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THE Ah RECEPTOR RECOGNIZES DNA BINDING SITES FOR THE B CELL TRANSCRIPTION FACTOR, BSAP: A POSSIBLE MECHANISM FOR DIOXIN-MEDIATED ALTERATION OF CD19 GENE EXPRESSION IN HUMAN B LYMPHOCYTES

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SUMMARY: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) inhibits murine and human B lymphocyte immunoglobulin production through an unknown mechanism. This study investigated the effect of TCDD on expression of the CD19 gene in a human B lymphocyte cell line. Northern blot analysis showed that TCDD treatment decreased steady state levels of CD19 mRNA by 67% in the IM-9 cell line. Using a gel mobility shift assay, we identified a DNA-binding complex in IM-9 nuclear extracts that by several criteria appears to be the Ah receptor. In addition, the Ah receptor complex recognized a DNA binding site for B cell lineage-specific activator protein (BSAP) in the promoter region of the human CD19 gene which is similar to the consensus Ah receptor DNA binding site. These results suggest that the AhR could interfere with BSAP-stimulated CD19 gene transcription by competition for a common DNA binding site.

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a persistent environmental contaminant which produces a wide spectrum of adverse effects in laboratory animals, including tumor promotion, reproductive and developmental toxicity, and immunotoxicity (1). With respect to immunotoxicity, TCDD alters both cell mediated and humoral immunity with varying sensitivity (2). In mice, TCDD administration results in decreased antibody responses to T-independent and T-dependent antigens (2-4). Furthermore, TCDD treatment of murine splenic and human tonsillar B lymphocytes *in vitro* inhibits immunoglobulin production elicited by a variety of stimuli (5-7). These studies and others (reviewed in ref. 2) support the conclusion that the B lymphocyte is the primary target in TCDD-mediated humoral immunosuppression.

TCDD binds with high affinity to an intracellular protein, the aryl hydrocarbon receptor (AhR), which functions as a ligand-activated transcription factor. Upon ligand binding, the

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Abbreviations used: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; DRE, dioxin response element; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; PMSF, phenyl-methyl sulfonyl fluoride; BSAP, B cell lineage-specific specific activator protein; kDa, kilodaltons.

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cytosolic AhR dissociates from heat shock proteins and translocates to the nucleus. The transcriptionally active AhR complex is a heterodimer composed of the ligand binding subunit and the ARNT protein, both members of the basic helix-loop-helix family of transcription factors (8-10). The AhR complex binds to specific DNA sequences termed dioxin response elements (DREs) containing the consensus sequence TNGCGTG (11). TCDD-dependent transcriptional induction of the cytochrome P-450 1A1 gene (*CYP1A1*), as well as genes for other drug metabolizing enzymes is mediated by binding of the AhR complex to DREs present in the 5' flanking region (12). Although few other AhR target genes have been identified, White and Gasiewicz (13) recently showed that the AhR bound *in vitro* to one of several DRE-like sequences in the coding region of the human estrogen receptor gene. Little is known concerning the regulation of gene expression by TCDD in lymphocytes or the molecular mechanism by which TCDD decreases B lymphocyte antibody production, but evidence supports a role for the AhR in the humoral immunotoxicity of TCDD (2, 4). Using ligand binding techniques, the AhR has been detected in cytosol and nuclear extracts of human tonsillar B lymphocytes (14) and a human B lymphocyte cell line (15); however, data regarding the DNA-binding properties of the AhR in a purified B lymphocyte population is lacking.

MATERIALS AND METHODS

Chemicals. TCDD was obtained from Midwest Research Institute (Kansas City, MO) through the National Cancer Institute Chemical Carcinogen Reference Repository. [γ^{32}P]dATP (3000 Ci/mmole) was purchased from Amersham Life Sciences (Arlington, IL) and [α^{32}P]dCTP was from ICN Biomedical (Costa Mesa, CA). All reagents for cell culture were from Gibco/BRL (Grand Island, NY), except fetal bovine serum (FBS), which was from Hyclone Laboratories (Logan, UT). Poly (dI-dC):(dI-dC) was obtained from Pharmacia Biotech (Piscataway, NJ). All other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise.

Cell culture. The IM-9 human B lymphoblast cell line (CCL 159) and the human hepatoma cell line HepG2 (HB8065) were both from the American Type Culture Collection (ATCC; Rockville, MD). The PJS-91 cell line was generated in our laboratory by Epstein-Barr virus transformation of peripheral blood lymphocytes isolated from a healthy male donor. The IM-9 and PJS-91 cell lines were maintained in RPMI 1640 medium supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, and 10% FBS. The HepG2 culture medium was minimum essential medium supplemented with 50 µg/ml gentamicin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% heat-inactivated FBS. Cells were treated with 25 nM TCDD in 0.1% DMSO or vehicle alone (0.1% DMSO).

Preparation of nuclear extracts. Cells were treated with 25 nM TCDD in serum free medium for 2 h at 37°C and crude nuclear extracts were prepared by a combination of previously described methods (16, 17). After treatment, the cells were washed sequentially with ice-cold phosphate-buffered saline and 10 mM HEPES, pH 7.6, resuspended in 3 volumes Buffer A (25 mM HEPES, pH 7.6, 1 mM DTT, 10 mM KCl, 3 mM $MgCl_2$) for 10 min at 0°C, and then homogenized with 10-20 strokes in a Dounce homogenizer. The nuclear pellet (3,500 x *g*, 15 min) was washed once with Buffer B (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 3 mM $MgCl_2$, 25% glycerol, 0.2 mM PMSF) containing 150 mM KCl, after which nuclear proteins were extracted with Buffer B containing 500 mM KCl for 1 h. The nuclear extract was centrifuged at 12,000 x *g* and the supernatant dialyzed against 100 volumes Buffer C (20 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 20% glycerol, 150 mM KCl, 0.2 mM PMSF) for 1h. The dialyzed extract was clarified by centrifugation (12,000 x *g* , 15 min) and stored in aliquots at -70°C.

Gel mobility shift assay. Complementary oligonucleotides synthesized by the University of Florida DNA Synthesis Core were annealed, end-labeled with [γ^{32}P]dATP and T4 polynucleotide kinase, and then purified on a 15% acrylamide gel. The ^{32}P-labeled probe used in

all experiments was the *CYP1A1* DRE oligonucleotide (Fig. 1) corresponding to a DRE at -968 to -997 in the upstream region of the human *CYP1A1* gene (18) to which the AhR binds (19). Nuclear extract protein was incubated in 15 mM HEPES, pH 7.9, 2 mM Tris-HCl, pH 7.5, 12% glycerol, 0.5 mM DTT, 0.5 mM EDTA, 100 mM KCl and 0.5-1 μg poly (dI-dC):(dI-dC) for 15 min at room temperature prior to addition of labeled probe (2-10 fmoles; 20-50,000 cpm), and then incubated another 15 min. Unlabeled double-stranded competitor oligonucleotides were added to the reaction mixture prior to the addition of labeled probe. In some experiments, goat anti-mouse AhR (20) or rabbit anti-human ARNT (21) antibody was added for 10 min at the end of the incubation with labeled probe. The reactions were electrophoresed through non-denaturing 4% acrylamide gels in 1X TGE buffer (25 mM Tris, 380 mM glycine, 2 mM EDTA) to separate protein:DNA complexes (17). Following electrophoresis, the gels were dried down and exposed to X-ray film at -70°C with intensifying screens. In competition experiments, the amount of specific protein:DNA complex present on the gel was quantitated by scanning the autoradiograph on a desktop laser scanner and digitizing the intensity of the images using NIH Image 1.52 software. The intensity of the AhR-dependent band at each competitor concentration was calculated as a percentage of the maximal DRE bound (i.e. in the absence of competitor). All experiments were repeated 2-3 times with at least two different nuclear extract preparations.

Northern blot analysis. Northern blots with 4 μg polyA$^+$ RNA were performed according to standard protocols except hybrization to radiolabeled cDNA probes was done at 68°C with QuikHyb solution (Stratagene; La Jolla, CA) according to the manufacturers instructions. The human CD19 probe was an EcoRI fragment of pB4-19 (22); and the human β-actin cDNA was from ATCC (no. 65128). Autoradiographs were quantitated by densitometry as described above for the gel shift assay.

RESULTS AND DISCUSSION

Our initial effort to identify genes potentially regulated by TCDD in B lymphocytes was made by searching published data on genes important in B lymphocyte function for DNA sequences which have homology to the consensus AhR binding site (TNGCGTG). This approach identified a subset of DNA binding sites for the transcription factor B cell lineage-specific activator protein (BSAP) which resemble the consensus binding site of the AhR (23). BSAP expression is essential for B lymphocyte development (24) and DNA binding sites for BSAP occur in regulatory regions of the immunoglobulin heavy chain gene locus and other genes expressed specifically in B lymphocytes (23, 25-27). The proposed consensus DNA recognition sequence for BSAP is 5' G/ANNCANTGNNGCGT/GG/AACC/GA/G-3' (23). The underlined nucleotides also conform to the consensus AhR "core" binding site (DRE) (11), and as shown in Fig.1, two binding sites for BSAP contain the GCGTG core sequence. The CD19-2 sequence is approximately 100 base pairs upstream from the transcription start site in the human CD19 gene (25), and the 5'Sγ2a sequence is ~2 kb upstream of a switch region 5' to the γ2a constant region in the mouse immunoglobulin heavy chain gene cluster (26). CD19 is a cell surface signal transducing protein expressed exclusively on B lymphocytes from the early stages of B cell development, but lost upon differentiation into plasma cells (see ref. 28 for review). Evidence supports a role for BSAP in the regulation of CD19 gene expression (24, 25), and we hypothesized that binding of the AhR to the DRE-like sequence in the CD19 promoter region could interfere with transcription by competing with BSAP for binding to this site.

TCDD treatment reduces steady state levels of CD19 mRNA in IM-9 cells. A 1.9 kb CD19 mRNA transcript was detected in the IM-9 human B lymphoblast cell line by Northern blot analysis (Fig. 2, *upper*). This is the appropriate size for a message encoding the 540 amino acid CD19 protein and is in close agreement with the 2.4 kb transcripts detected in other

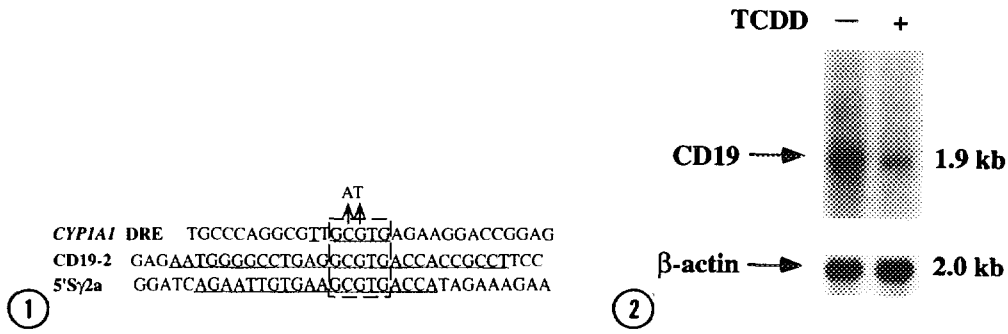

Figure 1. Sequences of oligonucleotides (upper strand only) used in this study. The *CYP1A1* DRE oligonucleotide contains an AhR binding site (*underlined*) in the 5' flanking region of the human *CYP1A1* gene. The mutant DRE oligonucleotide contains C to A and G to T mutations (*arrows*) in the GCGTG core. The CD19-2 and 5'Sγ2a oligonucleotides contain binding sites for BSAP as well as a region of similarity to the consensus binding sequence for the AhR (see text for details). The BSAP binding site identified by *in vitro* footprinting (25, 26) in the CD19-2 and 5'Sγ2a oligonucleotides is *underlined*. The GCGTG "core" sequence critical for AhR binding to the DRE (11) and the corresponding region of the CD19-2 and 5'Sγ2a oligonucleotides is *boxed*.

Figure 2. TCDD treatment reduces steady state levels of CD19 mRNA in the IM-9 human B lymphocyte cell line. IM-9 cells (2x 10^5/ml) were treated for 48 h with DMSO or 25 nM TCDD and polyA$^+$ RNA was isolated for Northern blot analysis. The blot was probed with a human CD19 cDNA (*upper*), stripped, and reprobed with a β-actin cDNA probe (*lower*).

B cell lines (22). In TCDD-treated IM-9 cells, the steady state level of CD19 mRNA was decreased by 67% relative to cells treated with vehicle alone, while β-actin mRNA levels were unchanged (Fig. 2, *lower*).

Gel mobility shift analysis of the AhR in human B lymphocytes. In Fig. 3, several bands representing nuclear protein bound to the *CYP1A1* DRE probe were present in nuclear extracts from TCDD-treated cells. In HepG2 cells, a human hepatoma cell line with high levels of the AhR (29) used here for comparison (Fig. 3, *left*), the AhR complex is the prominent band of lowest mobility which is induced by TCDD treatment (*large arrow*). Nuclear extracts from IM-9 and PJS-91 human B lymphoblast cell lines contain a *CYP1A1* DRE-binding complex of similar, but slightly lower mobility than the HepG2 AhR:DNA complex (Fig. 3, *center* and *right*). In contrast to the results seen in HepG2 cells, a substantial amount of this complex was present in nuclear extracts from IM-9 and PJS-91 cells exposed to DMSO vehicle alone. Others have reported low levels of the DNA-binding form of the AhR in the nucleus in the absence of TCDD treatment in MCF-7 breast cancer cells (13) and human dermal fibroblasts (19), however, the amount of AhR in the nucleus was still considerably induced by ligand. In our experiments, TCDD treatment only marginally increased the level of the nuclear DRE-binding complex in the IM-9 human B lymphoblast cell line, although the increase in the level of the nuclear DRE-binding complex in PJS-91 cells was more pronounced. Our results, together with those of others (13, 19), differ from the current model of AhR action where the unliganded receptor exists in an inactive form in the cytosol unable to bind DNA (11, 12). A possible explanation for these findings is that the DRE-binding complex in IM-9 and PJS-91 cells is in the nucleus constitutively or that ligand binding may be required for transcriptional activation but not for DNA-binding, a mechanism recently suggested for the estrogen receptor (30).

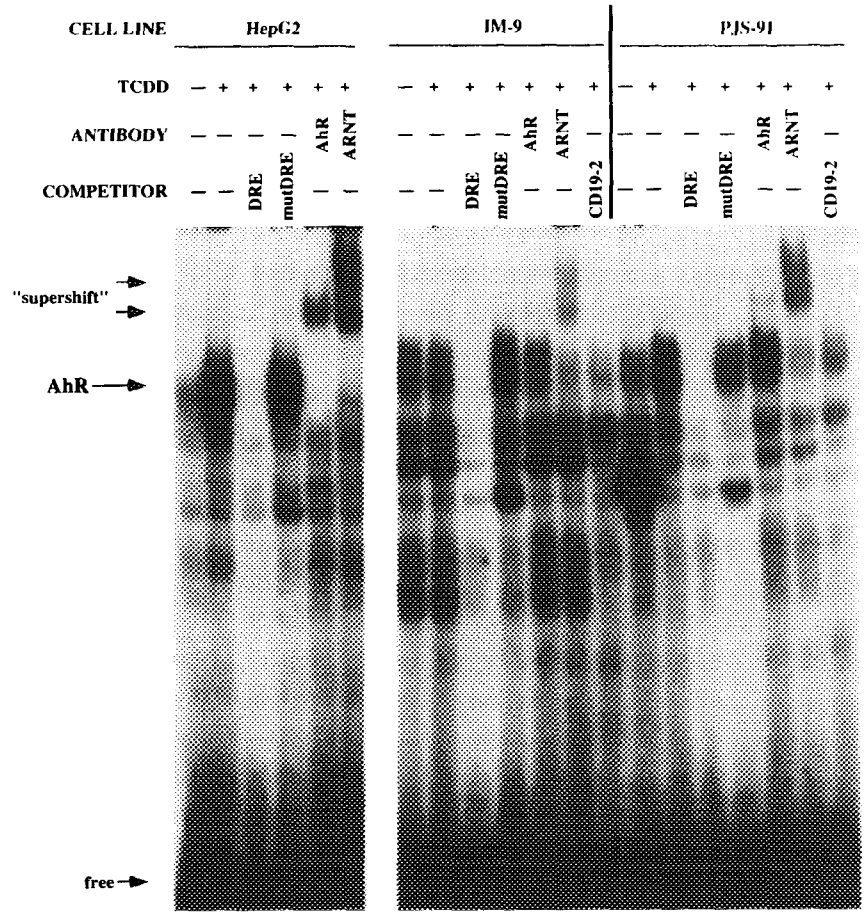

Figure 3. Gel mobility shift analysis of *CYP1A1* DRE-binding proteins in HepG2 cells and human B lymphocyte cell lines. Nuclear extracts from cells treated for 2 h with 25 nM TCDD were tested by gel shift assay for the presence of TCDD-inducible protein-DNA complexes using the ^{32}P-labeled *CYP1A1* DRE probe (Fig. 1). Unlabeled competitor oligonucleotides were present in 100-fold (DRE and mutDRE) or 625-fold (CD19-2) molar excess relative to the DRE probe. AhR or ARNT antibodies were added to the preformed DNA:protein complexes for 10 min prior to electrophoresis. The large arrow indicates the position of the AhR-dependent band and the small arrow indicates the supershifted AhR complex.

The formation of the AhR:DRE complex in all cell lines was completely inhibited by an excess of the unlabeled DRE oligonucleotide, but not by a DRE oligonucleotide containing a double mutation in the GCGTG core sequence (mutDRE) essential for AhR binding (11). Addition of an antibody specific for the conserved N-terminus of the mouse AhR (20) resulted in a DRE-binding complex of lower mobility (i.e., supershift, *small arrow*) in all three cell lines. However, in IM-9 and PJS-91 cells, only a fraction of the complex was supershifted by the AhR antibody compared to the complete supershift seen in HepG2 cells. In contrast, a human ARNT antibody (21) produced a strong supershift of the AhR complex in all three cell lines. These results indicate that one component of the DRE-binding complex in IM-9 and PJS-91 cells is the ligand binding subunit of the AhR, and that ARNT is a major component of the DRE-binding factor in

HepG2, IM-9 and PJS-91 cells. The partial supershift with the AhR antibody suggests that the human B lymphocyte AhR may be biochemically distinct from the AhR in HepG2 cells, or alternatively, that other as yet unidentified proteins may associate with ARNT to form the DRE-binding complex in human B cells. In line with these observations, a 108 kDa protein from untreated rat hepatic cytosol (31) and a 95 kDa protein from untreated Hepa-1 cytosol and nuclear extracts (32) were recently purified by DNA-affinity chromatography with a DRE-containing oligonucleotide.

Oligonucleotides corresponding to BSAP binding sites compete with the *CYP1A1* DRE for binding to the AhR. An unlabeled oligonucleotide corresponding to a BSAP binding site in the CD19 promoter region (Fig. 1, CD19-2) partially inhibited the formation of the AhR:DRE complex in the gel shift assay (Fig. 3). In competition experiments, increasing concentrations of unlabeled CD19-2 competed with the labeled DRE probe for binding to the AhR complex in IM-9 nuclear extracts (Fig. 4). In addition, another oligonucleotide containing a BSAP binding site (Fig.1, 5'Sγ2a) competed with the DRE for binding to the AhR over a similar concentration range (Fig. 4). The mutant *CYP1A1* DRE competed only slightly at the highest concentrations which is consistent with the importance of the intact GCGTG core sequence for AhR binding. Analysis of the competition curves yielded estimates of IC_{50} values of 0.8, 124, and 48 nM for the DRE, CD19-2, and 5'Sγ2a oligonucleotides, respectively. Non-specific competitor oligonucleotides containing a binding site for the Sp1 transcription factor or an octamer motif did not compete at a concentration of 600 nM, further indicating that this competition is not likely due to a large excess of DNA in the binding reaction (data not shown). Similar competition curves were obtained in parallel experiments using PJS-91 nuclear extracts (data not shown).

In summary, our results demonstrate that the AhR complex recognizes DNA binding sites for the B lymphocyte transcription factor, BSAP, albeit with lower affinity than the *CYP1A1* DRE. The CD19 gene is a probable target for BSAP (24, 25) although the contribution of the CD19-2 BSAP binding site used in this study to BSAP-regulated CD19 gene transcription is

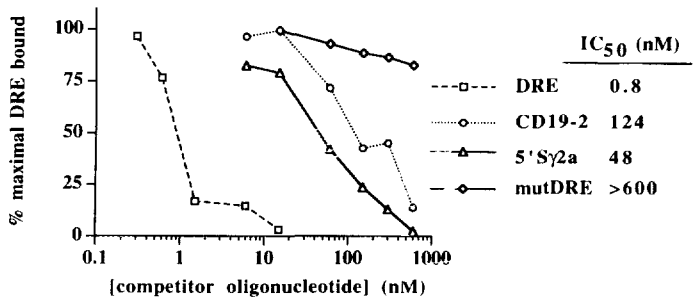

Figure 4. Competition curves for the *CYP1A1* DRE, mutant *CYP1A1* DRE, CD19-2, and 5'Sγ2a oligonucleotides in IM-9 nuclear extracts. The curves are generated with values from a representative competitive gel shift assay where increasing concentrations of unlabeled oligonucleotides were added to nuclear extracts before addition of a fixed amount of ^{32}P-labeled *CYP1A1* DRE probe. The concentration of unlabeled competitors (in fold molar excess) was 10-1000 except for the *CYP1A1* DRE which was 0.5-50. The IC_{50} is the concentration of unlabeled oligonucleotide at which binding to the DRE probe is reduced by 50% and was derived by least squares linear regression analysis of the linear portion of each curve.

unknown. We propose that the decrease in CD19 gene expression that occurs with TCDD exposure could result from the AhR acting to interfere with the binding of BSAP to a common DNA binding site in the CD19 promoter region. Further experiments are required to support this hypothesis and are currently in progress. The role of CD19 as a signal transducing molecule in B lymphocytes suggests that TCDD-mediated down regulation of CD19 expression may lead to altered cellular signalling and/or function.

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