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Aryl hydrocarbon hydroxylase induction in mammalian liver cell culture—IV. Stimulation of the enzyme activity in established cell lines derived from rat or mouse hepatoma and from normal rat liver

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IN ORDER to elucidate the mechanisms regulating membrane-bound mono-oxygenase activities,[1] we have used both mammalian cell culture[2,3] and genetic differences in the mouse[1,4] as advantageous experimental systems. In this laboratory we have examined in detail the induction of one drug-metabolizing enzyme—aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity—in fetal hamster and mouse cultures,[2] in several established cell lines,[5] and most recently in fetal rat hepatocytes.[3,6] The possible importance of this P_{450}-containing oxygenase in carcinogenesis and cytotoxicity has been recently reviewed.[7]

One advantage of fetal rat liver primary cultures (see Table 1) is that the basal and inducible hydroxylase activities are many times greater than those found in previous cultures that have been studied. Therefore, with the use of various inhibitors of cell metabolism such as actinomycin D and cycloheximide,[3,8] subtle changes in the oxygenase activity can be measured with confidence. The disadvantages with fetal rat hepatocytes, however, include: (1) the sacrifice of 12–24 pregnant rats in our laboratory each week; (2) the weekly expense for this number of animals; (3) our dependence upon animal suppliers for healthy animals of the correct gestational age; and (4) differences in the basal and inducible hydroxylase activities from week to week—presumably due to dissimilarities in the mean gestational age of the fetuses used, thereby causing different growth rates of the cultures and differences in yields of viable hepatocytes. Thus, if the hydroxylase is inducible equally well in hepatic established cell lines and if the kinetics of induction and the responses to various inducers in such established cell lines are shown to be similar to those parameters in fetal rat liver primary cultures, clearly such an experimental system would alleviate these disadvantages. In this report we describe the induction by various inducers of aryl hydrocarbon hydroxylase activity in five established cell lines—three derived from hepatomas and two from normal rat liver.

H-4-II-E, a rat cell line derived[9] from Reuber hepatoma H-35, was generously provided by Dr. E. Brad Thompson, National Cancer Institute, Bethesda, Md. MH_1 C_1, a clone of epithelial cells from the transplantable Morris hepatoma 7795 originally produced in the Buffalo strain of rat,[10] was purchased from the American Type Culture Collection Cell Repository, Rockville, Md. Hepa-1, a mouse cell line derived from the transplantable hepatoma BW 7756 originally produced in the C57/LJ mouse,[11] was kindly given to us in 1971 by Dr. Gretchen Darlington, Department of Biology, Yale University, New Haven, Conn. TRL-2-Cl-2 and ERL-2-Cl-3, cell lines[12] derived, respectively, from normal liver of 10-day-old and 8-week-old rats of the BD-6 strain, was generously provided by Dr. Yoji Ikawa, National Cancer Institute, Bethesda, Md; these two lines had been grown in McCoy's 5a medium with 10% fetal calf serum and were adapted in our laboratory to Eagle's minimal essential medium with 10% fetal calf serum. The BD-6 strain of rats was brought from Europe to this country by Dr. W. J. Burdette, M.D. Anderson Hospital and Tumor Institute, Houston, Texas, and are now commercially available from Flow Laboratories, Inc., Rockville, Md.

Waymouth MAB medium (specially ordered from Grand Island Biological Company, Grand Island, N.Y.) with 10% fetal calf serum was used during the induction studies with Hepa-1 cells, whereas H-4-II-E, MH_1 C_1, TRL-2-Cl-2, and ERL-2-Cl-3 cultures were grown in Eagle's minimal essential medium containing 10% fetal calf serum. Dissolving the inducers 3-methylcholanthrene (MC), benz[a]anthracene (BA), phenobarbital (PB), tryptamine (TR), or isoproterenol (ISO) in the growth medium was performed as previously described.[3,6] The remainder of the chemical reagents, tissue culture materials, and procedures for growing the cultures have been described before in detail.[2,3,6]

Hydroxylase activity was determined essentially as described previously.[4] One unit of aryl hydrocarbon hydroxylase activity is defined as that amount of enzyme catalyzing per min at 37° the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo[a]pyrene. The absolute limit of sensitivity for the assayed specific hydroxylase activity is estimated to be about 0·01 unit/mg of protein.

Table 1 shows the range of basal and inducible specific hydroxylase activities found in various cell cultures and in mouse tissues—under the same enzyme assay conditions in our laboratory. The magnitude of enzyme induction ranges from as little as 3- to 5-fold in mouse liver microsomes to greater than 10- or 50-fold in mouse kidney, fetal mouse liver and in the various cultures examined.

TABLE 1. RELATIVE BASAL AND MAXIMALLY INDUCIBLE ARYL HYDROCARBON HYDROXYLASE ACTIVITIES IN CULTURE AND IN THE INTACT ANIMAL

Mammalian cells in culture or intact animal tissue	Specific hydroxylase activity				Refs.
	Basal	BA or MC*	PB*	TR or ISO*	
Fetal mouse or hamster, secondary cultures	0·1–1·1	4–8	0·1–0·9		2
HTC, established rat hepatoma cell line	< 0·02	< 0·02	< 0·02		5
L cells, mouse established cell line	< 0·02	< 0·02	< 0·02		5
HeLa, human established cell line	< 0·02	0·1–0·5	< 0·02		5
3T3, mouse established cell line	< 0·02–0·10	1·4–3·4			13
Fetal rat hepatocyte, primary culture	1·0–18	60–140	50–110	80–140	3
Fetal C57BL/6N mouse liver, 20-days gestation	0·3–3·0	40–90			4
Weanling C57BL/6N mouse kidney	< 0·02–1·2	8–40	< 0·02–1·4	< 0·02–0·80†	4, 14
Weanling C57BL/6N mouse liver microsomes	480–780	1800–3600	950–1400	360–660†	4, 14

* Optimal or maximal doses of each compound in cell culture or administered to the intact animal can be found in the references cited.

† Unpublished data; 500 mg of tryptamine kg^{-1} was administered intraperitoneally for as long as 5 days without eliciting an increase in hydroxylase activity.

For those cultures or tissues in which the enzyme is inducible by polycyclic hydrocarbons but the basal enzyme is barely detectable or is below the threshold of detection by this assay, the actual magnitude of induction is highly variable and difficult to determine accurately.

The rate at which the induced hydroxylase activity accumulates in the various established cell lines treated with aromatic hydrocarbons, PB or biogenic amines is illustrated in Figs. 1 and 2. Note that

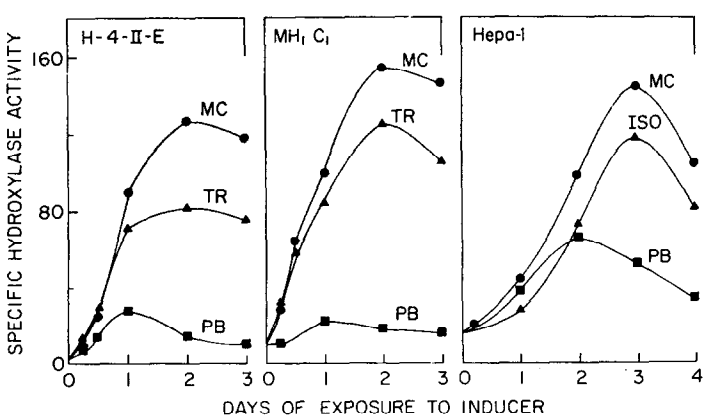

FIG. 1. Kinetics of aryl hydrocarbon hydroxylase induction in three established cell lines derived from rat Reuber hepatoma (H-4-II-E), rat Morris hepatoma (MH_1 C_1), and mouse hepatoma (Hepa-1) by 1 μM MC, 1 mM TR, 2 mM PB or 1 mM ISO in the growth medium. The basal enzyme specific activities in cells grown in control medium alone ranged in various experiments from 0·4 to 3·5 in H-4-II-E, from 3·2 to 8·8 in MH_1 C_1, and from 5·0 to 24 in the Hepa-1 line.

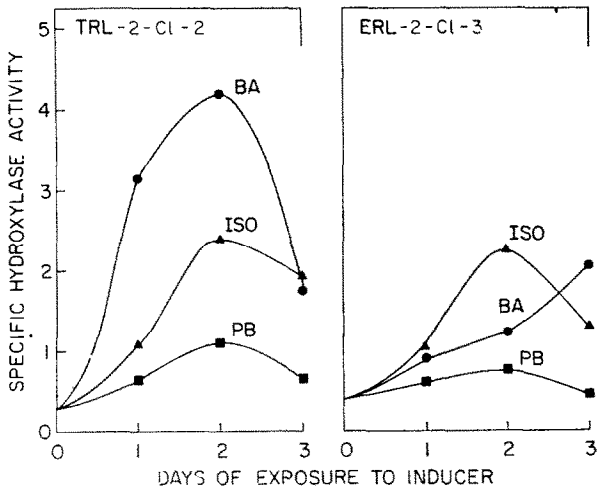

FIG. 2. Kinetics of aryl hydrocarbon hydroxylase induction in two established cell lines derived from normal liver of 10-day-old (TRL-2-Cl-2) and 8-week-old (ERL-2-Cl-3) rats by 13 μM BA, 0·5 mM ISO and 2 mM PB in the growth medium. The basal enzyme specific activities in cells grown in control medium alone ranged in various experiments from < 0·02 to 1·0 in TRL-2-Cl-2, and from < 0·02 to 0·64 in the ERL-2-Cl-3 cell line. The highest specific activities we have observed for the hydroxylase are: 35 with 13 μM benzo[a]pyrene as the inducer and 7·2 with BA as the inducer in the TRL-2-Cl-2 line; and 3·1 with 13 μM benzo[a]pyrene as inducer and 2·0 with BA as inducer in the ERL-2-Cl-3 line.

the maximally induced enzyme levels in Fig. 2 are at least 20 times less than those in Fig. 1. However, recently we have developed clones of the TRL-2-Cl-2 line having an inducible hydroxylase specific activity of more than 80. The general trend observed was that the polycyclic hydrocarbons were better inducers than the biogenic amines and that PB was the least effective type of inducer. PB is also less effective than the aromatic hydrocarbons or biogenic amines in the fetal rat hepatocyte primary cultures.[3] That PB did cause detectable increases in the hydroxylase activity is evidence that each of these five lines is of hepatic origin.[6] In each of these lines, the optimal inducer concentrations in the growth medium and the kinetics of enzyme induction by each type of inducer were strikingly similar to what has already been characterized in fetal rat liver cultures.[3,6] In addition, we have found* that the responses of the hydroxylase induction process to inhibitors of RNA and protein synthesis and the apparent independence of transcription and translation appear the same as these parameters described in fetal rat hepatocytes.[8] All five cell lines appear as homogeneous epithelial cells resembling normal hepatic cells. H-4-II-E cultures grow as individual cells and are the most rapidly proliferating of these five cell lines, with a generation time (t_G) of 18–22 hr. Hepa-1 cultures grow in colonies and the TRL-2-Cl-2 line appears as individual cells, and both grow less rapidly than H-4-II-E cells (t_G between 24 and 30 hr). MH_1C_1 cells grow in colonies and have a t_G of 35–45 hr. ERL-2-Cl-3 cultures are individual cells and at the present time grow in Eagle's medium very slowly (t_G greater than 40 hr).

These established cell lines offer several possibilities of experimental systems, depending on the question being asked. The Reuber H-4-II-E line has excellent growth properties and, because of quite low basal hydroxylase activity, has 20- to more than 100-fold increases in the enzyme activity after treatment with polycyclic hydrocarbons or biogenic amines; this line can also be maintained easily in suspension cultures—a distinct advantage if one wishes to harvest large quantities of cells for the isolation of a particular macromolecule or for spectrophotometric studies of cytochrome P_{450}. For studying characteristics of the control enzyme or of the induction process by PB, the Hepa-1 cell line would be the best choice. For comparison of enzyme induction parameters in normal liver with those in hepatoma, perhaps the TRL-2-Cl-2 line or fetal rat hepatocyte primary cultures might be compared with the rat Reuber or Morris hepatoma lines. The use of these established lines of cultured homogeneous cells may help elucidate the mechanisms of mono-oxygenase induction by the various types of inducers. We also suggest that such established cell lines might be useful as relatively simple assay systems for studying the carcinogenic, teratogenic or cytotoxic effects of various drugs or other xenobiotics (and their metabolites) in cell culture.

* I. S. Owens, J. E. Gielen and D. W. Nebert, manuscript in preparation.

In conclusion, aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity accumulates in five liver-derived cell lines treated with polycyclic hydrocarbons, biogenic amines or phenobarbital in the growth medium. The established lines are: H-4-II-E, derived from rat Reuber hepatoma H-35; MH_1C_1, from the rat transplantable Morris hepatoma 7795; Hepa-1, from the transplantable hepatoma BW 7756 originally developed in the C57/JL mouse; and TRL-2-Cl-2 and ERL-2-Cl-3, derived, respectively, from normal liver of 10-day-old and 8-week-old rats of the BD-6 strain. We feel that there are advantages in using such established cell lines instead of fetal rat hepatocyte primary cultures.

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REFERENCES

1. D. W. NEBERT, N. CONSIDINE and H. KON, *Drug Metab. Dispos.* **1,** 231 (1973).
2. D. W. NEBERT and L. L. BAUSSERMAN, *Ann. N.Y. Acad. Sci.* **179,** 561 (1971).
3. J. E. GIELEN and D. W. NEBERT, *J. biol. Chem.* **247,** 7591 (1972).
4. D. W. NEBERT and J. E. GIELEN, *Fedn Proc.* **31,** 1315 (1972).
5. W. F. BENEDICT, J. E. GIELEN and D. W. NEBERT, *Int. J. Cancer* **9,** 435 (1972).
6. J. E. GIELEN and D. W. NEBERT, *J. biol. Chem.* **246,** 5198 (1971).
7. J. W. DALY, D. M. JERINA and B. WITKOP, *Experientia* **28,** 1129 (1972).
8. D. W. NEBERT and J. E. GIELEN, *J. biol. Chem.* **246,** 5199 (1971).
9. H. C. PITOT, C. PERAINO, P. A. MORSE, JR. and V. R. POTTER, *Natn. Cancer Inst. Monogr.* **13,** 2299 (1964).
10. U. I. RICHARDSON, A. H. TASHJIAN and L. LEVINE, *J. Cell Biol.* **40,** 236 (1969).
11. H. P. BERNHARD, C. J. DARLINGTON and F. H. RUDDLE, *Devl Biol.*, in press.
12. Y. IKAWA, A. NIWA, L. TOMATIS, R. W. BALDWIN, A. F. GAZDAR and H. C. CHOPRA, *Proc. Am. Ass. Cancer Res.* **14,** 109 (1973).
13. W. F. BENEDICT, D. W. NEBERT, and E. B. THOMPSON, *Proc. natn. Acad. Sci. U.S.A.* **69,** 2179 (1972).
14. J. E. GIELEN, F. M. GOUJON and D. W. NEBERT *J. biol. Chem.* **247,** 1125 (1972).

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Effect of chemical sympathectomy on morphine antinociception and tolerance development in the rat

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IN A PREVIOUS paper,[1] it was reported from this laboratory that 6-hydroxydopamine (6-OHDA) which has been reported to cause a selective degeneration of the adrenergic neurons in the brain,[2–5] reduced the analgetic response to morphine in the mouse but did not affect materially the development of tolerance to morphine. Evidence was also provided indicating that the signs of morphine withdrawal were exacerbated by 6-OHDA. The present communication presents findings of a similar study performed in the rat.

Male Sprague–Dawley rats weighing 150–180 g from Horton Laboratories, Calif., were used. Chemical sympathectomy was effected by intraventricular injection[6] of 6-OHDA HBr (Regis Chemical Co., Chicago, Ill.), dissolved in nitrogen-saturated physiologic saline containing 0·1% ascorbic acid; control rats received the vehicle. Two dosage schedules of 6-OHDA were used.

In expt. 1, a dose of 0·5 mg/kg of 6-OHDA was injected for 2 consecutive days, and 5 days after the second dose, the analgetic response to morphine was determined. Four other animals in this series were utilized for estimation of brain norepinephrine (NE) and dopamine (DA) according to