

AN IN VITRO RAINBOW TROUT CELL BIOASSAY FOR
ARYL HYDROCARBON RECEPTOR-MEDIATED TOXINSCATHERINE A. RICHTER,*† VIRGINIA L. TIEBER,‡ MICHAEL S. DENISON§ and JOHN P. GIESY†
†Department of Fisheries and Wildlife, Pesticide Research Center, Institute for Environmental Toxicology.
‡Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824-1222, USA
§Department of Environmental Toxicology, Meyer Hall, University of California at Davis, Davis, California 95616, USA

(Received 31 January 1996; Accepted 18 July 1996)

Abstract—Halogenated aromatic hydrocarbons (HAHs) and other chemicals that act as aryl hydrocarbon (Ah) receptor (AhR) agonists cause a variety of toxic effects. In sac fry of many fish species, these effects include blue-sac disease and mortality. Because HAHs occur in complex mixtures, their toxicity in the environment is difficult to predict. A bioassay useful in predicting AhR-mediated toxicity to fish was developed using the RTH-149 rainbow trout hepatoma cell line. Stable transfection of this cell line with the pGudLuc1.1 plasmid, which contains a firefly luciferase reporter gene under the transcriptional regulation of dioxin responsive enhancers, has produced a recombinant cell line designated Remodulated Lightning Trout (RLT 2.0). The RLT 2.0 bioassay method detection limit for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is 4 pM. The responses of the RLT 2.0 bioassay to TCDD and several HAH congeners closely matched the responses observed *in vivo* in fish. The RLT 2.0 bioassay can provide an integrative measure of the total AhR-mediated toxic activity of complex mixtures to fish. The assay will be useful in screening environmental extracts, guiding chemical analysis, and interpreting the AhR-mediated mechanism of toxicity.

Keywords—TCDD Ah receptor Fish Bioassay PCB

INTRODUCTION

Halogenated aromatic hydrocarbons (HAHs) are a diverse group of chemicals, including the polychlorinated or polybrominated dibenzo-*p*-dioxins, dibenzofurans, biphenyls, naphthalenes, azobenzenes, and diphenyl ethers. Some were produced for industrial processes, whereas others are by-products of combustion or chlorination reactions [1-3]. This chemical family includes many congeners that are lipophilic, persistent, and toxic [3].

Halogenated aromatic hydrocarbons produce multiple toxic effects [1-3]. Most of these effects involve epithelial tissues, and many effects involve altered regulation of cell growth and differentiation [1-3]. The types and intensities of effects of HAHs vary among species [1-3]. In fish, exposure to HAHs may cause mortality, wasting syndrome, fin and gill lesions, hepatotoxicity, immunotoxicity, or reproductive impairment [4-7]. Fish eggs exposed to HAHs exhibit increased mortality at hatching and blue-sac disease, which results in mortality before the swim-up stage [7-9].

Halogenated aromatic hydrocarbons that assume a planar configuration and some nonhalogenated planar compounds, such as the polycyclic aromatic hydrocarbons (PAHs), share a common mechanism of action [1,3,10,11]. These compounds bind with varying affinities to the aryl hydrocarbon (Ah) receptor (AhR). After ligand binding, the AhR undergoes an activation process, during which it dimerizes with the Ah receptor nuclear translocator (ARNT) and acquires high affinity for specific DNA sequences, known as dioxin responsive enhancers (DREs) [10,12,13]. Binding of the HAH:AhR:ARNT complex to DREs results in stimulation of transcription of

adjacent genes [13]. Exposure to AhR-mediated toxins is often measured as an increase in aryl hydrocarbon hydroxylase (AHH) or 7-ethoxyresorufin-*O*-deethylase (EROD) activity. Aryl hydrocarbon hydroxylase and EROD are monooxygenase activities associated with cytochrome P4501A1, an AhR-regulated gene [10].

The involvement of the AhR in toxicity is supported by two lines of evidence. First, inbred mouse strains expressing defective AhR have decreased sensitivity to HAH toxicity [1,3,10]. Second, structure-activity relationships reveal that the potency of an HAH is correlated with the affinity of binding of that compound to the AhR [1,10].

Here we report on the development and characterization of a bioassay for AhR-mediated toxins. The bioassay uses a genetically engineered rainbow trout liver tumor cell line that contains the firefly luciferase reporter gene, the transcription of which is under the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-inducible control of several DREs. Exposure of these cells (designated Remodulated Lightning Trout [RLT 2.0] cells) to HAHs results in stimulation of expression of the luciferase gene, which can readily be detected using a light-generating substrate.

The objectives of this study were to characterize the RLT 2.0 cell line, investigate the relationship between luciferase induction and cytochrome P4501A1 expression, and establish the relevance of the RLT 2.0 bioassay to HAH toxicity in fish.

METHODS

Cell culture, plasmids, and transfections

RTH-149 cells (ATCC 1710, American Type Culture Collection, Rockville, MD, USA) were grown in basal Eagle's medium (Gibco/BRL, Grand Island, NY, USA) supplemented

* To whom correspondence may be addressed. The current address of C.A. Richter is Biochemistry Department, M716 Medical Sciences, University of Missouri-Columbia, Columbia, MO 65212, USA.

with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA) in an enriched CO₂ atmosphere at 21°C.

The plasmid pGudLuc1.1 was constructed by inserting the 1,810-bp *Hind*III fragment present in pMpap1.1 and pMcat5.9 [14,15] into the *Hind*III site of pGL2-Basic (Promega, Madison, WI, USA) upstream of the luciferase coding region. The insert contains a 1.3-kb fragment of the long terminal repeat from the mouse mammary tumor virus (MMTV-LTR), which contains the viral promoter but not the glucocorticoid responsive elements (GREs) normally present in the MMTV-LTR [16]. A 482-bp fragment of the regulatory region of the mouse cytochrome P4501A1 gene containing four DREs is inserted 284 bp upstream of the MMTV-LTR promoter start site [14,17,18]. Previous studies have shown that TCDD-inducible activity of a reporter gene controlled by this MMTV-LTR/DRE insert is dependent on the presence of an AhR-agonist, functional AhR in the cell line, and DRE sequences in the regulatory region [15].

To prepare stably transfected cells, 70% confluent plates of RTH-149 cells were cotransfected with 7 µg pGudLuc1.1 and 3 µg pSV₂neo using polybrene as previously described [19,20]. Cells were subcultured at a 1:30 dilution in medium containing 500 mg/L geneticin (G418) 3 d after transfection, and the G418 concentration was raised to 1,000 mg/L after 10 d. Colonies of surviving cells were isolated and screened for HAH-inducible luciferase activity. The RLT 2.0 cell line has been cultured with 1,000 mg/L G418 in the medium continuously.

For luciferase assays, cells were cultured in 96-well plates. For EROD assays, cells were cultured in 60-mm plates. Cells were dosed with the compound of interest dissolved in dimethylsulfoxide (DMSO) or isooctane. Dimethylsulfoxide and isooctane were used at a final concentration of 0.1% (v/v) and 1% (v/v) in medium, respectively.

Cell viability assays

Cytotoxicity of extracts, solvents, or test compounds may interfere with the RLT 2.0 luciferase assay. Cytotoxicity was assessed using the LIVE/DEAD® EukoLight Viability/Cytotoxicity Assay (Molecular Probes, Eugene, OR, USA). In this assay, calcein AM and ethidium homodimer are added to cells, and fluorescence is measured. The viability assay detects esterase activity characteristic of live cells. Esterases convert calcein AM to calcein, which can be detected by fluorescence (excitation 485 nm, emission 530 nm). The cytotoxicity assay detects dead or damaged cells. Ethidium homodimer enters only cells with damaged membranes and binds DNA to produce a fluorescent complex (excitation 530 nm, emission 645 nm). Fluorescence was measured using a CytoFluor 2300 fluorometer (Millipore, Bedford, MA, USA).

Luciferase assays

Cells were rinsed twice with phosphate-buffered saline (PBS), then harvested with 30 µl cell lysis buffer (Promega) per well. Aliquots (20 µl) of the cell lysates were mixed with 100 µl luciferase assay reagent (Promega) and light production was measured with a TD-20e luminometer (Turner Designs, Sunnyvale, CA, USA) set to 5-s delay and 30-s integration. Light production was expressed in luminescence units (lu) per sample.

EROD assays

The EROD assay was performed using the methods of Tillitt et al. [21], with modifications. RTH-149 cells were grown and

exposed to TCDD in DMSO (0.1% [v/v] in medium) in 60-mm plates. Plates were washed twice with PBS and harvested by scraping. Harvested cells were centrifuged, and the pellets were frozen at -70°C. Cells were thawed in Tris buffer containing 40 µM dicumerol, an inhibitor of diaphorase, which degrades resorufin [22]. Cells were then disrupted by sonication (25 pulses, 60% duty cycle, output control 7). The disrupted cell solutions were transferred to individual wells of a 96-well plate. 7-Ethoxyresorufin was added to the cell lysate, and the samples were warmed to 30°C. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was added to initiate the EROD reaction, which was allowed to proceed for 60 min at 30°C. Resorufin production was quantified by fluorescence (excitation 530 nm, emission 590 nm) on a CytoFluor 2300 fluorometer (Millipore).

Calculations

Median effective concentration (EC₅₀) values were estimated from Scatchard analyses. 2,3,7,8-Tetrachlorodibenzo-dioxin equivalence factors (TEFs) were calculated in two ways. First, TEFs were calculated as the ratio of the EC₅₀ for TCDD to the EC₅₀ for the compound of interest. Second, we calculated the concentration of each compound predicted to produce a response equal to 20% of the maximum response induced by TCDD. This concentration was designated EC₂₀ (TCDD). The TEFs were then calculated as the ratio of the EC₂₀ (TCDD) for TCDD to the EC₂₀ (TCDD) for the compound of interest.

Statistical power analysis was used to estimate the ability of the bioassay to distinguish between induced and control treatments [23]. The variance of the control group was measured in a single experiment. The type I error rate, α , was set at 0.05 and the type II error rate, β , at 0.20. The sample size per treatment was three. The smallest detectable difference in means was calculated using the methods of Oris and Bailer [23].

RESULTS

Transfection experiments

Transient transfection experiments confirmed that the luciferase reporter gene, MMTV-LTR promoter, and mouse DREs were functional in RTH-149 cells (data not shown). RTH-149 cells were cotransfected with pSV₂neo and pGudLuc1.1. Seven colonies survived to be tested for luciferase expression and induction. One clone (RTH-149-luc-T1) exhibited relatively little background luciferase expression and approximately 2.5-fold induction after exposure to 1 nM TCDD for 24 h. RTH-149-luc-T1 was designated Remodulated Lightning Trout (RLT 1.0).

The RLT 1.0 cell line exhibited changes over time in the shape of its dose-response curve for the luciferase reporter gene and in the maximum luciferase induction produced by TCDD treatment (data not shown). Because these changes were likely to be the result of a mixed cell population, the cell line was reisolated and designated RLT 2.0. A control chart monitoring the maximum luciferase induction produced by TCDD treatment for each new assay was used to confirm the continued purity of the reisolated cell line.

Characterization of the RLT 2.0 cell line

Characterization of the RLT 2.0 cell line included investigation of the relationships of luciferase induction with total cell mass, duration of exposure, and solvents. Protein concen-

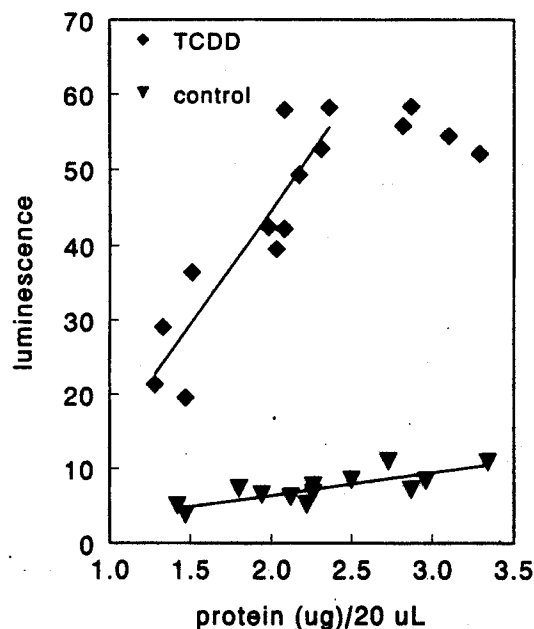


Fig. 1. Relationship between mass of cells and luciferase induction. Protein concentration is used as an index of total cell mass. Cells were treated for 4 d with 1% isooctane or 100 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in isooctane. Bovine serum albumin was used as a protein standard. Lines represent predictions from linear regression analysis (TCDD $R^2 = 0.811$; control $R^2 = 0.687$).

tration was used as an index of total cell mass. Luciferase activity increased in a linear relationship with cell mass for both TCDD and isooctane control cells, up to a protein concentration of approximately 2.5 $\mu\text{g}/20 \mu\text{l}$ (Fig. 1). The time course for luciferase induction in RLT 2.0 cells dosed with 100 nM TCDD was determined (Fig. 2). Induction increased with time up to approximately 6 d of exposure. After 4 d,

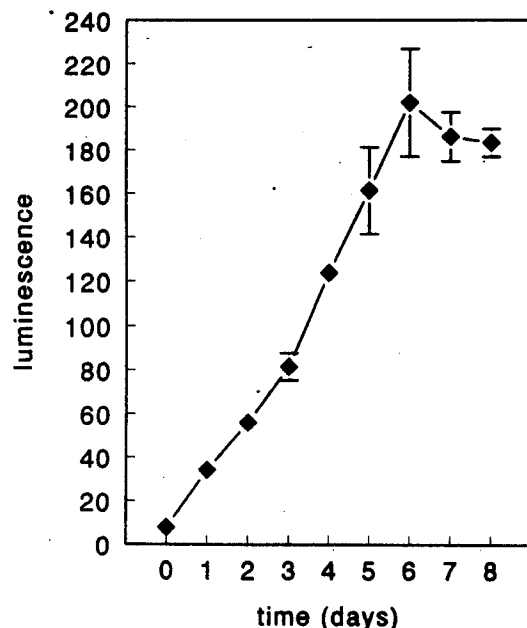


Fig. 2. Relationship between duration of exposure and luciferase induction. Cells were treated with 100 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in isooctane. Medium was renewed 8 d and 4 d before harvesting. Error bars represent one standard deviation from the mean ($n = 4$).

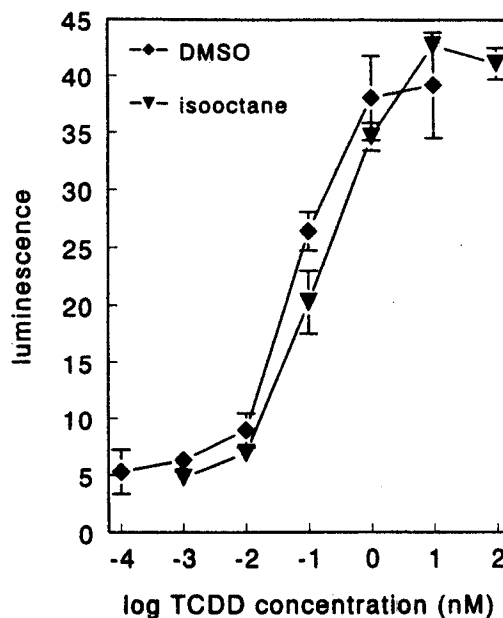


Fig. 3. Effects of solvent on luciferase induction. Cells were treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in dimethylsulfoxide (0.1%, v/v; 14.1 mM) or isooctane (1%, v/v; 60.4 mM) for 4 d. Error bars represent one standard deviation from the mean ($n = 3$).

variability increased. Cells were exposed to test compounds for 4 d in subsequent experiments. Dimethylsulfoxide and isooctane were compared as solvents to deliver the test compounds to the cells. Neither solvent produced a detectable difference in luminescence or in cell viability, relative to untreated cells. RLT 2.0 cells treated with TCDD delivered in these two solvents produced similar dose-response relationships (Fig. 3).

A cell viability assay controlled for cytotoxicity of the compounds and solvents tested. The viability assay could also detect large differences in the total number of cells per well. Treatments that showed evidence of cytotoxicity were excluded from the data set.

The discrimination power of the RLT 2.0 bioassay was determined by a power analysis [23]. The mean of 13 solvent control treatments was 9.4 lu per 20- μl sample, and the variance was 4.1. The least detectable difference in means was 4.12 lu. Therefore, the least detectable level of induction was 13.5 lu, or 1.4 times the control. A TCDD concentration of 4 pM was predicted to produce this response.

Comparison of luciferase and EROD induction

In order to compare the activity of the recombinant cell line reporter gene with the activity of a well-characterized, native, inducible gene, induction of luciferase activity in the RLT 2.0 cell line was compared to induction of EROD activity in the parent RTH-149 cell line (Fig. 4). The EROD activity was undetectable at TCDD concentrations less than 0.1 nM (the instrument detection limit was 0.05 pmol resorufin), so EROD induction relative to the control could not be measured. Although relative EROD to induction may be as great or greater than relative luciferase induction, absolute EROD activity was near the method detection limit of the assay and variable over the entire dose range. The least detectable difference in means for the EROD assay was 0.50 pmol resorufin/60 min/sample. Thus, EROD could not be used as an effective reporter gene

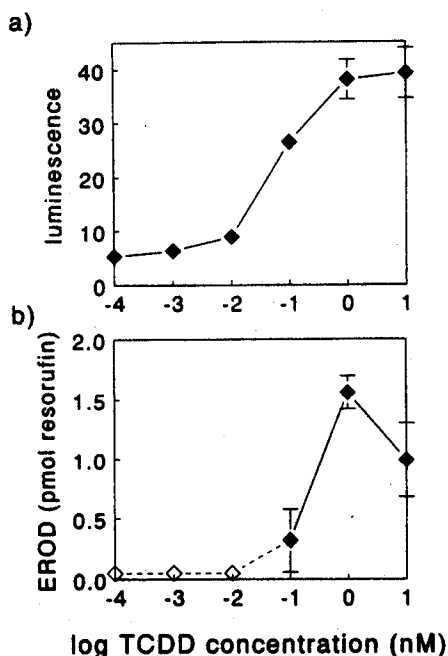


Fig. 4. Comparison of (a) luciferase induction in RLT 2.0 cells and (b) 7-ethoxyresorufin-*O*-deethylase (EROD) induction in RTH-149 cells, expressed in pmol resorufin/60 min/sample. Open symbols represent undetectable EROD activity, and are shown at the instrument detection limit of 0.05 pmol resorufin. Duration of exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was 4 d. Cells were exposed to TCDD in dimethylsulfoxide (0.1% v/v, in medium). Error bars represent one standard deviation from the mean ($n = 3$).

in RTH-149 cells. In contrast, luciferase activity in the RLT 2.0 cell line was an order of magnitude greater than the method detection limit of the assay and exhibited little variability.

Comparison of TCDD and PCBs

To assess the effects of TCDD and other AhR agonists in the RLT bioassay, TCDD; 1,2,3,7,8-pentachlorodibenzo-*p*-di-

oxin (1,2,3,7,8-PeCDD), 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PeCDF), and five polychlorinated biphenyl (PCB) congeners were tested (Fig. 5). The PCB congeners included three coplanar congeners (3,3',4,4',5-pentachlorobiphenyl, IUPAC PCB number 126; 3,3',4,4'-tetrachlorobiphenyl, IUPAC PCB number 77; and 3,3',4,4',5,5'-hexachlorobiphenyl, IUPAC PCB number 169), which are generally strong inducers of AhR-mediated responses, and two mono-*ortho*-substituted congeners (2,3',4,4',5-pentachlorobiphenyl, IUPAC PCB number 118, and 2,3,3',4,4'-pentachlorobiphenyl, IUPAC PCB number 105), which do not readily assume a planar conformation and are weak inducers [1,3]. 1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin, 2,3,4,7,8-PeCDF, and PCB 126 induced luciferase activity to the same maximum induced by TCDD; PCB 77 induced luciferase activity to approximately half the maximum induced by TCDD (Fig. 5). Polychlorinated biphenyl 169 was significantly less and induced luciferase activity significantly greater than the control only at the greatest concentration tested, 5.2 μ M (data not shown). Polychlorinated biphenyl 118 and PCB 105 did not induce luciferase activity at any concentration tested (data not shown). The greatest concentrations of PCB 118 and PCB 105 were 4.7 μ M and 6.2 μ M, respectively.

Comparison of RLT 2.0 TEFs with *in vivo* fish TEFs

The TEF values (Table 1) were calculated from the dose-response curves for TCDD and the PeCDD, PeCDF, and PCB congeners (Fig. 5). The TEF calculation based on the EC20 (TCDD) differed from the calculation based on the EC50s by less than 20% for the full agonists tested. The relative potencies of TCDD, 1,2,3,7,8-PeCDD, 2,3,4,7,8-PeCDF, and the PCB congeners were similar for the RLT 2.0 bioassay and an early life stage mortality assay [7]. In both assays, PCB 118 and PCB 105 failed to induce any response. Polychlorinated biphenyl 77 was approximately 10 times more potent in the RLT 2.0 bioassay than in the early life stage mortality assay (Table 1).

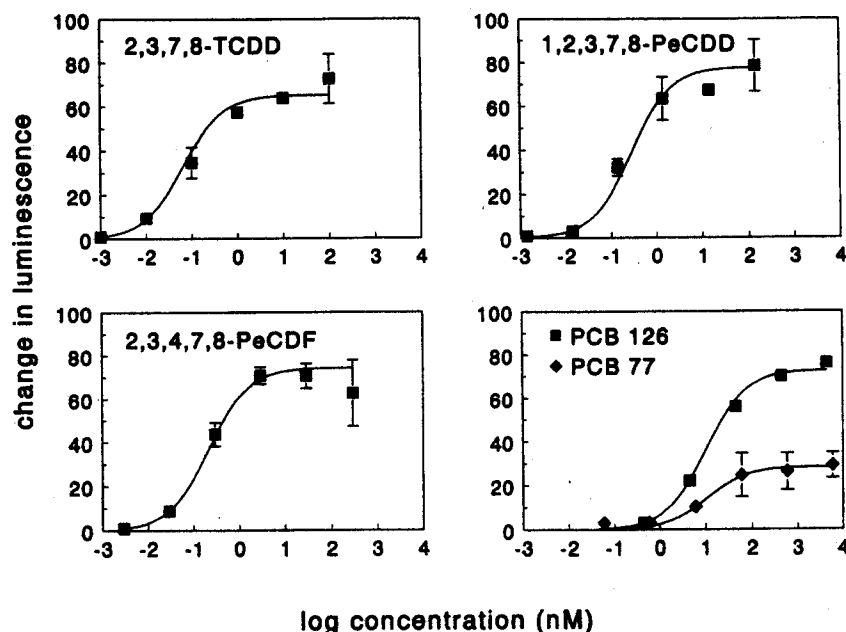


Fig. 5. Dose response of luciferase induction in RLT 2.0 cells. Cells were exposed to the test compounds in iso-octane (1% v/v, in medium) for 4 d. Lines represent responses predicted by Scatchard analysis. Error bars represent one standard deviation from the mean ($n = 3$).

Table 1. Responses of the RLT 2.0 bioassay to several halogenated aromatic hydrocarbon congeners, compared with responses of a rainbow trout early life stage (ELS) mortality assay [7]. Median effective concn. (EC50) and maximum values were calculated by Scatchard analysis. The maximum is the change in response relative to a solvent control, in luminescence units (lu). The RLT 2.0 TCDD equivalence factors (TEFs) were calculated from EC50s, and from the effective dose predicted to produce a response equal to 20% of the maximum response induced by TCDD (EC20 [TCDD])^a

	EC50 (nM)	Maximum (lu)	RLT 2.0 TEF (EC50)	RLT 2.0 TEF (EC20 [TCDD])	ELS mortality TEF [7]
2,3,7,8-TCDD	0.0643	65.3	1.0	1.0	1.0
1,2,3,7,8-PeCDD	0.286	77.8	0.225	0.279	0.73
2,3,4,7,8-PeCDF	0.217	74.3	0.296	0.347	0.359
3,3',4,4',5-PeCB (PCB 126)	10.2	72.7	0.00628	0.00717	0.005
3,3',4,4'-TCB (PCB 77)	10.8	28.2	0.00595	0.00173	0.00016

^a TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PeCDD = pentachlorodibenzo-*p*-dioxin; PeCDF = pentachlorodibenzofuran; PeCB = pentachlorobiphenyl; PCB = polychlorinated biphenyl; TCB = tetrachlorobiphenyl.

DISCUSSION

Transfection experiments

The function of the pGudLuc1.1 reporter system in the RTH-149 cell line was determined by transient transfection studies. The cells were able to produce functional luciferase, and their growth was not obviously affected. The MMTV-LTR promoter supported transcription of the luciferase gene in RTH-149 cells; thus the fish transcriptional machinery must recognize the regulatory elements in this construct. In addition, the fish AhR complex present in these cells [24] must recognize the mammalian DRE in the construct. This supports the hypothesis that the DRE sequence and DNA binding region of the AhR:ARNT complex are highly conserved among species [25]. In support of this is the recent work of Berndtson and Chen [26], which has demonstrated the presence of DRE sequences upstream of the TCDD-inducible rainbow trout CYP1A1 gene. Additional studies have indicated that mammalian AhR complex can bind to these trout DREs (M.S. Denison, unpublished results). These results support the utility of this reporter gene construct in examining AhR-dependent gene expression in various species.

Characterization of the RLT 2.0 cell line

The RLT 2.0 cell line was characterized to provide information needed in planning comparisons of luciferase and EROD activity, and investigations of HAH dose-response relationships. Protein concentration was used as an index of total cell mass. Protein is also correlated with cell density and the degree of confluence of the cells. Luciferase induction leveled off at protein concentrations greater than 2.5 µg/20 µl (Fig. 1). The samples containing greater than 2.5 µg/20 µl protein were taken from wells containing cells that had grown past confluence. The crowding of the cells may have inhibited luciferase production. Subsequent assays were performed with cells at approximately 90% confluence. The variability of the protein assay was too great to allow normalization of luminescence to protein concentration. Therefore, induction is reported as luminescence units per sample. Care was taken to introduce equal numbers of cells to each well in an assay. Although this method produced little variation within an assay, it allowed considerable variation in absolute luminescence units among assays. Relative induction was fairly consistent, at approximately eight times the control.

The time course was used to determine the optimum du-

ration of exposure for use in subsequent experiments. A duration of 4 d was chosen because it produced relatively great induction with little variation (Fig. 2). The relative induction and variation are important in determining the method detection limit of the bioassay. In addition, the cell growth medium must be renewed every 4 d. For durations of exposure longer than 4 d, medium renewal makes the test more labor-intensive, generates more waste, and raises concerns about changing the exposure of the cells to the test compound. In this experiment, the medium was renewed on day 4 of the exposure, without additional test compound or solvent. This may have contributed to the variation observed in 5-, 6-, 7- and 8-d treatments.

The time course of induction was significantly slower than that observed in mammalian cells [15,27,28]. This slower induction may result from the low levels of AhR present in RTH-149 cells as compared to most mammalian cells, or from species-specific differences in regulatory factors present in these cells. Temperature of incubation may also affect induction, because RTH-149 cells are grown at 21°C, whereas mammalian cells are generally grown at 37°C.

The solvents used to deliver test compounds to the cells did not affect induction (Fig. 3). In the absence of test compounds, neither solvent caused induction or effects on cell growth or viability at the concentrations used. Preliminary experiments showed that 1% (v/v) DMSO in medium negatively affected the viability of the cells (data not shown). Solvents could affect induction either by changing the kinetics of delivery of the test compound to the cells, or by affecting the cells directly. Effects of solvent on induction have been observed in assays of EROD activity in rat H4IIE cells [21].

Comparison of luciferase and EROD induction

The RLT bioassay was compared with an assay of EROD induction in the parent cell line, RTH-149 (Fig. 4). The two induction curves had similar thresholds between 0.01 and 0.1 nM. This suggests that the threshold of induction for both genes is dependent on activation of the AhR, and confirms that induction of the reporter gene is similar to induction of a native AhR-influenced gene.

Comparison of TCDD and PCBs

The dose-response relationships for TCDD and several HAH congeners were similar to those seen in tests of early life stage mortality in rainbow trout (Fig. 5) [7]. As expected, TCDD, 1,2,3,7,8-PeCDD, 2,3,4,7,8-PeCDF, and the non-*or-*

tho-substituted PCBs induced luciferase activity. However, there was no response to the mono-*ortho*-substituted PCBs, which neither induce CYP1A1 activity [29,30] nor produce early life stage mortality in rainbow trout [7]. This is a potential difference between fish and mammalian systems. At high concentrations, mono-*ortho*-substituted PCBs can induce responses in mammalian systems [21,27,31,32]. Given the abundance of mono-*ortho*-substituted congeners in environmental mixtures, they may be a source of AhR-mediated toxicity to mammals, and a source of error in using TEFs derived for mammals to predict toxicity to fish. The RLT 2.0 assay accurately represents toxicity to fish.

Comparison of RLT 2.0 TEFs with in vivo fish TEFs

The comparison of TEFs calculated from the RLT bioassay with TEFs calculated from *in vivo* fish bioassays was used to validate the RLT bioassay. Because the pattern of HAH potencies was the same for the RLT bioassay as for fish, the bioassay can be used as an approximate predictor of the responses of fish (Table 1).

Polychlorinated biphenyl 77 produced a complete dose-response curve with the same maximum as TCDD in the early life stage mortality assay [7]. However, PCB 77 acted as a partial agonist in the RLT 2.0 bioassay. Polychlorinated biphenyl 77 induced less than half the maximum luciferase activity induced by TCDD (Fig. 5). This partial agonist complicated the TEF calculations. It is not appropriate to base the calculation of the PCB 77 TEF on EC50 values, because at its EC50, PCB 77 induces less than half the response induced by TCDD at its EC50. We chose an arbitrary level of response, 20% of the maximum induced by TCDD, and calculated the concentration of each congener predicted to induce that response. These concentrations were then used to calculate TEFs. For example, the maximum response induced by TCDD was an increase of 65.3 lu. Twenty percent of this response is 13.1 lu. The concentration of TCDD predicted by Scatchard analysis to induce a response of 13.1 lu is 0.0161 nM. The concentration of PCB 77 predicted by Scatchard analysis to induce a response of 13.1 lu is 9.31 nM. Therefore, the TEF for PCB 77 is 0.00173. We validated this approach by comparing the TEFs calculated from EC50s and from the EC20 (TCDD). The results were similar for each of the full agonists tested (Table 1).

Utility of the RLT 2.0 bioassay

Because the RLT 2.0 bioassay can approximately predict AhR-mediated early life stage toxicity to fish, it can be useful in a variety of applications. The bioassay can be used to test compounds or extracts in situations in which *in vivo* testing is impractical, or as a supplement to chemical analysis. It can also be used to study the mechanism of action and the interactions of AhR-mediated toxins.

Because bioassays do not account for the pharmacokinetics and distribution of HAHs in fish, for species differences, or for fish physiology, we do not advocate the use of the RLT 2.0 bioassay for the development of TEFs for use in ecological risk assessment. Presumably, there are several steps between induction of AhR-activated genes and mortality. These steps are currently undefined, and are certainly not modeled by the RLT 2.0 bioassay. These differences between fish and fish cell cultures may account for the differences in TEF values calculated using the two systems (Table 1).

The RLT 2.0 bioassay is an appropriate alternative to *in*

vivo testing for screening of compounds, complex mixtures, or extracts. The rainbow trout egg injection early life stage mortality assay developed by Walker et al. [33] offers the advantages of realism, measurement of a toxicologically relevant endpoint, and delivery of a known amount of the test compound to the test organism. Early life stage mortality refers to the syndrome exhibited by rainbow trout and other salmonids when exposed to TCDD as eggs [7]. Exposure to TCDD causes increased mortality at hatching and development of blue-sac disease, which results in mortality before the swim-up stage [7-9]. Early life stage mortality is very sensitive to TCDD, with a median lethal dose (LD50) of 400 pg/g. However, results from the egg injection study are obtained 45 to 60 d after dosing [33], whereas results from the RLT 2.0 bioassay are obtained 4 d after dosing. The RLT 2.0 bioassay is appropriate for use as a screening tool that could test large numbers of samples for their potential toxicity to fish, and identify samples and compounds to be further tested in the egg injection assay.

Instrumental analysis of extracts and effluents can be enhanced by the RLT 2.0 bioassay. Prediction of total AhR-mediated toxicity of a sample by chemical analysis can be confounded by two factors. First, interactions among HAHs may lead to greater or lesser toxicity than predicted from an additive model. Second, some compounds that act through the AhR are not measured in routine analysis, because standards are unavailable or because the compounds have not yet been identified. The RLT 2.0 bioassay can be used in mass balance calculations to insure that chemical analyses have identified all the relevant components and interactions of the mixture. The RLT 2.0 bioassay can also be used to test defined mixtures in order to identify and characterize interactions involving AhR-mediated toxins. In fractionation studies, the components of a complex mixture are separated according to their chemical properties. The RLT 2.0 bioassay can be used to screen the separated fractions and direct further chemical analysis to identify sources of AhR-mediated toxicity. For example, pulp and paper mill effluents have recently been found to contain components that induce EROD activity and disrupt reproductive cycles in fish but are neither persistent nor chlorinated [34-38]. The RLT 2.0 bioassay could be useful in identifying the compound(s) responsible for these effects. It could be used to screen effluents, fractions of effluent, and putative AhR ligands found in the effluent through chemical analysis.

The RLT 2.0 bioassay is also appropriate for use in studies of the mechanism of action of AhR-mediated toxins to fishes. Because the RLT 2.0 bioassay measures induction of the luciferase reporter gene, it can be used in studies with metabolism inhibitors that inhibit EROD activity. These studies can be used to determine which form of a compound, the parent form or a metabolite, is active, and to determine if a compound is readily metabolized in fish liver cells.

In summary, the RLT 2.0 bioassay is useful as a screening tool that provides an integrative measure of the total AhR-mediated toxicity of a sample to fish. It can be used in conjunction with *in vivo* bioassays and with chemical analysis. In mechanism studies, the RLT 2.0 bioassay provides an index of the activation of the Ah receptor. Finally, the pGudLuc1.1 plasmid can be used to develop recombinant cell lines from different species, to allow direct comparisons of different species sensitivities to AhR-mediated toxins.

Acknowledgement—Jac M.M.J.G Aarts assisted in the preparation of

the pGudLuc1.1 plasmid. Frank M. D'Itri, William Helferich, and Stacey Kraemer assisted in the preparation of this manuscript. Dave DeWitt, Bill Smith, and Stacey Kraemer provided technical support and assistance. This research was supported, in part, by National Institutes of Health grant NIH-ES-04911, by National Institute of Environmental Health Sciences training grant T32-ES-07255, and by cooperative agreement CR822983-01-0 between the U.S. EPA Office of Water and Michigan State University. University sources of support included the Institute for Environmental Toxicology, Biotechnology Research Center, Department of Biochemistry, Department of Fisheries and Wildlife, and Pesticide Research Center.

REFERENCES

- Poland, A. and J.C. Knutson. 1982. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. *Annu. Rev. Pharmacol. Toxicol.* 22:517-554.
- Safe, S.H. 1986. Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. *Annu. Rev. Pharmacol. Toxicol.* 26:371-399.
- Safe, S. 1990. Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: Environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *Crit. Rev. Toxicol.* 21:51-88.
- Spitsbergen, J.M., J.M. Kleeman and R.E. Peterson. 1988. Morphologic lesions and acute toxicity in rainbow trout (*Salmo gairdneri*) treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Toxicol. Environ. Health* 23:333-358.
- Spitsbergen, J.M., K.A. Schat, J.M. Kleeman and R.E. Peterson. 1988. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or Aroclor 1254 on the resistance of rainbow trout, *Salmo gairdneri* Richardson, to infectious haematopoietic necrosis virus. *J. Fish Dis.* 11:73-83.
- Thomas, P. 1989. Effects of Aroclor 1254 and cadmium on reproductive endocrine function and ovarian growth in Atlantic croaker. *Mar. Environ. Res.* 28:499-503.
- Walker, M.K. and R.E. Peterson. 1991. Potencies of polychlorinated dibenzo-*p*-dioxin, dibenzofuran, and biphenyl congeners, relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, for producing early life stage mortality in rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* 21:219-238.
- Walker, M.W. and R.E. Peterson. 1994. Toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to brook trout (*Salvelinus fontinalis*) during early development. *Environ. Toxicol. Chem.* 13:817-820.
- Walker, M.K., J.M. Spitsbergen, J.R. Olson and R.E. Peterson. 1991. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity during early life stage development of lake trout (*Salvelinus namaycush*). *Can. J. Fish. Aquat. Sci.* 48:875-883.
- Whitlock, J.P., Jr. 1993. Mechanistic aspects of dioxin action. *Chem. Res. Toxicol.* 6:754-763.
- Hankinson, O. 1995. The aryl hydrocarbon receptor complex. *Annu. Rev. Pharmacol. Toxicol.* 35:307-340.
- Hoffman, E.C., H. Reyes, F-F. Chu, F. Sander, L.H. Conley, B.A. Brooks and O. Hankinson. 1991. Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science* 252:954-958.
- Denison, M.S., J.M. Fisher and J.P. Whitlock, Jr. 1988. The DNA recognition site for the dioxin-Ah receptor complex: Nucleotide sequence and functional analysis. *J. Biol. Chem.* 263:17221-17224.
- Fisher, J.M., K.W. Jones and J.P. Whitlock, Jr. 1989. Activation of transcription as a general mechanism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin action. *Mol. Carcinog.* 1:216-221.
- El-Fouly, M.H., C. Richter, J.P. Giesy and M.S. Denison. 1994. Production of a novel recombinant cell line for use as a bioassay system for detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-like chemicals. *Environ. Toxicol. Chem.* 13:1581-1588.
- Lee, F., C.V. Hall, G.M. Ringold, D.E. Dobson, J. Luh and P.E. Jacob. 1984. Functional analysis of the steroid hormone control region of mouse mammary tumor virus. *Nucleic Acids Res.* 12:4191-4206.
- Jones, P.B.C., D.R. Galeazzi, J.M. Fisher and J.P. Whitlock, Jr. 1985. Control of cytochrome P₁-450 gene expression by dioxin. *Science* 227:1499-1502.
- Jones, P.B.C., L.K. Durrin, D.R. Galeazzi and J.P. Whitlock, Jr. 1986. Control of cytochrome P₁-450 gene expression: Analysis of a dioxin-responsive enhancer system. *Proc. Natl. Acad. Sci. USA* 83:2802-2806.
- Kawai, S. and M. Nishizawa. 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. *Mol. Cell. Biol.* 4:1172-1174.
- Sussman, D.J. and G. Milman. 1984. Short-term, high-efficiency expression of transfected DNA. *Mol. Cell. Biol.* 4:1641-1643.
- Tillitt, D.E., J.P. Giesy and G.T. Ankley. 1991. Characterization of the H411E rat hepatoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons in environmental samples. *Environ. Sci. Technol.* 25:87-92.
- Lubet, R.A., R.W. Nims, R.T. Mayer, J.W. Cameron and L.M. Schechtman. 1985. Measurement of cytochrome P-450 dependent dealkylation of alkoxyphenoxazones in hepatic S9s and hepatocyte homogenates: Effects of dicumarol. *Mutat. Res.* 142:127-131.
- Oris, J.T. and J.A. Bailer. 1993. Statistical analysis of the *Ceriodaphnia* toxicity test: Sample size determination for reproductive effects. *Environ. Toxicol. Chem.* 12:85-90.
- Lorenzen, A. and A.B. Okey. 1990. Detection and characterization of [³H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin binding to Ah receptor in a rainbow trout hepatoma cell line. *Toxicol. Appl. Pharmacol.* 106:53-62.
- Bank, P.A., E.F. Yao, C.L. Phelps, P.A. Harper and M.S. Denison. 1992. Species-specific binding of transformed Ah receptor to a dioxin responsive transcriptional enhancer. *Eur. J. Pharmacol. Environ. Toxicol. Pharmacol. Sect.* 228:85-94.
- Berndtson, A.K. and T.T. Chen. 1994. Two unique *CYP1* genes are expressed in response to 3-methylcholanthrene treatment in rainbow trout. *Arch. Biochem. Biophys.* 310:187-195.
- Garrison, P.M., K. Tullis, J.M.M.J.G. Aarts, A. Brouwer, J.P. Giesy and M.S. Denison. 1996. Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-like chemicals. *Fundam. Appl. Toxicol.* 30:194-203.
- Postlind, H., T.P. Vu, R.H. Tukey and L.C. Quattrochi. 1993. Response of human CYP1-luciferase plasmids to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and polycyclic aromatic hydrocarbons. *Toxicol. Appl. Pharmacol.* 118:255-262.
- Gooch, J.W., A.A. Elskus, P.J. Kloepper-Sams, M.E. Hahn and J.J. Stegeman. 1989. Effects of *ortho*- and non-*ortho*-substituted polychlorinated biphenyl congeners on the hepatic monooxygenase system in scup (*Stenotomus chrysops*). *Toxicol. Appl. Pharmacol.* 98:422-433.
- Newsted, J.L., J.P. Giesy, G.T. Ankley, D.E. Tillitt, R.A. Crawford, J.W. Gooch, P.D. Jones and M.S. Denison. 1995. Development of toxic equivalency factors for PCB congeners and the assessment of TCDD and PCB mixtures in rainbow trout. *Environ. Toxicol. Chem.* 14:861-871.
- Anderson, J.W., S.S. Rossi, R.H. Tukey, T. Vu and L.C. Quattrochi. 1995. A biomarker, P450 RGS, for assessing the induction potential of environmental samples. *Environ. Toxicol. Chem.* 14:1159-1169.
- Sanderson, J.T., J.M.M.J.G. Aarts, A. Brouwer, K.L. Froese, M.S. Denison and J.P. Giesy. 1996. Comparison of Ah receptor-mediated luciferase and ethoxyresorufin-*o*-deethylase induction in H411E cells: Implications for their use as bioanalytical tools for the detection of polyhalogenated aromatic hydrocarbons. *Toxicol. Appl. Pharmacol.* 137:316-325.
- Walker, M.K., L.C. Hufnagle, Jr., M.K. Clayton and R.E. Peterson. 1992. An egg injection method for assessing early life stage mortality of polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls in rainbow trout, (*Oncorhynchus mykiss*). *Aquat. Toxicol.* 22:15-38.
- Munkittrick, K.R., G.J. Van Der Kraak, M.E. McMaster and C.B. Portt. 1992. Response of hepatic MFO activity and plasma sex steroids to secondary treatment of bleached kraft pulp mill effluent and mill shutdown. *Environ. Toxicol. Chem.* 11:1427-1439.
- Munkittrick, K.R., G.J. Van Der Kraak, M.E. McMaster, C.B. Portt, M.R. van den Heuvel and M.R. Servos. 1994. Survey of receiving-water environmental impacts associated with discharges from pulp mills. 2. Gonad size, liver size, hepatic EROD activity and plasma sex steroid levels in white sucker. *Environ. Toxicol. Chem.* 13:1089-1101.
- Servos, M.R., S.Y. Huestis, D.M. Whittle, G.J. Van Der Kraak and K.R. Munkittrick. 1994. Survey of receiving-water envi-

- ronmental impacts associated with discharges from pulp mills. 3. Polychlorinated dioxins and furans in muscle and liver of white sucker (*Catostomus commersoni*). *Environ. Toxicol. Chem.* 13: 1103-1115.
37. van den Heuvel, M.R., K.R. Munkittrick, G.J. Van Der Kraak, M.E. McMaster, C.B. Portt, M.R. Servos and D.G. Dixon. 1994. Survey of receiving-water environmental impacts associated with discharges from pulp mills. 4. Bioassay-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxic equivalent concentration in white sucker (*Catostomus commersoni*) in relation to biochemical indicators of impact. *Environ. Toxicol. Chem.* 13:1117-1126.
38. Zacharewski, T.R., K. Berhane, B.E. Gillesby and B.K. Burnison. 1995. Detection of estrogen- and dioxin-like activity in pulp and paper mill black liquor and effluent using in vitro recombinant receptor/reporter gene assays. *Environ. Sci. Technol.* 29:2140-2146.