Journal of Supramolecular Structure
and Cellular Biochemistry 16:269–279 (1981)
Mechanisms of Chemical Carcinogenesis 41–51

Identifying Human Cells Capable of Metabolizing Various Classes of Carcinogens

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Human cells that appear capable of metabolizing various classes of carcinogens have been identified using one of two methods: metabolism of tritiated benzo(a)pyrene to aqueous-acetone soluble forms or inhibition of cellular DNA synthesis. Each of the assay systems was optimized and the results on 15 human epithelial cell lines were compared. One or more cell lines were found to activate each of four classes of carcinogens examined: polycyclic hydrocarbons, aromatic amines, heterocyclic hydrocarbons, and nitrosamines. Cells that appeared capable of metabolizing polycyclic hydrocarbons or aromatic amines by these methods were also found to produce metabolites which were cytotoxic to cocultivated human xeroderma pigmentosum fibroblasts after a 48-hr exposure to the carcinogen.

Key words: metabolic activation, human cells, polycyclic hydrocarbons, aromatic amines, heterocyclic hydrocarbons, nitrosamines

Since every year new chemicals are introduced into our environment, it is important to have sensitive screening assays available to predict their biological effects. Thus, much effort has been placed on determining the potential carcinogenicity of environmental chemicals by developing short-term test systems which use induction of mutations in bacteria and mammalian cells as their basis for prediction [1]. However, evidence has accumulated which suggests that the majority of chemicals require enzymatic activation to produce metabolites which will react with cellular macromolecules. Since many bacteria and mammalian cells used in the short-term test systems are unable to activate these chemicals, microsomal systems are commonly used to supply activation [2–5].

Abbreviations used: BP, benzo(a)pyrene; XP, xeroderma pigmentosum; NF, normal skin fibroblasts; DMSO, dimethyl sulfoxide; BPDE I, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene; B(b)F, benzo(b)fluoranthene; B(j)F, benzo(j)fluoranthene; B(k)F, benzo(k)fluoranthene; AABP, 4-acetylaminobiphenyl; AAF, 2-acetylaminofluorene; MBOCA, 4,4′ methylene bis (2-chloroaniline); DEN, N-nitrosodiethylamine; DMN, N-nitrosodimethylamine; TCA, trichloroacetic acid; azapyrene, 2-azapyrene; azabenzanthracene, 1-azabenz(a)anthracene; azachrysene, 1-azachrysene.

Received April 22, 1981; accepted June 10, 1981.

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Cell-mediated systems have also been used to provide activation, but to a more limited extent [6–10]. However, recent studies have demonstrated that the activation of polycyclic hydrocarbons by subcellular fractions can produce metabolites which will react with DNA but which are not generally produced by intact cells [11–16]. The validity of short-term tests, however, is based on the assumption that the DNA adducts formed in the cells utilized for the test are representative of those found in mammals treated with the same parent compound. The results with the polycyclic hydrocarbons suggest that short-term assays utilizing subcellular fractions can be misleading as predictive indicators for at least this class of compounds.

Because the differences in the metabolic activation seem to arise because of cellular disruption, intact cells are to be preferred to subcellular fractions. Thus, it is important to be able to easily identify cells that are capable of metabolizing these chemicals. One such method which we used is that of Kouri et al [17] which measures the enzymatic conversion of tritiated BP to aqueous-acetone soluble products. While this assay is useful for the identification of cells capable of the metabolism of BP or related compounds, it is not useful for the identification of cells capable of metabolizing other classes of carcinogens. Therefore, we have examined a second, more general method which detects the formation of agents that damage DNA by measuring DNA synthesis inhibition after exposure to the carcinogen [18]. Because we wanted a source of human metabolizing cells with an infinite life span and with uniformly high levels of carcinogen-activating enzymes, we first examined human epithelial cell lines derived from tumors. Since these two screening assays can provide indications of metabolism, but give unequivocal evidence only of the inability of cells to metabolize, the cells which proved positive by either or both assays were examined further for biological evidence of metabolic activation. This was done by testing various carcinogens for mutagenicity, DNA binding, and/or cytotoxicity in human cells using a cell-mediated assay system with the candidate activating cells as the metabolizing cells [10]. We selected XP12BE cells as the target cells for this system because we and others have shown that they are abnormally sensitive to various compounds and UV light, due to the inability to excise DNA damage [19–22]. Therefore, these cells can be expected to accumulate DNA damage during the prolonged carcinogen exposure periods. This paper describes two screening methods for the identification of human cells that may be able to activate polycyclic hydrocarbons, aromatic amines, nitrosamines, and/or heterocyclic hydrocarbons and the use of these cell lines as metabolizing cells in the human cell-mediated cytotoxicity assay developed in this laboratory with XP12BE cells as target cells [10].

MATERIALS AND METHODS

The following chemicals were stored at –20°: BP (Sigma Chemical Co., St. Louis, MO), B(b)F, B(j)F, B(k)F, azapyrene, azachrysene, azabenzanthracene, BPDE I (Chemical Carcinogenesis Program, National Cancer Institute), MBOCA (RFR Corp., Hope, RI), DEN (Eastman Organic Chemicals, Rochester, NY), DMN (Aldrich Chemical Co., Milwaukee, WI), AAF, and AABP (generous gifts from Drs. J. A. and E. C. Miller). BP (56 Ci/mmol; Amersham

Corp., Arlington Heights, IL) and dThd (64 Ci/mmol; New England Nuclear, Boston, MA) generally labeled with tritium were stored at 4°. Glass-distilled DMSO, hexane, and acetone were purchased from Burdick and Jackson Laboratories, Muskegon, MI.

Cell Culture

Cells. The epithelial cell lines used in these studies were obtained from the Naval Biosciences Laboratory Naval Supply Center, Oakland, California (except for the PC-3 which was a gift from Dr. M. Edward Kaighn). The XP fibroblasts were derived from skin biopsies of an XP patient (XP12BE, complementation group A, obtained from the American Type Culture Collection, Rockville, MD). Normal human fibroblasts were derived from fresh foreskin tissue. All cells were grown at 37° in air atmosphere of 5% CO_2 and 95% humidity.

Culture medium. Cells were routinely cultured in minimum essential medium (Eagle's) with 50 μg gentamycin (Schering Corp., Kenilworth, NJ) per milliliter supplemented with either 15% fetal bovine serum (Grand Island Biological Co., Grand Island, NY or Sterile Systems, Inc., Logan, UT) for XP cells or 10% fetal bovine serum for NF cells and epithelial cell lines. All cells were stored in liquid N_2.

Freezing medium. Cells were suspended in minimum essential medium (Eagle's) supplemented with 17% fetal bovine serum, 50 μg gentamycin/ml, and 10% glass-distilled DMSO.

Hydrocarbon-metabolizing activity assay. The hydrocarbon-metabolizing activity of the cell lines was determined by measuring their ability to convert tritium labeled BP to aqueous-acetone soluble form(s) using the method of Kouri et al [17]. A 100-μl aliquot of the aqueous-acetone phase was added to 10 ml of scintillation fluid and counted in a liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). The counts per minute were converted to nanomoles of BP metabolites, using an external quench curve, an external ^{137}Cs standard, and the radiospecific activity of the tritium labeled BP. Control level activity resulting from incubation of BP for 24 hr in complete medium without cells was routinely subtracted from the levels with cells. For calculation of the enzymatic specific activity, total cellular protein was determined by the Lowry procedure, as modified by Oyama and Eagle [23]. Results were expressed as nanomoles of BP metabolized to an aqueous-acetone soluble form per mg total cellular protein.

DNA Synthesis Inhibition Assay

The inhibition of DNA synthesis in the cell lines to be examined was determined using a modification of the method described by Painter [18]. Cells to be tested were plated at 25–100 $\times$ 10^3 per 17-mm diameter multiwell dish (Falcon, Oxnard, CA) and allowed to attach for ~12 hr. Proximate carcinogens were dissolved in DMSO dried over molecular sieve type 3A (Matheson Coleman & Bell Manufacturing Chemists, Norwood, OH) and the carcinogen–DMSO solution was added to the complete medium to obtain the indicated final concentrations. The final DMSO concentration applied to each culture (including controls) was kept constant. After a 48-hr treatment period, the carcinogen

containing medium was replaced with complete medium containing tritium labeled thymidine at a final concentration of 5 μCi/ml. After 2 hr of incubation, the thymidine-containing medium was removed. The cells were rinsed with phosphate-buffered saline, removed in 0.05% trypsin-0.02% EDTA and collected on GF/C filters (Whatman LTD, England). The filters were rinsed two times with phosphate-buffered saline and two times with 10% TCA. Then the filters were placed in scintillation vials and the DNA hydrolyzed by the addition of 1 ml 0.5 N HCl and heated for 1 hr at 90°C before addition of scintillation fluid and counting in a Beckman Model A LS9000 liquid scintillation spectrometer. In these samples tritium was counted with ~23% efficiency.

When the ultimate reactive form of a carcinogen was used in the DNA synthesis inhibition assay, all procedures were identical except that an acetone solution of the carcinogen was added to the serum-free medium covering the monolayer of cells and the treatment time was 2 hr.

Cell-Mediated Cytotoxicity Assays

Target cells were plated in 35-mm-diameter dishes (Falcon Plastics, Oxnard, CA) and allowed to reach confluence. Appropriate numbers of lethally irradiated metabolizing cells were added and allowed to attach as previously described [10]. Carcinogens were dissolved as for the DNA synthesis inhibition assays. After a 24- or 48-hr carcinogen exposure, the cultures were trypsinized (0.25% trypsin) and assayed by cloning for the induction of cytotoxicity in the target XP cells as previously described [10].

RESULTS

Hydrocarbon-Metabolizing Activity Assay

Before screening cells for their ability to metabolize BP to aqueous-acetone soluble products, we examined the hydrocarbon metabolizing assay with respect to time of exposure to BP and number of cells treated (see Fig. 1). Since this assay was linear for at least 24 hr with cell numbers up to 8×10^5, we used a 24-hr treatment period and cell numbers no greater than 5×10^5.

Table I shows the results of screening 15 tumor-derived cell lines and four normal cell strains for hydrocarbon metabolizing activity. Hs835T cells served as a positive control and were run with every assay. Those cell lines above A549 in Table I showed consistently higher levels of metabolism and were considered good prospects for examination of cell-mediated cytotoxicity. Note the low activity of the normal cell strains. When confluent cultures of NF were exposed to BP at concentrations up to 30 μM for 48 hr and replated at low density for macroscopic colony formation, they showed little or no cytotoxic response (unpublished data). However, when confluent NF were cocultivated with increasing numbers of lethally irradiated Hs835T cells and treated for 48 hr with 4 μM BP, their survival (as reflected in colony-forming ability) was reduced to $< 10\%$ of control cells incubated with 4 μM BP in the absence of metabolizing cells (Fig. 2). Since the lethally irradiated metabolizing cells in the absence of BP do not produce or release compounds which are cytotoxic to the target cells (unpublished data), this suggests that the metabolizing cells are producing cytotoxic metabolites from BP.

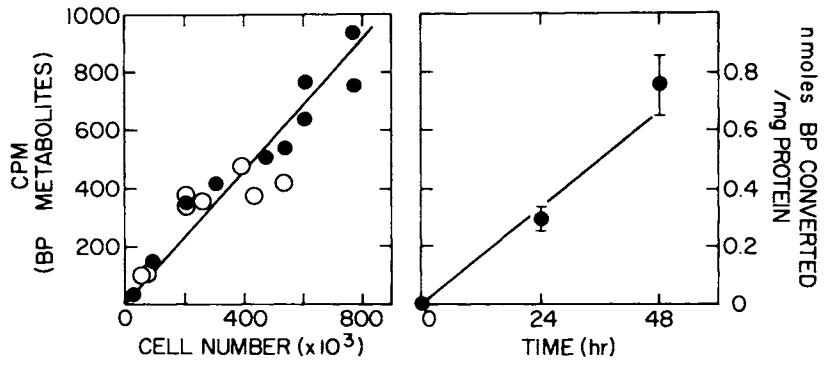

Fig. 1. (Left panel) Hydrocarbon metabolizing activity as a function of cell number. In two experiments (•, ○) PC-3 cells were plated at varying numbers in 60-mm petri dishes and allowed to attach. All dishes were treated for 24 hr with 4 μM BP supplemented with ^{3}H-BP (specific activity, 0.5 mCi/μmol). At the end of the treatment period, the media was extracted with hexane, as described under Methods and Materials, and the radioactivity in the aqueous-acetone phase was determined and plotted as a function of the number of cells in the dish at the end of treatment. The radioactivity in the aqueous-acetone phase from medium incubated 24 hr without cells (~200 cpm) has been subtracted. (Right panel) Hydrocarbon metabolizing activity as a function of time of exposure to BP. PC-3 cells were plated at 500 × 10^3 per 60-mm dish allowed to attach, and treated as described for the left panel for 24 or 48 hr. At the end of the treatment period the hydrocarbon metabolizing specific activity was determined as described under Materials and Methods and plotted as a function of time (•).

TABLE I. Hydrocarbon-Metabolizing Activity of Human Cells

Cell line designation	BP converted/ mg protein 24 hr (nmol)	Tissue of origin and diagnosis
Hs700T	27.8 ± 6.0[a]	Metastatic adenocarcinoma
Det 562	24.2 ± 13.5	Pharyngeal carcinoma
Hs703T	18.3 ± 3.2	Liver carcinoma
Hs835T	12.3 ± 3.6	From tumor produced by innoculation of Hs761T cells (kidney carcinoma) into nude mouse
A498	10.7 ± 0.4	Kidney carcinoma
A253	8.7 ± 4.4	Epidermoid carcinoma of the neck
A549	3.6 ± 0.4	Lung carcinoma
Hs696T	3.2 ± 0.4	Metastatic adenocarcinoma
Hs766	3.2 ± 0.4	Metastatic carcinoma of pancreas
Hs683T	2.4 ± 1.6	Glioma
HT1080	1.6 ± 0.8	Fibrosarcoma
JAR	0.8[b]	Choriocarcinoma
NF814.4[c]	0.8[b]	Normal skin fibroblasts
Hs695T	0.8[b]	Melanoma
A172	0.8 ± 0.4	Glioblastoma
FHS74INT	0.8 ± 0.4	Normal intestine
PC-3	0.8 ± 0.4	Bone marrow sample from patient with prostatic cancer
NF801.5[c]	0.4[b]	Normal skin fibroblasts
NF812.4[c]	0.4[b]	Normal skin fibroblasts

[a]Mean ± SD.
[b]Only one determination.
[c]Number to right of decimal indicates passage level.

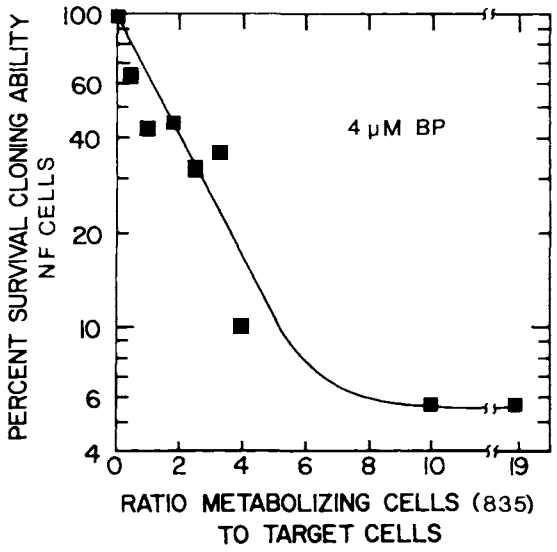

Fig. 2. Cytotoxic effect of 4 μM BP as a function of the ratio of metabolizing cells (Hs835T) to target cells (NF). NF were grown to confluence in 35-mm dishes. The number of cells was determined, and lethally irradiated Hs835T cells were seeded on top of the target cells at the indicated ratios and also into corresponding sets of dishes without target cells. After a 48-hr exposure to 2 ml of medium containing 4 μM BP, the cultures were replated at cloning densities. The number of metabolizing cells attached in each series was determined from the number of cells in the dishes without target cells. Cytotoxicity was determined from the cloning efficiency of target NF cocultivated with metabolizing cells divided by that number of NF cultured in the absence of metabolizing cells. The value is expressed as a percentage (■).

DNA Synthesis Inhibition Assay

To identify cells capable of activating chemical carcinogens which cannot be examined directly for metabolism because they are not available in radioactive labeled form or because the metabolites cannot be separated from the parent molecule by these procedures, we examined the ability of cells to activate them during a 48-hr exposure into forms capable of inhibiting DNA synthesis. As a preliminary step we determined the parameters of tritiated thymidine incorporation in exponentially growing untreated cells. The results (Fig. 3) indicated that at a density of $\lesssim 60 \times 10^4$ cells/dish incorporation was linear for at least 2 hr. Therefore, 2-hr incubation and 2.5 to 10×10^4 cells/dish were used in all subsequent assays to insure comparable results among different cell lines.

To determine whether this assay was sensitive enough to show a response at low concentrations of carcinogen, but concentrations at which cells show a cytotoxic or mutagenic response, we examined the DNA synthesis inhibition in NF after a 2 hr treatment with BPDE I, the ultimate reactive form of BP. The results (Fig. 4) show that at a concentration that would result in 37% survival, BPDE I decreased DNA synthesis to 40% that of the control. We showed previously that the level of DNA binding for this level of cytotoxicity is ~8 BPDE I residues per 10^6 nucleotides [20]. The Hs703T cells which were tested at the same time showed a similar response. As a positive control, we exam-

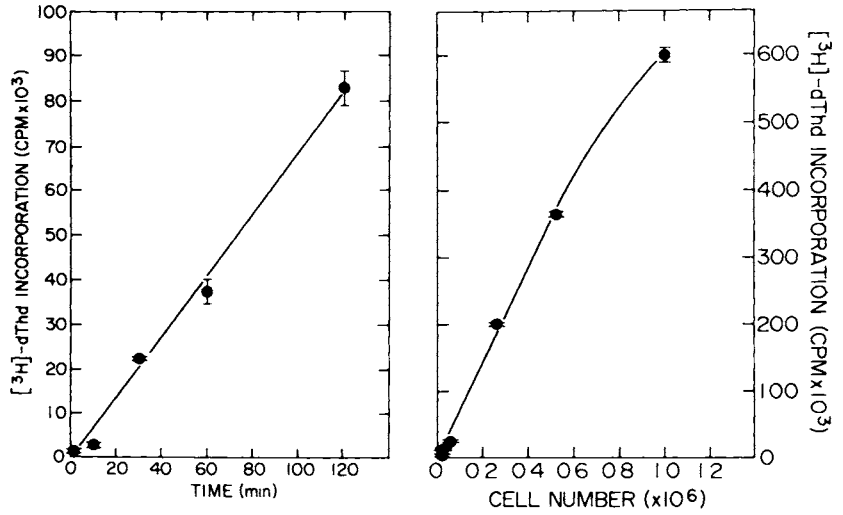

Fig. 3. (Left panel) Tritium-labeled thymidine incorporation into cellular DNA as a function of time. Exponentially growing untreated Hs703T cells (0.1×10^6) were exposed to tritiated thymidine at a concentration of 5 μCi/ml for the indicated times. The level of incorporation into cellular DNA was determined as described under Materials and Methods. (Right panel) Incorporation as a function of cell number. Untreated cells were plated at the indicated numbers, and DNA synthesis was measured as described.

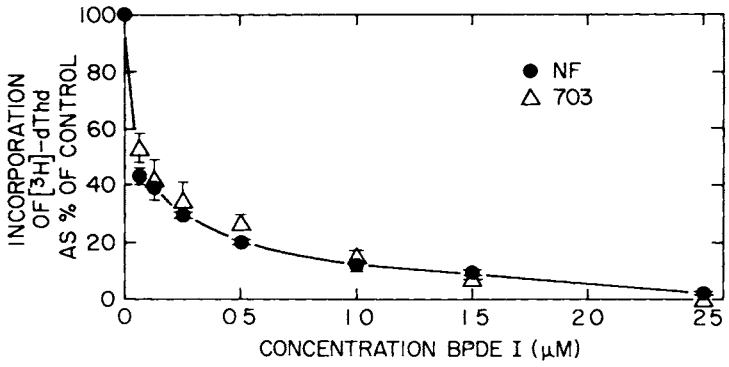

Fig. 4. Effect of the concentration of BPDE I on DNA synthesis in NF and Hs703T cells. NF or Hs703T cells were treated for 2 hr with various concentrations of BPDE I. At the end of the treatment period the inhibition of DNA synthesis was determined as described under Materials and Methods. The results for NF (•) and Hs703T cells (△) were plotted as a function of the treatment concentration of BPDE I.

ined two cell lines that we had already shown were capable of metabolizing BP (Hs835T and Hs703T) and one cell strain that cannot (XP12BE, unpublished data), for the effect of BP on cellular DNA synthesis. The results (Fig. 5) showed that inhibition of synthesis was correlated with the capability of the cells to metabolize BP to a biologically reactive form [10]. We concluded that the DNA synthesis inhibition assay could not only reflect metabolism to reactive forms, but also was sensitive at biological levels.

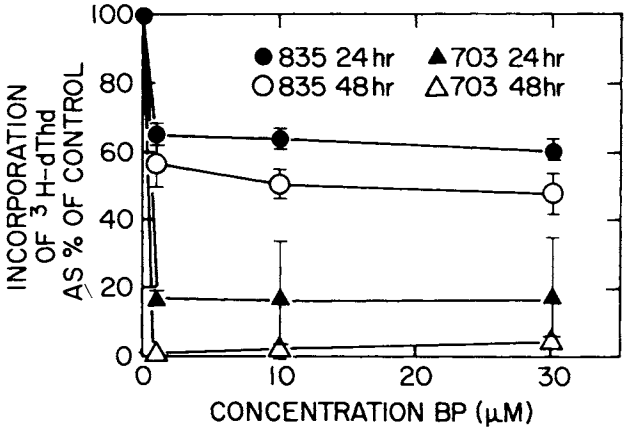

Fig. 5. The effect of the concentration of BP and time of exposure on cellular DNA synthesis in Hs835T cells and Hs703T cells. Hs835T cells and Hs703T cells were treated for 24 or 48 hr with increasing concentrations of BP. At the end of the treatment period inhibition of DNA synthesis was determined as described under Materials and Methods. The results for Hs835T cells (•, 24 hr; ○, 48 hr) and Hs703T cells (▲, 24 hr; △, 48 hr) were graphed as a function of BP concentration.

We examined the capability of human cells to metabolize other compounds using this assay. Four classes of compounds were studied: aromatic amines, nitrosamines, heterocyclic hydrocarbons, and other polycyclic hydrocarbons. For the polycyclic hydrocarbon study, we used Hs703T cells, a line that we showed had a high capacity for metabolizing polycyclic hydrocarbons, and three structurally related compounds, B(b)F, B(j)F, and B(k)F. The results of the DNA synthesis inhibition assay (Fig. 6A) suggested that Hs703T cells metabolized the compounds to biologically reactive forms. To confirm this, we examined them further using the cell-mediated cytotoxicity assay with XP as target cells (Fig. 6B) and found a concentration-dependent decrease in the survival of the target XP cells with B(b)F up to 0.75 μM. These results confirmed that cytotoxic metabolites were being produced by the metabolizing cells.

To identify cells capable of metabolizing aromatic amines, we examined the inhibition of DNA synthesis by AABP, AAF, and MBOCA in candidate metabolizing cells. The results (Fig. 7A) suggested that Hs700 cells are capable of activating this class of compounds. This conclusion was confirmed by the results of the cell-mediated cytotoxicity assay (Fig. 7B) which showed a decrease survival of the XP target cells with increasing concentrations of MBOCA up to 5 μM.

The cell line Hs703T was also tested for its ability to activate the heterocyclic compounds azapyrene, azachrysene, and azabenzanthracene. The results of DNA synthesis inhibition experiments (Fig. 8A) suggested that it can activate these compounds. Cell lines were also tested for their ability to activate the nitrosamines, DEN and DMN. Results with two of the cell lines tested suggested that both compounds could be activated. DEN produced greater DNA synthesis inhibition than DMN at similar concentrations (Fig. 8B).

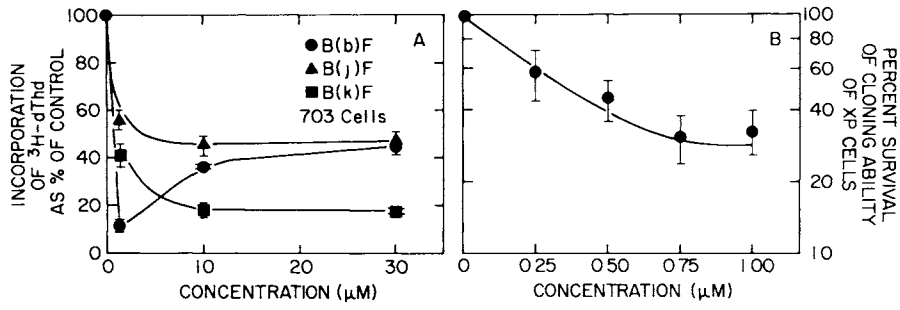

Fig. 6. (A) Comparison of the effect of concentration of B(b)F, B(j)F, and B(k)F on cellular DNA synthesis in Hs703T cells. The inhibition of DNA synthesis was determined after 48 hr treatment with various concentration of B(b)F (•), B(j)F (▲), or B(k)F (■). (B) The effect of the concentration of B(b)F on the survival of the cloning ability of target XP cells cocultivated with Hs703T cells. Lethally irradiated Hs703T cells were added to confluent cultures of XP cells. After a 48-hr exposure to the indicated initial concentrations of B(b)F, the cytotoxicity was determined as described in Figure 2.

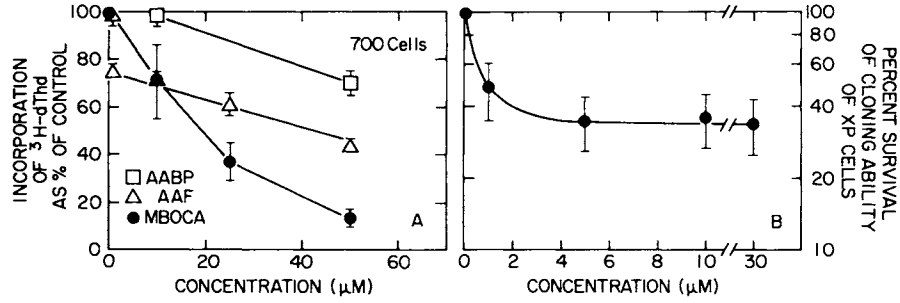

Fig. 7. (A) Comparison of the effect of the concentration of three aromatic amines: AABP (□), AAF (△), and MBOCA (•), on cellular DNA synthesis in Hs700T cells. Inhibition of DNA synthesis in Hs700T cells was determined as described after 48-hr treatment with various concentrations of AABP, AAF, and MBOCA. (B) The effect of the concentration of MBOCA on the survival of the cloning ability of target XP cells cocultivated with Hs700T cells. Lethally irradiated Hs700T cells were added to confluent cultures of XP cells. After a 48-hr exposure to the indicated initial concentrations of MBOCA, the cytotoxicity was determined as for Figure 2.

DISCUSSION

In this report, established human epithelial cell lines and fibroblast strains were monitored by two procedures for their ability to metabolize various carcinogens. Each of the techniques served as an excellent prescreening method for identifying cells capable of carcinogen activation. In the first procedure, which identified polycyclic hydrocarbon metabolizing cells, the conversion of tritiated BP to aqueous-acetone soluble products was determined using the method of Kouri et al [17]. Although this assay does not specifically determine the enzymatic activity responsible for production of BPDE I, the metabolite most likely to be the carcinogenic form of BP, the assay was useful because it reflected, in a qualitative way, the ability of the cells to produce cytotoxic

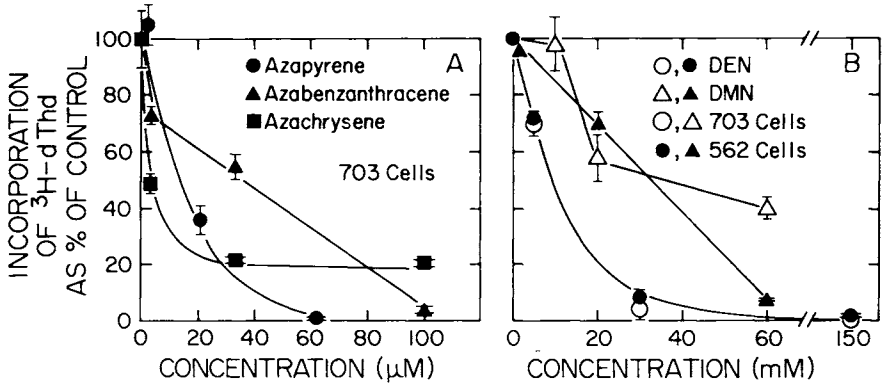

Fig. 8. Comparison of the effect of the concentration of azapyrene (●), azachrysene (■), and azabenzanthracene (▲) on cellular DNA synthesis in Hs703T cells (A) or DEN and DMN in Det 562 and Hs703T cells (B) after a 48-hr treatment.

metabolites. Cells with very low metabolizing activity, such as NF, showed little or no cytotoxic response when exposed to BP at concentrations up to 30 μM for 48 hr. However, when the NF, cocultivated with lethally irradiated Hs835T cells, which had 20 times the hydrocarbon-metabolizing capability, were exposed to 4 μM BP for 48 hr, their survival was reduced to $< 10\%$ that of the control. A similar observation was made with XP cells which showed no cytotoxic response to BP unless cocultivated with a metabolizing cell line, such as Hs835T, Hs703T, or A549 [10]. In fact, there was qualitative correlation between the cytotoxic response of XP cells and the level of enzymatic activity of the metabolizing cells in that Hs703T > Hs835T > A549. In other studies with the Hs835T cocultivated with the XP target cells we showed that there was a BP concentration-dependent increase in the frequency of 6-thioguanine-resistant colonies and in the level of metabolite binding to cellular DNA [10]. The predominant DNA adduct resulted from the reaction between BPDE I and N^2-deoxyguanosine [10]. This is consistent with the adducts observed in human tissue explants treated with BP [24–27]. Thus, the biological results (cytotoxicity, mutagenicity, and DNA binding) we observed using the cell-mediated system suggest that the hydrocarbon metabolizing activity assay is a very useful prescreen for identifying human cells that metabolize BP to biologically active form(s).

The second method, the inhibition of cellular DNA synthesis after treatment with carcinogens, was selected as a prescreen because we wanted an assay that reflected interaction of carcinogens with DNA. As discussed by Painter [28], DNA synthesis inhibition does not always have to reflect an irreversible interaction with DNA. However, we found that all the cells that showed carcinogen-dependent inhibition of DNA synthesis, which were subsequently tested for production of mutagenic and/or cytotoxic metabolites using the human cell-mediated assay, proved to be able to produce such metabolites. These results suggest that this assay is also a very useful prescreen for identifying cells with the potential for metabolizing carcinogens. In addition, the assay appears to be very sensitive. For example, as few as eight residues of BPDE I per 10^6 DNA nucleotides reduced DNA synthesis to 40% of normal. Although as discussed by Painter [28], the apparent sensitivity of the DNA

synthesis assay may vary depending upon the cells being tested, the relative cytotoxic response obtained with BP in target XP cells cocultivated with the metabolizing cells Hs835T or Hs703T [10] correlated with their relative BP metabolizing capability as predicted from the DNA synthesis inhibition assay.

In conclusion, we have successfully identified human cell lines that are capable of metabolizing BP to cytotoxic and mutagenic products using two screening methods. Work is in progress to determine whether other cell lines that appear capable of metabolizing aromatic amines, heterocyclic hydrocarbons, and/or nitrosamines are producing mutagenic products.

ACKNOWLEDGMENTS

This research was supported in part by Grant R805563 from the Environmental Protection Agency, ER-78-S-02-4659 from D.O.E., and ES 07076 from the DHHS Post-Doctoral Training Grant Awarded by the National Institute of Environmental Health Sciences. We thank E.M. Mahoney-Leo and K.J. Falahee for excellent technical assistance and our colleague Dr. N.R. Drinkwater for valuable discussions.

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