MOLECULAR PHARMACOLOGY, 11, 399-408

Induction of Aryl Hydrocarbon Hydroxylase Activity in Various Cell Cultures by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

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(Received January 13, 1975)

SUMMARY

NIWA, AKIRA, KUMAKI, KENJI & NEBERT, DANIEL W. (1975) Induction of aryl hydrocarbon hydroxylase activity in various cell cultures by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Mol. Pharmacol.*, **11**, 399-408.

The kinetics of aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase induction by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. (TCDD) among 10 established cell lines, as well as fetal primary cultures derived from hamster, rat, chick, rabbit, and four inbred strains of mice, and cultured human lymphocytes, is very similar to the time course of hydroxylase induction by 3-methylcholanthrene (MC) as the inducer. The TCDD-inducible process is sensitive to actinomycin D and cycloheximide at levels of inhibitor similar to those previously reported with MC in culture. The induced enzyme activity in cells treated with TCDD plus MC is not greater than that in cells exposed to either inducer alone. There exists no relationship between cytotoxicity by TCDD and the level of inducible hydroxylase activity in culture. Estimated ED_{50} values for the hydroxylase induction by TCDD range from about 0.12 nM in C57BL/6N mouse cultures and 0.23 nM in the H-4-II-E cell line to more than 100 nM in the VERO and HTC cell lines. No hydroxylase activity is detectable in the control, MC-, or TCDD-treated LB82 cell line. In several cell lines and in primary cultures, the responsiveness of hydroxylase induction to TCDD is between 250 and 900 times greater than that to MC. The responsiveness of hydroxylase induction to TCDD in C57BL/6N mouse-derived cultures is about 16 times greater than the responsiveness to TCDD in DBA/2N mouse-derived cultures; this difference in responsiveness to TCDD is very similar to that seen in these two mouse strains *in vivo*. A bioassay with H-4-II-E cells is suggested for the detection of minute (10^{-14} mole) levels of TCDD.

INTRODUCTION

Recent reviews (1-3) indicate the possible importance of aromatic hydroxylations of polycyclic hydrocarbons, drugs, and other environmental agents mediated by the membrane-bound monooxygenases to chemical carcinogenesis, pharmacology, and toxicology. Genetic differences in the induction of one such monooxygenase activity, the aryl hydrocarbon (benzo[*a*]-pyrene) hydroxylase system, have been demonstrated in fetal mouse cell cultures (4), in mice (5-7), and in cultured human lymphocytes (8). An increased incidence of MC[1]-initiated sarcomas in mice (9, 10) and, more recently, bronchogenic carcinoma in man (11) has been found in

[1] The abbreviations used are: MC, 3-methylcholanthrene; the hydroxylase, aryl hydrocarbon (benzo[*a*]-pyrene) hydroxylase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ED_{50}, that dose which elicits 50% of the maximal response.

individuals in whom the hydroxylase activity is more readily inducible by polycyclic hydrocarbons.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, a toxic contaminant formed during the commerical synthesis of the herbicide and defoliant 2,4,5-trichlorophenoxyacetic acid, is approximately 30,000 times more potent than MC as an inducer of the hydroxylase activity in rat liver (12). The enzyme activity in liver, kidney, bowel, lung, and skin of so-called "nonresponsive" mice is induced fully by TCDD but not by MC (13); however, the dose of TCDD required for maximal hydroxylase induction in mice genetically less "responsive" to MC is more than 10 times that necessary for mice genetically "responsive" to MC (14). Because TCDD is metabolized so slowly in the rat, the biological half-life of this potent inducer is about 17 days (15), and the induced hydroxylase activity and associated cytochrome P_1450 remain elevated for more than 35 days (12). It is therefore possible that TCDD may become a serious environmental contaminant for man. There is evidence (16) for the appearance of this toxic chemical in the food chain. TCDD induces the hydroxylase activity in cultured human lymphocytes at a concentration in the growth medium 40–60 times less than the concentration of MC necessary for maximal enzyme induction (17). The purpose of this report is to characterize in detail the hydroxylase induction by TCDD among numerous established cell lines and primary cell cultures from various species. Further, we suggest a bioassay which might be useful in detecting TCDD at levels well below 1 pmole in 3 ml of culture medium.

MATERIALS AND METHODS

The tissue culture materials and chemicals (18–20) used in this study were obtained from the sources cited. Trypan blue was purchased from Grand Island Biological Company. The established cell lines H-4-II-E, MA, E-3, HTC, and LB82 were generous gifts from Dr. E. Brad Thompson, National Cancer Institute. H-4-II-E is a rat cell line derived (21) from Reuber Hepatoma H-35 (22). MA is a cell line derived from normal adult liver of the BALB/cAnN mouse.[2] E-3 is a rat liver epithelial cell line[3] originally obtained from Drs. Arthur G. Schwarz and Harry Eagle, Albert Einstein College of Medicine. HTC is a hepatoma tissue culture line derived from an ascites tumor which in turn had been derived from the rat "minimal deviation" Hepatoma 7288c (23). LB82 is a bromodeoxyuridine-resistant, thymidine kinase-deficient subline (24) of the mouse L-cell established line. Originally the L line was established from subcutaneous areolar and adipose tissue from the C3H/An mouse. Hepa-1, a mouse cell line (25) derived from the transplantable hepatoma BW 7756 originally produced in the C57L/J mouse, was generously given to us in 1971 by Dr. Gretchen Darlington, Department of Biology, Yale University. TRL-2 and ERL-2, cell lines derived (26), respectively, from normal liver of 10-day-old and 8-week-old BD-6 rats, were kindly provided by Dr. Yoji Ikawa, National Cancer Institute. NRKE is a normal rat kidney epithelial cell line,[4] generously provided by Dr. Herbert K. Oie, National Dental Research Institute. The Chang liver cell line, derived from normal human liver (27), and the VERO cell line, derived (28) from African green monkey kidney, were purchased from the American Type Culture Collection Cell Repository. National Institutes of Health Animal Supply provided us with pregnant mice and golden Syrian hamsters. Taconic Farms, Inc. (Germantown, N. Y.), supplied us with pregnant Sprague-Dawley rats. Pregnant (New Zealand White) rabbits were purchased from the Frye Rabbitry (Germantown, Md.), and fertilized chicken eggs were obtained from Truslow Farms (Chestertown, Md.).

Stock solutions of 240 μg of TCDD per milliliter of *p*-dioxane and 8.0 mg of MC (Sigma Chemicals Company) per milliliter of dimethyl sulfoxide were diluted appropriately. The TCDD, provided generously

[2] D. Aviv and E. B. Thompson, personal communication.

[3] A. G. Schwarz, personal communication.

[4] H. K. Oie, personal communication.

by Dr. Alan Poland (University of Rochester School of Medicine and Dentistry), was originally from lot 851:144-II of Dow Chemical Company and had been shown by gas-liquid chromatography–mass spectrometry to be 98.6% pure, with 1.0% trichloro- and 0.4% pentachlorodibenzo-*p*-dioxin contaminants. The MC was recrystallized from benzene before use.

Established cell lines. TRL-2, ERL-2, NRKE, Chang liver, VERO, HTC, and LB82 cell lines were grown during this study in Eagle's minimal essential medium with 10% calf serum. H-4-II-E and Hepa-1 were grown in Eagle's minimal essential medium with 10% fetal calf serum; MA and E-3 lines were grown in Ham's F12 medium (Grand Island Biological Company) with 10% calf serum. All media contained 100 μg of streptomycin and 100 units of penicillin per milliliter. The cell lines were plated at a density of 0.7×10^6–1.5×10^6 cells/60-mm tissue culture dish, and all media were changed daily. Forty-eight or 72 hr after plating, when the cells were in the logarithmic growth phase, the inducer(s) and/or inhibitors were added. The time at which logarithmic phase was reached depended on the generation time and the number of cells initially plated; H-4-II-E had the longest doubling time, and VERO and LB82 the shortest. "Control medium" routinely contained 0.14% *p*-dioxane.

Primary cell cultures. Primary cultures derived from the entire fetus—minus the head, tail, and extremities—were routinely prepared as previously described (4, 18). Primary cultures derived from minced liver or from the combination of minced lung, bowel, and kidney of newborn or fetal mice were also prepared in a manner not previously described. With fetuses, or with newborns 6–12 hr following birth, nonhepatic (lung, kidney, and bowel combined) or hepatic tissue from one or two litters was minced and washed in Eagle's minimal essential medium. The minced tissue was suspended in minimal essential medium containing 40% fetal calf serum. The suspension was plated in 0.50 ml (about 1 mg of mouse tissue protein)/60-mm dish and incubated for 2 hr at 37°; 2.2 ml of Eagle's minimal essential medium containing 10% fetal calf serum were than added, and the cultures were incubated for about 22 hr at 37°. About 24 hr after the initial plating of cells, appropriate amounts of TCDD or MC were added to the cultures in a volume of 0.30 ml; the maximal final concentration of *p*-dioxane or dimethyl sulfoxide in the culture medium was 0.10% or 0.20%, respectively, unless otherwise indicated. After the second 24-hr period in a final volume of 3.0 ml of medium with appropriate doses of inducer, the cultures were harvested and assayed for the hydroxylase activity and protein content in the usual manner (4, 18).

Enzyme assay. Both the hydroxylase activity and protein concentration were determined in duplicate for the homogenate from cells scraped from one cell culture dish 60 mm in diameter by procedures previously described (4, 18). In a typical experiment, two values of hydroxylase specific activity were obtained from each of three dishes, the cells of which were harvested at each time point. One unit of aryl hydrocarbon hydroxylase activity is defined (4, 18–20) as that amount of enzyme catalyzing per minute at 37° the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo[*a*]pyrene recrystallized standard. The specific activity is expressed in units per milligram of protein of the total cellular homogenate.

RESULTS

Characterization of hydroxylase induction in established cell lines. Figure 1 shows the rate at which the hydroxylase activity accumulates in four established cell lines exposed to TCDD. For the Hepa-1 and H-4-II-E lines, the lag time, the length of rapid linear increase, and the maximal specific activities reached after 1–3 days of TCDD treatment are very similar to those respective parameters previously illustrated (20) after MC treatment. Note that the maximally inducible hydroxylase activity in the MA and NRKE lines is 10–30

times less than that in TCDD-treated Hepa-1 or H-4-II-E cultures.

In every cell line examined, the induction process was quite sensitive to rather low concentrations of actinomycin D or cycloheximide added simultaneously with the inducer TCDD. Typical results with the H-4-II-E line are illustrated in Fig. 2 and are comparable to results previously shown in MC-treated fetal rat primary hepatocyte cultures (19, 29) and the H-4-II-E and Hepa-1 lines (29).

The simultaneous administration to the rat (12) of maximally inducing doses of both TCDD and MC stimulates hepatic hydroxylase activity no more than either chemical alone. This same observation was made in cultured H-4-II-E cells (Table 1). Maximally induced specific activities with TCDD alone were 92–112, and with MC alone, 87–102. No combination of the two compounds at any concentration caused higher values. In fact, at the highest concentrations of both inducers together, the induced enzyme specific activity of 57 indicates a significant decrease; we feel that this probably reflects a cytotoxic effect.

Characterization of cytotoxicity and hydroxylase induction. In vivo, the extreme toxic effects of the chlorodibenzo-*p*-dioxins

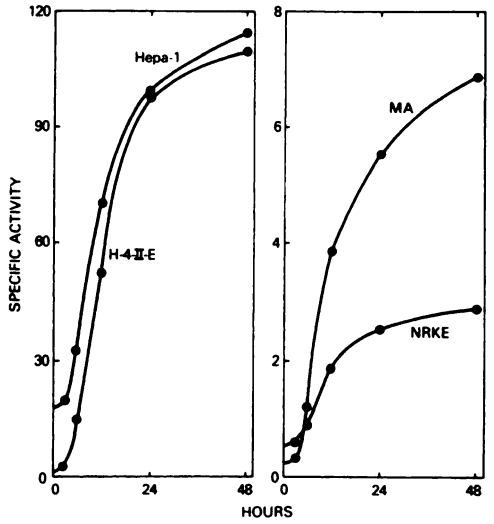

FIG. 1. *Kinetics of hydroxylase induction in four established cell lines by 100 nM TCDD in growth medium*

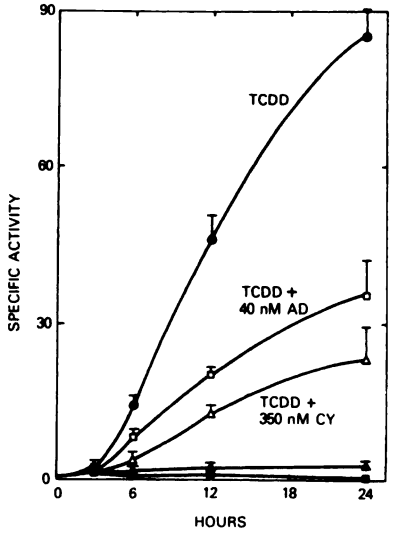

FIG. 2. *Effect of 40 nM actinomycin D (AD)* (□), *400 nM actinomycin D* (■), *350 nM cycloheximide (CY)* (△), *or 3.5 μM cycloheximide* (▲) *on rate of hydroxylase induction in H-4-II-E cells*

The inhibitors were added simultaneously with the inducer, 10 nM TCDD. The kinetics of induction by TCDD alone in this experiment is included for comparison. In this and subsequent figures, the symbol and associated bracket denote the mean and standard deviation of at least four determinations from two separate experiments.

are currently not well understood.[5] Table 2 shows that there exists no relationship between cytotoxicity by TCDD and the hydroxylase activity. An additional factor in the study of toxic effects by TCDD is the toxicity of the solvent alone. We determined that about 0.2% *p*-dioxane is the threshold at which solvent toxicity in all cell lines becomes significant; the extreme toxicity of 1.0% *p*-dioxane is shown in Table 2. The maximal solubility of TCDD in *p*-dioxane is 240 μg/ml (and considerably less than this in any other commonly used organic solvent). Therefore, in order to distinguish between toxicity in established cell lines from TCDD and that from the *p*-dioxane, the absolutely maximal TCDD concentration which can be used in cell culture is 1.5 μM. TCDD toxicity in primary cultures was seen at concentrations between 0.1 and 0.2 μM. In fetal

[5] For further relevant discussion, see the publication cited in ref. 16.

primary cultures, *p*-dioxane toxicity occurred at concentrations greater than 0.10%.

Estimation of ED_{50} for hydroxylase induction in cell lines by TCDD. Table 3 shows the estimated ED_{50} values for the TCDD-inducible hydroxylase activity in each of 11 established cell lines examined. The means by which we obtained these data for three representative cell lines are shown in Fig. 3: fractional response curves were plotted and, if the curve was symmetrical by probit analysis, a median effective dose for 50% of the maximal induction response could be accurately determined.

Estimation of ED_{50} for hydroxylase induction in fetal primary cultures and human lymphocytes by TCDD. Figure 4 shows that the magnitude of the hydroxylase induction by MC and the greatest response to MC on the second day after plating, in minced liver primary cultures derived from new born mice, is similar to that in primary mouse (4) or rat liver (18) cultures derived from fetal tissue. In primary cultures of fetal mouse, hamster, rat, chick, and rabbit, we found the time course of hydroxylase induction by TCDD to be virtually identical with that by MC. In all experiments, therefore, we added inducer to primary cultures 24 hr after plating and assayed the enzyme activity 24 hr later. From the six species, including man (Table 4), ED_{50} values for TCDD-inducible hy-

TABLE 1

Effect of various concentrations of TCDD and MC, alone or in combination, on hydroxylase activity in H-4-II-E cells

The cells were exposed to the compounds, alone or in combination and at the various concentrations shown, for 24 hr. Enzyme activity was then determined as described in MATERIALS AND METHODS. The values shown represent the average of duplicate determination on each of two duplicate tissue culture dishes.

MC	Specific aryl hydrocarbon hydroxylase activity					
	100 nM TCDD	10 nM TCDD	1 nM TCDD	0.1 nM TCDD	0.01 nM TCDD	No TCDD
μM	*units/mg cellular protein*					
10	57	70	74	75	81	87
1	71	83	86	87	98	102
0.1	74	75	60	64	68	76
0.01	90	78	56	15	15	6.9
0.001	92	86	76	13	3.6	1.1
0	112	92	94	19	0.83	0.54

TABLE 2

No relationship between inducible hydroxylase activity in several TCDD-treated cell lines and cytotoxicity

The cells were plated at 1.5 × 10^6/60-mm dish; 72 hr later, during logarithimic growth, *p*-dioxane, alone or with TCDD, was added as shown. Twenty-four hours later the surface of the cells was washed twice with phosphate-buffered NaCl and then treated for 10 min at 37° with 0.10% Viokase. The cells were collected by centrifugation at 800 × *g* for 5 min, resuspended in growth medium, and stained with trypan blue. Those cells not taking up the dye were regarded as "viable"; the percentage of viable cells was estimated after evaluating a minimum of 500 cells. Cells receiving the same treatment were harvested in the usual manner and assayed for aryl hydrocarbon hydroxylase specific activity, expressed in units per milligram of cellular protein.

Treatment	H-4-II-E		Hepa-1		TRL-2-Cl-2		TRL-2-Cl-3		LB82	
	Viable cells	Hydroxylase	Viable cells	Hydroxylase	Viable cells	Hydroxylase	Viable cells	Hydroxylase	Viable cells	Hydroxylase
	%		%		%		%		%	
1.0% *p*-dioxane	8.5	0.10	2.3	0.30	4.1	<0.10	6.2	0.15	2.1	<0.01
0.2% *p*-dioxane	89	0.90	92	19	87	0.32	91	0.68	92	<0.01
0.2% *p*-dioxane + 1.5 μM TCDD	46	31	12	13	5.9	0.20	9.0	0.20	31	<0.01
0.1% *p*-dioxane	95	1.2	96	24	93	0.40	97	0.80	96	<0.01
0.1% *p*-dioxane + 300 nM TCDD	77	82	96	92	89	1.5	93	7.3	98	<0.01
0.1% *p*-dioxane + 30 nM TCDD	98	91	93	67	93	1.2	93	5.2	97	<0.01

TABLE 3

Basal and inducible hydroxylase activities and ED_{50} for TCDD as inducer in 11 established cell lines

Between 0.7×10^6 and 1.5×10^6 cells were plated in 60-mm tissue culture dishes; 48 or 72 hr later, when the cells were in logarithmic growth, an ED_{50} was determined after 24-hr treatments of the cultures with varying amounts of TCDD. Because of the extreme difficulty in reproducibility of the hydroxylase assay when the maximally inducible specific activity is less than 1.00, we feel that the ED_{50} values for ERL-2, E-3, VERO, and HTC may not be accurate. No relationship ($r = -0.28$; $p > 0.10$) was found between the maximally inducible hydroxylase activity in TCDD-treated cells and the ED_{50} for TCDD.

Cell line	Specific aryl hydrocarbon hydroxylase activity			ED_{50} for TCDD	Origin of cell line
	Basal	MC-treated[a]	TCDD-treated[b]		
	units/mg cellular protein			*nm*	
H-4-II-E	0.50-2.0	108	118	0.23	Rat Reuber hepatoma
TRL-2-Cl-2	0.30-0.70	4.2	3.2	0.32	Normal rat liver
Hepa-1	15-30	112	114	0.45	Mouse hepatoma
MA	0.20-0.30	5.9	7.6	1.4	Normal rat liver
ERL-2	0.02-0.05	0.67	0.81	1.6	Normal rat liver
E-3	0.05-0.20	0.28	0.93	1.6	Normal rat liver
NRKE	0.30-0.60	1.5	2.9	2.6	Normal rat kidney epithelium
Chang liver	0.20-0.60	0.68	1.5	11	Human liver
VERO	0.05-0.30	1.1	0.93	110	Monkey kidney
HTC	<0.01-0.02	0.15	0.89	>200	Rat "minimal deviation" hepatoma
LB82	<0.01	<0.01	<0.01		Mouse fibroblasts

[a] Maximal enzyme activity found after exposure of cells to an optimal concentration of MC for 24 hr.
[b] Maximal enzyme activity found after exposure of cells to an optimal concentration of TCDD for 24 hr.

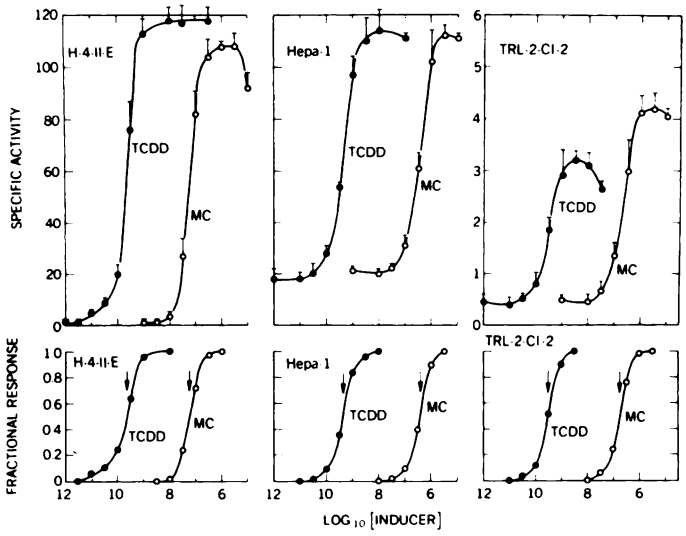

FIG. 3. *Basal and inducible hydroxylase activities and fractional response of induction, as a function of TCDD or MC concentration*

The vertical arrows denote the estimated ED_{50} for each inducer. The enzyme activities in H-4-II-E cells treated with 3.0 pM TCDD or 3.0 nM MC, and in Hepa-1 or TRL-2-Cl-2 cells treated with 10 pM TCDD or 10 nM MC, were not statistically different from the enzyme activities in these respective cell lines grown in control medium alone.

droxylase activity ranged from about 0.12 nM for the C57BL/6N mouse to about 6.4 nM for the AKR/N mouse; the responsiveness of hamster, rat, man, chick, and rabbit cultures to TCDD all fell between these two extreme values from the different inbred mouse strains. We realize that individual differences in the dose-response curve may exist between different individuals in the outbred human population (8, 17). Whether we used primary cultures derived from the whole fetus or fetal liver or primary minced cultures derived from liver or the combination of lung, bowel, and kidney from newborn mice, ED_{50} values for a particular strain were virtually identical; in other words, although the maximally induced hydroxylase activity varied by as much as 5-fold, the fractional response curves yielded reproducibly similar ED_{50} values characteristic for a given strain. The primary cultures were prepared in such a manner that 1.5×10^6 cells/60-mm dish could not be accurately determined beforehand. With all the cell lines, therefore, we determined the protein content equivalent to a quantity of cells: the value ranged from 3×10^6 cells/mg of protein for LB82 to 5×10^6 cells/mg of protein for H-4-II-E. Because the cell size

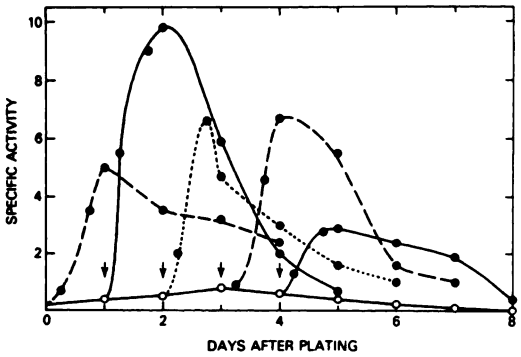

FIG. 4. *Kinetics of hydroxylase induction by MC in minced liver cultures from neonatal B6 mice*

The preparation and plating of these primary cells are described in MATERIALS AND METHODS. The vertical arrows indicate the five different times at which 1.0 μM MC was added to the growth medium. Each symbol represents the average specific enzyme activity from duplicate dishes treated with MC (●) or control medium alone (○). Dashed, solid, and dotted lines denote the beginning of addition of MC on different days.

TABLE 4

Estimated ED_{50} for TCDD as inducer of hydroxylase activity in primary cultures from six species

Primary cultures from fetuses of the indicated gestational ages were prepared as previously described (4, 18). Fetal rabbit and mouse cultures entered logarithmic growth 24 hr, fetal hamster cultures 48 hr, and fetal chick and rat cultures 72 hr after plating. During the period of logarithmic growth, varying concentrations of MC or TCDD were added in fresh growth medium, and the hydroxylase activity and protein content were determined 24 hr later. In all cases the ED_{50} is based on an estimation of 3×10^6 cells/60-mm dish.

Species	Normal gestational period	Estimated gestational age at which cultures were prepared	Specific aryl hydrocarbon hydroxylase activity			ED_{50} for TCDD
			Basal	MC-treated[a]	TCDD-treated[a]	
	days	*days*	*units/mg cellular protein*			*nM*
Chick	21	18	0.44-0.82	4.4	11.3	0.18
Hamster	16	12-14	0.16-0.60	6.7	5.9	0.24
Rat	21-23	16-18	0.82-1.3	9.4	6.4	0.62
Rabbit	30	26	0.13-0.22	2.8	3.2	1.2
Mouse	21	16-18				
C57BL/6N			0.45-0.80	5.1	4.5	0.12
C3H/HeN			0.41-0.70	4.9	2.5	0.76
DBA/2N			0.16-0.32	2.8	3.2	1.9
AKR/N			0.20-0.44	2.1	2.9	6.4
Human lymphocytes[b]			0.038-0.16	0.23	0.21	8.0

[a] Recorded values are the *maximal* enzyme activities found in several experiments after exposure of cells to optimal inducing concentrations of MC or TCDD for 24 hr.

[b] Prepared and assayed as recently described (8, 17).

of the primary cultures appeared morphologically similar to that of the various established cell lines, we estimated that 1 mg of protein from the primary cultures was equivalent to about 3×10^6 cells.

Representative dose-response curves from C57BL/6N and DBA/2N mouse cultures are illustrated in Fig. 5. It is evident that DBA/2N-derived cultures require more of either TCDD or MC to induce the enzyme activity than C57BL/6N-derived cultures. The slightly toxic effect of 10 μM MC, compared with 1.0 μM MC, is also shown in Fig. 5. It is therefore possible that, if TCDD were not toxic at levels of 0.2 μM or higher and if MC were not toxic at levels of 100 μM or higher, the maximally inducible hydroxylase activity in DBA/2N cultures would be the same as that in C57BL/6N cultures. Because 0.10 μM TCDD is toxic to the DBA/2N cultures, we realize that our estimated ED_{50} value may be slightly lower than the true ED_{50} value; however, by probit analysis our ED_{50} value is within the range predicted by a normal dose-response curve.

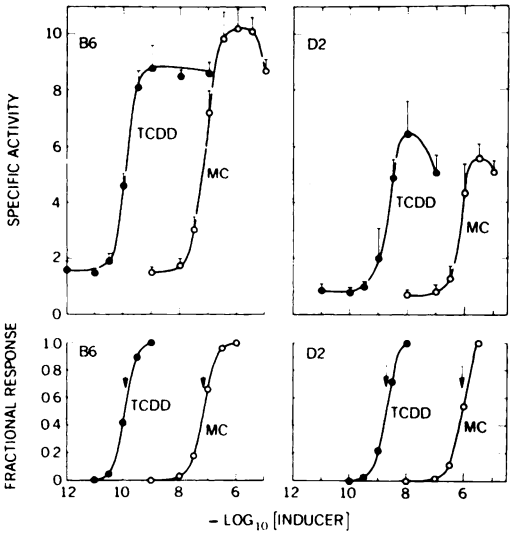

FIG. 5. *Basal and inducible hydroxylase activities and fractional response of induction, as a function of TCDD or MC concentration*

The vertical arrows denote the estimated ED_{50} for each inducer. Minced liver cultures, as described in MATERIALS AND METHODS and also used in the experiment shown in Fig. 4, were prepared from newborn C57BL/6N (B6) and DBA/2N (D2) mice. The enzyme activities in B6 cells treated with 10 pM TCDD or 10 nM MC, and in D2 cells treated with 300 pM TCDD or 100 nM MC, were not statistically different from the enzyme activities in these primary cultures grown in control medium alone.

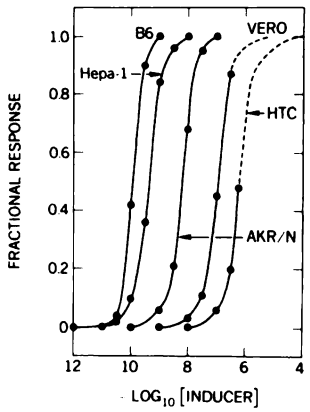

FIG. 6. *Fractional response of hydroxylase induction as a function of TCDD concentration in medium*

Although only fetal primary cultures from C57BL/6N (B6) and AKR/N inbred mice and the Hepa-1, VERO, and HTC established cells lines are illustrated, similar dose-response curves were generated for all the data shown in Tables 3 and 4. The slopes of the dose-response curves were not statistically significantly different for any of the cultures; the toxicity caused by TCDD at concentrations above 0.7 μM made it difficult for us to estimate accurate ED_{50} values for VERO or HTC cultures.

DISCUSSION

The estimated ED_{50} values for hydroxylase induction by TCDD among 11 established cell lines, fetal primary cultures derived from four strains of mice plus four other species, and cultured human lymphocytes ranged from 0.12 nM for the C57BL/6N mouse to more than 100 nM for several established cell lines. The problem of TCDD toxicity becomes important in the range of 0.7-1.0 μM TCDD (see Fig. 6). We therefore found it difficult to determine accurately the ED_{50} of any inductive response having an ED_{50} value greater than about 100 nM TCDD. By analyzing the shapes of the curves on probit paper and assuming a similar slope of the dose-response curve, especially for HTC cultures, we estimate that the ED_{50} values for the VERO and HTC lines are about 110

and 640 nM, respectively; however, the value for HTC remains in doubt and is expressed in Table 3 simply as ">200 nM." No detectable hydroxylase activity was ever found in the LB82 line. This finding is of particular importance, because bromodeoxyuridine treatment (24) of cell cultures may cause profound and lasting effects on various differentiated functions.

Of interest (Figs. 3 and 5) is that MC responsiveness in all cases examined was less than TCDD responsiveness, by a rather constant factor of between 250 and 900. For example, in H-4-II-E cells the ED_{50} for MC is about 57 nM and for TCDD its about 0.23 nM. These data differ somewhat from the 60-fold difference between MC and TCDD found (17) in human lymphocytes. It is difficult to be more precise than to estimate that the difference factor is approximately between 250 and 900, because of experimental variations such as cell growth and density and simply the approximation of drawing the dose-response curves. The reason for this 60-fold, 250-fold, or 900-fold difference in cell culture, compared with a difference of about 30,000 between MC and TCDD in the intact rat (12), is not understood. Also noteworthy with respect to TCDD-responsive hydroxylase induction is the approximately 16-fold difference between cultured C57BL/6N and DBA/2N cells (Fig. 5). This difference is very close to that observed (14) when the hepatic hydroxylase induction in these two strains is compared after TCDD administration *in vivo*.

What does an ED_{50} of 0.23 nM in culture signify? All cultures contained about 2×10^6 cells in 3.0 ml of medium at the time of assay. Therefore, if 0.23 nM equals 0.69 pmole of TCDD exposed to 2×10^6 cells, this means that the hydroxylase activity is half-maximally induced when there exist about 2.1×10^5 molecules/H-4-II-E cell. Because of the very low basal enzyme activity and the large rise in inducible hydroxylase activity in H-4-II-E cultures, we suggest that this cell line may be useful in a bioassay for detecting small amounts of TCDD. Under our experimental conditions, the hydroxylase activity in these cells is significantly induced above baseline levels at about 10 pM TCDD (i.e., about 9200 molecules/cell). This finding means that TCDD at about 1.0×10^{-14} mole/ml of culture medium causes a detectable rise in the enzyme activity in H-4-II-E cells. With the recent improvement of the hydroxylase assay in order to increase its sensitivity, as recently described (8, 17), and possibly with the cloning of even more sensitive H-4-II-E sublines, this detection level of TCDD in biological materials may be lowered even further.

This study also provides the groundwork for determining differences in the TCDD "receptor site," if such a specific, high-affinity binding site exists. Hence the affinity or the number of binding sites for TCDD in C57BL/6N or H-4-II-E subcellular fractions might be quantitatively greater than in DBA/2N, HTC, or LB82 subcellular fractions, if such specific binding of TCDD is related to its effect on the hydroxylase induction.

ACKNOWLEDGMENTS

We appreciate valuable discussions with Dr. Alan P. Poland concerning these experiments, and we acknowledge the help of Drs. Richard E. Kouri and Steven A. Atlas in the studies with cultured human lymphocytes.

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