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MODULATION OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN (TCDD)-MEDIATED MYELOTOXICITY BY THYROID HORMONES

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Abstract—Although binding by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to the *Ah* receptor is a prerequisite for toxicity, the events responsible for subsequent TCDD effects are essentially unknown. Several lines of evidence have indicated that thyroid hormones share common molecular properties with TCDD and can modulate its toxicity. In the present studies we employed suppression of murine bone marrow hematopoiesis by TCDD as an *in vitro* model to study the relationship between thyroid hormones and TCDD toxicity. Supraphysiological levels of thyroid hormone mimicked TCDD myelotoxicity, in that both were inhibited by a common antagonist, 1-NH_2-3,7,8-trichlorodibenzo-*p*-dioxin. Furthermore, myelotoxicity by both TCDD and thyroid hormone segregated with the *Ah* locus in congenic mice. These data provide evidence of a relationship between TCDD and thyroid hormones in that hormonal activity may help regulate TCDD toxicity.

The toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) results in specific pathological effects which can be characterized by alterations in cell proliferation and/or differentiation [1–3]. In addition, TCDD induces cytochrome P_1-450 mixed-function oxidases [4, 5]. Many of the toxic events and enzyme induction are initiated by binding of TCDD to the intracellular *Ah* receptor and nuclear translocation of the receptor–ligand complex [4, 5]. The biochemical events that regulate this interaction, although extensively investigated, are not well understood. Recently, several lines of evidence have indicated that thyroid hormones share many common molecular properties with TCDD and can modulate its toxicity [6–10]. We have reported previously that murine hematopoiesis, as measured by *in vitro* colony growth assays, provides a sensitive model to examine altered patterns of cell proliferation and differentiation induced by TCDD [11]. Utilizing this model, in concert with a specific antagonist for TCDD myelotoxicity, 1-NH_2-3,7,8-trichlorodibenzo-*p*-dioxin (NH_2-TriCDD; [11]), we provide evidence of a relationship between TCDD toxicity and thyroid hormones in that both mediate toxicity through similar events. These data imply that thyroid hormone activity can modulate TCDD toxicity at the level of the *Ah* receptor. The various mechanisms by which thyroid hormones may regulate toxicity mediated by TCDD are discussed.

METHODS

Mice. Female B6C3F1 (C57B1/6N × C3H/HeN) mice, 6–8 weeks of age and weighing 18–21 g, were obtained under the NCI production contracts from Charles River (Portage, MI). For genetic studies, C57BL/6J mice congenic at the *Ah* locus (B6.D2-Ah^{dd}) were used. These mice were originally obtained from Dr. A. Poland (McArdle Laboratory, University of Wisconsin) and are presently maintained by NIEHS as previously described [12]. C57BL/6J mice (Ah^{bb}) served as responsive controls for the B6.D2-Ah^{dd} mice.

Chemicals. Qualitative and quantitative confirmation of the TCDD (originally prepared by J. D. McKinney) stock solution was performed using a VG-Micromass AZB-2F high resolution mass spectrometer with a Varian 1400 gas chromatograph and a Finnigan INCOS 2300 data system. NH_2-TriCDD, obtained from P. W. Albro at NIEHS, was synthesized as previously described [13]. Thyroid hormones and thyroid binding prealbumin (TBPA) were obtained from Calbiochem, Inc. (San Diego, CA).

Stem cell assay. Bone marrow cells were aseptically collected from the femurs of B6C3F1 mice and pooled as previously described [1]. Single cell suspensions were prepared, and the number of nucleated cells was determined with a Coulter Counter. Cell viability, as assessed by trypan blue exclusion, was always greater than 95%. Granulocyte-macrophage progenitor cells (CFU-C) were assayed for their abilities to form colonies in 1.5% methylcellulose in the presence of 5% human AB serum, 20% fetal bovine serum, 2 mM L-glutamine, 0.5 μg/ml gentamycin, and 10% mouse lung conditioned medium (MLCM) as previously described [1]. TCDD was prepared for addition to cell cultures in serum using established methodology [1]. T_3 and/or T_4 was prepared in slightly alkaline EtOH and then added to serum which was used in preparing the complete culture medium. The final concentration of EtOH did not exceed 0.1%. NH_2-TriCDD was added to serum in *p*-dioxane so that the final concentration of *p*-dioxane did not exceed 0.001%. None of the vehicles at the concentrations used affected colony formation. Aliquots (1 ml) of the cell suspension

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Table 1. Influence of thyroid hormones on TCDD myelotoxicity

Hormone	TCDD (10 nM)	CFU-C/10^5 cells*	Percent suppression
Vehicle	−	116 ± 1†	
Vehicle	+	92 ± 1‡	21
10 μM T_3	−	114 ± 1†	2
10 μM T_4	−	114 ± 1†	2
10 μM T_3 + 2.5 μM T_4	−	95 ± 1‡	18
1 μM T_3 + 0.25 μM T_4	−	103 ± 2‡	11
0.1 μM T_3 + 0.025 μM T_4	−	110 ± 3	5
2.5 μM T_3 + 10 μM T_4	−	105 ± 2‡	10
2.5 μM T_3 + 2.5 μM T_4	−	101 ± 1‡	13
1 μM T_3 + 1 μM T_4	−	108 ± 3	7
10 μM D-T_3§ + 2.5 μM T_4	−	115 ± 1†	1
10 μM T_3	+	77 ± 2†‡	34
1 μM T_3	+	89 ± 2‡	23
0.1 μM T_3	+	93 ± 1‡	20
10 μM T_4	+	76 ± 2†‡	34
1 μM T_4	+	89 ± 2‡	23
0.1 μM T_4	+	92 ± 1‡	21

* Each value represents the mean ± SE of quintuplicate cell cultures.
† Significantly different from vehicle/TCDD at $P < 0.01$.
‡ Significantly different from control values at $P < 0.01$.
§ Unless otherwise indicated, T_3 and T_4 are the L-forms.

containing 10^5 cells were added to 10 × 35 mm culture plates and incubated for 7 days. The plates were stained with methylene blue, and total colonies containing 40 or more cells were counted using a stereomicroscope. Cells were obtained from a single pool and assayed in quintuplicate. All experiments were replicated on a different day to confirm the results, although only one replicate is shown. The significance of each treatment effect was determined by establishing confidence levels from replicate values of historical controls. Progenitor cell numbers in bone marrow cultures never varied more than 4% from the mean of replicate cultures which resulted in differences greater than 8% being significant at the $P < 0.01$ value.

RESULTS

The number of CFU-Cs in murine bone marrow cells cultured in the presence of triiodothyronine (T_3) and/or thyroxine (T_4), with and without 10 nM TCDD, is shown in Table 1. This concentration of TCDD consistently inhibited CFU-C formation by 20–24% from control values. Neither T_3 nor T_4, alone, inhibited colony formation at the concentrations tested (up to 10 μM). In contrast, the combination of T_3 and T_4 (4 to 1 ratio) at micromolar concentrations suppressed CFU-C formation in a dose-dependent manner, being approximately 1/100 as effective as TCDD. This particular ratio of T_3 to T_4 was selected because other studies estimated that the intracellular ratios of T_3 and T_4 in various cell types was 4 to 1 [14]. Suppression of CFU-C formation was not nearly as effective when T_3 and T_4 concentrations were altered such that T_4 was equal to or in excess of T_3. Furthermore, this response was stereospecific since suppression does not occur when L-T_3 is replaced by the hormonally less active D-form [15]. Although T_3 or T_4 alone did not influence

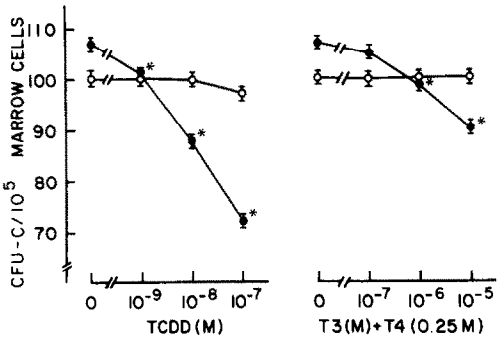

Fig. 1. CFU-C colony formation of bone marrow cells obtained from C57BL/6J mice congenic at the *Ah* locus cultured with either TCDD or thyroid hormones. Key: CFU-C colony formation in cultures of cells from *Ah* responsive C57B1/6 mice (●) and in cultures of cells from *Ah* nonresponsive C57B1/6 mice (○) treated with TCDD or thyroid hormone. Values which differ significantly from control at $P < 0.01$ are noted with an asterisk.

progenitor cell formation, 10 μM concentrations of either T_3 or T_4 were capable of enhancing myelotoxicity in the presence of TCDD (Table 1). To determine whether the myelotoxicity observed by supraphysiological levels of thyroid hormones was mediated through the *Ah* locus, bone marrow cells were utilized from C57BL/6J mice congenic at the *Ah* locus. Previous studies had shown that TCDD-induced myelotoxicity segregates with the *Ah*-locus and that the *Ah* receptor can be detected in bone marrow cell cytosol from *Ah* responsive (Ah^{bd} or Ah^{bb}) but not nonresponsive (Ah^{dd}) mice [1]. As shown in Fig. 1, bone marrow cells obtained from responsive mice (Ah^{bb}) were considerably more susceptible to suppression by TCDD, as well as to thyroid hormones, than cells from C57BL/6J non-

Table 2. Influence of NH_2-TriCDD on thydroid hormone-induced myelotoxicity*

Treatment	CFU-C/10^5 marrow cells	Percent suppression
Vehicle	116 ± 1	
10 nM TCDD	91 ± 2†	22
1 μM NH_2-TriCDD	111 ± 2	4
10 nM TCDD + 1 μM NH_2-TriCDD	114 ± 2	2
10 μM T_3 + 2.5 μM T_4	92 ± 2†	21
10 μM T_3 + 2.5 μM T_4 + 1 μM NH_2-TriCDD	111 ± 2	4
1 μM T_3 + 0.25 μM T_4	102 ± 2†	12
1 μM T_3 + 0.25 μM T_4 + 1 μM NH_2-TriCDD	111 ± 2	4

* NH_2-TriCDD was added to serum in *p*-dioxane and the serum was subsequently added to the culture medium so that the final concentration of *p*-dioxane did not exceed 0.001%. Other chemicals were added, and progenitor cell formation was determined. Data are presented as the mean ± SE of quintuplicate cell cultures.

† Significantly different from control at $P < 0.01$

responsive (Ah^{dd}) mice. While these data do not directly demonstrate that T_3 or T_4 binds to the *Ah* receptor, they do indicate that, like TCDD, thyroid hormone-induced myelotoxicity segregated with the *Ah* locus.

We recently reported that NH_2-TriCDD is a specific antagonist for TCDD induced myelotoxicity which competes with TCDD for binding to the *Ah* receptor [11]. To determine whether thyroid hormone-induced myelotoxicity, like that of TCDD, requires binding to the *Ah* receptor, we examined the combined effects of T_3 and T_4 with NH_2-TriCDD in bone marrow cell cultures. As shown in Table 2 and recently described in detail [11], addition of excess NH_2-TriCDD to the culture effectively blocked TCDD suppression of colony formation. Similarly, NH_2-TriCDD was also effective in blocking thyroid hormone-induced suppression of colony formation, suggesting that both TCDD and thyroid hormones competed with NH_2-TriCDD for binding to the receptor. These data add further support to the previously described studies with congenic mice which suggested that the myelotoxicity induced by TCDD or thyroid hormones is mediated by binding to the *Ah* receptor.

TBPA is a model for the thyroxine nuclear receptor [16]. Previous studies have demonstrated that compounds structurally related to TCDD, and presumably T_4, can compete for binding to TBPA [17]. If TCDD and thyroid hormones both recognize the same receptor, it might be expected that TBPA could bind both TCDD and thyroid hormones. Thus, the addition of exogenous TBPA into the culture system should decrease the availability of TCDD or thyroid hormones by serving as secondary sites of loss for binding to the operational receptor. As shown in Fig. 2, addition of exogenous TBPA to bone marrow cultures inhibited TCDD and hormone-induced myelotoxicity. That this competition is specific is suggested by the relatively low concentration of TBPA (100 nM) required to inhibit myelotoxicity and the lack of protection afforded by bovine serum albumin, presumably a low-affinity binding, and rat immunoglobulin which is not likely a transport protein for T_3 or T_4.

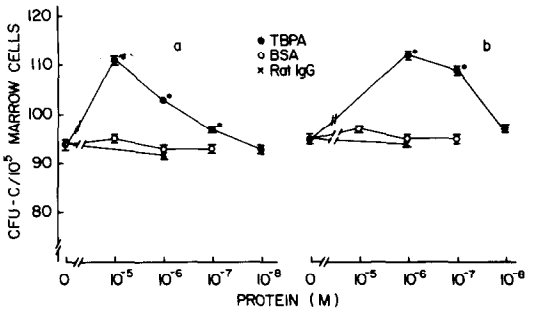

Fig. 2. CFU-C colony formation of bone marrow cells from B6C3F1 mice cultured in the presence of either 10 nM TCDD (Fig. 2a) or 10 μM T_3 plus 2.5 μM T_4 (Fig. 2b) with increasing concentrations of either TBPA, bovine serum albumin (BSA) or rat IgG. The number of CFU-C per 10^5 nucleated cells in control cultures (containing vehicles only) was 114 ± 2. Values which differ significantly from control at $P < 0.01$ are noted with an asterisk.

DISCUSSION

While it is generally agreed that many of the toxic manifestations associated with TCDD are initiated by binding to the *Ah* receptor, the regulation of this event and the mechanism(s) responsible for the various effects following nuclear translocation and gene expression have not been determined. Stimulated by the observation that TCDD and thyroid dysfunction cause similar nonspecific changes, including loss of body weight, bradycardia, alopecia, hirsutism, hyperkeratosis and altered levels of triglycerides and cholesterol [18], a number of investigators have noted various molecular and cellular relationships between thyroid hormone action and TCDD toxicity. While the structural resemblance between certain polychlorinated aromatic hydrocarbons and thyroid hormones was recognized early [19], molecular modeling studies have shown recently that T_4 and TCDD share common molecular properties which enable them to present a planar face and lateral halogens in interactions with proteins [7]. It has also been demonstrated that polychorinated biphenyls, which structurally resemble

TCDD, can compete with T_4 for binding to TBPA [17]. Cellular studies have provided additional support relating thyroid hormones and TCDD actions. Like TCDD, thyroid hormones at physiological concentrations induce hepatic mixed-function oxidase enzymes *in vitro*, whereas supraphysiological levels suppress these enzymes and hepatic P-450 content [20]. TCDD causes a dose-related depletion of serum and tissue T_4 levels in rats [9], although this may occur through increased biliary elimination of T_4 rather than decreased hormone production [21]. Recently, Rozman *et al.* [6], reported that chemical thyroidectomy effectively diminishes certain toxic responses of TCDD (e.g. body weight loss) whereas thyroidectomized-T_4 maintained rats are indistinguishable from TCDD-treated controls. Finally, the influence of thyroid hormones on cell functions, including immune responses, has been well documented [22, 23]. While physiological levels of thyroid hormones are required to maintain normal immune function, excessive amounts cause hyperplasia of lymphoid organs and immunosuppression similar to that seen with TCDD.

The present data provide several lines of evidence that thyroid hormones modulate TCDD myelotoxicity. Initially, it was observed that addition of T_3 or T_4 to cell cultures enhanced TCDD-induced myelotoxicity. Although the concentrations of T_3 and T_4 used in this study are 2 to 3 orders of magnitude greater than physiological levels, we were monitoring a toxic effect by thyroid hormones which should override normal control mechanisms. It would not be expected that physiological levels would be inhibitory in this system. Lamb *et al.* [10] have shown that co-administration to pregnant mice of exogenous T_3 or T_4 with TCDD also potentiates the induction of cleft palate in offspring when compared to incidences observed from separate administration of TCDD or thyroid hormones. Furthermore, differences were discernible between T_3 and T_4, which is consistent with their differences in hormonal activity. Although T_3 is normally 3- to 5-fold more biologically potent than T_4, we were not able to discern any differences in potency in the bone marrow until D-T_3 and L-T_3 were compared. The difference in hormonal activity between these two isomers is 10-fold [15], which is apparently large enough to discern in the bone marrow system (see Table 1). It was also demonstrated that myelotoxicity by combined T_3/T_4 treatment, like TCDD myelotoxicity, was inhibited by NH_2-TriCDD, an antagonist for *Ah* receptor binding. However, it should be noted that TCDD was several orders of magnitude more potent in inducing these responses than thyroid hormones. Finally, using congenic mice, we provided evidence that the ability of combined T_3/T_4 to induce myelotoxicity, like TCDD, segregates with the *Ah* locus.

Taken together, these observations suggest several possible hypotheses on the relationship between thyroid hormones and TCDD-mediated myelotoxicity. The first is that TCDD myelotoxicity is not dependent on thyroid hormone activity but, rather, is mediated by the binding of thyroid hormones to the *Ah* receptor. The ability of T_3 and/or T_4 to substitute for TCDD at supraphysiological concentrations is consistent with the similar molecular reactivity of thyroid hormones and TCDD [8]. This model also predicts that (1) there would be competition between thyroid hormones and TCDD at sites that are not "toxic" receptors and (2) administration of T_3 or T_4 would increase the availability of TCDD for binding to the *Ah* receptor. In this respect, addition of exogenous T_3 or T_4 would effectively compete with TCDD for binding to TBPA, thus increasing the availability of TCDD to bind to other sites.

Although the evidence is indirect, the results are also consistent with a second possibility in which multiple receptors are required for TCDD-induced myelotoxicity. In this respect, one of the authors (J.D.M.) has proposed a unifying hypothesis for TCDD and thyroid hormone interactions in which TCDD acts as a persistent thyroid hormone agonist [24]. This hypothesis advances a two-receptor model in which the planar aromatic system controls the initial binding to the *Ah* cytosolic receptor, and halogen substituents control subsequent nuclear events involving the nuclear thyroxine receptors. In this model, the combination and specific ratio of T_3 to T_4 (4 to 1) is thought to be important in maintaining the concentration gradient between the cytoplasm and the nucleus in order to maximize nuclear receptor occupancy and the associated biological response. Additional support for both of these models would be provided by the demonstration of thyroid hormone binding to the *Ah* receptor; however, workers have been unsuccessful, so far, in their attempts to displace [^{3}H]TCDD with unlabeled thyroxine competitor (W. F. Greenlee, personal communication, cited with permission).

A third hypothesis for the TCDD–thyroid hormone interactions is that the observed effects may be due to modulation of *Ah* receptor expression by hormone activity. For example, it has been shown that glucorcorticoid levels influence the binding capacity, as well as the binding affinity, of the nuclear T_3-binding site [25]. For that matter, synergistic effects have been demonstrated between thyroid hormones and glucocorticoids in the tadpole [26] and other species at dose levels that would suggest receptor modulation [27]. Synergistic interactions between TCDD and hydrocortisone also occur in the induction of cleft palate in mice [28]. In addition, there are a number of well-documented examples of the regulation of single and multiple clusters of genes through multiple hormonal interactions [29]. Thyroid hormones are involved in the ultimate expression of the target genes in several of these multihormonal regulating systems.

Regardless of the mechanism of action, these data provide evidence that thyroid hormones can induce and regulate toxicological processes associated with polyhalogenated aromatic hydrocarbons. Additional studies (e.g. using specific DNA probes) will be required to ascertain the direct relationship between hormone action and TCDD.

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