ACTIVATION OF THE AHR BY TCDD IN THE IMMUNE SYSTEM: CELLULAR TARGETS & CONSEQUENCES.

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Introduction

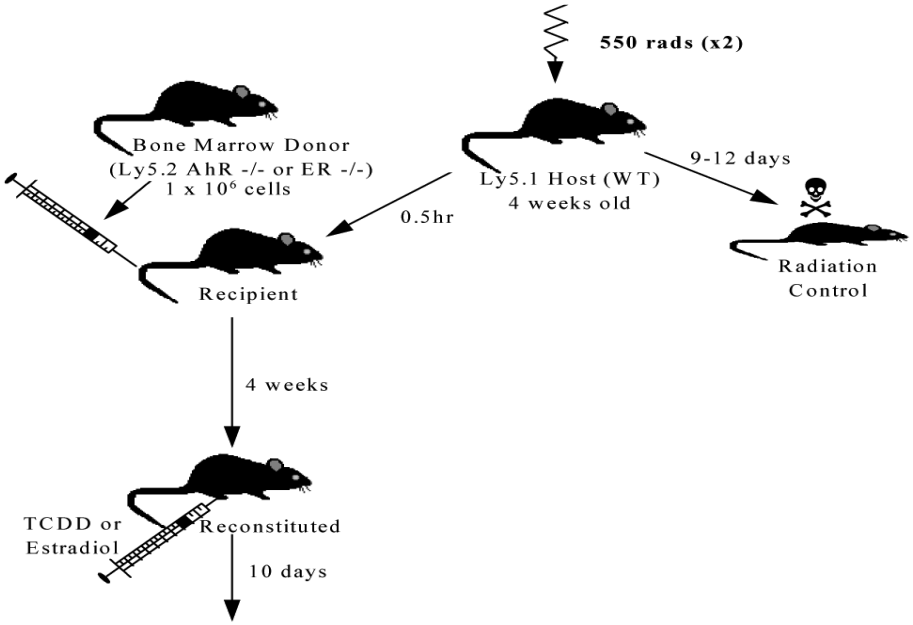
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) , and its related congeners affect the immune system, causing immunosuppression and thymic atrophy.(1). It is generally agreed that TCDD exerts its effects through the ligand activated transcription factor, the AHR, (2) and elimination of the receptor results in resistance to thymic atrophy as well as affecting other endpoints (3,4). Although several investigators suggested that the effects of TCDD on the immune system were mediated through activation of the AHR in non hemopoietic cells (5,6), the evidence for this was circumstantial. For example Greenlee and coworkers argued that TCDD caused thymic atrophy through activation of the AhR in thymic epithelium because pretreatment of cultured epithelium resulted in a reduction of thymocyte proliferative capacity on that epithelium in response to a T-cell mitogen. (5) . Vos and coworkers also suggested that the thymic epithelial elements were the major target of TCDD based on histopathological observations (6) and Esser suggested stromal elements were the major target of TCDD in vitro in fetal thymic organ cultures (FTOC) because pretreatment of stroma with TCDD in these cultures reduced proliferation of developing thymocytes.(7). We have developed a strategy whereby the specific cellular targets in which the AHR must be activated to cause a particular immune system effect can be identified (8). In this strategy, radiation chimeras are created in which the AHR is lacking in either hemopoietic elements, or in stromal (radiation insensitive) cells. This has allowed us to determine that AHR activation by TCDD must occur in radiation sensitive hemopoietic cells to cause thymic atrophy. (8) Furthermore this methodology allows us to ascertain if previously observed alterations in cellular phenotypes or gene expression actually have some relation to TCDD induced immune system outcomes. (8)

A derivative problem is whether the damage seen in various tissues after TCDD exposure is caused strictly by damage to cells of that tissue, or whether TCDD activation of the AHR might intensify inflammatory processes associated with the observed damage. The construction of the same radiation chimeras has allowed us to establish that inflammatory damage seen in liver of animals exposed to TCDD must be mediated by activation of the AHR in hemopoietic cells, and a lack of this receptor in hemopoietic cells ablates inflammatory damage in the liver while not blocking necrosis (9).

Materials and Methods

AHR-KO (KO/KO) mice and corresponding wild type (+/+) mice were derived from KO/+ heterozygotes on a 129SVXC57BL6N background.. Stable colonies of each were maintained by non brother-sister matings. In some cases B6Ly5.1 congenic mice were used as +/+ mice, in part to control for completeness of radiation reconstitution.

Radiation chimeras were produced by exposing 4-5 week old recipient mice to 110Gy split into two doses 4-5 hours apart. After the second dose, mice were given 10^6 cells from B6Ly5.1 or +/+ or KO/KO mice by tail vein injection. Radiation control mice were not given any cells, and died within two weeks. To make sure reconstitution of hemopoeitic compartments were complete, mice were not treated with TCDD (30μg/kg) until at least 4 weeks after radiation. That reconstitution had occurred and was .98% completewas confirmed utilizing the Ly5.1 and 5.2 (CD45) allelic markers to characterize the hemopoietic cells. The procedure is described in detail elsewhere (8) and is diagrammed below.



TCDD treated and control, vehicle treated mice were euthanized after 10 days, and thymus, bone marrow, spleen and liver were removed for analysis.

Thymocyte and bone marrow cell suspensions were made, and mononuclear cells were counted, and stained with fluorescent dye conjugated monoclonal antibodies, for assorted cell surface markers (CD4, CD8, CD3, CD44, CD25, Ter-119, Gr-1,Mac-1, c-kit, Sca-1, B220, (Pharmingen, San Diego) and CD45.2 (prepared in our laboratory) (8).

Confirmation of AHR activation by TCDD (or lack thereof in the case of K0/K0 mice) in recipient mice was accomplished by isolating total RNA from liver sections, and analyzing for CYP1A1 expression by Northern blotting.

Slides were prepared from 10% neutral-buffered formalin-fixed liver samples which were paraffin embedded, section at 5 μm and stained with hematoxylin and eosin. Each slide was independently

evaluated (coded and blinded) by two pathologists given duplicate sets (with different codings). Liver inflammation and necrosis were independently graded from 0 (for no effect) to 3 for severe effect.
Statistical analysis for significance for weight and cellularity differences was done using the two-tailed Student's t test for paired and unpaired variables. A Pearson correlaiton coefficient was generated to assess the degree of correlation in histopathology scoring between the two evaluators.

Results and Discussion

With the construction of transgenic mice lacking the AHR (AHR-KO's) (4,10), and the demonstration that many effects induced by TCDD, including thymic atrophy, were abolished (3) we decided to utilize well established techniques to construct radiation chimeras, in which the hemopoietic system is reconstituted with donor bone marrow. This technique allowed us to create mice in which the hemopoietic system can either have the AHR or lack it. Furthermore these hemopoietic systems can be established in mice either having or lacking the AHR.

CONSEQUENCES OF TCDD TREATMENT OF CHIMERAS

Chimera[a]	Thymic Atrophy[b]	CYP1A1 mRNA Induction	Decrease in %age of CD4+8+ immature cells[c]	Increase in %age of CD4-8+ mature cells[d]
+/+ → +/+	++++	++++	++	++
KO/KO → +/+	**No Effect**	++++	No effect	No effect
+/+ → K0/K0	++++	?Trace?	++	++
K0/K0 → K0/K0	**No Effect**	------	No effect	No effect

a) Donor mouse (and thus hemopoietic system) indicated first; Recipient second
b) Reduction in thymic weight and thymic cellularity (body wt. unaffected)
c) The percent of cells of the number remaining compared to vehicle treated controls (numbers of cells in all sets are reduced by TCDD in mice with a +/+ response)
d) This particular population has been identified as being less reduced, or even increased in FTOC systems (11,12)

Therefore it is clear that thymic atrophy and alterations in phenotypic subset proportions are strictly due to AHR activation in hemopoietic, radiation sensitive cells, and not in stromal epithelium. (or any other non radiation sensitive tissue in the organism)

Thymocytes, as well as bone marrow cells from the above mice were also analyzed for alterations in expression of stem cell markers. In the thymus, the CD4, CD8, CD3 negative population is largely of stem cell character. The sequence of development of these cells is from CD44+ CD25– ⇒ CD44+ CD25+ ⇒ CD44 – CD25+ ⇒ CD44 – CD25– ⇒ CD8+ CD4 –. In the bone marrow population the lin- stem cell compartment (those cells lacking B-cell, T-cell, granulocyte, macrophage, and erythrocyte markers) are developmentally characterized by expression of c-kit and Sca-1 antigens. The most pluripotent cells express both markers.

EFFECT OF TCDD ON STEM CELLS & LIVER DAMAGE

Chimera	% of CD44+25- Cells[a]	% of c-kit+ Sca-1+ cells[b]	Liver Necrosis	Liver Inflammation
+/+ → +/+	signif increase	sig. increase	moderate	moderate
KO/KO → +/+	no signif change	no sig. change	moderate	mild
+/+ → K0/K0	signif increase	ND[c]	mild	mild
K0/K0 → K0/K0	no sig increase	ND	mild	mild

a) Comparison to vehicle control of percent of cells in the CD3,4, 8 negative compartment (total taken as 100) with this phenotype. (the earliest stem cell)
b) Same as a) for cells in the lin- (CD3, 220, ter 119, CD8, Gr-1, Mac-1 negative) bone marrow compartment
c) Not done in this study

The above table demonstrates that TCDD activates the AHR in hemopoietic cells to arrest development of prethymic T-cells at the earliest stage (reducing subsequent stages). Similarly, if hemopoietic cells lack the AHR, TCDD does not induce inflammatory damage in the liver to the same degree as it when it is present in animals with AHR hemopoietic cells.

These studies, based on the reported data, demonstrate that direct activation of the AHR in the hemopoietic compartment is the main and necessary mediator of TCDD induced alterations. We are now working to precisely define which hemopoietic cells (developing lymphocytes, or dendritic and macrophage cells) are the ultimate targets of TCDD.

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References

1) Kerkvliet N; *Environ. Health Perspect.* **1995**, 103 (suppl)
2) Okey AB, Riddick DS, Harper PA; *Toxicol. Lett.***1994**, 70, 1
3) Fernandez-Salguero P, Hilbert DM, Rudikoff S, Ward JM, Gonzalez FJ; *Toxicol. Appl. Pharmacol.***1996**,140, 173
4) Fernandez-Salguero P, Pineau T, Hilbert, DM, McPhail T, Lee SST, Kimura S, Nebert DW, Rudikoff S, Ward JM, Gonzalez FJ; *Science. **1995***, 268,722
5) Greenlee WF, Dold KM, Irons RD Osborne R; *Toxicol. Appl. Pharmacol.***1985**, 79, 112
6) De Waal EJ, Schuurman H-J, Loeber JG, Van Loveren H, Vos JG *Toxicol. Appl. Pharmacol.***1992,** 115,80.
7) Kremer J, Gleichmann E and Esser C. *J. Immunol..***1994**, 153, 2778.
8) Staples JE, Murante FG, Fiore NC, Gasiewicz TA, Silverstone AE ;. *J. Immunol..***1998,** 160, 3844
9) Thurmond TS, Silverstone AE, Baggs RB, Quimby FW, Staples JE, Gasiewicz TA; *Toxicol. Appl. Pharmacol.***1999**, (in press)
10) Schmidt JV, Su GH-T, Reddy JK, Simon MC, Bradfield CA; *Proc.Nat.Acad. Sci. (USA)* **1996**, 93, 6731
11) Lai Z-W, Fiore NC, Gasiewicz TA, Silverstone AE; *Toxicol.Appl. Pharamcol.***1998**, 149, 167
12) Staples JE, Fiore NC, Frazier, DE Jr., Gasiewicz TA, Silverstone AE, *Toxicol.Appl. Pharamcol.***1998**, 151, 200.