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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: Segregation of Toxicity with the Ah Locus

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SUMMARY

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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related halogenated aromatic hydrocarbons (a) produce a similar pattern of toxic responses; (b) induce a battery of enzymes, including aryl hydrocarbon hydroxylase (AHH) activity; and (c) bind reversibly and with a high affinity to a cytosol protein that is the receptor for this enzyme induction response. To test the hypothesis that the toxic effects produced by these compounds are mediated by their binding to the induction receptor, we examined the genetic segregation of two toxic responses, with the Ah locus, which determines this receptor. The dose–response relationship for thymic atrophy produced by TCDD was examined in C57BL/6J mice, which have a high affinity receptor and are sensitive to enzyme induction, DBA/2J mice, which have a lower affinity receptor and are less sensitive to induction of AHH activity, and hybrid B6D2F$_1$/J mice. C57BL/6J mice were approximately 10-fold more sensitive to thymic involution than DBA/2J mice and the hybrid B6D2F$_1$/J were intermediate between the two parental strains. The progeny of a B6D2F$_1$/J × DBA/2J mating were phenotyped (as Aa or aa), and the mice heterozygous for the high affinity receptor were more sensitive to thymic atrophy. The capacity of other halogenated aromatic hydrocarbons to produce thymic atrophy corresponded to their capacity to bind to the induction receptor, and nonhalogenated compounds which are agonists for the receptor (3-methylcholanthrene, β-naphthoflavone) also produced this toxic response. TCDD produced a dose-related incidence of cleft palate formation in the fetuses of C57BL/6J mice. A single dose of 30 μg/kg of TCDD on day 10 of pregnancy produced an incidence of cleft palates of 54% in C57BL/6J fetuses, 13% in B6D2F$_1$/J fetuses and only 2% in DBA/2J fetuses. Five inbred strains of mice with a low affinity receptor developed only a 0–3% incidence of cleft palates from TCDD (30 μg/kg), while four of five inbred strains with a high affinity receptor developed a 50% or greater incidence. For TCDD and congeners, (a) the correspondence of the structure–activity relationship for receptor binding and for toxic potency and (b) the segregation of thymic involution and probably teratogenesis with the Ah locus indicate that binding of these compounds to the receptor is an essential step in their mechanism of toxicity.

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)[2] is an extraordinarily potent toxin, teratogen (1), and carcinogen (2) and serves as the prototype for a large series of halogenated aromatic hydrocarbons, all of which (i) are approximate isostereomers, (ii) produce a similar pattern of toxic responses, and (iii) elicit common biochemical responses (3).

TCDD and its congeners produce a characteristic pattern of toxic responses: a slow wasting syndrome, lymphoid involution, and teratogenesis and/or embryotoxicity are seen in most animal species, while hepatotoxicity, hyperkeratosis and chloracne, and an edematous syn-

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[2] The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AHH, aryl hydrocarbon hydroxylase; ED_{50}, the dose which produces one-half the maximal response; MOPS, morpholinopropanesulfonic acid.

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drome are expressed in a more limited number of species (4, 5). The cause of death is unknown.

TCDD and its congeners induce a variety of enzyme activities, primarily in the liver. The most studied of these responses is the induction of the hepatic microsomal monooxygenase activity, aryl hydrocarbon hydroxylase (AHH) (6). *The potency of halogenated aromatic hydrocarbon congeners to induce hepatic AHH activity correlates very closely with their toxic potency* (3). The induction of AHH activity by TCDD and congeners appears to be mediated by a cytosolic binding protein, which stereospecifically and reversibly binds these compounds with a high affinity (7) and mediates their specific nuclear binding (8). The cytosol binding protein appears to be the receptor for this induction response.

The induction receptor is determined by the Ah locus in mice (7). Certain inbred strains of mice, typified by C57BL/6J are sensitive to the induction of hepatic AHH activity by TCDD (ED_{50} = 1 nmol/kg) and have a high affinity cytosol binding protein (K_D = 0.27 nM for TCDD). Other inbred strains, typified by DBA/2J, are less sensitive to induction of hepatic AHH activity by TCDD ($ED_{50} \geq 10$ nmol/kg) and have a much lower affinity cytosol binding protein. The Ah locus (and hence the cytosol induction receptor) appears to control not only AHH activity, but the induction (and perhaps repression) of a battery of enzyme activities that are coordinately expressed.[3]

We suggest that any consideration of the mechanism of toxicity of TCDD and related halogenated aromatic hydrocarbons, should explain the similarity between the rank-ordered potency of these compounds to induce AHH activity and their potency as toxins. The similarity of these structure–activity relationships suggests that both processes are mediated through a common recognition site. We propose that the toxicity of TCDD and congeners is mediated through the induction receptor, that is, the initial event is their stereospecific recognition and binding to the cytosol binding species.

One obvious prediction of this model is that the toxicity of TCDD should segregate with the Ah locus. Mice that have a high affinity receptor and that are more sensitive to induction of AHH activity by TCDD should be more sensitive to the specific tissue toxicities of the compound.

In this report, we examine two toxic responses to TCDD, thymic atrophy and teratogenesis, in various inbred strains of mice and their segregation with the Ah locus.

[3] The original description of the Ah locus was based on phenotyping with polycyclic aromatic hydrocarbons (9, 10). Certain inbred strains of mice, typified by C57BL/6, when challenged with 3-methylcholanthrene, respond with the induction of hepatic AHH activity, and are termed aromatic hydrocarbon responsive. Other inbred strains, typified by DBA/2 mice, fail to respond, or responded very weakly to even large doses of 3-methylcholanthrene, and are termed nonresponsive. In crosses and backcrosses between C57BL/6 and DBA/2 mice, aromatic hydrocarbon responsiveness is inherited as a simple autosomal dominant. Further studies of other inbred strains have shown that the inheritance of AHH inducibility is controlled by multiple alleles at more than one locus (11, 12). All inbred strains of mice, those responsive or nonresponsive to polycyclic aromatic hydrocarbons, are inducible by TCDD, and vary only in their degree of sensitivity (13). In this report, we refer to mice as having a greater or lesser sensitivity to TCDD induction of AHH activity or TCDD toxicity, or a high or low affinity induction receptor, and avoid the use of responsive and nonresponsive (to aromatic hydrocarbons).

MATERIALS AND METHODS

Materials. 2,3,7,8-[1,6-^{3}H]Tetrachlorodibenzo-*p*-dioxin was synthesized as previously described (7) and had an initial specific activity of 39 Ci/mmol. The compound was 95% chemically pure; the impurity consisted of radiolabeled trichloro- and pentachlorodibenzo-*p*-dioxin. The other halogenated dibenzo-*p*-dioxins and dibenzofurans were synthesized as previously reported (14, 15).

3,4,5,3′,4′,5′-Hexabromobiphenyl and 2,4,5,2′,4′,5′-hexabromobiphenyl were purchased from En Chem Environmental Division, RFR Corporation, Hope, R.I.

TCDD and many of its congeners are extremely toxic materials and should be handled with special precautions as previously outlined (7), including the use of disposable gloves and bench top coverings and as much disposable plastic ware as possible. Plastic animal cages, which housed TCDD-treated mice, were soaked in an aqueous solution of 1% Triton X-100 prior to general washing. The liquid waste was mixed with fuel oil and pyrrolyzed and the solid waste, containing much lower TCDD concentrations, was buried in a landfill.

Pyrene and β-naphthoflavone were purchased from Aldrich Chemical Company (Milwaukee, Wis.) and 3-methylcholanthrene was purchased from K+K Laboratories, ICN Pharmaceuticals (Plainview, N.Y.). Zoxazolamine was a generous gift of McNeal Laboratories, Inc. (Fort Washington, Pa.) and activated charcoal (PX-21) was a generous gift of Amoco Research (Chicago, Ill.). MOPS was purchased from Calbiochem (La Jolla, Calif.).

Animals. All inbred strains of mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and housed either in hanging wire-frame cages or in plastic cages on hardwood bedding (Beta-Chip, Northeastern Products Corp., Warrenburg, N.Y.). The animals were maintained on a diurnal cycle of 12 hr light/12 hr darkness and permitted water and food (Mouse Breeder Blox, Wayne Laboratory Animal Diets, Allied Mills, Inc., Chicago, Ill.) *ad libitum.*

Thymic involution. The mice in each experiment were of the same age (5 to 6 weeks old) and of the same sex. Both age and sex affect the thymus weight. Each mouse was weighed to the nearest 0.1 g and administered TCDD or a congener dissolved in *p*-dioxane (0.4 ml/kg) intraperitoneally. 3-Methylcholanthrene, pyrene, β-naphthoflavone, and the brominated biphenyls were dissolved in corn oil (8 ml/kg). The controls received injections of the appropriate solvent. At a subsequent time, usually 5 or 6 days (indicated in the legends to the figures), the animals were killed and the body and thymus weight taken. Occasionally the thymus was examined histologically, after formalin fixation, sectioning, and hematoxylin and eosin staining.

Zoxazolamine phenotyping. In the genetic segregation experiment in Fig. 2B progeny of B6D2F$_1$/J × DBA/2J cross were injected intraperitoneally with β-naphthoflavone (80 mg/kg) in corn oil for 2 days. Forty-eight hours after the last injection the mice were administered zox-

azolamine (225 mg/kg) dissolved in corn oil (15 ml/kg) intraperitoneally and the interval from the loss of the righting reflex to recovery was measured. Heterozygote (Aa) mice were paralyzed for 0–10 min and homozygous mice (aa) for greater than 45 min. The few animals with intermediate sleeping times were discarded. The mice were permitted to recover from the drug exposure for 2 weeks prior to the challenge with TCDD to examine thymus atrophy.

AHH and 7-ethoxycoumarin-O-deethylase activities. In the experiment shown in Table 3, the enzyme activities were measured in the thymus and liver on the 10,000*g* supernatant fraction as previously described (16, 17). In the thymus, both AHH and 7-ethoxycoumarin-*O*-deethylase activity were assayed in tissue equivalent to 10 mg wet wt, incubated for 30 min at pH 7.5. In the liver, both enzyme activities were measured in the tissue equivalent to 1 mg wet wt, incubated for 10 min at pH 7.2. The benzo[*a*]pyrene concentration was 100 μM and the 7-ethoxycoumarin concentration was 500 μM. All assays were performed in duplicate.

Cytosol binding. The 100,000*g* supernatant fraction of mouse thymus and liver was prepared in MDEN buffer (MOPS 25 mM, dithiothreitol 1 mM, EDTA 1 mM, sodium azide 0.02%) containing 5% glycerol, pH 7.4. One-milliliter fractions of liver cytosol, and 0.6-ml fractions of thymus cytosol, both at a protein concentration of approximately 2 mg/ml, were incubated with varying concentrations of [^{3}H]TCDD (1 to 12.5 × 10^4 dpm/ml) or [^{3}H]TCDD plus a 200-fold molar excess of 2,3,7,8-tetrachlorodibenzofuran added in a total volume of 10 μl *p*-dioxane for 30 min at 20°. A suspension of charcoal:dextran (3%:0.03%) in buffer equal to one-half the incubation volume was added and the mixture incubated for another 5 min. The suspension was centrifuged and a sample of the supernatant fraction quantified by liquid scintillation spectrometry. All assays were performed in duplicate. From the total binding ([^{3}H]TCDD) minus the nonspecific binding ([^{3}H]TCDD + 2,3,7,8-tetrachlorodibenzofuran), we calculated the specific binding (the high affinity, displaceable pool) (7). Specific binding is shown in Fig. 4.

Teratogenesis studies. Female nulliparous mice, 9 to 20 weeks old (usually 10 to 14 weeks), were maintained in moderately crowded conditions to inhibit the estrus cycle (18). The females were then placed one or two per plastic cage on soiled male bedding for 48 to 52 hr. The female mice were moved to a cage with fresh bedding (noon to 4 PM) and an experienced male mouse added. Each subsequent morning the mice were checked for vaginal plugs and this was designated day 0 of pregnancy. On the morning of day 10, the pregnant animals received a subcutaneous injection in the flank region of TCDD dissolved in *p*-dioxane (0.4 ml/kg). On day 18, the mice were sacrificed and scored for number of dead or resorbed fetuses and cleft palates (using a dissecting microscope).

RESULTS

The administration of a single dose of TCDD (1 × 10^{-7} mol/kg) to C57BL/6J mice produced a decrease in the size of the thymus that was maximal by 6 days (Fig. 1). The thymus weight fell from a control value of 50.3 ± 4.8 to 16.8 ± 2.0 mg. The data in Fig. 1 and throughout the paper are presented as the ratio of thymus to body weight rather than absolute thymus weight, to correct for variation in body weights. Histologic examination of the thymus tissue revealed a loss of the cortical cells without obvious cell necrosis, in agreement with reports by others who have noted either no necrosis or minimal focal cell necrosis (4, 19, 30).

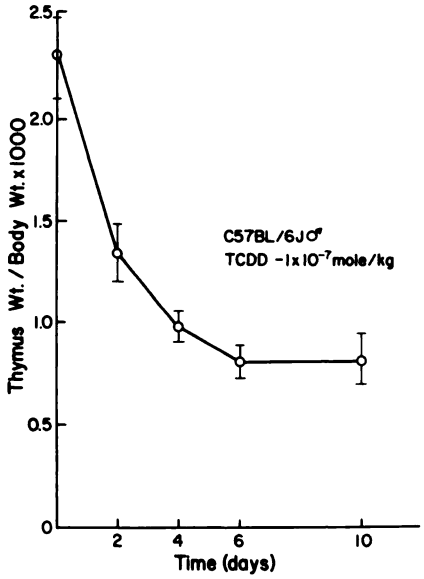

FIG. 1. *Time course of thymic involution following TCDD*
A single intraperitoneal injection of 1 × 10^{-7} mol/kg of TCDD dissolved in *p*-dioxane was administered to C57BL/6J male mice 2, 4, 6, and 10 days prior to sacrifice. Control mice (zero time) received only *p*-dioxane 10 days prior to sacrifice. All animals were killed on the same day, at 48 days of age, and their thymus and body weights determined. Each point is the mean ± standard error of the values of seven mice.

If the toxicity of TCDD is mediated through the induction receptor, then toxicity should segregate with the Ah locus, which determines the receptor. C57BL/6J mice, which have a high affinity receptor and which are sensitive to hepatic AHH induction by TCDD, should be sensitive to the toxicity of TCDD, and conversely DBA/2J mice, which have a low affinity receptor and are less sensitive to AHH induction, should be less sensitive to TCDD toxicity.

In Fig. 2A are shown the dose–response curves for TCDD-induced thymus atrophy in C57BL/6J, DBA/2J and the hybrid B6D2F_1/J mice. C57BL/6J mice are more sensitive to thymic involution than DBA/2J by approximately an order of magnitude, and B6D2F_1/J mice show intermediate sensitivity. To determine if this toxic effect of TCDD formally segregates with the Ah locus, we mated B6D2F_1/J female mice (heterozygous, Aa, for the locus) with DBA/2J mice (aa) and after weaning, the offspring (either Aa or aa) were phenotyped by use of zoxazolamine sleeping times. As shown in Fig. 2B, the heterozygous mice were more sensitive to TCDD than were mice homozygous (aa) at the Ah locus. The results for male and female mice are presented separately because sex has a substantial effect on the ratio of thymus to body weight in control animals.

If the toxicity of halogenated aromatic hydrocarbons is mediated through the induction receptor, the structure–activity relationship for congeners to bind to the

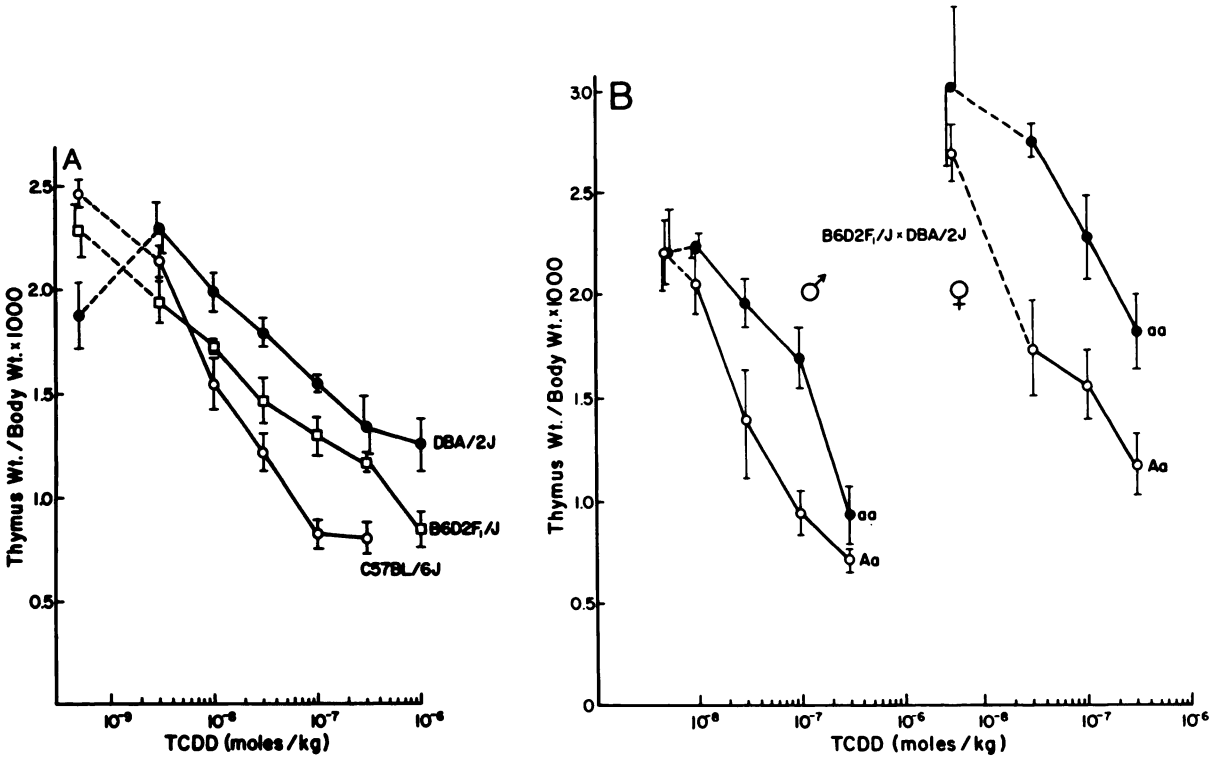

FIG. 2. *Dose-response curves for the thymus atrophy produced by TCDD in C57BL/6J, DBA/2J, B6D2F$_1$/J, and backcross mice*

(A) C57BL/6J, DBA/2J, and B6D2F$_1$/J male mice, 36 or 37 days old, were administered a single intraperitoneal injection of varying doses of TCDD dissolved in *p*-dioxane and killed 6 days later, and their thymus and body weights measured. (B) The offspring of B6D2F$_1$/J female × DBA/2J male mice, were phenotyped by zoxazolamine paralysis times (see MATERIALS AND METHODS) as responsive to β-naphthoflavone (Aa) or nonresponsive (aa). Two weeks later at 39 ± 2 days of age the animals were given a single injection of varying doses of TCDD and killed 6 days later. Control animals received only *p*-dioxane and their values are connected to the treated animals by a dashed line. Each point is the mean ± standard error of five to eight animals.

receptor (and to induce AHH activity) should correspond to their capacity to produce a toxic effect. As seen in Fig. 3, TCDD and 2,3,7,8-tetrachlorodibenzofuran, which have high affinities for the receptor (7), produce thymic atrophy, whereas 1,3,6,8-tetrachlorodibenzo-*p*-dioxin and 2,7-dichlorodibenzo-*p*-dioxin, neither have affinity for the receptor nor produce thymic atrophy.

Two broad classes of compounds are agonists for the induction receptor: (1) the halogenated aromatic hydrocarbons (dibenzo-*p*-dioxins, dibenzofurans, azo- and azoxybenzenes, biphenyls) and (2) the polycyclic aromatic hydrocarbons and other nonhalogenated aromatic compounds (e.g., β-naphthoflavone). None of the toxic responses characteristic of TCDD and its congeners have been reported to occur with the administration of polycyclic aromatic hydrocarbons. We examined the effect of these latter compounds on thymic involution.

3-Methylcholanthrene produced a dose-related thymus atrophy in C57BL/6J mice as seen in Table 1. A single dose of 3-methylcholanthrene of 3×10^{-4} mol/kg (80 mg/kg) produced a near maximal effect (comparable to the effect of TCDD at 3×10^{-7} mol/kg) and no greater thymic loss was produced by the compound given at this daily dose for 4 days. Pyrene, a polycyclic aromatic hydrocarbon which does not bind to the receptor, even at a very high dose (4.9×10^{-4} mol/kg), failed to produce any significant decrease in thymus size. In another experiment β-naphthoflavone, administered at a dose of 2.9×10^{-4} mol/kg (80 mg/kg) for 4 days, produced a modest

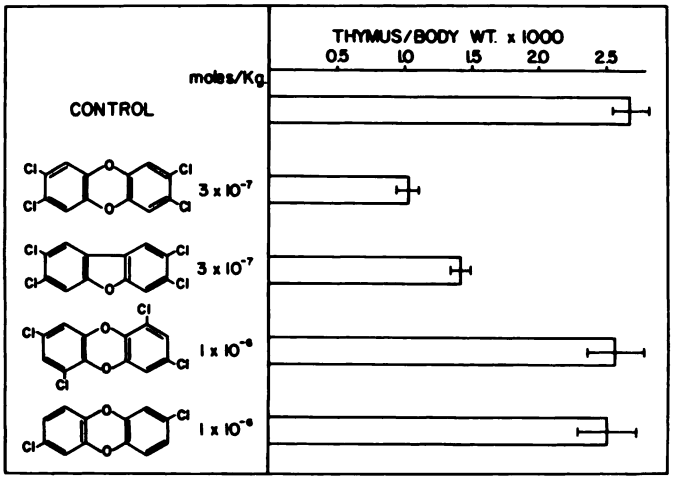

FIG. 3. *The effect of four halogenated aromatic hydrocarbon congeners in the thymus*

C57BL/6J mice, 35 days of age, were administered a single intraperitoneal injection of a halogenated aromatic compound dissolved in *p*-dioxane or the vehicle alone (0.4 ml/kg). Five days later the animals were killed and their body and thymus weights determined. Each bar represents the mean ± standard error of the values for a group of eight animals.

atrophy of the thymus. The planar brominated biphenyl congener 3,4,5,3′,4′,5′-hexabromobiphenyl, an agonist for the induction receptor (20), produced substantial thymic involution, but the 2,4,5,2′,4′,5′-hexabromo- congener,

TABLE 1

Thymus atrophy induced by 3-methylcholanthrene and other compounds in C57BL/6J and DBA/2J mice

Five-week-old C57BL/6J and DBA/2J male mice, were administered a single intraperitoneal dose of corn oil (8 ml/kg) or the indicated compounds dissolved in corn oil and killed 5 days later. The total body weight and thymus weight were recorded. Each value is the mean ± standard error of seven to nine animals.

	Dose	Thymus wt	Thymus wt / body wt × 10^3
	(mol/kg)	*(mg)*	
C57BL/6J			
Control[a]	—	63.7 ± 1.5	3.19 ± 0.07
3-Methylcholanthrene	7.5×10^{-5}	56.0 ± 3.4	3.01 ± 0.15
3-Methylcholanthrene	1.5×10^{-4}	44.4 ± 3.4	2.39 ± 0.18
3-Methylcholanthrene	3.0×10^{-4}	33.7 ± 1.6	1.94 ± 0.09
Pyrene	4.9×10^{-4}	58.2 ± 3.4	3.05 ± 0.14
3,4,5,3′,4′,5′-Hexabromobiphenyl	1.6×10^{-4}	23.0 ± 2.5	1.39 ± 0.15
2,4,5,2′,4′,5′-Hexabromobiphenyl	1.6×10^{-4}	58.8 ± 2.6	2.96 ± 0.12
Control[b]	—	55.2 ± 2.7	2.55 ± 0.13
3-Methylcholanthrene	3.0×10^{-4}/ daily × 4 days	23.0 ± 1.9	1.18 ± 0.09
β-Naphthoflavone	2.9×10^{-4}/ daily × 4 days	37.8 ± 2.4	1.88 ± 0.11
DBA/2J			
Control[a]	—	43.1 ± 1.8	2.49 ± 0.06
3-Methylcholanthrene	3.0×10^{-4}	30.0 ± 1.6	2.28 ± 0.06
3-Methylcholanthrene	9.3×10^{-4}	32.8 ± 0.9	1.92 ± 0.11
3-Methylcholanthrene	9.3×10^{-4} daily × 4 days	27.0 ± 1.8	1.70 ± 0.12
3,4,5,3′,4′,5′-Hexabromobiphenyl	1.6×10^{-4}	40.5 ± 2.2	2.43 ± 0.15

[a] Control animals received corn oil by intraperitoneal injection 8 ml/kg once.

[b] Control animals received corn oil intraperitoneal injection 8 ml/kg/day for 4 days.

shows no receptor affinity and produced no thymus atrophy.

DBA/2J mice are much less sensitive to AHH induction by polycyclic aromatic hydrocarbons (9, 10). A single dose of 3-methylcholanthrene (3×10^{-4} mol/kg), which produces a near maximal response in C57BL/6J mice, has little or no effect in DBA/2J mice, although a repeated daily dose of 3×10^{-4} mol/kg for 4 days or a single large dose of 9.3×10^{-4} mol/kg of 3-methylcholanthrene produced a moderate thymic involution (Table 1). 3,4,5,3′,4′,5′-Hexabromobiphenyl, at a dose that produces thymic involution in C57BL/6J mice, was ineffective in DBA/2J mice.

Receptor binding, TCDD uptake, and monooxygenase induction in the thymus. The TCDD–cytosol binding protein was initially described in mouse and rat liver. Recently, Carlstedt-Duke (21) has identified a high affinity TCDD binding species in a number of tissues of rat, including the thymus. If the receptor mediates the induction response and the toxic effects produced by TCDD, then presumably the receptor should be present in all tissues in which these responses occur. We measured the specific binding of [^{3}H]TCDD in the cytosol of liver and thymus from C57BL/6J and DBA/2J mice (Figs. 4A and B). We observed high affinity saturable binding of [^{3}H]TCDD in both tissues from C57BL/6J mice, and much lower, virtually absent, specific binding in DBA/2J thymus and liver. The insolubility of the radioligand prevented us from using higher concentrations in an attempt to demonstrate saturable binding in DBA/2J mice.

Following the intraperitoneal administration of [^{3}H]TCDD (1×10^{-8} mol/kg, 39 Ci/mmol), we observed a rapid uptake of TCDD in the liver of mice, and about a 10-fold lower concentration of radiolabel accumulating in other tissues—thymus, lung, and kidney (Table 2). While in the hepatic tissues accumulation of [^{3}H]TCDD was considerably greater in C57BL/6J mice than DBA/2J mice, no strain difference in uptake was seen in other tissues.

We administered TCDD or 3-methylcholanthrene to

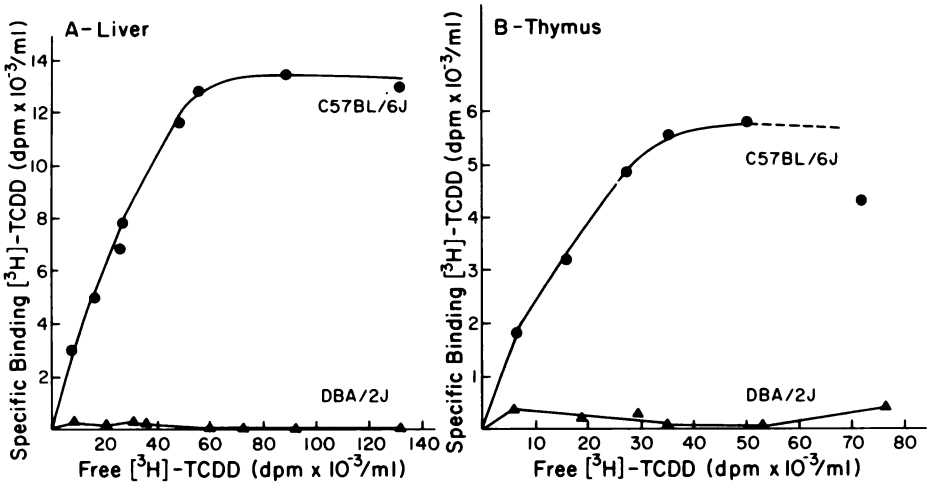

FIG. 4. *Specific binding of [^{3}H]TCDD to cytosol of liver and thymus from C57BL/6J and DBA/2J mice*

The assays were performed as described in METHODS using a 1-ml sample of liver cytosol and a 0.6-ml sample of thymus cytosol. (A) *Liver*: The protein concentration for C57BL/6J mice was 1.77 mg/ml and for DBA/2J mice, 1.69 mg/ml. (B) *Thymus*: The protein concentration for C57BL/6J mice was 1.69 mg/ml and for DBA/2J mice, 1.53 mg/ml.

C57BL/6J mice and measured monooxygenase activity in the liver and thymus, 12 or 24 hr later (Table 3). TCDD and 3-methylcholanthrene produced a substantial induction of hepatic AHH and 7-ethoxycoumarin-*O*-deethylase activities. In the thymus, the basal AHH activity was barely measurable above the zero time blank fluorescence, and equivalent to approximately 0.05% of the activity in control liver. TCDD and 3-methylcholanthrene produced a modest increase in thymus AHH activity. 7-Ethoxycoumarin-*O*-deethylase activity is more easily measured and more reliable than AHH activity in the thymus (the fluorescence of the product formed by basal enzyme activity is twice that of the zero time blank). TCDD produced a rather small increase (50%) in this thymus monooxygenase activity.

The main conclusions drawn from these studies are: (1) the cytosol induction receptor is present in the thymus; (2) following intraperitoneal administration, TCDD reaches the thymus, although tissue uptake is an order of magnitude lower than in the liver; and (3) monooxygenase activity is present in the thymus and modestly inducible by TCDD, but control and induced activities are three orders of magnitude lower than the corresponding hepatic enzyme activities.

Teratogenesis. Among the most sensitive toxic effects of TCDD are teratogenesis, embryotoxicity and fetal loss. Courtney and Moore (22) noted that in mice TCDD produces primarily two teratogenic effects, cleft palates and hydronephrosis, and that C57BL/6J mice were considerably more sensitive to these effects than DBA/2J mice. We chose to study only cleft palate formation, which is easily scored without the necessity of histology. Pregnant C57BL/6J and DBA/2J mice, from mating with their respective males, and pregnant C57BL/6J mice, from mating with DBA/2J males, were administered a single subcutaneous dose of TCDD dissolved in *p*-dioxane or the vehicle alone on day 10 after vaginal plugs, killed on day 18, and the fetuses inspected. As seen in Table 4, there was some fetal wastage, but this was not related to the dose of TCDD. There were no cleft palates in any of the control fetuses. TCDD, at a dose of 3 μg/kg, produced no cleft palates and at a dose of 10 μg/kg, only a 3% incidence of cleft palates among live fetuses in C57BL/6J mice. A dose of 30 μg/kg TCDD produced a 54% incidence in C57BL/6J mice, only a 2% incidence in DBA/2J mice, and an intermediate 13% incidence in the B6D2F$_1$/J fetuses. At a dose of 50 μg/kg, TCDD

TABLE 2

Tissue uptake of [^{3}H]TCDD in C57BL/6J and DBA/2J mice

C57BL/6J and DBA/2J males, 33 days old were given a single intraperitoneal dose of [^{3}H]TCDD (1×10^{-6} mol/kg, 8.21×10^6 dpm/kg). The animals were killed, 3, 8, and 24 hr later; the thymus, lung, kidney, and liver were removed and weighed; and a portion of each was digested in Protosol and the radioactivity quantified. Each value is the mean ± standard error of the determination on tissues from five animals.

		Thymus uptake	Lung uptake	Kidney uptake	Liver uptake
		(dpm × 10^{-5}/g tissue)			
3 hr	C57BL/6J	1.85 ± 0.25	2.71 ± 0.21	2.05 ± 0.28	22.3 ± 2.6
	DBA/2J	0.59 ± 0.15	1.00 ± 0.19	0.65 ± 0.12	6.3 ± 0.10
8 hr	C57BL/6J	1.73 ± 0.12	2.05 ± 0.09	1.48 ± 0.14	25.5 ± 2.6
	DBA/2J	1.07 ± 0.13	1.56 ± 0.16	1.15 ± 0.13	11.6 ± 1.1
24 hr	C57BL/6J	1.44 ± 0.29	1.32 ± 0.12	1.00 ± 0.16	42.6 ± 2.7
	DBA/2J	2.34 ± 0.52	1.09 ± 0.24	0.92 ± 0.31	18.1 ± 1.7

TABLE 4

Cleft palate production by TCDD in C57BL/6J, DBA/2J, and B6D2F$_1$/J mice

Nulliparous female mice, 10 to 20 weeks of age, were mated with males and inspected daily for vaginal plugs. On day 10 of pregnancy, the animals were given a single subcutaneous dose of TCDD (3, 10, or 30 μg/kg) dissolved in *p*-dioxane or the solvent alone (0.4 ml/kg). On day 18 the animals were killed and scored for dead fetuses or resorbed embryos and cleft palate formation.

	Treatment	Number of litters	Dead or resorbed fetuses	Cleft palates/Live fetuses	Percentage cleft palates
	(nmol/kg)				
C57BL/6J	Control	7	7	0/48	0
	TCDD 9.3	8	0	0/59	0
	TCDD 31	11	2	2/67	3
	TCDD 93	13	12	52/96	54
B6D2F$_1$/J	Control	10	0	0/60	0
	TCDD 9.3	8	0	0/57	0
	TCDD 31	6	1	0/41	0
	TCDD 93	12	0	11/88	13
DBA/2J	Control	12	7	0/64	0
	TCDD 9.3	13	3	0/78	0
	TCDD 31	13	2	0/70	0
	TCDD 93	16	0	2/96	2

TABLE 3

Effect of the administration of TCDD and 3-methylcholanthrene on AHH and 7-ethoxycoumarin-O-deethylase activity in thymus and liver of C57BL/6J mice

C57BL/6J, 4-week-old male mice, were administered a single intraperitoneal dose of *p*-dioxane, TCDD in *p*-dioxane, corn oil, or 3-methylcholanthrene dissolved in corn oil. The values for the *p*-dioxane and corn oil-treated animals did not differ and only the former are shown. The animals were killed 12 or 24 hr after dosing and thymus and liver AHH and 7-ethoxycoumarin-*O*-deethylase activities measured (see MATERIALS AND METHODS). AHH and 7-ethoxycoumarin-*O*-deethylase activity were measured in separate experiments. Each value is the mean ± standard error of determinations on four animals.

	Dose	Time interval	AHH activity		7-Ethoxycoumarin-O-deethylase activity	
			Thymus	Liver	Thymus	Liver
	(mol/kg)	*(hr)*	*(pmol/mg/min)*		*(pmol/mg/min)*	
Control		24	0.003 ± 0.001	7.6 ± 1.1	0.052 ± 0.004	39.3 ± 1.7
TCDD	3.0×10^{-7}	12	0.022 ± 0.002	49.3 ± 5.0	0.085 ± 0.010	163 ± 12.8
TCDD	3.0×10^{-7}	24	0.051 ± 0.007	72.4 ± 7.9	0.093 ± 0.016	273 ± 5.7
3-Methylcholanthracene	3.0×10^{-4}	24	0.012 ± 0.002	81.3 ± 1.7	0.061 ± 0.006	144 ± 11.7

TABLE 5

Cleft palate formation by TCDD in ten inbred strains of mice

Nulliparous female mice, 9 to 20 weeks of age, were mated and inspected daily for vaginal plugs. On day 10 of pregnancy, the animals were given a single subcutaneous dose of TCDD (93 nmol/kg, 30 μg/kg), dissolved in *p*-dioxane (0.4 ml/kg). The animals were killed on day 18 and the fetuses inspected for cleft palates. C57BL/6J, A/J, BALB/cByJ, SEC/1ReJ, and CBA/J mice have a high affinity cytosol receptor; DBA/2J, RF/J, AKR/J, SWR/J, and 129/J mice have a low affinity cytosol receptor.

Strain	Number of litters	Dead or resorbed fetuses	Cleft palates/Live fetuses	Percentage cleft palates
C57BL/6J	13	12	52/96	54
A/J	7	16	37/51	73
BALB/cByJ	9	11	26/40	65
SEC/1ReJ	4	6	19/20	95
CBA/J	12	5	0/61	0
DBA/2J	16	7	2/96	2
RF/J	9	18	1/34	3
AKR/J	8	1	0/38	0
SWR/J	7	3	0/37	0
129/J	6	3	0/29	0

produced an 85% incidence of cleft palates (44/52 fetuses) in C57BL/6J mice (data not shown). We wished to test the segregation of susceptibility to cleft palate with the Ah locus, but phenotyping fetuses proved difficult.[4] As an alternative, we examined the strain distribution of cleft palate formation in eight other strains administered a single dose of 30 μg/kg of TCDD. The results of the experiments along with those of C57BL/6J and DBA/2J are shown in Table 5. No attempt was made to measure the incidence of cleft palates in solvent-injected control mice. In DBA/2J, RF/J, AKR/J, SWR/J, and 129/J, the five strains with a low affinity receptor, TCDD produced a 0–3% incidence of cleft palates. Four of the five strains with a high affinity receptor, C57BL/6J, A/J, BALB/cByJ and SEC/1ReJ, developed a 54–95% incidence in response to TCDD. CBA/J was the only strain with a high affinity receptor that developed no cleft palates. CBA/J mice are also resistant to cortisone-induced cleft palates (23). The strain distribution suggests that TCDD-evoked cleft palate formation segregates with the Ah locus.

[4] We planned to mate B6D2F$_1$/J and DBA/2J mice, treat the pregnant mice with TCDD, and examine the fetuses for cleft palates and their phenotype. The two phenotypes from this cross, heterozygous, high affinity receptor (Aa) and homozygous, low affinity receptor (aa) can be easily distinguished by specific binding of [^{3}H]TCDD to fetal liver cytosol. However, the low incidence of cleft palate formation produced by TCDD (30 μg/kg) in B6D2F$_1$/J meant examining and phenotyping a large number of fetuses. A higher dose of TCDD (50 μg/kg) did not increase the incidence of cleft palates in B6D2F$_1$/J mice. Alternatively we considered mating B6D2F$_1$/J mice with C57BL/6J, and distinguishing the fetal phenotypes (AA and Aa) by the amount of high affinity specific binding in fetal liver. There is a gene–dose effect and one can demonstrate that C57BL/6J mice have about twice as much high affinity cytosol binding as B6D2F$_1$/J mice. However, in comparing the amount of fetal liver cytosol binding, there is some overlap between individuals of the two phenotypes (AA and Aa).

DISCUSSION

In this report we examined two characteristic toxic effects produced by TCDD, thymic involution and teratogenesis, in inbred strains of mice, and asked if these toxic responses segregate with the Ah locus. In crosses and backcrosses between C57BL/6J and DBA/2J mice, sensitivity to thymic atrophy produced by TCDD segregated with the Ah locus. Other halogenated aromatic hydrocarbons, and most interestingly nonhalogenated aromatic hydrocarbons (3-methylcholanthrene and β-naphthoflavone), which bind to the induction receptor and induce AHH activity, also produced thymic involution. Cleft palate formation produced by TCDD followed the strain distribution for the Ah locus in 9 of 10 inbred strains of mice.

Thymus. One of the most characteristic toxic responses produced by TCDD is the involution of lymphoid tissue (thymus, lymph nodes, and spleen) observed in mice, rats, and guinea pigs, which in young animals is accompanied by suppression of cellular immune responses (4, 19, 24–26). TCDD produces a dose-related atrophy of the cortical cells of the thymus (4). The response is not mediated through the corticosteroid hormones, because TCDD produced thymus atrophy in adrenalectomized animals (26). While there has been considerable investigation of this toxic response it is not clear precisely what steps in the cellular immune response are affected, nor which cell population is susceptible. We have found cell culture of mouse lymphomas (bearing Thy, TL, and θ antigens, and susceptible to corticosteroids) were not susceptible to TCDD in culture.[5] Despite our ignorance of the mechanism of thymic atrophy, the data suggest that the response is mediated through the receptor.

A rough estimate of the dose of TCDD that produced one-half the maximal thymic involution in C57BL/6J mice is about 10^{-8} mol/kg, and about a 10-fold higher dose is required for DBA/2J mice (Fig. 2A). The dose of TCDD which produces half-maximal induction of hepatic AHH activity in C57BL/6J mice is 1×10^{-9} mol/kg and in DBA/2J mice is approximately 1×10^{-8} mol/kg. The differential sensitivity between strains of mice is seen for both hepatic enzyme induction and thymic sensitivity. The 10-fold higher dose needed for the thymus effect may arise from the greater concentration of TCDD in the liver compared with thymus and other nonhepatic tissues (Table 2).

Teratogenicity. TCDD is one of the most potent embryotoxic–teratogenic compounds known (1, 22, 27). Courtney and Moore (22) reported that the administration of TCDD to pregnant mice produced cleft palates and kidney abnormalities (hydronephrosis) in the fetuses. Fortuitously, these authors used two inbred strains of mice, C57BL/6J and DBA/2J, and noted that the former were more sensitive to the teratogenic effects of TCDD than were the latter. For the modest number of chlorinated dibenzo-*p*-dioxins that have been tested for teratogenicity (1, 28), their rank-ordered potency to produce teratogenicity corresponds to the binding affinity for the induction receptor.

[5] Knutson, J. and A. Poland. Manuscript in preparation.

In this report, we have restricted our observations to a single embryonic effect, cleft palate formation. In 9 of 10 inbred strains studied, the susceptibility to cleft palate formation produced by TCDD followed the strain distribution of the Ah locus. The 5 strains with a low affinity receptor developed a 0 to 3% incidence of cleft palates from a dose of TCDD of 30 μg/kg, while 4 of 5 strains with a high affinity receptor developed a 50% or greater incidence. The one exception were CBA/J mice, which have a high affinity receptor but developed no cleft palates. The reason for this is unknown. CBA/J mice are also resistant to cleft palate formation by corticosteroids (23) and may have overriding genetic or developmental factors that make them resistant to cleft palate formation from a variety of stimuli.

The distribution of strain sensitivity to cleft palate formation is suggestive that this toxic response segregates with the Ah locus. Confirmation of these results with formal segregation experiments proved difficult because of phenotyping. As an alternative, one might use mice congenic for the Ah locus or recombinant inbred lines.

The aggregate evidence suggests that the toxicity of TCDD and related halogenated aromatic hydrocarbons is mediated through the induction receptor: (a) the potency of the chlorinated dibenzo-*p*-dioxin (1, 29), dibenzofuran, and biphenyl congeners (30) to produce specific toxic responses (chick edema, chloracne, teratogenesis, or their lethal potency [LD_{50}]) corresponds to their affinity for the induction receptor; (b) as shown in this report, thymic involution and probably cleft palate formation produced by TCDD segregate with the Ah locus.

The Ah locus controls not only the induction of AHH activity but a battery of enzymes that are coordinately expressed (6). In view of our present ignorance about the pleiotropic effects of the Ah locus (e.g., the number and function of the genes regulated, the overall physiological purpose of this coordinated response, the existence of an endogenous inducing ligand) it is difficult to consider a precise mechanism for the toxicity of TCDD. We propose that the stereospecific binding of the chlorinated aromatic hydrocarbons by the induction receptor, and presumably the sustained expression (or repression) of one or more genes controlled by the receptor leads to the toxic responses characteristic of these compounds. There is no reason to believe that the induction of AHH activity per se is responsible for toxicity. For instance, the thymus, a target organ, has a high concentration of cytosol receptor, but a constitutive AHH activity three orders of magnitude lower than that in liver and only modestly induced by TCDD.

It might be argued that all compounds that bind to the induction receptor and act as agonists (presumably evoking the same pleiotropic response) should produce the same pattern of toxicity. Yet, none of the characteristic toxic effects produced by TCDD have been observed with polycyclic aromatic hydrocarbons, which are agonists for the induction receptor. Unexpectedly, we found 3-methylcholanthrene and β-naphthoflavone did produce thymic atrophy. It remains to be determined if the polycyclic aromatic hydrocarbons can produce any of the other toxic responses characteristic of TCDD. As a corollary consideration it is possible that some of the toxic effects produced by polycyclic aromatic hydrocarbons, may not be due to the metabolism and covalent binding of these compounds, but their action as agonists on the induction receptor.

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