in vitro data of PAH in MEC to assess their toxic potential in human breast, five PAHs with

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graded toxicity in rats were compared for their effects in rat and human MEC. The relationship between in vivo and in vitro toxic effects was examined. The reliability of the in vitro tests to assess human risks was evaluated.

## MATERIALS AND METHODS

The five PAHs, i.e., 7,12-dimethylbenz(a)anthracene (DMBA) benzo[a]pyrene (B[a]P), 20-methylcholanthrene (MCA), 1,2-benzanthracene (BA), and benzo[e]pyrene (B[e]P), were obtained from Sigma Chemical Co. (St. Louis, MO). They were dissolved in dimethylsulfoxide (DMSO) at a concentration of 1 mg/ml and stored at −70°C before being used in the assays. The complete medium (CM) is M199 medium supplemented with 10% fetal calf serum, insulin (5 μg/ml), ovine prolactin (5 μg/ml), 17β-estradiol (0.005 μg/ml), progesterone (0.5 μg/ml), hydrocortisone (0.5 μg/ml), cholera toxin (55 ng/ml), penicillin streptomycin (300 μg/ml), 10 mM HEPES buffer, and sodium bicarbonate. The reagents and media were purchased from the following sources: collagenase from Cooper Biomedical (Malvern, PA); 5′-bromo-2′-deoxyuridine and Hoechst 33258 from Sigma Chemical Co. (St. Louis, MO); $^3$H thymidine from New England Nuclear (Boston, MA); Gurr buffer, pH 7, from George T. Gurr, (London, SW6, England); Liebowitz-15 (L-15) medium and other additives of CM from GIBCO (Grand Island, NY).

### Mammary Cell Isolation

MECs were isolated from the mammary tissue of inbred 50–60 day-old female Lewis rats and from human breast tissue obtained at the autopsy of young adult women by the method of Gould [1982]. In brief, the mammary tissues were minced and dissociated with collagenase (144 units/mg, 1 mg/ml for 100 mg mammary tissue) by stirring gently for 2–3 hr at 37°C. After dissociation, the cell suspension was poured through a sterile nylon filter to remove undissociated tissue. The cells in the filtrate were washed and finally resuspended in complete medium (CM). The suspension, which was composed of fragments of mammary gland ducts, cell clumps, and single cells along with red blood corpuscles, was transferred to 75 cm$^2$ tissue culture flasks and allowed to settle for 40–60 min at 37°C. The supernatant which contained mostly ductal fragments was then removed and transferred to a new flask and placed in an atmosphere of 5% $CO_2$–95% air at 37°C. Near-confluent cultures of MEC which grew out of the ductal fragments were obtained after 7 days of incubation. These cells were removed with 0.5% trypsin–0.2% EDTA and used to initiate cultures for experiments.

### Labeling Index Depression Assay

To initiate the assays, MEC were seeded onto 22 mm plastic coverslips at 1 × 10$^4$/sq cm in 6 well tissue culture plates and incubated at 37°C in M199 medium.

Two days after seeding, the cells were exposed to medium containing PAHs at a final concentrations of 5 μg/ml (probably at or near PAH saturation concentrations) for 24 hr at 37°C. DMSO was used as a solvent control. At the end of treatments, the cells were washed 3 times with CM and then incubated in fresh CM.

To assay for the influence of the PAHs on DNA synthesis, the cultures were incubated in medium containing $^3$H-TdR (5 μCi/ml, 2 Ci/mmol) for 4 hr. On day 1, 3, or 7 following treatments, the cells were rinsed once in CM and twice in HBSS, fixed in ethanol, air-dried, and coated with Ilford L-4 nuclear tract emulsion (diluted 1:1 in distilled water) for autoradiography. The exposed autoradiograms were developed after 4 days at 4°C in dark boxes, stained with hematoxylin, dehydrated, and mounted in Coverbond (Scientific Products), and the $^3$H-TdR labeling index was determined. Three to 4 autoradiograms were examined for each experimental condition and a labeling index (L.I.) ± S.D. was calculated.

Additional cultures, set up and treated with PAH in an identical manner, were used at days 1, 3, and 7 following treatments for determination of viability. For measuring percent viable cells, the cells were dissociated with trypsin-EDTA and suspended in the medium. The viability of MEC in suspension was determined by the percent of cells that excluded trypan blue.

### Sister Chromatid Exchange (SCE) Assay

For SCE assays, 10$^6$ Chinese hamster V79 cells were added to 100 mm culture dishes, which either contained M199 medium (control) or had been seeded with 10$^6$ MEC 2 days before the co-cultivation. Four hours later, the control and co-cultivated cells were exposed to medium containing 1 μg/ml PAH for 24 hr. After exposure to PAHs, the cultures were washed 3 times with HBSS, dissociated selectively with 3 ml of 0.5% trypsin–0.2% EDTA, and washed, diluted, and subcultured into a T75 flasks at 1–2 × 10$^6$ viable cells/flask. Bromo-deoxyuridine (BudR) at a final concentration of 10 μM was also included in CM and the cultures were incubated in the dark at 37°C for 30–60 hr allowing for completion of 2 mitotic cycles. It was consistently observed that PAH treatment inhibited the cell growth and the cells needed long period to complete mitotic cycles. Colcemid (0.1 μg/ml) was presented to arrest mitotic cells during the final 2 hr. Following trypsinization to harvest the cells, the V-79 cells were treated with 0.075 M hypotonic solution for 8 min at 37°C and then fixed in 1:3 acetic acid:methanol. Preparation of chromosomes on slides and differential staining by FPG method were done as described previously [Hsu et al., 1978]. Data in Table III represented counts from a minimum of 40 metaphases and the SCE were scored by 1 person.

**TABLE I. Effect of Exposure to Different PAH Compounds on the DNA Synthesis of Rat MEC***

| Compound | Concentration (μg/ml) (%) | $^3$HTdR labeling index ($\bar{x}$(%) + SD) on indicated day after exposure | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Exp. #1 | | | Exp. #2 | | | Exp. #3 | | |
| | | Day 1 | Day 3 | Day 7 | Day 1 | Day 3 | Day 7 | Day 1 | Day 3 | Day 7 |
| None | 0 | 31 ± 6 | 20 ± 2 | 19 ± 4 | 27 ± 4 | 23 ± 3 | 19 ± 1 | 29 ± 7 | 26 ± 2 | 20 ± 5 |
| DMSO | 0.5 | 27 ± 6 | 20 ± 2 | 18 ± 1 | 24 ± 5 | 21 ± 4 | 20 ± 1 | 18 ± 5 | 20 ± 4 | 21 ± 5 |
| DMBA | 5 | 29 ± 9 | 9 ± 3 (55)[a] | 10 ± 1 | 27 ± 5 | 11 ± 2 (48) | 14 ± 1 | 18 ± 2 | 10 ± 1 (50) | 10 ± 1 |
| B[a]P | 5 | 21 ± 1 | 8 ± 1 (60) | 11 ± 3 | 25 ± 6 | 8 ± 1 (62) | 11 ± 4 | 22 ± 3 | 11 ± 0 (45) | 12 ± 0 |
| MCA | 5 | 30 ± 11 | 13 ± 0 (35) | 11 ± 1 | 25 ± 2 | 11 ± 5 (48) | 14 ± 3 | 20 ± 4 | 12 ± 4 (40) | 11 ± 2 |
| BA | 5 | 29 ± 7 | 14 ± 7 (30) | 18 ± 0 | 18 ± 6 | 24 ± 4 | 23 ± 2 | 29 ± 4 | 21 ± 5 | 21 ± 4 |
| B[e]P | 5 | — | — | — | 24 ± 5 | 23 ± 3 | 18 ± 4 | — | — | — |

*The data analysis was done by analysis of variance (1 way) as described in Materials and Methods. *Exp. #1, #2, and #3 of day 1:* Comparisons between pairs of means within each experiment for day 1 rendered $P > 0.05$. Thus, the differences between each group are not significant. *Exp. #1 on day 3:* DMBA and B[a]P < untreated and DMSO; DMBA and B[a]P < BA, $P<0.05$; *Exp. #1 on day 7:* DMBA and B[a]P < untreated and DMSO, $P<0.05$. *Exp. #2 on day 3:* DMBA and MCA < untreated, DMSO, BA, and B[e]P; b[a]P < untreated, DMSO, BA and B[e]P, $P<0.05$; *Exp. #2 on day 7:* DMBA and B[a]P < untreated, DMSO, BA, and B[e]P; MCA < DMSO and BA, $P<0.05$. *Exp. #3 on day 3:* DMBA and B[a]P < untreated, DMSO, and BA; MCA < untreated, $P<0.05$. *Exp. #3 on day 7:* DMBA, B[a]P, and MCA < untreated, DMSO and BA $P<0.05$.

[a]( ) = % depression in L.I.

### Statistical Analysis

Since all of the experiments reported here contained several groups, analysis of variance (1-way) was used to test the null hypothesis of identical population means [Snedecor and Cochran, 1967]. When a significant F value was obtained at $P \leq 0.01$, significant differences between pairs of means within each experiment were identified by means of the Newman-Keuls test at a significance level of $P \leq 0.05$.

## RESULTS

### Effect of PAH on DNA Synthesis of MEC

The labeling indices of rat MEC cultures measured at days 1, 3, and 7 after exposure to different PAHs are shown in Table I. The depression of L.I. was maximal at day 3. Exposures to the potent carcinogens DMBA and B[a]P resulted in marked depression in L.I. (45–62%), while the less potent carcinogen MCA produced a slightly smaller (35–48%) depression in L.I. In contrast, the non-carcinogenic PAHs, BA and B[e]P, did not significantly depress the L.I. of rat MEC relative to DMSO-treated control cultures. The cells on the coverslips for labeling index assay were all viable. As shown in Figure 1, there is no significant difference in viability between PAH-treated and control MEC. Cell viability on 1–7 days after exposure to the agents was approximately 90% in all groups. Although the percent viability for MEC on the coverslips did not drop significantly on days 1–7, the total cell counts measured at day 7 after treatments were 1.5–2-fold lower (data not shown) ($P<0.05$) in DMBA-, B[a]P-, and MCA-treated groups than in DMSO-treated groups. In contrast, total cell counts in the BA- and B[e]P-treated cultures were not significantly lower than those of DMSO-treated control cultures.

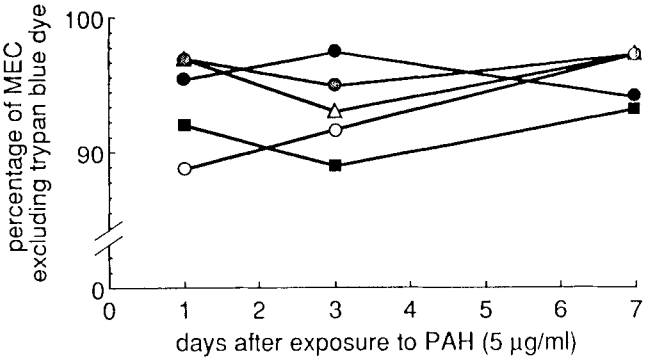


**Fig. 1.** Percent of viable cells in culture after exposure to various PAHs (5 μg/ml). Cells ($1 \times 10^4$/sq cm) were plated in 6-well dishes and were treated with B[a]P (●), MCA (stippled circles), BA (△). DMBA (■), or DMSO (solvent control, ○) for 24 hr. At days 1, 3, and 7 posttreatment, the cultured cells were harvested. Percent viability was determined by using trypan blue exclusion test.

Similar experiments with human MEC cultures showed that the L.I. was depressed after exposure to carcinogenic PAHs in the same way as rat MEC (Table II). In the first experiment, the labeling indices of the DMBA and B[a]P groups were significantly lower (50–80%) than the L.I. of the DMSO group on day 3 after treatments. MCA was not tested in the human MEC cultures. In the second experiment, DMBA- and B[a]P-treated cultures had significantly lower (88%) labeling indices than the DMSO control on both day 1 and day 3 after treatment. The L.I. for BA was also significantly lower than the DMSO control on day 1. The L.I. of the BA-treated cells, although lower than the DMSO control on day 1 and 3 (38 and 30%) after treatment was, nevertheless, significantly higher than the DMBA and B[a]P groups.

**TABLE II. Effect of Exposure to Different PAH Compounds on the DNA Synthesis of Human MEC***

| Compound | Concentration (μg/ml) (%) | $^3$HTdR labeling index (x̄(%) + SD) on indicated day after exposure | | | |
|---|---|---|---|---|---|
| | | Case 1 | | Case 2 | |
| | | Day 1 | Day 3 | Day 1 | Day 3 |
| DMSO | 0.5 v/v | 6 ± 3 | 10 ± 2 | 8 ± 1 | 10 ± 3 |
| DMBA | 5 | 6 ± 3 | 5 ± 2 (50)[a] | 1 ± 0 (88) | 1 ± 0 (90) |
| B[a]P | 5 | 4 ± 1 | 2 ± 0 (80) | 1 ± 0 (88) | 1 ± 0 (90) |
| BA | 5 | — | — | 5 ± 0 (38) | 7 ± 1 (30) |

**Case #1: day 1:* no comparisons are significant; *day 3:* DMBA and B[a]P < DMSO, at $P<0.05$. *Case #2: day 1:* DMBA and B[a]P < DMSO and BA, BA < DMSO at $P<0.05$; *day 3:* DMBA and B[a]P < DMSO; BA ~ DMSO, at $P<0.05$.

[a]( ) = % depression in L.I.

## MEC-Mediated SCE Assay

Induction of SCE in V-79 cells exposed to PAH in the presence and absence of rat MEC is shown in Table III. Analysis of data in rat MEC-mediated SCE assay showed that DMBA and B[a]P, the two most potent animal carcinogens, also have the highest mean numbers of SCE per chromosome in V-79 cells. DMBA mediated by rat MEC was more effective than B[a]P in the induction of SCE. In the absence of MEC, the numbers of SCE in V-79 cells exposed to PAHs were lower than those in the presence of MEC; the frequencies did not differ among treatment groups. The order of activity to induce SCE in rat MEC-mediated assay is DMBA > B[a]P > MCA > BA > B[e]P. B[e]P is the least reactive and showed no significant SCE induction over that of control DMSO.

Experiments carried out with human MEC are also shown in Table III. The mean numbers of SCE/chromosome in V-79 cells exposed to DMBA and B[a]P in the presence of MEC were significantly higher than those in DMSO control and in cultures of V-79 cells alone. It was interesting that B[a]P at 1 μg/ml in the presence of human MEC induced more SCE than DMBA (Table III).

**TABLE III. MEC-Mediated SCE in V-79 Cell Exposed to PAH**

| Compound | Concentration (μg/ml) (%) | SCE frequencies/chromosome (mean ± S.D.)[a] | | |
|---|---|---|---|---|
| | | V-79 cells only | V-79 cell + rat MEC | V-79 cell + human MEC |
| DMSO control | 0.5 | — | 0.3 ± 1.0 | 0.4 ± 0.1 |
| DMBA | 1 | 0.3 ± 0.2 | 6.0 ± 1.0 | 3.6 ± 2.1 |
| B[a]P | 1 | 0.4 ± 0.1 | 3.0 ± 1.0 | 4.5 ± 1.7 |
| MCA | 1 | 0.4 ± 0.1 | 1.4 ± 0.4 | — |
| BA | 1 | 0.4 ± 0.1 | 0.7 ± 0.5 | — |
| B[e]P | 1 | 0.4 ± 0.1 | 0.4 ± 0.2 | — |

[a]A total of 40 metaphases from 4 or more cultures per data point.

## DISCUSSION

The MEC were obtained as an "enriched population of epithelial cells" by the method of Gould [1982] and showed over 95% of cells were positive to keratin by immunocytochemical staining. In the present study, five PAHs of graded carcinogenic potency were evaluated 1) for their effects on DNA replication of rat and human MEC and 2) for the induction of SCE in Chinese hamster V-79 cells mediated by MEC.

DMBA and B[a]P are known potent mammary carcinogens [Huggins et al., 1961; Huggins and Yang, 1962] whereas BA is a weak carcinogen, inactive in rat mammary gland system [Cavalieri et al., 1980; Cavalieri and Rogan, 1985]. In rat, DMBA is a much stronger carcinogen than B[a]P [Cavalieri et al., 1980]. The order of the activities is DMBA > B[a]p > MCA. In this in vitro study, DMBA, B[a]p, and MCA were active in both increasing SCE of V-79 cells and in depressing L.I. of MEC, while B[e]P and BA did not show significant effects over DMSO-treated controls (Tables I and III). However, based on quantitative comparison of the in vitro effects for the 5 PAHs, there is no significant difference in L.I. depression among DMBA-, B[a]P-, and MCA-treated MEC. SCE induction mediated by rat MEC has a better correlation with in vivo carcinogenicity of the PAH in rats. The mechanism of L.I. depression is not clear. In all 3 repeated experiments, labeling indices were determined on days 1, 3, and 7. L.I. depression was observed on day 3 and day 7. Synchronization of MEC during the cell cycle and low $^3$H thymidine incorporation at G phase seemed unlikely to explain the L.I. depression in MEC exposed to B[a]P, DMBA, or MCA.

In the cell-mediated SCE assay, MEC activated PAH and released the proximate or ultimate toxins to co-cultivated V-79 cells. These active metabolites interacted with macromolecules or bound to DNA and induced SCE or mutation in the co-cultivated cells. In a study utilizing Chinese hamster ovary cells, a linear relationship between the induction of SCE and induction of mutations had been reported by Carrano et al. [1978]. The pattern of MEC-mediated SCE induction by the 5 PAHs is consistent with the observations of others using the cell-mediated mutation assay in which

DMBA, B[a]P, and MCA, but not BA or B[e]P, were effective in inducing mutations in V-79 cells [Gould, 1980; Gould et al., 1986]. Such a correlation indicated that binding of PAH metabolites with DNA or macromolecules is a common event that leads to induction of SCE and mutation. It is interesting to note that B[a]P mediated by human MEC induced a higher frequency of SCE than DMBA; a higher mutagenic effect of B[a]P over DMBA has previously reported in human MCE mediation assays by Gould et al. [1986]. Thus, our SCE results confirm the notion that B[a]P has a stronger genotoxic effect than DMBA in human MEC.

The toxic responses induced by a xenobiotic are largely determined by its rate of absorption, distribution, metabolism, and excretion [Klassen et al., 1986]. The correlation of in vitro and in vivo toxic effects in several PAHs indicates that metabolism of xenobiotics in MEC is the most important factor in determining toxic or carcinogenic potential of PAH. Absorption, distribution, excretion, or serum concentration of PAH is comparable in animal mammary gland and may not play a major role for species difference in toxicity or carcinogenicity. Thus, comparison of in vitro effects between human and animal MEC may allow us to predict the susceptibility of human breast cells to PAHs.

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