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2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN-INDUCED THYMIC ATROPHY AND LYMPHOCYTE STEM CELL ALTERATIONS BY MECHANISMS INDEPENDENT OF THE ESTROGEN RECEPTOR

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Abstract—2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) has both agonist and antagonist effects on estrogen-mediated activities and estrogen receptor (ER) levels in epithelial tissues following exposure. We previously demonstrated that TCDD alters bone marrow lymphocyte stem cells, including prothymocytes, as measured by functional assays and alterations in the lymphocyte stem cell-specific markers terminal deoxynucleotidyl transferase (TdT) and recombinase activating gene-1 (RAG-1). We have also shown that 17β-estradiol valerate (E_2V) affects lymphocyte stem cells by reducing TdT and RAG-1 mRNA. It has been suggested that the effect of TCDD on these lymphocyte stem cells may be mediated directly or indirectly through estrogenic action and/or the ER. Studies were designed to evaluate whether endogenous estrogens or the ER mediate TCDD-elicited bone marrow alterations and thymic atrophy. Ovariectomy did not alter the sensitivity of mice to TCDD-induced thymic atrophy or to a reduction in TdT biosynthesis in bone marrow cells compared with either intact or sham-operated mice. The pure estrogen antagonist ICI 164,384 blocked E_2V-induced uterine hypertrophy, thymic atrophy and reductions in lymphocyte stem cell markers. However, the antiestrogen failed to protect against TCDD-elicited thymic atrophy or bone marrow alterations in intact animals. The results are consistent with the hypothesis that the effects of TCDD on the thymus and/or bone marrow are mediated by mechanisms independent of estrogens or the ER.

Key words: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; thymic atrophy; lymphocyte stem cells; estrogen; estrogen receptor; estrogen antagonist

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD§) and estrogen are agents with well documented immunomodulatory properties [1, 2]. One of the primary targets of TCDD and estrogen immunotoxicity is the thymus [3, 4]. Although it has been demonstrated that TCDD-induced thymic atrophy is due to events initiated by TCDD binding to the *a*ryl *h*ydrocarbon receptor (AhR) [5], and, by analogy, it is likely that estrogen also elicits atrophy via the estrogen receptor (ER) [6], the actual direct cellular targets mediating these alterations are unknown. The ability of these agents to directly affect thymic epithelium [7–9] and/or thymocytes [9–11] has been reported. Recent studies have shown that alterations in the lymphoid-specific stem cell populations likely contribute to the thymic atrophy induced by TCDD and estrogens [12–14]. Normal T-cell development in the neonate or adult requires the migration of extrathymic lymphocyte stem cells from the fetal liver or bone marrow to the thymus [15, 16]. Donskoy and Goldschneider [17] recently reported that the bone marrow provides thymocyte precursor cells throughout postnatal life. Thus, agents that affect thymocyte stem cells could alter intrathymic T-cell development and the mature T-cell repertoire. The determination of the mechanism(s) whereby TCDD or estrogen causes this involution will likely lead to a greater understanding of the more subtle and longer term effects of these agents on the immune system.

Many similarities exist between the actions of TCDD and estrogens on the thymus. We observed that maximal atrophy induced by a single injection of TCDD or 17β-estradiol valerate (E_2V) was not attained in mice until at least 12 days following treatment, and this atrophy persisted for at least as long as 24 days [18]. In the normal thymus, thymocytes develop through a variety of stages from cells lacking the surface markers CD4 and CD8 ($CD4^-8^-$) to cells expressing both cell markers ($CD4^+8^+$). These $CD4^+8^+$ cells are the major immature intrathymic subpopulation and develop into the more mature single positive $CD4^+8^-$ or $CD4^-8^+$ cells that migrate to the periphery to compose the functionally mature T-cell repertoire

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§ Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; E_2V, 17β-estradiol valerate; ICI, ICI 164,384; TdT, terminal deoxynucleotidyl transferase; RAG-1 recombinase activating gene-1; AhR, *a*ryl *h*ydrocarbon receptor; ER, estrogen receptor; DES, diethylstilbestrol; OvX, ovariectomy; and 1× SSC, 0.15 M sodium chloride + 0.015 M sodium citrate.

[19]. Flow cytometric analysis of the thymocyte subpopulations at the time of maximal atrophy in TCDD- or E_2V-treated mice revealed that TCDD (30 μg/kg) reduces all thymocyte subpopulations [12, 13], whereas E_2V (75 mg/kg) results in a more selective loss of the immature $CD4^+8^+$ thymic subset [18]. However, responsive mouse strains exposed to TCDD at doses greater than 60 μg/kg [11, 20, 21], or to high dose (>50 mg/kg) estradiol administration [18, 22–24], display a preferential depletion of the $CD4^+8^+$ subset. We found that although a single injection of E_2V at a lower dose (5 mg/kg) can cause a similar degree of atrophy, the relative reduction of the $CD4^+8^+$ thymocyte subpopulation was less than observed at a higher dose (>50 mg/kg) [18]. These results imply that multiple cellular targets with differing dose dependencies may exist for TCDD and estrogen.

Bone marrow hypocellularity also occurs following *in vivo* exposure to either TCDD or estrogen [25–27]. This loss of bone marrow cells has been correlated with inhibition of hematopoiesis, including reductions in progenitor cells of the granulocyte-macrophage (CFU-GM) lineage and "pluripotent" cells (CFU-S) [26, 27]. More recently, our laboratories determined that TCDD markedly affects lymphocyte stem cells in bone marrow, including cells with prothymocyte activity at doses causing minimal or no effect on either CFU-GM or CFU-S [13, 28]. Bone marrow cell populations from TCDD-treated mice manifest reductions in the lymphocyte stem cell-specific enzyme terminal deoxynucleotidyl transferase (TdT), reduction of both TdT and recombinase activating gene-1 (RAG-1) mRNA expression, and a decreased ability to reconstitute the thymic compartment of irradiated syngeneic animals [12, 13, 18, 28]. Similarly, bone marrow cell populations from animals treated with E_2V were depleted significantly of populations expressing TdT and RAG-1, with reductions in TdT biosynthesis and TdT and RAG-1 mRNA. For both agents, the time course of thymic atrophy correlated with reductions in both TdT and RAG-1 in the bone marrow [18, 29].

The perinatal animal has been shown to be especially sensitive to the immunomodulatory effects of both TCDD and estrogen [30–33]. Fine *et al.* [12, 28] showed that exposure to TCDD *in utero* affects prethymic development, and reduces prothymocyte activity and lymphocyte precursors in bone marrow and fetal liver. Similarly, Holladay *et al.* [14] found that diethylstilbestrol (DES), a nonsteroidal estrogenic compound, reduces TdT-expressing cell populations and prothymocyte activity of fetal liver cells. Therefore, thymic atrophy induced by both TCDD and estrogen could be due, in part, to the reduced capacity of these lymphocyte stem cells to repopulate thymic tissue. This lymphocyte stem cell population might be the most sensitive and specific hematopoietic target for these two agents during fetal development.

The connection between the biologic actions of TCDD and estrogen was first noted by Kociba *et al.* [34] who found an increased incidence of liver cancer in rats exposed to TCDD. However, these animals were protected from spontaneously arising mammary and uterine tumors. More recently, Lucier *et al.* [35] observed the hepatic tumor promoting ability of TCDD to be much greater in intact than in ovariectomized animals. Umbreit and Gallo [36] suggested that TCDD and estrogen may act by similar mechanisms, because TCDD causes similar physiologic and pathologic responses as observed with hypo- and hyperestrogenemia. For example, TCDD causes a reduction of estrogen-induced uterine growth and development in the female reproductive system [37, 38]. However, TCDD does not bind directly to steroid hormone receptors [39, 40], unlike the estrogen antagonist ICI 164,384 (ICI), which has high affinity for the ligand binding site on the ER [41]. Thus, TCDD may exert tissue-specific estrogenic and antiestrogenic effects in other ways [36, 37], such that the effect of TCDD on the thymus and bone marrow-derived stem cells might be mediated directly or indirectly through estrogenic action and/or the estrogen receptor. In this paper we describe studies, using ovariectomized mice and the estrogen antagonist ICI, to determine the possible roles of estrogens and/or the ER in TCDD-induced thymic atrophy and alterations of lymphocyte stem cell populations in bone marrow.

MATERIALS AND METHODS

Animals. Normal, sham-operated and ovariectomized BALB/c mice were obtained from Charles River (Raleigh, NC) at 28 days of age. Mice were ovariectomized (OvX) or sham-operated (sham-OvX) when they were 23 days old. For antiestrogen studies, normal immature (22-day-old) female BALB/cJ mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were randomly assigned to treatment groups (3–6 mice/group), housed in cages containing Beta Chip™ hardwood bedding (Northeastern Products Corp., Warrensburg, NY), and given food and water *ad lib.* Animals were maintained at a constant temperature and humidity on a 12:12 hr light:dark cycle, and acclimated for 1 week prior to treatment.

Chemicals. TCDD (Cambridge Isotopes, Cambridge, MA) and β-estradiol-17-valerate (Sigma Chemical Co., St. Louis, MO), were diluted to an appropriate concentration in olive oil. ICI 164,384 [*N* - *n* - butyl - *N* - methyl - 11 - (3,17β - dihydroxy-oestra-1,3,5 - (10) - trien - 7α - yl)undecanamide], a gift of Dr. A. E. Wakeling (ICI Pharmaceuticals, England), was prepared in 95% ethanol and diluted in olive oil.

Animal treatment. For ovariectomy studies, normal, sham-operated, and ovariectomized mice, 5 weeks of age, received a single i.p. injection of either TCDD (30 μg/kg) or olive oil (vehicle-treated; 0.1 mL/0.02 kg). Mice were killed by CO_2 overdose 12 days after injection, thymus and hind leg bones were removed immediately, and thymus wet weight was determined. The dose and time of TCDD exposure have been found to be optimal for elicited thymic atrophy [18].

To determine whether antiestrogen reverses estrogen-induced uterine hypertrophy or thymic atrophy, two different doses of E_2V, 15 μg/kg or 5 mg/kg, respectively, were utilized in a similar

Table 1. E_2V dose-response experiment

E_2V* (μg/kg)	Rel. uterine wt†,‡ (mg/g body wt, wet wt)	Rel. thymus wt†,‡ (mg/g body wt, wet wt)
Control§	1.4 ± 0.5	3.2 ± 0.4
1.0§	1.7 ± 0.4	3.6 ± 0.7
5.0§	3.0 ± 1.3‖	2.9 ± 0.9
10§	2.1 ± 0.2‖	3.4 ± 0.5
15§	3.7 ± 2.0‖	3.1 ± 0.8
50§	4.3 ± 1.2‖	3.1 ± 0.4
200¶	—**	2.9 ± 0.2
1,000¶	—	2.0 ± 0.3‖
5,000¶	—	1.7 ± 0.4‖
75,000¶	—	1.7 ± 0.5‖
5,000††	—	1.1 ± 0.1‖
10,000††	—	1.2 ± 0.2‖
25,000††	—	1.4 ± 0.7‖
50,000††	—	1.1 ± 0.6‖
75,000††	—	1.1 ± 0.2‖

* Three separate experiments were performed to determine appropriate uterotrophic dose and/or thymic atrophy dose.

† N = 3–6 mice per dose group; mice were injected s.c. at 30–36 days of age.

‡ Individual uterine and thymus weights from each mouse were divided by the body weight of each mouse to determine relative organ weight. Values are expressed as means ± SD.

§ Thymus examined 4 days after injection.

‖ Significantly different ($P < 0.05$) from control, as determined by ANOVA and Student's *t*-test.

¶ Thymus examined 6 days after injection.

** Not determined.

†† Thymus examined 12 days after injection.

dosing protocol. The dose of 15 μg/kg E_2V induced an approximate 1.5- to 3-fold increase in relative uterine weight, with little or no observed decrease in thymus weight after 4 days. Preliminary evidence showed 1 mg/kg E_2V caused statistically significant thymic atrophy after 6 days, but not after 12 days (data not shown), whereas 5 mg/kg E_2V reduced relative thymus weights by approximately 50% after 6 days and 70% by 12 days (Table 1). Thus, in the studies described, the lower E_2V dose (15 μg/kg) was used to examine the effect of ICI on uterine weights and the higher dose (5 mg/kg) was used to examine the effect of ICI on thymic atrophy. Normal, 4-week-old, female BALB/cJ mice received either a single s.c. injection of E_2V (15 μg/kg or 5 mg/kg) or ICI (7.5 mg/kg) or a single i.p. injection of TCDD (30 μg/kg) (day 0). Control (vehicle-treated) mice received olive oil (0.1 mL/0.02 kg) alone by s.c. or i.p. injection. Also beginning on day 0, and subsequently on days 1 through 5, all mice received an additional s.c. dose of either olive oil (0.1 mL/0.02 kg) or ICI (7.5 mg/kg). The dose of ICI used in our studies was based upon the studies of Wakeling and Bowler [42]. Animals were killed by CO_2 overdose on day 6 of the experiment, and thymus, uterus, and hind leg bones were removed immediately. Thymic and uterine wet weights were determined and normalized to animal body weight, and the averaged control values were then taken as 100%. No exceptional animal weight gain or loss, or visible behavioral alterations were observed in any of the treatment groups relative to controls in either the ovariectomy or antiestrogen studies.

The doses of estrogen used in these experiments were designed to induce either uterine hypertrophy or thymic atrophy. The estrogen dose used to induce thymic atrophy in our studies has been shown to raise blood estrogen levels approximately 10- to 20-fold higher than fluctuating physiologic levels in a pregnant mouse 1 day following administration [43, 44]. However, by days 10–14 post-administration (the point at which estrogen-induced maximal atrophy is reached [18, 29]), blood estrogen levels are approximately equivalent to normal maximal proestrous levels [43, 44].

Cell isolation. Thymuses were removed and dissected free of nodes and blood vessels, and the individual thymuses were weighed and pooled. Thymocytes were released by mashing the pooled organs in a 100 × 60 mm petri dish with a sterile 10 mL plastic syringe plunger in cold Minimum Essential Medium (MEM) with Hanks' Salts (GIBCO, Grand Island, NY) containing 5% fetal bovine serum (FBS) (Sigma) until organs were completely dissociated. Tissue was triturated and the debris was eliminated by passing the cell suspension through 75-gauge Nylon mesh. To obtain bone marrow cells, femur and tibia were dissociated free of attached tissues, both ends of the bone were cut at the epiphysis, and the shafts were flushed with MEM + 5% FBS. Cells were pelleted and then incubated with ACK red cell lysis buffer (0.17 MNH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA) for 2 min to remove contaminating red blood cells from the bone marrow cell suspensions. MEM + 5% FBS was added, and then leukocytes were pelleted by centrifugation, washed once, and resuspended with MEM + 5% FBS for cell counting. Cell yield and viability were determined by enumeration of multiple samples of pooled cells in each group by trypan blue dye (0.08%) exclusion and light microscopy (400×, Neubaur hemocytometer). Viability was greater than 90% in all experimental samples, and the standard deviation was less than 5%.

TdT protein biosynthesis analyses. Metabolic synthesis of TdT protein in bone marrow cells and thymocytes was evaluated by immunoprecipitation as previously described [12, 13]. Briefly, 1–2 × 10^7 thymocytes or bone marrow cells were washed with calcium- and magnesium-free HBSS (GIBCO), resuspended in 2.0 mL MEM (without methionine) with Earle's Balanced Salt Solution and supplemented with 10% dialyzed FBS, 50 μM β-mercaptoethanol (Sigma), 2 mM L-glutamine (GIBCO), 1 mM sodium pyruvate (GIBCO), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma). [^{35}S]-Methionine (ICN Biomedicals, Inc., Irvine, CA) (100 μCi/10^7 cells) was added to cell cultures that were incubated for 2.5 to 3 hr at 37° and 5% CO_2. The nonadherent cells were recovered and washed with calcium- and magnesium-free HBSS, and the cell pellets were frozen and stored at −80° until analysis. Cells were lysed and ultra-centrifuged, and the supernatants were precipitated with an excess of rabbit anti-calf TdT polyclonal antiserum which was

shown to cross-react with mouse TdT (a gift of M. S. Coleman, University of North Carolina, Chapel Hill, NC). Actin was co-precipitated by anti-actin antibodies that normally occur in the serum. Protein was precipitated from an equal amount of biosynthesized protein as indicated by equal amounts of [^{35}S]methionine label incorporated into acid-precipitable material. Immunoprecipitates were analyzed by separation on SDS–PAGE gels and autoradiography. Quantitative values were obtained by scanning densitometry by "Pict file" analysis using a Microtek Color/Grey Scanner and the Adobe Photoshop computer program (Adobe Systems, Inc., Mountain View, CA) run on a Macintosh IIci computer to import the autoradiogram image. The "NIH Image" software program (NIH Research Service Branch, NIH, Bethesda, MD) was used to quantify mean band densities and areas. Scanning was done at 200 dpi resolution and 150% scaling. The amount of TdT (60 kDa) was normalized to the amount of actin (45 kDa), and the averaged control values for a particular exposure determined to be in the linear range were then taken as 100%. Analyses were performed in duplicate, and all values, normalized to controls, were within 20% of each other.

TdT and RAG-1 RNA analyses. Total cellular RNA was isolated from bone marrow cells and thymocytes using the guanidinium thiocyanate method of Chomczynski and Sacchi [45] and quantified by optical density at 260 nm. Samples (5 μg) of thymocyte RNA and samples of bone marrow RNA (20 μg) were separated by size in 1% agarose-formaldehyde 14 cm horizontal gels, alkaline fragmented, and transferred to "Magnagraph" Nylon membranes (MSI, Westborough, MA) using a Bio-Rad Transblot Apparatus (Mountain View, CA) and Tris–acetate buffer. RNA was bound to the membrane by vacuum oven baking or in some cases by UV cross-linking.

TdT and RAG-1 cDNA probes were excised from plasmid DNA as previously described [12, 46, 47]. cDNA inserts were ^{32}P-labeled by random oligonucleotide priming using the Amersham "Multiprime DNA Labeling System" (Amersham, Arlington, IL) and [^{32}P]dCTP (NEN, Boston, MA). The murine pTdT 20 probe [46] and the murine pRAG-1 [47] (gift of D. Schatz, Yale University, New Haven, CT) were labeled to equal levels, and blots were probed simultaneously for both TdT mRNA (2.2 kb) and RAG-1 mRNA (6.1 kb) using equal amounts of input radioactivity in a Hybrid Minihybridization System Oven (National Labnet, Woodbridge, NJ) at 65° overnight. Nylon membrane blots were washed twice at 65° for 5 min with 2× SSC and once for 30–40 min at the same temperature in 0.1% SDS and 0.1× SSC. Autoradiograms were obtained by serially exposing the probed filter at −70° to obtain exposures within the linear range of the film (from 1 day to 3 weeks). After development of the autoradiograms, the RAG-1 and TdT probes were removed by stripping according to the manufacturer's directions (Magnagraph, MSI), except at 70° instead of the recommended 65°. Blots were then reprobed for β-actin (2.0 kb) (probe a gift of Dr. J. Hamlin, University of Virginia; originally from Dr. P. Aponte, Stanford University). RAG-1, TdT, and actin autoradiograms were quantified as described above, and compared values were normalized to the average of the control values. RNA analyses were performed in triplicate, and all values, normalized to controls, were within 20% of each other.

Statistics. All experiments were performed wholly or in part at least twice, and the most comprehensive data set for each study is shown. Although there was some variation (<30%) of both the control and experimental data between experiments, the same trend, e.g. no effect of ICI treatment on TCDD-induced thymic atrophy, was consistent. The statistical difference between control and treatment groups was determined using a randomized complete block analysis of variance (ANOVA). Student's *t*-test was used to evaluate differences between individual E_2V or TCDD treatment groups to their

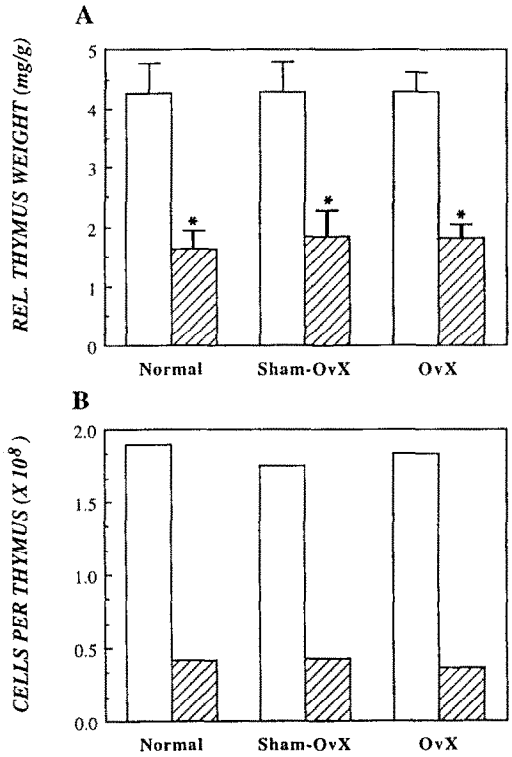

Fig. 1. Effect of TCDD on thymus atrophy in normal, sham-operated and ovariectomized mice. Thymuses were removed from TCDD-treated (▨) and vehicle-treated (□) animals 12 days after treatment; the relative weights and total cellularity per thymus were determined as described in Materials and Methods. (A) Relative thymus weights (mg/g) of vehicle-treated controls were 4.3 ± 0.5 for OvX (N = 5), 4.3 ± 0.5 for sham-OvX (N = 5), and 4.3 ± 0.3 for normal mice (N = 5). Values for relative thymus weights are presented as means ± SD. (B) Average thymus cellularity values of pooled vehicle-treated control mice were: 1.9×10^8 (OvX), 1.8×10^8 (sham-OvX), and 1.8×10^8 (normal). The numbers of TCDD-treated mice in this experiment were: OvX (N = 7), sham-OvX (N = 8), and normal (N = 8). Key: (*) significantly different ($P < 0.05$) from control, as determined by ANOVA and Student's *t*-test.

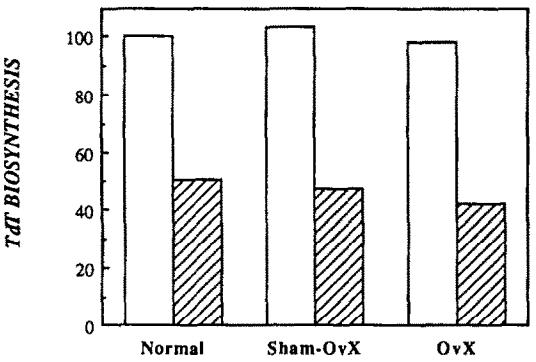

Fig. 2. Effect of TCDD on TdT biosynthesis in bone marrow from OvX, sham-OvX and normal mice. Bone marrow cells were isolated, pooled and assayed in duplicate by immunoprecipitation for [^{35}S]methionine-labeled TdT protein, as described in Materials and Methods. The data are presented as a percent of control (vehicle-treated) (□) for OvX, sham-Ovx, and normal mice (N = 5 for vehicle-treated and 7 or 8 for TCDD-treated (▨) groups). Normalized bone marrow TdT/actin ratios for vehicle-treated controls were 0.600 for OvX, 0.630 for sham-OvX, and 0.610 for normal mice.

respective vehicle-treated controls. Results were considered statistically significant at $P < 0.05$.

RESULTS

Effect of ovariectomy on TCDD-induced thymic atrophy. To determine the influence of endogenous estrogens on TCDD-induced thymic atrophy, we used prepubertal mice that were ovariectomized at 23 days of age. Mice were examined 12 days after administration of a single i.p. injection of 30 μg/kg TCDD. Surgical removal of the ovaries from immature female mice or sham-operation did not affect either thymus weights or cellularity (Fig. 1). Following TCDD treatment, however, significant thymic atrophy occurred equivalently in the groups of normal, sham-OvX, and OvX mice. This atrophy was manifest as a greater than 60% reduction in thymus weight and a greater than 75% decrease in thymus cellularity compared with respective vehicle-treated controls (Fig. 1). TCDD-induced thymus atrophy in normal, sham-OvX, and OvX groups was correlated with at least a 50% reduction (as compared with control) of TdT biosynthesis capacity in bone marrow cells isolated from these mice (Fig. 2). However, little or no loss of TdT biosynthesis in thymocytes from the treated mice was observed (data not shown). The apparent greater sensitivity of the marrow cells as compared with thymocytes, at least as determined by TdT biosynthesis, is consistent with our previous studies [12, 13, 29].

Effect of antiestrogen on uterine hypertrophy and thymic atrophy. Although the above ovariectomy study suggests that endogenous estrogens do not affect or mediate the action of TCDD on the thymus and/or bone marrow, it is possible that extragonadal estrogens [48] may mediate TCDD's effects. It is also possible that TCDD treatment may by some

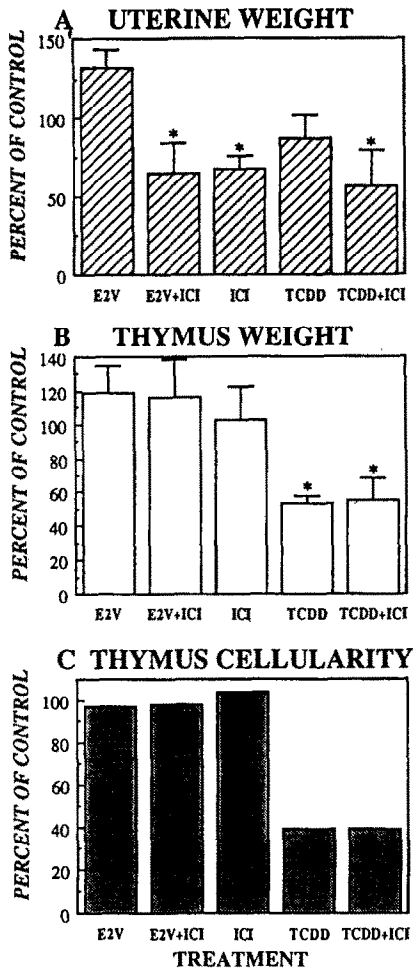

Fig. 3. Effects of E_2V (15 μg/kg), TCDD and ICI on uterus and thymus. Normal, 4-week-old, female BALB/cJ mice received either a single s.c. injection of E_2V (15 μg/kg) or ICI (7.5 mg/kg) or a single i.p. injection of TCDD (30 μg/kg) (day 0). Control (vehicle-treated) mice received olive oil (0.1 mL/0.02 kg) alone by s.c. or i.p. injection. Also beginning on day 0, and subsequently on days 1 through 5, all mice received an additional sc dose of either olive oil (0.1 mL/0.02 kg) or ICI (7.5 mg/kg). Animals were killed by CO_2 asphyxiation at day 6 and the following parameters determined: (A) relative uterine weight (mg/g), (B) relative thymus weight (mg/g), and (C) pooled thymus cellularity. The data are presented as percent of control (vehicle-treated) equivalent to the means ± SD. The absolute relative uterine weight for E_2V and TCDD control groups was 0.98 ± 0.22 (N = 3–5 animals for each group). Absolute relative thymus weights for E_2V and TCDD control groups were 4.13 ± 0.45 and 4.48 ± 0.42, respectively (N = 3–5 animals for each group). The average absolute number of cells per thymus for E_2V and TCDD control groups was 1.92×10^8 and 2.16×10^8 (N = 3–5 animals for each group). Key: (*) significantly different ($P < 0.05$) from control, as determined by ANOVA and Student's *t*-test.

mechanism increase the functional activity of the ER despite substantially lower endogenous levels of estrogen. To test these possibilities, we examined the ability of the specific estrogen antagonist, ICI,

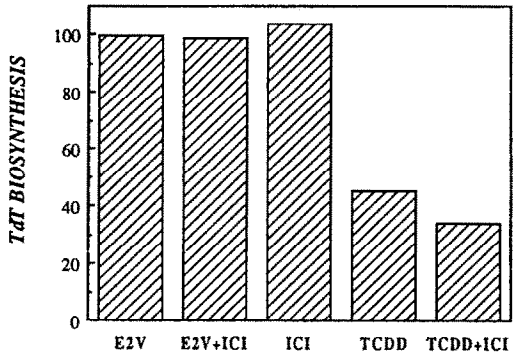

Fig. 4. Effects of E_2V (15 μg/kg), TCDD and ICI on TdT biosynthesis in bone marrow cells. Animals were treated as described in the legend of Fig. 3. Bone marrow cells were isolated, pooled and assayed in duplicate by immunoprecipitation for [^{35}S]methionine-labeled TdT protein, as described in Materials and Methods. The data presented here are from a single experiment and are representative of four different experiments where ICI was used in conjunction with E_2V or TCDD. The values are presented as a percent of control (vehicle-treated) for each of the treatment groups (N = 3–5 animals for each group). Normalized bone marrow TdT/actin ratios for vehicle-treated controls were 0.612 for the E_2V group and 0.573 for the TCDD group.

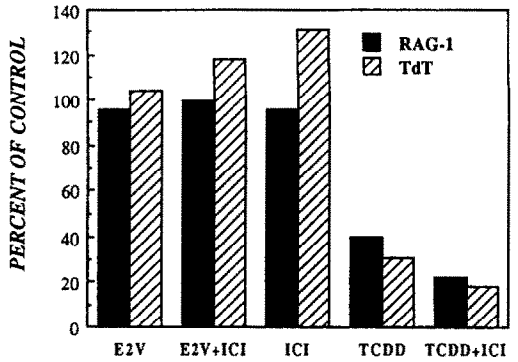

Fig. 5. Effects of E_2V (15 μg/kg), TCDD and ICI on TdT and RAG-1 mRNA expression in bone marrow. Animals were treated as described in the legend of Fig. 3. Bone marrow cells were isolated and pooled, and RNA was extracted and analyzed in triplicate with specific cDNA probes for TdT and RAG-1, as described in Materials and Methods. The data presented here are from a single experiment (the same as in Fig. 4) and are representative of four different experiments where ICI was used in conjunction with E_2V or TCDD. The values are presented as a percent of control (vehicle-treated) for respective treatment groups (N = 3–5 animals for each group). Normalized bone marrow TdT/actin and RAG-1/actin ratios for vehicle-treated controls were 1.047 (TdT) and 0.808 (RAG-1) for E_2V-treated, and 1.392 (TdT) and 0.847 (RAG-1) for TCDD-treated mice.

to block TCDD- and/or E_2V-induced uterotrophic changes or thymic atrophy. Due to limitations on the amount of ICI that could be obtained, it was first necessary to precisely define a minimal dosing regimen that could effectively block both E_2V-induced uterotrophic effects and thymic atrophy in immature female BALB/cJ mice. We determined our ICI dosing protocol from that described by Wakeling and Bowler [42].

As a positive control it was necessary to demonstrate the efficacy of the ICI dosing regimen to inhibit an estrogenic response. Initially, an E_2V dose–response study was performed to determine the minimal dose required to induce an increase in uterine weight, but not thymic atrophy (see Table 1). The E_2V dose of 15 μg/kg was selected for use in further experiments requiring an E_2V-induced uterotrophic/no-atrophy response. Although variable, this dose typically increased relative uterine weight by 130–260% within 4–6 days post-injection, with little or no effect on thymus weight compared with control. This dose of E_2V was administered either alone, or in combination with the estrogen antagonist, ICI, to normal adolescent mice (4 weeks of age). E_2V increased the relative uterine weight in the experiment shown (Fig. 3) to 130% of vehicle-treated control mice, without any significant reduction of thymus weight or cellularity. ICI treatment alone reduced relative uterine weight by 40%, but had no significant effect on relative thymic weight or cellularity. ICI also significantly reduced the E_2V-induced increase in relative uterine weight by greater than 50%. The expression of lymphoid-specific stem cell markers, TdT and RAG-1, in the bone marrow of these animals was not decreased by this dose of E_2V, ICI, or E_2V + ICI (Figs. 4 and 5), consistent with the lack of a significant effect on thymic weight.

The specific antiestrogenic effect of ICI on E_2V-elicited thymic atrophy was demonstrated further in another study. A single s.c. injection of 5 mg/kg E_2V caused a similar amount of thymic atrophy within 12 days (Table 1), and reduced bone marrow TdT and RAG-1 gene expression, as have been observed with multiple or single higher doses of E_2V [18, 22–24]. A comparable dose of E_2V has been shown to raise estrogen blood levels 1 day post-injection approximately 10- to 20-fold higher than normal fluctuating physiologic levels found in a pregnant mouse [43, 44]. However, 10–14 days post-administration (the point at which maximal thymic atrophy is observed) blood estrogen levels are approximately equivalent to normal maximal proestrous levels [43, 44]. ICI reversed the atrophy induced by E_2V (Fig. 6, A and B). In addition, ICI, in this protocol, also inhibited the E_2V-induced reduction of bone marrow TdT and RAG-1 mRNA expression (as compared with vehicle-treated control animals, Fig. 6C). These experiments strongly support the hypothesis that E_2V causes thymic atrophy and affects bone marrow lymphocyte stem cells directly through the ER.

Effect of ICI on TCDD-induced thymic atrophy. Finally, we examined whether TCDD could induce thymus atrophy or reduce bone marrow TdT biosynthesis, or TdT and RAG-1 mRNA expression

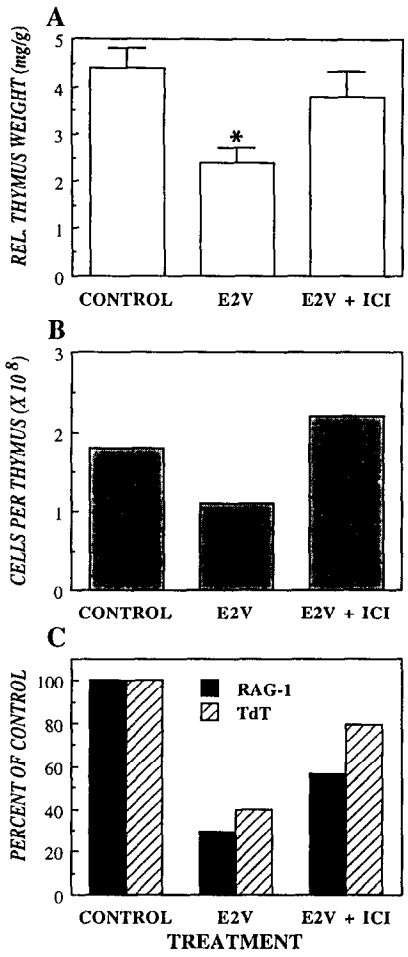

Fig. 6. Effects of E_2V (5 mg/kg) and ICI on (A) relative thymus weight, (B) pooled thymus cellularity, and (C) bone marrow TdT and RAG-1 mRNA expression. Normal, 4-week-old, female BALB/cJ mice received either a single s.c. injection of E_2V (5 mg/kg) or ICI (7.5 mg/kg) or a single i.p. injection of TCDD (30 μg/kg) (day 0). Control (vehicle-treated) mice received olive oil (0.1 mL/0.02 kg) alone by s.c. or i.p. injection. Also beginning on day 0, and subsequently on days 1 through 5, all mice received an additional sc dose of either olive oil (0.1 mL/0.02 kg) or ICI (7.5 mg/kg). Animals were killed by CO_2 asphyxiation at day 6. The data presented here are from a single experiment. The graphs are expressed as actual numbers (means ± SD) or as percent of control (vehicle-treated) for vehicle-treated and treatment groups (N = 6 animals for each group). Key: (*) significantly different ($P < 0.05$) from control, as determined by ANOVA and Student's *t*-test. Normalized bone marrow TdT/actin and RAG-1/actin ratios for vehicle-treated control mice were 1.276 and 0.583, respectively.

in the presence of the estrogen antagonist ICI. The thymic atrophy inducing dose of TCDD (30 μg/kg) [which produced a degree of atrophy equivalent to that of 5 mg E_2V/kg (Table 1)] was administered to mice and followed by daily doses of ICI or vehicle for 6 days. TCDD alone caused a slight reduction in relative uterine weight (20%), whereas TCDD in combination with ICI caused an additional reduction in relative uterine weight to 50% of control values (Fig. 3A). TCDD also significantly reduced the relative thymus weight by greater than 45%, and thymus cellularity by greater than 60% compared with control (Fig. 3, B and C). However, ICI did not inhibit TCDD-induced thymus atrophy. Furthermore, the atrophy induced by TCDD correlated with a reduction in bone marrow TdT biosynthesis (>55%) and TdT and RAG-1 (>60%) gene expression whether given alone or in combination with ICI (Figs. 4 and 5). Thus, ICI blocked the tissue-specific uterotrophic response (Fig. 3) and thymic atrophy elicited by E_2V (Fig. 6), but had no effect on TCDD-induced thymic atrophy or reduction of the bone marrow parameters that could contribute to thymic atrophy. These results strongly suggest that TCDD does not act through the ER to induce thymic atrophy.

DISCUSSION

TCDD has both estrogenic and antiestrogenic properties that affect a variety of estrogen-responsive tissues [36]. It has been known for many years that the thymus and T-cell-mediated immune functions in animals are influenced by endogenous or exogenous estrogens [3, 32, 49]. Since both TCDD and estrogen cause thymic atrophy in animals, we examined the possibility that TCDD acts through an immuno-endocrine axis to cause this pathologic change. The present study revealed that the development of thymic atrophy in mice administered TCDD is not mediated through estrogenic effects on thymus or bone marrow. Ovariectomy had no effect on the ability of TCDD to elicit either thymic atrophy or alterations in bone marrow TdT activity. Furthermore, the estrogen antagonist ICI was able to block E_2V-induced uterotrophic effects and thymic atrophy, but had no effect on TCDD-elicited alterations in the thymus and bone marrow. Thus, TCDD causes thymic atrophy and changes in lymphoid stem cell populations regardless of the estrogen status of the mice.

Our studies with E_2V and ICI show that the estrogenic effects upon hormonally responsive tissues or cells are mediated via the ER. ICI has been shown to act as a pure estrogen antagonist. That is, ICI is devoid of any measurable estrogenic activity upon binding to the ER [41, 50]. The induction of uterine hypertrophy by E_2V in our studies was reduced with ICI treatment, as previously shown by Wakeling and Bowler [42]. The dose of E_2V used in our experiments to induce uterine hypertrophy did not cause thymic atrophy or reduce TdT biosynthesis or TdT and RAG-1 mRNA expression in the bone marrow. ICI alone had no significant effect on any of these parameters. However, previous experiments have shown that an E_2V dose (75 mg/kg) that causes alterations in thymus weight, cellularity, and cell phenotype also reduces these bone marrow parameters [18]. In our present study, a lower dose of E_2V (5 mg/kg) was utilized (which induced an equivalent amount of thymic atrophy compared with 75 mg/kg E_2V), and ICI effectively reversed not only thymic atrophy, but also the E_2V-elicited reduction of TdT and RAG-1 expression in bone

marrow. The inhibition of E_2V-induced TdT or RAG-1 mRNA depletion by ICI could be due to direct effects on transcriptional regulation of the messages [51], or, as we prefer to suggest, the inhibition of cell differentiation, proliferation or cell death induced by E_2V treatment. This is the first study to show that ICI can inhibit estrogen-induced thymic atrophy and lymphocyte stem cell alterations. Earlier work utilized the partial estrogen agonist and antagonist, tamoxifen, to block estrogen-induced thymus atrophy, bone marrow hypocellularity, and reduction of CFU-S activity [52, 53]. These findings support our earlier work indicating that estrogen acts on distinct molecular targets through the ER to cause bone marrow alterations that may be related to thymic atrophy [18].

TCDD causes thymic atrophy and this atrophy is associated with a reduction of bone marrow TdT synthesis, TdT and RAG-1 mRNA expression, and a decreased capacity of these cells to repopulate the thymus compartment of irradiated syngeneic animals [12, 13, 28]. Genetic evidence supports the concept that the binding of TCDD to the Ah receptor initiates events that lead to thymic atrophy [5]. Furthermore, the TCDD-elicited alterations in bone marrow TdT and RAG-1 appear to be dependent on the Ah receptor [54]. Similarly, work by other investigators [52, 53, 55] and the present data also support the concept that estrogen binds to the ER to initiate events leading to thymic atrophy and bone marrow alterations. However, TCDD does not bind to the ER [40], nor do steroids bind to the AhR [56]. Furthermore, because ICI specifically binds to the ER to inhibit estrogenic action, any direct or indirect action of estrogen in TCDD-induced thymic atrophy or bone marrow alterations should have been inhibited. Thus, although E_2V and TCDD induce similar alterations in bone marrow and thymus, they apparently do so through different mechanisms, at least at the receptor level.

TCDD and E_2V cause thymic atrophy with similar kinetics, although E_2V has a more selective effect on the $CD4^+8^+$ population in the thymus [18]. Both agents cause thymic atrophy by a slow developing but ultimately persistent reduction in thymic weight and cellularity. We have postulated that the atrophy induced by these agents is due in part to their alterations of bone marrow-derived lymphocyte stem cells [12, 13, 29]. The lymphoid-specific stem cell markers, TdT and RAG-1, expressed by these bone marrow cells, represent a common target for both TCDD and E_2V. In addition, we have observed in the present and additional [18] studies that the TCDD or E_2V doses that cause reductions in these lymphoid stem cell markers are those that also induce thymic atrophy. These results imply that the actions of TCDD and E_2V are specific for TdT and RAG-1 expressing cells, and this is consistent with the ability of these compounds to affect lymphocyte stem cell development. Whether this is due to direct or indirect killing of stem cells or their precursors, inhibition of precursor differentiation, or an unusual specific effect on those genes involved in recombinase activities has yet to be determined. Although TdT and RAG-1 have been suggested to be in identical populations in the adult thymus [57, 58], we have observed recently that SCID (severe combined immunodeficiency) mice can have normal levels of TdT mRNA on a per cell basis in their atrophied thymuses, but have significantly reduced levels of RAG-1 compared with normal and heterozygous littermates.* Furthermore, it has been well established that during B-cell differentiation cells involved in the rearrangement of immunoglobulin light chain elements lack TdT [59]. These observations indicate that the RAG-1 and TdT gene products are independently regulated and can be found in different cell populations. To date, the effects of estrogens on functional prothymocyte activity have not been examined, as they have for TCDD [12, 13]. However, Holladay *et al.* [14] have reported recently that the non-steroidal synthetic estrogen DES affects prothymocyte activity in a dose-dependent manner. We interpret this and our current results as evidence for estrogen acting in the bone marrow to contribute to thymus atrophy.

In summary, as we have reported elsewhere [18], E_2V exerts effects upon bone marrow cells that are quite similar to TCDD. However, we report here that TCDD-induced thymic atrophy and bone marrow effects are not influenced by estrogenic effects or the ER. These results are also consistent with the interpretation that TCDD and estrogen treatment, through their individual intracellular receptors, initiate events leading to alterations in the bone marrow-derived lymphocyte stem cells responsible for colonizing the thymus, which could result in reduced thymic seeding and contribute to the development of thymic atrophy.

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