



Development of an *In Situ* Toxicity Assay System Using Recombinant Baculoviruses

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ABSTRACT. A new method for experimentally analyzing the role of enzymes involved in metabolizing mutagenic, carcinogenic, or cytotoxic chemicals is described. *Spodoptera frugiperda* (SF-21) cells infected with recombinant baculoviruses are used for high level expression of one or more cloned enzymes. The ability of these enzymes to prevent or enhance the toxicity of drugs and xenobiotics is then measured *in situ*. Initial parameters for the system were developed and optimized using baculoviruses engineered for expression of the mouse soluble epoxide hydrolase (msEH, EC 3.3.2.3) or the rat cytochrome P4501A1. SF-21 cells expressing msEH were resistant to *trans*-stilbene oxide toxicity as well as several other toxic epoxides including: *cis*-stilbene oxide, 1,2,7,8-diepoxyoctane, allylbenzene oxide, and estragole oxide. The msEH markedly reduced DNA and protein adduct formation in SF-21 cells exposed to [³H]allylbenzene oxide or [³H]estragole oxide. On the other hand, 9,10-epoxyoctadecanoic acid and methyl 9,10-epoxyoctadecanoate were toxic only to cells expressing sEH, suggesting that the corresponding fatty acid diols were cytotoxic. This was confirmed by showing that chemically synthesized diols of these fatty acid epoxides were toxic to control SF-21 cells at the same concentration as were the epoxides to cells expressing sEH. A recombinant baculovirus containing a chimeric cDNA formed between the rat P4501A1 and the yeast NADPH-P450 reductase was also constructed and expressed in this system. A model compound, naphthalene, was toxic to SF-21 cells infected with the rat P4501A1/reductase chimeric baculovirus, but was not toxic to cells infected with a control virus. This susceptibility could be reversed by co-infecting SF-21 cells with either a human or a rat microsomal EH virus along with the P4501A1/reductase virus. These results demonstrate the usefulness of this new system for experimentally analyzing the role of enzymes hypothesized to metabolize endogenous and exogenous chemicals of human health concern. *BIOCHEM PHARMACOL* 51;4:503–515, 1996.

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The metabolism of drugs and xenobiotics has been shown to be one of the most important factors in determining the biological and toxicological effects of exposure. However, the enzymes involved are diverse and interact *in vivo* in ways that are difficult to define using purified enzymes. With the advent of cDNA expression technology it is now possible to directly manipulate the expression of drug- and xenobiotic-metabolizing enzymes in a variety of eukaryotic cells. Although still less

than perfect, these *in vitro* systems provide a more realistic approach for defining the role of individual enzymes, and the interaction of these enzymes, in metabolizing xenobiotics *in vivo*.

There are several heterologous expression systems that have been used for expressing enzymes involved in drug and xenobiotic metabolism [see reviews in Refs. 1 and 2]. These include both prokaryotic and eukaryotic systems. Prokaryotic systems are easy to use, there is a large variety of cloning and expression vectors, the genetics are well understood, and they are inexpensive. However, *in situ* expression of membrane bound proteins (such as cytochrome P450) is very inefficient, apparently because of the lack of intercellular membranes [3]. In addition, there is a high probability that the proteins will not be active *in situ* due to lack of glycosylation and incorrect folding. Eukaryotic systems include yeast expression systems [4], vaccinia virus systems [5], COS cell-based transient expression systems [6], and stably transfected cell lines in which

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recombinant plasmid expression is maintained *in situ* by antibiotic selection (human lymphoblastoid cells [2], V79 cells [7], Chinese hamster CHL cells [8], and MCF-7 breast cancer cells [9]). Most of these expression systems have focused on cytochrome P450-mediated activation of promutagens. There have been two reports in which UDP-glucuronosyltransferase and γ -glutamyltransferase, respectively, were expressed in V79 cells [10, 11], and two recent reports describing glutathione S-transferase expression in MCF-7 cells [9, 12].

All of these systems have advantages and disadvantages, depending on the ultimate goal of the research. The yeast system provides high expression levels but may contain endogenous P450 activity [4]. The vaccinia system is not yet designed to co-express multiple cDNA inserts and also requires worker immunization and safety precautions [5]. The yeast and vaccinia virus systems express at the highest levels, with the lymphoblastoid cell system roughly an order of magnitude lower, and the transient based expression systems and V79 cells another order of magnitude lower. To date, the most widely used system for cytotoxicity and promutagen activation studies is the lymphoblastoid system [13–15].

Studies using the expression systems mentioned above have been extremely useful by providing insights into the role of specific P450s in activating promutagens [16] and in the analysis of enzymes in protecting cells from cytotoxicity and adduct formation. They also demonstrate that heterologous expression systems are valuable alternatives to animal use in toxicology research and testing. However, as our knowledge and expertise advance from these initial studies, it is clear that significant improvements can be made to extend the use of *in vitro* toxicity assay systems to other classes of chemicals and enzymes in order to increase their utility and validity. Specific problems with these systems include: relatively low expression levels; complicated vector construction for expressing multiple cDNAs in the same cell; lack of familiarity within the general scientific community; relatively limited cloning options; and expense of use and maintenance.

The baculovirus expression system [17] is a potential alternative method that has not been fully developed for use as a system for *in situ* metabolite production, cytotoxicity studies, or studies of chemically induced genetic damage. There are two major reasons for this. The first is that because baculovirus infection eventually causes host cell death and is considered a transient expression system, it has been assumed that it is not useful for measuring *in situ* endpoints such as cytotoxicity or adduct formation. The second is that infected host cells have very little endogenous NADPH reductase, and therefore infected cells are unlikely to express active cytochrome P450 *in situ*. In this paper we systematically examined these two assumptions. We found that there exists a 24- to 48-hr window during which both heterologous enzyme expression and cell viability are high. In addition, by using a chimeric enzyme formed by fusing a rat cytochrome P450 to a yeast NADPH-reductase, high P450 activity can be obtained *in situ*. Using optimized conditions, we then validated the use of this system for experimentally testing hypotheses related to drug and xenobiotic metabolism.

MATERIALS AND METHODS

Chemicals

Test compounds were of reagent grade from the Aldrich Chemical Co., Milwaukee, WI (TSO,* CSO, diepoxybutane, allylbenzene 2',3'-oxide, estragole, and diepoxybutane), from the Sigma Chemical Co., St. Louis, MO (octadecenoic acid, 9,10-epoxyoctadecanoic acids and methyl esters, MTT, XTT, propidium iodide), or were synthesized as described below.

Cloning and Expression of Recombinant msEH in the Baculovirus System

The original *SpeI/XhoI* full-length cDNA fragment of msEH (psEH-2 [18], accession number L05781) was modified by polymerase chain reaction (PCR) to delete all 3' sequences past the stop codon. We amplified a 491 bp fragment encompassing the unique *Hind III* site at bp 1212 and the TAG stop codon at bp 1665. The 5' primer was 5'-GAGGCTGAAC-TGGAGAAGAAC-3' (bp 1174–1195 of psEH-2) and the 3' primer was 3'-CACTGGAGGTTCTAAATCGAGCTCGG-5' (bp 1648–1665 of psEH-2) containing a flanking 5' *Xho I* site for subsequent cloning. The PCR product was digested with *Hind III* and *Xho I* and cloned into the unique *Hind III* and *Xho I* sites of psEH-2. Three independent clones were confirmed by restriction digest and sequencing. One of these clones (in pBluescript SK II+, Stratagene, La Jolla, CA) was then modified by site-directed mutagenesis [19] to remove the internal *Eco RI* site of psEH-2 at bp 132 (GAA \rightarrow GAG; both code for Glu), and in a second mutagenesis reaction, to add a unique *Eco RI* site 22 bp below the *Kpn I* site of the vector. The doubly modified plasmid was checked by restriction digest and sequencing across the mutations, then cut with *Eco RI* and inserted into *Eco RI* digested pAcUW21 forming the baculovirus co-transfection plasmid pAcUW21-msEH. The recombinant baculovirus was obtained by cotransfection of pAcUW21-msEH with BacPac6 viral DNA (Invitrogen, San Diego, CA) according to standard methods [20]. Two independent recombinant clones were plaque purified and analyzed for expression of msEH in infected cells. At various times post-infection, cells were collected by centrifugation, washed with PBS, and then were used directly for enzyme assays. Cell supernatants were prepared by lysing with three cycles of freeze/thaw in an ethanol dry-ice bath and then centrifuging at 16,000 g for 10 min at 4°. msEH enzyme activity was measured with TSO using standard methods [21]. Construction of the human sEH baculovirus was described previously [22]. Construction of human and rat microsomal EH baculoviruses was done essentially as described above. Protein concentration was

* Abbreviations: TSO, *trans*-stilbene oxide; CSO, *cis*-stilbene oxide; SF-21, *Spodoptera fugiperda*; sEH, soluble epoxide hydrolase; msEH, mouse soluble epoxide hydrolase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt; BSTFA, bis(trimethylsilyl)trifluoroacetamide; LR/EI/MS, low resolution electron impact mass spectrometry; MOI, multiplicity of infection; and EROD, ethoxyresorufin deethylase.

measured using the BCA reagent (Pierce) with bovine serum albumin as a standard.

Cloning and Expression of the Recombinant Rat P4501A1/Yeast Reductase Fusion cDNA in the Baculovirus System

The cDNA coding for the rat P4501A1/yeast reductase fusion protein was excised from pAFCR1 [23] with *Hind* III, filled in with Klenow polymerase, and blunt-end ligated into *Sma* I digested pAcAB3 transfer vector (Pharminggen, San Diego, CA). This site is downstream from the p10 promoter. Recombinant baculoviruses were then constructed by co-transfection with BacPak 6 DNA as described above for the msEH virus.

P4501A1 activity was measured *in situ* using ethoxyresorufin (Molecular Probes, Eugene, OR). Whole cells were collected at various times post-infection and washed three times by gentle centrifugation and aspiration in PBS. The final cell pellet was resuspended in 100 μ L of 100 mM KH_2PO_4 buffer (pH 8.0). The reaction was started by adding 15 μ L of a stock ethoxyresorufin solution (2.5 μ M in 1.25% Tween-80), and the fluorescence was measured on a Perkin-Elmer fluorimeter (excitation 510 nm/emission 586 nm) as described [24].

Cell Culture and Toxicity Assays

Cells were routinely infected in 25 mL of complete medium (96% EX-CELL 401 with L-glutamine, JRH Biosciences, Lenexa, KS; 3% heat-inactivated fetal bovine serum, Intergen, Purchase, NY; and 1% Pen/Strep antibiotics, Sigma) in a 50-mL spinner flask with 1×10^6 SF-21 cells/mL. At various times post-infection, toxicity assays were performed in 24-well tissue culture plates or in 16×125 mm glass test tubes by adding 0.5 mL of infected cells to the wells or tubes followed by the test compound in 8 μ L of DMSO. The cells were incubated for 24 hr at 27°, and then assayed for viability by incubation for 2 hr at 27° with MTT as described [25, 26]. The cells were then lysed overnight at room temperature in the dark to dissolve the insoluble formazan product. The MTT hydrolysis product was quantified by transferring the contents of the wells or tubes to 96-well plates and measuring the O.D. at 560 nm. Viability assays with propidium iodide were performed either on a Beckton Dickinson flow cytometer or with a Beckman fluorimeter (emission at 590 nm, excitation at 530 nm) 1 hr after adding propidium iodide (Sigma) to the cells (20 μ M final concentration). All of the toxicity assay data presented in this paper are representative results from at least two completely independent experiments.

Epoxyde Synthesis

9,10-Epoxyoctadecanoic acid was synthesized using an *in situ* oxone epoxidation of oleic acid [27]. This method had a 100%

yield for 9,10-epoxyoctadecanoic acid production. The ^1H NMR (CDCl_3) of 9,10-epoxyoctadecanoic acid showed signals at 0.88 (t, 7.0 Hz, 3 H), 1.20–1.55 (m 24 H), 1.62 (tt, 7.0 Hz, 2 H), 2.35 (t, 7.0 Hz, 2 H), and 2.92 (bs, 2 H). 9,10-Dihydroxyoctadecanoic acid was produced by acid hydrolysis of 9,10-epoxyoctadecanoic acid. In short, after dissolving 130 mg of 9,10-epoxyoctadecanoic acid in 4 mL of tetrahydrofuran: H_2O (3:1), 1 mL of 5% aq. HClO_4 was added, and the solution was stirred until complete hydrolysis was achieved as evident by TLC. The fatty acids were recovered by adding 3 mL of saturated brine and extracting the solution using 3×10 mL diethyl ether. The recombined organic phases were then washed with saturated brine, dried over anhydrous Na_2SO_4 , and concentrated under vacuum. The concentrated clear oil was then applied to a silica column and eluted using a gradient of 35% ethyl acetate in hexane (1% acetic acid) to 45% ethyl acetate in hexane (1% acetic acid). Eighty milligrams of pure 9,10-epoxyoctadecanoic acid was obtained. The ^1H NMR (CDCl_3) of 9,10-dihydroxyoctadecanoic acid showed signals at 0.8 (t, 6.0 Hz, 3 H), 1.10–1.48 (m, 27 H), 1.55 (tt, 6.0 Hz, 2 H), 2.26 (t, 7.5 Hz, 2 H), and 3.32 (m, 2 H). 9,10-Epoxyoctadecanoic acid was methylated using trimethylsilyldiazomethane and then derivatized to its bistrimethylsilyl ether using BSTFA for GC/MS analysis. LR/EI/MS m/z (fragment, relative intensity) 443 ($\text{M}^+ - \text{MeO}$, 2.7%), 259 ($\text{M}^+ - \text{C}_9\text{H}_{18}\text{OTMS}$, 62%), 215 ($\text{M}^+ - \text{C}_{10}\text{H}_{18}\text{O}_3\text{TMS}$), 155 (25.5%), 109 (26.0%), 73 ($\text{C}_2\text{H}_5\text{OSi}$).

Regioselectivity of ^{18}O -9,10-Epoxyoctadecanoic Acid Hydrolysis with Wild-Type and Recombinant msEH

The ^{18}O -labeled epoxides were synthesized by a previously published procedure [28]. Stock solutions (100 mM) of ^{18}O -9,10-epoxyoctadecanoic acid and ^{18}O -methyl 9,10-epoxyoctadecanoate were prepared in *N,N*-dimethylformamide. Enzymatic hydrolysis of the latter substrates was achieved by addition of 1 μ L substrate (stock) to 100 μ L sodium-phosphate buffer (100 mM, pH 7.4) containing 100 μ g/mL BSA and 6.0 μ g/mL wild-type msEH or 3.9 μ g/mL recombinant msEH. Controls contained 100 μ g/mL BSA to ensure that no hydrolysis had taken place due to the interaction of substrates with buffer and BSA. All enzymatic and control experiments were incubated in a 37° water bath for 30 min, after which saturated NaCl (100 μ L) was added to quench the hydrolysis. Acidic hydrolysis of the same epoxides was performed by introducing 2 μ L of the substrates (stock) into a 5% perchloric acid solution (100 μ L). After a 30-min incubation at 37° in a water bath, saturated NaCl (100 μ L) and enough 10% KOH was added to neutralize the solution. The enzymatic and chemical hydrolysis products were extracted similarly by addition of 450 μ L of ethyl acetate. The ethyl acetate was withdrawn and placed in a conical reaction vessel, isoctane (5 μ L) was added as trap solvent, and the solvent was evaporated under nitrogen. Each sample was derivatized 30 min prior to GC/EI/MS analysis with BSTFA (50 μ L). All enzymatic products and acid-catalyzed hydration products showed a single peak clearly distinct from the *erythro*-diol and indistinguishable from the

/hreo-diol. The masses monitored by single ion monitoring were 215, 217, 259, and 261. The ratio of the 215/261 tandem was compared with that of the 217/259 tandem after correction for the isotopic impurities. GC/EI/mass spectra were obtained using a VG TRIO2 spectrometer equipped with a VG-11-250 data system. GC separation was performed on a Hewlett Packard 5890A gas chromatograph, fitted with a DB-5 (30 m) capillary column directly attached to the mass spectrometer.

Measurement of Adducts

Adduct formation was measured using [^3H]allylbenzene oxide and [^3H]estragole oxide. The unlabeled and tritium-labeled epoxides were synthesized as described [29, 30]. Two-day post-infection cells were washed three times with $1\times$ PBS by centrifugation, resuspended in PBS, and exposed to 20 μCi of [^3H]allylbenzene oxide or [^3H]estragole oxide in 50 mL polypropylene tubes for 2 hr at 26°. Cells were then washed three times by centrifugation to remove unbound labeled compounds. Cell pellets were resuspended in 500 μL of Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH [31]). RNA, DNA, and protein fractions were then isolated according to the manufacturer's directions. Pellets were washed for at least 24 hr in 80% ethanol (RNA and DNA) or 100% ethanol (protein).

RESULTS

We cloned the msEH and expressed this enzyme in the baculovirus system. The recombinant msEH was purified by affinity chromatography using standard techniques [21]. The purified enzyme was analyzed by SDS gel electrophoresis and western blotting and found to be indistinguishable from the wild-type msEH [32]. Kinetic constants for the recombinant enzyme using TSO (K_m : $2.9 \pm 0.6 \mu\text{M}$; V_{max} : $1212 \pm 109 \text{ nmol/min/mg protein}$) were also similar to the non-recombinant enzyme [33].

As an additional verification that the recombinant msEH was indistinguishable from the wild-type msEH, we compared the regioselectivity of enzyme attack of ^{18}O -9,10-epoxyoctadecanoic acid and ^{18}O -methyl 9,10-epoxyoctadecanoate. Acidic hydrolysis of ^{18}O -9,10-epoxyoctadecanoic acid and ^{18}O -methyl 9,10-epoxyoctadecanoate resulted in a 1:1 distribution of carbon 9 and carbon 10 labeled diol, a testament to the chemical equivalence of the two epoxide carbons. However, 68.5% of the enzymatic attack occurred at carbon 10 with wild-type msEH, indicating regioselective control on the hydrolysis of the epoxide. The results of enzymatic hydrolysis with ^{18}O -methyl 9,10-epoxyoctadecanoate were identical to the results obtained with its corresponding free acid. The recombinant msEH, under the same experimental conditions, yielded diols with the same distribution of ^{18}O atom on carbon-9 and 10, indicating identical regioselectivity as compared with wild-type msEH.

We found that from 72 to 144 hr post-infection, high levels of msEH activity were present in SF-21 cells (Fig. 1A). From

this observation, we hypothesized that during this time SF-21 cells should be resistant to the toxic effects of epoxides that could be metabolized by the enzyme. Initial experiments to test this hypothesis were performed by exposing aliquots of cells to TSO each day during the first 3 days of virus infection (Fig. 1B). At both 0 and 1 day post-infection, during which time there was essentially no msEH expressed in infected SF-21 cells (Fig. 1A), the cells were highly susceptible to TSO. However, at 2 and 3 days post-infection when SF-21 cells were expressing high levels of msEH, the cells were highly resistant to TSO. This same result was obtained when using propidium iodide uptake as an indicator of cell death. This resistance to TSO could be reversed (not shown) at day 3 by adding 500 μM (2S,3S)-(-)-3-(4-nitrophenyl)-glycidol, a stereospecific inhibitor of msEH ($I_{50} = 1.1 \mu\text{M}$ [34]). The 2R,3R enantiomer ($I_{50} > 600 \mu\text{M}$), however, had no effect.

Initial toxicity assays were performed using SF-21 cells infected with the msEH virus at an MOI of 1 (1 virus per cell). To experimentally optimize this variable, we performed TSO toxicity assays at various MOIs and used a recombinant baculovirus that expresses β -galactosidase (LacZ) as a control. Figure 2 shows that the MOI had little effect on the results, although higher MOIs caused a general reduction in assay sensitivity. The increase in O.D. with decreasing MOI was most likely due to growth of uninfected SF-21 cells. At high MOIs (10:1 and 1:1), nearly all of the cells are infected with virus within 1 hr and stop dividing within approximately 12 hr [20]. At lower MOIs, some cells remain uninfected and continue to divide. The results in Fig. 2B suggest, however, that after 48 hr all of the cells become infected from budded viruses produced during the initial infection. Based on these results, we routinely used an MOI of 0.1 for subsequent toxicity assays.

SF-21 cells are derived from the ovary cells of *Spodoptera fugiperda* [20]. For an *in vitro* toxicity assay system to be useful for predicting results *in vivo*, it is important to determine whether baculovirus-infected SF-21 cells are more or less sensitive to cytotoxic compounds than are other eukaryotic cells. To test this, we exposed HeLa cells, uninfected SF-21 cells, LacZ-infected cells and msEH-infected cells to TSO and measured cell viability using the standard MTT assay. We found (not shown) that all cells were equally susceptible to TSO, except for SF-21 cells infected with the msEH virus, which were resistant. We repeated these toxicity assays using diepoxycane, CSO, allylbenzene oxide, and estragole oxide and obtained similar results (not shown). These results suggest that virus-infected SF-21 cells are representative eukaryotic cells that are no more or less susceptible to the toxic compounds tested than are other cells.

The results above suggest that msEH can protect cells from epoxide-mediated cytotoxicity. One potential mechanism by which epoxides can cause cytotoxicity is by forming adducts with cellular proteins, DNA, or RNA. To test this hypothesis we exposed virus-infected SF-21 cells to [^3H]allylbenzene oxide and [^3H]estragole oxide and then compared the amount of insoluble tritium label remaining in the protein, DNA, and RNA fractions of the cells. The results (Table 1) show that for both compounds msEH markedly reduced the amount of ra-

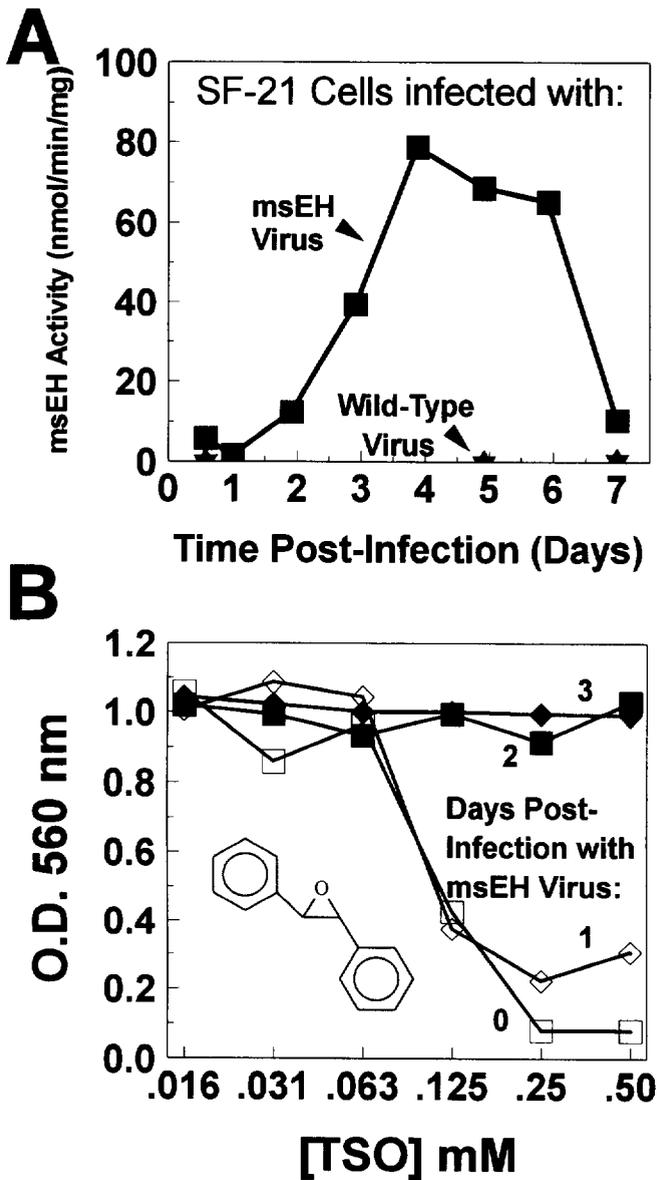


FIG. 1. (A) msEH expression in SF-21 cells infected with the msEH virus or the wild-type AcNPV virus. Activity was measured with TSO. Activity in SF-21 cells expressing the wild-type AcNPV virus was essentially undetectable using this assay (<0.1 nmol/min/mg). (B) Viability of SF-21 cells (expressed as the optical density at 560 nm) exposed to TSO during a 3-day time course of infection with the msEH virus. Viability was measured with the standard MTT assay as described in Materials and Methods.

dioactivity found in the DNA and protein fractions but not in the RNA fraction of the cells. The finding of relatively higher counts in the protein fraction compared with the DNA fraction is consistent with the hypothesis that these compounds act as general cytotoxins by forming adducts with cellular proteins.

Previous results have shown that monoepoxidized C_{18} fatty acids and esters are excellent substrates for msEH [35]. In addition, some fatty acid epoxides have been implicated in the pathology of dioxin exposure [36]. We therefore experimentally tested the hypothesis that msEH could protect SF-21 cells

from monoepoxidized C_{18} fatty acid and ester toxicity. Surprisingly, we found that SF-21 cells expressing either the hsEH or msEH were much more sensitive to these compounds than were SF-21 cells expressing the control β -galactosidase enzyme (Fig. 3, panels A, B, C, and D). Although the results in Fig. 3 are given for the *cis*-epoxides, we found similar results using the *trans*-epoxides (not shown). *threo*-Diols synthesized by acid hydrolysis of the corresponding *cis*-epoxides were toxic to all cell types tested (Fig. 3, panels E and F). We found similar results using HeLa cells or using propidium iodide uptake as a measure of cell viability: the epoxides were less toxic than were the chemically synthesized diols (not shown).

For this system to be widely used for *in vitro* toxicity testing, ideally it should be capable of expressing any enzyme for which

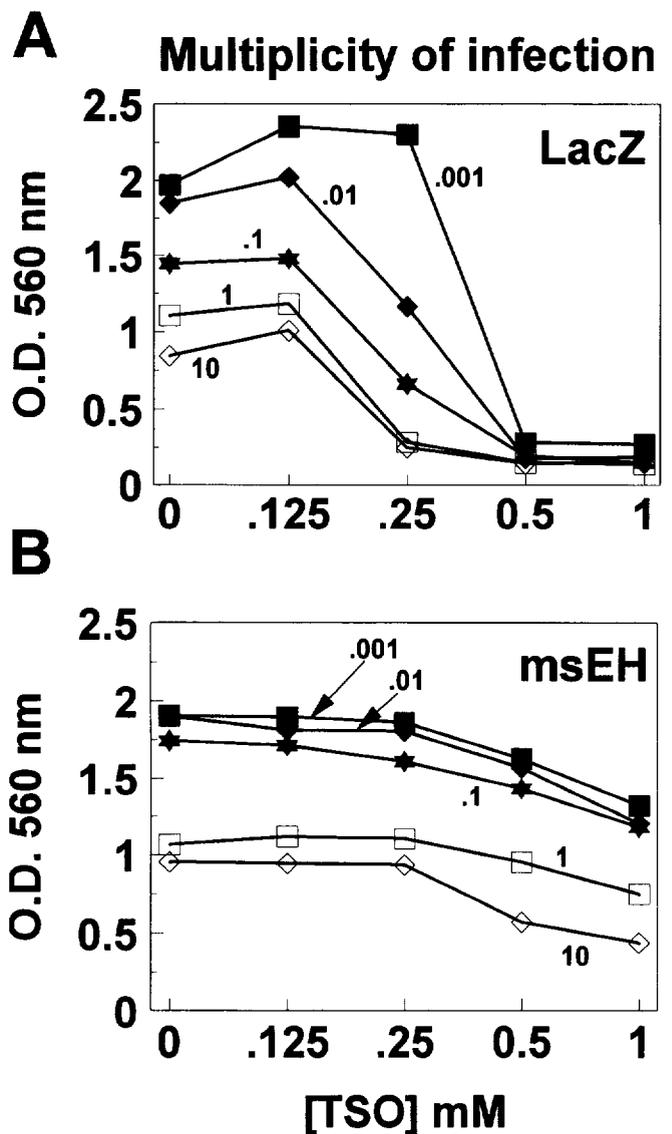


FIG. 2. Effects of the MOI on viability of SF-21 cells exposed for 24 hr to TSO at 3 days post-infection. (A) Viability of SF-21 cells infected with the LacZ control virus. The MOI for each treatment is indicated on each curve. (B) Viability of SF-21 cells infected with the msEH virus. Symbols are as in panel A. For both graphs, viability was measured using the standard MTT assay.

TABLE 1. Adduct formation in SF-21 cells exposed to radioactive epoxides

Cell fraction	Epoxide* (cpm)			
	³ H]Allylbenzene oxide		³ H]Estragole oxide	
	msEH	LacZ	msEH	LacZ
RNA	51 ± 6	74 ± 4	52 ± 3	97 ± 17
DNA	60 ± 3	241 ± 11	72 ± 3	426 ± 48
Protein	77 ± 12	3640 ± 165	109 ± 24	6685 ± 1042

* Values listed are the mean cpm (±SD, N = 3) in the pellets of each of the corresponding cell fractions. msEH is the mouse soluble epoxide hydrolase virus; LacZ is the control virus expressing β-galactosidase. The specific activity of [³H]allylbenzene oxide was 0.861 mCi/mmol; 20 μCi was used in each experiment. The specific activity of [³H]estragole oxide was 2.01 mCi/mmol; 20 μCi was used in each experiment. These are results from one of three separate experiments, all of which led to similar conclusions.

a cDNA is available, including enzymes that can activate compounds *in situ*. An important group that belongs to this latter class of enzymes is the cytochrome P450 isozymes. Previous workers have cloned and expressed P450 isozymes in the baculovirus system and have used homogenized cells for preparing reconstituted activation systems. However, previous workers have not been able to produce activity *in situ*, most likely due to the lack of NADPH-P450 reductase present in virus-infected cells. To circumvent this problem, we cloned and expressed the pAFCR1 fusion cDNA in the baculovirus expression system. The pAFCR1 clone contains the rat P4501A1 fused to the yeast NADPH-P450 reductase. This clone has been characterized thoroughly and shown to be active in a yeast expression system [23]. We initially screened four independent baculovirus isolates and checked each for expression of protein and immunological cross-reactivity, using a P4501A1 monoclonal antibody. The results (Fig. 4A) show that all four isolates expressed a unique protein having the predicted molecular mass of 131 kDa, and all cross-reacted with a monoclonal antibody specific for P4501A1 proteins (Fig. 4B). Lower molecular mass bands are most likely due to degradation products.

Previous workers have shown that hemin added to the culture medium can increase the level of P450 activity and P450 protein from a baculovirus expression system [38, 39]. Although those results were obtained using isolated microsomes and adding reductase and NADPH, they point out that SF cells have insufficient endogenous hemin. Since hemin can be toxic to SF cells [39], we used our MTT assay protocol to optimize the level of hemin in the culture medium. We found that exogenously added hemin became cytotoxic above a concentration of 1 μg/mL, but that from 0 to 1 μg/mL hemin appeared to slightly increase the viability of cells infected with either the LacZ virus or the P4501A1/reductase virus. We therefore routinely used hemin at a concentration range of 0.5 to 1.0 μg/mL in the culture medium when expressing the P4501A1/reductase or control viruses.

Using the optimum concentration of hemin determined above, we infected SF-21 cells with the P4501A1/reductase virus and measured EROD activity, cell viability, and protein

expression during the course of infection (Fig. 5). The EROD measurements were obtained with whole cells that were prepared only by washing with PBS. The results showed a peak of EROD activity at 3 days post-infection, followed by a steady decline (Fig. 5A). Cell viability increased from day 1 to day 2, then leveled off at day 3, followed by a steady decline in cell viability due to viral infection [20]. The loss in cell viability from day 3 to day 6 closely matched the decline in EROD activity during this time. SF-21 cells infected with LacZ showed the same decline in cell viability from day 3 through day 6, but had no measurable EROD activity.

Expression of the fusion protein (Fig. 5B) was clearly visible on a Coomassie stained gel at day 2, then gradually increased to day 5, followed by a decrease at day 6. Comparison of Fig. 5A and 5B shows that even though EROD activity and cell viability were decreasing after day 3, P4501A1/reductase protein levels continued to increase until day 5. This suggests that EROD activity is dependent on cell viability as well as protein expression, and is consistent with the hypothesis that *in situ* production of NADPH is likely to be a limiting factor coinciding with cell necrosis after day 3.

As mentioned above, the decrease in cell viability at 3 days post-infection is due to cell necrosis caused by virus infection, and is an inherent characteristic of the baculovirus expression system [20]. Figure 5A clearly demonstrates, however, that a 24-hr window (from day 2 to day 3 post-infection) existed during which both EROD activity and cell viability were high, and suggests that during this time cells would be highly susceptible to compounds that could be activated by P4501A1. To test this hypothesis, we exposed SF-21 cells to naphthalene, a model P4501A1 substrate, at 2 days post-infection and measured cell viability 24 hr later. The results (Fig. 6) show that naphthalene was toxic only to cells infected with the P4501A1 virus.

An additional feature of baculovirus expression is the ability to co-infect individual cells with more than one virus. To explore this possibility in our toxicity assay system, we infected five aliquots of SF-21 cells with either the P4501A1 virus or the control LacZ virus, with each aliquot containing one of five additional recombinant viruses. We then exposed the cells to naphthalene at 2 days post-infection and determined whether one or more of these five viruses could rescue cells from naphthalene toxicity. The results (Fig. 7) show that both the human and rat microsomal EH could reverse the effects of the P4501A1 virus, whereas the human or mouse sEH had little effect. SF-21 cells from each of these co-infections were analyzed by SDS-PAGE (not shown) to verify that the indicated proteins were expressed.

DISCUSSION

In this paper, we describe a new, generally applicable methodology for experimentally defining *in vitro* the role of enzymes in metabolizing endogenous or exogenous chemicals that are hypothesized to have an impact on human health. This experimental system makes use of the baculovirus expression

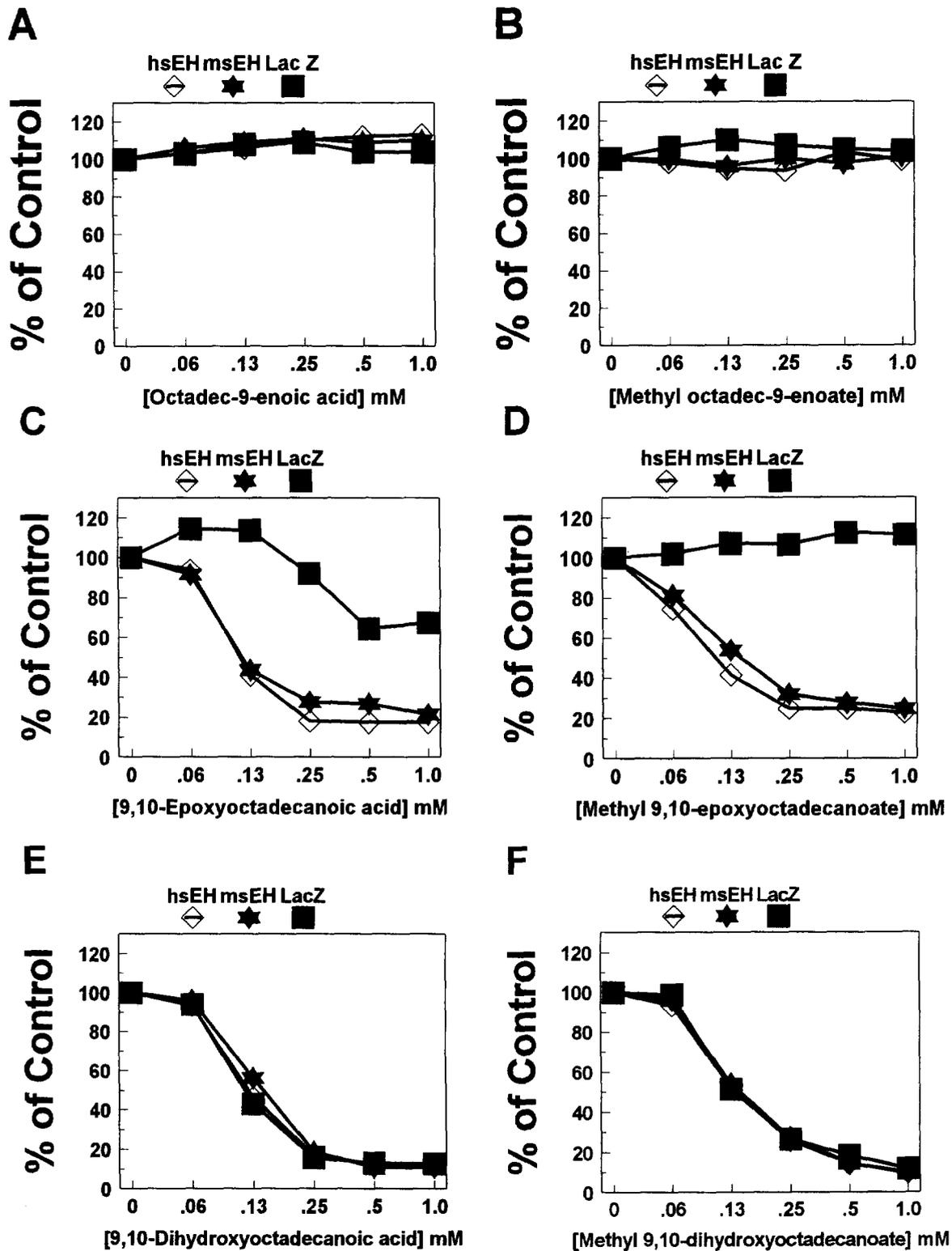


FIG. 3. Toxicity assays using C₁₈ fatty acids and methyl esters. hsEH is the human sEH; msEH is the mouse sEH. (A and B) Octadecenoic acid and methyl octadecenoate are not toxic to SF-21 cells expressing human or mouse sEH, or LacZ. (C and D) 9,10-Epoxyoctadecanoic acid and methyl 9,10-epoxyoctadecanoate are more toxic to cells expressing sEH than to cells expressing LacZ. (E and F) The 9,10-diols of octadecanoic acid and methyl octadecanoate are toxic to SF-21 cells infected with either of the sEH viruses or the LacZ virus. For all compounds, cells were exposed for 24 hr at 3 days post-infection with the indicated concentration of C₁₈ fatty acids or methyl esters. Viability was measured using the standard MTT assay.

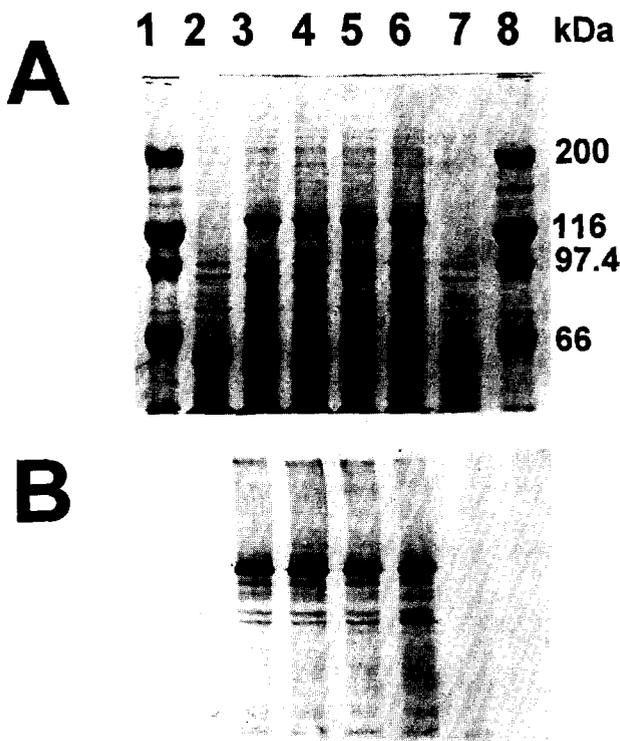


FIG. 4. Expression of the rat P4501A1/reductase fusion enzyme in SF-21 cells. Four independent recombinant viruses were used to infect cells. At 4 days post-infection, cells were collected, washed three times by centrifugation, and directly lysed in SDS sample buffer. (A) SDS gel stained with Coomassie blue. Lanes 1 and 8 show molecular weight markers. Lanes 2 and 7 show the total proteins expressed in SF-21 cells infected with the msEH virus. msEH is 62 kDa. Lanes 3 through 6 show the total proteins expressed in SF-21 cells infected with each of four independent rat Cyp1A1/reductase viruses. The predicted molecular mass of the P4501A1/reductase chimeric protein is 131 kDa (1209 amino acid residues [23]). (B) Western blot of the same samples shown in (A) using a monoclonal antibody developed against the scup P4501A1 [37].

technology that has been in continual development as an expression system for the last 20 years. A distinct advantage of this heterologous expression system compared with other heterologous systems used in toxicology is that it in itself is not new; it is a widely used model expression system. The new approach taken in this study is to use this system as a means for easily expressing various combinations of drug- and xenobiotic-metabolizing enzymes in a eukaryotic cell line, and at the same time, and in the same cells, for quantifying the toxicological results of these experimental manipulations. To the best of our knowledge, this is the first report of the use of this system for *in situ* toxicity testing.

We tried several modifications of the standard toxicity assay in order to optimize assay conditions. In addition to MTT, we tried XTT [41] in this assay system. Because XTT metabolism by viable cells produces a soluble end product, we thought XTT might be more convenient to use. SF-21 cells, however, did not metabolize XTT significantly even after a 12-hr incubation at 27° (results not shown). We also tried to further automate the assay by growing and treating infected cells in

96-well plates. Although the results were essentially the same (not shown), this modification actually proved to be less reliable and more time consuming due to problems with evaporative loss and the difficulty of adding test compounds in extremely small volumes of solvent.

An important variable that influenced assay repeatability was the density of the cells prior to baculovirus infection. Replicate experiments with cells that were at different densities prior to infection gave slightly different results, although the overall patterns were similar. We subsequently found that optimum repeatability and sensitivity occurred when cultures were rapidly growing and were at a density of $3\text{--}4 \times 10^6$ cells/mL just prior to infection with the recombinant viruses.

In preliminary experiments, we measured the linearity of the MTT assay as a function of time and number of cells and used optimized conditions for toxicity tests. We also measured the toxicity of DMSO and ethanol to infected SF-21 cells and

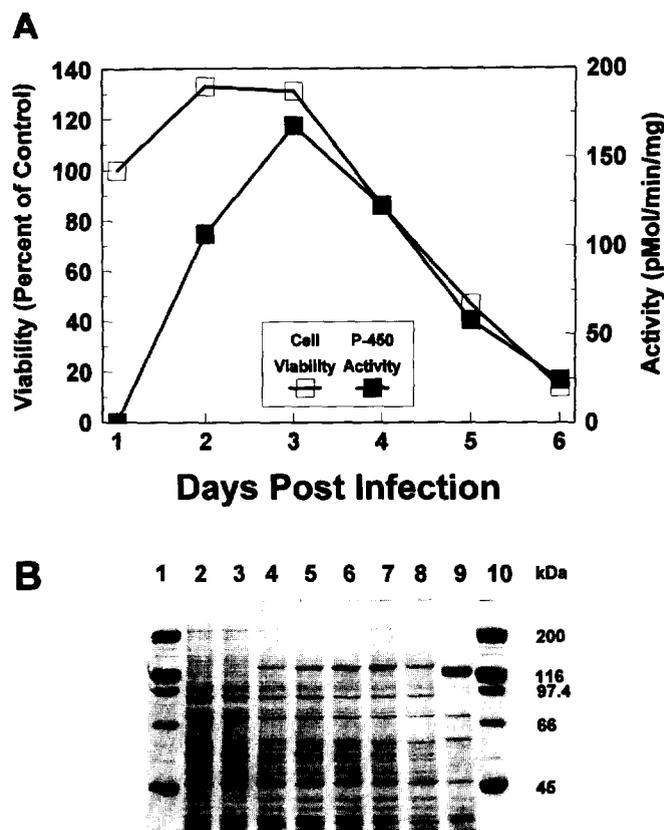


FIG. 5. Expression of the rat P4501A1/reductase during infection of SF-21 cells. (A) EROD activity was measured each day for 6 days (with 1 $\mu\text{g}/\text{mL}$ hemin final concentration added to the medium) in whole, unlysed cells after washing three times with $1 \times \text{PBS}$. At the same time cell viability was measured using the standard MTT assay. (B) Coomassie-stained SDS gel showing the total protein (40 $\mu\text{g}/\text{lane}$) in cells collected during the infection time course shown in (A). Lanes 1 and 10 are molecular weight markers. Lane 2 is SF-21 cells at 1 day post-infection with the LacZ virus. Lanes 3 through 8 are SF-21 cells at days 1 through 6, respectively, infected with the P450/reductase virus. Lane 9 is SF-21 cells at 6 days post-infection with the LacZ virus. The molecular mass of LacZ is 115 kDa [40].

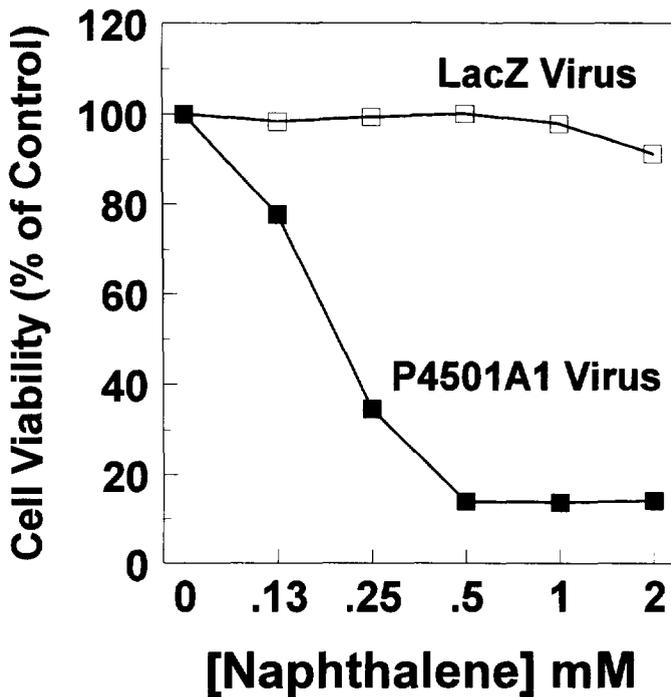


FIG. 6. Viability of SF-21 exposed to naphthalene for 24 hr at 2 days post-infection with the P4501A1 virus or control, LacZ virus. Hemin concentration was 1 $\mu\text{g}/\text{mL}$ for both treatments. Viability was measured with the standard MTT assay as described in Materials and Methods.

found that there was no effect of either solvent on baseline toxicity values when used below 3.2%.

No *in vitro* toxicity assay system is ideal for every experimental design, and the baculovirus expression system described here is no exception. The human lymphoblastoid cell system developed by Crespi *et al.* [13, 14] is the only system that is commercially available, and is the system used most frequently. This is an ideal system for specific types of chemicals and for long-term, low-dose exposure studies for which the baculovirus system clearly would not be appropriate. However, in terms of questions designed to understand mechanisms of xenobiotic metabolism, the baculovirus expression system has several advantages, the most significant of which is the relatively high expression levels. With low expression systems, endogenous enzyme activity can be a significant concern. Enzyme activity assays require the use of large numbers of cells or a concentrated cell homogenate. The low expression levels can also make metabolite analysis difficult and sensitivity low. Minor metabolites would not be detectable. With low expression levels the differences between the control and test cells become more difficult to verify. More replicates are required, and assay replicate variability must be controlled carefully.

Another drawback associated with low expression levels in other systems is that they can only be used for analyzing compounds that become extremely reactive once they are metabolized. Typical compounds used are aflatoxin, benzo[a]pyrene, 2-amino-3-methylimidazole[4, 5-*f*]quinoline, NNK [4-(methylnitrosamino)-1-(3-pyridyl)-4-butanol], NDEA (*N*-nitrosodi-

ethylamine), NNA [1-*N*-methyl-*N*-nitroso)-1-(3-pyridyl)-4-butanol], and similar aromatic amines and nitrosamines [2]. Because these compounds are highly reactive following P450 activation, very little enzyme is required to produce a cytotoxic or mutagenic dose. Granted, these are extremely important compounds and understanding their routes of metabolism is necessary for predicting their effects in humans. However, it is clear that it would be advantageous to be able to broaden the range of compounds for which these *in vitro* assays are useful. The one or two orders of magnitude greater expression levels available with a baculovirus-based system allow researchers to test hypotheses with all of the above compounds, plus a potentially huge number of chemicals that may be an order of magnitude less toxic, but are nevertheless of human health concern in occupational and industrial settings. In addition, high level expression systems may prove invaluable for conducting metabolism studies in which stereospecific analysis requires the need for larger amounts of analyte.

There are several other advantages of a baculovirus-based toxicity assay system. There are baculovirus transfer vectors available that have as many as 23 possible cloning sites, 7 for the polyhedrin promoter and 16 for the P10 promoter. This makes the system very compatible with currently used cDNA

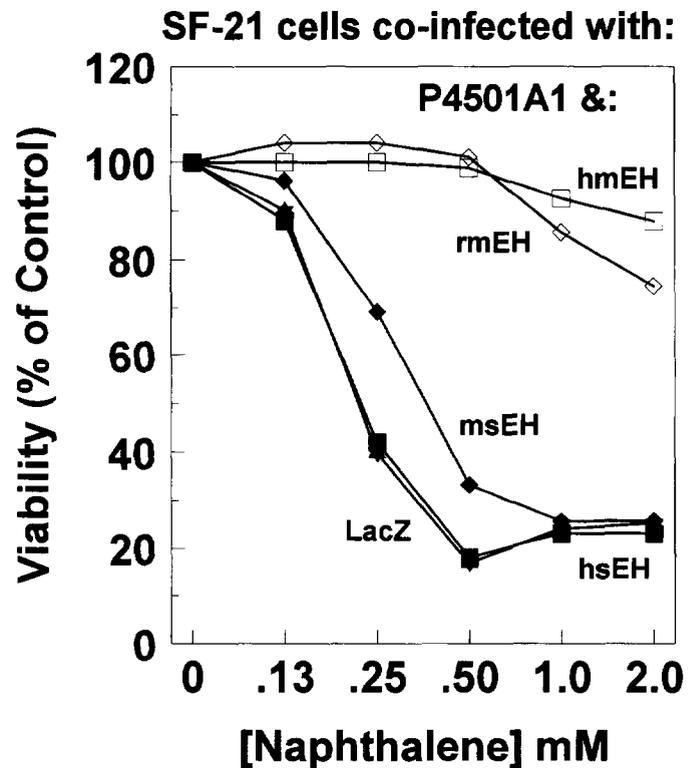


FIG. 7. Effects of epoxide hydrolase virus co-infection on the toxicity of naphthalene in P4501A1/reductase-infected cells. Cells were co-infected with the indicated viruses at an MOI of 1. Control infections contained the LacZ virus (instead of the P4501A1/reductase virus) along with one of each of the five other viruses listed. Abbreviations: hmEH, human microsomal EH; rmEH, rat microsomal EH; msEH, mouse soluble EH; and hsEH, human soluble EH.

cloning vectors and provides a tremendous amount of flexibility for constructing recombinant viruses containing one, two, or even more inserts. The baculovirus system can be used to express as many as four inserts at high levels [42]. Because more than one enzyme can be expressed at the same time in the same cell using this system, metabolic activation, metabolic detoxification, or both processes can be evaluated using any of the above endpoints. In many currently available systems, the cells must be continually passaged and subjected to constant exposure to drugs for maintenance of the expressed plasmid. With every cell passage, therefore, there is the possibility of revertants and gradual loss of activity. An advantage of the baculovirus-based system, is that cells are maintained without selection agents. The baculovirus expression system is much less expensive to use than are mammalian cell systems. Medium can be routinely used with 3% serum rather than 10%, and the cells can be adapted to serum-free medium. CO₂ is not required, expensive selection agents such as G418, hygromycin, or histidinol are not needed, and the cells can be grown at room temperature without the need for an incubator. Because the stock baculoviruses are stable at 4° in the dark for up to 1 year [43], no liquid nitrogen or -80° freezer space is required. This saves a considerable amount of time and space and eliminates the problems of mutations associated with continual propagation of recombinant organisms under selection pressure. The assay is very quick and automated compared with other systems that use clonogenic survival as an endpoint. New methods have been developed for producing recombinant baculoviruses at frequencies approaching 100% [44]. This makes the construction of recombinant viruses extremely easy and straightforward. The viruses pose no safety risk, and the system offers the ability to measure several toxicological endpoints (e.g. cytotoxicity and adduct formation).

The host cells that are infected by baculoviruses are insect cells rather than mammalian cells, and it could be argued that this is a disadvantage for an *in vitro* toxicity assay system. However, SF-21 cells are eukaryotic cells, and they provide a eukaryotic environment that facilitates proper folding, disulfide bond formation and other post-translational modifications required for the biological activity of eukaryotic proteins. These include signal cleavage, proteolytic cleavage, N-glycosylation, O-glycosylation, acylation, amidation, phosphorylation, prenylation, and carboxymethylation. The sites of these post-translational modifications are usually at identical sites on the proteins produced in insect and mammalian cells [20]. This argues that SF-21 cells are typical of most eukaryotic cells. Indeed, we found that the toxicity of all of the epoxides used in this study was very similar to that found using HeLa cells.

Baculovirus infection results in cessation of host gene expression by 24–36 hr post-infection. After this time, host gene expression is primarily, if not exclusively, of viral origin [20, 45]. The infrastructure and integrity of the cells are intact, but protein expression is determined solely by the coding sequences contained in the virus genome. For experimentally testing mechanisms of metabolism, therefore, this system is ideal. However, virus infection will eventually kill the host

SF-21 cells, and because of this, the baculovirus expression system is considered a transient expression system. In this study, we showed that there is a 24- to 48-hr time frame (from 48 to 96 hr post-infection) during which virus-infected SF-21 cells are actively expressing recombinant protein; the cells show no significant signs of virus-specific toxicity.

We have validated this new *in vitro* toxicity assay system using msEH as a model epoxide hydrolase, and the rat P4501A1 as a model cytochrome P450. sEH was discovered approximately 20 years ago, and its endogenous role remains unknown. sEH is found in nearly all animals and in all tissues that have been studied. Because of its broad substrate specificity, sEH is hypothesized to be an important enzyme for protecting cells from the cytotoxic and genotoxic effects of many of the endogenous and exogenous carcinogenic epoxides to which we are exposed. There is, however, little direct experimental evidence supporting this. Previous work has relied on sEH inhibitors to test this hypothesis. These inhibitors, however, are themselves toxic, are never completely specific, and may have other unknown effects. In a previous study [18], we expressed msEH in the COS-7 mammalian cell line but found very low levels of enzyme activity. This suggests that COS-7 cells can down-regulate msEH expression and may be typical of many other mammalian cell lines. In addition, we found evidence of endogenous sEH expression in COS cells [18], but have not detected sEH activity in infected SF-21 cells. The baculovirus expression system, therefore, represents an ideal *in vitro* model system for analyzing enzymes that may be expressed poorly in other eukaryotic expression systems.

Because the msEH had not been expressed in the baculovirus system prior to this study, it was important to provide qualitative and quantitative data verifying the similarity of the recombinant and wild-type enzymes. We compared several physical and kinetic parameters. All of these studies suggest that the recombinant and wild-type msEH are very similar. The results obtained from the regioselectivity study suggest that the geometry and the environment about the active site of the wild-type and recombinant msEH are identical.

Using the baculovirus expression system, we demonstrated that sEH can protect eukaryotic cells from cytotoxicity and adduct formation caused by exposure to several toxic epoxides. In addition, we show for the first time that diols produced from C₁₈ fatty acid monoepoxides are more toxic to SF-21 cells and HeLa cells than are the parent epoxides. Since these fatty acid epoxides are excellent substrates for msEH [35], this implies that like many P450 isozymes, sEH can metabolize and thereby activate a compound that is normally non-toxic. This hypothesis is supported by our results showing that the diols of fatty acid epoxides were toxic to SF-21 cells at the same concentrations as were the epoxides to cells expressing either the mouse or human sEH. These results contradict the widely held belief that epoxides are always more toxic than their corresponding diols and demonstrate the utility of the baculovirus system for providing leads in this area.

The significance of this finding is not yet clear; however, these results point out a previously unknown and potentially important route of fatty acid-mediated toxicity. Fatty acid ep-

oxides could come from a variety of sources including the diet [46, 47], lipid peroxidation [48], and P450 catalyzed epoxidation [49, 50]. Although the concentrations causing toxicity are relatively high, at lower doses these diols may have potent physiological effects. Our results suggest that other C₁₈ fatty acid epoxides also might be metabolized by sEH to form more toxic compounds. The monoepoxides of linoleic acid, for example, are termed leukotoxins and have been associated with adult respiratory distress syndrome, disseminated intravascular coagulation, and cardiovascular shock in burn patients [51–53]. These compounds also are excellent substrates for sEH [34], and, based on our results here, we hypothesize that the diols rather than the parent epoxides may be the toxic agents involved.

We also validated this assay system using the rat P4501A1 as a model cytochrome P450. Theoretically, this same approach could be used for any P450 isozyme, making this a very general and flexible system for P450 expression. Naphthalene was used as a model compound since it was shown previously to be activated by rat cytochrome P4501A1 to the arene epoxide, and then further metabolized to the corresponding diol by microsomal EH [54–56]. In addition, naphthalene is known to be cytotoxic to Clara cells *in vivo* [57] and *in vitro* [58]. Our results show that although naphthalene is not a highly toxic compound, its metabolism can be studied experimentally using the baculovirus system. The concentrations causing toxicity in the baculovirus system are very similar to the concentrations causing toxicity in isolated Clara cells [58]. We tried several other substrates as negative controls with the P4501A1 virus since we thought it was possible that with the high expression levels in this system, compounds that normally are not considered good substrates could be metabolized to some extent and produce false positives. The compounds we tried were thalidomide and safrole. Thalidomide is known to be metabolized by a P450 enzyme expressed only in fetal tissues [59]. The mechanism of safrole activation is unknown but is not due to P4501A1 activity [60]. Both of these compounds gave the expected negative results at a concentration of 1 mM.

The next step is to further develop this expression system to its full potential by co-expressing these and other important enzymes in order to determine their role in activating and detoxifying chemicals that affect human health. By analyzing the metabolites produced in these experiments we can better understand the relationship between toxicity and xenobiotic metabolism.

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