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Identification of Functional Aryl Hydrocarbon Receptor and Aryl Hydrocarbon Receptor Nuclear Translocator in Murine Splenocytes

Courtney E. Williams, Robert B. Crawford,**
Michael P. Holsapple† and Norbert E. Kaminski‡*

*Department of Pharmacology & Toxicology and Department of Pathology, Michigan State University, East Lansing, MI 48824; and †Dow Chemical Company, Midland, MI 48674, U.S.A.

ABSTRACT. The objective of the present studies was to determine whether the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (ARNT) protein are present and functional in B6C3F1 (C57BL/6 × C3H) mouse splenocytes. Northern analysis of poly(A) RNA isolated from splenocytes revealed transcripts of approximately 6.6 kb which hybridized to the AhR complementary DNA (cDNA) probe. Anti-AhR antibodies identified two major cytosolic forms of the AhR in splenocytes, approximately 95 and 104 kDa, corresponding to the codominately expressed Ahr^b alleles in the B6C3F1 mice. Northern analysis utilizing an oligomer probe for ARNT identified three messenger RNA (mRNA) transcripts, approximately 5.6, 2.0, and 1.1 kb, in spleen which was consistent with the banding pattern observed in the B6C3F1 mouse liver. Western blotting confirmed the presence of the approximately 87 kDa ARNT protein in splenocytes. Protein quantitation by slot blot analysis demonstrated approximately 2.0-fold more AhR in liver than in splenocytes. Interestingly, ARNT was approximately 2.4-fold more abundant in splenocytes than in liver. Consistent with these results, comparison by quantitative reverse transcriptase-polymerase chain reaction analysis of AhR and ARNT transcripts in liver and splenocytes demonstrated approximately 2.3-fold more AhR transcripts in liver than in splenocytes and approximately 3.2-fold more ARNT transcripts in splenocytes than in liver. In addition, comparisons between AhR and ARNT transcripts isolated from the liver and splenocytes indicated a greater number of ARNT transcripts as compared with AhR in both preparations. TCDD treatment of splenocytes induced binding of the AhR nuclear complex to the dioxin-responsive enhancer (DRE) as detected by the electrophoretic mobility shift assay. These findings confirm that the AhR and ARNT are present in mouse splenocytes and are capable of binding to the DRE. BIOCHEM PHARMACOL 52;5:771–780, 1996.

KEY WORDS. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD; AhR; ARNT; RT-PCR; DNA binding

The AhR§ is a 95–110 kDa cytosolic protein that is believed to mediate the effects of dioxin and related compounds [1–5]. In the absence of ligand, the receptor is associated with hsp90 [6–8]. Ligand binding induces conformational changes in the AhR by displacing hsp90, enabling the receptor–ligand complex to translocate to the nucleus where it is transformed into a DNA binding protein [6–12]. This transformation involves dimerization of the receptor–ligand complex with a structurally related 87 kDa protein called the ARNT protein [11, 13, 14]. The heterodimer can regulate gene expression by binding DREs located upstream of sensitive genes such as cytochrome P_1450 *Cyp1a1*, glutathione S-transferase, and menadione oxidoreductase [1, 2, 13–18]. This mechanism has been primarily elucidated by studying the induction of drug-metabolizing enzymes, such as *Cyp1a1*, by halogenated aromatic hydrocarbons in liver and liver-derived cell lines.

Of the wide range of biological effects produced upon exposure of animals to TCDD and related compounds, including a general wasting syndrome, lymphoid involution (especially the thymus), pancytopenia, hepatomegaly and hepatotoxicity, chloracne and hyperkeratosis, gastric lesions, urinary tract hyperplasia, tumor promotion, teratogenicity and embryotoxicity, and decreased spermatogenesis (reviewed in Refs. 1 and 2), immune suppression appears to be one of the most sensitive consequences of TCDD exposure. In fact, studies investigating the effects of TCDD

‡ Corresponding author: Dr. Norbert E. Kaminski, Department of Pharmacology & Toxicology, B-330 Life Sciences Bldg., Michigan State University, East Lansing, MI 48824. Tel. (517) 353-3786; FAX (517) 353-8915.

§ *Abbreviations:* AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; cDNA, complementary DNA; DRE, dioxin-responsive enhancer; EMSA, electrophoretic mobility shift assay; hsp90, heat shock protein 90; IS, internal standard; mRNA, messenger RNA; rcRNA, recombinant RNA; RT-PCR, reverse transcriptase-polymerase chain reaction; sRBC, sheep red blood cell; SSPE, sodium chloride, sodium phosphate, EDTA; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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on immunocompetence have identified varying degrees of immunological change in virtually every species when TCDD is used at doses that do not produce obvious signs of toxicity (reviewed in Ref. 19). In spite of this rather extensive database, little is known about the actual molecular mechanism responsible for the immunotoxic effects produced by TCDD. For the most part, it is widely presumed that immunotoxicity by TCDD is induced, at least in part, through an AhR-mediated mechanism. This premise is supported by a number of lines of indirect evidence including: (a) results with congenic mice showing that B6 mice expressing the wild-type phenotype Ah^{bb} allele are more sensitive to TCDD than are congenic B6 Ah^{dd} mice [20]; (b) structure–activity relationship studies demonstrating that, with few exceptions, high affinity AhR ligands are more immunosuppressive than low affinity ligands [21]; (c) an increased sensitivity to immune suppression by TCDD in B6 mice (Ah high-responsive) versus DBA/2 mice (Ah low-responsive) following acute exposure to TCDD [20, 22, 23]; and (d) the identification of nuclear [^{3}H]TCDD by sucrose density gradient centrifugation in human tonsilar cells, thus suggesting the translocation of the AhR–TCDD complex to the nucleus [24]. However, these findings are tempered by the fact that: (a) although identified in lymphoid tissues, the AhR and ARNT have not been demonstrated in immunocompetent cells; (b) employment of the electrophoretic mobility shift assay using rat and guinea pig spleen extracts has not shown binding of the AhR–ARNT heterodimer to the DRE following TCDD treatment [25]; (c) the low affinity AhR ligand, 2,7-dichlorodibenzo-*p*-dioxin and TCDD produce comparable inhibition of the anti-sRBC IgM antibody forming cell response following subchronic treatment of mice *in vivo* and following direct addition to naive splenocytes *in vitro* [26]; and (d) subchronic TCDD treatment produces a marked immunosuppression in both DBA/2 and B6C3F1 mice [27].

In light of the importance placed on an AhR-mediated mechanism for TCDD-mediated toxicity, the objective of the current studies was to determine if AhR and ARNT are present in B6C3F1 (C57BL/6 × C3H) mouse splenocytes and, if so, can this lymphoid-derived cell preparation serve as an adequate cellular model to study the involvement of the AhR in mediating the effects of TCDD on the immune system. The reason for using this model is 2-fold. First, the spleen is a common source of lymphoid tissue in immunotoxicological assessments and, second, B6C3F1 mice are AhR-high responders. To test this model, we determined if the components thought to be essential for an AhR-mediated mechanism (i.e. the AhR and ARNT) were present and functional in B6C3F1 spleen. In these studies, splenocytes rather than whole spleen were used to eliminate any confounding results that may be attributable to the tissue of the splenic capsule. This was deemed especially important in light of previous findings that demonstrated comparable binding of [^{3}H]TCDD in the splenic capsule and isolated splenocytes, suggesting that the AhR may be present in the connective tissue that surrounds the spleen [28].

MATERIALS AND METHODS

Chemicals

TCDD was purchased from AccuStandard Inc. (New Haven, CT). The certificate of product analysis stated the purity of TCDD to be 100% by GC/MS.

Animals and Cell Line

Virus-free female B6C3F1 mice, 5–6 weeks of age, were purchased from the Frederick Cancer Research Center (Frederick, MD). On arrival, mice were randomized, transferred to plastic cages containing a sawdust bedding (5 mice/cage), and quarantined for 1 week. Mice were provided with food (Purina Certified Laboratory Chow) and water *ad lib*. Animal holding rooms were kept at 21–24° and 40–60% relative humidity with a 12-hr light/dark cycle. The mouse hepatoma cell line, Hepa 1c1c7, was provided to our laboratory by Dr. Michael S. Denison (University of California, Davis). Hepa 1c1c7 cells were cultured in α-Minimum Essential Medium supplemented with 100 U of penicillin/mL, 100 U of streptomycin/mL, 2 mM L-glutamine (Gibco BRL, Grand Island, NY), and 10% bovine calf serum (Hyclone, Logan, UT).

Probes

The plasmid pSportAhR (ATCC 63215) developed by Bradfield and coworkers [4] contains a 3.12 kb insert of the cDNA for the mouse *Ahr* gene that was cloned from the Hepa 1c1c7 cell line. The AhR cDNA probe was a 1.87 kb fragment cut with restriction enzymes *Hin*dIII and *Bam*HI from pSportAhR and corresponds to the 3′ end of the cloned *Ahr* gene. The 75 base ARNT oligomer probe was synthesized using an Applied Biosystem DNA synthesizer and purified by HPLC (Macromolecular Structure Facility, MSU). This oligomer represents nucleotides 360–435 of the human ARNT cDNA clone sequenced by Hoffman *et al.* [13].

Northern Blot Analysis

Total RNA was isolated using a modified method of Chomczynski and Sacchi [29]. Briefly, mouse spleen and liver were homogenized in denaturing solution (4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 100 mM 2-mercaptoethanol) and extracted twice with phenol:chloroform:isoamyl alcohol (1:1:24). Nucleic acids were precipitated and resuspended in water. Poly(A) RNA was purified by PolyATtract (Promega, Madison, WI), precipitated, resuspended in water, and quantitated spectrophotometrically. Poly(A) RNA was fractionated in a 1.2% agarose-formaldehyde gel, transferred to nylon membrane (Amersham, Arlington Heights, IL), and cross-

linked to the membrane using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Blots were prehybridized for 2–6 hr and hybridized overnight at 42° in hybridization solution (50% formamide, 5× SSPE, 10× Denhardt's solution, 2% SDS, 7% dextran sulfate, yeast transfer RNA at 130 μg/mL, and labeled probe), and then washed twice for 5 min in 2× SSPE with 0.5% SDS, twice for 15 min in 1× SSPE with 1% SDS, and twice for 15 min in 0.1× SSPE with 0.1% SDS if needed. Blots were then exposed to Reflection film (Dupont NEN, Boston, MA) at −80° in the presence of intensifying screens. Mouse spleen without capsule was prepared by carefully separating the splenic pulp from the splenic capsule. The spleen cells were homogenized in denaturing solution, and mRNA was isolated as described above.

Liver Protein Lysate Preparation

Livers were perfused with HEDGM (25 mM HEPES (pH 7.5), 1 mM EDTA, 2 mM dithiothreitol, 10% (v/v) glycerol, 20 mM sodium molybdate) through the portal vein, isolated, pooled, and minced in buffer. The last wash was passed through a cheesecloth, and 1.5% (w/v) of HEDGM/LAP (HEDGM with 100 μM leupeptin, 40 U/mL aprotinin, and 200 μM phenylmethylsulfonyl fluoride) was added. The sample was homogenized and centrifuged at 21,000 *g*, and supernatant was collected and then centrifuged at 105,000 *g*. The supernatant was aliquoted and stored at −80° prior to use in the western and slot blot analysis. Protein concentrations were determined by the bicinchoninic acid protein assay (Sigma, St. Louis, MO).

Splenocyte Protein Lysate Preparation

Single spleen cell suspensions without red blood cells were prepared as previously described [30]. After the last wash, one cell volume of HEDM/LAP was added and homogenized with a tight-fitting pestle. An equal volume of HED2GM/LAP (HEDM/LAP with 20% glycerol) was added and centrifuged at 105,000 *g* for 1 hr at 4°. The supernatant was aliquoted and stored at −80° prior to being used in the western and slot blot analysis. Protein concentrations were determined as described above.

Western Blot Analysis

Cell lysates were prepared and subsequently resolved by denaturing SDS–PAGE on a continuous 7.5% polyacrylamide slab gel. The electrophoresed proteins were transferred to nitrocellulose (Amersham) by semi-dry electrophoretic transfer (Bio-Rad, Hercules, CA). Protein blots were blocked in BLOTTO buffer [5% low fat dry milk in Tween-20 Tris-buffered saline (TTBS)] for 1–2 hr at 22°, washed in TTBS, dried, and stored in cellophane at 4°. Primary antibodies to the Ah receptor (17-10B) and ARNT protein (20-9B), previously characterized by Pollenz *et al.* [12], were gifts from Dr. Richard S. Pollenz (Medical University of South Carolina). Immunochemical staining was carried out as previously described [12]. Detection was performed using the enhanced chemiluminescence (ECL) method (Amersham). Western blot results were confirmed with anti-AhR and anti-ARNT monoclonal antibodies provided by Dr. Gary Perdew (Purdue University).

Slot Blot Analysis

Varying amounts of cell protein lysates were filtered directly onto nitrocellulose membranes using a Bio-Dot SF microfiltration apparatus (Bio-Rad). The same procedures used in the western blot analysis were employed for the detection of AhR and ARNT proteins in the slot blots. The negative control consisted of cell lysates that were incubated with the secondary antibody but not with the primary antibody. Slot blots were analyzed using a model GS-670 Imaging Densitometer (Bio-Rad). The band intensity of the negative control blots were designated as the background absorbance and subtracted from values determined in the quantitation of the AhR and ARNT.

Quantitative RT-PCR

All enzymes used in the generation of the internal standards were purchased from Promega. *Taq* DNA polymerase was purchased from Perkin Elmer (Foster City, CA). Primers for both the ISs and the genes of interest were synthesized using an Applied Biosystems DNA synthesizer and purified by HPLC. Total RNA was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH) as described by Chomczynski [31, 32]. To avoid any DNA contamination, RNA samples were incubated with RNase-free DNase for 15 min at 37° in 10 mM $MgCl_2$, 1 mM dithiothreitol, 25 U RNasin, 10 mM Tris, 1 mM EDTA, then phenol:chloroform extracted, and precipitated in isopropanol. Three pooled tissue preparations were made from B6C3F1 mice. Competitive RT-PCR was performed as outlined in Gilliland *et al.* [33, 34], except that the rcRNA was used as an IS instead of genomic DNA. ISs were generated as previously described [35] and contain specific PCR primer sequences for the AhR or ARNT (PCR product sizes: AhR, 385 bp; AhR-IS, 256 bp; ARNT, 340 bp; ARNT-IS, 255 bp). Briefly, known amounts of total RNA and IS rcRNA were reverse-transcribed simultaneously, in the same reaction tube, into cDNA using oligo$(dT)_{15}$ as primers. A PCR master mixture consisting of PCR buffer, 4 mM $MgCl_2$ for the AhR reaction and 2 mM $MgCl_2$ for the ARNT reaction, 6 pmol each of the forward and reverse primers, and 2.5 U *Taq* DNA polymerase was added to the cDNA samples. Samples were then heated to 94° for 4 min and cycled 35 times at 94° for 15 sec, 55° for 30 sec, and 72° for 30 sec after which an additional extension step at 72° for 5 min was included. PCR products were electrophoresed in 3% NuSieve 3:1 gels (FMC Bioproducts, Rockland, ME) and visualized by ethidium bromide staining. Quantitation was performed by assessing the optical density for both of

the DNA bands (i.e. IS versus target gene) using a Gel Doc 1000 video imaging system (Bio-Rad). The number of transcripts was calculated from a standard curve generated by using the density ratio between the gene of interest and the different IS concentrations used [34]. The point at which the ratio of IS to mRNA is equal to one signifies the "cross-over" point which represents the amount of AhR or ARNT molecules present in the initial RNA sample. Three separate RNA isolations were analyzed for each of the tissues. Primer sequences were chosen using GeneWorks, IntelliGenetics, Inc. (Real Mountain View, CA).

Primer sequences for the AhR are:
forward primer = TCATGGAGAGGTGCTTCAGG
reverse primer = GTCTTAATCATGCGGATGTGG

Primer sequences for the ARNT protein are:
forward primer = TTCCGATTCCGATCTAAGACC
reverse primer = TGTTCTGATCCTGCACTTGC

No significant homology was detected when each of these sequences was searched in the Genebank database, and the PCR products were observed as a single band of the expected size on an ethidium bromide-stained agarose gel (data not shown).

Hepa 1c1c7 Nuclear Protein Preparation

Hepa 1c1c7 cells were grown to near confluence in P100 tissue culture-treated petri dishes (Becton Dickinson, Franklin Lakes, NJ), medium was replaced with α-MEM supplemented with 100 U of penicillin/mL, 100 U of streptomycin/mL, 2 mM L-glutamine and 5% bovine calf serum and incubated with DMSO (0.1%) or 30 nM TCDD in DMSO for 2 hr at 37°. Cells were removed from the petri dishes with 0.5 M EDTA in PBS and centrifuged at 270 *g* for 10 min. The cell pellet was then incubated in 10 mM HEPES (pH 7.5) for 15 min on ice, and centrifuged at 270 *g* for 10 min. One cell volume of HED/LAP (HEDGM/LAP without glycerol and sodium molybdate) was added to the cell pellet and homogenized with a loose fitting pestle. Nuclei were pelleted by centrifuging at 1000 *g* for 15 min, resuspended in 1 pellet volume of HEDK/LAP (HED/LAP with 400 mM KCl), and incubated with agitation for 30 min at 4°. After incubation, glycerol was added to the cell lysate to 10% (v/v) and centrifuged at 105,000 *g*. The supernatant was aliquoted and stored at −80° prior to use in the EMSA. Protein concentrations were determined as described above.

Splenocyte Nuclear Protein Preparation

Single spleen cell suspensions without red blood cells were prepared as described above and incubated in RPMI 1640 supplemented with 100 U of penicillin/mL, 100 U of streptomycin/mL, 2 mM L-glutamine, and 5% bovine calf serum plus vehicle (0.1% DMSO) or 30 nM TCDD for 2 hr at 37°. Nuclear protein was prepared as described above with the Hepa 1c1c7 cell line.

Synthetic DRE Oligonucleotides

A complementary pair of synthetic DNA fragments corresponding to the 26 bp AhR binding site of mouse DRE3 [36] were synthesized using an Applied Biosystems DNA synthesizer and purified by HPLC. The DRE oligonucleotide was annealed and end labeled using T4 polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, IN) and [γ-^{32}P]ATP (Dupont NEN).

EMSA

Nuclear protein preparations were used in the EMSA as previously described [11, 14] with a few modifications. Briefly, nuclear extracts (6 μg protein) were incubated with poly(dI–dC) (Boehringer Mannheim Biochemicals) at room temperature for 15 min. Radiolabeled DRE oligomer was added (20,000 cpm) and incubated at room temperature for another 15 min. The binding of protein to the DNA was resolved by a 4.0% nondenaturing PAGE gel, dried on 3MM filter paper (Whatman, Hillsboro, OR), and autoradiographed. Final reaction concentrations for the Hepa 1c1c7 cells were as follows: 25 mM HEPES (pH 7.5), 1 mM EDTA, 2 mM dithiothreitol, 10% glycerol, 110 mM KCl, and 1.8 μg poly(dI–dC). The splenocyte reaction mixture was identical to that used for the Hepa 1c1c7 cells with the exception of a modest change in the poly(dI–dC) concentration (100 ng/vol). Where indicated, 50-fold excess of cold DRE oligomer was added to the reaction.

RESULTS

Northern Blot Analysis of Mouse Splenocytes

Transcripts for the AhR and ARNT have been identified recently in rat spleen by ribonuclease protection analysis [37]. In the present experiments, splenic RNA was probed for the AhR and ARNT transcripts. Liver RNA from the same mouse strain was isolated as a comparative control since the AhR and its putative mechanism of action have been primarily studied using hepatic tissue or hepatic cell lines. Northern analysis for the AhR using a 1.87 kb mouse cDNA probe identified a transcript of approximately 6.6 kb in mouse spleen and liver (data not shown). Splenocytes devoid of connective tissue were examined by northern analysis for the AhR to address the possibility that the transcripts observed from the spleen may have originated primarily from the connective tissue encapsulating the spleen. Transcripts detected in the splenocytes matched the liver control (Fig. 1). Northern analysis using a 75 base oligomer synthesized from human cDNA for ARNT [13] revealed three transcripts of approximately 5.6, 2.0, and 1.1 kb in both the spleen and liver (data not shown).

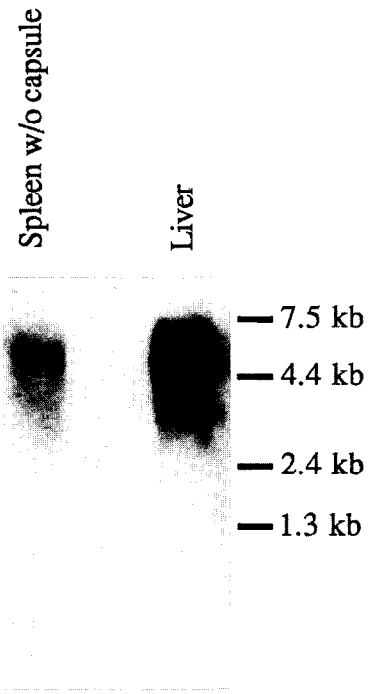


FIG. 1. Northern blot analysis of AhR mRNA expression in splenocytes and liver. Splenocytes were separated from connective tissue (capsule) as detailed in Materials and Methods. Total RNA was then extracted from the splenocytes and liver by using a guanidinium isothiocyanate method. Poly(A) RNA was isolated from the total RNA using the PolyATtract mRNA isolation system. Poly(A) RNA from the spleen without capsule (6 μg), and liver (2 μg) was loaded onto a 1.0% formaldehyde gel and electrophoresed at 100 V for 4 hr. The gel was blotted onto a nylon membrane and hybridized with a ^{32}P-labeled 1.87 kb fragment from the cloned mouse *Ahr*. This figure is representative of two separate experiments.

Western Analysis of Mouse Splenocytes

Based on the identification of transcripts for both the AhR and ARNT in mouse splenocytes, western blot analysis was conducted on whole cell lysates prepared from splenocytes devoid of connective tissue and red blood cells. Antibodies to the AhR revealed two major proteins of approximately 95 and 104 kDa in splenocytes and liver (Fig. 2). These two sizes correspond, respectively, to the codominant expression of the Ahr^{b-1} (C57BL/6) and Ahr^{b-2} (C3H) alleles in the B6C3F1 (C57BL/6 × C3H) mice [38]. An approximately 70 kDa protein, previously identified as a proteolytic fragment of the 95 kDa AhR in C57BL/6 mice [39], was detected in liver of the B6C3F1 mice. Antibodies to ARNT identified an approximate 87 kDa protein in both tissues (Fig. 3).

Slot Blot and Quantitative RT-PCR Analysis of Mouse Splenocytes

Slot blot analysis of whole cell lysates prepared from the splenocytes and liver, which were quantitated by densitometry, indicated that there was approximately 2.0 ± 0.5-fold (from two experiments) more AhR protein present in liver than in splenocytes (Fig. 4), whereas approximately 2.4 ± 0.05-fold (from two experiments) more ARNT protein was identified in splenocytes than that observed in liver (Fig. 5). Quantitative comparisons by RT-PCR of the AhR and ARNT transcripts in liver and splenocytes demonstrated approximately 2.3-fold more AhR transcripts in liver as compared with splenocytes and approximately 3.2-fold more ARNT transcripts in splenocytes as compared with liver (Table 1). In addition, a greater number of transcripts for ARNT as compared with AhR were identified in both the liver and splenocytes (Table 1).

EMSA of Mouse Splenocytes

With the identification of both the AhR and ARNT proteins in splenocytes, the functionality of these proteins was assessed by EMSA. Hepa 1c1c7 cells, a cell line extensively characterized with respect to the induction by TCDD of nuclear translocation of AhR and the subsequent binding of the AhR/ARNT heterodimer to the DRE, served as a comparative control. Following a 2-hr treatment of Hepa 1c1c7 cells or splenocytes with 30 nM TCDD, the ligand–AhR/ARNT nuclear complex was confirmed to be functional in both nuclear preparations as demonstrated by its ability to bind to the DRE motif. This is shown in Fig. 6,

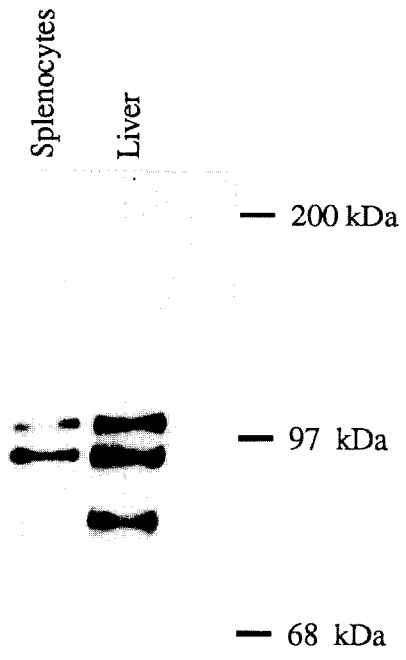


FIG. 2. Western blot analysis of the AhR in splenocytes and liver. One hundred micrograms of protein from whole cell lysates was loaded in each lane and resolved on 7.5% SDS–PAGE gels, transferred to nitrocellulose, and incubated with 2 μg/mL of anti-AhR, 17-10B. Antibody binding was visualized by staining the blot with donkey anti-rabbit horseradish peroxidase-linked immunoglobulins. This figure is representative of more than three separate experiments.

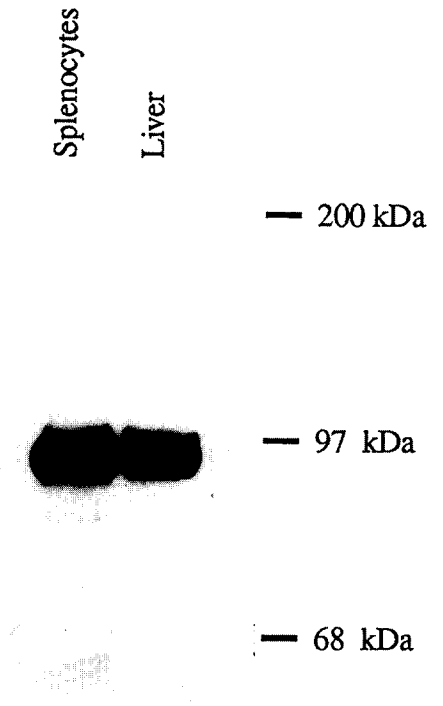
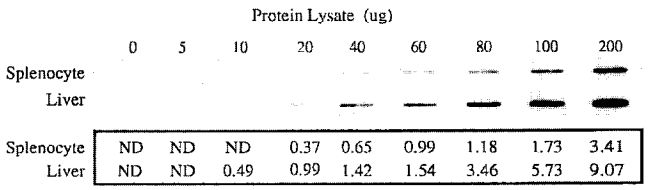


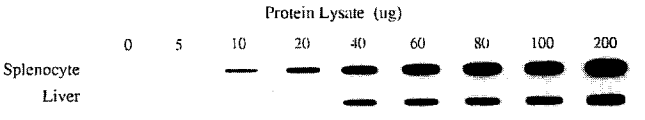
FIG. 3. Western blot analysis of ARNT in splenocytes and liver. One hundred micrograms of protein from whole cell lysates was loaded in each lane and resolved on 7.5% SDS–PAGE gels, transferred to nitrocellulose, and incubated with 2 μg/mL of anti-ARNT, 20-9B. Antibody binding was visualized by staining the blot with donkey anti-rabbit horseradish peroxidase-linked immunoglobulins. This figure is representative of more than three separate experiments.

lane 3, for TCDD-treated Hepa 1c1c7 cells and in lane 5 for TCDD-treated splenocytes. TCDD-inducible DRE binding was not observed in nuclear protein preparations from either Hepa 1c1c7 cells or splenocytes treated with vehicle (Fig. 6, lanes 2 and 4). Denison and Yao [36] have identified a constitutive protein–single-stranded DNA complex that migrates faster than the TCDD-inducible protein–



Splenocyte	ND	ND	ND	0.37	0.65	0.99	1.18	1.73	3.41
Liver	ND	ND	0.49	0.99	1.42	1.54	3.46	5.73	9.07

FIG. 4. Slot blot analysis of the AhR in splenocytes and liver. Increasing concentrations of protein from whole cell lysates were filtered directly onto a nitrocellulose membrane. The membrane was incubated with 2 μg/mL of anti-AhR, 17-10B. Antibody binding was visualized by staining the blot with donkey anti-rabbit horseradish peroxidase-linked immunoglobulins. Slot blots were analyzed using a densitometer. The table contains values derived from adjusted volume (O.D. × area) which reflect the relative intensity of each band. The regression coefficients for spleen and liver were 0.996 and 0.957, respectively. This figure is representative of two separate experiments.



Splenocyte	ND	0.81	2.70	5.50	11.63	15.57	19.15	19.80	27.46
Liver	ND	ND	1.00	3.84	5.42	5.95	7.01	7.69	11.45

FIG. 5. Slot blot analysis of ARNT in splenocytes and liver. Increasing concentrations of protein from whole cell lysates were filtered directly onto a nitrocellulose membrane. The membrane was incubated with 2 μg/mL of anti-ARNT, 20-9B. Antibody binding was visualized by staining the blot with donkey anti-rabbit horseradish peroxidase-linked immunoglobulins. Slot blots were analyzed using a densitometer. The table contains values derived from adjusted volume (O.D. × area) which reflect the relative intensity of each band. The regression coefficients for spleen and liver were 0.955 and 0.987, respectively. This figure is representative of two separate experiments.

DNA complex. We similarly observed this same constitutive complex in nuclear protein preparations from Hepa 1c1c7 cells and splenocytes (Fig. 6, complex labeled C in lanes 2, 3, and 5). However, the gel retardation pattern observed with the TCDD-treated splenocytes was different from that observed with the Hepa 1c1c7 cells in that the TCDD-inducible protein–DNA complex resolved as a rather broad band in comparison to the complex formed in Hepa 1c1c7 cells (Fig. 6, band labeled A and B in lane 5). Addition of excess unlabeled DRE abrogated the TCDD-induced mobility shift, indicating specificity of binding of the AhR/ARNT complex to the DRE (Fig. 6, lane 6).

DISCUSSION

Although there is general agreement that the immune system is a sensitive target organ for the toxic effects of TCDD in most animal species, the molecular mechanism responsible for these effects on immunocompetent cells is poorly understood. Considerable emphasis has been placed on establishing a role for the AhR in mediating the diverse effects that TCDD and structurally related compounds produce on the immune system, most of which are inhibitory. As already established for a number of dioxin-sensitive genes of which *Cyp1a1* has served as a prototype, it has been speculated that immune suppression may be similarly mediated through deleterious changes in transcription, by the binding of the AhR/ARNT heterodimer to a DRE motif present in the promoter region of genes critical for immunocyte activation. However, not only have no target genes in immunocompetent cells been identified, but the direct identification of the fundamental components necessary for an AhR-mediated mechanism, specifically the AhR and ARNT protein, has not been demonstrated convincingly in immunocompetent cells. In this report, we confirmed the presence and functionality (i.e. binding of the AhR-ARNT heterodimer to the DRE) of the AhR and ARNT in Ah-responsive B6C3F1 splenocytes.

As summarized in the introduction, a number of previous

TABLE 1. Quantitative analysis of AhR and ARNT mRNA as determined by RT-PCR

Tissue	AhR (molecules/25 ng RNA)	ARNT (molecules/25 ng RNA)	ARNT:AhR ratio
Liver	$9.4 \times 10^4 \pm 1.4 \times 10^3$*	$4.7 \times 10^6 \pm 7.3 \times 10^5$	50
Splenocytes	$4.2 \times 10^4 \pm 6.0 \times 10^3$	$1.5 \times 10^7 \pm 1.5 \times 10^6$	357

* Mean ± SEM of molecules of target gene RNA/25 ng tissue RNA from three separate experiments.

studies that have capitalized on genetic differences in mouse strains and/or on differences in structure–activity relationships have indirectly implicated the involvement of the AhR in the immunotoxicity by TCDD [20–23]. Additionally, Lorenzen and Okey [24] have demonstrated specific and saturable binding of [^{3}H]TCDD in cellular cytosol from human tonsils, suggesting the presence of cytosolic AhR. In these studies, the authors went on to identify the translocation of protein bound [^{3}H]TCDD to the nucleus by sucrose gradient centrifugation. Photoaffinity-labeled AhR has also been demonstrated in the cytosolic fraction of whole spleen from B6 mice [40], and transcripts for the AhR and ARNT were identified in whole tissue preparations from spleen and thymus in the rat [37]. Although these findings were suggestive that immunocompetent cells possess the AhR and ARNT, it is unclear from the preparations that were used whether the actual source of the AhR and ARNT was from the immunocompetent cells or the contaminating non-immunocompetent cells also present in the preparations. This possibility was of concern based on biodistribution studies by Neumann and coworkers [28] in which they treated mice with [^{3}H]TCDD and demonstrated a comparable amount of [^{3}H]TCDD in splenic capsule as that found associated with splenocytes. Further, these results were highly reminiscent of previous findings in which Greenlee and coworkers [41, 42] demonstrated that although the thymus possesses a significant amount of AhR, it is in fact localized primarily in the thymic epithelium and that the thymic epithelium is the primary target for TCDD.

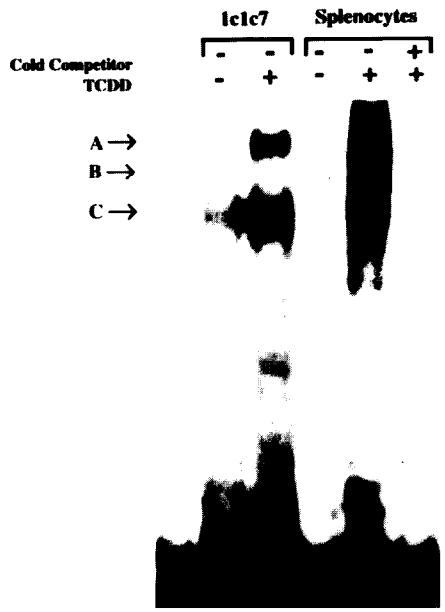


FIG. 6. Binding of Hepa 1c1c7 (1c1c7) or splenocyte nuclear proteins to a dioxin-responsive element. Six micrograms of nuclear protein from Hepa 1c1c7 cells, vehicle (lane 2) or TCDD-treated (lane 3), or B6C3F1 splenocytes, vehicle (lane 4) or TCDD-treated (lanes 5 and 6), were incubated with a ^{32}P-labeled 26 bp DRE oligonucleotide. Protein–DNA complexes were resolved using the EMSA. Unlabeled competitor DRE oligonucleotide was added at a 50-fold excess (lane 6) to show specific interaction of the proteins with the DRE oligonucleotide. Lane 1 is the DRE oligonucleotide without nuclear protein. Letters A and B identify TCDD-inducible protein–DNA complexes, and letter C identifies constitutive protein–single-stranded DNA complexes. This figure is representative of more than three separate experiments.

In the present studies, to avoid the possibility of confounding results, splenocytes devoid of capsule and red blood cells were evaluated by northern and western analysis and by quantitative RT-PCR for both AhR and ARNT. Northern analysis of poly(A) RNA isolated from splenocytes revealed a single band, approximately 6.6 kb, to which the AhR cDNA probe hybridized. As previously shown, B6C3F1 mice possess two forms of the AhR which are codominately expressed. These two forms of the AhR correspond to the *Ahr*$^{b\text{-}1}$ (C57BL/6J) and *Ahr*$^{b\text{-}2}$ (C3H) alleles [38]. In agreement with these previously reported results in the B6C3F1 mouse, we identified by western blot two major AhR proteins of approximately 95 and 104 kDa in splenocyte lysates. Northern analysis of mRNA isolated from spleen for ARNT revealed the presence of three mRNA transcripts of approximately 5.6, 2.0, and 1.1 kb. An identical banding profile for ARNT was observed in RNA isolated from liver. The presence of ARNT in mouse spleen was confirmed by western blot analysis as an 87 kDa protein. It is important to emphasize that we have used splenocyte suspensions in our analysis, which would include both lymphocytes and macrophages. Although we cannot rule out a contribution by macrophages, we believe our results are most consistent with a profile of activity in lymphocytes, primarily because macrophages constitute only about 5% of the splenocyte content from the B6C3F1 mouse [43, 44]. As such, we believe our results provide the most direct evidence to date for the presence of the AhR and the ARNT protein in mouse-derived lymphocytes.

Not surprisingly, because of the well-studied association of the AhR with the actions of TCDD in the liver, our quantitation of AhR by RT-PCR and slot blot analysis revealed greater amounts of message and protein for the receptor in liver than in spleen. However, it is quite intriguing that a greater amount of ARNT message and protein (approximately 3.2- and 2.4-fold, respectively) was present in splenocytes than in liver. Equally intriguing is the fact that more ARNT than AhR mRNA was expressed in the liver and splenocytes. A difference in the relative ratio of ARNT:AhR mRNA was also observed between the two preparations. These observations are in agreement with previous results by Carver and coworkers [37] in which they showed by RNase protection a trend towards greater expression of transcripts for ARNT as compared with AhR in rat spleen, thymus, and placenta; however, it is notable that in these studies the authors did not consider this difference in expression to be significant in the lymphoid tissues. Carver and coworkers went on to speculate that the disparate levels of the AhR and ARNT may be indicative of other biological roles for ARNT [37]. An alternative explanation for greater amounts of ARNT than AhR in some tissues is that this skewed ratio would increase the likelihood that the ligand-bound receptor would find its necessary binding partner (i.e. the ARNT protein), especially in tissues where there are low amounts of AhR. In support of this alternative possibility Li and coworkers [45] have demonstrated, in wild-type mouse hepatoma cells transfected with ARNT cDNA, an increase in magnitude, but not sensitivity, of the transcriptional response to TCDD. From this result, they concluded that the intracellular concentration of ARNT influences the response of a target gene to TCDD [45].

The previous demonstration that protein bound [^{3}H]TCDD translocates from cytosol to the nucleus in human tonsilar cells [24] is especially significant to our present studies since this was the first evidence suggesting that lymphoid cells may possess "functional" AhRs. However, the report by Lorenzen and Okey [24] was inconsistent with results from studies by Denison and coworkers [25] in which they were unable to show binding of the AhR/ARNT heterodimer to the DRE by electrophoretic mobility shift using TCDD-treated splenic cytosol from a variety of animal species including rat, guinea pig, and hamster. Taken together, the available results indicated that it was not enough to demonstrate specific binding by radiolabeled TCDD, or even the presence of the AhR in lymphoid cells, without questioning whether the AhR is "functional" in lymphocytes. In the present studies, we tested the functionality of the AhR/ARNT heterodimer by its ability to bind to the DRE following treatment of splenocytes with TCDD. Furthermore, this binding was demonstrated to be specific for the DRE motif as indicated by the ability of unlabeled DRE to compete for the binding of the heterodimer with ^{32}P-labeled DRE. These results confirm that the AhR and ARNT are, in fact, functional in B6C3F1-derived lymphocytes. We suspect that one reason this same result was not observed by Denison and coworkers may be due to the fact that the optimum conditions for AhR/ARNT binding to the DRE are modestly different for liver cytosolic preparations than that found in splenic preparations. In addition, we identified DRE binding in TCDD-treated splenocytes that resolved by EMSA as a rather broad band in comparison to DRE binding in TCDD-treated Hepa 1c1c7 cells. This is due to the formation of more than one TCDD-inducible protein–DNA complex in splenocytes as supported by: (1) the fact that B6C3F1 mice express two different AhRs of approximately 95 and 104 kDa; and (2) recent results by Bank *et al.* [46] demonstrated two distinct DRE binding complexes in hepatic cytosol from mouse and several other species.

While we have demonstrated that lymphocytes possess functional AhR and ARNT, these results do not necessarily confirm the role of an AhR-mediated mechanism for TCDD immunotoxicity. Several previously reported observations have challenged the exclusivity of this model and still need to be resolved. These include the observation that (a) B6C3F1 and DBA/2 mice, when treated subchronically with TCDD, exhibit a comparable magnitude of immune suppression, and (b) the low affinity AhR ligand, 2,7-dichlorodibenzo-*p*-dioxin, and TCDD produce a comparable inhibition of the anti-sRBC IgM antibody forming cell response following subchronic treatment of mice *in vivo* and following direct addition to naive splenocytes *in vitro* [26]. Nevertheless, we believe that the present studies clearly establish B6C3F1 splenocytes as a suitable model to begin to elucidate the involvement of the AhR in mediating immunotoxicity by TCDD and structurally related compounds. Additionally, this model should be useful in resolving the observations identified above that are inconsistent with an AhR-mediated mechanism of immune suppression.

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