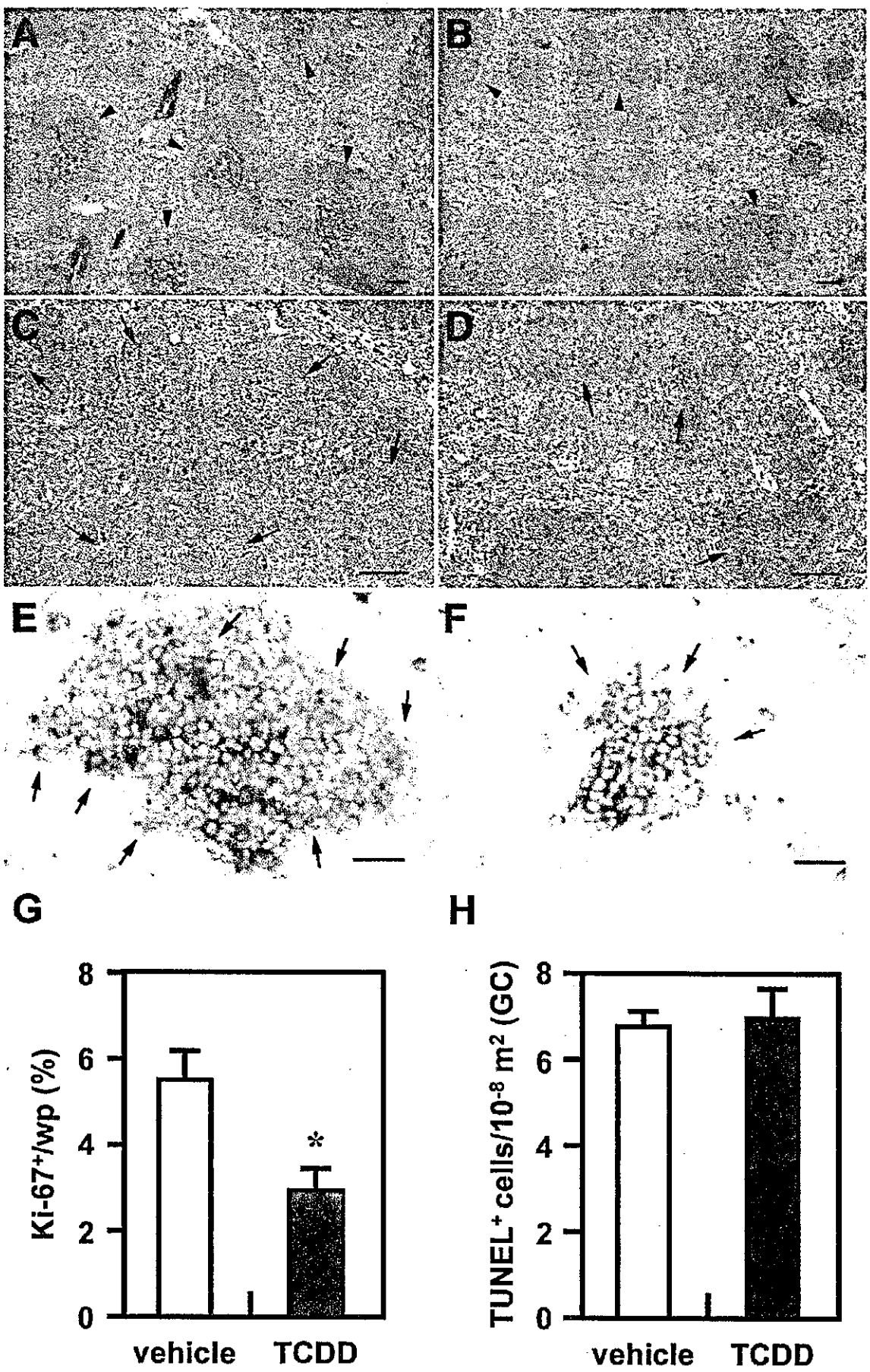
A
B
C
D
E
F
G
H
Ki-67+/wp (%)
8
6
4
2
0
*
vehicle
TCDD
TUNEL+ cells/10-8 m2 (GC)
8
6
4
2
0
vehicle
TCDD

treated mice (Fig. 4G), which indicates that cellular proliferation in the GCs was significantly suppressed by TCDD exposure. Next, cellular apoptosis was assessed by the TUNEL method; the cells were double-stained with PNA to detect GC. Again, reduced GC size was detected in the TCDD-treated mice; however, TUNEL$^+$ cells were detected in the GCs in both the vehicle-control mice and the TCDD-treated mice on day 7 (Figs. 4E and 4F). The frequency of TUNEL$^+$ cells to the area of GCs suggests that programmed cell death was not significantly promoted by exposure to TCDD (Fig. 4H). These results suggest that reduced cellular proliferation in GCs by TCDD administration contributes to reduced GC formation in the spleen.

TCDD Exposure Suppresses High-Affinity AFC Generation in the Spleen and High-Affinity IgG1 Level in the Plasma

Since TCDD exposure suppressed GC formation in the spleen, we used NP-CG to determine whether the generation of hapten-specific high-affinity AFC is reduced by TCDD exposure. High-affinity AFCs were shown to be early products of GCs and subsequently accumulate to BM (Smith *et al.*, 1997). We thus evaluated the frequency of high-affinity AFC in addition to the total (high and low) AFCs in the spleen and BM on days 10 and 14 by ELISPOT (Fig. 5). As is shown in Figure 5A, a large number of total NP-specific AFCs was detected in the spleen of the control mice on day 10, and these cells were decreased on day 14. In these control mice, the number of total AFC in the bone marrow was much smaller than that in the spleen. As compared with the vehicle-control group, the number of total AFCs was reduced in the spleen of the TCDD-treated group (by 90 and 64% on days 10 and 14, respectively). In parallel with the total AFCs, high-affinity NP-specific AFCs were abundant in the spleen of the control mice on day 10 and decreased on day 14 (Fig. 5B). TCDD exposure was clearly shown to reduce the number of high-affinity AFCs on days 10 (by 96%) and 14 (by 65%). These reductions in the high-affinity AFCs by TCDD exposure were more prominent than those observed in the total splenocytes (by 51% on day 10 and by 41% on day 14). The numbers of bone marrow cells in the NP-CG–immunized mice were not altered by TCDD exposure (data not shown).

We further examined whether high-affinity NP-specific IgG1 production was suppressed in the mice immunized

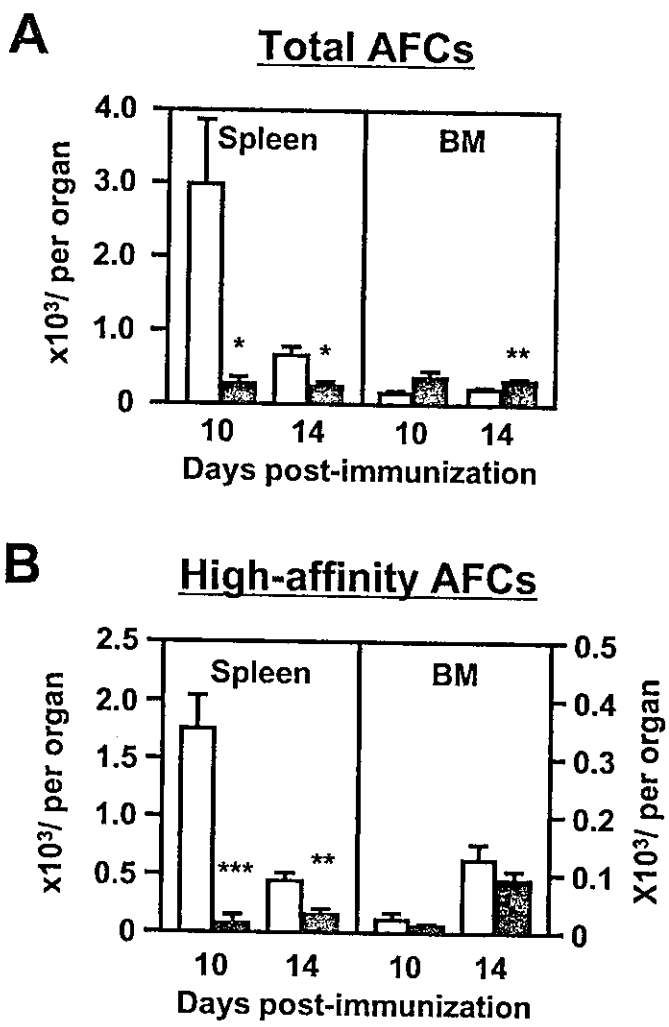

FIG. 5. Effects of TCDD on the generation of total and high-affinity NP-specific AFCs in the spleen and BM. C57BL/6N mice were administered a single oral dose of 20-μg/kg TCDD (closed column) or corn oil as a vehicle (open column) and then were immunized with 100 μg of NP-CG/alum on day 0. Total (A) and high-affinity (B) NP-binding AFCs in the spleen and BM were measured by ELISPOT using NP25-BSA and NP2-BSA as the coating antigens, as described in the Materials and Methods section. Data are presented as means ± SE. (n = 6). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

with NP-CG by TCDD exposure on days 10 and 14 after immunization (Fig. 6). Total NP-specific IgG1 production was, in the same manner as anti-OVA antibody production

FIG. 4. Histopathology of GC in the spleen. The mice were treated as described in Figure 1. (A, B) Splenic sections from vehicle-control mice (A) and TCDD-treated mice (B) on day 14 postimmunization were stained with PNA (arrowhead) and then counterstained with hematoxylin. These images are representative of a minimum of four animals analyzed per group. Bar = 100 μm. (C, D) Proliferating cells in GCs were identified immunohistochemically using anti-Ki-67 Ab (arrow) on day 7 postimmunization in the vehicle-control (C) and TCDD-treated (D) groups. The sections were counterstained with hematoxylin. Bar = 100 μm. (E, F) Apoptotic cells in the GCs were visualized using a histochemical method in the vehicle-control (E) and TCDD-treated (F) groups. Sections of spleen from individual mice on day 7 postimmunization were labeled by the TUNEL method (brown, arrow) in order to identify apoptotic cells, followed by staining with PNA to label the GCs (blue). Bar = 25 μm. (G) The following values were calculated: the area occupied by the Ki-67$^+$ cell clusters and the area of white pulp in the splenic sections obtained from the vehicle-control mice (open column) and TCDD-treated mice (closed column). The data represent the average ratio of the area occupied by the Ki-67$^+$ cell clusters to the area of the white pulp in the spleen. Data are presented as means ± SE (n = 4–5). *$p < 0.05$. (H) The frequency of TUNEL$^+$ cells in GCs in the vehicle-control mice (open column) and TCDD-treated mice (closed column) were calculated. Data are presented as means ± SE (n = 3).

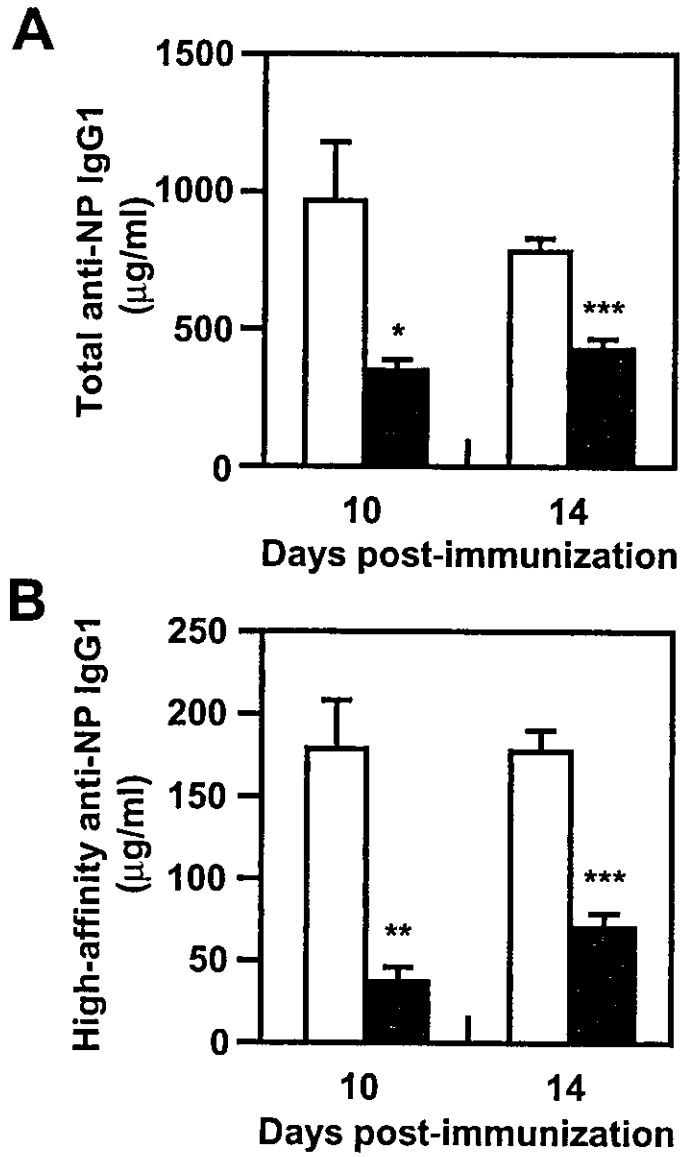

FIG. 6. Suppression of total and high-affinity NP-specific IgG1 production by TCDD. The mice were treated as described in Figure 5. The plasma samples were obtained on days 10 and 14 postimmunization. The levels of total (A) and high-affinity (B) anti-NP IgG1 in the plasma (diluted 1:2700) were measured by ELISA using NP25-BSA and NP2-BSA as the coating antigens, respectively, as described in the Materials and Methods section. Data are presented as means ± SE ($n = 6$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(Fig. 1), suppressed by TCDD (by 64% on day 10 and by 45% on day 14, respectively; Fig. 6A). Furthermore, high-affinity NP-specific IgG1 production was confirmed to be greatly suppressed by TCDD (by 80% on day 10 and by 61% on day 14; Fig. 6B). These results indicate that TCDD exposure suppresses both the generation of high-affinity AFCs in the spleen and high-affinity antibody production in the primary humoral immune response.

DISCUSSION

In this paper, we demonstrated that TCDD suppresses the formation of GCs where antigen-activated B cells proliferate and mature into high-affinity B cells. TCDD has been reported to induce apoptosis in thymocytes and activation-induced cell death in activated T cells (Camacho *et al.*, 2002; Kamath *et al.*, 1997). On the other hand, the suppression of GC formation found in the present paper is not likely to have been caused by apoptosis, but instead was exerted by the inhibition of cellular proliferation. Previous studies have indicated that TCDD suppresses helper T-cell function in an antibody-producing responses (Lundberg *et al.*, 1992; Tomar and Kerkvliet, 1991). Since the antigen-specific helper T-cells interact with the antigen-specific B cells and activate these cells before the formation of GCs, the suppressed T-cell function may be involved in the inhibition of cellular proliferation in the GC formation. T-cell–derived cytokine production was reported to be suppressed by TCDD (Kerkvliet *et al.*, 1996). Recently, we demonstrated, using the same experimental procedure as used in the present paper, that TCDD exposure suppresses the production of helper T-cell–derived cytokines (Ito *et al.*, 2002). The suppression was observed from day 4, which was prior to the GC formation, suggesting that reduced T-cell function is involved in the inhibition of antibody production by TCDD.

Previous studies have shown that TCDD inhibits IgM production by altering later stages of B-cell maturation into antibody production. That is, TCDD hardly affected the proliferation of activated B cells but inhibited their IgM production (Luster *et al.*, 1988; Wood and Holsapple, 1993). In contrast, the present paper shows that TCDD suppresses B-cell proliferation. The difference between the previous studies and ours is thought to be dependent on the experimental systems that were used. In the studies by Luster *et al.* (1998) and Wood and Holsapple (1993), cellular proliferation was examined using isolated B cells directly exposed to TCDD. In contrast, in the present paper, the cells were activated and exposed to TCDD *in vivo*, where many cell types, including T cells, as described above, seems to be involved in the suppression of B-cell proliferation. On the other hand, TCDD has been reported to stimulate the interaction of AhR with p27 kip1 and Rb proteins that are involved in cell-cycle regulation (Kolluri *et al.*, 1999; Puga *et al.*, 2000). These direct pathways may also function in B cells activated and affected by other cell types *in vivo* and may inhibit cellular growth by causing cell cycle arrest. Further studies will be required to fully describe the molecular mechanism of TCDD toxicity in GC formation and the following antibody production.

In this paper, we also demonstrated that TCDD suppresses the high-affinity antibody response. High-affinity AFCs are generated in the GCs of lymphoid organs such as the spleen, and they migrate to the BM, where they survive for several months in mice. In the present paper, we recognized the following time-course: A greater amount of high-affinity AFCs

was observed in the spleen on day 10, with a decrease by day 14; in contrast, only a small number of high-affinity AFCs was observed in the BM on day 10, with an increased number observed on day 14 in the vehicle-control mice. However, TCDD greatly suppressed the number of high-affinity AFCs in the spleen on day 10 and also showed a similar suppressive effect on day 14. Since high-affinity AFCs were shown to be early products of GCs (Smith *et al.*, 1997), the inhibition of GC formation from its onset by TCDD seems to be responsible for the decreased high-affinity AFC generation. In the present paper, the levels of high-affinity antibodies in the plasma were also confirmed to be reduced after TCDD exposure. Since the affinity of antibodies is augmented more than 20 times during the affinity maturation (Takahashi *et al.*, 1998), the suppression of high-affinity antibody production by TCDD is thought to hamper an effective immune reaction.

GCs are also considered to play an essential role for the generation, maturation, and selection of high-affinity memory B cells (MacLennan, 1994). Memory B cells have a long life-span and are responsible for a robust secondary antibody response following re-exposure to an antigen (Ahmed and Gray, 1996). Actually, the inhibition of GC formation has been shown to result in an attenuation of the secondary antibody response (MacLennan, 1994). Therefore, the present results suggest that the suppressive effects of TCDD on GC formation during the primary immune response may influence repeated responses to the same antigen over a long period of time. In support of this possibility, we have previously reported that a single-dose oral administration of TCDD followed by OVA/alum immunization attenuated the secondary antibody response of mice boosted with OVA 3 weeks after the primary immunization (Fujimaki *et al.*, 2002; Nohara *et al.*, 2002). As an example showing a longer-lasting effect of TCDD on the immune system, perinatal exposure of rats to TCDD is reported to cause persistent suppression of a delayed-type hypersensitivity reaction that was observed until 19 months after birth (Gehrs and Smialowicz, 1999). The inhibition of GC formation by TCDD that we reported in this paper may inhibit memory B-cell generation and afford another example of a long-lasting effect of TCDD.

In summary, the present paper demonstrates for the first time that TCDD exposure has suppressive effects on the high-affinity antibody response to antigens in the primary humoral immune response. TCDD was suggested to suppress this response by inhibiting the GC formation at an early stage, before the antibody production from high-affinity AFCs could take place.

ACKNOWLEDGMENTS

We thank Dr. T Azuma (Tokyo University of Science) for the generous gift of standard high-affinity anti-NP IgG1 antibodies. We also thank Dr. N. Nishimura for her kind advice on the immunohistochemical method, Dr. T. Yonemoto for his useful discussion, M. Matsumoto and T. Takano for their technical assistance, and K. Nakazawa for her secretarial assistance. This study was supported in part by AstraZeneca Research Grant 2001 to K.N. and Health and Labour Sciences Research Grants to C.T.

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Chemosphere 52 (2003) 655–662

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Functional activation of arylhydrocarbon receptor (AhR) in primary T cells by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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Received 16 September 2002; received in revised form 3 January 2003; accepted 20 January 2003

Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) exerts diverse adverse health effects by activating the transcription factor arylhydrocarbon receptor (AhR). The activated AhR induces the expression of various genes having xenobiotic responsive elements (XREs) in their enhancer regions, such as the gene for cytochrome P-450 1A1 (CYP1A1). The immune system is sensitively affected by TCDD, while the precise mechanism of how TCDD acts in each immune cell type remains to be determined. The results of previous studies on AhR activation in B cell lines, T cell clones, and thymocytes, which mainly consist of immature T cells, suggested that AhR in mature T cells is inactive, whereas that in B cells and immature T cells act functionally. In the present study, we investigated whether or not TCDD induces the CYP1A1 gene by functionally activating AhR in primary mature T cells in mice. When the splenocytes that contain mature T and B lymphocytes as their predominant cell types or the thymocytes were cultured in the presence of TCDD, each of them showed a similar magnitude of CYP1A1 induction with a peak induction at 4 h. Both mature T cells and B cells that had been separated from total splenocytes also showed CYP1A1 induction at the same magnitude with a peak induction at 4 h. Gene expression of CYP1A1 was observed at 0.1 nM or greater concentrations of TCDD in splenocytes and separated T cells. The induction of CYP1A1 in T cells was confirmed in mice exposed to TCDD. These results indicate that TCDD induces the functional activation of AhR in primary mature T cells in mice.

Keywords: TCDD; CytochromCYP1A1; T cell; B cell

1. Introduction

Dioxins are wide spread and persistent environmental contaminants. The most toxic congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), has been reported to exert diverse adverse effects, such as immunological, reproductive, and neurobehavioral toxicities (Birnbaum and Tuomisto, 2000; Tohyama, 2002). Most of the biological effects of TCDD are thought to be mediated by the activation of ligand-dependent transcription factor AhR (Schmidt and Bradfield, 1996). Upon entering cells, TCDD binds AhR in the cytoplasm, and the ligand/receptor complex translocates to the nucleus, where it heterodimerizes with another transcription factor, arylhydrocarbon receptor nuclear translocator (ARNT). The AhR/ARNT complex then binds to the specific DNA sequence XRE, and alters the expressions of

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0045-6535/03/$ - see front matter
doi:10.1016/S0045-6535(03)00112-7

various genes, including the induction of one of the most sensitive genes, CYP1A1 (Schmidt and Bradfield, 1996; Wilson and Safe, 1998). However, the genes responsible for individual toxicities remain to be identified.

TCDD induces thymus atrophy and suppresses both humoral and cellular immunity (Holsapple et al., 1991; Vos et al., 1997/98; Kerkvliet, 2002). In a recent study using chimeric mice with various combinations of hemopoietic and stromal components from wild-type and AhR-deficient mice (Staples et al., 1998), thymus atrophy is shown to be mediated by AhR activation in the thymocytes but not in the stromal cells. Lowered cytotoxic T cell activity and suppressed antibody production to allogeneic tumor cells and sheep red blood cells (SRBC) are also reported to be dependent on AhR activation (Vorderstrasse et al., 2001), while the primary target cell type has not been determined in these reactions. With regard to the suppressive effect of TCDD on antibody production, primary effects on both B cells and T cells have been reported (reviewed by Holsapple et al., 1991; Kerkvliet, 2002). Holsapple and coworkers studied the effects of TCDD on a system in which unprimed splenocytes obtained from vehicle-control or TCDD-administered mice were primed in vitro in the presence or absence of TCDD. In the in vitro immunization system, these researchers showed that TCDD directly affects B cells, rather than T cells or macrophages, in the suppression of antibody production (Holsapple et al., 1991). In murine splenocytes (Williams et al., 1996) and human peripheral lymphocytes (Vanden Heuvel et al., 1993; Lang et al., 1994), TCDD was shown to induce the functional activation of AhR. TCDD was also reported to induce CYP1A1 in several B cell lines (Waithe et al., 1991; Masten and Shiverick, 1996). These studies suggest that TCDD activates AhR in B cells and modulates their function. On the other hand, Kerkvliet et al. indicated that TCDD primarily affects regulatory T cells in the antibody production elicited in vivo (Kerkvliet and Brauner, 1987; Tomar and Kerkvliet, 1991). Our recent studies further suggested that the decreased level of cytokines produced by antigen-activated T cells and Th2 cells is involved in the suppression of antibody production by TCDD (Fujimaki et al., 2002; Nohara et al., 2002; Ito et al., 2002). TCDD is shown to activate AhR and induce CYP1A1 gene expression in thymocytes (Germolec et al., 1996; Stephen et al., 1997). However, another previous study showed that AhR in a T cell clone is functionally inactive (Lawrence et al., 1996), which suggests that mature T cells do not directly respond to TCDD.

Since AhR activation in primary T cells has not been examined so far, we have investigated whether AhR in primary T cells in mice can elicit the AhR-mediated gene expressions upon TCDD exposure by measuring CYP1A1 expression as an indicator of the functional activation of AhR.

2. Materials and methods

2.1. Animals

Female C57Bl/6N mice were purchased from Clea Japan Inc. (Tokyo). They were maintained under controlled conditions with a temperature of 24 ± 1 °C, humidity of $45 \pm 5\%$ and a 12/12 h light/dark cycle. They were subjected to acclimatization for at least three days before use. The animals were handled throughout this study according to the guidelines for animal care of NIES.

2.2. Cell preparation

Single cell suspensions of the thymus and spleen were prepared as described previously (Nohara et al., 2002). The number of cells was counted with a hemocytometer following staining with trypan blue.

2.3. Separation of T and B cells from splenocytes

T and B cells were separated from splenocytes with a MiniMACS separation system (Miltenyi Biotec Inc., Auburn, CA) according to the manufacturer's instructions. Splenocytes were labeled with biotinylated anti-mouse CD3ε (Pharmingen, San Diego, CA) followed by Streptavidin MicroBeads (Miltenyi Biotec Inc.) for T cell separation, or labeled with MACS CD45R (B220) MicroBeads (Miltenyi Biotec Inc.) for B cell-separation.

In order to assess their purity, the separated cells were stained with phycoerythrin-conjugated (PE-) anti CD4 plus fluorescein isothiocyanate-conjugated (FITC-) anti CD8, or FITC-anti B220 (B cell marker), and measured using a FACSCalibur (B–D) as described previously (Nohara et al., 1997). All of the monoclonal antibodies were purchased from Pharmingen (San Diego, CA).

2.4. Primary cell culture

Total splenocytes or thymocytes were cultured at 2×10^7 viable cells/3 ml complete medium containing 0.05% DMSO with or without 10 nM TCDD in a tissue culture dish with a 6 cm-diameter. T cells or B cells isolated from total splenocytes were cultured at 1×10^7 viable cells/1.5 ml in a 6-well plate. TCDD in DMSO (purity, 99%) was purchased from Cambridge Isotope Laboratories (Andover, MA). After the culturing, the

cells were detached from the dish by pipetting and used to prepare total RNA as described below.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using ISOGEN (Nippon Gene, Toyama) according to the manufacturer's protocol. The quality of total RNA was checked by electrophoresing it and confirming the existence of clear bands representing 28S and 18S ribosomal RNAs. Semi-quantitative RT-PCR was performed using an RNA LA PCR kit (AMV) ver1.1 (TaKaRa Biomedicals, Tokyo) as reported previously (Nohara et al., 2000a). Five hundred ng of total RNA was reverse-transcribed using oligo dT-adaptor primer following the manufacturer's instructions. PCR was carried out with β-actin or hypoxanthine phosphoribosyltransferase (HPRT), the housekeeping gene, to standardize the mRNA concentration. PCR cycles were titrated to document the linearity of the signal strength, and PCR products were quantified during the exponential phase of amplification. For this purpose, the cDNA of interest was first amplified for several cycles, and then the primers for a housekeeping gene were added to the reaction mixture followed by additional cycles. The samples were heated at 94 °C for 2 min and cycled at 94 °C for 0.5 min, 60 °C for 0.5 min, and 72 °C for 0.5 min, and further extended at 72 °C for 10 min after the final cycle. The total nember of cycles for each genes were as follows: 30 for CYP1A1, 33 for AhR and ARNT, and 26 for β-actin and HPRT. In some experiments, the housekeeping gene was amplified in a separate tube. PCR products were quantified as reported previously (Nohara et al., 2000a). The sequences of primers are listed in Table 1.

2.6. TCDD administration

In order to confirm the CYP1A1 induction in T cells in vivo, TCDD was administered to mice. TCDD in nonane (purity 99%, Cambridge Isotope Laboratories) was diluted with corn oil (Sigma, St. Louis, MO) at a concentration of 2 μg/ml and given orally to three mice from each group at a single dose of 20 μg/kg body weight. One or three days later, splenocytes and T cells were prepared from the spleens. The cells from three mice in each group were combined, and the total RNAs were prepared as described above.

3. Results

3.1. Expression of AhR, ARNT and CYP1A1 mRNAs in thymocytes and splenocytes following in vitro TCDD exposure

Previous studies reported that administering TCDD to rats induces CYP1A1 expression in thymocytes, but not in splenocytes (Germolec et al., 1996; Stephen et al., 1997). Another study showed that in vitro exposure of TCDD induces activation of AhR in murine splenocytes (Williams et al., 1996). In the present study, we first compared the magnitude and time-course of CYP1A1 expression in murine thymocytes and splenocytes exposed to TCDD in vitro. As shown in Fig. 1, the CYP1A1 expression of thymocytes was markedly induced as of 2 h after the addition of 10 nM TCDD. The expression peaked at 4 h and gradually decreased thereafter until 24 h. The CYP1A1 expressions in splenocytes were similar to those of thymocytes in terms of the magnitude and the time course.

We also examined AhR and ARNT mRNAs in the thymocytes and splenocytes cultured in the presence or absence of TCDD (Figs. 2 and 3). In up to 24 h of culture, similar amounts of AhR (Fig. 2) and ARNT (Fig. 3) mRNA were detected in cultures of thymocytes or splenoytes irrespective of the presence or absence of TCDD (Fig. 2).

3.2. Induction of CYP1A1 gene by TCDD in primary T and B cells

Next, we examined CYP1A1 induction in primary T cells and B cells that were separated from the total splenocytes. The purities of the T cells and B cells were both approximately 95%, and their viabilities were around 90%. TCDD was shown to induce the CYP1A1 gene in T cells and B cells, and the patterns of the two

Table 1
Oligonucleotide primers used for PCR analysis

Gene	5′ Primer	3′ Primer	Product size
	5′ 3′	5′ 3′	
CYP1A1	ccatgaccgggaactgtgg	tctggtgagcatcctggaca	344
AhR	acataacggacgaaatcctgacc	tcaactctgcaccttgcttagga	710
ARNT	gctataatcattcccaggtttct	cattgttgtaggtgttgctttgg	768
HPRT	Gctggtgaaaaggacctct	cacaggactagaaaatat	249
β-actin	cctctatgccaacacagt	agccaccgatcacaacag	153

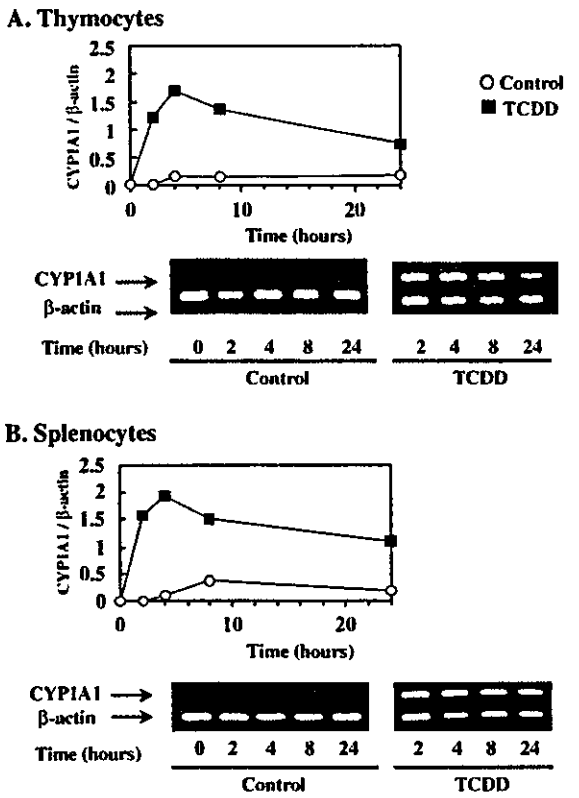

Fig. 1. Expression of CYP1A1 mRNA in thymocytes (A) and splenocytes (B) cultured in the presence of 10 nM TCDD. Total RNA was prepared, reverse-transcribed and amplified by PCR for CYP1A1 in conjunction with β-actin as an internal standard as described in Section 2. The expression of CYP1A1 was quantified by densitometrically scanning gel images and normalized to β-actin mRNA. This figure is representative of two separate experiments.

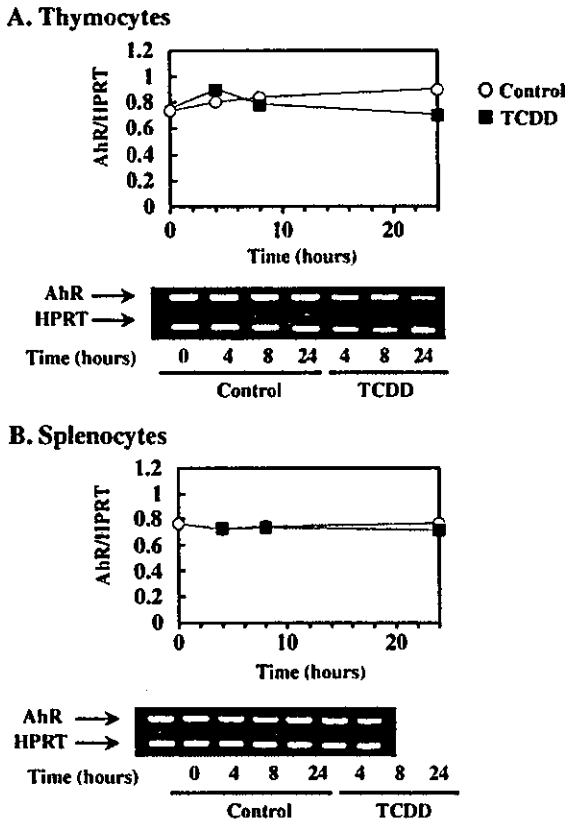

Fig. 2. Expression of AhR mRNA in thymocytes (A) and splenocytes (B) cultured in the presence of 10 nM TCDD. The expression of AhR gene was quantified as described in the legend for Fig. 1, using HPRT as an internal control. This figure is representative of three separate experiments.

time-courses were similar to each other (Fig. 4) and also similar to that of total splenocytes (Fig. 1).

3.3. Dose-dependency of CYP1A1 induction by TCDD

Since our results showed that TCDD at 10 nM induces the CYP1A1 gene in both splenocytes and T cells as described above, we next examined the dose-dependency of the gene expression at 4 h. Whereas TCDD at 0.01 nM did not induce CYP1A1 expression in splenocytes, 0.1 nM or higher doses of TCDD markedly induced the gene (Fig. 5). TCDD also caused CYP1A1 expression at 0.1 nM and higher concentrations of TCDD in the T cells.

3.4. CYP1A1 induction in T cells following in vivo exposure to TCDD

The CYP1A1 induction was also confirmed in splenocytes and T cells prepared from mice to which TCDD had been administered. One day and three days after TCDD administration, T cells as well as splenocytes showed significant amounts of AhR mRNA (Fig. 6).

All the results described above indicated that AhR in T cells is functionally activated by TCDD exposure in vitro and in vivo.

4. Discussion

TCDD is reported to suppress T cell functions, such as cytotoxic T cell activity (Hanson and Smialowicz, 1994; De Krey and Kerkvliet, 1995) and delayed-type hypersensitivity (Gehrs and Smialowicz, 1999). Previous studies (Kerkvliet and Brauner, 1987; Tomar and Kerkvliet, 1991; Lundberg et al., 1992; Shepherd et al., 2000) and our recent studies (Fujimaki et al., 2002; Nohara et al., 2002; Ito et al., in press) have suggested that T cells are also responsible for the suppression of antibody production by TCDD. However, it has not been clearly shown if TCDD can directly affect T cells, or if it indi-

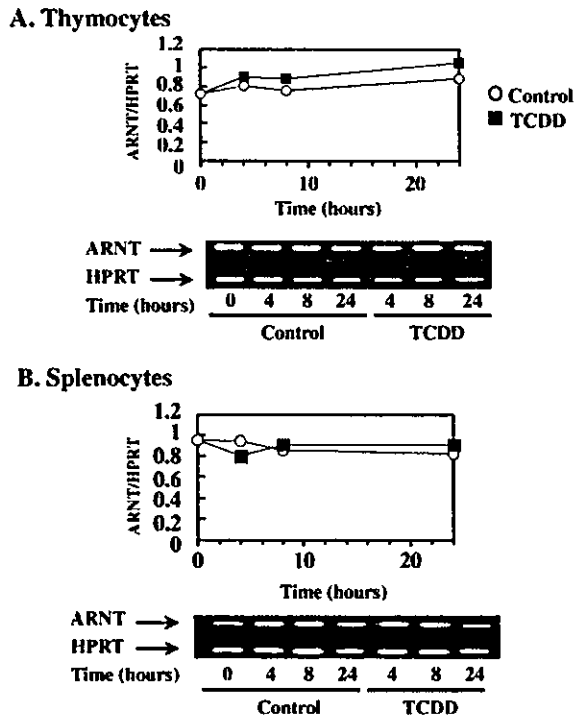

Fig. 3. Expression of ARNT mRNA in thymocytes (A) and splenocytes (B) cultured in the presence of 10 nM TCDD. The expression of ARNT gene was quantified as described in the legend for Fig. 2. This figure is representative of two separate experiments.

rectly suppresses their function by inhibiting the functions of other cell types. Although many kinds of lymphoid cell lines have been reported not to express AhR (Hayashi et al., 1995; Salomon-Nguyen et al., 2000), several human B cell lines such as BCR-5 and PJS-91 express AhR and elicit CYP1A1 induction in response to TCDD (Waithe et al., 1991; Masten and Shiverick, 1996). In a murine B cell line, CH12.LX, TCDD induces the binding of the AhR/ARNT complex to XRE and inhibits LPS-induced IgM secretion (Sulentic et al., 2000). TCDD also induces the binding of AhR/ARNT to the XRE sequence or CYP1A1 gene expression in human and murine monocytic cell lines such as U937, HEL/s and IC21 (Hayashi et al., 1995; Lawrence et al., 1996). On the other hand, a previous study reported that T cell clones that were established from TCDD-responsive mouse strains expressed AhR, but that their AhR did not bind to XRE and was functionally inactive (Lawrence et al., 1996). These findings suggest that TCDD activates AhR in B cells but not in T cells, and that the effects on T cells are indirect. In murine primary T cells, however, we have shown here that TCDD directly activates AhR and induces CYP1A1 at the same magnitude as in primary B cells. Our results precluded the possibility that T cells cannot be a direct target of

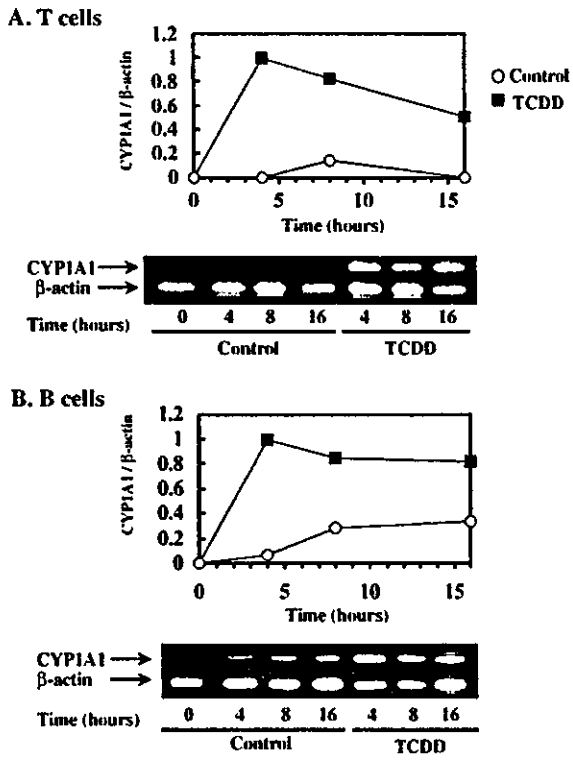

Fig. 4. TCDD induces CYP1A1 mRNA in T cells to the same extent as in B cells. T cells (A) and B cells (B) were isolated from the spleen and cultured in the presence of 10 nM TCDD. The expression of CYP1A1 gene was quantified as described in the legend for Fig. 1. This figure is representative of two separate experiments.

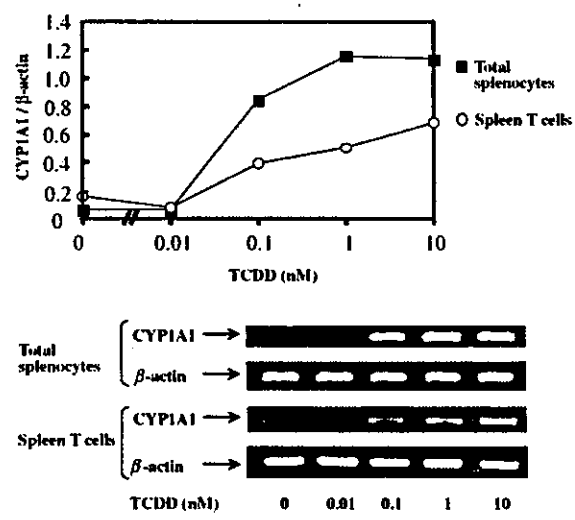

Fig. 5. Dose-dependency of CYP1A1 induction by TCDD. Total splenocytes and T cells isolated from splenocytes were cultured with 0–10 nM TCDD for 4 h. The expression of CYP1A1 gene was quantified as described in the legend for Fig. 1. This figure is representative of two separate experiments.

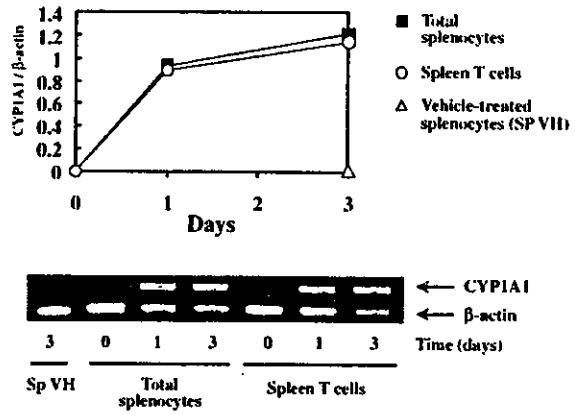

Fig. 6. Expression of CYP1A1 mRNA in T cells prepared from TCDD-exposed mice. Mice were administered a single oral dose of 20 μg/kg, and total splenocytes and T cells were prepared one or three days later as described in Section 2. The expression of CYP1A1 gene was quantified as described in the legend for Fig. 1.

TCDD-induced immunotoxicity because of their inability to elicit AhR-dependent gene expressions following TCDD exposure.

In terms of AhR activation in primary splenocytes, previous studies have reported that rat splenocytes are insensitive to TCDD exposure. In vitro exposure to a 0.1 nM or higher concentration of TCDD was shown to induce CYP1A1 expression and its enzyme (ethoxyresorufin-O-deethylase) activity in rat thymocytes, but not in splenocytes (Stephen et al., 1997). In another study, the oral administration of 100 μg TCDD/kg to rats resulted in the induction of CYP1A1 in the whole spleen, whole thymus and isolated thymocytes, but not in the isolated splenic lymphocytes or blood lymphocytes (Germolec et al., 1996). Previously, we orally administered TCDD to pregnant rats on gestational day 15 and examined CYP1A1 induction in the spleen and thymus of the offspring. We detected the induction of CYP1A1 mRNA in the spleen of offspring on postnatal day 5 following maternal exposure to 800 ng/kg of TCDD; however, the induction in the spleen was very weak and about 100-fold less than in the thymus (Nohara et al., 2000b). This finding also supports the observation that rat splenocytes are much less sensitive to TCDD than are the thymocytes. On the other hand, it is reported that TCDD exposure in vivo induces functional activation of AhR in murine splenocytes (Williams et al., 1996). In the present study, we further showed that TCDD exposure in vitro and in vivo induces CYP1A1 gene expression in the murine splenocytes and in T cells, and that the magnitude of the gene expression induced by TCDD in the splenocytes was at a level similar to that in the thymocytes. TCDD is reported to suppress a plaque-forming cell (PFC) response by splenocytes to SRBC in mice with 0.7 μg/kg of ED50, while it did not suppress the response in rats at doses up to 30 μg/kg (Smialowicz et al., 1994). This insensitivity in T cell-dependent antibody production to TCDD may be partly related to the low responsiveness of splenocytes to TCDD in rats.

In the present study, we also examined the time-course and dose dependency of CYP1A1 gene expression in the lymphoid cells after TCDD exposure in vivo. TCDD rapidly induced CYP1A1 gene within 2 h after TCDD exposure in vitro, and the induction peaked at 4 h in the murine splenocytes and thymocytes. Similar kinetics were also observed in the T cells and B cells. The splenocytes and spleen T cells showed CYP1A1 induction in the presence of 0.1 nM or higher concentrations of TCDD in the culture. The induction level of CYP1A1 protein and the enzyme activity in lymphoid cells are shown to be much lower than in the liver (Germolec et al., 1996; Dey et al., 2001). TCDD was also reported to induce CYP1A1 activity within 2 h at concentrations as low as 0.01 nM in the murine hepatoma Hepa-1 cell line (Riddick et al., 1994). These studies suggest that hepatocytes respond to TCDD in a time-course similar to that seen in lymphoid cells, but more sensitively and vigorously than lymphoid cells. The murine liver contained 2.0-fold more AhR protein and 2.3-fold more receptor mRNA than did the spleen (Williams et al., 1996), which is thought to contribute to the higher sensitivity and abundance in the induction of CYP1A1 by TCDD in the liver. Other factors involved in the formation and activation of AhR/ARNT heterodimer, like the states of phosphorylation (Berghard et al., 1993; Park et al., 2000), the coactivator CBP/p300 (Kobayashi et al., 1997) the binding to which is competed by other transcription factors such as NF-κB (Ke et al., 2001), and the degradation of translocated AhR (Davarinos and Pollenz, 1999; Ma, 2001) may be related to the different magnitude of CYP1A1 induction in the liver and the lymphoid cells. In Hepa-1 cells, TCDD is known to rapidly deplete AhR protein without altering the receptor mRNA levels (Giannone et al., 1998). The mechanism of this depletion may cause the suppression of CYP1A1 expression after the peak induction at 4 h observed in the present study.

In the experiments shown in Fig. 5, T cells isolated from splenocytes and cultured for 4 h showed weaker CYP1A1 induction than did the splenocytes. On the other hand, the freshly isolated T cells showed CYP1A1 induction at the same magnitude as the splenocytes (Fig. 6). These apparent discrepancies may be due to a difference in the state of the T cells in the two experiments. That is, in the experiment shown in Fig. 5, T cells were isolated and then cultured in the presence of TCDD to induce CYP1A1. On the other hand, in the experiment shown in Fig. 6, T cells were exposed to TCDD in vivo and their RNA was examined immediately after the

isolation of cells. Thus, the isolated T cells in the experiment shown in Fig. 5 was possibly not be able to fully respond to TCDD during the culture after the isolation procedure. Alternatively, the disparity may be due to the difference in the concentrations of TCDD to which the cells were exposed in vitro and in vivo. Further study is needed to clarify the level of CYP1A1 induction in T cells and total splenocytes including other cell types.

In summary, we have shown that TCDD directly induces functional activation of AhR in primary mature T cells in mice. This finding supports the possibility that T cells can be a direct target in the suppression of antibody production by TCDD.

Acknowledgements

The authors wish to thank Dr. S. Tsukumo for his useful discussions. They also thank Ms. M. Matsumoto for her secretarial assistance.

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Distinct response to dioxin in an arylhydrocarbon receptor (AHR)-humanized mouse

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Edited by Ronald M. Evans, The Salk Institute for Biological Studies, San Diego, CA, and approved March 19, 2003 (received for review December 26, 2002)

There are large inter- and intraspecies differences in susceptibility to dioxin-induced toxicities. A critical question in risk assessment of dioxin and related compounds is whether humans are sensitive or resistant to their toxicities. The diverse responses of mammals to dioxin are strongly influenced by functional polymorphisms of the arylhydrocarbon receptor (AHR). To characterize responses mediated by the human AHR (hAHR), we generated a mouse possessing hAHR instead of mouse AHR. Responses of these mice to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3-methylcholanthrene were compared with the responses of naturally sensitive (C57BL/6J) and resistant (DBA/2) mice. Mice homozygous for h*AHR* exhibited weaker induction of AHR target genes such as *cyp1a1* and *cyp1a2* than did C57BL/6J ($Ahr^{b\text{-}1/b\text{-}1}$) mice. DBA/2 ($Ahr^{d/d}$) mice were less responsive to induction of *cyp* genes than C57BL/6J mice. hAHR and DBA/2 AHR exhibit similar ligand-binding affinities and homozygous h*AHR* and $Ahr^{d/d}$ mice displayed comparable induction of AHR target genes by 3-methylcholanthrene. However, when TCDD was administered, a greatly diminished response was observed in homozygous h*AHR* mice compared with $Ahr^{d/d}$ mice, indicating that hAHR expressed in mice is functionally less responsive to TCDD than DBA/2 AHR. After maternal exposure to TCDD, homozygous h*AHR* fetuses developed embryonic hydronephrosis, but not cleft palate, whereas fetuses possessing $Ahr^{b\text{-}1}$ or Ahr^{d} developed both anomalies. These results suggest that hAHR may define the specificity of the responses to various AHR ligands. Thus, the hAHR knock-in mouse is a humanized model mouse that may better predict the biological effects of bioaccumulative environmental toxicants like TCDD in humans.

human | C57BL6/J | DBA/2 | CYP1A1

Polycyclic aromatic hydrocarbons (PAH) and halogenated aromatic hydrocarbons (HAH), including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), benzo[*a*]pyrene, and polychlorinated biphenyls, are ubiquitous environmental toxicants whose chemical stability and lipophilicity make them highly persistent in the environment and in living organisms. These groups of chemicals cause various toxicological and biological responses, typified by teratogenesis, thymic atrophy, severe epithelial disorders, wasting syndrome, tumor promotion, and induction of xenobiotic-metabolizing enzymes in experimental animals (1, 2). The toxicities of these compounds are mediated by a conserved signaling pathway (1–4) through binding to and activation of the arylhydrocarbon receptor (AHR). AHR activation in turn mediates a transcriptional response for genes regulated by this transcription factor (5–8). Despite strong conservation of this pathway, there are wide inter- and intraspecies differences in the toxicological responses to AHR ligands (9–11). The molecular basis for these species and strain differences appears to relate to polymorphisms in AHR. Factors influencing susceptibility to the toxicity of TCDD have been studied in several animal models. There is a 10-fold difference in susceptibility between the dioxin-sensitive C57BL/6 and the resistant DBA/2 strains of mice that can be explained by polymorphic variations in the ligand-binding domain and in the C-terminal region of the AHR molecule of each strain (9, 12–14). Response to TCDD in the Long–Evans (sensitive) and Han/Wistar rats (resistant) differs by >1,000-fold due to a critical point mutation in the transactivation domain in the AHR of the Han/Wistar rat (15–17).

The effects of TCDD on humans are less well understood, although high incidences of chloracne, teratogenicity, and abortion have been associated with high blood concentrations of dioxin and related compounds in residents of regions where industrial accidents or extensive use of dioxin-containing defoliants have resulted in human exposures (3). Increased levels of dioxin in the body have been reported recently to be associated with abnormal sex ratio of newborns nearly 25 years after the accident in Seveso, Italy (18). Because the AHR primarily mediates the pleiotropic manifestations of dioxin exposure, characterization of the structural and functional properties of the human AHR (hAHR) is critical for understanding the types and magnitudes of human responses to various PAH/HAHs.

To date, *in vitro* characterization of the hAHR has provided ambiguous insights into human sensitivity to dioxin. The dissociation constant (K_d) of hAHR for TCDD was comparable to that of TCDD-resistant DBA/2 AHR (9, 19), suggesting that humans might be resistant to TCDD. By contrast, high homology of the human receptor to the AHR of the guinea pig, which is the most sensitive animal to TCDD, suggests a high responsiveness of humans to the toxin (20). Ligand specificity of hAHR was also examined and compared with those of zebrafish and rainbow trout AHRs using polychlorinated dibenzo-*p*-dioxin, dibenzofuran, and biphenyl congeners as test ligands. These studies revealed that mono-ortho polychlorinated biphenyls activated hAHR but were not very effective in activating either zebrafish or rainbow trout AHRs (21).

Assessment of human responses *in vivo* to unintended exposures to various PAH/HAHs has been hampered by limited exposure assessments and toxicological follow-up. Observational studies after intentional exposures have not been and should not be conducted. To gain stronger insight into the hazards to human health posed by compounds interacting with the hAHR *in vivo*, we generated a mouse model that harbors the hAHR cDNA instead of the mouse *AhR* gene. This mouse may reveal a humanized susceptibility to chemical toxicities. In response to challenges with 3-methylcholanthrene (3-MC) and TCDD, two prototypical AHR ligands, the hAHR knock-in

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: AHR, aryl hydrocarbon receptor; hAHR, human AHR; h*AHR*, human AHR knock-in allele; Ahr^{d}, DBA/2 *Ahr* allele; $Ahr^{b\text{-}1}$, C57BL/6 *Ahr* allele; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 3-MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbons; HAH, halogenated aromatic hydrocarbons; ES, embryonic stem; GD, gestation day.

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mouse displayed a distinct response profile compared with control animals harboring either the C57BL/6 *Ahr* allele ($Ahr^{b\text{-}1}$) (TCDD-sensitive C57BL/6J AHR) or the DBA/2 *Ahr* allele (Ahr^{d}) (TCDD-resistant DBA/2 AHR) in the same C57BL/6J genetic background. Although gene expression responses mediated by hAHR from 3-MC were comparable to that by DBA/2 AHR, the homozygous human AHR knock-in allele (h*AHR*) mouse was the weakest responder to TCDD among the three strains examined. These results suggest that hAHR molecules expressed in mice retain a functional human specificity that can be distinguished from the murine AHR and provide important insights into the toxicological susceptibility of humans to AHR ligands released into the environment.

Materials and Methods

Construction of the hAHR Knock-in Vector. The hAHR knock-in vector was constructed by using 129SV/J mouse *Ahr* genomic clones and hAHR cDNA as described (22). A 2-kb *Bam*HI/*Hph*I fragment containing the 129SV/J *Ahr* promoter was ligated to the hAHR cDNA (9, 22). The *neo* gene cassette was fused to the 3′ end of the hAHR cDNA in a reverse orientation, followed by a 6.5-kb *Hind*III/*Eco*RI fragment of the 129SV/J *Ahr* gene. This construct was ligated to the thymidine kinase cassette on the 5′ end.

Generation of hAHR Knock-in Mice. The knock-in vector was electroporated into E14 embryonic stem (ES) cells (23). A pair of primers (sense, GTATGCATTACCATGCTCCCATTCTGCTGG; antisense, ACATCTTGTGGGAAAGGCAGCAGGCTAGCC) was used for PCR screening. After confirmation by Southern blot analysis, positive clones were injected into blastocysts. Heterozygous hAHR knock-in mice were backcrossed into a C57BL/6J background up to the seventh generation and interbred to yield heterozygous and homozygous h*AHR* and wild-type $Ahr^{b\text{-}1/b\text{-}1}$ mice. The genotype of each pup was determined by PCR, with a common sense primer; 5′-ATGAGCAGCGGCGCCAACAT-3′, an antisense primer for endogenous *Ahr* allele; 5′-GCTAGACGGCACTAGGTAGG-3′, and an antisense primer for targeted allele; 5′-CAGGTAACTGACGCTGAGCC-3′. PCR amplification was carried out for 30 cycles under the following conditions; 94°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec.

Chemicals and Animals. TCDD (99.5% pure) and 3-MC were purchased from Cambridge Isotope Laboratories (Andover, MA) and Wako Pure Chemical (Osaka), respectively. D2N-*Ahrd* mice and inbred C57BL6/J mice were procured from The Jackson Laboratory. *Ahr*-null mutant mice used in this study were generated by Y.F.-K (22).

RNA Blotting Analyses. We isolated total RNA by using ISOGEN (Nippon Gene, Tokyo) and purified polyA RNA by using an Oligotex-MAG mRNA purification kit (Takara Biotechnology, Tokyo). For detection of Ahr mRNA, 5 μg of polyA RNA per lane was applied, and a portion of mouse $Ahr^{b\text{-}1}$ cDNA (*Bpu*1102I-*Kpn*I; 734-bp) encoding the PAS domain was used for a probe. This nucleotide sequence is conserved with 83% homology to the corresponding hAHR cDNA (12, 24). To examine the inducibility of *CYP1A1* and *CYP1A2*, 6-week-old littermates ($Ahr^{b\text{-}1/b\text{-}1}$ and homozygous h*AHR*) and D2N-*Ahrd* ($Ahr^{d/d}$) mice were given a single i.p. injection of 80 mg/kg 3-MC or 100 μg/kg TCDD. Mice were killed by cervical dislocation 24 h after injection. Ten micrograms of total RNA per lane was hybridized with the appropriate mouse cDNA probes (25).

RT-PCR Analyses of hAHR and Murine Ahr mRNA Expression in Embryos. Total RNA was isolated from palate and kidney of gestation day (GD)18.5 fetus by using ISOGEN. One microgram of the total RNA was reverse-transcribed into cDNA with Superscript-II reverse transcriptase (Life Technologies, Gaithersburg, MD) and random hexamers at 42°C for 50 min. The resulting cDNAs were subjected to 30 cycles of PCR by using the specific primers for the gene for the h*AHR* (5′ primer, 5′-GTAAGTCTCCCTTCATACC-3′; 3′ primer, 5′-AGGCACGAATTGGTTAGAG-3′), mouse *Ahr* (5′ primer, 5′-CTTTGCTGAACTCGGCTTGC-3′; 3′ primer, 5′-TTGCTGGGGGCACACCATCT-3′) and GAPDH (5′ primer, 5′-CCCCTTCATTGACCTCAACTACATGG-3′; 3′ primer, 5′-GCCTGCTTCACCACCTTCTTGATGTC-3′). The reaction was performed under the following conditions: 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec.

Immunohistochemical Analysis of hAHR Expression. Immunohistochemical analysis was performed as described (26). Lungs were fixed in 0.1 M phosphate buffer containing 4% paraformaldehyde for 24 h and embedded in paraffin. Sections were incubated with anti-AHR antibody in 1:200 dilution, which reacts with both human and mouse AHR (N-19; Santa Cruz Biotechnology). AHR immunoreactivity was visualized with the avidin-biotin-peroxidase system (Vector Laboratories).

TCDD Treatment and Evaluation of Teratogenesis. TCDD treatment was performed as described (22). On GD12.5, pregnant mice were given TCDD by i.p. administration at a dose of 40 μg/kg body weight (27). On GD18.5, the fetuses were taken out and fixed in 4% paraformaldehyde. The palatal structure was examined by cutting between the upper and lower jaws. The kidneys were sliced longitudinally and stained with hematoxylin/eosin. The presence and severity of hydronephrosis in each kidney was examined under a microscope as previously described (28) by using severity scores ranging from 0 to 3+ (0, normal kidney; 1+, slight decrease in length of papilla; 2+, marked decrease in length of papilla with some loss of renal parenchyma; 3+, complete absence of papilla, shell of kidney remaining with only a small amount of renal parenchyma). For statistical analysis, pairwise comparisons were made by Mann–Whitney *U* test, by using StatView for Macintosh version 5.0 (SAS Institute, Cary, NC).

Results

Replacement of the Mouse *Ahr* Gene with hAHR cDNA. We hypothesize that the specific functional characteristics of the hAHR molecule form the principal basis for the pattern of human responses to xenobiotics that interact with the AHR. To characterize responses mediated by hAHR, we generated a mouse possessing hAHR instead of murine AHR. hAHR cDNA was introduced into the mouse *Ahr* locus by homologous recombination, thereby disrupting the mouse *Ahr* gene (Fig. 1*A*). The cDNA was recombined so that hAHR is expressed under the control of the endogenous mouse *Ahr* promoter. Sixteen independent G418-resistant ES clones were obtained of 240 by PCR screening, and seven clones were further confirmed as correctly targeted ES cells by genomic DNA blot analysis. *Eco*RI-digested genomic DNA from the three representative positive clones (nos. 14, 25, and 58) revealed 11.0- and 6.2-kb fragments derived from the intact and targeted alleles, respectively, when hybridized with the 5′-external probe (Fig. 1*B*).

These three clones harboring h*AHR* were used for the generation of chimeric offspring. The male chimeras were mated with C57BL/6J females to obtain heterozygotes of the h*AHR* allele. They were subsequently bred into a C57BL/6J genetic background through the seventh generation, and the backcrossed heterozygous animals were interbred to yield hAHR homozygous mutant mice. The transmission of the targeted allele to the offspring was confirmed by genomic DNA blot analysis, and the genotype was determined by PCR by using tail DNA as a template (Fig. 1 *C* and *D*). Of 124 offspring obtained

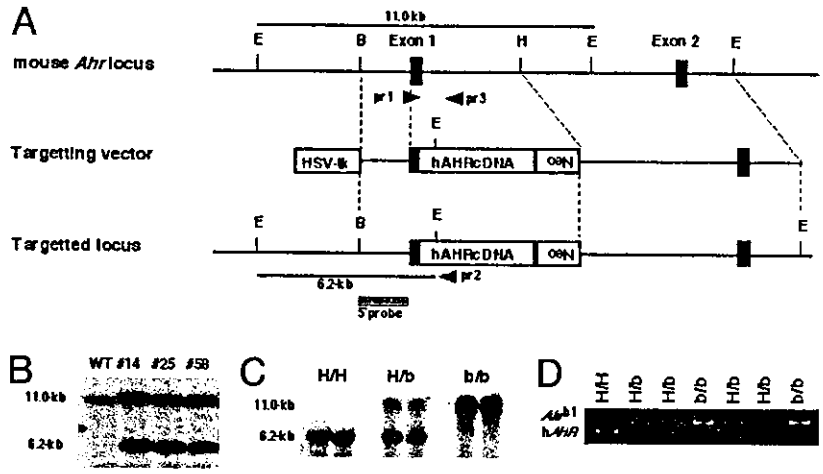

Fig. 1. Generation of the hAHR knock-in mouse. (*A*) Strategy for hAHR cDNA knock-in by homologous recombination. E, H, and B are restriction sites for *Eco*RI, *Hind*III, and *Bam*HI, respectively. Neo indicates the neomycin-resistance gene, and HSV-tk is the thymidine kinase gene under control of the herpes simplex virus promoter. The 5′-genomic probe used for DNA blot analysis is indicated by the hatched box. The positions of wild-type (pr 3) and mutant allele-specific (pr 2) primers and the common primer (pr 1) used in the genotyping PCR are indicated by arrowheads. The *Eco*RI restriction fragments detected with the 5′-genomic probe in the wild-type and targeted allele are denoted by horizontal bars. (*B*) DNA blot analyses of three recombinant ES clones. Genomic DNA was prepared from the ES clones (nos. 14, 25, and 58), and aliquots (10 μg) were digested by *Eco*RI. *Eco*RI digestion generated 11.0- and 6.2-kb bands for the wild-type and targeted alleles, respectively, by using the 5′-genomic probe. (*C*) Genotyping of the *Ahr* gene by DNA blot analysis. Genomic DNA was extracted from the tails of heterozygous and homozygous h*AHR* mice and wild-type $Ahr^{b\text{-}1/b\text{-}1}$ mice and digested by *Eco*RI for DNA blot analysis. (*D*) Genotyping of littermates from the intercrosses of heterozygotes. PCR fragments of wild-type amplified with pr1 and pr3 ($Ahr^{b\text{-}1}$; 280 bp) and mutant allele with pr1 and pr2 (h*AHR*; 240 bp) as depicted in *A*. H/H, H/b, and b/b indicate homozygous and heterozygous h*AHR* mice and wild type ($Ahr^{b\text{-}1/b\text{-}1}$), respectively.

from heterozygous matings, wild-type ($Ahr^{b\text{-}1/b\text{-}1}$), heterozygous, and homozygous h*AHR* mutant mice numbered 29, 71, and 24, respectively, conforming to the expected Mendelian inheritance ratio. Homozygous h*AHR* mice were viable, and no abnormalities were observed.

Expression of hAHR in hAHR Knock-in Mice. The expression of hAHR and mouse Ahr mRNAs was examined by RNA blot analysis by using polyA RNAs isolated from major AHR-expressing organs including liver, lung, kidney, intestine, and thymus (Fig. 2*A*). A cDNA fragment encoding the PAS domain of C57BL/6 AHR, which shows 83% homology with the corresponding human molecule, was used as a common probe for detecting both mouse Ahr and hAHR mRNAs. The larger band detected in heterozygous h*AHR* mice and wild-type $Ahr^{b\text{-}1/b\text{-}1}$ mice corresponds to the 5.4-kb transcript derived from the endogenous $Ahr^{b\text{-}1}$ gene, and the shorter 5.0-kb transcript observed in heterozygous and homozygous h*AHR* is derived from the hAHR knock-in allele. This result establishes that, whereas the homozygous h*AHR* mouse lacks mRNA for murine *Ahr*, it expresses mRNA for h*AHR*. Further, the level of expression of hAHR mRNA is comparable to that of endogenous murine Ahr mRNA in the other strains.

The embryonic expressions of mouse Ahr and hAHR mRNAs were examined by RT-PCR at the stage of GD18.5. As observed in the RNA blot analysis of adult tissues, the hAHR mRNA was expressed in the embryonic palate and kidney of homozygous and heterozygous h*AHR* mice. The abundance was comparable with that of the mouse Ahr mRNA expressed in $Ahr^{b\text{-}1/b\text{-}1}$ and heterozygous h*AHR* mice (Fig. 2*B*). These results demonstrate that hAHR mRNA is transcribed under the control of the mouse *Ahr* promoter in both adult and embryonic hAHR knock-in mice.

To ascertain that hAHR protein is expressed from the knock-in allele, immunohistochemical analysis was performed on lung sections obtained from hAHR knock-in homozygous mouse and the *Ahr*-null mutant (22). Intense signals were detected in the alveolar epithelial cells of hAHR knock-in animals (Fig. 2*C*). The signal intensity of *Ahr*-null mutant lung (Fig. 2*D*) was as faint as the hAHR knock-in lung without the antibody (data not shown). Thus, hAHR protein is expressed from the knock-in allele.

The hAHR Knock-in Mouse Displays a Distinct Induction Profile of AHR Target Genes to Different AHR Ligands. The response of the hAHR knock-in mouse to two prototypical AHR ligands, 3-MC and TCDD, was examined. To characterize the distinct properties, if any, of the hAHR, two strains of control mice were used for the analysis. One strain is a wild-type mouse in the C57BL/6J genetic background, which possesses AHR with high affinity for TCDD. The other strain is a congenic mouse, D2N-*Ahrd*, possessing AHR with low affinity (from DBA/2 mouse) in the C57BL6J genetic background. Because the hAHR knock-in mouse was backcrossed into C57BL/6J, these two strains of mouse enabled us to compare the characteristics of hAHR to those of C57BL/6J and DBA/2 AHR in the same genetic background.

Robust expression of the *CYP1A1* and *CYP1A2* genes was observed in the liver of $Ahr^{b\text{-}1/b\text{-}1}$ mice after administration of 3-MC, whereas the magnitudes of induction in homozygous h*AHR* and $Ahr^{d/d}$ mice were much weaker and comparable to each other (Fig. 3*A*). The relative mean band intensities for *CYP1A1* were 1.0 and 0.9 and were 1.0 and 1.1 for *CYP1A2* in homozygous h*AHR* and $Ahr^{d/d}$ mice, respectively. After treatment with TCDD, the induction of the two genes was strongest in $Ahr^{b\text{-}1/b\text{-}1}$ mice, intermediate in $Ahr^{d/d}$ mice, and weakest in homozygous h*AHR* mice (Fig. 3*B*). The fold inductions in homozygous h*AHR*, $Ahr^{d/d}$, and $Ahr^{b\text{-}1/b\text{-}1}$ mice were 1.0, 4.9, and 14.6 for *CYP1A1*, and 1.0, 5.7, and 8.4 for *CYP1A2*, respectively.

When the responses of $Ahr^{b\text{-}1/b\text{-}1}$ and $Ahr^{d/d}$ mice were compared, the *CYP1A1* expression levels were higher in $Ahr^{b\text{-}1/b\text{-}1}$ than in $Ahr^{d/d}$ mice, which is consistent with previous reports (9, 12, 13). It is noteworthy that the responsiveness of homozygous h*AHR* mice to 3-MC was almost comparable to that of $Ahr^{d/d}$ mice, whereas the responsiveness to TCDD was much weaker. The differential response between $Ahr^{d/d}$ and homozygous h*AHR* mice was unexpected, because a previous study indicated

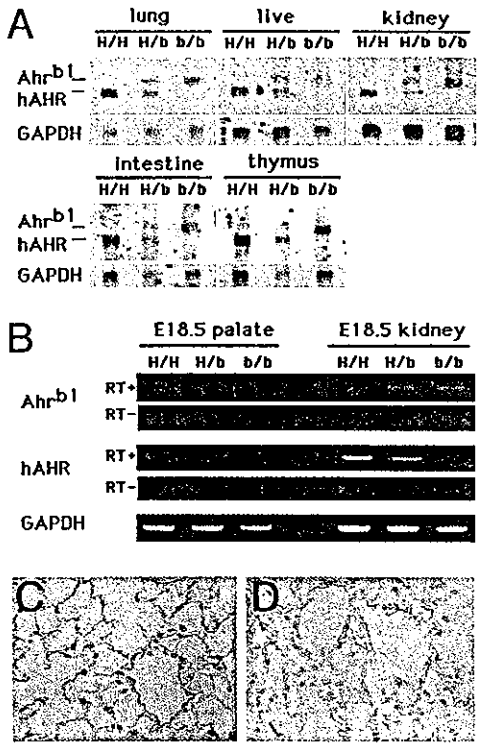

Fig. 2. Expression of hAHR in multiple tissues of the hAHR knock-in mouse. (*A*) RNA blot analysis of polyA RNA (5 μg/lane) extracted from five representative organs of homozygous and heterozygous h*AHR* mice and $Ahr^{b\text{-}1/b\text{-}1}$ mice. Human and mouse *Ahr* transcripts (hAHR and $Ahr^{b\text{-}1}$, respectively) are indicated (*Left*). The same membrane was rehybridized with ^{32}P-labeled cDNA of mouse *GAPDH*. H/H, H/b, and b/b are described in the Fig. 1 legend. (*B*) RT-PCR analyses of hAHR and murine Ahr mRNA expression in kidney and palate of GD18.5 fetuses. The reverse transcription was conducted either in the presence (+) or absence (−) of reverse transcriptase. PCR products representing the transcripts derived either from h*AHR* or $Ahr^{b\text{-}1}$ are indicated on the left. (*C* and *D*) Immunohistochemical analysis of hAHR protein in the lung of a homozygous h*AHR* mouse. Immunoreactivity of AHR protein was observed in the alveolar epithelial cells of homozygous h*AHR* lung (*C*), whereas no immunoreactivity was observed in the lung of $Ahr^{-/-}$ mouse (*D*). Original magnifications, ×400 (*C* and *D*).

that hAHR and DBA/2 AHR exhibit similar dissociation constants for TCDD binding as measured *in vitro* (9, 19). This result suggests that ligand binding does not fully define the integrated function of hAHR.

hAHR Knock-in Mouse Is Relatively Resistant to TCDD-Induced Teratogenicity. The responses to TCDD mediated by hAHR are weaker than that by DBA/2 and C57BL/6 AHR when measured

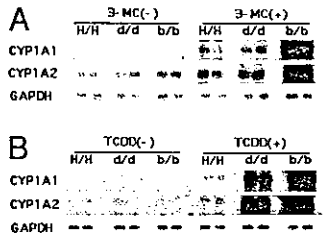

Fig. 3. Inducible expression of AHR target genes. Northern blot analysis of AHR-regulated *CYP1A1* and *CYP1A2* was performed. Six-week-old homozygous h*AHR*, $Ahr^{d/d}$, and $Ahr^{b\text{-}1/b\text{-}1}$ mice were treated with 80 mg/kg 3-MC (*A*) or 100 μg/kg TCDD (*B*). Total hepatic RNA was isolated 24 h after treatment and subjected to Northern analysis (10 μg/lane). Equal loading was confirmed by the abundance of *GAPDH* transcripts.

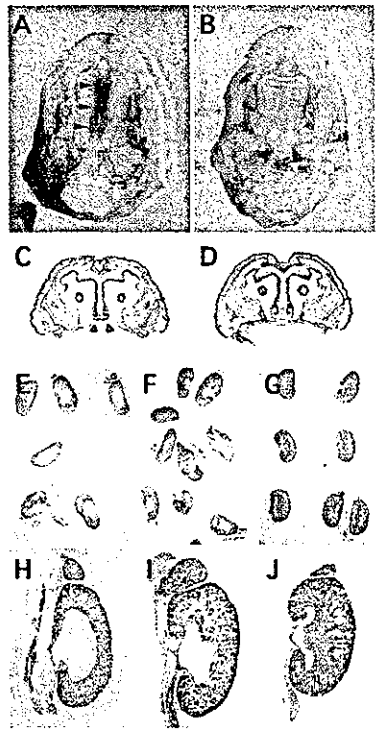

Fig. 4. Fetal teratogenesis after maternal administration of TCDD. (*A* and *C*) Cleft palate in an $Ahr^{b\text{-}1/b\text{-}1}$ fetus is shown. Filled arrowheads in *A* and open arrowheads in *C* indicate the failure of palatine shelves to fuse. Note that homozygous h*AHR* fetuses showed no cleft palates after TCDD treatment (*B* and *D*). (*E*, *F*, *H*, and *I*) Fetal hydronephrosis induced by TCDD. $Ahr^{b\text{-}1/b\text{-}1}$ (*E* and *H*) and homozygous h*AHR* (*F* and *I*) fetuses are shown. (*G* and *J*) Unaffected kidneys from untreated $Ahr^{b\text{-}1/b\text{-}1}$ fetuses are shown.

as inducibility of *CYP1A* family genes. Teratogenicity is a more integrated and complex toxicological manifestation of TCDD action. The most prominent teratogenic effects of TCDD on mouse fetus are cleft palate and hydronephrosis, both of which depend completely on AHR expression (29). The frequency and severity of these teratogenic effects of TCDD were examined in hAHR knock-in fetuses. Homozygous hAHR knock-in females were mated with males of the same genotype and given a single i.p. dose of 40 μg of TCDD per kg of body weight at GD12.5. $Ahr^{b\text{-}1/b\text{-}1}$ and $Ahr^{d/d}$ females were treated in the same way as controls. All dams were weighed to monitor the normal continuation of the pregnancy and killed at GD18.5 to remove fetuses for examination of cleft palate and hydronephrosis.

As reported previously, cleft palate was observed in 100% of the wild-type $Ahr^{b\text{-}1/b\text{-}1}$ fetuses exposed to TCDD (Fig. 4 *A* and *C* and Table 1) (22). By contrast, none of the treated homozygous h*AHR* fetuses showed abnormal palatogenesis (Fig. 4 *B* and *D* and Table 1). An intermediate frequency (30%) of cleft palate was observed in the $Ahr^{d/d}$ fetuses. Differences in the severity of cleft palate were not apparent in any of the symptomatic fetuses of any genotype. This anomaly was most frequent in $Ahr^{b\text{-}1/b\text{-}1}$, intermediate in $Ahr^{d/d}$, and least frequent in homozygous h*AHR* mice, in accordance with the transcriptional inducibility of AHR target genes, which was strongest in $Ahr^{b\text{-}1/b\text{-}1}$, intermediate in $Ahr^{d/d}$, and weakest in homozygous h*AHR* mice. Thus, a strong correlation between the incidence of cleft palate in each strain and the intrinsic transcriptional activity of their respective AHR molecules was observed.

Hydronephrosis, another teratogenic effect of TCDD, is characterized by a dilated renal pelvis. The severity of this anomaly in the fetal kidney was scored from 0 (normal) to 3 (severest)

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Table 1. Incidence of anomalies caused by TCDD in homozygous h*AHR*, $Ahr^{b\text{-}1/b\text{-}1}$, and $Ahr^{d/d}$ fetuses

Genotype of fetuses	TCDD dose, μg/kg	Dams examined, *n*	Fetuses examined, *n*	Fetuses with: Cleft palate *n*	Cleft palate %*	Hydronephrosis *n*	Hydronephrosis %*	Hydronephrosis Severity: 0–3.0†
$Ahr^{b\text{-}1/b\text{-}1}$	0	2	13	0	0	2	12.5	0.19 ± 0.10‡
$Ahr^{b\text{-}1/b\text{-}1}$	40	5	29	29	100	26	89.7	2.54 ± 0.14§
$Ahr^{d/d}$	0	2	15	0	0	2	13.3	0.20 ± 0.10‡
$Ahr^{d/d}$	40	5	30	9	30	25	81.7	1.98 ± 0.14§
Homo-h*AHR*	0	2	16	0	0	1	6.3	0.03 ± 0.03‡
Homo-h*AHR*	40	5	37	0	0	30	81.1	1.19 ± 0.01§

*Percentage of fetuses with each anomaly of all fetuses examined.
†The criteria for severity scores are described in *Materials and Methods*. Data are expressed as mean ± SE.
‡Significant difference between TCDD-treated and -untreated fetuses of each genotype ($P < 0.0001$).
§Significant difference between TCDD-treated homozygous h*AHR* fetuses and $Ahr^{b\text{-}1/b\text{-}1}$ or $Ahr^{d/d}$ fetuses ($P < 0.0001$).

according to criteria described previously (28). When kidneys scored at 1, 2, or 3 were counted as hydronephrotic, 89.7% of the $Ahr^{b\text{-}1/b\text{-}1}$ offspring suffered from this teratogenic outcome after TCDD treatment (Fig. 4 *E* and *H*, and Table 1 for TCDD-treated animals; Fig. 4 *G* and *J*, and Table 1 for untreated animals). A similar incidence was observed in a previous study (22). $Ahr^{d/d}$ and homozygous h*AHR* fetuses also displayed this teratogenic effect with incidences of 81.7% and 81.1%, respectively (Fig. 4 *F* and *I*, and Table 1). Thus, there is no substantial difference in the incidence of hydronephrosis among the mice expressing the three distinct *Ahr* (h*AHR*) genes. When severity score values were compared among the TCDD-treated fetuses, they averaged 2.54, 1.98, and 1.19 for the $Ahr^{b\text{-}1/b\text{-}1}$, $Ahr^{d/d}$ and homozygous h*AHR* genotypes, respectively (Table 1). Therefore, hydronephrosis observed in the homozygous h*AHR* fetuses was significantly less severe compared with that in either $Ahr^{b\text{-}1/b\text{-}1}$ or $Ahr^{d/d}$ fetuses. Nonetheless, the average score of TCDD-treated homozygous h*AHR* fetuses (1.19) was still significantly higher than that of untreated homozygous h*AHR* fetuses (0.03), clearly demonstrating that the TCDD-activated hAHR mediates renal teratogenesis in mice. Although the magnitude of *CYP* gene induction is dramatically different depending on the *Ahr* genotype, the incidence of hydronephrosis is surprisingly comparable among the three strains. These results revealed that differences between human and murine AHR allowed for the emergence of discrete biological effects; e.g., hydronephrosis, but not cleft palate in homozygous h*AHR* mice.

To exclude the possibility that maternal factors affect the teratogenic manifestations on the fetuses, heterozygous h*AHR* parents were used to obtain homozygous h*AHR* and $Ahr^{b\text{-}1/b\text{-}1}$ fetuses. Heterozygous mothers were treated with TCDD as described above, and fetuses were examined for both cleft palate and hydronephrosis. As described in Table 2, the incidence of cleft palate was 100% and 0% in $Ahr^{b\text{-}1/b\text{-}1}$ and homozygous h*AHR* fetuses, respectively, which is identical to the results presented in Table 1. The incidence and severity (mean score) of hydronephrosis were 100% and 2.47 for $Ahr^{b\text{-}1/b\text{-}1}$ and 66.6% and 1.17 for homozygous h*AHR* fetuses, respectively. Again, a more moderate effect in the homozygous h*AHR* fetuses is suggested, the severity difference being statistically significant. Therefore, we conclude that the TCDD-induced teratogenic effects are independent of maternal genotypes, and that fetal AHR activity is critical for determining the outcomes.

Discussion

One of the central issues in the uncertainty surrounding risk assessments for TCDD and its structural analogs is whether humans are relatively sensitive or resistant to the toxicities of this class of compounds. Because the pleiotropic adverse effects induced by these toxins involve multiple processes, the human response is generated by the summation and integration of the properties inherent to the human components, including expression level, ligand-binding affinity, and transcriptional activity of the AHR, as well as the variety, function and activity of the AHR target genes. Through numerous preceding studies, the primary structure of the AHR protein has been regarded as one of the most critical factors determining the susceptibility and specificity of responses of animals to various PAH/HAHs including dioxin. On the basis of several observations *in vitro*, polymorphic variation in the *Ahr* gene is considered the primary basis for differences in sensitivity to TCDD among strains of mice (9–11). In this study, we attempted to establish an *in vivo* system to evaluate the specific function of the hAHR protein to better evaluate its role in determining possible patterns of human responses to PAH/HAHs.

For this purpose, we adopted a knock-in strategy to introduce hAHR cDNA into the mouse *Ahr* genomic locus by homologous recombination. This strategy offers an obvious advantage compared with a transgenic method, because the introduced sequence is transcribed under the same regulatory mechanisms of the replaced gene (30). As desired, expression levels of the h*AHR* transcript were almost the same with those of endogenous mouse

Table 2. Incidence of anomalies caused by TCDD in fetuses from heterozygous h*AHR* parents

Genotype of fetuses	TCDD dose, μg/kg	Dams examined, *n*	Fetuses examined, *n*	Fetuses with: Cleft palate *n*	Cleft palate %*	Hydronephrosis *n*	Hydronephrosis %*	Hydronephrosis Severity: 0–3.0
$Ahr^{b\text{-}1/b\text{-}1}$	40		9	9	100	9	100.0	2.47 ± 0.14†
Hetero-h*AHR*	40	7	25	12	48	22	88.0	2.46 ± 0.13
Homo-h*AHR*	40		12	0	0	8	66.6	1.17 ± 0.01†

*Percentage of fetuses with each anomaly out of all fetuses examined.
†Significant difference between homozygous h*AHR* and $Ahr^{b\text{-}1/b\text{-}1}$ fetuses ($P < 0.0001$).

Ahr mRNA in multiple AHR-expressing tissues of adult mice and GD18.5 embryos. The hAHR protein was detected by immunostaining in the lungs of homozygous h*AHR* mice.

A possible explanation of the relative resistance of the hAHR knock-in mouse to TCDD lies in the qualitative difference between the human and mouse AHR molecules. Assuming that the abundance of the hAHR protein is the same as that of the endogenous mouse AHR, our results imply that the hAHR-mediated response to TCDD *in vivo* is much lower than that of DBA/2 AHR, although previous reports showed that their affinities to TCDD, as measured *in vitro*, are almost the same (9, 19). Alignment of the primary amino acid sequences of the two molecules indicates the considerable divergence in the C-terminal regions (9) and the deletion analysis differently localized the transcriptional activity within the regions (31). Such structural diversity of the C-terminal region might lead to species-specific interaction behaviors with transcriptional cofactors. TCDD-activated hAHR may not recruit coactivators as efficiently as the DBA/2 counterpart. One possibility must be noted that the incompatibility between TCDD-activated hAHR and the mouse coactivators may cause the reduced response of hAHR knock-in mouse to TCDD.

hAHR was not detectable by immunoblot analysis with the current antiserum, and its abundance relative to the constitutive level of mouse AHR protein could not be determined. Considering this lack of quantitative information, limited protein accumulation might account for the attenuated responsiveness of hAHR knock-in mice to TCDD. hAHR may have an intrinsically shorter life than mouse AHR at physiological expression levels *in vivo*.

The susceptibility of embryonic kidneys of homozygous h*AHR* mice to the teratogenic effects of TCDD is noteworthy. The pathogenesis of this renal lesion induced by TCDD involves hyperplasia of the ureteric epithelium, resulting in an occlusion of the ureter and subsequent hydronephrosis (32). Adverse effects on the kidney and urinary tract have also been reported in humans exposed to TCDD (33). However, studies in Ben Tre Province in Vietnam, where defoliant containing dioxin was sprayed extensively, revealed little increase in the prevalence of cleft lip and/or palate compared with that observed in Japan (34), suggesting that hAHR is less potent to mediate the manifestation of cleft palate, and that a higher dose might be required for it. Consistent with these human reports, our analysis showed that hAHR, although expressed in mice, mediated the development of hydronephrosis induced by TCDD, but not cleft palate at our experimental dose. Thus, the knock-in animal seems to mimic some aspects of the human responses to PAH/HAHs.

An intriguing utilization of our knock-in mouse strategy would be as an *in vivo* system for the qualitative and quantitative assessment of possible human responses to various PAH/HAHs. In this study, D2N-*Ahrd* mice responded more strongly to TCDD than to 3-MC, whereas the hAHR knock-in mice responded almost equally to these two compounds. These results clearly show that the relative efficacy profiles, examined by TCDD and 3-MC, are different between D2N-*Ahrd* and our hAHR knock-in mouse. Therefore the efficacy profile specific to hAHR can be displayed by analyses of the responses of the hAHR knock-in mouse to an array of PAH/HAHs. Because environmentally relevant levels of exposure to dioxin and related compounds have garnered much concern in terms of their possible effects on reproductive, neurobehavioral, and immunological functions of humans, our hAHR knock-in mouse will serve as a humanized model mouse, exhibiting the human-specific responses to PAH/HAH congeners. This mouse should help define the range of biological and toxicological effects that could be expected to affect humans and thereby reduce some uncertainty in risk assessments of these persistent environmental contaminants.

We thank Drs. H. Ueda and S. Kimura at the Environmental Health Department of the Ministry of the Environment for valuable suggestions and support to initiate this project, and Dr. T. Kensler for the discussion and critical reading of the manuscript. We also thank Dr. N. Morito, Ms. N. Kaneko, and R. Kawai for help. This work was supported by grants from Exploratory Research for Advanced Technology Environmental Response Project (M.Y.), the Ministry of Education, Science, Sports and Culture (H.M. and M.Y.), the Ministry of Health, Labor, and Welfare (H.M., C.T., and M.Y.), Japan Society for the Promotion of Science–Research for the Future Program (M.Y.), Core Research for Evolutional Science and Technology (Y.F.-K., S.O., Y.A., C.T., and M.Y.), Program for Promotion Basic Research Activities for Innovative Biosciences (H.M. and S.T.), and Special Coordination Fund for Promoting Science and Technology (H.M.).

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Toxicology in Vitro 17 (2003) 259–269

Toxicology in Vitro

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Effects of 3,3′,4,4′,5-pentachlorobiphenyl, a coplanar polychlorinated biphenyl congener, on cultured neonatal mouse testis

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Accepted 12 July 2002

Abstract

3,3′,4,4′,5-Pentachlorobiphenyl (PCB126), a congener with a planar configuration, has been established to have relatively strong toxicities similar to those of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) via aryl hydrocarbon receptors. We investigated the effects of this coplanar PCB on mammalian early spermatogenesis and steroidogenesis in a mouse neonatal testicular organ culture system. Testes collected from newborn mice were subjected to organ culture in medium containing 0, 10, 100 or 1000 nM PCB126. Histochemical analysis revealed that the BrdU-labeling indices of both spermatogenic cells and Sertoli cells were unchanged in all testis specimens exposed to the coplanar PCB. CYP1A1 and steroidogenic enzymes (P450scc, P450c17, 3β-HSD and 17β-HSD) mRNA levels were determined by semiquantitative RT-PCR. The CYP1A1 mRNA level in cultured testis was significantly increased by PCB126 in a dose-dependent manner. Although mRNA levels of 3β-HSD and 17β-HSD were unchanged, the P450scc mRNA level was significantly down-regulated by PCB126 in a dose-dependent manner. In contrast, the P450c17 mRNA level was significantly higher in 1000 nM PCB126-exposed testis than in control testis. These results suggest that the coplanar PCB does not alter the proliferative activity of spermatogenic cells and Sertoli cells in neonatal testis, but that it directly affects the expression of steroidogenic enzyme genes.

Keywords: Coplanar PCB; Testis; Steroidogenic enzyme

1. Introduction

Coplanar polychlorinated biphenyls (coplanar PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are categorized as dioxins and related compounds since these compounds show various adverse toxicity similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) through a common pathway via a ligand-dependent transactivating factor, named aryl hydrocarbon receptor (AhR). In particular, in utero and lactational exposure to dioxins cause adverse effects on the male reproductive system, such as reduced sperm count (Mably et al., 1992a; Wilker et al., 1996; Gray et al., 1997) and reduced reproductive organ size (Mably et al., 1992b; Roman et al., 1998a). Peterson and co-workers reported a series of studies on male Holtzman rat offspring born from dams administered a single oral dose of TCDD on gestational day (GD) 15 (Mably et al., 1992a) and found statistically significant

Abbreviations: ABP, androgen binding protein; AhR, aryl hydrocarbon receptor; BrdU, 5-bromo-2′-deoxyuridine; coplanar PCBs, coplanar polychlorinated biphenyls; DMEM, Dulbecco's modified Eagle's medium; DNase I, Deoxyribonuclease I; DSP, daily sperm production; GD, gestational day; PCB126, 3,3′,4,4′,5-pentachlorobiphenyl; PCDDs, polychlorinated dibenzo-*p*-dioxins; PCDFs, polychlorinated dibenzofurans; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive element

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0887-2333/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0887-2333(03)00011-0