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Relationships between Benzo(*a*)pyrene-DNA Adduct Levels and Genotoxic Effects in Mammalian Cells[1]

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ABSTRACT

The effectiveness of benzo(*a*)pyrene [B(a)P]-DNA binding as an internal dosimeter was evaluated. Data were obtained from concurrent studies, measuring B(a)P induced genotoxic effects and DNA adducts in several short-term bioassay systems: cytotoxicity, gene mutation, and sister chromatid exchange in Chinese hamster V79 cells; cytotoxicity, gene mutation, and chromosome aberrations in mouse lymphoma L5178Y TK$^{+/-}$; cytotoxicity and enhanced virus transformation in Syrian hamster embryo cells; and cytotoxicity and morphological transformation in C3H10T½CL8 mouse embryo fibroblasts. Both total B(a)P-DNA binding and specific B(a)P-DNA adducts were measured. N^2-(10β-[7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene]yl)deoxyguanosine [BPDE I-dGuo] was one of the major adducts identified in all bioassay systems. DNA binding and genotoxic responses varied significantly between bioassays. Each genetic end point was induced with a differing efficiency on a per adduct basis. However, the relationships between frequency of genetic effect or morphological transformation and B(a)P-DNA binding or BPDE I-dGuo were linear within a given assay. In order to compare biological end points of diverse frequencies in diverse biological systems, a doubling adduct level, expressed as the number of BPDE I-dGuo adducts per unit of DNA required to double the induced frequency of biological response, was applied to the data.

INTRODUCTION

The rapid development of short-term tests which can detect the mutagenic and potentially carcinogenic activity of chemicals has provided the capability to examine the comparative effects of chemicals in a variety of genetic toxicology bioassay systems. For chemicals such as B(a)P,[4] a large data base is available concerning its effects in bacteria, mammalian cells, and rodents including its ability to induce gene mutation, chromosome effects, SCE, DNA damage and repair, oncogenic cell transformation, and cancer (2). B(a)P is a ubiquitous environmental pollutant which is found in the air, water, and soil (2). Because of this widespread exposure, it has been reported that certain human populations have residing levels of B(a)P-bound DNA adducts in their tissues (3, 4). In a limited study, roofing tar and foundry workers were found to have measurable quantities of B(a)P-bound DNA adducts in their WBC (4). In a large number of experimental test systems the major B(a)P-bound DNA adduct is the BPDE I-dGuo-adduct. This adduct has been found in bacterial, mammalian, and human cells as well as in the rodent tissues which are susceptible to B(a)P carcinogenesis, *e.g.*, skin, lung, and forestomach (5–18).

In comparing mutagenic and carcinogenic effects of B(a)P in a variety of bioassay systems, a major problem is determining the actual dose to the target tissue or organ from the exposure dose. The use of DNA adducts as an internal dosimeter can obviate many variables such as absorption, distribution, metabolic activation, detoxification, and excretion which contribute to this problem.

In order to explore comparisons between *in vitro* bioassay results in genetic toxicology test systems, we have measured B(a)P adducts in cultures of several types of mammalian cells and have quantitated the genetic and cell-transforming effects induced by this agent.

MATERIALS AND METHODS

Chemicals and Biochemicals. Alkaline phosphatase (*Escherichia coli* type III) was purchased from Sigma Chemical Co., St. Louis, MO; venom phosphodiesterase, spleen phosphodiesterase, and DNase I were from Worthington Biochemical, Freehold, NJ, and *Neurospora crassa* endonuclease was from Miles Biochemicals, Elkhart, IN. All media were from Grand Island Biological Co., Grand Island, NY. [*G*-^{3}H]B(a)P (17–14 Ci/mmol) obtained from Amersham (Arlington Heights, IL) was dried in a stream of dry nitrogen and dissolved in a solution of B(a)P (Gold Label; Aldrich Chemical Co., Milwaukee, WI) in acetone. The specific activity ranges of the dosing solutions were: 0.328–5.59 Ci/mmol in the SHE viral enhancement studies; 0.856–1.94 Ci/mmol in the C3H10T½ cell studies; 0.0198–3.93 Ci/mmol with SHE-V79 studies; and 0.321–0.340 Ci/mmol in the mouse lymphoma L5178Y cell studies. In all cases, except the mouse lymphoma cell studies, the specific activity was inversely varied with the final concentration of B(a)P.

Cocultivation and Treatment of V79 Cells and Irradiated SHE Cells. Primary SHE cells were prepared (19) by trypsinization of eviscerated and decapitated embryos after 13 to 14 days of gestation. Cells were resuspended in modified Dulbecco's medium supplemented with 10% heat-inactivated FBS and $NaHCO_3$ (0.22 g/100 ml), irradiated with 5000 rads of radiation, seeded at 2×10^6 cells/75-cm^2 flask, and allowed to attach for 4–6 h. V79 cells [selected against spontaneous TG^r by the method of Bradley *et al.* (20)] were plated on the SHE cells at 0.5×10^6 cells/flask in Williams E medium with 10% FBS. After 20 h, B(a)P was added and 24 h later the medium was removed, and the flasks were rinsed twice with phosphate-buffered saline. Flasks were then rinsed with 3 ml of 0.008% trypsin, the trypsin was removed, and 1 ml of trypsin was added to the flask. After incubation for 40 s at room temperature, the flask was tapped once to dislodge the V79 cells but

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[4] The abbreviations used are: B(a)P, benzo(*a*)pyrene; C3H10T½, C3H10T½CL8; HPLC, high pressure liquid chromatography; TG^r, 6-thioguanine resistant; TFT^r, trifluorothymidine resistant; SHE, Syrian hamster embryo; SCE, sister chromatid exchange; BPDE I-dGuo, N^2-(10β-[7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene]yl)deoxyguanosine; BPDE II-dGuo, N^2-(10α-[7β,8α,9β-trihydroxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene]yl)deoxyguanosine; SA7, simian adenovirus SA7; TK, thymidine kinase; FBS, fetal bovine serum; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; PAH, polycyclic aromatic hydrocarbon.

not the irradiated SHE cell feeder layer substrate. The V79 cells in the trypsin solution were removed and added to an equal volume of cold Williams E medium with 10% FBS. The V79 cell suspension was then triturated to ensure a single cell suspension and a sample was removed and counted in a hemacytometer to determine the total cell count and the percentage of contamination with SHE cells. The cell mixture was centrifuged and resuspended to 10^6 cells/ml and reseeded for cytotoxicity, mutagenesis, and SCE analyses. B(a)P-DNA adducts were isolated from V79 cells treated with [^{3}H]B(a)P in parallel experiments using the same batch of cells described above. These cells were frozen in 10% glycerol in Williams E medium with 10% FBS for subsequent DNA adduct analyses.

V79 Cell Mutagenesis. After treatment, V79 cells (100 cells/60-mm dish in 5 ml of Williams E medium with 10% FBS) were seeded for determining cloning efficiency. Cells were also plated at 100,000 cells/75-cm^2 flask, allowed to grow for 4 days, split, and plated in Williams E medium with 10% FBS on day 6 posttreatment. On day 8 following chemical treatment, cells were trypsinized, centrifuged, and plated for cloning efficiency and mutant selection. Cloning efficiencies were determined by seeding 100 cells/60-mm dish in 5 ml of complete medium. After 8 days the dishes were fixed, stained, and scored (21).

Cells for mutant selection were seeded at 100,000 cells/100-mm dish in 10 ml of complete medium containing 10 μg/ml of 6-thioguanine. After 10 days the dishes were fixed, stained, and scored for TG^r mutant colonies.

SCE Analysis. For SCE analysis, 8×10^5 V79 cells isolated from the SHE cell-mediated system as described above were plated in 6 ml of Williams E medium with 10% FBS. After 3–4 h for cell attachment, 15 μM 5-bromo-2′-deoxyuridine was added and the cells were incubated for 24 h (two cell cycles) in complete darkness. Colchicine (0.5 μg/ml) was added for the last 2 h. The cells were then trypsinized, centrifuged, and treated with 0.075 M KCl hypotonic solution for 8 min and repeatedly fixed in methanol:acetic acid (3:1). Slides were prepared and stained by fluorescence-plus-Giemsa methods (22, 23). SCE frequencies were analyzed in 35–40-second division cells/culture (24).

Cultivation and Treatment of L5178Y Mouse Lymphoma Cells. The TK$^{+/-}$ 3.7.2C line of L5178Y mouse lymphoma cells were maintained in Fisher's medium for leukemic cells of mice, supplemented with penicillin, streptomycin, sodium pyruvate, Pluronic F-68, and heat-inactivated horse serum (25–27). The mutagenesis studies were performed according to the methods of Clive *et al.* (26) as modified by Moore and Clive (27). Cells were suspended in medium containing 3% horse serum at a density of 0.6×10^6 cells/ml. Aroclor 1254-induced rat hepatic S-9 was used for activation and NADP (2.4 mg/ml) and isocitric acid trisodium salt trihydrate (4.5 mg/ml) were used as cofactors (26). B(a)P (dissolved in acetone) was added at the indicated concentrations to a 10-ml cell culture. Following a 4-h treatment time, cells were centrifuged, washed, and resuspended in fresh medium. Treated cells were analyzed for both gross chromosome aberrations and mutation at the *TK* locus. In parallel experiments cells from TK$^{+/-}$ 3.7.2C culture were treated with [^{3}H]B(a)P. The treatment procedure was the same as above except that five 10-ml cell cultures (0.6×10^6 cells/ml) were treated at each indicated concentration of B(a)P. Following the 4-h treatment, cells were centrifuged, washed twice with fresh medium, resuspended in 10% glycerol in medium, and frozen for DNA adduct analyses.

Analysis of Chromosome Aberrations. Gross chromosome aberrations in TK$^{+/-}$ 3.7.2C cells were analyzed 22 h after the midpoint of the 4-h treatment. Following 2 h in 0.1 μg/ml Colcemid and 10 min in 0.075 M KCl hypotonic solution, the cells were fixed in acetic acid:methanol (1:3). Slides were prepared, heat dried at 60°C for 1 min, and stained with 4% Gurr-Giemsa (Bio/Medical Specialities, Santa Monica, CA). Slides were coded and 100 metaphases were scored for chromosome number and chromosome and chromatid events (excluding gaps).

Mutation at the Thymidine Kinase Locus. Treated cells were counted daily, maintained in log phase, and allowed 2 days for expression of newly induced mutants. Cells were cloned in agar-supplemented (0.37%) growth medium. Cells (1×10^6) were selected for TK-deficient mutants (TFT^r mutant colonies) using 1 μg/ml of trifluorothymidine (28). Plating efficiency was determined by cloning 600 cells without selection. Colonies were counted after 10 days incubation in 5% CO_2 at 37°C. Survival was calculated according to the methods of Clive and Spector (25) and included a measure of growth both in suspension and during the cloning period.

Chemical Enhancement of SA7 Virus Transformation of SHE Cells. Primary SHE cells were prepared as described above and enhancement assays performed according to the method of Hatch *et al.* (19). Cells were resuspended in modified Dulbecco's medium supplemented with 5% FBS and 0.22 g/100 ml of $NaHCO_3$. Approximately 5×10^6 cells in 5 ml of medium were plated into 60-mm dishes and cultured for 3 days. The medium was changed approximately 8 h prior to treatment and then treated with B(a)P dissolved in acetone (final concentration, 0.5%) for 18 h. The cells were washed and SA7 virus was added to SHE cells ($3–4 \times 10^7$ plaque-forming units/culture) and adsorbed for 3 h. The virus-inoculated cells were removed with trypsin, resuspended in medium with 10% FBS, and plated into ten 60-mm dishes at 2×10^5 cells/dish. After 2 days, transformation assay plates were refed with 0.1 mM $CaCl_2$-containing medium and after 6 days were overlaid with 3 ml of medium containing 0.3 g/100 ml of Bacto-Agar (BBL Microbiology Systems, Cockeysville, MD). At weekly intervals, 3 ml of additional agar-containing medium were added. Cells were fixed in 10% buffered formalin and stained with 0.03% Giemsa 25 to 30 days from the beginning of the experiment. Cytotoxicity was determined by plating 700 cells/60-mm dish into each of 5 dishes. After 8 to 9 days of incubation the colonies were fixed in 10% buffered formalin and stained with 0.02% crystal violet. The cloning efficiencies of virus-inoculated cells under these conditions were usually 8–15%.

Enhancement was expressed as the ratio between the transformation frequency (number of SA7 foci/10^6 surviving cells) of treated cells and the transformation frequency of control cells (19). In parallel studies for determination of B(a)P-DNA adducts, SHE cells from the same batch were treated with [^{3}H]B(a)P as described above except SA7 virus was not added. After 18 h of exposure, the cells were washed, trypsinized, and frozen in medium with 20% FBS and 10% glycerol for subsequent DNA adduct analyses.

C3H10T½ Cell Morphological Transformation. C3H10T½ cells (29) were seeded at a density of 1000 cells/60-mm dish (24 dishes/concentration) in Eagle's basal medium supplemented with L-glutamine and 10% heat-inactivated FBS and treated 24 h later with B(a)P dissolved in acetone (0.5% final concentration). The carcinogen was removed 24 h later by changing the medium, and 25 μg/ml of garamycin (Schering Corp., Kenilworth, NJ) were added. Medium was changed weekly until the end of the experiment. When the cells reached confluence, the FBS concentration was reduced to 5% (30). After 6 weeks, the dishes were washed with 0.9% NaCl, fixed with methanol, stained with Giemsa, and scored for morphological transformation. Morphological transformation was scored as type II and type III foci/10^3 survivors (31). In these studies, 24 dishes/concentration were scored at the completion of each experiment. Cytotoxicity was determined for each experimental group with the same protocol as used in the transformation assay, except that each dish was seeded with 200 cells (6 dishes/experimental point) and the dishes were stained 6 to 10 days after treatment. In parallel studies for B(a)P-DNA adducts, cells from the same batch were seeded at 7.5×10^4 cells/dish and on attaining log phase growth were treated with [^{3}H]B(a)P for 24 h. They were harvested by trypsinization and frozen in medium with 20% FBS and 10% glycerol for DNA adduct analyses.

Determination of the Binding of [^{3}H]B(a)P to the DNA of Cultured Cells. Cells from frozen samples were digested with 0.1% sodium dodecyl sulfate, 10 mM Tris-HCl (pH 7.9), 10 mM EDTA, and 200 μg/ml proteinase K (Boehringer-Mannheim, Indianapolis, IN) for 1 h at 37°C. The lysate (adjusted to 0.4 M NaCl) was extracted three times with phenol:chloroform:8-hydroxyquinoline (1:1:0.001). The DNA was spooled with the addition of 2.5 volumes of −20°C ethanol and washed two times with 70% ethanol in water. The precipitate was then dissolved in a 0.5 ml solution of 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, and 0.4 M NaCl and treated with RNase A (25 μg/ml) for 2 h at 37°C. The sample was extracted with phenol, ethanol was added, and the DNA was spooled, washed, and redissolved in 10 mM Tris-HCl (pH 7.9), 100 mM NaCl, and 1 mM $MgCl_2$. Binding levels were determined by

quantitation of DNA by UV spectroscopy and covalently bound B(a)P by the levels of radioactivity as determined by liquid scintillation counting. The DNA was enzymatically hydrolyzed as reported by Yamasaki *et al.* (32). The hydrolyzed DNA samples were chromatographed on a Sephadex LH-20 column using a stepwise elution of water and 80% methanol in water. The B(a)P-nucleoside products present in the methanol eluant were characterized by HPLC using a Waters C_{18}-μBondapak column (Waters Associates, Milford, MA) developed with a 35–60% nonlinear methanol-water gradient at 50°C at a 1-ml/min flow rate over 100 min. The UV markers included in the chromatography were BPDE I-dGuo, BPDE II-dGuo, and tetraols derived from BPDE I.

RESULTS

Genotoxicity in Chinese Hamster V79 Cells Cocultured with Irradiated SHE Cells. Chinese hamster lung V79 cells treated with B(a)P for 24 h were used to study several biological end points concomitantly: cytotoxicity; gene mutation (TG^r mutant colonies); SCE; and DNA binding (Table 1). Since B(a)P is not sufficiently metabolized by V79 cells, they were cocultivated with irradiated SHE cells for increased metabolic activation. B(a)P was only moderately cytotoxic (Fig. 1*A*). After an initial decrease in survival at 0.125 μg/ml, the lowest concentration tested, there was no further decrease in survival at concentrations up to 15 μg/ml (data above 2 μg/ml not shown).

The HGPRT mutant frequency was monitored by resistance to 6-thioguanine (TG^r). Maximal mutant frequency was obtained at 0.5 μg/ml B(a)P in each of two independent experiments (Fig. 1*B*). Above 0.5 μg/ml, the mutant frequency declined and then reached a plateau which continued to 10 μg/ml (data above 2 μg/ml not shown).

SCE frequency, expressed as the mean SCE/cell (Fig. 1*C*), increased with B(a)P concentration to 0.5 μg/ml and then reached a plateau. No additional SCEs were observed in cells treated with B(a)P between 2 and 10 μg/ml.

B(a)P-DNA-binding levels (Fig. 1*D*) ranged from 1 μmol B(a)P/mol DNA at 0.065 μg/ml to 8.3–10 μmol B(a)P/mol DNA at 1 μg/ml. At concentrations between 1 and 10 μg/ml, the binding levels ranged between 6 and 9 μmoles B(a)P/mol DNA (data above 2 μg/ml not shown).

Several B(a)P-DNA adducts were determined by HPLC analysis (Fig. 2). Peak 4 coeluted with BPDE I-dGuo, which represented 20% of the total binding. Peak 5 coeluted with BPDE II-dGuo. Peak 1 has been observed by Ashurst *et al.* (17) in B(a)P-treated mouse skin and by Sebti and Baird (6) in B(a)P-treated V79 cells and is consistent with a 9-hydroxy-B(a)P metabolite bound to an unidentified deoxyribonucleoside. Although 7S BPDE I-dGuo eluted in the same area as Peak 3, this adduct did not coelute with the marker. Pruess-Schwartz and Baird (33) observed this adduct in early-passage Wistar rat embryo cell cultures and confirmed that it is not a BPDE I product. The other adducts in Fig. 2 have not been identified. This profile is similar to those seen in SHE cultures treated with B(a)P (34) and is consistent with the report of Sebti *et al.* (35) in which SHE cells were used to activate B(a)P.

Table 1 *Genetic toxicology systems and end points examined*

Cell type	Metabolic activation system	End point scored
Chinese hamster lung, V79	Irradiated SHE cells	Cytotoxicity TG^r mutants SCE DNA binding
Mouse lymphoma, L5178Y $TK^{+/-}$ 3.7.2C	Aroclor 1254-induced rat liver S-9	Cytotoxicity TFT^r mutants Chromosome aberration DNA binding
Primary hamster embryo, SHE	Endogenous	Cytotoxicity Chemical-enhanced SA7 virus transformation DNA binding
Mouse embryo, C3H10T½	Endogenous	Cytotoxicity Morphological transformation DNA binding

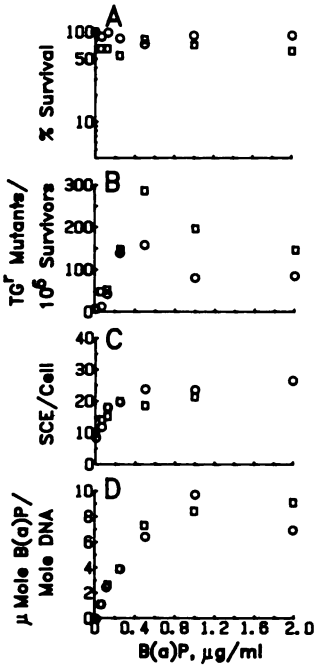

Fig. 1. Responses of V79 cells cocultivated with irradiated SHE cells treated with B(a)P for 24 h. *A*, cytotoxicity; *B*, mutation (TG^r mutant colonies); *C*, SCE; *D*, DNA binding levels. □, ○, results from two independent studies.

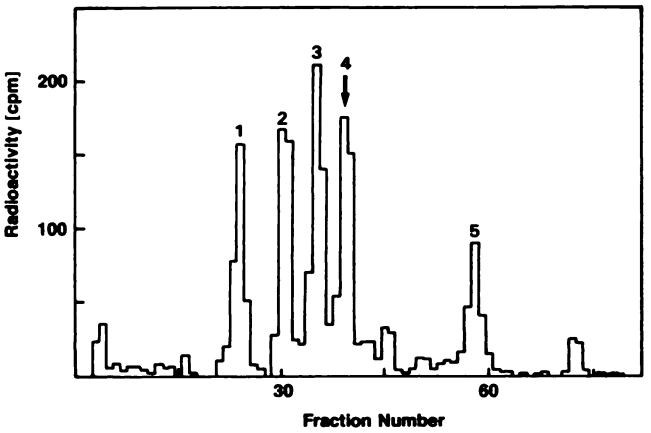

Fig. 2. HPLC profile of B(a)P-deoxynucleoside adducts from V79 cells cocultivated with irradiated SHE cells. Cells were exposed to 1 μg/ml of B(a)P for 24 h. *Peak 1* is tentatively identified as a 9-hydroxy-B(a)P metabolite-deoxyribonucleoside adduct; *Peaks 2* and *3* are unidentified; *Peak 4* contains BPDE I-dGuo; *Peak 5* contains BPDE II-dGuo.

Genotoxicity in L5178Y/$TK^{+/-}$–3.7.2C Mouse Lymphoma Cells. Cytotoxicity, gene mutation (TFT^r mutant colonies), chromosome aberration, and DNA binding were measured concomitantly in L5178Y mouse lymphoma cells (Table 1). Cytotoxicity was evaluated using the method of Clive and Spector (25) (Fig. 3*A*). Survival decreased with B(a)P concentration to approximately 12% at 6.0 μg/ml.

A nonlinear increase in mutant frequency determined by TFT^r was observed at concentrations of B(a)P from 2.5 to 6.0 μg/ml (Fig. 3*B*).

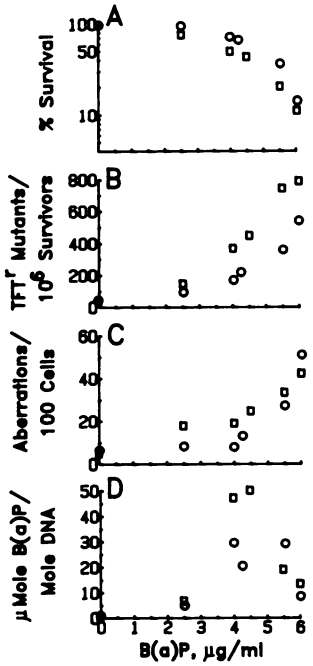

Fig. 3. Responses of L5178Y TK$^{+/-}$ mouse lymphoma cells incubated with B(a)P and an Aroclor 1254-induced rat liver S-9 fraction for 4 h. *A*, cytotoxicity; *B*, mutation (TFT^r mutant colonies); *C*, chromosome aberrations; *D*, DNA binding levels. □, ○, results from two independent studies.

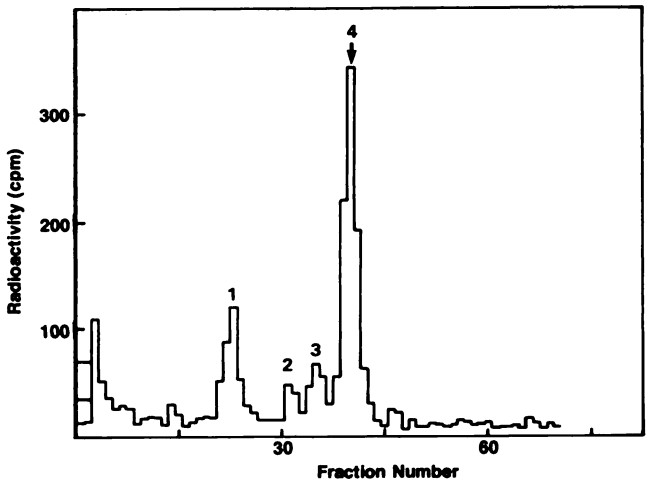

Fig. 4. HPLC profile of B(a)P-deoxynucleoside adducts from L5178Y TK$^{+/-}$ mouse lymphoma cells. Cells were exposed to 4.5 μg/ml of B(a)P and an Aroclor 1254-induced rat liver S-9 for 4 h. See Fig. 2 for peak identification.

Chromosome aberrations, expressed as aberrations per 100 cells scored, also increased nonlinearly with B(a)P concentration (Fig. 3*C*). At 6.0 μg/ml, approximately 40–50 aberrations/100 cells were observed.

Unlike the genetic end points, the B(a)P-DNA binding levels reached a maximum at 4–4.5 μg/ml of 30–50 μmol B(a)P/mol DNA in two experiments. The binding level decreased to 9–13 μmol B(a)P/mol DNA at 6.0 μg/ml.

In addition to measuring the levels of B(a)P-DNA binding, the individual adducts were separated by HPLC and quantitated (Fig. 4). Peak 4 coeluted with the BPDE I-dGuo marker and was the predominant adduct formed (14% of total binding). The other peaks were minor products and correspond to those described in Fig. 2. This profile is consistent with published profiles using induced rat liver S-9 as a metabolic activation system (36).

Enhancement of SA7 Virus Transformation in SHE Cells. In the SHE cell SA7 virus transformation system, the SHE cells were isolated, cultured, and exposed to B(a)P; and SA7 virus was subsequently added. The addition of B(a)P prior to exposure to SA7 virus results in an enhanced level of virus transformation. Three separate experiments were performed.

Primary SHE cells in the presence of the SA7 virus were very sensitive to the cytotoxic effects of B(a)P as compared to the other culture systems studied (Fig. 5*A*). At 1 μg/ml less than 25% of the cells survived.

When the enhancement ratio was examined as a function of B(a)P concentration, a 1.5–3-fold increase in the enhancement ratio occurred at the lowest concentrations of B(a)P tested (Fig. 5*B*). A plateau was observed in the initial portion of the concentration response curve [between 0.1 and 0.6 μg/ml B(a)P]. At doses above 0.6 μg/ml a positive concentration response was observed.

B(a)P-DNA binding in SHE cells showed a linear dose-response relationship up to 1.0 μg/ml (Fig. 5*C*). Specific B(a)P-DNA adducts were isolated by HPLC. The profiles were similar to those shown in Fig. 2 and as reported by Ivanovic *et al.* (34) in SHE cells. BPDE I-dGuo represented 22% of the total binding.

Morphological Transformation of C3H10T½ Cells. The C3H10T½ morphological transformation system developed by Reznikoff *et al.* (29), was also utilized for these studies. Due to the nature of the established protocols, this was the only test system in which different cell densities were used for the end points, transformation, cytotoxicity, and B(a)P-DNA binding levels. Three separate studies were performed.

Colony survival was decreased to 50% by 0.3 μg/ml and did not decrease with increasing concentrations (Fig. 6*A*). Morphological transformation, measured by the appearance of type II and type III foci, increased with concentration reaching a maximum of approximately 12 type II and type III foci/10^3 cells (Fig. 6*B*).

Similarly, the B(a)P-DNA binding levels in C3H10T½ cells also increased with concentration up to 1.2 μg/ml in the two

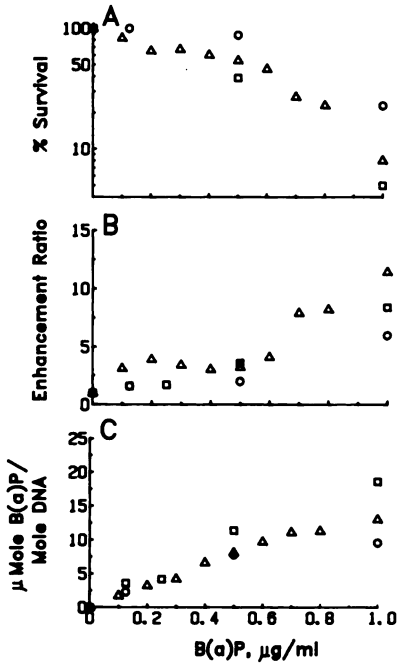

Fig. 5. Responses of SHE cells exposed to B(a)P for 18 h and then to SA7 virus. *A*, cytotoxicity; *B*, enhancement of SA7 virus transformation; *C*, DNA binding levels. □, ○, △, results from three independent studies.

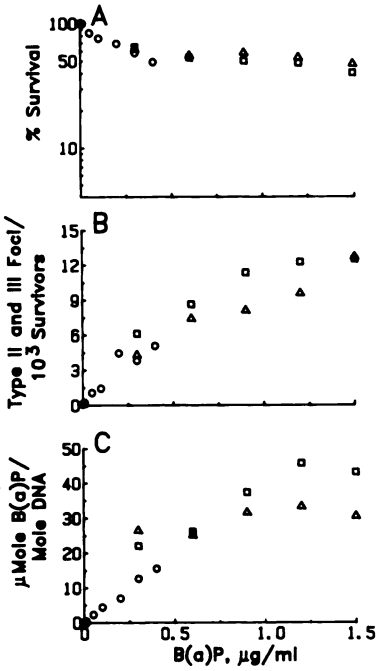

Fig. 6. Responses of C3H10T½ cells exposed to B(a)P for 24 h. *A*, cytotoxicity; *B*, morphological transformation; *C*, DNA binding levels. □, ○, △, results from three independent studies.

experiments (Fig. 6*C*). Maximum binding levels were 33 and 46 μmol B(a)P/mol DNA.

The B(a)P-DNA adducts were separated by HPLC and were similar to those reported for C3H10T½ cells by Brown *et al.* (7) in which the predominant adduct was BPDE I-dGuo (59% of the total binding).

DISCUSSION

In our studies BPDE I-dGuo was found in all four cell types examined and represented 14 to 59% of the total B(a)P bound to DNA. In C3H10T½ cells and mouse lymphoma L5178Y cells, BPDE I-dGuo was the major adduct. In all cell systems, the proportion of DNA adducts attributed to the BPDE I-dGuo adduct did not vary with B(a)P concentration (data not shown) as reported by Eastman *et al.* (9) and Theall *et al.* (10). The BPDE I-dGuo adduct has been found in the DNA of *Salmonella typhimurium* (5), in mammalian cell cultures (1, 6–9), in human cell cultures (3, 4, 10, 11), in explant tissues (12–14), and in rodents exposed *in vivo* (15–18). Several investigators have linked specific mutational effects with the formation of BPDE I-DNA adducts (37–39).

The extent of DNA binding is a function of the concentration of B(a)P, the time of measurement, the generation time of the cells, and the extent of DNA repair/persistence of the adducts. With each cell type, the time of measurement of DNA binding was associated with the time of B(a)P exposure using standard published bioassay protocols. Under those conditions, differing B(a)P-DNA binding levels were generated in the three cell types which use similar B(a)P exposure concentrations and exposure times. SHE cells treated with 0.1–1.0 μg/ml B(a)P for 18 h showed a binding response of 1–17 μmol B(a)P/mol DNA. A slightly lower response was observed in V79 cell cultures, 1–9.8 μmol B(a)P/mol DNA, over the same concentration range and treated for 24 h. The slightly lower binding of B(a)P to V79 cells DNA may be a result of the competition of V79 cell DNA and SHE cell DNA for the active forms of B(a)P (40). In C3H10T½ cells the total adduct formation ranged from 0.4 to 46 μmol B(a)P/mol DNA over an exposure range of 0.001 to 1.5 μg/ml and an exposure time of 24 h. Higher levels of B(a)P bound to DNA could be a result of increased cytochrome P-448 (41) in the cells or a slower repair process. In support of the latter explanation, 60% of the initial BPDE I-dGuo adduct in C3H10T½ cells remained 68 h after a 24 h exposure to B(a)P (7).

Mouse lymphoma cells, utilizing a liver homogenate for activation, displayed lower DNA binding than the other culture systems at low (1–2 μg/ml) concentrations and a 4-h treatment time. Since the cells could be exposed to higher concentrations of B(a)P, maximum binding levels of approximately 50 μmol B(a)P/mol DNA were reached. However, above 5 μg/ml, B(a)P adduct levels decreased. This phenomenon was also noted in human epidermal keratinocyte cultures exposed to toxic levels of B(a)P.[5] The reason for the decrease at higher concentrations is unclear but may be related to the high cytotoxicity observed at these concentrations. It is also possible that the unusual decrease is because of differential metabolism and then subsequent inhibition. Shen *et al.* (42) have shown that B(a)P phenols can inhibit the formation of the B(a)P diol-epoxides. The higher B(a)P binding levels in L5178Y cells can also be a result of the shorter exposure time which gives insufficient time for DNA repair.

The shapes of all the concentration responses curves were curvilinear. In V79 cells, SCE and mutation were measured concomitantly yet the SCE response increased and plateaued, and the mutation (TG^r) response reached a maximum level and began to decrease with higher concentrations. Both the mutation (TFT^r) and chromosomal aberration end points in mouse lymphoma cells displayed a nonlinear concentration response pattern. Since TFT^r can result from either single gene mutation or chromosomal aberration (43), the exponential-like shape of this mutation response may reflect the increase in chromosomal aberrations at the higher concentrations. In the SHE/SA7 cells, there is an initial increase in enhancement which remains constant until a substantially higher concentration is reached and then the response becomes linear with a steep slope. These results are suggestive of the effects expected from two or more subpopulations of cells in a heterogeneous mixture of primary cells or from a multistep transformation process. In the C3H10T½ cells a linear concentration-response relationship exists with a general plateauing at the highest dose. Although it is recognized that cell density affects the transformation frequency (44), the effect of a 50% reduction in survival at the highest B(a)P concentrations could not appreciably alter the transformation frequencies.

The first description of the relationship between DNA binding, DNA adducts, and carcinogenic effects for PAH was published by Brookes and Lawley (45). They described a linear-like relationship between the binding of [^{3}H]PAH to the DNA of mouse skin and Iball's index for 5 PAH. Newbold *et al.* (46) extended this approach to construction of mutation frequency-DNA binding curves for 7-bromomethylbenz(*a*)anthracene, and the *anti*-isomer of BPDE (BPDE I). They demonstrated linear to curvilinear relationships between total DNA binding in V79 cells and mutation frequency (8-azaguanine resistance) in the same cell type. The correlations between cytotoxicity, mutation frequency, and BPDE I-DNA binding have also been examined in normal diploid human fibroblasts and excision repair-deficient xeroderma pigmentosum cells. From these studies it was concluded that BPDE I-dGuo was responsible for the observed

[5] Unpublished results.

biological effects and that a linear relationship existed between BPDE I-DNA binding and induced mutation frequency (6-thioguanine resistance) in the normal cells (47, 48). Similar linear relationships between mutation frequency and BPDE I- or BPDE II-DNA binding have been reported in *S. typhimurium* strains TA98 and TA100 (49). *In vivo* studies of B(a)P binding and tumorigenesis have been performed on mouse skin (17, 50) and in other organs (Refs. 15, 16, 51, 52, and references therein) giving mixed results. B(a)P-DNA binding was observed in tissues both susceptible and nonsusceptible to the tumorigenic effects of B(a)P. The study of the relationships between carcinogen-DNA adducts and mutagenesis and cancer is not limited to B(a)P. van Zeeland *et al.* (53) have reported on the relationships between O^6-ethylguanine formation in V79 cells (6-thioguanine resistance), in *E. coli* (Nalidixic acid resistance), and in mice (specific locus) induced by ethylating agents. Along similar lines, Aaron and Lee (54) have reported a linear relationship between the frequency of sex-linked recessive lethals in *Drosophila melanogaster* and ethylations per sperm cell. With respect to a cancer end point, Swenberg *et al.* (55) have investigated the relationships between O^4-ethyldeoxythymidine levels in hepatic tissues, hepatic initiation, and hepatocellular carcinoma induction in rats exposed to diethylnitrosamine.

In the studies presented here, the shapes of the genetic or morphological transformation response-B(a)P concentration relationships are curvilinear; however, a linear relationship can be obtained between the genotoxic effect and B(a)P-DNA binding (Fig. 7). This linearization has been previously observed in studies with BPDE I in V79 cells, normal diploid human fibroblasts, and *Salmonella typhimurium* (46–49). The normalization of concentration by use of DNA adducts as an internal dosimeter of exposure to the DNA also tends to lessen the interexperimental variation as observed by the correlation coefficients. These effects may be due to the elimination of the variation with respect to metabolic activation as described by Hoel *et al.* (56) using pharmacokinetic models. The comparison of the genotoxic effect-DNA binding relationships for the 6 end points indicates that DNA binding levels range from 0.2 to over 50 μmol B(a)P/mol DNA while the range of genetic effect or morphological transformation frequency is 5×10^{-5}/cell to 15/cell. Thus the genotoxic response varies over 7 orders of magnitude whereas the DNA-binding response spans only 2 orders of magnitude. These differences are not unanticipated because the DNA binding is measured across the whole genome while the target size for the genotoxic end points varies. Of the genotoxic end points, only SCE and chromosome aberration have the whole (or most of the) genome as its target while mutation at single loci evaluate genetic damage at only a small part of the genome.

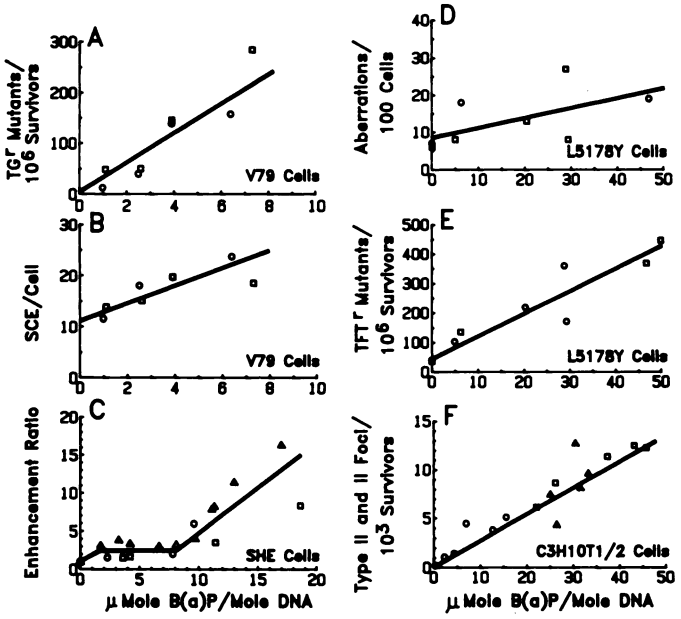

Fig. 7. Genotoxic activity of B(a)P as a function of B(a)P-DNA binding. *A*, mutation and *B*, SCE in V79 cells, cocultivated with irradiated SHE cells, treated with B(a)P for 24 h. Correlation coefficients, 0.89 (*A*), 0.75 (*B*). □, ○, two independent studies; *C*, enhancement of SA7 virus transformation in SHE cells treated with B(a)P for 18 h. □, ○, △, results from three independent studies; *D*, chromosome aberration in L5178Y cells incubated with B(a)P and an Aroclor 1254-induced rat liver S-9 fraction for 4 h. Correlation coefficient, 0.47. □, ○, results from two independent studies; *E*, mutation (TFT^r) in L5178Y cells incubated with B(a)P and an Aroclor 1254-induced rat liver S-9 fraction for 4 h. Correlation coefficient, 0.88. □, ○, results from two independent studies; *F*, morphological transformation in C3H10T½ cells treated with B(a)P for 24 h. Correlation coefficient, 0.91. □, ○, △, results from three independent studies.

These differences in magnitude of responses of genetic or morphological transformation frequency make comparisons between the end points difficult. A system which allows comparison of the divergent genotoxic effects would be helpful if the short-term bioassays are to be applied to quantitative risk assessment analysis. One approach to these comparisons is to apply the doubling dose concept used in studies on radiation and chemically induced genetic effects (57, 58). Since the mechanisms of spontaneous mutational events appear to be different from induced events, the traditional doubling dose analysis based on a doubling of spontaneous frequencies was modified. Instead a doubling of induced frequencies is used. Thus, a doubling adduct level is the number of adducts per unit of DNA required to double the induced frequency of the genetic or transformation effect measured. This modification based on the formula

$$\text{Doubling adduct level} = \frac{\ln 2}{\Delta \ln(\text{effect})/\Delta \text{ adducts}}$$

assumes an exponential relationship between genetic effects and DNA adducts. However, as observed in Fig. 7, these relationships were linear. The initial part of a linear relationship transposed to a semilogarithmic scale cannot be distinguished from an exponential relationship expressed semilogarithmically. Therefore, theoretically, the initial slope of this linear relationship expressed semilogarithmically can be evaluated using this equation.

The doubling adduct levels expressed as μmol BPDE I-dGuo/mol DNA (95% confidence limits) were: TG^r in V79 cells, 0.18 (0.094–2.6); SCE in V79 cells, 0.34 (0.22–1.0); TFT^r in L5178Y TK$^{+/-}$ cells, 2.8 (1.7–6.3); and morphological transformation in C3H10T½ cells, 3.7 (2.5–7.1). These values vary 20-fold indicating again the uniqueness of each bioassay system. The doubling adduct levels for SCE and mutational end points measured in V79 cells are similar. However, a comparison of the two mutation assays, TG^r in V79 cells and TFT^r in L5178Y cells shows greater than a 10-fold difference. The differences observed in some of the doubling adduct levels probably reflect, for example, the extent of modification needed to produce different genotoxic effects, the target size, and the number of copies of the gene(s) in the DNA as well as the equilibria between adduct formation and adduct removal.

We believe that the potential use of this concept is in the area of comparative mutagenesis and carcinogenesis. However, much more work needs to be done to validate this approach and caution must be exercised before its application. The doubling adduct level has the advantage of allowing quantitative comparisons of genotoxic end points to be made without major

concern for differences in metabolism, detoxification, excretion, and absolute frequency of response. These comparisons can be between different species, and genotoxic end points *in vitro* and *in vivo*.

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