A Metabolically Competent Human Cell Line Expressing Five cDNAs Encoding Procarcinogen-Activating Enzymes: Application to Mutagenicity Testing

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A human B-lymphoblastoid cell line, designated MCL-5, constitutively expressing human cytochrome P-450 CYP1A1 and also expressing five transfected human cDNAs encoding drug-metabolizing enzymes, has been developed. cDNAs encoding CYP1A2, CYP2A6, and microsomal epoxide hydrolase (mEH) were introduced by using a vector conferring hygromycin B resistance, and cDNAs encoding CYP2E1 and CYP3A4 were introduced by using a vector conferring resistance to 1-histidinol. MCL-5 cells stably expressed all five cDNAs and the native CYP1A1 as determined by measurement of form-specific enzyme activity levels. The mutagenicity of seven model procarcinogens to MCL-5 cells was examined at the hypoxanthine guanine phosphoribosyltransferase (hprt) and thymidine kinase (tk) loci. Exposure to benzo[*a*]pyrene (BP), 3-methylcholanthrene (3MC), *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), aflatoxin B_1, (AFB_1), 2-(acetylamino)fluorene (AAF), or benzidine (BZD) induced a statistically significant increase in mutant frequency. Linear interpolation of the concentration of procarcinogen necessary to produce a doubling of the mutant fraction at the hprt locus in MCL-5 cells and the parent AHH-1 cell line revealed that, for each of the chemicals examined, except BZD, MCL-5 cells were significantly more sensitive than the parent AHH-1 cells. The increase in sensitivity to mutagenicity ranged from 3-fold for AAF to greater than 40 000-fold for NDMA. MCL-5 cells have great potential as a screening system for the analysis of human procarcinogen/promutagen activation.

Introduction

The cytochromes P-450 are a superfamily of hemoproteins, many of which are capable of metabolizing xenobiotics such as drugs, procarcinogens, and enviromental pollutants (reviewed in ref 1). These enzymes are found at high concentrations in liver, and some P-450 forms are expressed in extrahepatic tissues. For unknown reasons, mammalian cell lines, even those of liver origin, have lost most or all P-450 expression. In general, the only P-450 expressed to any degree is the inducible CYP1A1. This limitation in P-450 expression has restricted studies of P-450-mediated metabolism to either primary cells or tissue fractions. For example, rodent liver subcellular fractions are commonly used as extracellular activating systems in mammalian cell and bacterial assays designed to detect genotoxic effects of promutagens and procarcinogens (reviewed in ref 2). The composition of these subcellular fractions in terms of specific P-450 forms has not been well characterized.

Recently, several laboratories have successfully introduced P-450 cDNAs into mammalian cell lines (*3–11*). These cDNA-expressed cytochrome P-450 enzymes are useful for intracellular activation of promutagens and procarcinogens. When the cells to be transfected are properly chosen, the resulting cell lines expressing human P-450s can also serve as target cells for measuring the induction of gene locus mutations. We have focused on introducing human P-450s into human cells on the assumption that human cells expressing human P-450s will be a better predictive model of human susceptibility to the toxic and mutagenic effects of carcinogen exposure than nonhuman systems (*5–8*).

While we have successfully cotransfected CYP2A6 and mEH cDNAs on a single vector into a single cell line (*6*), virtually all other cell lines were developed by transfecting a single P-450 cDNA into the target cell line. Cell lines expressing individual P-450s have proven quite useful for examining P-450 form specific activation of procarcinogens. However, no single cell line can be used to accurately study different classes of procarcinogens because different P-450s have unique substrate specificities. Therefore, to screen potential procarcinogens/promutagens, a number of cell lines, each expressing a single P-450 form, would be needed. Such a screening approach would be costly and time consuming. As an alternative approach, it would be more desirable to use a small number of cell lines expressing multiple P-450 forms. However, to develop such cell lines, it is useful to know the human P-450 forms involved in human procarcinogen metabolism.

Specific inhibitory antibodies and correlation of procarcinogen activation with levels of P-450 form specific enzyme assays were used to determine the human liver P-450 form(s) primarily responsible for procarcinogen activation. These studies have revealed that three P-450s, CYP1A2, CYP2E1, and CYP3A (CYP3A4 being generally the most abundant), are primarily responsible for the activation of a variety of polycyclic aromatic hydrocarbons, nitrosamines, aromatic amines, mycotoxins, and other procarcinogens (*12–16*). Similar results were also obtained with vaccinia virus expressed human P-450 forms (*17–19*).

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An important role for CYP2A6 in the activation of some nitrosamines has also been established by using the human lymphoblast cDNA expression system (*6*, *8*). It is noteworthy that CYP2A6 has not been purified from human tissue sources and cDNA expression has been the only means to study its catalytic activities. CYP1A1 and mEH[1] are known to be important in the activation of polycyclic aromatic hydrocarbons. However, CYP1A1 is very poorly expressed in human liver. These studies have been reviewed (*20*).

The current state of knowledge for human P-450 mediated procarcinogen activation suggests that a cell line expressing CYP1A1, CYP1A2, CYP2A6, CYP2E1, and CYP3A4 as well as mEH has the potential to be a useful tool for screening many of the procarcinogens to which humans are exposed. In the present report we describe the development of such a cell line via the transfection of multiple human cDNAs.

Experimental Procedures

Caution! *The following chemicals are hazardous and should be handled carefully*: *AFB_1, BP, 3MC, NDMA, NDEA, AAF, and BZD.* Handling was performed in accordance with National Toxicology Program Health and Safety Requirements.

Cells, Tissue Culture, and DNA Introduction. AHH-1 TK+/− is a human B-lymphoblastoid cell line. L3 is a variant of AHH-1 TK+/− with higher native CYP1A1 activity and a lower background mutant fraction at the tk locus (*6*). Cells were maintained in RPMI medium 1640 supplemented to 9% (v/v) horse serum. Protoplast fusions were performed according to Yoakum (*21*). Cells bearing recombinant plasmids were maintained in either medium containing 100–200 μg/mL hygromycin B (pMF6-based vectors), 2 mM 1-histidinol, without 1-histidine (pEBVHistk-based vectors), or both.

Enzymes and Reagents. Restriction endonucleases, Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from New England Biolabs, Beverly, MA. Hygromycin B was purchased from Calbiochem, San Diego, CA. Unlabeled and ^{3}H-labeled benzo[*a*]pyrene 4,5-oxide were obtained from the National Cancer Institute Chemical Carcinogen Repository. 6β-Hydroxytestosterone standard was obtained from Steraloids, Inc., Wilton, NH. 6-Hydroxychlorzoxazone standard was a generous gift of Dr. G. P. Guengerich. These two standards were found to be greater than 95% pure by reverse-phase HPLC with absorbance detection. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were not analyzed for purity by the investigators.

Cytotoxicity and Mutagenicity Assays. Cytotoxicity was estimated by measuring growth after procarcinogen treatment. After cultures have resumed exponential growth, the cumulative growth of the mutagen-treated cultures was divided by the cumulative growth of the negative control cultures to yield relative survival. Induction of mutation at the hprt and tk loci was measured by previously published protocols (*22*) with the minor modification that each replicate culture contained 3×10^7 cells. Human lymphoblasts were exposed to the mutagen for 28 h. After a 7-day phenotypic expression period, the mutant fraction was measured by plating 5×10^6 or 7.5×10^6 cells in two or three 96-well microtiter plates in the presence of 6-thioquanine (0.6 μg/mL) or trifluorothymidine (4 μg/mL) and 500 cells in two 96-well microtiter plates without selection. AFB_1, BP, 3MC, AAF, and BZD were dissolved in DMSO; NDMA and NDEA were dissolved in water for delivery to the cell cultures.

DNA Manipulations. Bacterial transformations, plasmid DNA isolations, and construction of the expression vectors were performed according to standard protocols (*23*).

Enzyme Assays. Enzyme activities were measured in whole cells. Coumarin 7-hydroxylase activity was measured according to the method of Greenlee and Poland (*24*) by adding 100 μM coumarin directly to the cell cultures and measuring fluorescence production after a 2-h incubation. Microsomal epoxide hydrolase was measured according to the procedure of Glatt et al. (*25*) using 5×10^6 cells in 0.5 mL and incubation for 30 min. 7-Ethoxyresorufin deethylase activity (*26*) was measured in whole cells as described (*27*). Endogenous CYP1A1 was induced by pretreatment with 100 nM dibenz[*a*,*h*]anthracene for 24 h. All other enzyme assays utilized 1×10^7 cells in a 1-mL incubation and incubation for 60 min. Microsomal incubations utilized 0.1 mg (coumarin hydroxylase) or 0.4 mg (all others) of microsomal protein in 0.4 mL total volume. Acetanilide 4-hydroxylase (*28*), chlorzoxazone 6-hydroxylase (*29*), and testosterone hydroxylase (*30*) activities were measured as described.

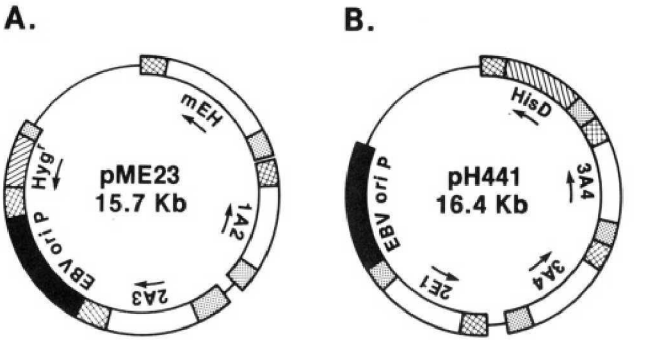

Figure 1. Schematic map of the pME23 (panel A) and pH441 (panel B) vectors. The thin line represents pBR322 sequences, the black box represents the Ori P sequences derived from EBV, the hatched boxes represent the hygromycin B resistance gene (panel A) or the *Escherichia coli* His D gene (panel B), the stippled boxes represent the herpes simplex virus thymidine kinase gene promoters, the cross-hatched boxes represent the herpes simplex virus thymidine kinase gene polyadenylation signals, and the open boxes represent the cDNAs. Identities of the cDNAs and direction of transcription are indicated on the figures.

Immunoblot Analysis. Samples of microsomal protein (MCL-5 cells) and whole cell lysate (vaccinia virus infected HepG2 cells) were subjected to SDS–PAGE according to Laemmli (*31*) and transferred to nitrocellulose membranes by using an electrotransfer blotter (Integrated Separation Systems, Hyde Park, MA). The filters were incubated with polyclonal antibodies against CYP1A2 (*32*), CYP2A2 (*33*), CYP3A1 (*34*), and CYP2E1 (*35*). The blots were developed using goat anti-rabbit IgG conjugated with alkaline phosphatase (KPL, Gaithersburg, MD).

Vector Constructions. The vectors were based on pMF6, which confers resistance to hygromycin B (*6*), and on pEBVHistk, which confers resistance to 1-histidinol (*36*). With appropriate selection, the pMF6 and pEBVHistk vectors are maintained at 5 and 40 copies per diploid cell DNA, respectively. The constructions were performed as follows:

The pMF6-based construct, designated pHEPtk1 (*6*) and containing independently expressed CYP2A6 and mEH cDNAs, was digested with *Hin*dIII and treated with DNA polymerase Klenow fragment. *Cla*I linkers were added to the blunt-ended DNA, and the construct was then treated with calf intestine phosphatase. The vector was ligated to the 2.6-kb *Nar*I fragment of pMF6/IA2 (*7*) which contains a complete CYP1A2 cDNA transcriptional unit. The identity of the construct was verified by restriction mapping, and the resulting plasmid was designated pME23 (Figure 1A).

The pEBVHistk-based construct, designated pH44 (*37*) and containing two CYP3A4 transcriptional units to enhance the expression level, was subjected to a *Nar*I partial digest and phosphatase treatment. The vector was ligated to the 2.6-kb *Nar*I fragment of p257Atk2 (*36*) which contains a complete CYP2E1 transcriptional unit. The identity of the construct was verified by restriction mapping, and the resulting plasmid was designated pH441 (Figure 1B).

Results and Discussion

Construction of the Cell Line. A total of five human cDNAs encoding CYP1A2, CYP2A6, CYP2E1, CYP3A4,

[1] Abbreviations: AAF, 2-(acetylamino)fluorene; AFB_1, aflatoxin B_1; BP, benzo[*a*]pyrene; BZD, benzidine; hprt, hypoxanthine guanine phosphoribosyltransferase; 3MC, 3-methylcholanthrene; mEH, microsomal epoxide hydrolase; NDEA, *N*-nitrosodiethylamine; NDMA, *N*-nitrosodimethylamine; tk, thymidine kinase.

Table I. Enzyme Expression in MCL-5 Cells and Comparison to Expression with a Single cDNA per Vector[a]

assay	primary P-450 form(s)	enzyme activity, pmol/(10^6 cells·min) time in culture, days 6	37	63	individual
7-ethoxyresorufin deethylation (basal)	1A1 and 1A2	0.40 ± 0.02	0.40 ± 0.01	0.27 ± 0.02	n/a[b]
7-ethoxyresorufin deethylation (induced)	1A1 and 1A2	1.71 ± 0.13	1.68 ± 0.05	1.35 ± 0.07	n/a
7-ethoxyresorufin deethylation (induced—basal)	1A1	1.32 ± 0.11	1.29 ± 0.04	1.09 ± 0.09	1.6 (*6*)
acetanilide 4-hydroxylation	1A2	1.40 ± 0.06	1.52 ± 0.11	1.36 ± 0.11	1.2 (*32*)
coumarin 7-hydroxylation	2A6	1.02 ± 0.11	1.82 ± 0.08	1.59 ± 0.18	1 (*8*)
chlorzoxazone 6-hydroxylation	2E1	1.47 ± 0	1.52	1.18 ± 0.28	0.5[c]
testosterone 6β-hydroxylation	3A4	0.98 ± 0.04	0.97 ± 0.08	1.22 ± 0.12	1.4 (*32*)
benzo[*a*]pyrene 4,5-oxide hydrolase	mEH	1.04 ± 0.08	1.14 ± 0.07	1.24 ± 0.11	1.8 (*6*)
N-nitrosodimethylamine cytotoxicity (100 ng/mL)	2E1	0.13 ± 0.03[d]	0.11 ± 0.01[d]	0.10 ± 0.02[d]	

[a] A culture of transfected cells were scaled up sufficiently to establish a freezer stock of several hundred vials. The freezer stock was established 24 days after transfection with pME23 and 12 days after the bulk culture began to grow in hygromycin B. "0" time represents establishing a culture from this freezer stock. Independent vials were thawed at different times. Cultures that were propagated for 6, 37, and 63 days were tested, and all enzyme activities were assayed simultaneously. Acetanilide 4-hydroxylase, coumarin 7-hydroxylase, chlorozoxazone 6-hydroxylase, testosterone hydroxylase, and benzo[*a*]pyrene 4,5-oxide hydrolase activities were not detectable in untransfected cells. Listed are the assay used, the P-450 form(s) primarily responsible for this activity, and the values (mean and standard deviation of duplicates) for cells cultured for 6, 37, and 63 days. The column labeled "individual" gives the enzymatic activity observed for the same P-450 transfected either as a single cDNA with the same means of vector selection or in untransfected cells (7-ethoxyresorufin deethylase, CYP1A1 only). Literature references for these values are given in parentheses where available. NDMA cytotoxicity was measured after a 28-h exposure to 100 ng/mL NDMA. [b] n/a, not applicable. [c] Unpublished observation. [d] Relative survival.

and mEH were incorporated into two independent expression vectors. Both vectors contain the OriP sequences derived from the Epstein-Barr virus (*38*). This type of vector is stably maintained as an extrachromosomal plasmid in the presence of appropriate selection. We designed these constructions so that the total P-450 content would not be more than that previously observed to be stably supported in this system (e.g., cells bearing CYP2A6 in the pEBVHistk vector contain about 1 pmol of P-450 per million cells).

In preliminary studies, we observed that certain P-450 cDNAs were more efficiently expressed than others. We sought to construct cell lines expressing approximately equivalent levels of several cDNAs. To achieve this end, we introduced the two relatively efficiently expressed P-450 cDNAs (CYP1A2 and CYP2A6) into the low copy number vector (pME23) and introduced the two inefficiently expressed P-450 cDNAs (CYP2E1 and CYP3A4) into the high copy number vector (pH441). The plasmids were introduced into the L3 derivative (*6*) of AHH-1 TK+/− cells sequentially. pH441 (Figure 1B) was introduced into L3 cells via protoplast fusion. Bulk cell populations were initially grown for 10 days in media without l-histidine and containing 0.5 mM l-histidinol. The l-histidinol concentration was then gradually increased to 2 mM, and when the cells were growing well, clonal populations were isolated by plating at low cell density in 96-well microtiter plates. CYP3A4, CYP2E1, and native CYP1A1 enzymatic activities and the structure and copy number of the vector were verified in the clonal populations.

One clonal population containing pH441 was subsequently transfected with pME23 (Figure 1A), and a bulk population resistant to both 2 mM l-histidinol and 200 μg/mL hygromycin B was selected. After transfection with pME23, 30 μg/mL 5-aminolevulinic acid was added to the media to help support the heme synthesis necessary for P-450 expression. The bulk transfected population was designated MCL-5, indicating that it is a metabolically competent lymphoblast with five (5) cDNAs.

cDNA Expression and Stability of the Cell Line. Given the multiplicity of related vectors and the large number of herpes simplex virus tk gene derived promoters and polyadenylation signals (4 per vector), we were concerned about the stability of the cell line and the efficiency of expression of each cDNA. To verify continued expression of each cDNA and native CYP1A1 and to examine stability of the cell line, we measured a series of cytochrome P-450 form specific enzyme activities (Table I). We also compared the levels of the activities in MCL-5 cells to those observed in cells transfected with a single cDNA with the same means of selection.

The level of native CYP1A1 in MCL-5 cells was measured by determining 7-ethoxyresorufin deethylase activity after dibenz[*a*,*h*]anthracene induction and subtraction of the basal level which contains a contribution from CYP1A2. The activity was found to be comparable to that observed in dibenz[*a*,*h*]anthracene-induced, untransfected L3 cell line (*6*). Acetanilide 4-hydroxylase (CYP1A2-specific), coumarin 7-hydroxylase (CYP2A6-specific), testosterone 6β-hydroxylase (CYP3A4-specific), and microsomal epoxide hydrolase activities in MCL-5 cells were comparable (within a factor of 2) to those observed in cells transfected with pMF6/IA2 (*7*), p91Dtk [pMF6/2A6 (*8*)], pH44 [pEBVHistk with two CYP3A4 cDNAs (*37*)], and p167Dtk2 [pMF6/mEH (*6*)], respectively. The levels of chlorzoxazone 6-hydroxylase (CYP2E1 specific) in MCL-5 cells were about 3-fold higher than in cells bearing pEBVHis/2E1. On the basis of these assays and comparisons, we have concluded that all cDNAs as well as the native CYP1A1 were being adequately expressed. It also appears that there was no substantial promoter interference in these vector constructs.

The stability of expression was analyzed at the enzyme level by monitoring the levels of the above enzyme activities as a function of time of growth in cell culture. The levels of activity did not vary substantially as a function of culture time (Table I). The maximum difference for any activity after 2 months in culture was a factor of approximately 1.5. We also examined NDMA cytotoxicity in the same experiment. Exposure to 100 ng/mL NDMA reduced relative survival to about 0.1 for all time points. This observation indicates that, in addition to the bulk levels of CYP2E1 enzyme activity being unchanged, at least 90% of the cells were expressing CYP2E1 [CYP2A6 would not appreciably activate NDMA at this concentration (*8*)]. The 2-month time frame analyzed indicates that this cell line is sufficiently stable to permit many applications including long-term, low-dose exposure experiments (*39*, *40*) and transfer of the cell line to independent laboratories.

The levels of expression of transfected CYP1A2, CYP2A6, CYP2E1, and CYP3A4 were measured by using

Table II. Microsomal Cytochrome P-450 Contents and Enzyme Activities[a]

cytochrome P-450 form	P-450 content, pmol/mg of protein	substrate	sp act., pmol/(mg of protein·min)
CYP1A2	3	acetanilide	34 ● 3
CYP2A6	2.7	coumarin	28 ± 2
CYP2E1	2.3	chlorzoxazone	24.5 ± 0.4
CYP3A4	1.4	testosterone	25 ± 5

[a] Microsomal P-450 contents were determined by quantitative Western immunoblot using spectrophotometrically quantitated, vaccinia virus vector produced cytochromes P-450 as standards. This comparison assumes low or comparable apoprotein levels in MCL-5 cells and vaccinia virus vector produced materials. P-450 content in MCL-5 cells was estimated by comparing band intensities on the Western blot. Such quantitations contain an uncertainty of 20–50%. Values for specific enzyme activities represent the mean and standard error of the mean for triplicate determinations.

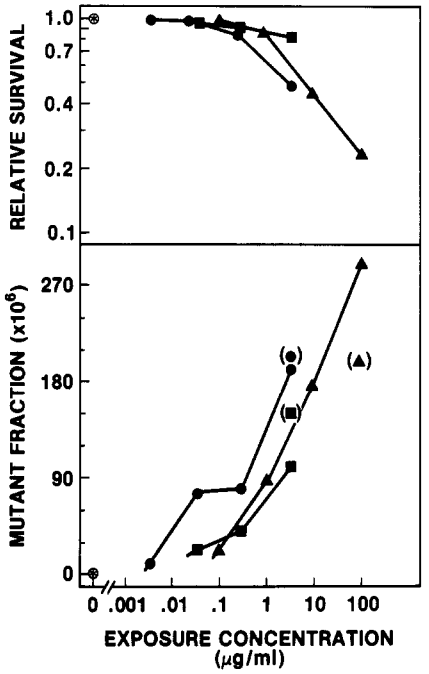

Figure 2. Mutagenicity of benzo[*a*]pyrene, 3-methycholanthrene, and *N*-nitrosodiethylamine at the hprt and tk loci in MCL-5 cells. Cells were exposed to the indicated concentrations of the mutagens for 28 h. Each chemical was tested in at least two independent experiments with at least duplicate cultures in each experiment. Mean values are plotted. All concentrations were assayed for mutant fraction at the hprt locus; the highest concentration was also assayed for mutant fraction at the tk locus. The tk locus data points are enclosed in parentheses. Procarcinogens are as follows: circles, benzo[*a*]pyrene; squares, 3-methycholanthrene; and triangles, *N*-nitrosodiethylamine. The mean negative control mutant fractions were 2.8×10^{-6} and 3.6×10^{-6} at the hprt and tk loci, respectively. The circled stars are the negative control mutant fraction and relative survival.

quantitative Western immunoblotting of the microsomal fraction isolated from MCL-5 cells. The specific P-450 contents were compared to catalytic activities toward P-450 form specific substrates. The results are presented in Table II. P-450 contents ranged from 1.4 to 3 pmol of P-450/mg of microsomal protein. The levels of catalytic activities toward acetanilide (CYP1A2), coumarin (CYP2A6), and testosterone (CYP3A4) were within 20–40% of that predicted on the basis of our previous analyses of substrate turnover by these enzymes (*37*). The level of catalytic activity toward chlorzoxazone (CYP2E1) is in good agreement with that predicted on the basis of

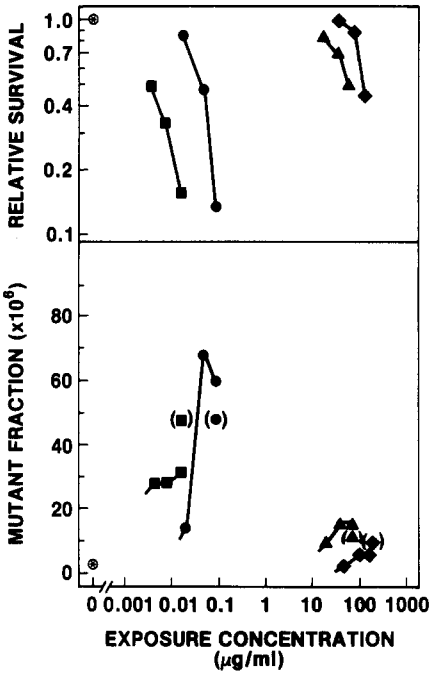

Figure 3. Mutagenicity of aflatoxin B_1, *N*-nitrosodimethylamine, 2-(acetylamino)fluorene, and benzidine at the hprt and tk loci in MCL-5 cells. Experiments were performed and data plotted as described in Figure 2. Procarcinogens are as follows: circles, *N*-nitrosodimethylamine; squares, aflatoxin B_1; triangles, 2-(acetylamino)fluorene; and diamonds, benzidine. The circled stars are the negative control mutant fraction and relative survival.

studies with vaccinia virus expressed CYP2E1.[2] The specific P-450 contents and catalytic activities were lower than observed in human liver microsomes. However, the level of P-450 expression was ample to perform efficient activation of the structurally diverse procarcinogens to human cell mutagens.

Procarcinogen Activation by MCL-5 Cells. In order to examine the ability of MCL-5 cells to activate procarcinogens, concentration–response relationships were determined for seven model procarcinogens: BP, 3MC, NDMA, NDEA, AFB_1, AAF, and BZD. Mutant fractions were determined at the hprt locus for all procarcinogen concentrations and were also determined at the tk locus for the highest procarcinogen concentration. The results are presented in Figures 2 and 3.

The negative control mutant fractions at the hprt and tk loci in MCL-5 cells were comparable to those observed in the parent L3 cell line. This indicated that the expression of the cytochrome P-450s did not substantially affect the spontaneous mutation rate (which could possibly happen through the generation of activated oxygen species). This observation is consistent with our previous observations that P-450 expression does not affect background mutant frequency (*5–8*).

BP induced a mutagenic response at an exposure concentration of 3 ng/mL, and a monotonic increase in mutant fraction was observed over a 1000-fold concentration range (Figure 2). BP was not particularly cytotoxic; at 3 μg/mL BP relative survival reduced to 60%. MCL-5 cells were 3- to 10-fold more sensitive to BP than MCL-1 cells. MCL-1 cells are an L3 derivative which express native CYP1A1 and transfected CYP2A6 and mEH under hygromycin B selection (*6*). This observation is consistent with CYP1A1 and mEH being primarily involved in ac-

[2] F. Gonzalez and R. Peter, unpublished observation.

tivating this procarcinogen in addition to suggesting a role for another transfected P-450. For example, Shimada et al. (*14*) have reported that CYP3A can activate BP-7,8-diol in human liver microsomes. Other transfected P-450 forms may also play a role in this activation.

NDEA induced a mutagenic response at an exposure concentration of 100 ng/mL and was active over a 1000-fold concentration range including noncytotoxic exposure concentrations (Figure 2). We have previously demonstrated that both CYP2A6 and CYP2E1 activate NDEA (*8*); however, the mutant fractions observed in MCL-5 cells were about 2-fold higher than predicted on the basis of the sum of the mutant fractions in individual 2A3/Hyg cells and 2E1/Hol cells (*8*). The higher mutant fractions in MCL-5 cells may be due to the apparently higher levels of CYP2E1 as measured by chlorzoxazone 6-hydroxylase activity and NDMA sensitivity (Table I).

3MC induced a significant increase in mutant fraction at an exposure concentration of 30 ng/mL, and a monotonic increase in mutant fraction was observed over a 100-fold concentration range (Figure 2). Again, for this chemical, substantial increases in mutant fraction were observed in the absence of significant cytotoxicity.

AFB_1 induced a significant increase in mutant fraction at an exposure concentration of 5 ng/mL (Figure 3). Unlike BP, 3MC, and NDEA, AFB_1 was quite cytotoxic at low concentrations. We have previously reported AFB_1 to be activated to a mutagen in 1A2/Hyg, 3A4/Hol, and 2A3/Hyg cells (*32*), with 1A2/Hyg cells being about 5-fold more sensitive than 3A4/Hol cells and about 70-fold more sensitive than 2A3/Hyg cells. The parent cell line, AHH-1 TK+/−, is also sensitive to high concentrations of AFB_1 (*7*). The sensitivity of MCL-5 cells was consistent with the sum of individual P-450 activation capacities.

NDMA induced a significant increase in mutant fraction at an exposure concentration of 20 ng/mL (Figure 3). This chemical was also quite cytotoxic. MCL-5 cells were about 3-fold more sensitive to NDMA than 2E1/Hol cells (*8*), which is consistent with the higher levels of chlorzoxazone 6-hydroxylase activity in MCL-5 cells. CYP2A6 plays a minor role in the activation of NDMA (1000-fold less active than CYP2E1), which probably did not significantly contribute to the increased mutagenic response.

AAF induced a significant increase in mutant fraction at an exposure concentration of 10 μg/mL (Figure 3). AAF has been reported to be activated by CYP1A2 and CYP1A1 (*41*). The response in MCL-5 cells was greater than that observed in the parent AHH-1 cell line and thus was consistent with one of the transfected activities or the enhanced native CYP1A1 activity in L3 cells mediating the activation. Further testing with cell lines expressing single cDNAs will be required to establish which form(s) was (were) responsible.

BZD induced a very modest increase in mutant fraction (2- to 3-fold, Figure 3). The response in MCL-5 cells was similar to that observed in the parent AHH-1 cell line, indicating that metabolism by the introduced activities was probably not the rate-limiting step for the activation of this compound to a mutagen. Cellular acetylation capacity may be rate limiting for BZD mutagenicity (*42*).

In general, there was good agreement between measurements at the hprt and tk loci in the magnitude of the mutagenc response observed (Figures 2 and 3). Values were all within a factor of 2. This indicates that the tk locus can be used for many screening applications with a significant savings in time and materials because of the shorter phenotypic expression time at the tk locus relative to the hprt locus.

Table III. Comparison of the Sensitivities of MCL-5 Cells and the Parent AHH-1 TK+/- Cell Line[a]

carcinogen	calcd concn necessary to double the mutant fraction, ng/mL: MCL-5	calcd concn necessary to double the mutant fraction, ng/mL: AHH-1 TK+/−	increase in sensitivity, x-fold
benzo[*a*]pyrene	1.2	1000 (*8*)	830
3-methylcholanthrene	4.4	nd[b]	n/a[c]
N-nitrosodiethylamine	13	>100000 (*8*)	>7700
N-nitrosodimethylamine	5	>200000 (*8*)	>40000
aflatoxin B_1	0.6	1000 (*8*)	1700
2-(acetylamino)fluorene	8000	25000 (*5*)	3.1
benzidine	86000	70000[d]	0.8

[a] Values were calculated by linear interpolation between the lowest promutagen concentration tested which yielded more than a doubling in mutant fraction and either the next lower promutagen concentration or the negative control. Values for AHH-1 TK+/- cells were calculated primarily from previously published data, and references are provided in parentheses. Fold increase in sensitivity was calculated by dividing the values for AHH-1 TK+/- by the values for MCL-5 cells. [b] nd, not done. [c] n/a, not applicable. [d] Unpublished observations.

In order to further illustrate the effects of increased procarcinogen activation on mutagenic response, we have compared the sensitivity of the MCL-5 cell line to that of the parent AHH-1 cell line which contains only lower levels of native CYP1A1 activity (Table III). For this comparison we have calculated via linear interpolation the concentrations of mutagens necessary to yield a doubling in mutant fraction at the hprt locus. This comparison was possible for all chemicals except 3MC, for which we have no data in the AHH-1 cell line. Both AHH-1 and MCL-5 cells have comparable negative control mutant fractions at the hprt locus [$(2.5–3) \times 10^{-6}$]. For all procarcinogens except BZD the MCL-5 cell line was significantly more sensitive than AHH-1 cells. The nitrosamines were about 10000-fold more active, BP and AFB were about 1000-fold more active, and AAF was 3-fold more active in MCL-5 cells. Only BZD gave comparable responses in both cell lines. On the basis of the mutagenic responses observed and the comparisons to AHH-1 cells, the procarcinogen-activating capacity was substantially improved by transfecting the cDNAs into the L3 derivative of AHH-1 TK+/- cells. Indeed, MCL-5 cells were very sensitive to the mutagenic effects of two polycyclic aromatic hydrocarbons, two nitrosamines, a mycotoxin, and an aromatic amide. These results indicate that this cell line can activate a wide spectrum of procarcinogens. The full capacity of MCL-5 can only be established by testing additional procarcinogens in the system.

The understanding of human procarcinogen activation and the P-450 forms responsible for this activation is still quite limited. Therefore, it is reasonable to expect that additional P-450s, not examined in this study, will be identified as being primarily responsible for the activation of other procarcinogens. The development of the MCL-5 cell line establishes the feasibility of introducing multiple human P-450 cDNAs into human lymphoblast cells. Additional P-450s could be incorporated into the MCL-5 cells either with the same vectors or with a third selectable marker. Alternatively, other cell lines having different sets of P-450 cDNAs could be developed.

The goal of this study was to construct a cell line expressing multiple cDNAs and not to construct a specific P-450 profile which mimics a particular tissue (e.g., liver). Accordingly, our results should not be interpreted as indicative of quantitative human tissue susceptability to procarcinogen exposure. However, as more is learned

about specific P-450 profiles in particular tissues, this study can be viewed as a paradigm for the construction of cells with specific enzyme profiles tailored for human risk assessment.

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The Dimethyl Ester of *meso*-2,3-Dimercaptosuccinic Acid and Its Interactions with Cd^{2+} and Rabbit Liver Metallothionein I

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The ion association complex formed with the cadmium chelate of the dimethyl ester of *meso*-2,3-dimercaptosuccinic acid and the tetramethylammonium cation, $[(Me)_4N]_2[Cd(DiMeDMSA)_2]$, has been synthesized. The structures of the ion association complex and of the ligand (*meso*-DiMeDMSA) were determined by single-crystal X-ray diffraction. The two methyl ester groups and the two sulfhydryl groups in *meso*-DiMeDMSA were in a staggered conformation. The anion $[Cd(DiMeDMSA)_2]^{2-}$ is essentially a distorted tetrahedron, with a mononuclear CdS_4 kernel. The structure of the $[Cd(DiMeDMSA)_2]^{2-}$ anion in solution was found to be similar to its structure in the solid state by multinuclear (^1H, ^{13}C, ^{113}Cd) magnetic resonance spectroscopy. In the presence of rabbit liver metallothionein I (MT), *meso*-DiMeDMSA was found to coordinate Zn^{2+} and Cd^{2+} ions. ^1H NMR spectroscopy proved to be an effective analytical technique for monitoring the competition between *meso*-DiMeDMSA and the protein for these metal ions. This was accomplished by probing the tertiary structure of MT with ^1H NMR spectroscopy after incubation with *meso*-DiMeDMSA. In addition, the ^1H NMR spectra of rabbit liver metallothionein I demonstrated that MT is more susceptible to oxidation at physiological pH after the metal ions have been removed. It appears that intra- as well as intermolecular disulfide cross-linkages are formed upon oxidation. When rabbit liver metallothionein I was incubated with *meso*-DiMeDMSA in a 20:1 (*meso*-DiMeDMSA:MT) mole ratio,, 32% of the cadmium and 87% of the zinc bound to MT were removed.

Introduction

The fate and distribution of cadmium in the body are closely related to metallothionein (MT),[1] a low molecular weight, metal binding protein. The synthesis of MT is induced by cadmium, and the subsequent binding of the cadmium ions to MT is a major detoxifying mechanism for this heavy-metal ion (*1*). For example, when rats were given $CdCl_2$, the biliary excretion of cadmium was decreased by more than 99% 2 days after the induction of metallothionein synthesis (*2*). Bile is an important route for the excretion of cadmium (*3*). The treatment of chronic cadmium intoxication, however, has not been very successful [see the review by Jones and Cherian (*4*)]. This seems to be related to the inability of some chelating agents to cross cell membranes or their inability to compete effectively with metallothionein for the Cd^{2+} ions. The dimethyl ester of *meso*-DMSA (*meso*-DiMeDMSA) increased the biliary excretion of cadmium via the bile (*5-7*); this strongly indicates that *meso*-DiMeDMSA crosses the cell membrane and competes effectively with metallothionein for cadmium.

From these results it is possible to infer that *meso*-DiMeDMSA will coordinate Cd^{2+} ions in the presence of metallothionein. But structural information about the types of complexes formed by *meso*-DiMeDMSA and Cd^{2+} ions, and direct chemical evidence that *meso*-DiMeDMSA will coordinate Cd^{2+} ions in the presence of metallothionein, has been lacking. Cadmium and thiolato ligands can form complexes that are either mononuclear (*8*, *9*) or polynuclear (*10-14*). The formation of polynuclear complexes has stimulated a large amount of research in the area of metal-thiolate chemistry.

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[1] Abbreviations: MT, rabbit liver metallothionein I; *meso*-DMSA, *meso*-2,3-dimercaptosuccinic acid; *meso*-DiMeDMSA, dimethyl ester of *meso*-DMSA; $(Me)_4N^+$, tetramethylammonium cation.

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