Carcinogenesis vol.11 no.12 pp.2205–2210, 1990

Inhibition of growth by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in 5L rat hepatoma cells is associated with the presence of Ah receptor

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The role of the Ah receptor in mediating the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was investigated in 5L rat hepatoma cells containing TCDD-inducible cytochrome P450IA1 activity and in variants lacking cytochrome P450IA1 and Ah receptor. TCDD inhibited growth of the wild-type 5L cells, but not of the Ah receptor deficient variants. The two strong Ah receptor ligands 3,3′,4,4′-tetrachlorobiphenyl (3,3′,4,4′-TCB) and benz[*a*]anthracene (BA) exerted toxic effects in 5L cells that resembled those of TCDD. The poor Ah receptor ligand 2,2′,4,4′-tetrachlorobiphenyl was not toxic in 5L cells. The concentrations of TCDD, 3,3′,4,4′-TCB or BA required for the toxic response were similar to those that elicited P450IA1 induction. The present results suggest strongly that interaction with the Ah receptor is a necessary link in the chain of events leading to toxic effects in 5L cells upon exposure to TCDD.

Introduction

The environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD*) has been intensively studied during the last two decades with the aim of understanding its high toxicity and carcinogenicity. The toxic effects of TCDD include body weight loss, thymic involution, teratogenesis, chloracne and porphyria (1–3). TCDD also acts as a promoter of tumor formation (4,5). The mechanism(s) by which TCDD elicits toxic and carcinogenic responses are still not known.

One of the most thoroughly studied properties of TCDD and related compounds is their high-affinity binding to a specific cytosolic protein, the Ah receptor (6). Following transfer of the ligand receptor complex to the nucleus and interaction with regulatory sequences of nuclear DNA (7–9) a number of genes, the so-called Ah gene battery, are activated (10,11). One of the most prominent genes is that for cytochrome P450IA1 (for nomenclature of the P450 superfamily see ref. 12). A number of *in vivo* studies suggest that the Ah receptor is involved in TCDD toxicity (1–3). For example, the toxic effects of TCDD segregate with the expression of functional Ah receptor in certain mouse strains (13). The idea of a receptor-mediated mechanism of action has gained further support from structure–activity relationships and the correlation of the toxicity of a series of polyhalogenated aromatic hydrocarbons with their potency to induce P450I activity (3). However, there are also some reports which suggest that binding of TCDD to proteins other than the Ah receptor might be important for evoking biological responses to the dioxin (14–16).

In the past, studies on the mechanism of TCDD toxicity have been severely hampered by the lack of cellular test systems, particularly continuous cell lines (17), which are amenable to selection and genetic engineering. Recently, we have shown that toxic effects of TCDD can be detected readily in 5L cells, which are descendants of H4IIEC3 rat hepatoma cells (18,19).

The present study was aimed at clarifying the role of the Ah receptor in the toxic effects of TCDD in 5L cells. First, we investigated TCDD toxicity in 5L cells and in some of their variants deficient in cytochrome P450IA1 activity and presumably Ah receptor. Second, we tested the effects of 3,3′,4,4′-tetrachlorobiphenyl (3,3′,4,4′-TCB) and benz[*a*]anthracene (BA), representatives of halogenated and non-halogenated ligands of the Ah receptor respectively, on the growth of 5L cells in comparison to those of the very poor ligand 2,2′,4,4′-tetrachlorobiphenyl (2,2′,4,4′-TCB) (20,21). Third, we compared the concentrations of the test compounds that elicited toxic responses with those that induced cytochrome P450IA1. The results suggest strongly that the presence of Ah receptor is necessary for the toxicity of TCDD in 5L cells.

Materials and methods

Cell culture

The dedifferentiated 5L Reuber hepatoma cells (19) were provided by Dr M.Weiss and were subcultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 7.5% CO_2, 92.5% air at 37°C. Cells were trypsinized when nearly confluent and seeded for experiments at a density of 150 000 cells/65 cm^2 culture dish or 3000 cells/well of a 96 well microtiter plate. They were grown in the absence of the test compounds for 24 h, if not stated otherwise. The 5L variant clones 5L-rBP-8, 5L-rBP-12, 5L-r1, 3-DNP-1 and 5L-r1,3-DNP-3 were selected for their growth in medium containing benzo[*a*]pyrene (B[*a*]P) or 1,3-dinitropyrene (1,3-DNP) (F.Kiefer and F.J Wiebel, in preparation).

Preparation of xenobiotic-containing media and treatment of cells

TCDD was obtained in toluene and stored at room temperature Aliquots were dried under a stream of nitrogen and redissolved in DMSO. BA was dissolved in DMSO; stock solutions of TCBs were prepared in acetone. Appropriate dilutions of the stock solutions were added to the growth medium to give a final solvent concentration of 0.1%.

Preparation of cell homogenates

Monolayers were rinsed twice with ice-cold Dulbecco's PBS and cells were collected by scraping into 1 ml of Tris buffer (50 mM, pH 7.6) with a rubber policeman. Samples were frozen and stored at −80°C.

Determination of aryl hydrocarbon hydroxylase (AHH) activity

The enzyme activity was assayed as described previously (22). The amount of protein in cell homogenates was determined according to Lowry *et al.* (23).

Determination of DNA

The amount of DNA was measured using the bisbenzimide dye H33258 according to Labarca and Paigen (24) with minor modifications. Cells suspended in 100 μl of PBS were disrupted by addition of 400 μl double-distilled water. Samples were kept for 60 min after addition of 1.5 ml high salt buffer (2.5 M NaCl, 62.5 mM sodium phosphate, pH 7.4) and then 0.5 ml of high salt buffer containing 5 μg/ml H33258 was added. The extent of fluorescence was determined at 360 nm

*Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 3,3′,4,4′-TCB, 3,3′,4,4′-tetrachlorobiphenyl; BA, benz[*a*]anthracene; 2,2′,4,4′-TCB, 2,2′,4,4′-tetrachlorobiphenyl; FBS, fetal bovine serum; B[*a*]P, benzo[*a*]pyrene; 1,3-DNP, 1,3-dinitropyrene; AHH, aryl hydrocarbon hydroxylase; HEDG, buffer containing 25 mM HEPES, 1,5 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol; TCDF, 2,3,7,8-tetrachlorodibenzofuran.

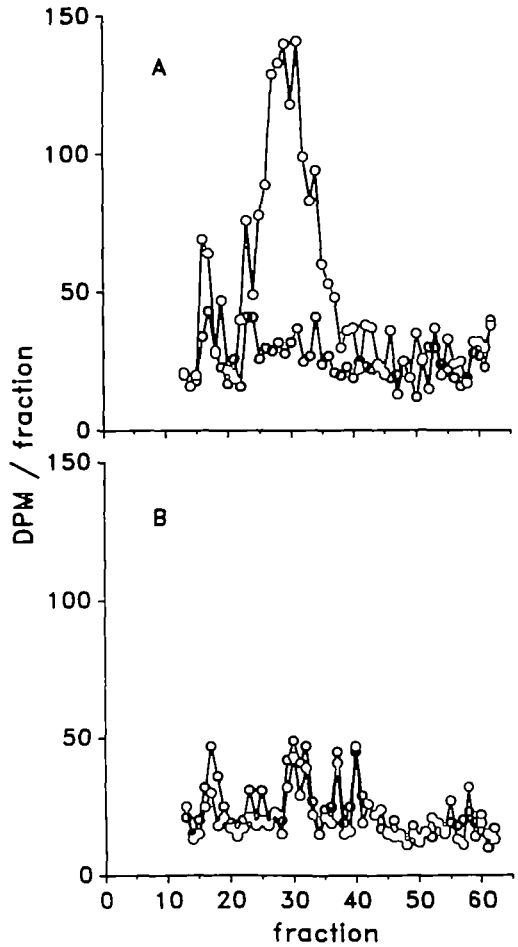

Fig. 1. Specific binding of [^{3}H]TCDD to the cytosols from 5L wild-type cells (**A**) and from clone 5L-rBP-8 (**B**). Cytosol (500 μl) containing 0.5 mg/ml protein from wild-type cells or the variant clone were incubated for 2 h with 5 nM [^{3}H]TCDD in the presence (○ —— ○) or absence (○ —— ○) of a 500-fold excess of unlabelled TCDF. Samples were subjected to gel-permeation chromatography and radioactivity was determined in the fractionated eluate. The specific binding of [^{3}H]TCDD is represented by the difference in radioactivity bound in the absence or presence of TCDF. Similar results were obtained from two independent experiments.

excitation and 450 nm emission. Calf thymus DNA was used as a standard.

The amount of DNA from cultures grown on microtiter plates was measured directly in the culture wells. The growth medium was removed and cells were disrupted by addition of 60 μl double-distilled water. DNA content/well was determined in a total volume of 300 μl as described above.

Determination of protein content and neutral red uptake in cultures on microtiter plates

The protein content/well was assessed by staining with Kenacid Blue R according to Knox *et al.* (25) with the modification that cells were fixed in a solution of 50% methanol and 1% acetic acid. The uptake of neutral red was measured as described by Borenfreund and Puerner (26).

Preparation of cytosol

Nearly confluent cultures of 5L cells grown on 65 cm^2 culture dishes were rinsed twice with ice-cold PBS. Cells were harvested as described above and collected by centrifugation at 50 *g*. The pooled cell pellets from 10–12 dishes were homogenized in 1 ml HEDG buffer (25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol, 10% v/v glycerol) by 10 strokes at 1500 r.p.m. using a Potter–Elvehjem homogenizer. Homogenates were centrifuged at 100 000 *g* for 1 h and supernatants snap frozen in liquid nitrogen.

Determination of the Ah receptor

Preparations of cytosol were assayed for binding of [^{3}H]TCDD following the procedure described by Poland *et al.* (6). Briefly, 400–500 μl cytosol containing 0.5–1 mg/ml protein were incubated for 2 h with 5 nM [^{3}H]TCDD in the presence or absence of a 500-fold excess of unlabelled 2,3,7,8-tetrachlorodibenzofuran (TCDF). Unbound ligands were removed by treatment with dextran-coated charcoal (1 mg/ml charcoal/0.1 mg/ml dextran) [^{3}H]TCDD-protein complexes were separated by high-pressure gel permeation chromatography using a Superose 6 column and HEDG buffer lacking glycerol as eluent at a flow rate of 0.2 ml/min at 4°C. Fifty fractions of 0.4 ml were collected starting with the void volume of the column (5 ml). Specific binding to cytosolic protein is expressed as the difference in the radioactivity profiles of incubations in the presence or absence of TCDF.

Materials

Materials were obtained from the following sources: powdered cell culture media, antibiotics and trypsin from Biochrom (Berlin, FRG); FBS from Gibco (Eggenstein, FRG); cell culture dishes from Falcon (Heidelberg, FRG); 96 well culture plates from Nunc (Wiesbaden, FRG); TCDF, 2,2',4,4'-TCB and 3,3',4,4'-TCB from Promochem (Oberwesel, FRG); TCDD from Supelco (Bellefonte, PA, USA); the chromatography column Superose 6 from Pharmacia, (Freiburg, FRG), calf thymus DNA from Serva (Heidelberg, FRG); BA and Kenacid Blue R from Sigma (Deisenhofen, FRG), and the bisbenzimide dye H33258 from Riedel deHaen (Hannover, FRG). [^{3}H]TCDD (sp. act. 960 kBq/nmol) was a generous gift from Dr A.Poland and was purified by HPLC prior to use.

Results

Ah receptor levels and sensitivity to TCDD toxicity of 5L cells and variant clones

[^{3}H]TCDD specifically bound to cytosol from 5L cells eluted in a single peak from the gel permeation column (Figure 1A).

Table I. TCDD toxicity, AHH activities and content of Ah receptor in 5L wild-type cells and variant clones[a]

Cells	Cells per plate (10^6)[b]			AHH activity (pmol/mg × min)[c]		Ah receptor (fmol/mg)[d]
	Control	TCDD	(%)[e]	Control	TCDD	
5L wild-type	1.50 ± 0.07	0.85 ± 0.02	(57)	3.4 ± 0.6[f]	106 ± 3[f]	84 ± 19[f]
5L-rBP-8	1.07 ± 0.03	1.21 ± 0.06	(113)	<0.1	<0.1	<3
5L-rBP-12	1.09 ± 0.03	0.90 ± 0.05	(83)	<0.1	<0.1	<3
5L-rI,3-DNP-1	1.64 ± 0.21	1.58 ± 0.04	(97)	<0.1	<0.1	<3
5L-rI,3-DNP-3	1.57 ± 0.07	1.53 ± 0.06	(97)	<0.1	<0.1	<3

[a]Cells were seeded at a density of 150 000/65 cm^2 dish and grown for 48 h. Then they were exposed to 1 nM TCDD for 24 h.
[b]The number of cells was determined by using a Coulter Counter. Values are means ± SD from triplicate determinations of one of two experiments that gave similar results.
[c]AHH activity/mg of total cellular protein.
[d]Amount of the Ah receptor/mg of cytosolic protein.
[e]Percentage of controls.
[f]Means ± SD from three independent experiments.

Similar elution profiles were obtained for cytosols from liver of adult rat (data not shown). The specific TCDD binding was taken to represent the Ah receptor. The level of Ah receptor in 5L cells amounted to 84 fmol/mg cytosolic protein (Table I). In contrast to 5L wild-type cells, the variant clone 5L-rBP-8 lacked detectable Ah receptor (Figure 1B). This was also true for three other 5L variant clones, 5L-rBP-12, 5L-rl,3-DNP-1 and 5L-r-1, 3-DNP-3 (Table I). The presence and absence of Ah receptor in the test cell lines was associated with the expression or lack of AHH activity. Thus, 5L cells contained appreciable constitutive AHH activities which were highly inducible by TCDD, whereas the Ah receptor deficient variants were lacking these functions (Table I).

Exposure to TCDD for 24 h inhibited strongly the growth of 5L wild-type cells as determined by the number of cells (Table I) but did not cause signs of overt toxicity such as detachment of cells. The dioxin had no significant effect on the growth of the four Ah receptor-deficient variant clones.

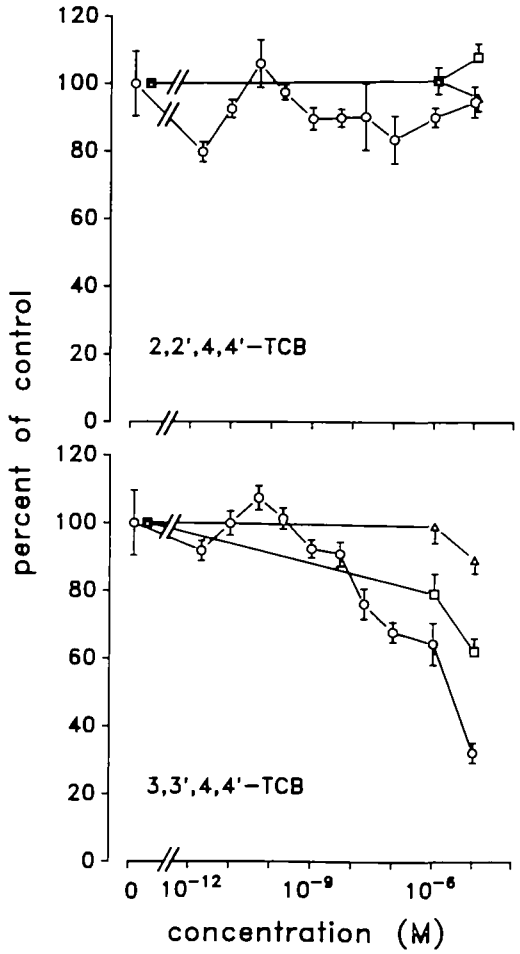

Fig. 2. Effect of isomeric tetrachlorobiphenyls on growth and vitality of 5L cells. Cells were treated with various concentrations of 2,2',4,4'-TCB or 3.3',4,4'-TCB for 48 h. The amount of DNA (○——○) or protein (□——□) and the uptake of neutral red (△——△) were determined as described in Materials and methods The results are shown relative to the solvent-treated control. Control values of DNA or protein content per well showed a 6-fold increase over the 48 h of treatment. Values are means ± SD from determinations in four culture wells Similar results were obtained in a second independent experiment

Toxicity of TCBs and BA in 5L cells

The toxicity of the test compounds was examined by determining their effects on the amount of cellular DNA or protein as a measure for cell growth, and the uptake of neutral red as a measure for cell vitality. 3,3',4,4'-TCB reduced the increase in the DNA content of 5L cells by >60% (Figure 2). The effect on the increase in protein content was less pronounced. The uptake of neutral red was only slightly reduced by 3,3',4,4'-TCB.

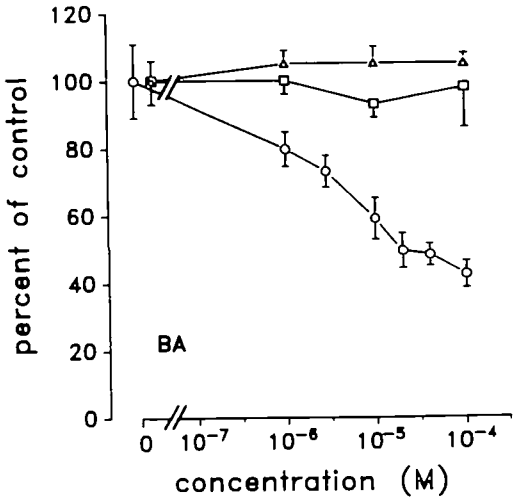

Fig. 3. Effect of BA on growth and vitality of 5L cells. Cells were treated with various concentrations of BA for 48 h. The amount of DNA (○——○) or protein (□——□) per culture and the uptake of neutral red (△——△) were determined as described in Materials and methods. For details and control values refer to legend of Figure 2. Values are means ± SD from determinations in four culture wells.

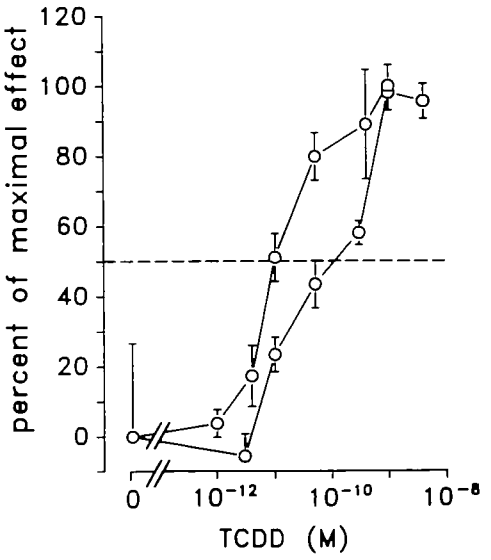

Fig. 4. Inhibition of growth and induction of AHH activity by TCDD. Growth inhibition (○——○) was determined on a 96 well microtter plate as the relative difference in DNA content from TCDD-treated and control cultures after 48 h exposure to the dioxin. Growth inhibition of 100% represents the maximum difference effected by 1 nM TCDD, which is a reduction by 57% of the control. Induction of AHH activity (○——○) was measured after 24 h TCDD treatment on 20 cm^2 culture dishes. Values are shown relative to the maximum induced activity of 106 ± 3 pmol/mg × min (100%) and the basic level of 3 4 ± 0.6 pmol/mg × min (0%). Values are means ± SD from three culture plates or four culture wells respectively in one of three experiments that gave similar results.

Exposure to the isomeric 2,2′,4,4′-TCB (Figure 2) did not inhibit the increase in DNA or protein content or effect the uptake of neutral red. In order to ascertain that the two TCBs differ truly in their affinities to the Ah receptor of 5L cells, we determined their ability to compete with 3 nM [^{3}H]TCDD for receptor binding. Half-maximum competition was achieved by 0.2 μM 3,3′,4,4′-TCB, whereas 2,2′,4,4′-TCB did not compete effectively at concentrations of up to 10 μM.

BA inhibited the growth of 5L cells to a similar degree as 3,3′,4,4′-TCB. The amount of DNA in cultures exposed to BA for 48 h was 40% of that in solvent-treated controls (Figure 3). BA had no significant effect on the protein content or the uptake of neutral red.

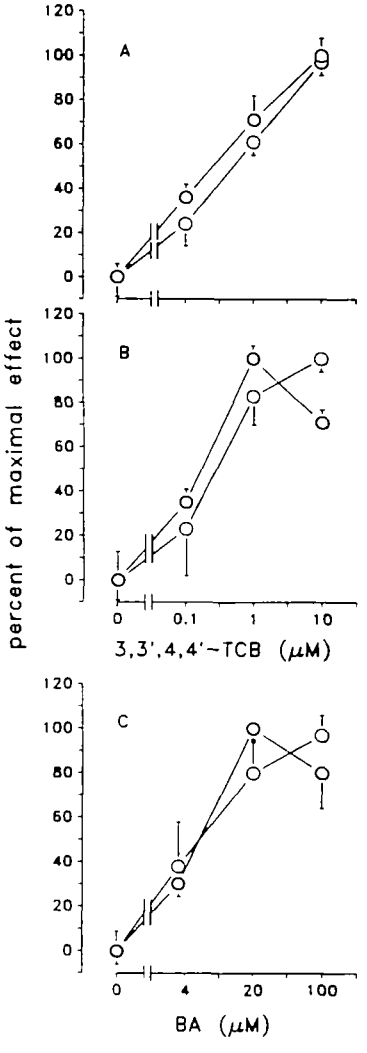

Fig. 5. Inhibition of growth and induction of AHH activity by various concentrations of BA or 3,3′,4,4′-TCB. Cells grown on 20 cm^2 culture dishes were treated for 24 h (**A**) or 48 h (**B**) with 3,3′,4,4′-TCB, or for 48 h with BA (**C**). Growth inhibition (○ —— ○) was determined as the relative difference in the DNA content of homogenates from treated and control cells. AHH activity (○ —— ○) was determined in the same homogenates. Values are presented relative to the maximum effects. Maximum induced AHH activities were 80 ± 2 and 39 ± 3 pmol/mg × min at 24 and 48 h respectively for 3,3′,4,4′-TCB; 76 ± 3 pmol/mg × min at 48 h for BA. Maximum inhibition of growth, i.e. reduction in the amount of DNA/culture, was 48 and 70% of untreated cells at 24 and 48 h respectively for 3,3′,4,4′-TCB, and 56% at 48 h for BA. Values are means ± SD from three replicate cultures. Similar results were obtained in a second independent experiment

Concentration dependency of growth inhibition and induction of AHH activity

The dose–response curves for induction of AHH activity and inhibition of growth by TCDD were nearly parallel (Figure 4). The EC_{50} concentrations for AHH induction were consistently lower than those for growth inhibition, the former being as low as 10 pM, the latter as high as 30–100 pM.

Since preliminary experiments had shown that exposure to 3,3′,4,4′-TCB for 48 h elicited morphological signs of cytotoxicity, the effects of this compound were tested also after exposure for 24 h. As shown in Figure 5(A) and 5(B), the dose–response curves for growth inhibition and AHH induction were similar after both periods of treatment and yielded EC_{50} concentrations that differed by less than a factor of 2. 2,2′,4,4′-TCB neither affected DNA content of cultures nor induced AHH activity (data not shown).

Exposure to BA (Figure 5C) caused a half-maximum inhibition of growth at the same concentration that was required for half-maximum induction of AHH activity.

Discussion

The present results suggest strongly that the Ah receptor is an essential factor in mediating the toxicity of TCDD in 5L cells. This is supported by three lines of evidence: (i) TCDD toxicity correlates with the presence of the Ah receptor; (ii) other ligands of the Ah receptor have toxic effects resembling those of the dioxin; and (iii) the toxic concentrations of the test compounds are similar to those that induce P450IA1.

The correlation of TCDD toxicity with the presence of the Ah receptor was shown by the finding that variant 5L clones, which lacked detectable Ah receptor and inducible P450IA1 activity, were insensitive to TCDD. The AHH activity in 5L cells most likely represents cytochrome P450IA1, since the related cytochrome P450IA2, which also hydroxylates B[*a*]P, has not been found in rat Reuber hepatoma cells (27, L.Corcos, personal communication). At present, we cannot exclude the possibility that the toxic effect of TCDD is a consequence of the highly induced P450IA1 activity. The inducing chemicals, for example, might be converted to reactive metabolites, or the induced enzyme might catabolize components required for cell proliferation (28). However, we consider it unlikely that P450IA1 plays a direct role in TCDD toxicity. TCDD is metabolized at only an extremely low rate and does not bind to DNA to a significant degree (29,30). Furthermore, TCDD changes the progression of cells through the growth cycle rapidly, suggesting an immediate effect (18).

The second argument for the importance of the Ah receptor in TCDD toxicity stems from the finding that the two ligands of the Ah receptor, 3,3′,4,4′-TCB and BA, in contrast to the poorly binding 2,2′,4,4′-TCB, exert toxic effects in 5L cells. In principle, the effects are similar to those of TCDD in that the test compounds inhibit cell growth, increase the protein to DNA ratio and do not decrease cell vitality (M.Göttlicher and F.J.Wiebel, in preparation). In the case of 3,3′,4,4′-TCB this is valid only for concentrations up to 1 μM. At 10 μM this compound causes signs of overt toxicity such as detachment of cells after 24–48 h of treatment. These toxic effects might be due to metabolic activation and macromolecular binding of 3,3′,4,4′-TCB (31), which, in contrast to TCDD, is readily metabolized. Thus, direct cytotoxicity appears to be superimposed on the 'TCDD-like' cytostatic effect of 3,3′,4,4′-TCB.

The third indication for the involvement of the Ah receptor is given by the fact that TCDD, 3,3′,4,4′-TCB, and BA all cause

half-maximal inhibition of growth at concentrations similar to those inducing P450IA1, whereas the biological potencies of the compounds differ by five orders of magnitude. The finding that TCDD requires up to one order of magnitude higher concentrations for inhibiting cell growth than inducing P450IA1 may argue against the Ah receptor as mediator of both toxicity and P450IA1 induction. However, the apparent discrepancy would be resolved if toxicity and P450IA1 induction are mediated by different genes of the Ah gene battery which respond to different concentrations of TCDD. Such a phenomenon has been observed, for example, for the induction of certain gene products by steroid hormones via the glucocorticoid receptor (32).

The presence of Ah receptor, although necessary, appears not to be sufficient for the susceptibility of cells to TCDD toxicity. Thus, there are many cell lines, including several hepatoma lines related to 5L cells, that possess Ah receptor as indicated by their high inducibility for P450IA1 but are insensitive to TCDD toxicity (17,18). Clearly, additional conditions have to be met for cells to become sensitive to TCDD. Most likely, a particular status of regulation, possibly in conjunction with specific conditions of culture, render 5L cells susceptible to the activation of critical genes via the Ah receptor in analogy to the conditional response of cells to hormones or growth factors (33,34). Preliminary evidence suggests that changes in the composition of the growth medium aggravate the toxicity of TCDD in 5L cells and evoke signs of toxicity in other hepatoma lines that are insensitive under standard growth conditions (unpublished observation). The observation that the mere presence of the Ah receptor does not correlate with the toxicity of TCDD has also been made *in vivo* in rodents (35). Different species can differ by more than three orders of magnitude in sensitivity to TCDD, although comparable levels of the Ah receptor are present.

In conclusion, interaction of TCDD with the Ah receptor appears to be the first link in a chain of events leading to the toxic response. Cell lines such as 5L offer a powerful system for dissecting this sequence of events and clarifying the role of the Ah receptor at the molecular level. It remains to be established whether and to what extent the observations made in cultured cells can be extrapolated to TCDD toxicity and tumor promotion *in vivo*.

Acknowledgements

The excellent technical assistance of F Kiefer is gratefully acknowledged The work was partially supported by the Deutsche Forschungsgemeinschaft (fellowship to M.G.) and by the Bundesministerium für Forschung und Technologie (07DIX10 0, P C.)

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Received on July 12, 1990; revised on September 12, 1990; accepted on September 13, 1990