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XENOBIOTIC-METABOLIZING ENZYME ACTIVITY IN HUMAN NON-SMALL-CELL DERIVED LUNG CANCER CELL LINES

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Abstract—Human lung cancer cell lines in culture were investigated for the expression of monooxygenase and other xenobiotic-metabolizing enzyme activities. Two brochiolo-alveolar carcinoma derived cell lines (NCI-H322 and NCI-H358) and two small-cell carcinoma derived cell lines (NCI-H128 and NCI-H69) were used. Previous work has shown that NCI-H322 has ultrastructural features of Clara cells while NCI-H358 shows characteristics of alveolar type II cells [Schuller *et al.*, *Proc. Am. Ass. Cancer Res.* **26**, 27 (1985)]. NCI-H128 and NCI-H69 show very poor differentiation of cytoplasmic organelles. Cytochrome P-450 levels were spectroscopically detectable only in NCI-H322. Both NCI-H322 and NCI-H358, but not NCI-H69 and NCI-H128, exhibited aryl hydrocarbon hydroxylase (using benzo[*a*] pyrene as substrate) and ethoxycoumarin *O*-deethylase activities. These activities were highly inducible following pretreatment with the polycyclic aromatic hydrocarbons (PAH) β-naphthoflavone or benzo[*a*] anthracene. The PAH produced a 2-fold increase in spectroscopically detectable cytochrome P-450 levels in NCI-H322. Following induction, cytochrome P-450 was also spectroscopically detectable in NCI-H358. No aldrin epoxidase activity was present in either untreated or pretreated cell lines. Pretreatment with phenobarbitone or dexamethasone did not induce the aryl hydrocarbon hydroxylase activity in either NCI-H322 or NCI-H358. The ethoxycoumarin *O*-deethylase activity in β-naphthoflavone-pretreated NCI-H322 and NCI-H358 was inhibited in a concentration-dependent manner by ellipticine, α-naphthoflavone, cimetidine or metyrapone. Untreated NCI-H322 and NCI-H358 also contained cytochrome b_5, NADPH cytochrome *c* reductase and epoxide hydrolase activities. None of these enzyme activities measured was detectable in the untreated or pretreated small-cell derived cancer cell lines (NCI-H128 and NCI-H69). These data show that the two bronchio-alveolar carcinoma derived cell lines (NCI-H322 and NCI-H358) exhibit cytochrome P-448-dependent monooxygenase activity and may thus prove useful to study the processes of xenobiotic activation in human lung.

Mammalian cells in culture provide a useful model for the study of the toxic/carcinogenic effects and the mechanism of action of various chemicals [1]. The usefulness of these cells for such studies depends on their expression of xenobiotic-metabolizing enzymes, especially the cytochrome P-450-dependent monooxygenase enzyme system which is involved in the activation of numerous chemicals to their reactive intermediates [2, 3]. The xenobiotic-metabolizing enzyme activity has been studied in a number of continuous mammalian cell cultures, both from normal tissue and from tumors [1]. The cell line A-549, derived from a human lung adenocarcinoma, has been reported to contain low aryl hydrocarbon hydroxylase (AHH†) activity which is inducible by benzo[*a*]anthracene (BA) [1]. This study utilizes two human bronchiolo-alveolar carcinoma derived cell lines whose ultrastructural characterization has been reported by Schuller *et al.* [4]. NCI-H322 consists of cells with Clara cell features [4] and NCI-H358 expresses morphological and functional (phospholipid synthesis) features of alveolar type II cells [4, 5]. These two cell lines have been shown to metabolize the lung toxin 4-ipomeanol [6] and the lung carcinogen *N*-nitrosodiethylamine [7]. They also exhibit prostaglandin endoperoxide synthetase activity, as measured by conversion of exogenous arachidonic acid to prostaglandin E_2 [8]. The well-defined ultrastructural characterization of these cell lines allows correlation between the biochemical spectrum of the cell line and the cell type comprising the tumor of origin. For comparison, two small-cell derived cancer cell lines (NCI-H69 and NCI-H128) which show very poor differentiation of cytoplasmic organelles [4] and which do not metabolize 4-ipomeanol [6] or *N*-nitrosodiethylamine [7] were also used in this study.

MATERIALS AND METHODS

Chemicals. RPMI 1640 and fetal bovine serum were obtained from Biofluids (Maryland, U.S.A.) and HyClone Laboratories (Utah, U.S.A.) respectively. NADPH, benzo[*a*]anthracene, cytochrome *c*, metyrapone, ellipticine, cimetidine and ethoxycoumarin were obtained from the Sigma Chemical

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† Abbreviations: AE, aldrin epoxidase; AHH, aryl hydrocarbon hydroxylase; ANF, α-naphthoflavone; BA, benzo[*a*]anthracene; BNF, β-naphthoflavone; BP, benzo[*a*] pyrene; DMSO, dimethyl sulfoxide; EC, ethoxycoumarin; ECOD, ethoxycoumarin *O*-deethylase; and PAH, polycyclic aromatic hydrocarbon.

Co. (Missouri, U.S.A.). α-Naphthoflavone and β-naphthoflavone were obtained from the Aldrich Chemical Co. (Wisconsin, U.S.A.). Benzo[*a*]pyrene was obtained from Roth (Karlsruhe, F.R.G.) and aldrin from Riedel-de-Haan (Seelze, F.R.G.).

Preparation of the cells for incubation. The two cell lines NCI-H322 and NCI-H358, which grow as monolayers, were maintained in RPMI 1640 medium supplemented with glutamine (2 mM), fetal bovine serum (10%) and gentamycin (50 mg/ml) at 37° in an atmosphere of 95% air/5% CO_2. They were subcultured at a density of approximately 1.2×10^3/sq. cm, and the spent medium was replaced by fresh medium on days 1 and 4. The cells were used when the monolayer had reached near confluency. The two cell lines derived from small-cell carcinoma (NCI-H128 and NCI-H69) which grow as floating aggregates were also maintained as described above. The cells were serially subcultured during the course of the study which was carried out on cell cultures of passage number between 15 and 30.

The cells were exposed to 50 μM β-naphthoflavone (BNF) [9], 20 μM BA [10] or 20 μM BA plus 5 μM dexamethasone [10] for 24 hr. Exposure to 2 mM phenobarbitone [10] or 5 μM dexamethasone [10] was carried out for 72 hr. The medium was replaced daily. Except for phenobarbitone, which was dissolved in the incubation medium, all compounds were added in dimethyl sulfoxide (DMSO) to a final solvent concentration of not more than 0.1%.

Prior to use, the cell monolayer was washed twice with Hanks' balanced salt solution. In the presence of salt solution, the cells were scraped off at 4° and transferred to 50 ml centrifuge tubes. Floating aggregates which do not require scraping were transferred directly to centrifuge tubes. Following centrifugation at 800 rpm and a further wash in Hanks' balanced salt solution, the cells were resuspended in the appropriate buffer, and enzyme activities were determined by the following procedures. AHH was measured by a modification [11] of the method of Nebert and Gelboin [12]. The reaction mixture contained 100 μM benzo[*a*]pyrene (BP) in 25 μl methanol, 5 mM $MgCl_2$ and 200–400 μg of cellular protein in a final volume of 2 ml of 50 mM Tris–HCl buffer, pH 7.6. Aldrin epoxidase (AE) was measured by the method of Wolff *et al.* [13]. The reaction mixture contained 0.2 mM aldrin in 20 μl methanol, 5 mM $MgCl_2$ and 100 μg of cellular protein in a final volume of 0.2 ml of 80 mM phosphate buffer, pH 7.4. Ethoxycoumarin *O*-deethylase (ECOD) was measured as described by Edwards *et al.* [9]. The reaction mixture contained 200 μM ethoxycoumarin (EC) in 25 μl DMSO, 5 mM $MgCl_2$ and 200–400 μg of cellular protein in a final volume of 2 ml of 100 mM Krebs–Henseleit buffer, pH 7.4. For all assays described above, the incubation time was 60 min. NADPH cytochrome *c* reductase was measured by direct spectrophotometry by the method of Phillips and Langdon [14], using homogenized cells. The reaction mixture contained 80 μM cytochrome *c*, 0.3 mM NADPH and 1 mg of cellular protein in a final volume of 1 ml of 0.3 M phosphate buffer, pH 7.7. Epoxide hydrolase was measured by the method of Oesch *et al.* [15]. The reaction mixture contained 25 nmoles [^{3}H]styrene oxide in 25 μl acetonitrile, 120 mM KCl and 1.0 to 1.2 mg of cellular protein in a final volume of 0.5 ml of 0.1 M Tris buffer, pH 9.5. The incubation time was 15 min. Cytochrome P-450 was measured by the method of Omura and Sato [16], cytochrome b_5 by the method of Strittmatter *et al.* [17] and protein by the method of Lowry *et al.* [18].

Inhibition studies were carried out on BNF-induced cells. The cells were harvested, and the cell suspension was prepared as previously described. Ellipticine (concentration range 0.1 to 5.0 μM), α-naphthoflavone (ANF) (concentration range 0.1 to 50 μM) and cimetidine (concentration range 0.1 to 10 mM) were added in DMSO, and metyrapone (concentration range 0.1 to 10 mM) was added in acetone. The final concentration of the solvent was never more than 0.1%. The substrate (EC) was added following a 5-min preincubation with the inhibitor. The incubation time was 60 min. The cell viability, as determined by trypan blue exclusion, was measured at time 0 and at the end of the incubation period (60 min) for AHH, ECOD, and AE.

Statistical analysis was carried out using Student's *t*-test for non-paired samples. A value of $P < 0.05$ was considered significant.

RESULTS

Xenobiotic-metabolizing enzymes in untreated cells. Only NCI-H322 had any spectroscopically detectable levels of cytochrome P-450 (Table 1). The peak of the reduced cytochrome P-450–CO complex was at 448 nm. Both NCI-H322 and NCI-H358 contained basal AHH activity (measured as BP hydroxylase) and *O*-deethylase activity (measured as ECOD) (Table 1). The presence in the incubate of the inhibitor of glucuronide conjugation, salicylamide (0.5 mM), did not have any effect on AHH and *O*-deethylase activities (data not shown). AE activity was not detectable. The reaction rate for each of the above assays was maximal at the substrate concentration used. A range of concentrations (20–150 μM for BP; 25–300 μM for EC; and 20–250 μM for aldrin) was used to arrive at the optimal concentration. NADPH cytochrome *c* reductase and epoxide hydrolase activities and cytochrome b_5 were present in both cell lines (Table 1). All values in NCI-H322 were significantly higher ($P < 0.005$) than the respective values in NCI-H358. None of the enzyme activities were detectable in the human small-cell derived cancer cells lines NCI-H69 and NCI-H128 (data not shown). The limits of detectability for the various assays were as follows: spectroscopic determination of cytochrome P-450 and cytochrome b_5, 10.0 pmole/mg protein; ECOD, 0.05 pmole/min/mg protein; AE, 0.1 pmole/min/mg protein; BP hydroxylase, 0.025 pmole/min/mg protein; NADPH cytochrome *c* reductase, 0.8 pmole/min/mg protein; epoxide hydrolase, 0.03 nmole/min/mg protein.

Scraping of the cells prior to incubation caused a 10–15% loss of viability, as measured by trypan blue exclusion. This loss of viability was similar for NCI-H322 and NCI-H358. There was a further loss of cell viability (12 ± 2%) over the 60-min incubation period. This occurred to the same extent (i) in the

Table 1. Cytochrome P-450 and drug-metabolizing enzyme activities in the human cancer cell lines NCI-H322 and NCI-H358

	Cell line	
	NCI-H322[1]	NCI-H358
Cytochrome P-450 (pmoles/mg protein)	14.3 ± 1.2*	ND†
(BNF induced)‡	30.1 ± 2.27* (2)	10.2 ± 0.08
Aryl hydrocarbon hydroxylase (pmoles/min/mg protein)	0.26 ± 0.07*	0.14 ± 0.01
(BNF induced)‡	5.84 ± 0.18* (22)	2.31 ± 0.03 (16)
Ethoxycoumarin *O*-deethylase (pmoles/min/mg protein)	0.21 ± 0.012*	0.09 ± 0.006
BNF induced)‡	3.43 ± 0.11* (16)	1.41 ± 0.07 (16)
Aldrin epoxidase	ND	ND
Cytochrome b_5 (pmoles/mg protein)	31.8 ± 1.71*	14.6 ± 1.06
NADPH cytochrome *c* reductase (pmoles/min/mg protein	4.73 ± 0.38*	2.15 ± 0.18
Epoxide hydrolase (nmoles/min/mg protein)	0.34 ± 0.02*	0.27 ± 0.01

Values are means ± S.D. from duplicate determinations using triplicate cultures and were carried out using untreated cells unless otherwise specified. Assays were carried out as described in Materials and Methods. The numbers in parentheses are the fold induction compared to untreated cells.

* Significantly different ($P < 0.005$) from respective value in NCI-H358 cells.

† Not detectable.

‡ Cells were exposed to BNF (50 μM) for 24 hr.

different buffers used for the various enzyme assays and (ii) in the four cell lines studied.

Effects of potential enzyme inducers on cytochrome P-450-dependent monooxygenase activity. The BP hydroxylase and ECOD activities in NCI-H322 were highly induced by pretreatment with BNF (50 μM) (22- and 16-fold induction respectively) (Tables 1 and 2). Similarly, BNF caused a 16-fold induction of the enzyme activities in NCI-H358 (Tables 1 and 2). BP hydroxylase activity was also induced by BA (20 μM) in both NCI-H322 (24-fold) and NCI-H358 (17-fold) (Table 2) (ECOD activity was not measured after BA induction). Pretreatment with BNF or BA was carried out for 24 hr (the two compounds were dissolved in DMSO). In the concentration used (0.1%), DMSO alone had no effect on the enzyme activity. Exposure to phenobarbital (2 mM for 72 hr) did not affect the monooxygenase activity in NCI-H322 (measured as AHH, Table 2, or ECOD, data not shown). There was a 1.4-fold increase in AHH activity in NCI-H358 (there was no change in ECOD, data not shown) after exposure to phenobarbital. However, this increase is not significant when compared to the induction after pretreatment with the polycyclic hydrocarbons. Likewise, exposure to dexamethasone (5 μM for 72 hr) did not affect AHH or ECOD activity in NCI-H358. The corticosteroid reduced the activity of AHH by 70% in NCI-H322 (Table 2). Exposure to dexamethasone plus BA partially suppressed the

Table 2. Aryl hydrocarbon hydroxylase activity in human cancer cell lines NCI-H322 and NCI-H358

	AHH activity (pmoles/min/mg protein)	
Treatment	NCI-H322	NCI-H358
Untreated	0.26 ± 0.07	0.14 ± 0.01
Dexamethasone* (5 μM)	0.08 ± 0.01	0.13 ± 0.01
Phenobarbital* (2 mM)	0.21 ± 0.06	0.20 ± 0.01
β-Naphthoflavone† (50 μM)	5.84 ± 0.18 (22)	2.31 ± 0.03 (16)
Benz[*a*]anthracene† (20 μM)	6.13 ± 0.15 (24)	2.43 ± 0.03 (17)
Benz[*a*]anthracene + (20 μM) Dexamethasone† (5 μM)	3.45 ± 0.05 (13)	

Activity was measured using benzo[*a*]pyrene as substrate. Values represent the mean and range from determinations from duplicate cultures. One of two experiments which gave similar results is shown. Cells were prepared and assays were carried out as described in Materials and Methods. The incubation time was 60 min. The numbers in parentheses are the fold induction compared to untreated cells.

* Exposure time 72 hr.

† Exposure time 24 hr.

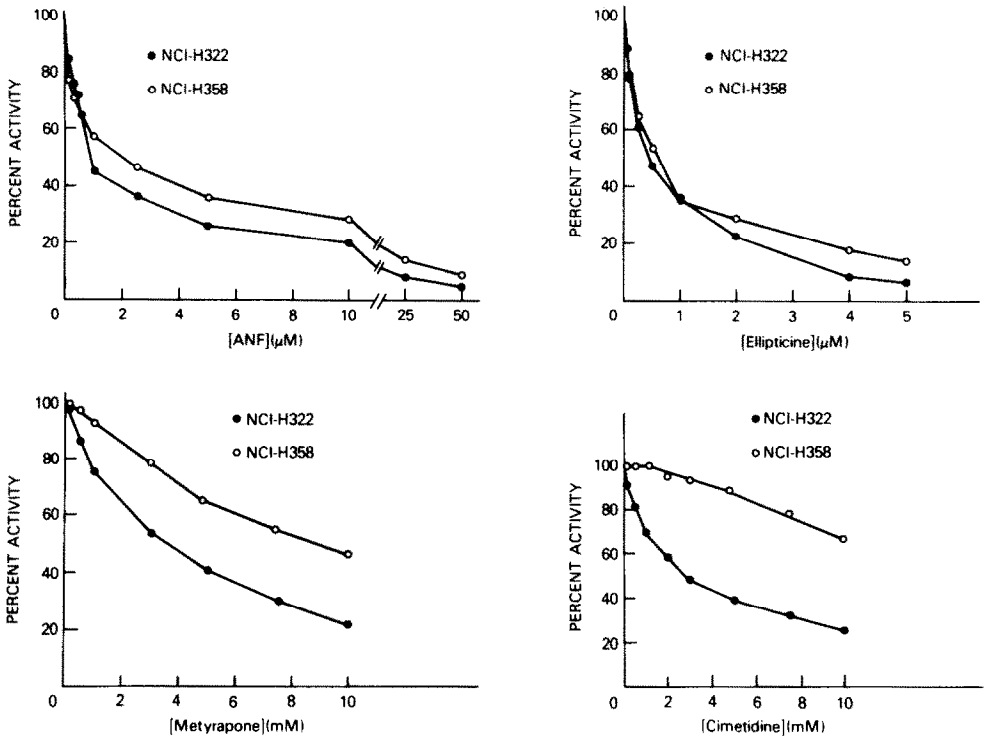

Fig. 1. Effects of various inhibitors of the cytochrome P-450-dependent monooxygenase enzyme system on ethoxycoumarin *O*-deethylase (ECOD) activity. Studies were carried out on cells exposed to BNF (50 μM) for 24 hr. After harvesting the cells, the inhibitors were added in DMSO (ellipticine, ANF and cimetidine) or acetone (metyrapone). Incubation time was 60 min. Values are means from duplicate determinations using triplicate cultures.

induction by BA in both cell lines (Table 2). No monooxygenase or other activity was detected following pretreatment of the human small-cell derived cell lines NCI-H69 and NCI-H128 with the inducing agents shown in Table 2.

Effects of potential inhibitors of the monooxygenase enzyme system on ECOD. Inhibition of monooxygenase activity was monitored using BNF-induced cells and ECOD as an index of activity. Ellipticine was the most potent inhibitor in both NCI-H322 and NCI-H358, with almost complete enzyme inhibition at a concentration of 5 μM (Fig. 1). As with ellipticine, inhibition by ANF was parallel in the two cell lines, with very low ECOD activity being present at an ANF concentration of 50 μM (Fig. 1). Metyrapone and cimetidine were less potent inhibitors. For both inhibitors, a concentration of 10 mM produced an 80% drop in ECOD activity in NCI-H322 (Fig. 1). At the same inhibitor concentration, ECOD inhibition was significantly less ($P < 0.005$) in NCI-H358 compared to NCI-H322 (Fig. 1). In Fig. 1, 100% activity represents the activity in incubates containing the respective vehicle for the inhibitor (ellipticine, ANF and cimetidine in DMSO, metyrapone in acetone).

DISCUSSION

This study shows the predominance of the polycyclic aromatic hydrocarbon (PAH) inducible cytochrome P-488-dependent, as opposed to the cytochrome P-450-dependent, monooxygenase in the two human non-small-cell derived cancer cell lines NCI-H322 and NCI-H358. *In vivo* this cytochrome P-448-dependent monooxygenase enzyme system is most active in liver, but it is also present in extrahepatic tissues of many species including the lung of rodents [19, 20] and of man [21]. A number of observations have shown that oxidative metabolism of various xenobiotics occurs in rabbit Clara cells and alveolar type II cells [22]. These authors have reported higher enzyme activity in Clara cells than in alveolar type II cells. These observations are borne in the present study, where the Clara cell-derived HCI-H322 had a higher activity for all the variables measured when compared to the alveolar type II cell derived NCI-H358. To our knowledge, there have been no reports of the presence of monooxygenases in specific cell types of human lung. These data also correlate with the results obtained for the metabolic activation of 4-ipomeanol [6] and *N*-nitrosodiethylamine [7] by these cell lines, where metabolism was higher in NCI-H322 than in NCI-H358. The small-cell derived NCI-H69 and NCI-H128 were devoid of any of the enzyme activities measured. These cells do not have any prostaglandin endoperoxide synthetase activity [8], in contrast to the non-small-cell derived NCI-H322 and NCI-H358 which convert arachidonic acid to prostaglandin E_2 [8]. NCI-H128 and NCI-H69 do not metabolize 4-ipomeanol [6] or *N*-nitrosodiethylamine [7]. Thus, it appears that there is an overall correlation between the differentiation (as

assessed by morphological criteria) [4] and the enzyme activity in the cell lines studied.

As previously mentioned, the spectrum of monooxygenase-associated enzyme activities in the two cell lines (i.e. presence of AHH and ECOD activities, and absence of AE activity) together with the induction by PAH but not by dexamethasone or phenobarbitone all point to the predominance of the cytochrome P-448-dependent, as opposed to the cytochrome P-450-dependent, monooxygenases. The cytochrome P-448-dependent monooxygenases have been reported previously to predominate in established cells in culture derived from liver, lung, kidney and skin [10, 23] and in extrahepatic tissues [20, 24]. The levels of constitutive and PAH-induced monooxygenase activities in the two cell lines are similar to those of various continuous cell lines such as 3T3 (a mouse fibroblast cell line) or BHK (a cell line from hamster kidney) which are routinely used for cyto- and genotoxicity studies [1]. The activities in the two cell lines studied here, NCI-H322 and NCI-H358, were considerably higher than those in A549, a continuous cell line derived from a human lung adenocarcinoma [1]. AE has been established as marker reaction for the cytochrome P-"450"-dependent monooxygenases which are predominantly located in the liver [12, 25]. Low AE activities have also been observed in rat lung and are thought to be mediated by a cytochrome P-450 form different from those in liver [26]. To date, AE activity has only been detected in a few cell lines of hepatic origin [9, 27]. Thus, it is not surprising that this monooxygenase activity is not detectable in the two lung cancer cell lines.

The inhibition studies also point to the predominance of cytochrome P-448. ANF preferentially binds to cytochrome P-448 and preferentially blocks monooxygenase activities associated with such forms [28]. Ellipticine is a better inhibitor (by more than 10-fold) of AHH activity than ANF or metyrapone [29]. Monooxygenase activities known to be associated with different isozymes of cytochrome P-450 are all markedly inhibited by ellipticine to about the same extent [30]. Cimetidine and metyrapone inhibited the monooxygenase-dependent ECOD to different extents in NCI-H322 and NCI-H358. The wide spectrum of drug metabolism that can be inhibited by cimetidine is reminiscent of ellipticine [31]. On the other hand, metyrapone *in vitro* preferentially inhibits basal and phenobarbitone-induced AHH [28, 32]. The difference in the pattern of inhibition by cimetidine and metyrapone in NCI-H322 compared to NCI-H358 may be due to a different overall pattern of cytochrome P-450 isozymes in these two non-small-cell lines.

NADPH cytochrome *c* reductase, which is an essential component of the monooxygenase complex, was expressed in the two non-small-cell lines NCI-H322 and NCI-H358. However, the level of activity was very low when compared to the activity previously reported in continuous cultures of mammalian cells derived from liver, kidney and hepatomas [1]. Epoxide hydrolase activity was comparable to the values reported in Reuber hepatoma cells [33]. This enzyme, which like the cytochrome P-450 isozymes is a microsomal enzyme [14], is important in the detoxification and toxification of various PAH [34, 35].

In conclusion, the results showed that NCI-H322 and NCI-H358 cells, which retain many properties of Clara cells and alveolar type II cells respectively [4, 5], possess cytochrome P-448-dependent monooxygenase activity. Furthermore, other microsomal enzymes such as cytochrome b_5, NADPH cytochrome *c* reductase and epoxide hydrolase are also expressed. The two cell lines also contain glutathione and glutathione-*S*-transferase activity, but no UDP-glucuronosyltransferase activity (Wiebel *et al.*, personal communication). They may, thus, offer a useful model system to follow the capacity of human lung to activate toxins and/or carcinogens.

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