2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) Affects Thymocyte Development in Multiple Ways without Apoptosis

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ABSTRACT: Many mechanisms have been proposed to explain the induction of thymic atrophy by TCDD and related PHAHs. We have obtained considerable evidence that apoptosis is **not** a major mechanism in TCDD induced atrophy, while inhibition of thymocyte development in the earliest stages is a more likely mechanism. We also have obtained evidence that TCDD activation of the AhR in epithelial part of the stromal components of the immune system **does not** play a major role in atrophy induction but rather atrophy must be due to direct activation of the AhR in developing hemopoietic tissues.

INTRODUCTION: One of the effects of TCDD and related PHAHs on the immune system that is mediated through the Ah Receptor (AhR) is the induction of thymic atrophy at levels well below that associated with lethal toxicity[1-3]. While atrophy inducing levels of these chemicals are often greater than those associated with suppression of the formation of specific plaque-forming cells in the primary immune response[3-5], they still are one of the more accurately quantifiable immune system effects of these agents. In some rat strains, for example, thymic atrophy is observed at doses in which effects on the SRBC PFC assay are not detectable[6]. However, despite a large number of studies and hypothesis, the mechanism(s) by which TCDD and related compounds induce thymic atrophy is still in dispute.

Some have suggested that thymic atrophy is due to a direct effect on thymocytes by inducing apoptosis or sensitizing thymocytes to apoptosis[7-9]. Others have suggested that epithelial stromal elements in the thymus are induced by TCDD to express or suppress production of soluble factors or surface co-receptors that are involved in thymic development or proliferation, or cell death[10-12]. We and other groups have argued that reduction of extrathymic precursor cells or inhibition of stem cell development are decisive in atrophy induction[12-16]. Still another group has suggested that TCDD induces an acceleration of thymic development, resulting in a smaller thymus by disturbing the equilibrium between thymocyte formation and maturation and emigration[17,18]. We have examined several of these hypotheses, and conclude that thymic atrophy induction by TCDD is mainly due to inhibition of development in the prethymic and early intrathymic stem cell compartment, primarily

mediated by activation of the AhR in hemopoietic cells, with neglible contributions from AhR activation of genes in epithelial components of thymic and bone marrow stroma.

EXPERIMENTAL METHODS: C3HeN (Taconic Labs, NY) and C3HeN bcl-2 transgenic mice[19] were bred from founder mice provided as a gift by Dr. S.J. Korsmeyer (Washington Univ. Mo.); RAG-KO mice and SCID mice on C57 Bl6 backgrounds were purchased from Jackson Labs (Me.). AhR-KO mice(B6 x129Sv, Ly5.2) and genetically matched AhR^{bb} controls[20] were bred from founders provided by Drs. F.J. Gonzalez and P. Fernandez- Salguero (NCI, NIH, Md). C57Bl/6J Ly5.1 congenics were maintained in our own colony. Chimeric B6Ly5.1 mice containing hemopoietic systems lacking the AhR were constructed by lethally irradiating the B6Ly5.1 AhR^{bb} strain, injecting, i.v., 10^6 bone marrow cells from the AhR-KO mouse, and waiting 4 weeks before TCDD treatment. The absolute reconstitution by the KO mouse or a positive genetically matched animal was verified by phenotyping lymphocytes for Ly5.2 ($CD45^b$). TCDD was administered by I.P. injection in olive oil. Fetal thymic organ cultures (FTOCs) were established as previously described[21], and treated with TCDD dissolved in 1,4-dioxane at concentrations of 0.5-10nM. Thymii were weighed to 0.1 ± 0.1 mg accuracy. Cell suspensions were prepared as previously described and 3 color immunofluorescent analysis was carried out with commercial antibodies on a BD Facstar Plus using the LySySII acquisition programs. Analysis was also done in the LySyS II program[22].

The TUNEL assay using the ONCOR (MD.) ApopTag plus kit followed the manufacturer's protocol, but the cells were not washed with detergent at the end of the procedure to avoid excessive cell lysis. The Pulse Field Gel electrophoresis (PFGE) assay to quantitate double strand DNA breaks in small numbers of cells has been described[23], and was used to supplement the other two assays by electrophoreses of DNA from 10,000 cells through 1% agarose at 60V, with a pulse time of 300s. DNA was quantitated by transfer to nylon membranes, hybridization against ^{32}P-labelled total mouse genomic DNA, and quantitation using a Phosphor Imager.

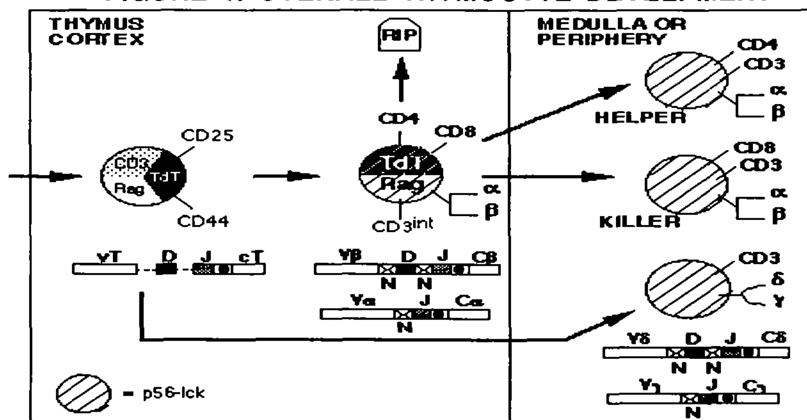
RESULTS AND DISCUSSION

TCDD does not cause thymic atrophy by apoptosis.

Apoptosis has been confirmed as a process in normal thymocyte development[24]. Cells in the CD4+CD8+(DP) set undergo apoptotic cell death either during negative selection, or by failure to be positively selected (Figure 1). An *in vitro* study by McConkey proposed that TCDD activated a calcium dependent apoptosis mechanism that lead directly to apoptosis[7]. Although the dose levels in these studies were considerably higher than those observed by us to cause atrophy in the thymus *in vivo*,[13,14] it was possible that *in vitro* sensitivity is altered as it is in many other cases. However, we could not detect apoptosis in even 1% of cells using the standard oligonucleosome DNA fragmentation assay from 2 days after treatment of animals with TCDD, although we could easily detect such fragmentation with corticosteroids. We had also established that atrophy did not begin to occur in young adult mice until 3 days after exposure, despite the presence of maximal levels of TCDD[22]. Recently,

another group has claimed that they could detect an increase of apoptosis in thymocytes in B6 mice exposed to TCDD 12 hrs.*in vivo*, but only if the thymocytes were then cultured *in vitro* for 24 hours[9]. Repeating this protocol with C3HeN and bcl-2 transgenics using not only a standard oligonucleosomal fragmentation assay, but also the "TUNEL"-flow cytometric assay, and the PFGE assay which allows detection of fragmentation at very low levels, failed to detect any apoptosis at 24 hours *in vivo* or any increase above background apoptosis after 24 hours in culture.

FIGURE 1. OVERALL THYMOCYTE DEVELPMENT



In related experiments, with transgenic mice containing the bcl-2 anti-apoptosis gene under the control of the p56lck-promoter (see above), TCDD induced atrophy although dexamethasone induced atrophy was totally inhibited. Bcl-2 expression is reduced, normally, in DP thymocytes supposedly contributing to their sensitivity to apoptosis but in the transgenic animals it is expressed at high levels in DP thymocytes.

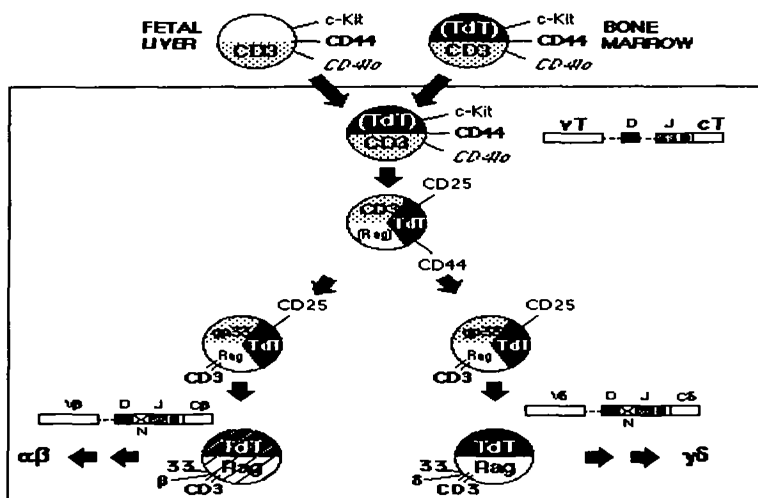
Therefore, because we could not detect any increase in apoptosis in thymocytes exposed to TCDD *in vivo* either immediately after extraction, or cultured for 24 hours *in vitro*, and because bcl-2 provides no significant protection from TCDD, we feel there is little support for either a selective apoptosis of DP thymocytes or a general apoptosis in all major subsets in TCDD induced atrophy.

Inhibition of Differentiation

We have previously provided evidence that the "prothymocyte" (the thymocyte precursor found in the fetal liver or adult bone marrow) was the target for TCDD induced thymic atrophy[13,14]. We could detect a reduction in RNA markers in fetal liver or bone marrow associated with lymphocyte stem cells (the recombinase activating genes and TdT genes) after TCDD exposure[22]. In association with these markers we also found a reduced capacity to repopulate the thymus by bone marrow or fetal liver hemopoietic cells. In further studies, we have discovered in FTOCs (a system in which atrophy can be induced without consideration of stem cell influx), and in SCID and

RAG mouse thymuses (which cannot develop DP or mature SP thymocytes), an arrest of development in the earliest stages of double negative thymocyte development. That is, definition of early stages in thymocyte development, utilizing the CD44, CD25 markers (see Figure 2) provide evidence for inhibition of differentiation at a stage prior to expression of theCD25 marker and pre-T-cell receptor expression.

FIGURE 2. EARLY STEPS IN THYMOCYTE DEVELOPMENT



TCDD Activates the AhR in Hemopoietic Cells to Cause Thymic Atrophy
Chimeric mice containing normal epithelial tissues and hemopoietic elements lacking the AhR were constructed through lethal irradiation and bone marrow reconstitution. These mice and appropriate chimeric controls (AhR^{bb} B6 X129Sv marrow into Ly 5.1 mice)were treated with 30µg/kgTCDD as well as mice constructed in parallel with normal AhR containing marrow cells. No significant atrophy was detected in the AhR-KO mice compared to the control chimeras which showed normal atrophy. These findings eliminate the possibility that it is thymic epithelium or epithelial elements in stroma of other lymphopoietic tissues that are the proximal targets for TCDD activation of the AhR to cause atrophy. That epithelial tissues were not affected by radiation, CYP1A1 induction in liver cells was confirmed in the treated mouse groups. Therefore TCDD must activate genes in radiation sensitive hemopoietic targets to cause thymic atrophy.

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