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Fetal Thymus Organ Culture as an *in Vitro* Model for the Toxicity of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and Its Congeners

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SUMMARY

Fetal thymuses from C57BL/6 (B6) and DBA/2J (D2) mice from gestation day 14 or 15 were explanted and grown for 2 and 6 days in culture in the presence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and a number of its congeners, known ligands of the *Ah* receptor (*Ah*, designating genetic locus for *a*ryl *h*ydrocarbon responsiveness). TCDD and 2,3,7,8-tetrachlorodibenzofuran (TCDBF) showed the same toxicity to B6 thymuses with a 50% inhibition of lymphoid development (EC_{50}) at 10^{-10} M concentration. 3,3′,4,4′-Tetrachloroazoxybenzene (TCAOB) was only 2–10 times less effective, while the EC_{50} of 3,3′,4,4′-tetrachlorobiphenyl (TCB) was around 10^{-8} M (100 times higher than that of TCDD). TCBs with chlorine atoms in the position close to the biphenyl bridge were nontoxic even at 10^{-5} M concentration. Thymuses exposed to TCDD, TCDBF, and TCAOB *in vivo* at teratogenic doses given to the mothers and explanted 24–48 hr later were smaller and inhibited in their early *in vitro* growth, but recovered slowly (less rapid for TCDD) as judged by lymphoid cell counts and [^{3}H]thymidine incorporation. These results indicate a good correlation for this group of compounds between their activity as ligands of the *Ah* receptor and toxicity *in vitro*. Other ligands of the *Ah* receptor, namely 3-methylcholanthrene and β-naphthoflavone, were inactive at the highest concentrations tested (10^{-6} M). Thymuses from D2 mice, considered *Ah* receptor-defective, were nonsensitive to TCDD at the concentrations used (up to 3×10^{-8} M) after 2 days in culture, indicating more than 100 times lower sensitivity as compared to B6 thymuses. After 6 days in culture, their sensitivity was however only 1 order of magnitude lower than that of B6 thymuses. Therefore "low sensitivity" of D2 thymuses may be at least partially overcome by prolonged exposure to TCDD *in vitro*.

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and some related halogenated aromatic hydrocarbons produce a similar pattern of toxic responses, of which thymic atrophy (hypoplasia in developing individuals) is probably one that is inducible in more species than any other toxicity (see Ref. 1 and articles by Goldstein and McConnell, respectively, in Ref. 2 for review). There is convincing evidence that toxicity of the TCDD[1] congeners segregate with the *Ah* locus (1, 4–6), which regulates the induction of P_1-450-dependent drug-metabolizing enzyme systems, but also a number of other coordinately expressed enzyme activities. A cytosolic receptor protein (*Ah* receptor) first isolated from the liver is viewed as the gene product of this locus (7–9). This receptor protein has been found in a number of extrahepatic tissues as well, including the thymus (10–12), and in high concentration in murine embryonic tissues sensitive to the teratogenic action of TCDD (mandible and secondary palates) already in the organogenetic period (13). Some strains of mice (such as DBA and AKR) are considered nonresponsive to the toxic effects of polycyclic hydrocarbons and less sensitive to TCDD and its congeners. They are not easily induced with respect to P_1-450-dependent monooxygenase activities and they appear to have a defective *Ah* receptor (1, 3, 11).

A puzzling phenomenon in TCDD toxicology is the fact that it has been virtually impossible to find overt toxicity to cultured cells, although most cell types respond *in vitro* with induction of monooxygenase activities (14–16). Knutson and Poland (17) eventually found a mouse teratoma cell line (XB-cells) that seems to be a workable model for hyperkeratosis, one of the most char-

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[1] The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDBF, 2,3,7,8-tetrachlorodibenzofuran; TCAOB, 3,3′,4,4′-tetrachloroazoxybenzene; TCB, tetrachlorobiphenyl; FCS, fetal calf serum; PBS, phosphate-buffered saline.

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acteristic responses to TCDD *in vivo* in primates. They could observe a dose-dependent keratinization of these cells by TCDD and its congeners.

As thymic hypoplasia is a toxic response found in virtually all species, and since the resulting immunodeficiency may cause serious long term problems in exposed individuals, an *in vitro* model for this toxicity would be most advantageous. It might help to explain the relatively specific toxicity to the thymus as compared to other tissues by TCDD. It would help to assort different ligands of the *Ah* receptor with respect to toxicity. In addition, it could serve as a screening system for new potentially thymotoxic compounds.

Thymic hypoplasia in developing rodents has been shown to be most easily induced if they were exposed to TCDD already during prenatal life (18). Previous works established a good morphological and functional development of the fetal murine thymus in organ culture (19, 20). This suggested the possibility of evaluating the effects of TCDD and congeners in such an organ culture system. The results show a good correlation for this group of chemicals between activity as ligands of the *Ah* receptor, and inhibition of lymphoid development.

MATERIALS AND METHODS

Mice. Mice, approximately 3 months old, of the C57BL/6 (B6) (Anticimex, Sollentuna, Sweden) and DBA/2JBom (D2) (G1. Bomholtgaard, Ry, Denmark) strains were used in this study. The animals were kept in air-conditioned rooms at a temperature of 23° and a 12-hr daylight cycle, and were given a pellet diet (nr R3, Ewos, Södertälje, Sweden) and tap-water *ad libitum*. Males and females were mated overnight and the following morning (day 0) the females were checked for vaginal plugs.

Chemicals. TCDD and TCDBF were gifts from C. Rappe, Department of Organic Chemistry, Umeå University, Umeå, Sweden. TCAOB was synthesized by Göran Sundström and 3,3′,4,4′-, 2,2′,4,5′- and 2,2′,5,5′-TCB were synthesized by Åke Bergman, both at the Wallenberg Laboratory, University of Stockholm, Sweden. 7,8-Benzoflavone (β-naphthoflavone) and 3-methylcholanthrene were purchased from Sigma Chemical Co., St. Louis, MO. 1,4-Dioxane was from Merck, Darmstadt, West Germany. All test substances were dissolved in the solvent (1,4-dioxane if not otherwise stated) and added to culture medium at least 24 hr before they were used, and these media were shaken for several hours to ensure proper distribution of the test substances in the medium.

Organ culture technique. The procurement of thymuses from day 14 (B6 and D2) and day 15 (D2) embryos and the organ culture technique used have been described in detail previously (19, 20). In brief, plastic 35 × 10-mm tissue culture dishes were equipped with a 60-mesh stainless steel bridge with a matching piece of Millipore filter, of pore size 0.45 μm (Millipore Corporation, Bedford, MA), situated in the gas-medium interface. Two ml of RPMI 1640 tissue culture medium supplemented with L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and heat-inactivated (56° for 30 min) 10% FCS (Flow Laboratories, Irvine, U. K.), and containing the test substance, was added to each culture dish. Three randomly chosen thymic anlagen were placed on the same bridge in each culture. Cultures were established in triplicate for each concentration of the test substances. They were incubated at 37° in gas-tight boxes with a water-saturated atmosphere of 5% CO_2 in air. Control cultures always contained the same amount of the used vehicle (0.16 μl of dioxane/ml of medium). Most experiments were repeated at least once.

Quantitation of lymphoid cell number. The thymuses in organ culture were removed from the Millipore filters at the indicated times and transferred to small plastic homogenizers containing 150 μl of Ca- and Mg-free PBS. After gentle homogenization of the thymuses, 50 μl of the cell suspension was immediately transferred to a small tube and mixed with an equal volume of 0.2% trypan blue in PBS. The concentration of viable lymphocytes was determined one minute later by counting in a hemocytometer. From these cell counts, the number of lymphocytes per thymic anlage was calculated. In some experiments, the number of lymphoid cells outside the thymic anlagen (media plus filters) was determined in the media after thorough rinsing of filters after the anlagen had been removed. The filters were stained with Giemsa. Less than 5% of the total number of lymphoid cells were found to be outside the thymuses, without any difference between control and treated cultures.

Incorporation of [^{3}H]thymidine. Thymuses were removed from the Millipore filters at the times indicated and each thymus was gently homogenized in 150 μl of FCS-free medium. The number of viable lymphocytes was determined in an aliquot of 20 μl of the cell suspension (see above).

The rest of the cell suspension was transferred to a 96-well microtiter plate. A total of 2 μCi of [^{3}H]thymidine with specific activity of 26 Ci/mmol (Amersham International, England) in a 20 μl of FCS-free medium was added to each well containing cell suspension. After 4 hr at 37°, the pulsed cells were harvested using a Skatron multiple cell culture harvester (Skatron, Lierbyen, Norway). This procedure includes collection of the cells on glass fiber filters, followed by washing with water. The radioactivity of the filters was measured in a liquid scintillation counter (LKB Wallac), and expressed as counts per min per cell. Microautoradiography of thymuses, similarly incubated in [^{3}H] thymidine, revealed a distinct nuclear uptake (results not shown).

In vivo administration of test substances. In separate experiments, B6 mice on day 12 or 13 of gestation were administered intraperitoneally TCDD, TCDBF, or TCAOB at dose levels of 20 μg, 200 μg, and 6 mg/kg body weight, respectively. Control animals were given vehicle alone (dioxane, 320 μl/kg body weight). On day 14 of gestation, fetal thymuses were removed and cultured as above, however without addition of test substances. In one experiment, serum was collected from thus treated (TCDD and dioxane on day 13 of gestation) animals and thymuses from additional nontreated animals were then cultured in serum from the TCDD- and dioxane-treated animals.

RESULTS

Concentration-response studies and comparisons of different Ah receptor ligands. Thymuses from gestational day 14 B6 fetuses in organ culture showed a concentration-dependent decrease (or, more exactly, inhibition of the time-dependent increase) in the number of lymphoid cells when treated with TCDD, TCDBF, TCAOB, and 3,3′,4,4′-TCB (Fig. 1, A and B; Table 1). The concentration-response curve was rather flat and not always linear, and even at high concentrations a cell number roughly corresponding to the cell number at the start of the culture period was observed.

To calculate an exact effective concentration causing 50% inhibition of thymic lymphoid development (EC_{50}) under these conditions is not possible. The approximate EC_{50} values given in Table 1 indicate the concentration resulting in an increase in cell number, half of that observed in control cultures. The latter was calculated from the cell number of control cultures at harvest, reduced by the cell number at maximal inhibition (approximately the cell number at start of culture).

After 2 days in culture (Fig. 1A), no major differences in EC_{50} ($\sim 10^{-9}$ M) values between TCDD and TCDBF were observed, while TCAOB was an order of magnitude less potent. At 6 days (Fig. 1B), TCDD and TCDBF were equally potent ($EC_{50} \sim 10^{-10}$ M), while TCAOB seemed

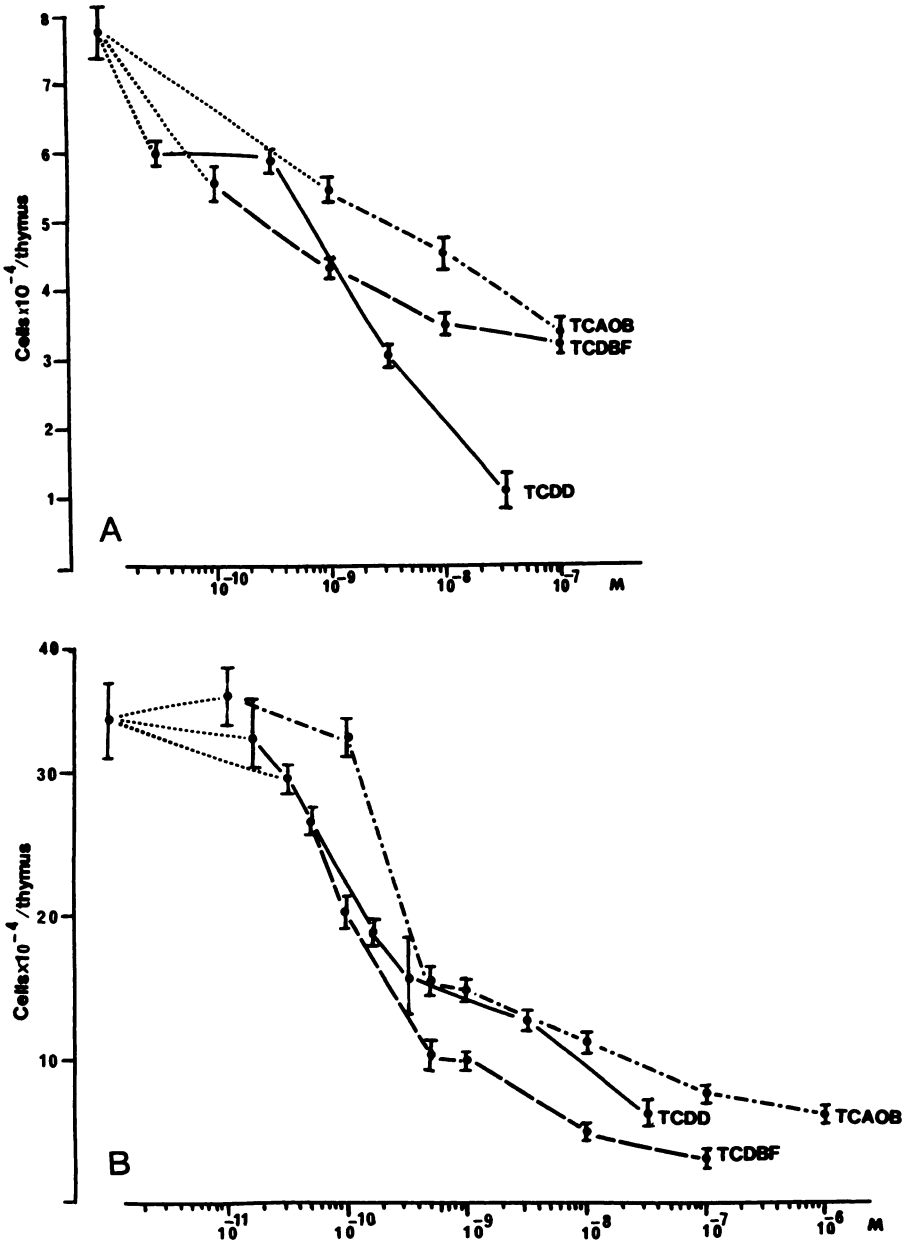

FIG. 1. *Concentration-response curves for the inhibition of lymphoid development in B6 thymuses, induced by TCDD, TCDBF, and TCAOB*
Thymuses were explanted on day 14 of gestation and grown for 2 days (A) and 6 days (B) in culture (three thymuses in each organ culture). The test substances were added to the growth medium 24 hr before start of culture, dissolved in 1,4-dioxane (0.16 μl/ml of medium). At harvest of cultures, thymuses were gently homogenized and lymphoid cells were counted. Control cultures received only 1,4-dioxane. ·····, connection of control and test cultures. Each point is the mean of three cultures (nine thymuses) ± SE.

only slightly less active ($EC_{50} \sim 2 \times 10^{-10}$ M). 3,3′,4,4′-TCB was considerably less potent with an EC_{50} value of 10^{-8} M at 6 days, and 2,2′,4,5′-TCB and 2,2′,5,5′-TCB were inactive at concentrations up to 10^{-5} M (highest tested; Table 1). β-Naphthoflavone and 3-methylcholanthrene, which were tried on several occasions, with the use of various solvents (dioxane, dimethyl sulfoxide, amyl alcohol), did not show any toxicity at 10^{-6} M or lower (Table 1). 3-Methylcholanthrene was most successfully dissolved in amyl alcohol for this purpose, but was not toxic when tried at 10^{-5} M concentration on one occasion. However, there are doubts about its solubility in the growth medium at this high concentration.

In general, the number of lymphoid cells in the fetal thymus *in vivo* depends on the rate of immigration of stem cell, cell proliferation, lymphocyte emigration, and a high rate of intrathymic cell death. In this particular experiment, it cannot be determined with certainty whether the decreased cell number caused by TCDD and its congeners was due to reduced cell proliferation or to increased cell death. However, the number of dead cells upon cell counting was low, and histological examination of intact anlagen at the end of the culture period did not reveal an increase in number of necrotic cells in treated cultures (results not shown). Nor could any differential

TABLE 1

Effects on lymphoid development in thymuses from B6 fetuses, explanted on day 14 of gestation and grown for 2 to 6 days in culture

Approximate EC_{50} values to TCDD and a number of other halogenated aromatic hydrocarbons, and β-naphthoflavone and 3-methylcholanthrene. Data were taken from Fig. 1, A and B, and from similar concentration-response diagrams (not shown), and calculated as indicated under Results.

Substance	EC_{50} time in culture	
	2 days	6 days
		M
2,3,7,8-TCDD	$\sim10^{-9}$	$\sim10^{-10}$
2,3,7,8-TCDBF	$\sim10^{-9}$	$\sim10^{-10}$
3,3′,4,4′-TCAOB	$\sim10^{-8}$	$\sim2 \times 10^{-10}$
3,3′,4,4′-TCB		10^{-8}
2,2′,4,5′-TCB		$>10^{-5}$[a]
2,2′,5,5′-TCB		$>10^{-5}$[a]
β-Naphthoflavone		$>10^{-6}$[a]
3-Methylcholantrene		$>10^{-6}$[a]

[a] Highest concentration tested.

in vitro loss of lymphocytes by emigration into the medium be detected.

Effects of TCDD on thymuses from D2 versus B6 mice. Thymuses from D2 fetuses explanted on day 14 or 15 of gestation and grown in culture for 2 days did not respond significantly with inhibition of lymphoid development (reduced cell number as compared to controls), even at a concentration of 3×10^{-8} M (highest tested) (Fig. 2A). This means that the D2 thymuses were more than 2 (probably 3 orders of magnitude less sensitive than B6 thymuses at this short incubation interval. As can be seen from Fig. 2A, D2 thymuses explanted on day 15 showed similar cell numbers on culture day 2 as those of B6 thymuses explanted on day 14 (of similar size at explantation as well). Later experiments in the D2 strain were therefore concentrated on thymuses from day 15. After 6 days in culture, there was a dose-dependent inhibition of lymphoid development comparable to that of thymuses from B6 fetuses, however with approximately 10 times lower sensitivity (Fig. 2B).

In vivo treatment followed by growth in vitro: reversibility of inhibited thymic lymphoid development. We know relatively little about dose-effect relationships of different congeners of TCDD with respect to thymic hypoplasia in fetuses after *in vivo* treatment. Likewise, we have no information on the placental transfer of these congeners. We thus used the data we had from before (5, 21, 22) on dose-response relationships with respect to cleft palate formation, and thus administered to B6 mice on day 12 of gestation the ED_{50} doses for day 12 (for TCDD, 20 μg; TCDBF, 200 μg; TCAOB, 6 mg/kg body weight). On day 14, fetal thymuses were explanted as before and grown without addition of test substances. Fig. 3 shows that, at the doses employed, there had been a nearly maximal inhibition of thymic lymphoid development as measured after 2 days in culture, comparable to that found at high concentrations added *in vitro* (Fig. 1A) for all three substances. This growth inhibition had started already *in vivo* as judged from the size of these thymuses at explantation. However, when the thymuses were allowed to grow for 6 days in culture, the cell number of the TCDBF- and TCAOB-treated thymuses approached those of controls. This was, however, not true for the TCDD-treated thymuses. Table 2 shows that the rate of [^{3}H]thymidine incorporation was inversely proportional to the number of cells per thymus at day 2 as well as day 6. This suggests a recovery of lymphoid development *in vitro* of the *in vivo* treated thymuses and that the TCDD-

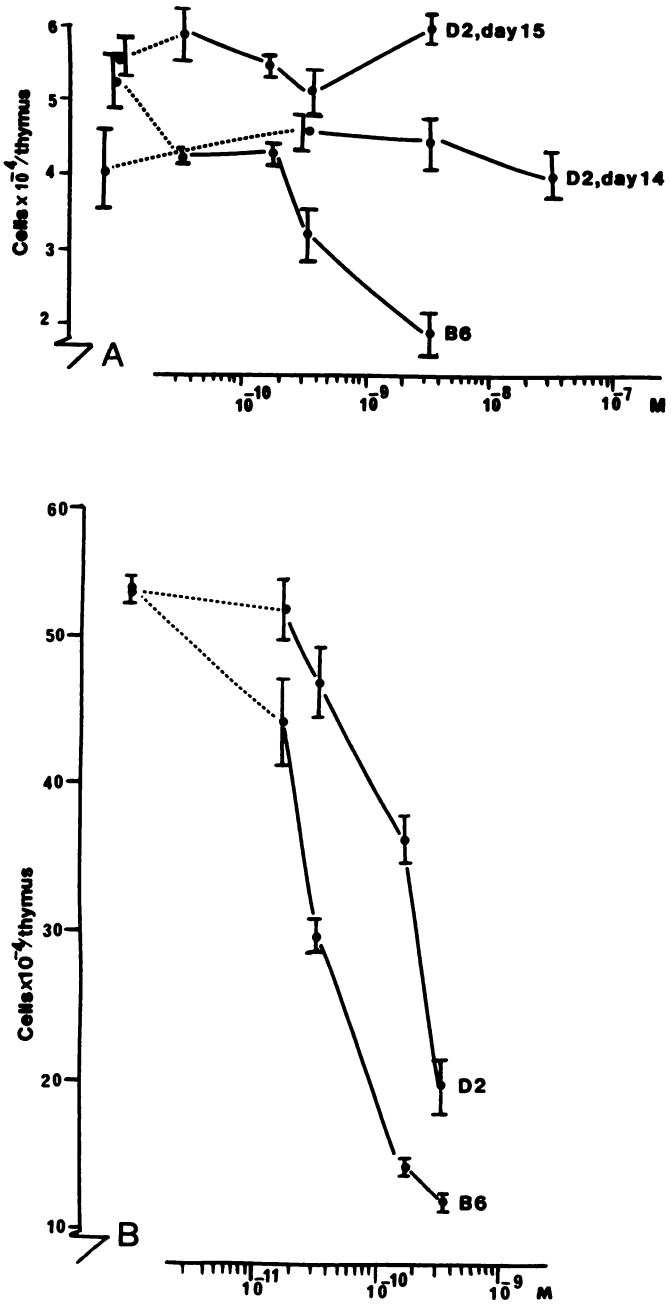

FIG. 2. *Concentration-response curves for the inhibition of lymphoid development in D2 versus B6 thymuses*

Procedure described in Fig. 1. A is after 2 days and B is after 6 days in culture. A, experiments were performed on D2 thymuses explanted both at day 14 and day 15. As the size of the day 15 D2 thymuses at explantation as well as at harvest was close to that of day 14 B6 thymuses, the culture for 6 days (B) was concentrated on day 15 D2 thymuses in comparison with day 14 B6 thymuses. Each point is the mean of three cultures (nine thymuses) ± SE.

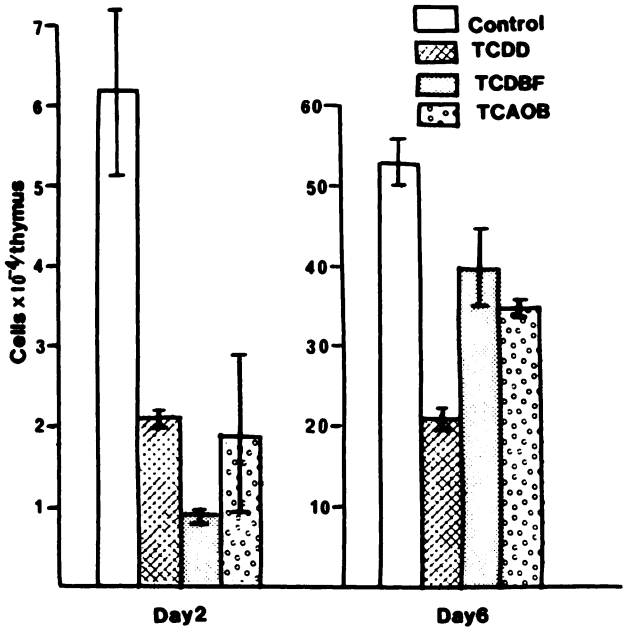

FIG. 3. *Lymphoid cell number of fetal B6 thymuses grown in vitro after in vivo treatment with TCDD, TCDBF, and TCAOB*

B6 mice on day 12 of gestation were treated intraperitoneally with TCDD (20 μg/kg body weight) TCDBF (200 μg/kg), or TCAOB (6 mg/kg). Control animals were given the vehicle 1,4-dioxane, 320 μl/kg). At day 14, the fetuses were removed and the thymuses were explanted and cultured as described in Fig. 1. The cultures were discontinued after 2 days (left) and 6 days (right). Each bar represents the mean of three cultures (nine thymuses) ± SE.

treated thymuses were slowest in recovery. The results are consistent with earlier observations (23) that thymuses subjected to growth inhibiting radiation or oxygen deficiency *in vitro* may recover as determined by increased cell number and [^{3}H]thymidine incorporation. In control cultures, [^{3}H]thymidine incorporation declines already after day 2 of culture (19).

In order to obtain more information on this, B6 mice were treated with TCDD (20 μg/kg body weight) *in vivo* (in this case on day 13), and thymuses were explanted on day 14 and cultured for up to 13 days. As can be seen in Fig. 4, the TCDD-treated thymuses slowly increased their cell number and reached the day 6 control value only after 13 days in culture. The cell number of control thymuses reached a plateau (the same cell number at 6 and 9 days in culture), which is in accordance with earlier results (19, 20, 23). For unknown reasons, the control cultures in our experiments had lost a substantial number of cells by day 13. These results thus show that *in vivo* exposure to TCDD for a 24-hr period at day 13 of gestation is enough to cause thymic hypoplasia, but that a slow recovery in terms of cell number may occur if the thymuses are no longer exposed to TCDD.

In similar experiments where thymuses were instead pulsed with TCDD (3×10^{-10} M) *in vitro* for 3 days and the medium was then exchanged for medium without TCDD, the TCDD-treated thymuses did not reach the cell number of the control thymuses at any time point. However, as for the *in vivo* treatment experiments, these cultures also showed a partial recovery as measured by increased [^{3}H]thymidine incorporation (results not shown). Pulsing the cells for only 1 day with TCDD at 3×10^{-10} M concentration did not decrease the cell number as compared to controls either at 2 or at 6 days of culture.

Culturing thymuses in serum from TCDD-treated mice. Fig. 5 shows that culturing thymuses from nontreated B6 mice in medium containing 8% serum from mice treated with 20 μg TCDD per kg body weight decreased the cell number to about 50% by day 6 of culture, which is approximately the same inhibition as was caused by culturing thymuses in 3×10^{-10} M TCDD media. This would indicate that the serum contained around 4×10^{-9} M (~1 ng/ml) TCDD at 24 hr after treatment. Inhibition of lymphoid development in the fetal thymuses from the dams of which the serum was taken was slightly greater (thymuses grown without test substance *in vitro*).

DISCUSSION

The three major contributions of this study to the discussion around the toxicity of TCDD and its congeners are 1) that an *in vitro* system has been found, which can mirror an essential toxicity of this group of chemicals, under a period when the organ (thymus) is in a developmental phase of utmost importance for the future immune system, 2) that TCDD and congeners seem to act directly on the thymus and not secondarily via, e.g., nutritional and hormonal disturbances, and 3) that three

TABLE 2

Cell number and [^{3}H]thymidine incorporation (counts per min) in lymphoid cells of fetal B6 thymuses explanted on day 14 of gestation and grown for 2 and 6 days, respectively, in vitro

The mothers were treated 48 hr before explanation with TCDD, TCDBF, and TCAOB as indicated. Mean of three cultures (nine thymuses) ± SE. Figures within parentheses represent % of control.

	Day 2		Day 6	
	Cells × 10^{-4}/thymus	cpm × 10^2/cell	Cells × 10^{-4}/thymus	cpm × 10^2/cell
Control (dioxane)	6.3 ± 1 (100)	2 ± 1 (100)	53 ± 3 (100)	1.6 ± 0.3 (100)
TCDD (20 μg/kg body wt.)	2.1 ± 0.1 (33)	14 ± 5 (700)	21 ± 1 (40)	5.2 ± 0.4 (325)
TCDBF (200 μg/kg body wt.)	0.9 ± 0.1 (14)	44 ± 5 (2200)	40 ± 5 (76)	2.5 ± 0.1 (156)
TCAOB (6 mg/kg body wt.)	1.9 ± 1 (30)	15 ± 7 (750)	35 ± 1 (66)	2.4 ± 0.1 (150)

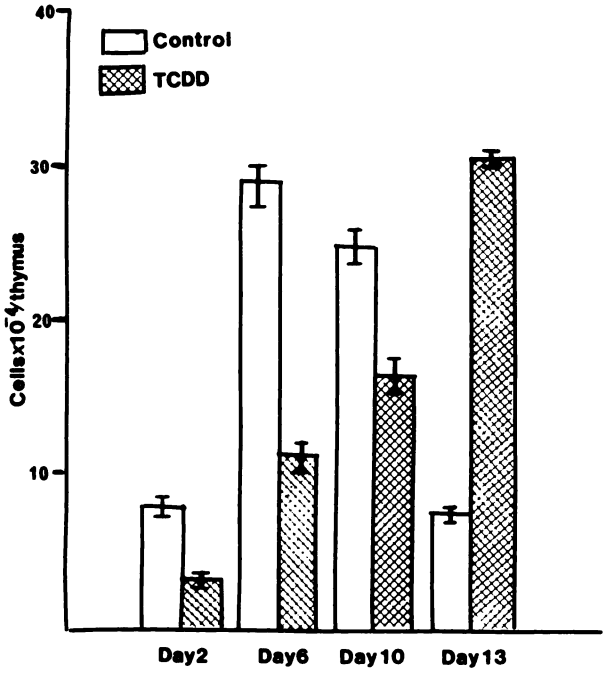

FIG. 4. *Lymphoid cell number of fetal B6 thymuses grown in vitro after in vivo treatment with TCDD*

The procedure was as described in Fig. 3 with the exception that the mice were treated at day 13 (24 hr before explantation), and that some cultures were allowed to grow for longer periods of time (10 and 13 days). Each bar represents the mean of three cultures (nine thymuses) ± SE.

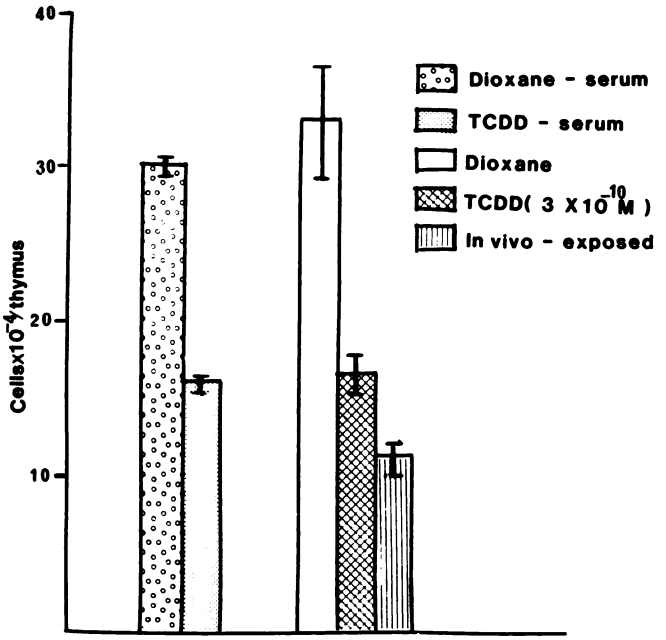

FIG. 5. *Effect on in vitro lymphoid development of fetal B6 thymuses by serum from TCDD-treated mice*

Mice on day 13 of gestation were treated with TCDD (20 μg/kg body weight) or 1,4-dioxane (320 μl/kg) and 24 hr later, serum from the dams was collected. Day 14 fetal thymuses from additional nontreated mice were then grown in FCS-free culture medium containing 8% serum from either the dioxane-treated (Dioxane-serum) or the TCDD-treated (TCDD-serum) mice. Other thymuses were grown in normal medium (containing 10% fetal calf serum) with vehicle (Dioxane) or TCDD (3×10^{-10} M) added. Also grown in this latter medium were the fetal thymuses from the *in vivo* TCDD-treated (*in vivo*-exposed) animals. Each point indicates the mean of three cultures (nine thymuses) ± SE.

ligands of the *Ah* receptor (namely TCDD, TCDBF, and TCAOB) which have approximately the same affinities for the receptor (24) show an *in vitro* toxicity that corresponds very well to their relative affinity. This strongly indicates that the 10- and 100-fold (or even higher) general toxicity and teratogenicity of TCDD as compared to TCDBF and TCAOB, respectively (1, 2, 5, 22), are mainly due to the more rapid metabolism and/or excretion of the latter two substances. It is, however, notable that thymuses pulse-exposed *in vivo* and then grown *in vitro* showed slower recovery if they had been treated with TCDD as compared with TCDBF and TCAOB. If this may reflect a stronger binding of TCDD to the *Ah* receptor, a higher persistence in the tissues or a more profound damage is not clear.

Of the three tetrachlorobiphenyls tested, 3,3′,4,4′-TCB has an affinity for the *Ah* receptor, although approximately 100 times lower than that of TCDD (25), and also showed around 100 times lower toxicity in our culture system. 2,2′,4,5′- and 2,2′,5,5′-TCB are not ligands of the receptor and showed no toxicity at the highest concentration tested (10^{-5} M).

One objection against the theory of the *Ah* receptor as a mediator of the toxicity of TCDD and congeners has been that other ligands of this receptor, such as polycyclic hydrocarbons, do not express the toxicities of TCDD. However, 3-methylcholanthrene and β-naphthoflavone were shown to decrease thymic weight when administered to mice (4), and Vos and collaborators (26) recently showed 3-methylcholanthrene and benzo(*a*)pyrene to be acnegenic in a rabbit ear test. These both toxicities, however, required 3 to 4 orders of magnitude higher doses of the polycyclic hydrocarbons, as compared to TCDD. Again, differences in metabolism and/or excretion may account for some of these differences, however probably not for the 10,000 times lower activity of the polycyclic hydrocarbons in a rabbit ear test. Our difficulties in getting a toxic response in the form of lower cell numbers in the thymus even at 10^{-6} M concentrations of 3-methylcholanthrene and β-naphthoflavone also suggest that these compounds are not very toxic as such, and that part of their toxicity *in vivo* is caused by reactive metabolites. It should be noted that other investigators have obtained maximal aryl hydrocarbon hydroxylase activities in different cell lines at a concentration of 10^{-7} M of 3-methylcholanthrene (15). Besides, β-naphthoflavone and 3-methylcholanthrene may induce keratinization in the mouse teratoma cell line XB in culture if added at very high concentrations (~1 μM) (17).

Although B6 and D2 mice differ genetically at several loci in addition to the *Ah* locus, these two strains are often compared when toxicity of polycyclic hydrocarbons and TCDD are being studied. Weaned B6 mice have been shown about 10 times more sensitive to TCDD with respect to thymic involution, as compared to D2 mice (receptor-defective) (4). As expected, thymuses explanted from D2 fetuses showed a lower sensitivity to TCDD *in vitro* as compared to those from B6 fetuses,

although much more so when cultured for short periods of time (2 days) as compared to longer culture periods (6 days). In an *in vitro* situation, the test substance will be available to the cell for a long period of time. It is possible that under such circumstances an interaction of the TCDD with the genome may occur even with a defective receptor, followed by a repression of transcription [RNA polymerase suppressed in thymus in contrast to liver by TCDD (27)]. The role of the *Ah* receptor in TCDD toxicity is on the whole little understood. Could its role be to concentrate the TCDD at the "critical site" in the genome more rapidly (*in vitro* as well as *in vivo*), and more longstanding at higher concentrations *in vivo*? In a live animal, where plasma and cellular concentrations may fluctuate depending on the experimental situation, a functioning receptor would thus tend to increase the toxicity of TCDD.

Some investigators have claimed that D2 mice exhibit the *Ah* receptor which is, however, detectable in the nucleus only, *and* provided that high doses of TCDD have been administered *in vivo* (11). The D2 receptor would thus show different characteristics as compared to that of B6 mice. Whitlock and Galeazzi (28) have presented evidence that the *Ah* receptor of hepatoma cell lines may be predominantly nuclear, by keeping the tissue to buffer ratio high upon isolation of the receptor. In the perspective of these two reports, our results on the increasing sensitivity with time of the D2 thymuses are of interest and suggest some kind of "activation" of the nuclear D2 receptor or some other regulatory mechanism under the conditions of an *in vitro* culture.

It is interesting to note that B6 thymuses from fetuses of mothers treated *in vivo*, although they are smaller at explantation and have fewer cells even after 10 days in culture, will gradually reach the same number of lymphoid cells as control cultures at their plateau level. This indicates that the damage to the thymus is quantitatively reversible once TCDD is removed from the environment. It remains, however, to be determined if such lymphoid cells show the same characteristics in terms of cell surface markers and cellular functions.

It has been much discussed whether the TCDD effect on thymus might be secondary to hormonal, nutritional, or other factors induced by TCDD in the body (29, 30). Our results show that TCDD has a direct effect on the development of the thymus in culture, which most likely parallels that seen *in vivo*. This is to our knowledge the first demonstration that an intact tissue cultured *in vitro* may respond to TCDD very similarly to that response occurring *in vivo* at concentrations as low as 10^{-10} M, and that strain differences in TCDD toxicity can be observed *in vitro*. This model should lend itself to realistic studies of maturation of different subtypes of T-lymphocytes, nutritional and hormonal dependency of the TCDD toxicity, and also which cell type in the thymus (notably thymic epithelium and the lymphocytes) is the target cell for the TCDD. With the rapid development of knowledge on thymic growth factors, it may be possible to get closer to the mechanism of toxicity of TCDD and its congeners.

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