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EXPRESSION OF GLUTATHIONE *S*-TRANSFERASE AND PHENOL SULFOTRANSFERASE, BUT NOT OF UDP-GLUCURONOSYLTRANSFERASE, IN THE HUMAN LUNG TUMOR CELL LINES NCI-H322 AND NCI-H358

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Abstract—The expression of xenobiotic-metabolizing enzymes was studied in the human lung tumour cell lines NCI-H322 and NCI-H358. These cells are derived from adenocarcinomas and exhibit features of Clara cells and alveolar type II cells, respectively. Examination of the *in vitro* activities showed that both cell lines lack UDP-glucuronosyltransferase against the substrates 3-hydroxybenzo[*a*]pyrene (3-OH-BaP) and 4-hydroxybiphenyl (4-OH-Bph) and that *in vitro* conjugation of sulfate with 3-OH-BaP was only just detectable. In contrast, both cell lines showed fairly high levels of glutathione-*S* transferase activity with the substrate 1-chloro-2,4-dinitro-benzene (54.4 and 83.0 nmol/min × mg protein, respectively) and of glutathione (81 and 41 nmole/mg protein, respectively).

The metabolic capacity of intact NCI-H322 and NCI-H358 cells was examined using benzo[*a*]pyrene (BaP) and 3-OH-BaP as substrates. The cell lines formed sulfate conjugates from 3-OH-BaP (4.5 and 0.4 pmol/min × mg protein, respectively) but did not produce any detectable glucuronides. When cultures of the two cell lines were exposed to BaP, phenolic products accumulated in the growth medium. NCI-H322 cells also formed some sulfate conjugates, whereas such conjugates were barely detectable in the medium of NCI-H358 cells. In contrast A549, a human pulmonary adenocarcinoma cell line known to contain UDP-glucuronosyltransferase activity, efficiently conjugated 3-OH-BaP to glucuronic acid and converted the primary phenolic products formed from BaP to glucuronides.

Thus the NCI-H322 and NCI-H358 cells are exceptional in that they possess no or very little glucuronosyltransferase activity but exhibit appreciable monooxygenase activity. The cell lines may therefore be of interest for examining the biological effects of potentially toxic chemicals which are otherwise detoxified by glucuronic acid conjugation. The cells may also be useful as test systems for evaluating the potential cytotoxicity and genotoxicity of chemicals to human lung.

Cells in continuous culture which express specific xenobiotic-metabolizing enzymes [1] are being used increasingly in pharmacological and toxicological studies. Cell lines derived from human tissues are of particular interest when examining the metabolism and biological effects of xenobiotics in an attempt to estimate human risk.

Recently, two cell lines derived from human bronchiolo-alveolar carcinomas, NCI-H322 and NCI-H358, have been shown to contain a number of enzymes involved in the activation of chemicals. They possess cytochrome P-450-dependent monooxygenases [2, 3], cytochrome *c* reductase [2], and prostaglandin endoperoxide synthetase E_2 [4]. NCI-H322 cells have the morphological and functional features of Clara cells whereas NCI-H358 cells have the features of alveolar type II cells [5, 6].

The steady state level of reactive intermediates depends equally on the rates of formation and degradation, so that the presence of "inactivating" enzymes is a major determinant of the biological response to noxious chemicals. In the present study we examined NCI-H322 and NCI-H358 cells for their expression of the 3 major types of inactivating enzymes, glutathione *S*-transferase (GSTase) (EC 2.5.1.18), UDP-glucuronosyltransferase (GTase) (EC 2.4.1.17) and phenol sulfotransferase (STase) (EC 2.8.2.1).

3-Hydroxybenzo[*a*]pyrene (3-OH-BaP)‡ and 4-hydroxybiphenyl (4-OH-Bph) served as marker substrates for GTase activities. 3-OH-BaP is metabolized by one or more forms of the transferase [7] called by its operational name UDP-glucuronosyltransferase 1 (GTase/1) [8]. GTase/1 is inducible by polycyclic aromatic hydrocarbons and occurs in virtually all mammalian tissues [9]. 4-OH-Bph is a substrate for a second transferase form, UDP-glucuronosyl-transferase 2 (GTase/2), which is inducible by phenobarbital [9, 10] and has been observed in only a few tissues, notably the liver and small intestine [9].

‡ Abbreviations used: BaP, benzo[*a*]pyrene; GSH, glutathione; GSTase, glutathione *S*-transferase; GTase, UDP-glucuronosyltransferase; GTase/1, UDP glucuronosyltransferase activity directed toward the substrate 3-hydroxybenzo[*a*]pyrene; GTase/2, UDP-glucuronosyltransferase activity directed toward the substrate 4-hydroxybiphenyl; 3-OH-BaP, 3-hydroxybenzo[*a*]pyrene; 4-OH-Bph, 4-hydroxybiphenyl; STase, phenol sulfotransferase.

STase was also assayed with 3-OH-BaP as substrate. At present it is not known whether, and to what degree, an overlap exists between the many STase forms for this substrate. 1-Chloro-2,4-dinitrobenzene was used to monitor for conjugation with glutathione (GSH). This substrate is known to be metabolized by most of the GSTase forms isolated so far [11].

Two additional cell lines were investigated as "positive" and "negative" controls for GTase expression: A549 cells, another human lung adenocarcinoma cell line which has previously been shown to express GTase/1 activity, and the Chinese hamster lung cells V79 which lack detectable GTase activity [12].

MATERIALS AND METHODS

Cell cultures. 2×10^5 cells were seeded in 100 mm or 60 mm Falcon plastic dishes. NCI-H322 and NCI-H358 cells were grown in RPMI 1640 medium, A549 and V79 cells in Dulbecco's Minimum Essential Medium, each containing 10% fetal calf serum, 100 units penicillin and 100 μg/ml streptomycin, at 37° in an atmosphere of 93% air and 7% CO_2.

Preparation of media containing benz[a]anthracene or substrates. Benz[*a*]anthracene was added to complete growth media in dimethylsulfoxide to give a final concentration of 20 μM and 0.1% of the solvent. Preliminary experiments had shown that the Phenol Red contained in standard growth media interferes with the determination of fluorescent metabolites. Therefore, we used a Phenol Red free modified Hams's F12 medium [13], which supported the growth of the 4 cell lines examined and was readily available in this laboratory, when monitoring the formation of fluorescent metabolites by intact cells. 3-OH-BaP or benzo[*a*]pyrene (BaP) were added to the modified Ham's F12 medium in methanol to give a final concentration of 50 μM and 0.1% of the solvent. 4-OH-Bph was added in dimethylsulfoxide to give final concentrations of 0.05, 0.1, 0.2 and 0.5 mM and a maximum of 0.8% of the solvent.

Enzyme assays. Monolayers were rinsed twice with cold Dulbecco's phosphate buffered saline and cells were collected and stored at −80° as described previously [14]. Enzyme activities were determined by the following procedures: hydroxylation of BaP according to Wiebel *et al.* [14], GTase/1 with the substrate 3-OH-BaP according to Singh and Wiebel [15]. The amount of cellular protein in the reaction mixture of the GTase/1 assay was 0.05–0.12 mg. The reaction was carried out for 60 min. GTase/2 with the substrate 4-OH-Bph was measured according to Bock *et al.* [9] using 0.15–0.21 mg of cellular protein and incubating the samples up to 60 min; STase with the substrate 3-OH-BaP was assayed according to Wiebel *et al.* [16] with the modification that a 30,000 *g* supernatant was used; GSTase with the substrate 1-chloro-2,4-dinitro-benzene was determined according to Kamisaka *et al.* [17] as described by Summer and Wiebel [18].

Determination of fluorescent products formed by intact cells from benzo[a]pyrene, 3-OH-BaP, or 4-OH-Bph. Monolayer cultures (60 mm dishes) approaching confluency were exposed to the substrates added in 2 ml of medium. After various times 0.2 ml aliquots of the medium were withdrawn and examined for the appearance of fluorescent products. For the determination of BaP-glucuronides the medium was extracted with methanol:chloroform (1:2, v/v) as described for the *in vitro* GTase/1 assay [15], and the fluorescence in the aqueous phase determined at 378 nm excitation and 425 nm emission. The identity of the glucuronides was established from their fluorescence spectra. They were quantified as described previously [15]. For the determination of BaP-sulphates the medium was extracted with 25 volumes of propanol:hexane (1:3, v/v) and the fluorescence in the aqueous phase determined at 388 nm emission and 415 nm excitation [16]. BaP-phenols were extracted into 2 ml of propanol:hexane (1:3, v/v). 1.0 ml of the organic phase was vigorously shaken with 1.0 ml of 1 N NaOH and the amount of BaP-phenols in the alkaline phase assessed as described previously [14]. The formation of 4-OH-Bph-glucuronides was examined following the *in vitro* assay developed by Bock *et al.* [9].

Protein determination. Cellular protein was determined by the method of Lowry *et al.* [19] using bovine serum albumin as standard.

Materials. RPMI 1640 medium was purchased from Biochrom KG, Berlin, F.R.G.; fetal calf serum from the Grand Island Biological Company, Karlsruhe, F.R.G.; 4-OH-Bph from EGA-Chemie, Steinheim/Albuch, F.R.G.; 1-chloro-2,4-dinitrobenzene, *p*-nitrophenosulfate, and adenosin-3′,5′-diphosphate from Sigma, Taufkirchen, F.R.G. 3-OH-BaP was a generous gift from the National Cancer Institute Standard Chemical Carcinogen Repository, Bethesda, MD, U.S.A. Sources of the other materials are given in refs 12 and 15.

RESULTS

In vitro *activities of xenobiotic-metabolizing enzymes*

The capacities for *in vitro* hydroxylation of BaP and the inducibility of the hydroxylase by benz[*a*]-anthracene, were similar in both cell lines (Table 1) and of the same order of magnitude as those observed previously [2]. Neither NCI-H322 nor NCI-H358 cells expressed detectable GTase activity (GTase/1) directed towards 3-OH-BaP (Table 1). The level of detectability for the transferase was 0.1 pmol/min × mg protein, i.e. 4 orders of magnitude lower than the specific activity observed in certain rat hepatoma cells [1]. Exposure of NCI-H322 or NCI-H358 cells to benz[*a*]anthracene for 24 hr did not elicit any GTase/1 activity. It was thought possible that the lung cell lines contain β-glucuronidase since it has been found to be present in human alveolar type II cells [20]. Therefore we added the β-glucuronidase inhibitor D-saccharolactone (5 mM) to the reaction mixture. However, the cell lines still failed to produce measurable amounts of BaP-glucuronides.

The test for GTase/2 activity (against the substrate 4-OH-Bph) was also negative in both bell lines (Table 1). The background fluorescence in this assay corresponded to a conjugation rate of 20 pmol 4-OH-Bph/min × mg protein, i.e. the level of detect-

Table 1. *In vitro* activities of xenobiotic-metabolizing enzymes in human lung cell lines NCI-H322 and NCI-H358

Enzyme	Specific activity*†¶ NCI-H322	NCI-H358
BaP-hydroxylase¶		
untreated	0.10 ± 0.01§	0.20 ± 0.02
benz[*a*]anthracene-treated‡	2.66 ± 0.14	4.17 ± 0.20
UDP-glucuronosyltransferases		
GTase/1¶		
untreated	<0.1	<0.1
benz[*a*]anthracene-treated	<0.1	<0.1
GTase/2†	<0.02	<0.02
Sulfotransferase*	<0.75	n.d.‖
Glutathione *S*-transferase†	53.4 ± 9.5	83.0 ± 27.0

* pmol/min × mg cytosolic protein.
† nmol/min × mg cellular protein.
‡ Cells were treated with 20 μM benz[*a*]anthracene for 24 hr. Other conditions as described in Materials and Methods.
§ Values give the mean ± S.D. of determinations on triplicate cultures.
‖ n.d. = not determined.
¶ pmol/min × mg cellular protein.

ability was 200 times lower than that for glucuronic acid conjugation with 3-OH-Bap.

The formation of sulfate conjugates with 3-OH-BaP was tested in the 30,000 *g* supernatant of NCI-H322 cells (Table 1). The specific fluorescence observed was 50–100% above the background level, corresponding to a specific transferase activity of about 0.75 pmol/min × mg protein.

In contrast to the GTases and STases, appreciable activities of GSTase were found in both NCI-H322 and NCI-H358 cells, i.e. 53 and 83 nmol/min × mg protein, respectively (Table 1). The two cell lines also contained substantial amounts of glutathione, i.e 81.7 ± 4.9 and 41.5 ± 4.2 nmol/mg protein, respectively (mean ± S.D. for triplicate determinations).

Conjugation of phenols by intact cells

In order to increase the level of detectability of the glucuronidation and sulfatation reaction we exposed cultures of intact NCI-H322 and NCI-H358 cells to 3-OH-BaP (or 4-OH-Bph) for several hours and measured the amount of water-soluble metabolites accumulating in the growth medium (Fig. 1). The amount of fluorescent material retained in the cells was found to be only a minor fraction of the total water-soluble products formed during several hours of exposure to the phenols (3-OH-BaP). We included A549 and V79 cells in these studies as models of GTase/1-containing and GTase/1-deficient cells.

As expected, intact A549 cells formed products which were not extractable into methanol:chloro-

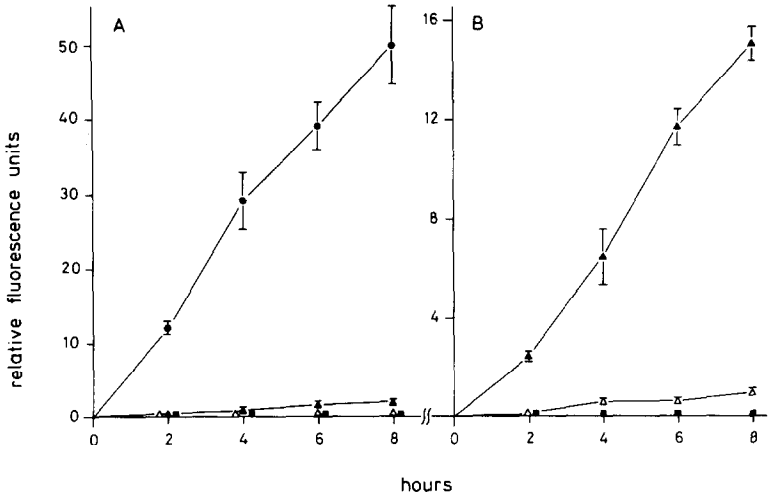

Fig. 1. Time course of formation of water-soluble 3-OH-BaP products by human and hamster lung cells: ▲, NCI-H322; △, NCI-H358; ●, A549; ■, V79. Cultures (60 mm dishes) approaching confluency were exposed to 50 μM 3-OH-BaP. At various times aliquots of the medium were withdrawn and assayed for the presence of fluorescent products in the aqueous phase as described in Materials and Methods. (A) Fluorescence at 378 nm excitation/425 nm emission; (B) fluorescence at 388 nm excitation/415 nm emission. For the fluorescence spectra of the water-soluble 3-OH-BaP products formed by NCI-H322 and A549 cells see Fig. 2. Note the difference in the scales of the ordinates. Numbers give the mean ± standard deviation of determination on triplicate cultures.

form (Fig. 1A) and had the fluorescent characteristics of BaP-3-glucuronide (Fig. 2A; cf. [15, 21]). The rate of glucuronidation was approximately 70 pmol/min × mg protein. No glucuronides were detectable in cultures of V79 cells. Some water-soluble, fluorescent material appeared in the medium of NCI-H322 cells. The total amount was less than 2% of that of the fluorescent products formed by A549 cells (Fig. 1A). NCI-H358 cells did not produce any metabolites with a fluorescence at 378 nm excitation and 425 nm emission.

The fluorescence spectra of the water-soluble metabolites contained in the medium of NCI-H322 cells resembled those of BaP-3-sulfate, and not BaP-3-glucuronide (Fig. 2B), with maxima at 388 nm excitation and 415 nm emission [22]. When the medium was extracted with propanol:hexane to assay for the more hydrophilic BaP-3-sulfate it became apparent that the NCI-H322 cells were capable of producing considerable amounts of this conjugate. The rate of BaP-3-sulfate formation was approximately 4.5 pmol/min × mg protein over a period of at least 8 hr (Fig. 1B). Under these conditions of extraction some STase activity was also detectable in NCI-H358 cells (Fig. 1B).

The fluorescence spectra of BaP-3-sulfate and BaP-3-glucuronide differ to such an extent (cf. Fig. 2) that the presence of greater amounts of glucuronide in a sulfate:glucuronide mixture would be readily recognizable. A rough estimate indicates that NCI-H322 cells—if they possess any GTase/1 at all—conjugate at most 0.05 pmol of 3-OH-BaP/min × mg protein to glucuronic acid.

No glucuronides of 4-OH-Bph were detectable in the medium of NCI-H322 cells which had been exposed to the phenol (0.05–0.5 mM) for up to 48 hr.

Formation of conjugates from BaP by intact cells

In order to examine the fate of phenols formed within the cells we exposed NCI-H322 cells to BaP and monitored the appearance of fluorescent phenols and conjugates in the medium. Intact A549 cells formed water soluble product(s) with the fluorescence characteristics of BaP-3-glucuronide, which accumulated linearly in the medium for 24 hr (Fig. 3B). Only minute amounts of fluorescent BaP-phenols were detected in the medium from these cells (Fig. 3A). Quite different results were obtained with NCI-H322 and NCI-H358 cells. These cells produced fluorescent organic-soluble products (Fig. 3A) which were extractable into alkali and had a fluorescence spectrum similar to that of 3-OH-BaP (spectrum not shown). The accumulation of these phenolic products was linear in NCI-H358 cells but levelled off in NCI-H322 cells after 8 hr. No material with the fluorescence characteristics of BaP-3-glucuronide was detectable. After a lag of about 8 hr NCI-322 cells produced considerable amounts of some metabolites which were not extractable into propanol:hexane (Fig. 3B) and had an excitation spectrum similar to that of BAP-3-sulfate. The maximum of the emission wavelength, however, was shifted to 410 nm (data not shown) indicating the presence of water-soluble BaP-products other than BaP-3-sulfate. NCI-H358 cells produced only very small amounts of these fluorescent water-soluble products.

Cytotoxicity of 3-OH-BaP

When NCI-H322, NCI-H358 or V79 cells were exposed to 3-OH-BaP (50 μM) they rounded up after 4–8 hr and began to detach after a further 16 hr (data not shown). In contrast, A549 cells did not show any

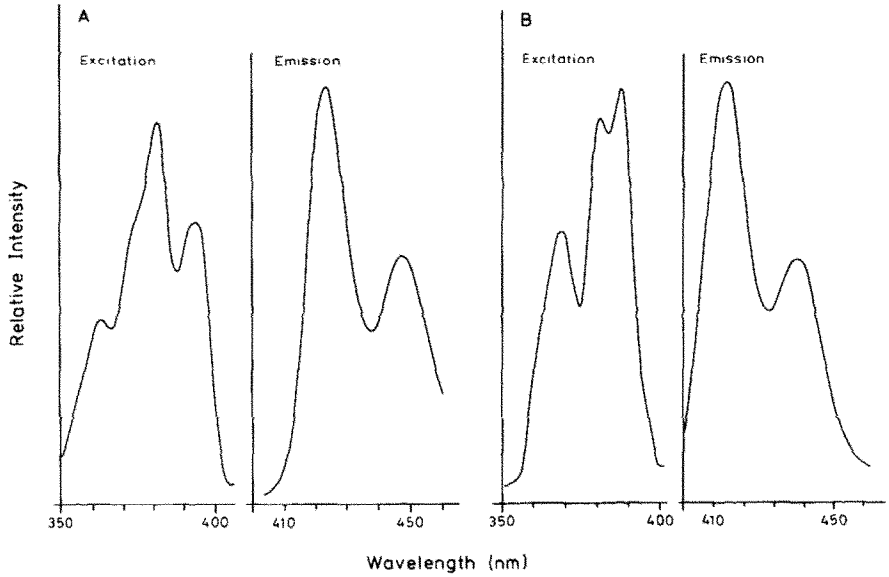

Fig. 2. Fluorescence spectra of water-soluble products formed by intact human lung cells from 3-OH-BaP: (A) cultures of A549 cells; (B) cultures of NCI-H322 cells. Conditions as described in Fig. 1. Growth medium from cultures exposed to 3-OH-BaP for 8 hr. Fluorescence spectra were recorded at (A) 378 nm excitation/425 nm emission and (B) 388 nm excitation/415 nm emission.

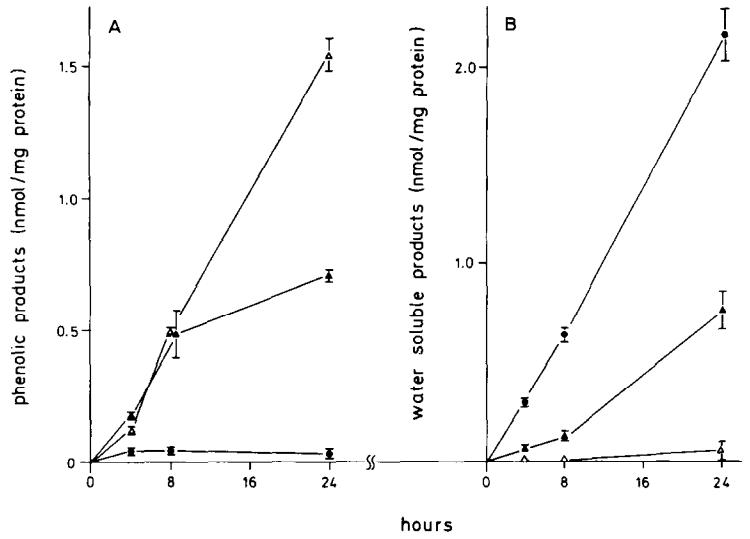

Fig. 3. Time course of formation of phenolic and water-soluble products from BaP by intact NCI-H322 and A549 cells. Cultures of NCI-H322 cells (▲), NCI-H358 cells (△) and A549 cells (●) were exposed to benz[*a*]anthracene (20 μM) for 18 hr. The medium was then replaced by fresh medium containing BaP (50 μM). Aliquots of the medium were withdrawn at various times and assayed for the presence of fluorescent products in the aqueous phase as described in Materials and Methods. (A) Phenolic products appearing in the medium were quantified by measuring the fluorescence in the alkaline extract of the organic-soluble phase at 392 nm excitation/522 nm emission and equating the fluorescence with that of 3-OH-BaP. (B) The amounts of water-soluble products formed by NCI-H322 and NCI-H358 cells were determined by the appearance of fluorescence with wavelength maxima at 388 nm excitation/415 nm emission ("sulfate conjugates"), that of water-soluble products formed by A549 cells by measuring at the fluorescence maxima of 378 nm excitation/425 nm emission ("glucuronic acid conjugates"). Numbers give the mean ± standard deviation of determinations on triplicate cultures. Other conditions as described in Fig. 1.

overt sign of toxicity over a 24 hr observation period. Exposure to BaP (50 μM) had no apparent effect on the morphology of the 4 cell lines.

DISCUSSION

The most striking aspect of the present findings on xenobiotic-metabolizing enzymes in NCI-H-322 and NCI-H358 is their apparent lack of GTase activity. These are the first continuous cell lines reported, apart from the cat cell line C81 [12], which are proficient in the oxidation of xenobiotics but deficient in their conjugation with glucuronic acid.

GTases

GTase/1 has been found in most cell lines [12] in correspondence with its virtually ubiquitous distribution *in vivo*. Generally, the specific activity of GTase/1 *in vivo* and in cultured cells [12] greatly exceeds that of the cytochrome P-450-dependent monooxygenases safeguarding the removal of the reactive nucleophilic products. As shown here this is also the case in A549 cells in which the ratio of glucuronidation of 3-OH-BaP to hydroxylation of BaP was approximately 35:1. A few cell lines such as V79, NC37BaEV [12], or C1300 (unpublished observation) do not contain detectable GTase/1 activity. However, these lines also lack monooxygenase activity alleviating the need for the inactivation pathway.

The lack of GTase/2 in the lung cell lines NCI-H322 and NCI-H358 is not surprising since the activity of this enzyme *in vivo* is localized primarily in the liver [9]. In continuous cell cultures GTase/2 has only been observed in highly differentiated lines of hepatic origin (Wiebel, unpublished observation).

The possibility that NCI-H322 and NCI-H358 cells contain GTase/1 activities which are below the level of detectability of the assay used cannot be discounted. However, such activities would certainly be too low to have a significant impact on the metabolism of the marker substrate 3-OH-BaP. This is indicated by the accumulation of phenolic products from BaP by intact NCI-H322 cells, in contrast with the efficient removal of these phenols by the GTase/1-containing A549 cells. Another line of evidence for the functional lack of GTase/1 activity in NCI-H322 and NCI-H358 cells comes from their sensitivity to the cytotoxic effects of 3-OH-BaP. The cytotoxicity of 3-OH-BaP is most likely mediated by its derivatives, presumably quinones and quinoles, which generate reactive oxygen species by redox cycling [23]. BaP-quinoes have been shown to be conjugated to glucuronic acid [24–26] thereby interrupting the redox cycling and preventing the cytotoxity of the phenol [21]. Our preliminary results show that the GTase/1-containing A549 cells tolerate the presence of 3-OH-BaP whereas NCI-H322 and NCI-H358 cells are incapable of detoxifying the phenols and their derivatives.

STase

Although STase activity can be detected in intact NCI-H322 and NCI-H358 cells it is clearly not suf-

ficient to prevent the accumulation of phenolic products formed from BaP nor to counteract the cytotoxicity of 3-OH-BaP (50 μM). However, the STase activity may suffice to inactivate the—presumably—small amounts of cytotoxic products arising from BaP via its phenolic metabolites. As shown by Nemoto *et al.* [22] appreciable amounts of sulfate conjugates are formed when BaP-quinones (the putative cytotoxic agents) are incubated with the 105,000 *g* supernatant of rat liver in the presence of the appropriate cofactors.

GSTase

Conjugation with GSH is a major pathway for the inactivation of reactive electrophiles. The amount of GSH and the activity of GSTase in NCI-H322 and NCI-H358 cells was similar to those observed in other continuous cell lines of both hepatic and extrahepatic origin, including A549 cells [18]. The GSTase activities are also comparable to those observed in preparations of human lung [27].

Relationship of enzyme activities in cultured cells and in vivo

It is open to question whether, or to what extent the balance of activating and inactivating pathways observed in NCI-H322 and NCI-H358 cells reflects that of cells in human lung *in vivo*. The data available on BaP-metabolism in human respiratory tissue has been derived from organ cultures, which show the sum of BaP-products formed by the many cell types contained in these tissue specimens. However, it is striking that organ cultures of human peripheral lung [28], bronchus or trachea [29] produce only very small amounts of glucuronides from BaP. The major water-soluble products in these cultures have been identified as conjugates with sulfate and GSH. This contrasts with the conjugation pattern found in rodent respiratory tissue in which glucuronides and not sulfate conjugates are the major water-soluble detoxification products [28, 29]. Epithelial cells from other human tissues, e.g. bladder and skin in early passage culture [30, 31] or explant cultures of colon [29], also produce few or no glucuronides from BaP. Peripheral lung has a particularly low overall conjugating ability [29]. Poor glucuronidation, however, is not a general property of human tissues. Human liver and kidney, for example, form appreciable amounts of glucuronides [32, 33]. Also, several lines derived from human bronchial carcinomas conjugated 1-naphthol and 3-OH-BaP predominantly to glucuronic acid [34].

Conclusion

NCI-H322 and NCI-H358 cells, which have the properties of Clara cells and alveolar type II cells, respectively, are of interest for toxicological studies for two reasons. Firstly, the cell lines are exceptional in that they express monooxygenase activity but lack detectable GTase/1 activity. Thus they offer versatile tools for analysing the role of glucuronic acid conjugation in the inactivation of chemicals in intact cells. Secondly, the cell lines appear to mimic conditions of human lung *in vivo*, both with respect to their capacity for activation by cytochrome P-450-dependent monooxygenases, and to their deficiency for inactivation by glucuronidation. They may thus be useful for assessing the cytotoxic potential of chemicals to human lung.

Note added in proof: The cloning efficiency of NCI-H322 cells was strongly reduced after exposure to concentrations of BaP above 1 μM for 24 hr in spite of the fact that no cytotoxicity was detectable by morphological criteria.

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