図 1. PCB118 および PCB114 によるマウス肝臓の CYP2B9, CYP2B10 および CYP2C55 遺伝子の発現レベル； 野生型（TTR +/+）および TTR 欠損マウス（−/−）

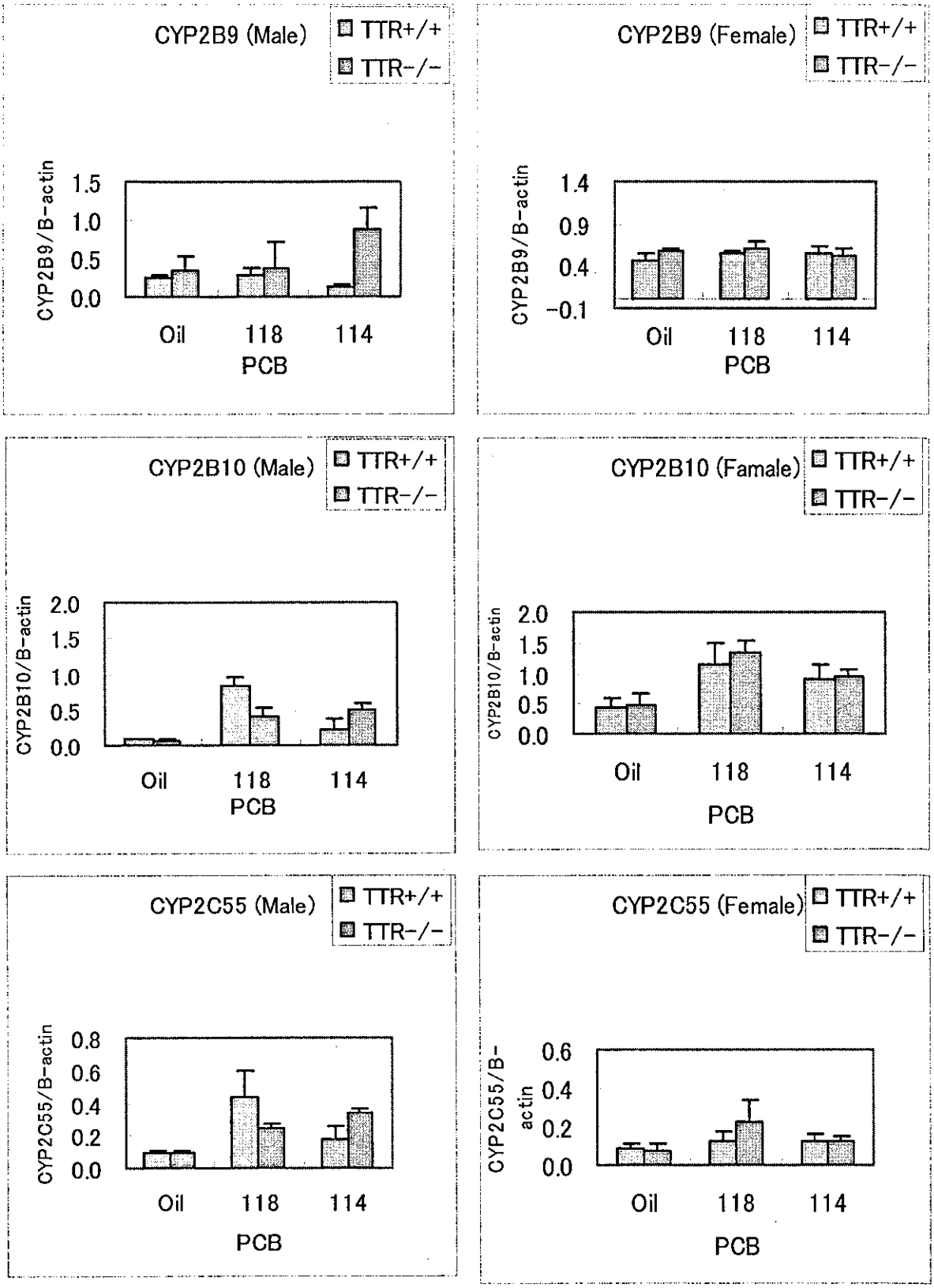


図 2. PCB153 によるマウス血清 T4 の変化　（野生系（TTR ＋/＋）および TTR 欠損マウス（－/－）

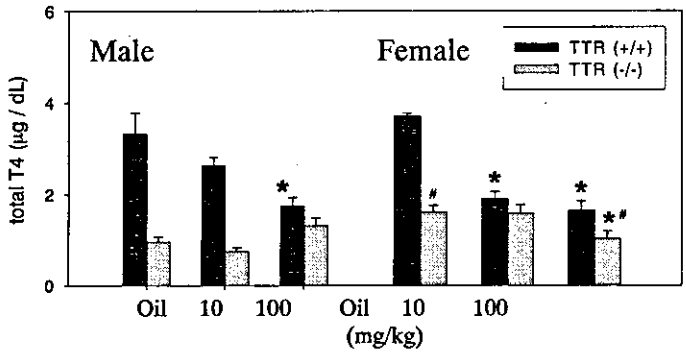
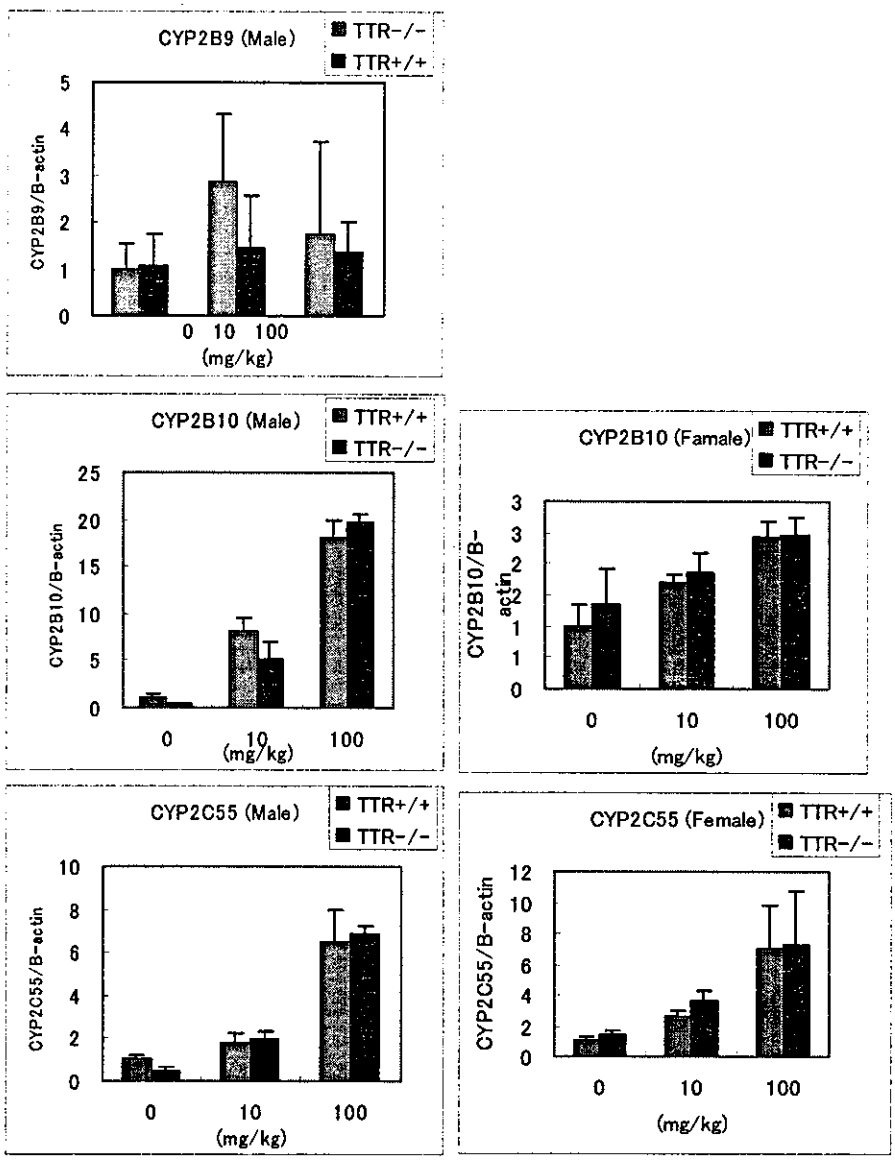


図 3.　PCB153 投与マウス肝臓の CYP2B9, CYP2B10 および CYP2C55 遺伝子の発現レベル　（野生型（TTR +/+）および TTR 欠損マウス（−/−））



厚生労働科学研究費補助金（化学物質リスク研究事業）
分担研究報告書

PCB と TCDD の毒性発現機構の比較解析のための脳における影響評価系の開発

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研究要旨

PCB の毒性を TCDD 類似毒性と非 TCDD 毒性に分類するための評価法を確立することを目的に、先ず以下のように、TCDD の毒性発現機構を解析した。体重当り 5 μg/kg の TCDD への周産期曝露が胎仔脳における遺伝子発現をどう変化させるかを、DNA マイクロアレイ法で網羅的に解析して見出した、発現量が増加、あるいは減少する遺伝子群につき、昨年度に引き続き解析を進めた。この結果、2 種類のケモカイン遺伝子の発現量が増加することを明らかにした。今後、コプラナーPCB への同様の曝露により、これら TCDD への曝露で発現量が変化する遺伝子群の発現がどう変化するかを Real-time RT-PCR 法で解析し、PCB の胎仔脳における遺伝子発現への影響を、TCDD に類似したものと類似しないものに分類する。

A. 研究目的

PCB の毒性を 2,3,7,8-tetrachloro-dibenzo-p-dioxin（TCDD）類似毒性と非 TCDD 毒性に分類するための評価法を確立することを目的に、先ず TCDD の毒性発現機構の解析を進めることにした。TCDD は、発癌性、免疫毒性、生殖毒性など人体に種々の毒性をもつことが懸念されている（Tohyama, 2002）。また、疫学的にヒトの脳の発達や高次機能に影響することが示唆されており（Vreugdenhil, et al., 2002）、げっ歯類においては、脳神経系に種々の生化学的変化を惹起することが確かめられている(Cheng, et al., 2002, Haavisto, et al., 2001, Hays, et al., 2002, Ikeda, et al., 2002, Kakeyama, et al., 2001, Pitt, et al., 2000, Unkila, et al., 1995)。さらに、妊娠ラットへの投与が胎仔脳の形態や成長後の行動に影響することが報告されている（Hojo, et al., 2002, Zareba, et al., 2002）。しかし、このような脳神経毒性が惹起される分子機構は未だ明らかでない(Kakeyama and Tohyama, 2003, Petersen, et al., 2000)。そこで先ず、体重当り 5 □g/kg の TCDD への周産期曝露が胎仔脳における遺伝子発現をどう変化させるかを、DNA マイクロア

レイ法で網羅的に解析し、発現量が変化する遺伝子群につき解析を進め、次にコプラナーPCB への同様の曝露により、これら TCDD への曝露で発現量が変化する遺伝子群の発現がどう変化するかを Real-time RT-PCR 法で解析し、PCB の胎仔脳における遺伝子発現への影響を、TCDD に類似したものと類似しないものに分類する。

本年度は、体重当り 5 μg/kg の TCDD への周産期曝露が胎仔脳における遺伝子発現をどう変化させるかを、昨年度に引き続き、DNA マイクロアレイ法で網羅的に解析し、発現量が増加、あるいは減少する遺伝子群につき、解析を進めた。

B. 研究方法

1　TCDD

実験動物には、Cambridge Isotope Laboratories から購入した TCDD(50 μg/ml、ノナン溶液)を、TCDD の含量が 0.5 μg/ml となるようにコーン油に溶解させ、マウス用胃ゾンデで強制経口投与した。対照群にはコーン油を同様に投与した。

2　実験動物および飼育条件

C57BL/6N マウスは日本クレア株式会社から購入した。マウスの飼育およびマウスを用いた実験は、山梨大学の動物実験専門委員会の承認を得て行った。

3　実験計画
体重当り 0 μg/kg または 5 μg/kg の TCDD を、妊娠 12.5 日目の 6 匹の C57BL/6N マウスに投与し、6 日後、これらマウスの雌雄の胎仔、それぞれ 1 匹ずつ、計 12 匹の脳 RNA の量や種類を、6 匹の対照非投与妊娠マウスの雌雄の胎仔、それぞれ 1 匹ずつ、計 12 匹の脳 RNA と DNA マイクロアレイ(CodeLink; Amersham 社)で比較解析した。各胎仔の雌雄の判定は、各胎仔の体の一部から DNA を抽出し、Y 染色体上の Sry 遺伝子を PCR 法で確認することにより行った。解析には、子宮内で異性に挟まれた位置にない胎仔を用い、各サンプルあたり 2 枚のマイクロアレイを用いて解析した。なお、マイクロアレイ解析は、エコジェノミクス株式会社と共同で行った。次に、マイクロアレイ解析で発現量に差異を認めた遺伝子については、Real-time RT-PCR 法で差異を再検討した。さらに、差異を確認した遺伝子について、脳切片を用いて、組織レベルでの発現部位、及び発現量を In situ hybridization 法により解析した。

4　分析方法
4.1　DNA マイクロアレイ
CodeLink DNA マイクロアレイ(1 枚あたり、約 1 万種の異なる cDNA に相当する 30 塩基長のオリゴヌクレオチドを結合させたもの（Amersham 社製）を、各サンプルあたり 2 枚用いて解析した。

4.2　In situ hybridization
雄雌共に増加が認められた遺伝子ケモカイン□について in situ hybridization で脳内の mRNA の局在と TCDD 曝露による量の変化を調べた。
cRNA プローブは、【^{35}S】UTP でラベルしたものを作製した。胎仔の脳を採取し、ドライアイスパウダー上で凍結し、-80ºC で保存した。20 μm の切片をクライオスタットで作製し、固定は 4%パラホルムアルデヒドで 15 分間行った。これを proteinase K（2 μg/ml）で 30 分間、37ºC で処理し、アセチル化、脱水を行った後、55℃で 16 時間ハイブリダイゼーションを行った。ハイブリダイゼーション後、切片を RNase A で 30 分処理し、0.1×SSC で 60℃で 30 分間洗浄した。シグナルの検出は、X 線フィルムに 10 日間曝露させて行った。

C.　結果
1　DNA マイクロアレイ解析
TCDD 投与により雄雌共に発現量が増加した遺伝子を 7 種、減少した遺伝子を 1 種見出した。また、雄のみで増加した遺伝子を 27 種類、減少した遺伝子を 39 種類、雌のみで増加した遺伝子を 64 種類、減少した遺伝子を 10 種類見出した（1.5 倍以上の増加、または 0.6 倍以下の減少を、変化が見られたものとした。なお、2.0 倍以上の増加、または 0.5 倍以下の減少を、変化が見られたものとした場合には、TCDD 投与により、雌雄共に発現量が増加、または減少する遺伝子は無く、雄のみで発現量が増加した遺伝子を 2 種類、減少した遺伝子を 19 種類、また、雌のみで発現量が増加した遺伝子を 19 種類、減少した遺伝子を 2 種類認めた。）。

2　Real-time RT-PCR 法による解析
DNA マイクロアレイ解析によって、変化が認められた遺伝子のいくつかについて Real-time RT-PCR 法で変化を検討した。内部コントロールには、Cyclophilin 遺伝子を用いた。このうち、CYP1B1 遺伝子と 2 種類のケモカイン遺伝子については、DNA マイクロアレイの結果と同様の結果が得られた（図 1）。ケモカイン遺伝子群は、炎症時の白血球の遊走に関与する(Sallusto, et al., 2000)が、最近、脳神経系の形成に関与していることが報告されている(Cartier, et al., 2005, Tran and Miller, 2003)。そこで、他のケモカイン遺伝子についても、Real-time RT-PCR 法で調べた結果、変化が認められないものや、発現量が TCDD 投与群で、減少しているものが認められた。現在、DNA マイクロアレイの結果、変化が認められた他の遺伝子についても Real-time RT-PCR 法で変化を確認中である。

3　In situ hybridization
雄雌共に発現量の増加が認められたケモイカイン□遺伝子について、TCDD 曝露群及び非曝露群、各 5 匹ずつの胎仔脳を in

situ hybridization 法で調べた結果、非投与群ではかすかなシグナルしか検出できないが、TCDD 投与群では、脳室周囲、及び脳表面周囲に、より強いはっきりしたシグナルが認められ、組織レベルでも mRNA 量の増加を確認した。

D. 考察

本研究では、PCB の毒性発現機構を TCDD 類似毒性と非 TCDD 毒性に分類する評価法を確立することを目的に、先ず TCDD への周産期曝露が胎仔脳における遺伝子発現をどう変化させるかを、DNA マイクロアレイ法で網羅的に解析し、2 種類のケモカイン遺伝子の発現量が増加することを明らかにした。
ケモカイン遺伝子群は、主に炎症時の白血球の遊走に関与している(Sallusto, et al., 2000)が、最近、ノックアウトマウスを用いた研究により、ケモカイン遺伝子群のうちの一つが、海馬歯状回の形成に必須であることが報告され(Lu, et al., 2002)、脳神経系の形成にも関与していることが示唆されている(Cartier, et al., 2005, Tran and Miller, 2003)。これらケモカイン遺伝子群のうち、2 種類の発現量が TCDD 投与により雌雄ともに増加していた。この結果は、胎仔期における TCDD への曝露が、ケモカインを介して脳神経毒性を惹起する可能性を示唆する。今後、この変化を経時的、あるいは曝露量を変化させて調べ、TCDD による変化の詳細を明確にする。そしてこの変化を、コプラナーPCB 曝露による変化と比較し、その差異を明らかにすることによって、コプラナー PCB の胎仔脳におけるケモカイン遺伝子発現への影響を、TCDD に類似したものと類似しないものに分類することを目指す。

E. 結論

本研究では、PCB の毒性発現機構を TCDD 類似毒性と非 TCDD 毒性に分類する評価法を確立することを目的に、先ず TCDD への周産期曝露が胎仔脳における遺伝子発現をどう変化させるかを、DNA マイクロアレイ法で網羅的に解析し、2 種類のケモカイン遺伝子の発現量が増加することを、明らかにした。この結果は、胎仔期における TCDD への曝露が、ケモカインを介して脳神経毒性を惹起する可能性を示唆する。

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G. 健康危険情報

特に無し

H. 知的財産権の出願・登録状況

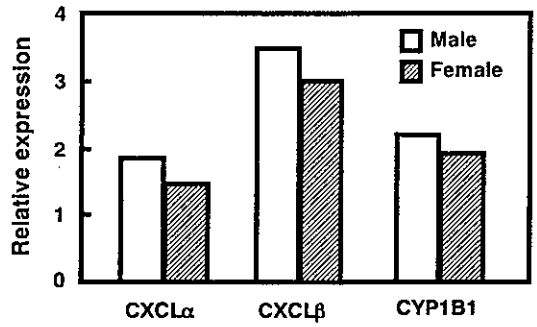
1. 特許取得
特に無し
2. 実用新案取得
特に無し
3. その他
特に無し

I. 図とその説明

図 1　Real-time RT-PCR 法によるサイトカインおよび CYP1B1 の解析。TCDD 曝露及び非曝露、雌雄各 6 匹の胎仔全脳から得られた RNA を用い、非曝露群の mRNA 量を 1 とした時の、曝露群の相対的な mRNA 量を示した。
CXCLa：ケモカインの 1 種
CXCLß：ケモカインの 1 種
CYP1B1：シトクローム P450 1B1

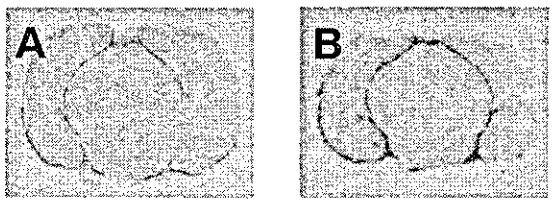
図 2. In situ hybridization 法による解析
C57Bl/6N マウスの妊娠 12.5 日目に TCDD を投与し、妊娠 18.5 日目の胎仔の脳におけるケモカイン（CXCLa）mRNA の発現を【35S】UTP 標識 cRNA プローブを用いて行った In situ hybridization の結果。
A：　対照、　B：　TCDD 曝露

図 1　Real-time RT-PCR 法による解析



TCDD 曝露及び非曝露、雌雄各 6 匹の胎仔全脳から得られた RNA を用い、非曝露群の mRNA 量を 1 とした時の、曝露群の相対的な mRNA 量を示した。
CXCLa：ケモカインの 1 種
CXCLa：ケモカインの 1 種
CYP1B1：シトクローム P450 1B1

図 2. *In situ* hybridization 法による解析



Ⅲ．研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号・ページ	出版年
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Constitutively Active Aryl Hydrocarbon Receptor Expressed Specifically in T-Lineage Cells Causes Thymus Involution and Suppresses the Immunization-Induced Increase in Splenocytes[1]

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The aryl hydrocarbon receptor (AhR) is a transcription factor belonging to the basic helix-loop-helix-PER-ARNT-SIM superfamily. Xenobiotics, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, bind the receptor and trigger diverse biological reactions. Thymocyte development and T cell-dependent immune reactions are sensitive targets of AhR-dependent 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity. However, the exact role of the AhR in T cells in animals exposed to exogenous ligands has not been clarified because indirect effects of activated AhR in other cell types cannot be excluded. In this study, we generated transgenic (Tg) mice expressing a constitutively active mutant of AhR under the regulation of a T cell-specific CD2 promoter to examine AhR function in T cells. The mRNAs of the constitutively active mutant of AhR and an AhR-induced gene, CYP1A1, were expressed in the thymus and spleen of the Tg mice. The transgene expression was clearly detected in the thymocytes, CD4, and CD8 T cells, but not in the B cells or thymus stromal cells. These Tg mice had a decreased number of thymocytes and an increased percentage of CD8 single-positive thymocytes, but their splenocytes were much less affected. By contrast, the increase in number of T cells and B cells taking place in the spleen after immunization was significantly suppressed in the Tg mice. These results clearly show that AhR activation in the T-lineage cells is directly involved in thymocyte loss and skewed differentiation. They also indicate that AhR activation in T cells and not in B cells suppresses the immunization-induced increase in both T cells and B cells. ***The Journal of Immunology*****, 2005, 174: 2770–2777.**

Xenobiotics, such as polycyclic aromatic hydrocarbons and halogenated aromatic hydrocarbons, bind and activate the aryl hydrocarbon receptor (AhR),[3] a transcription factor belonging to the basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) superfamily (1, 2), and elicit diverse biological and physiological responses (3–6). These findings suggest that the AhR functions physiologically as a ligand-dependent transcription factor, whereas the endogenous ligands and intrinsic role of the AhR have yet to be identified. The decreased fertility and abnormalities found in various organs, including the liver, spleen, vascular structures, ovary, mammary gland, and bone marrow lymphocytes, in AhR-deficient mice (7–12) also imply intrinsic roles of the AhR in normal developmental processes. In the absence of ligands, the AhR exists in the cytoplasm in an inactivated form complexed with a dimer of heat shock protein 90 and the immunophilin homologue hepatitis B virus X-associated protein 2 (13). Upon binding with ligands, such as its most potent ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the AhR becomes activated, dissociates from the protein complex, and translocates into the nucleus, where the receptor dimerizes with another basic helix-loop-helix-PAS transcription factor, aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR/ARNT heterodimer specifically binds DNA sequences, called xenobiotic responsive elements (XREs), distributed in the enhancer regions of various genes, including one of the most sensitive targets, CYP1A1, and modulates their expression (14). The receptor complex also interacts with various nuclear proteins, such as retinoblastoma, NF-κB, and estrogen receptors (15–17). However, determination of the functions of the AhR requires identification of the genes and proteins that it modulates and the cell types in which the individual biological or physiological reactions occur.

The immune system is one of the sensitive targets of TCDD (6). Although a major portion of TCDD toxicities, such as thymus involution, suppressed CTL activity, and reduced Ab production, have been demonstrated to be mediated through the AhR by studies in AhR-deficient mice (18–20), the precise mechanisms of AhR function, including the primary cellular targets and biological reactions involved in these toxic effects, remain to be clarified. The thymus involution induced by administration of TCDD or other AhR ligands to mice is characterized by decreases in tissue weight and cell number that are mainly attributable to a decrease in $CD4^+CD8^+$ double-positive (DP) cells, the predominant population of thymocytes. Skewing of thymocyte differentiation toward CD8 single-positive (SP) T cells is another peculiar feature of the

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[3] Abbreviations used in this paper: AhR, aryl hydrocarbon receptor; PAS, PER-ARNT-SIM; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ARNT, aryl hydrocarbon receptor nuclear translocator; XRE, xenobiotic responsive element; DP, double-positive; SP, single-positive; FTOC, fetal thymus organ culture; Tg, transgenic; CA-AhR, constitutively active mutant of AhR; h, human; DIG, digoxigenin; 7-AAMD, 7-aminoactinomycin D; DN, double negative.

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response to TCDD exposure (18, 21, 22). All of these features are reproduced in vitro by direct addition of TCDD to fetal thymus organ culture (FTOC) (19, 23, 24), indicating that the target cells responsible for the alterations are present in the thymus. The results of previous studies that have included histological examination have led to the hypothesis that thymic stromal cells, and not thymocytes themselves, are the direct targets of TCDD that induce thymus involution (25). This hypothesis was supported by a study showing that the stroma of a fetal thymus reaggregation culture treated with an AhR-binding halogenated aromatic hydrocarbon, not the thymocytes, induced thymus involution (24). By contrast, a recent study in which chimeric mice having AhR-deficient hemopoietic cells and wild-type stromal cells or vice versa were exposed to TCDD demonstrated that the AhR in the hemopoietic compartment, that is in the thymocytes or their precursor cells, is responsible for the TCDD-induced thymus involution (18).

The results of other studies have also shown or suggested that the AhR in T cells plays an essential role in TCDD-induced immunotoxicity. A recent study by Kerkvliet et al. (26) in a mouse graft-vs-host model injected with $AhR^{+/+}$ or $AhR^{-/-}$ T cells showed that AhR activation in T cells is critical to the suppression of CTL activity by TCDD. In our own study examining the effect of TCDD on OVA-specific Ab production in mice (27), TCDD exposure suppressed the increase in T cell number in the spleen and production of IL-2 and Th2-type cytokines before the inhibition of Ag-induced Ab production, suggesting that the AhR activation in T cells causes suppression of T cell activation and subsequent immune reactions leading to Ab production. However, it is difficult to determine the specific role of AhR activation in T cells alone in TCDD-exposed mice, because the AhR in all cell types, including B cells and APCs, is simultaneously activated, and indirect effects cannot be excluded. Chimeric mouse models and a T cell transfer system produced by using AhR-deficient mice or their cells are very useful tools for studying the primary cell target of TCDD, but they are inconvenient, because generation of chimeric mice and reconstitution by T cell transfer require highly specialized techniques. In addition, when chimeric mice are used, it must be borne in mind that their hemopoietic cells contain precursor cells not only for T cells but for B cells and APCs as well (28).

To investigate the role of AhR activation in TCDD-induced immunotoxicity, in the present study we generated transgenic (Tg) mice that specifically express a constitutively active mutant of AhR (CA-AhR) in T-lineage cells by expressing a CA-AhR with a minimal deletion in the PAS-B domain (29) under the regulation of a CD2 promoter. The AhR mutant constitutively localizes to the nucleus, heterodimerizes with ARNT, and activates transcription by binding XRE sequences in a ligand-independent manner (29, 30). The results of the present study demonstrate that AhR activation in T-lineage cells alone directly induces the thymocyte changes. They also show that the increase in number of splenocytes after immunization is suppressed in the Tg mice, whereas resting splenocytes in nonimmunized mice are much less affected, suggesting that the AhR plays a role in the growth of activated and proliferating T cells.

Materials and Methods

Generation of Tg mice

The CA-AhR expression construct (VA hCD2-CA-AhR) was generated by subcloning PAS B-domain-deleted mouse AhR cDNA with poly(A) signal (29) into the *EcoRI/BamHI* site of the VA human CD2 (hCD2) minigene, an improved version of a human CD2 minigene-based vector (31). Tg founder mice were obtained by microinjecting the transgene expression construct into C57BL/6J × DBA/2 eggs as described previously (32). In some lines, VA hCD2-GFP was coinjected with VA hCD2-CA-AhR. One line carrying both CA-AhR and GFP constructs (line A) and two lines with the CA-AhR construct alone (lines K and N) were chosen for further studies and subsequently were backcrossed into C57BL/6J mice. Founders and subsequent littermates were genotyped by PCR of tail DNA using primers for VA hCD2-CA-AhR (5′-GAACAGAGAGTTTGTCCAGC-3′, located in hCD2 promoter, and 5′-CTTCCAAAGGTAAGCATAAGAGTC-3′, located in N terminus of CA-AhR). Integrated CA-AhR copy number was determined by Southern blot analysis. Genomic DNA from a tail sample was digested with *EcoRI* and *PstI*, separated by agarose gel electrophoresis, blotted onto a Hybond filter (Amersham), and hybridized with a digoxigenin (DIG)-labeled probe. The DIG-labeled probe was synthesized from the *HincII* digestion fragment of pEB6CAG-CA-AhR-GFP (29) with a DIG-high prime DNA labeling and detection starter kit I (Roche Diagnostics) and was detected with CSPD as a substrate according to the manufacturer's instructions. Heterozygous ($CA\text{-}AhR^{+/-}$) mice were used for experiments after crossing into C57BL/6 mice for two to six generations. Their nontransgenic ($CA\text{-}AhR^{-/-}$) littermates (designated as wild type) were used as controls.

Cell preparation

Single cell suspensions of thymus and spleen were prepared by forcing cells in RPMI 1640 medium supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (complete medium) through a stainless-steel mesh. Spleen cells and bone marrow cells prepared from thigh bones were treated with ammonium chroride/EDTA solution (0.83% NH_4Cl, 0.1% $KHCO_3$, 0.37% EDTA (pH 7.4)) for 2 min at room temperature to eliminate RBCs and then were washed with PBS (33). Cells were counted with a hemocytometer after staining with trypan blue.

RT-PCR

Total RNA was isolated from cells or tissues with an RNeasy Mini kit (Qiagen). After checking the quality of the RNA by electrophoresis, RT-PCR was performed with an RNA LA PCR kit (AMV) ver1.1 (TaKaRa Biomedicals) according to the manufacturer's instructions. The amplification was conducted by heating at 94°C for 2 min, cycling at 94°C for 30 s, 60 or 66°C for 30 s, and 72°C for 30 s, and then extension at 72°C for 10 min after the final cycle. The primer sequences and annealing temperatures for each gene are shown in Table I. The primers for detecting mRNA expression of CA-AhR were designed to span the region coding for PAS B domain to distinguish PCR products between CA-AhR and wild-type AhR. The PCR products were separated with a 1.2% Synergel (Diversified Biotech) containing 0.5 μg/ml ethidium bromide, and the gel images were captured and visualized using an Electrophoresis Documentation and Analysis System 290 (Eastman Kodak).

Flow cytometry

Cells were stained with mAbs against lymphocyte surface markers or streptavidin-allophycocyanin (BD Pharmingen) for 20 min on ice. After staining, the cells were washed, treated with 7-aminoactinomycin D (7-AAMD; Sigma-Aldrich) to label dead cells, and measured with a FACSCalibur (BD Biosciences). Live cells were gated and analyzed (22). The following mAbs, all purchased from BD Pharmingen, were used: PE-conjugated anti-CD4 (anti-CD4-PE, clone GK-1.5), FITC-conjugated anti-CD8

Table I. *List of primers used for RT-PCR*

Description	Primer Sequence (5′-3′)	Annealing Temperature (°C)	Product Size (bp)
CA-AhR	TTACCTGGGCTTTCAGCAGT	66	506
	AACTGGGGTGGAAAGAATCC		
CYP1A1	CCATGACCGGGAACTGTGG	60	344
	TCTGGTGAGCATCCTGGACA		
Adseverin	GTGCTTCTAAGCATTTCCCC	60	121
	GAGTGAATGGCATCCAAGTG		
CD4	AAGGGCTCTCCCTGAGAGTC	60	104
	AAAGAGGAAAAAAGGGGAAGG		
Spatial	GAAGGTGACAGCGAAAATCA	60	112
	AAGGCATTAGACAGGTTGGG		
β-Actin	GAGGCCCAGAGCAAGAGAG	60	225
	GGCTGGGGTGTTGAAGGT		
HPRT	GCTGGTGAAAAGGACCTCT	60	249
	CACAGGACTAGAACACCTGC		

(anti-CD8-FITC, clone 53-6.7), biotinylated anti-CD8 (anti-CD8-biotin, clone 53-6.7), anti-CD3-PE (clone 145-2C11), anti-CD19-biotin (clone ID3), anti-B220-FITC (clone RA3-6B2), anti-CD127(IL-7Rα)-biotin (clone B12-1), and anti-CD61-PE (clone 2C9.G3). Biotinylated rat IgG2a was used as an isotype-matched control.

Immunization

OVA (albumin, chicken egg, grade VII) was purchased from Sigma-Aldrich. Alum-precipitated OVA (OVA/alum) was prepared as follows (27, 34). OVA (1 mg/ml) in PBS was mixed with an equal volume of 9% (w/v) $AlK(SO_4)_2$, and pH of the mixture was adjusted to 6.5 with KOH. The precipitate was washed three times with PBS and then resuspended in PBS at 0.5 mg/ml. Mice were i.p. immunized with the OVA/alum (100 μg OVA/mouse).

TCDD treatment

TCDD (50 μg/ml in nonane) purchased from Cambridge Isotope Laboratories was diluted with corn oil to adjust it to a dose volume of 10 μl/g body weight. TCDD was administered to the mice orally.

Fetal thymus organ culture

Line A heterozygous Tg mice backcrossed into C57BL/6J mice for five generations were mated, and homozygous CA-AhR$^{+/+}$ Tg mice were obtained. Male CA-AhR$^{+/+}$ mice were mated with female C57BL/6J mice, and thymuses were collected from fetuses on gestation day 16.5. One or two lobes of the thymuses were placed on a nitrocellulose filter (45-μm pore size) set in a 24-well culture plate with 1 ml of complete medium and were cultured for 4 days (35). To deplete them of thymocytes, the lobes were cultured in the presence of 1.35 mM 2-deoxyguanosine (Sigma-Aldrich) for 4 days (36, 37).

Results

Generation of T cell-specific CA-AhR Tg mice

We used the VA hCD2 vector to generate Tg mice expressing a CA-AhR mutant (Fig. 1*A*) specifically in T-lineage cells. We chose one line carrying both CA-AhR and GFP constructs (line A) and two lines with only the CA-AhR construct (lines K and N) functioning under the control of the VA hCD2 vector for the subsequent experiments. The transgene-positive mice were mated with C57BL/6 mice and maintained as heterozygotes. Heterozygous mice were used in all experiments unless otherwise specified, and their nontransgenic (CA-AhR$^{-/-}$) littermates (designated as wild type) were used as controls. Integrated CA-AhR copy numbers were determined by Southern blotting to be 2 for line A, 6–7 for line K, and 9–11 for line N. All of the lines were fertile, exhibited a normal sex ratio at birth, showed no increase in mortality after birth, and appeared healthy.

Fig. 1*B* shows CA-AhR mRNA expression in various organs in line A Tg mice. CA-AhR mRNA was detected in the thymus and spleen as expected, and was also found in the lung and, to a very minor extent, in the kidney. Expression of the AhR-responsive gene CYP1A1 was also detected in the thymus and spleen in the Tg mice in contrast with their wild-type littermate mice (Fig. 1*C*). In the lung, CYP1A1 mRNA was detected in the wild-type mice. The lung is reported to express the highest level of AhR mRNA among the tissues examined, including the thymus and spleen, in the mice (38). Recently, endogenous ligand was isolated from porcine lung (39). Thus, the lung may contain abundant AhR and endogenous ligand may activate the receptor and induce CYP1A1. The expression of CYP1A1 in the lung was further increased in the Tg mice (Fig. 1*C*). Expression of CA-AhR and CYP1A1 mRNA was also confirmed in the thymus and spleen of lines K and N (see Fig. 4).

Expression of the transgene in immune cells was measured by flow cytometry analysis of the GFP expression in line A Tg mice (Fig. 2). Thymocytes showed a broader peak of the GFP-positive population (Fig. 2*A*). CD4 and CD8 T cells in the spleen were confirmed to be GFP-positive, and B cells did not express GFP (Fig. 2*B*).

We then investigated whether the bone marrow cells of the CA-AhR Tg mice expressed the transgene and, as shown in Fig. 3, the CD3$^-$CD127$^+$ (IL-7Rα-expressing) lymphocyte progenitor fraction (40) was found to be faintly GFP-positive (Fig. 3*C*, R3). Although a previous study reported that the VA hCD2 vector functions in megakaryocytes as well as T-lineage cells (32), CD61$^+$ megakaryocytes (41) in the bone marrow did not express the transgene (Fig. 3*C*, R4 and R5).

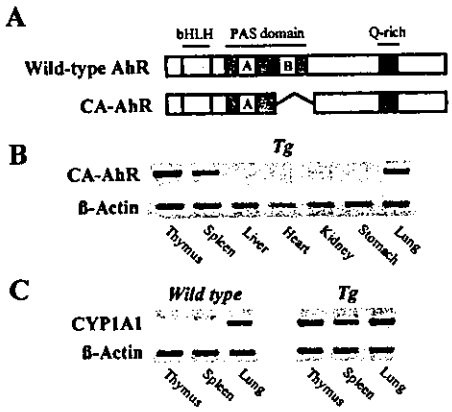

FIGURE 1. Generation of T cell-specific CA-AhR Tg mice. *A*, Schematic representation of the wild-type mouse AhR and the CA-AhR mutant lacking the minimal PAS B motif. *B*, Different tissues from line A heterozygous Tg mice were examined for CA-AhR mRNA expression by RT-PCR. *C*, Functional activation of the CA-AhR was confirmed by detection of CYP1A1 expression by RT-PCR.

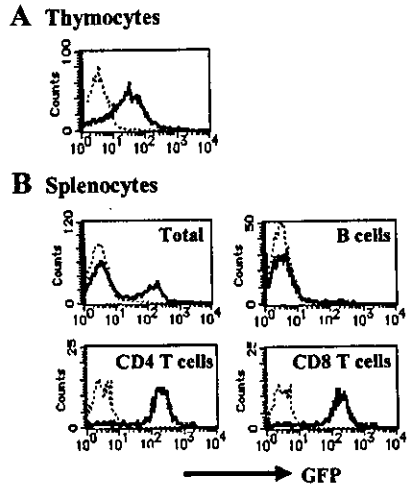

FIGURE 2. Transgene expression in thymocytes and splenocytes. *A*, Thymocytes prepared from line A heterozygous mice were stained with 7-AAMD and analyzed with a FACSCalibur flow cytometer. 7-AAMD-negative live cells were gated and expression of coinjected GFP was analyzed. *B*, Splenocytes from line A heterozygous mice were stained with a combination of anti-CD19-biotin/streptavidin-allophycocyanin and anti-CD3-PE or a combination of anti-CD8-biotin/streptavidin-allophycocyanin and anti-CD4-PE and then were stained with 7-AAMD and analyzed with a FACSCalibur. CD4 T cells, CD8 T cells, and CD19$^+$ B cells in the 7AAMD-negative live cells were gated, and GFP expression was analyzed. The staining obtained in Tg mice is represented by the bold line, and the staining in wild-type mice is represented by the dotted line.

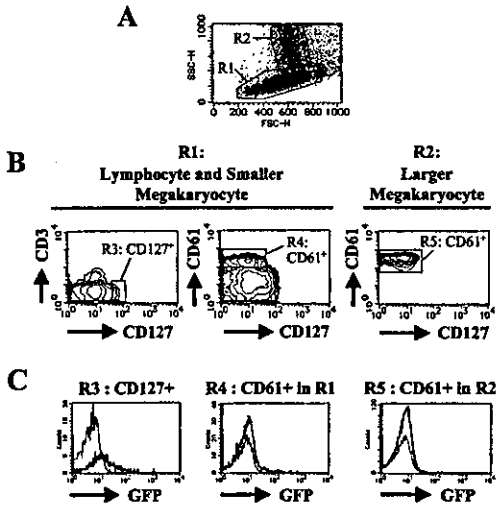

FIGURE 3. The transgene is faintly expressed in CD127$^+$ cells in the bone marrow. Bone marrow cells from line A heterozygous mice were stained with anti-CD127-biotin/streptavidin-allophycocyanin and either anti-CD3-PE or anti-CD61-PE and then were stained with 7-AAMD and analyzed with a FACSCalibur. *A*, Side scatter vs forward scatter of the cells analyzed. Region 1 (R1), including lymphocytes and smaller size megakaryocyte, and R2, including larger size megakaryocytes (41), in the 7-AAMD-negative live cells are indicated in the dot plot. *B*, R3, including CD127$^+$ cells, and R4, including CD61$^+$ cells in R1, and R5, including CD61$^+$ cells in R2, are indicated in the contour plot. *C*, Expression of GFP as analyzed by gating R3–R5 is shown in the histogram plot. The staining obtained in Tg mice is represented by the bold line, and the staining in wild-type mice is represented by the thin line.

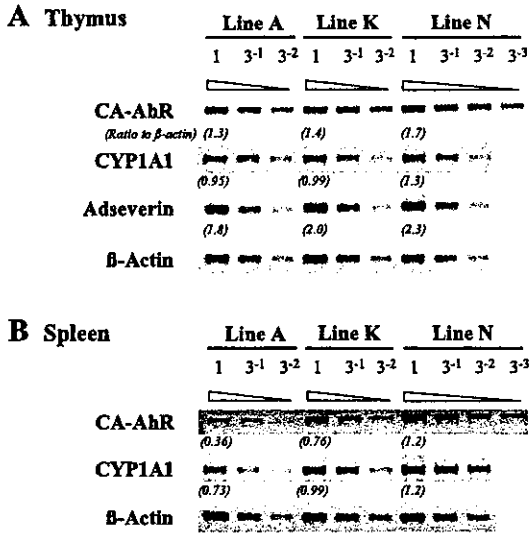

FIGURE 4. Comparison of CA-AhR and target gene expression in the thymus and spleen from line A, line K, and line N heterozygous mice. Total RNA was prepared from the thymus and spleen of the three lines with the RNeasy mini kit. cDNAs prepared from 20 ng of total RNA and serial dilutions (3^{-1}–3^{-3}) were amplified by PCR using primers for CA-AhR, CYP1A1, adseverin, or β-actin as a housekeeping gene. The expression of genes was quantified by densitometrically scanning gel images, and the values normalized to β-actin mRNA are indicated in parentheses. The numbers of PCR cycles for CA-AhR in thymus and spleen were 32 and 34, respectively, 26 and 32 for CYP1A1, and 20 for β-actin in both tissues. Mice were used after crossing into C57BL/6 mice for six generations in line A and for three generations in lines K and N.

CA-AhR expression induces target gene mRNA in both thymus and spleen, but reduces thymocyte number alone

Expression of the CA-AhR transgene and its target genes and phenotypic changes in the thymus and spleen were examined in the three lines. RT-PCR analyses showed that CA-AhR and CYP1A1 expression in the thymuses and spleens of lines A, K, and N were increased according to the integrated CA-AhR gene numbers (Fig. 4). Expression of adseverin, which was reported to be induced by TCDD in mice thymuses in an AhR-dependent manner (37), was also increased according to the transgene numbers.

The thymus weight was reduced in all three lines, by 36% in line A, 70% in line K, and 63% in line N (Fig. 5*A*). The thymocyte number was reduced by 49% in line A, 96% in line K, and 92% in line N (Fig. 5*A*). The thymocyte population defined by CD4 and CD8 expression was also affected in the Tg mice, with reduced percentages of CD4$^+$CD8$^+$ DP cells and increased percentages of CD8 SP and double negative (DN) cells (Table II). The ratios of CD4 SP/CD8 SP were significantly reduced in the Tg mice (Table II). The remarkable increases in the percentage of DN cells in line K and N were parallel to the large decreases in the total cell numbers.

By contrast, spleen weight was unaffected by the expression of CA-AhR (Fig. 5*B*). Splenocyte number was significantly reduced only in line N (by 40%), in which CA-AhR is most highly expressed (Fig. 5*B*), and the percentages of CD4 T cells, CD8 T cells, and B cells were unchanged, even in line N (data not shown). All of these findings are consistent with those observed in the thymuses exposed to TCDD (18, 21, 22).

We further confirmed that the CA-AhR is not expressed in the stromal cells and that CA-AhR expression in T-lineage cells alone is capable of inducing the thymus alteration in FTOC. mRNA expression was examined in whole thymus and stroma obtained by culturing thymus tissues in the presence of 2-deoxyguanosine to deplete it of thymocytes. As expected, CD4 mRNA was detected only in whole thymus, and Spatial mRNA, which is specifically expressed in thymic stromal cells (42), was detected in both the

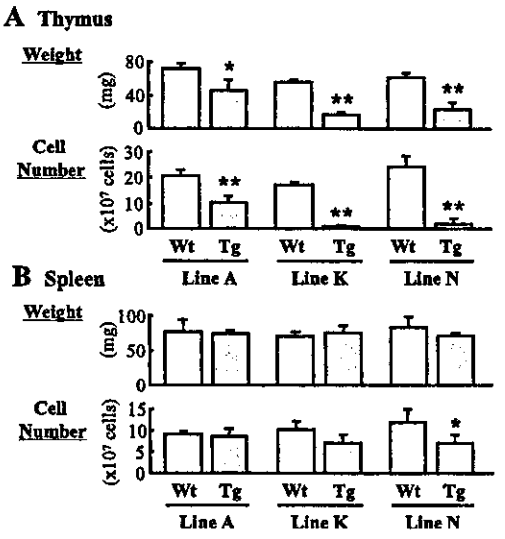

FIGURE 5. CA-AhR expression in T-lineage cells reduces thymus weight and cell number, but affects the spleen less. Thymus and spleen from female heterozygous Tg mice and nontransgenic littermate wild-type mice of line A (8 wk old, n = 4/each group), line K (10 wk old, n = 5), and line N (8–9 wk old, n = 5) were examined. Mice were used after crossing into C57BL/6 mice for two generations in line A and for three generations in lines K and N. The differences between Tg mice and wild-type mice were analyzed by Student's t test. The data are expressed as mean ± SD. *, $p < 0.05$; **, $p < 0.01$.

Table II. *CA-AhR expression affects thymocyte population*[a]

	Line A (n = 4)		Line K (n = 5)		Line N (n = 5)	
	Wt	Tg	Wt	Tg	Wt	Tg
	(%)		(%)		(%)	
DN	4.7 ± 0.5[b]	6.4 ± 1.2	4.2 ± 1.1	52.6 ± 7.8	6.8 ± 2.8	27.9 ± 14.1
DP	82.2 ± 1.7	79.3 ± 1.4	87.2 ± 1.8	33.5 ± 7.6	83.2 ± 2.4	53.1 ± 15.9
CD4 SP	10.0 ± 1.5	9.5 ± 2.0	6.9 ± 0.7	8.7 ± 1.2	7.0 ± 0.8	10.2 ± 0.9
CD8 SP	3.1 ± 0.2	4.9 ± 0.4	1.8 ± 0.4	5.2 ± 0.5	3.1 ± 0.3	8.8 ± 1.2
CD4/CD8[c]	2.6 ± 1.1	2.0 ± 0.5	4.0 ± 0.7	1.8 ± 0.6**	2.3 ± 0.3	1.2 ± 0.1**

[a] Thymocytes from female heterozygous (CA-AhR$^{+/-}$) Tg mice and nontransgenic (CA-AhR$^{-/-}$) littermate mice (Wt) (8–10 wk old) were examined by flow cytometry.
[b] The data was expressed as means ± S.D. The differences between CD4/CD8 ratio in Tg mice and Wt mice were evaluated with Wilcoxon rank sum test. **, $p < 0.01$.
[c] Ratio of CD4 SP cells/CD8 SP cells.

whole thymus and the stroma (Fig. 6). CA-AhR was detected only in whole thymus, not in the stroma, in this system (Fig. 6). Thus, AhR activation in thymocytes alone was demonstrated to cause the cellular loss and population changes in the thymus.

CA-AhR suppresses the increase in spleen weight and splenocyte number caused by immunization

We previously reported finding that TCDD administration to mice immunized with OVA suppressed the immunization-induced increase in spleen weight and splenocyte number (27, 34). Consistent with these findings, the increase in spleen weight and splenocyte number observed in wild-type mice after immunization with OVA was suppressed in line A Tg mice (Fig. 7), although their spleen was unaffected when not immunized, as stated above (Fig. 5). Interestingly, increases of both CD4 T cells and B cells were significantly suppressed despite the specific expression of CA-AhR in T cells (Fig. 7). The number of CD8 T cells was also fewer in the Tg mice than in the wild-type mice, although the difference was not significant. Simultaneous suppression of the T and B cell increase was also observed in OVA-immunized and TCDD-exposed wild-type mice (27).

To estimate how much TCDD induces the corresponding level of AhR activation, CYP1A1 expression in the thymus and spleen of line A Tg mice and TCDD-exposed wild-type mice was compared. As shown in Fig. 8, the level of expression of CYP1A1 mRNA in the thymus of the Tg mice was slightly higher than its level of expression in wild-type mice exposed to a single dose of 20 μg/kg TCDD. CYP1A1 expression in the spleen of the Tg mice was less than in wild-type mice exposed to 20 μg/kg TCDD, which seems plausible because only T cells express CYP1A1 mRNA in the spleen of Tg mice, whereas both T and B cells express CYP1A1 mRNA in TCDD-exposed wild-type mice (43). These results show that the function of the activated AhR in line A heterozygous mice and AhR activated by 20 μg/kg TCDD are roughly comparable. In our previous study, 20 μg/kg TCDD suppressed the splenocyte number to 60% of the number in unexposed control mice 1 wk after immunization (27). The same dose of TCDD reduced the thymus weights to 40–60% (44). The fact that a similar extent of suppression was observed in the thymus and spleen of the Tg mice indicates that the major portion of the effect of TCDD in these organs is attributable to AhR activation in the T cells.

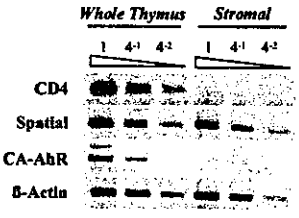

FIGURE 6. CA-AhR is expressed in the thymocytes, but not in the stromal cells, in the thymus. line A male CA-AhR$^{+/+}$ mice were mated with female C57BL/6J mice, and thymuses were collected from fetuses on gestational day 16.5. The thymuses were cultured for 4 days on a nitrocellulose filter floated on complete medium. To deplete them of thymocytes and obtain stromal cells, the lobes were cultured in the presence of 1.35 mM 2-deoxyguanosine. Fifteen or 16 lobes were pooled from each treatment group and were used to prepare RNA. cDNAs prepared from 20 ng of total RNA and serial dilutions (4^{-1}, 4^{-2}) were amplified by PCR

Discussion

To prove the role of activated AhR in T cells in TCDD-induced immune suppression, we generated Tg mice in which expression of CA-AhR cDNA is controlled by an improved version of hCD2 promoter and their T cells specifically express activated form of AhR. We obtained three lines bearing different copy numbers of the transgene, ranging from 2 to 10. All three lines showed expression of CA-AhR and an AhR-induced target gene in the thymus and spleen. In line A mice, which coexpress GFP in addition to CA-AhR under the control of the same CD2 promoter, the transgene was confirmed to be strongly expressed in the thymocytes, CD4, and CD8 T cells, but not in the thymus stromal cells or in the B cells. CD2 is principally expressed on T cells and NK cells in humans (45), but low expression has also been reported on subsets of other cell types, including B cell progenitors (46, 47). Consistent with the observation, faint expression of the transgene was detected on the CD3$^-$CD127$^+$ lymphocyte progenitor cells in our

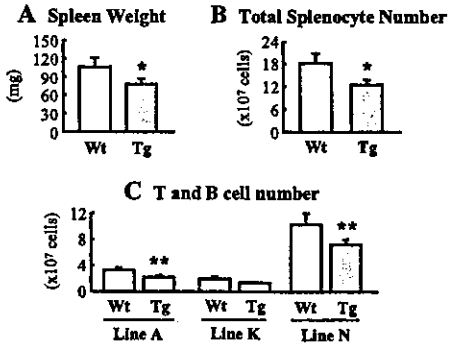

FIGURE 7. CA-AhR expression suppresses the increase in spleen weight and both CD4 T cell and B cell numbers after immunization. Line A female heterozygous Tg mice and littermate wild-type mice (8 wk old, n = 4) were immunized with OVA/alum, and their spleens were examined 7 days later. Mice were used after crossing into C57BL/6 mice for five generations. The differences between the Tg mice and wild-type mice were analyzed by Student's t test. The data are expressed as mean ± SD. *, $p < 0.05$; **, $p < 0.01$.

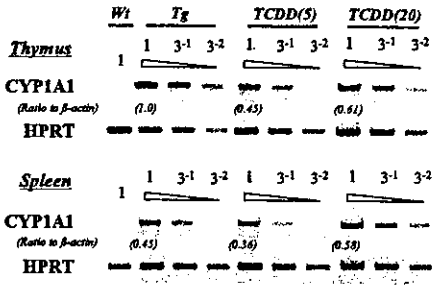

FIGURE 8. Comparison of CYP1A1 induction by CA-AhR in Tg mice and by TCDD exposure. Female C57BL/6 mice were given 5 or 20 μg/kg TCDD, and 3 days later total RNAs were prepared from thymocytes and splenocytes. CYP1A1 induction was compared with its induction in female line A heterozygous mice. cDNAs prepared from 20 ng of total RNA and serial dilutions (3^{-1}, 3^{-2}) were amplified by PCR using primers for CA-AhR or hypoxanthine phosphoribosyltransferase as a housekeeping gene. The expression of genes was quantified by densitometrically scanning gel images, and the values normalized to β-actin mRNA are indicated in parentheses. In both tissues, 30 PCR cycles were used for CYP1A1 and 20 for hypoxanthine phosphoribosyltransferase.

Tg mice. The transgene was also found to be expressed in the lung and, to a very minor extent, in the kidney in the Tg mice. Although the mechanism for the expression of CA-AhR in those tissues is unknown, presence of cells expressing CA-AhR in those tissues, such as T cells in the lung, may partly contribute to the transgene expression. Albeit the expression of the vector is found in other cell types, these CD2-based vectors, including the VA hCD2 vector, have been proved to be very useful to study specific functions of molecules in T cells. Likewise, the Tg mice we developed in the present study enable a new approach to explore the effect of AhR activation in T cells in the immune suppression. Lymphocyte progenitor cells have been reported to be affected by TCDD, and its effect was suggested to contribute to a loss of thymocytes (48, 49). Although the expression of CA-AhR in the lymphocyte progenitor cells was much fainter than that in the thymocytes and T cells (Figs. 2 and 3), the effects of low expression of CA-AhR may need to be considered.

All three lines of our Tg mice were characterized by thymus involution, including reduced thymocyte number and increased percentage of CD8 SP cells, the same as observed in TCDD-exposed mice. The fact that direct exposure of FTOC to TCDD reproduces the thymus involution induced by TCDD exposure in vivo (19, 24) shows that TCDD directly affects the thymus, in which the target cells are present. However, the results of previous studies have suggested that two types of cells in the thymus, thymocytes (18, 28) and stromal cells (24, 25), are the primary targets. The results of the present study in the Tg mice demonstrate that AhR activation in T-lineage cells alone can cause the thymus alterations, including loss of thymocytes and increase in percentage of CD8 SP thymocytes, without AhR activation in the stromal cells. Tomita et al. (50) recently produced T cell-specific ARNT-deficient mice in which the ARNT gene is disrupted under the control of T cell-specific $p56^{lck}$ proximal promoter, and showed that the thymus of the Tg mice is resistant to TCDD. Their results are consistent with our own showing that the AhR/ARNT heterodimer in the thymocytes, but not stromal cells, is essential for the occurrence of thymus involution.

Whereas thymus undergoes involution upon TCDD exposure, the splenocytes and splenic T cells of nonimmunized animals are unaffected by TCDD (18, 51). The same finding was observed in the spleen of our T cell-specific CA-AhR Tg mice, even though the CA-AhR was fully expressed in both the spleen T cells and the thymocytes. Although we examined the expression of CYP1A1 and adseverin as sensitive AhR-dependent target genes to estimate the extent of AhR activation, the genes responsible for the thymus involution remain to be identified. Previous studies have suggested suppression of thymocyte proliferation (24, 28) and induction of apoptosis (52) as the biological process involved in the thymus atrophy caused by TCDD. Our own recent study demonstrated that CA-AhR expression in Jurkat T cells inhibits cell growth by inducing both apoptosis and cell cycle arrest (29). Several genes in these CA-AhR-expressing Jurkat T cells that are related to apoptosis or cell cycle arrest, such as Fas, cyclin G_2, and growth arrest and DNA damage-inducible protein 34, were shown to be up-regulated in an XRE-mediated transcription-dependent manner (29), and these genes may be responsible for the loss of thymocytes.

In contrast with the nonimmunized mice in which splenocytes were less affected by AhR activation, as described above, the increase in splenocyte number after immunization was suppressed in the CA-AhR Tg mice, suggesting that the AhR/ARNT heterodimer inhibits cell growth in activated and proliferating T cells, but not in resting T cells. From this point of view, the effect of AhR activation in thymocytes may be also attributable to the effect on activated or proliferating cells: in thymus atrophy, the suppression of DN cell proliferation (28) and the loss of DP cells (52) are suggested to be responsible for cellular loss, DN thymocytes are vigorously proliferating cells, and DP cells receive an activation signal via their T cell receptors. Thus, activation state of the cell seems to affect the sensitivity of T-lineage cells to AhR activation.

The results of the present study also demonstrated that AhR activation in T cells alone suppresses the increase in both T and B cells in the spleen after OVA immunization. In terms of primary target cells of TCDD toxicity in immune reaction, Kerkvliet et al. (26) recently showed that AhR in both CD4 and CD8 T cells is necessary for full suppression of CTL response by TCDD in a mouse acute graft-vs-host model in which T cells, or CD4 or CD8 subsets, from $AhR^{+/+}$ and $AhR^{-/-}$ C57BL/6 mice were injected into C57BL/6 × DBA/2 F_1 host mice. Consistently, our results indicated that AhR activation in T cells is involved in changes in immune reaction. We previously reported that TCDD administration to OVA-immunized mice suppresses the growth of T and B cells and the production of Th2-type cytokines before suppression of Ab production (27, 34), which suggested that TCDD inhibits Ab production by suppressing T cell activation and the subsequent Th2-cell differentiation. The results of the present study strongly support the hypothesis that activation of the AhR directly inhibits cellular activation of the T cells and their subsequent proliferation and differentiation, leading to the suppression of T cell help on B cell proliferation. Alternatively, indirect effect of $CD4^+CD25^+$ regulatory T cells may be involved in the immune suppression. The regulatory T cells are positively selected in the thymus when their TCR receives a signal with intermediate strength (53). TCDD exposure to thymus is shown to affect thymocyte selection, possibly through up-regulation of Notch 1 (54) or activation of the ERK pathway (35) in the thymocytes, and these mechanism may alter the selection of $CD4^+CD25^+$ regulatory T cells. Further study of our Tg mice will clarify whether the suppression of T cell growth by AhR activation inhibits Th2-type cytokines and following Ab production by B cells, and it will also provide a clue for solving the mechanism of inhibition.

Andersson et al. (55) recently produced Tg mice expressing a CA-AhR mutant (30), which has a structure very similar to the one we used, under the control of an Ig H chain enhancer ($E\mu$), which promotes transgene expression in both B- and T-lineage cells (56). In addition to exhibiting thymus atrophy, their mice have a reduced life span and develop tumors in the glandular part of the stomach

(55). The Tg mice generated in our study, in contrast, are fertile and do not exhibit any overt phenotype differences except thymus atrophy, showing that AhR activation in T cells is not responsible for the stomach tumors.

Although T cells contain functional AhR and directly respond to TCDD (57), phenotypic changes caused by TCDD in T cells, such as changes in proliferation and differentiation, are difficult to detect in vitro, possibly because of optimized culture conditions that compensate for the effects of TCDD (6). The Tg mice expressing CA-AhR in T cells will be a useful model for investigating the role of activated AhR in the T cells. In particular, immunization of the Tg mice is expected to show suppression of various immune reactions, including Ab production and CTL activity, the same as observed in TCDD-exposed mice. Dioxins are persistent environmental contaminants and as such animals are continually exposed to them. TCDD maternally exposed is transferred to fetus and pups through the placenta or milk and activates their AhR (58, 59). Our Tg mice express CA-AhR mRNA in the fetal thymuses (data not shown) and the expression continues in the T-lineage cells after birth. Thus, these Tg mice will also be a useful model for clarifying the effect of persistent activation of AhR in T cells. Studies using our CA-AhR Tg mice should shed light on the role of the AhR in T cells in immune suppression by TCDD and also in physiological reactions.

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Disclosures

The authors have no financial conflict of interest.

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Search for the target genes involved in the suppression of antibody production by TCDD in C57BL/6 mice

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Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) suppresses antibody production through activation of a transcription factor, the aryl hydrocarbon receptor (AhR). To explore the genes that are involved in the suppression of antibody production by TCDD, we investigated TCDD-induced changes in gene expression in the CD4 T cells and B cells of C57BL/6 mice immunized with ovalbumin (OVA) plus alum as an adjuvant. Changes in gene expression were analyzed with Affymetrix oligonucleotide microarrays. The results showed that OVA-immunization alone up-regulated expression levels of many genes in the CD4 T cells as early as 3 h after immunization, with 55 up-regulated and 5 down-regulated. At 24 h, 42 genes were found to be up-regulated and 30 down-regulated. Fewer genes were affected in the B cells than in the CD4 T cells. In contrast to the up-regulation of genes induced by immunization in the CD4 T cells, administration of TCDD to mice 3 h prior to the immunization mainly caused down-regulation of genes in the CD4 T cells when compared with immunization alone, with 1 being up-regulated and 4 down-regulated at 3 h after immunization and 3 up-regulated and 34 down-regulated at 24 h. In particular, at 3 and 24 h, TCDD suppressed expression of three and seven genes, respectively, that were up-regulated by immunization. Another characteristic of the TCDD-induced changes in gene expression was the suppression of many genes encoding proteins that are involved in GTP-binding protein-linked signaling in CD4 T cells. These results suggest that the inhibition of immunization-induced gene expression and modulation of G-protein-linked signaling in CD4 T cells are responsible for the TCDD-induced suppression of antibody production.

Keywords: TCDD; Gene expression; CD4 T cell; B cell; Antibody production

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1. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic halogenated aromatic hydrocarbon, induces a variety of adverse biological and physiological reactions in mammals [1–3], including immunotoxicity, such as thymus atrophy and suppression of antibody production and cytotoxic T cell activity [4,5]. Recent studies in aryl hydrocarbon receptor (AhR)-deficient mice have shown that the majority of these TCDD-induced toxicities are mediated through the AhR, a ligand-activated transcription factor [6–10]. Upon binding with TCDD, the AhR translocates from the cytoplasm to the nucleus and dimerizes with another transcription factor, the AhR nuclear translocator (Arnt). The AhR–Arnt complex binds with a *cis*-acting DNA enhancer sequence, the xenobiotic responsive element (XRE), located in the enhancer region of target genes, and modulates gene expression [11,12]. Comprehensive study of changes in expression of these genes has recently been made possible by the development of technologies, such as gene microarrays and serial analysis of gene expression technique [13–16]. However, the effects of TCDD greatly vary, depending on the type of species, tissues, cells, and the state of the cells, and genes and proteins responsible for the individual TCDD toxicities remain to be identified.

We previously investigated the effect of TCDD on T-cell-dependent antibody production in mice [17,18]. When mice were immunized with ovalbumin (OVA) plus alum as an adjuvant, antigen-specific IgM and IgG1 production was detected in the plasma on day 7 and day 10, respectively, after immunization [17]. In the series of reactions leading to antibody production, B cell expansion and differentiation into antibody-producing cells require the help of Th2 cells, the effector cells differentiated from naïve CD4 T cells and characterized by the production of cytokines, such as IL-4, IL-5, and IL-6 [19]. In our previous study, Th2-type cytokine production and an increase in spleen T cells and B cells following immunization were observed prior to antibody production [17], whereas simultaneous administration of TCDD at the time of immunization suppressed the Th2-derived cytokine production and the increase in T cells and B cells and resulted in inhibition of OVA-specific IgM and IgG1 production [18]. These results suggest that the suppression of T cell activation, differentiation to Th2 cells, and Th2-type cytokine production by TCDD adversely affect reactions involved in antibody production.

In the present study, we used Affymetrix oligonucleotide microarrays to investigate the gene expression profiles of CD4 T cells prepared from TCDD-exposed and OVA-immunized mice. We particularly focused on early gene expression, i.e., 3 and 24 h after immunization, to identify genes that are involved in the induction of TCDD-induced antibody suppression. Gene expression in B cells prepared at the same times was investigated for comparison. The results revealed characteristic TCDD-induced changes in gene expression in immunized CD4 T cells and B cells.

2. Materials and methods

2.1. Mice

Five-week-old female C57BL/6J mice were obtained from Clea Japan (Tokyo) and maintained in our animal facility under controlled conditions at a temperature of 24±1 °C, humidity of 50±10%, and a 12:12-h-light/dark cycle. Mice were allowed to acclimate for 1 week and were handled in a humane manner according to the NIES guidelines for animal experiments.

2.2. Experimental protocol

TCDD (50 μg/ml in nonane, 98% pure) was purchased from Cambridge Isotope Laboratories (Andover, MA). The TCDD in nonane solution was diluted with corn oil to obtain a dose volume of 10 μl/g body weight. Ovalbumin (OVA; chicken egg, grade VII) was purchased from Sigma (St. Louis, MO). Alum-precipitated OVA (OVA/alum) was prepared by mixing 1 mg/ml of OVA in PBS with an equal volume of 9% (w/v) $AlK(SO_4)_2$ and adjusting the pH of the mixture to 6.5 with KOH [17,18]. The precipitate was washed three times with PBS and then resuspended in PBS at 0.5 mg OVA/ml. Corn oil or TCDD (20 μg/kg) was orally administered to 6-week-old mice, and, 3 h later, they were intraperitoneally immunized with OVA/alum (100 μg OVA/mouse). The animals'