Quantitative Analysis of Constitutive and 2,3,7,8-Tetrachlorodibenzo-\(p\)-dioxin-induced Cytochrome P450 1B1 Expression in Human Lymphocytes

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Abstract

Exposure to 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin (TCDD or dioxin) results in a broad spectrum of biological responses, including altered metabolism, disruption of normal hormone signaling pathways, reproductive and developmental effects, and cancer. Cytochrome P450 1B1 (CYP1B1) is a dioxin-inducible gene that is active in the formation of 4-hydroxyestradiol, a potentially genotoxic catechol estrogen. Therefore, the analysis of CYP1B1 in humans may be useful in establishing relationships between dioxin exposure and adverse health effects. In this study, we examined the expression of CYP1B1 in human peripheral blood lymphocytes of unexposed individuals using a quantitative reverse transcription-PCR method. Absolute CYP1B1 RNA levels varied more than 30-fold in uncultured mononuclear cells obtained from 10 individuals. In vitro treatment of mitogen-stimulated lymphocytes with TCDD for 1–5 days of culture resulted in a peak induction of CYP1B1 after 3 days. The induction of CYP1B1 RNA levels after 3 days of culture was dose-dependent, exhibited a maximum response above 10 nm TCDD, and varied greatly among different individuals. However, the half maximal dose required for this induction was similar between individuals and comparable to that observed in the MCF-7 and HepG2 human cell lines. These observations indicate that CYP1B1 exhibits variable constitutive expression and is inducible \textit{in vitro} by TCDD in human lymphocytes and that the magnitude of induction varies within the population. These data define the suitability of CYP1B1 for use as a mechanistically based biomarker in ongoing molecular epidemiological studies of human populations exposed to dioxins and related chemicals that bind the aromatic hydrocarbon receptor.

Introduction

TCDD \(^3\) is a human carcinogen (1), the persistence of which in the environment and presence in the food chain results in chronic exposure to human populations and bioaccumulation in human tissues (2). Epidemiological studies of populations exposed to high levels of TCDD or dioxin-like compounds, either occupationally or accidentally, are invaluable in determining the relationships between human exposure to these compounds and adverse health outcomes, such as chloracne and cancer (3, 4). TCDD is the most potent member of a family of structurally related compounds that are believed to have a common mechanism of action that involves initial binding to a cytosolic protein known as the AHR (5). Binding to and activation of the AHR results in the altered expression of a wide variety of genes, including the cytochrome P450 genes \(\text{CYP1A1 and CYP1B1}\). The specific deleterious effects associated with exposure to dioxin are believed to be due to species- and tissue-specific expression of dioxin-regulated genes (6). The analysis of the expression of these dioxin-inducible genes in human populations may aid in determining dose-response relationships for human exposure to dioxins \textit{in vivo}, assessing the variability of human response, and comparing the effect of dioxin across species. The interindividual variability of response is important in human health risk assessment because a high degree of variability may indicate the presence of subpopulations that are more sensitive to chemical exposures.

Measurements of human tissues for biomarkers of chemical exposure have historically been made using tissues that are easily obtained through minimally invasive procedures, such as hair, blood, urine or peripheral blood lymphocytes. The induction of \(\text{CYP1A1-associated aryl hydrocarbon hydroxylase activity in mitogen-activated human lymphocytes was first described over 25 years ago (7, 8).} \) \(\text{CYP1A1}\) is the most well-characterized of the dioxin-inducible genes (9) and has been shown to be inducible by TCDD in human lymphocytes (10). The magnitude of induction of \(\text{CYP1A1}\) gene expression by AHR agonists in mitogen-activated human peripheral blood lymphocytes has been correlated with an increased risk of lung cancer in some studies (11). Consequently, the induction of \(\text{CYP1A1}\) in human lymphocytes has been used in numerous studies as a biomarker of exposure to polycyclic aromatic

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3 The abbreviations used are: TCDD or dioxin, 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin; AHR, aromatic hydrocarbon receptor; \(\text{CYP1A1, cytochrome P450 1A1; CYP1B1, cytochrome P450 1B1; FBS, fetal bovine serum; PHA, phytohemagglutinin; PWM, pokeweed mitogen; DEPC, diethyl pyrocarbonate; RTP-PCR, reverse transcription-PCR; RTP, reverse transcriptase primer; IS, internal standard.} \)
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hydrocarbons (12, 13). CYP1A1 is currently being evaluated in our laboratory as a potential biomarker of exposure in risk assessment for potentially susceptible populations exposed to TCDD. However, the relationship between induction of CYP1A1 by TCDD and the development of adverse health effects has not been fully established.

CYP1B1 is another cytochrome P450 gene the expression of which is increased following exposure to TCDD or AHR agonists in both humans and rodents (14–18). Although less well-characterized than CYP1A1, CYP1B1 is a TCDD-inducible gene that could be involved in the mechanism of carcinogenesis for both endogenous and environmental carcinogens and therefore may be a relevant biomarker for evaluating TCDD exposure and gene induction in humans. Specifically, CYP1B1 is active in the metabolism of 17β-estradiol and in the bioactivation of polycyclic aromatic hydrocarbons and arylamines (19–21). The metabolic formation of the catechol estrogen 4-hydroxyestradiol by CYP1B1 has been implicated in the formation and development of benign tumors in humans (22, 23).

CYP1B1 is constitutively present in uncultured human peripheral blood leukocytes and has been qualitatively detected but not quantitated in these cells (14). The objective of the present study was to quantitatively measure constitutive and TCDD-induced CYP1B1 expression in human peripheral blood lymphocytes. We determined the dose response and the time course of induction of CYP1B1 expression by TCDD in mitogen-stimulated lymphocytes. Mitogen stimulation was used because it enhances AHR expression in lymphocytes, thereby increasing the magnitude of AHR-dependent responses (24, 25). However, mitogen stimulation results in activation of human lymphocytes and relative changes in cell population subsets. Therefore, we also measured constitutive CYP1B1 expression in RNA isolated from normal, uncultured lymphocytes. From our results, we compared levels of gene expression between individuals to determine variability within populations for constitutive as well as TCDD-induced expression. These results are used to establish the suitability of CYP1B1 as a measurable end point in peripheral blood lymphocytes in studies of dioxin-exposed human populations.

Patients and Methods
Study Subjects. Approximately 60 ml of venous blood from each of 10 locally recruited donors from North Carolina were collected in heparinized tubes under a contractual agreement with CODA, Inc. (Research Triangle Park, NC; contract NO1-ES-43576). These donors included six women from 24 to 52 years of age (average, 38 years), two of whom were smokers, and four men from 23 to 29 years of age (average, 25 years), two of whom were smokers.

Lymphocyte Isolation and Culture. Mononuclear cells were isolated from fresh whole blood within 2 h of collection using Ficoll density gradient centrifugation (Histopaque 1077; Sigma Chemical Co., St. Louis, MO) according to the manufacturer’s instructions. Isolated cells were cryopreserved in basal culture medium [RPMI 1640, 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Life Technologies, Inc., Gaithersburg, MD) supplemented with 7.5% cell culture grade DMSO (American Type Culture Collection, Rockville, MD) and 20% FBS (Hyclone, Logan, UT)]. The cell cultures were frozen at a density of 1 × 10^6 cells/ml in a Cryomed 700A programmed cell freezer and thereafter stored in vapor phase liquid nitrogen. Cryopreserved mononuclear cells were thawed, diluted in basal culture medium supplemented with 10% (v/v) FBS, centrifuged at 200 × g for 10 min, decanted and resuspended in fresh medium. Cell concentrations were determined and adjusted to 2 × 10^6 cells/ml with stimulation medium (basal culture medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol (Sigma), 1.25 μg/ml PHA (Murex Diagnostics Ltd., Norcross, GA), and 0.15% (v/v) PWM (Life Technologies)). Aliquots of 2–6 × 10^6 cells were immediately removed from each cell suspension, centrifuged, lysed by repeated pipetting in 1 ml of TRI REAGENT (Sigma), and stored at −70°C. These cell preparations normally consist primarily of lymphocytes (>80%), monocytes (<10%), granulocytes, erythrocytes, and platelets (each <5%). For ease of nomenclature, these cell preparations are herein referred to as uncultured lymphocytes. The remaining cells were added to 75-cm² tissue culture flasks at a density of 2 × 10^6 cells/ml, and then one volume of stimulation medium, containing twice the desired final concentration of TCDD, was added. Cell cultures were maintained at 37°C, 5% CO₂ and 95% relative humidity until harvest, 1–5 days later. Harvested cell suspensions were transferred to tubes and centrifuged 300 × g for 10 min. Cells were lysed by repeated pipetting in 1 ml of TRI REAGENT (Sigma), and stored at −70°C. These cell preparations are termed mitogen-stimulated lymphocytes.

MCF-7 and HepG2 Cell Culture. MCF-7 (ATCC HTB-22) and HepG2 (ATCC HB-8065) cells were obtained from the American Type Culture Collection and maintained as frozen stocks or propagated in culture at 37°C, 5% CO₂ and 95% relative humidity for a maximum of 20 passages. Both cell lines were maintained in MEM (phenol red-free for MCF-7 cells) with Earle’s salts and nonessential amino acids, supplemented with 10 μg/ml bovine insulin (MCF-7 cells only), 1 mM sodium pyruvate, 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 10% FBS (for HepG2, heat-inactivated at 56°C for 30 min). All medium components were obtained from Life Technologies, except for the FBS used with MCF-7 cells, which was purchased from Hyclone. Cells in log phase growth were harvested by trypsinization and seeded to 6-well plates. When cells reached approximately 50% confluency (after 4–6 days), they were treated for 24 h with 0, 0.1, 0.5, 2.5, 10, or 25 nM TCDD. For both MCF-7 and HepG2 cells, aliquots of the same TCDD stock dilutions used for lymphocyte cell cultures were added to maintenance medium in which cells had been growing for 48 h. After the 24-h treatment period, the medium was removed, and cells were lysed directly in the plate well following the addition of 1 ml of TRI REAGENT; lysates were stored in tubes at −70°C until RNA extraction.

TCDD Treatment. TCDD solutions were prepared by adding a constant amount of TCDD-containing FBS to the cell culture medium. The final concentrations used in the cell treatments included 0.1, 0.5, 2.5, 10, and 25 nM TCDD. The TCDD-FBS stock solutions were prepared essentially as described previously (26). Briefly, a 1 mg/ml solution of TCDD in toluene was spiked with [14C]TCDD (33mCi/mmol), both >99% pure and from Cambridge Isotope Laboratories (Andover, MA), to give a ~2 mM solution with a specific activity of ~5 μCi/μmol. An aliquot of this TCDD solution was added to a glass tube with glass homogenizing beads, and the toluene was evaporated under a stream of nitrogen. FBS was then added to the tube, mixed with the beads, and adsorbed with TCDD overnight at 4°C. The TCDD adsorbed FBS was collected and filter-sterilized, and the TCDD concentration was determined by scintillation counting. From this ~0.75 μM TCDD-FBS stock, serial dilutions were made in FBS to achieve the desired TCDD stock.
Figure 1. Quantitative RT-PCR analysis of CYP1B1 RNA levels in human peripheral blood lymphocytes. Total RNA (0.1 μg), isolated from lymphocytes treated with 10 μM TCDD and cultured for 3 days, was reverse transcribed and co-amplified by PCR in the presence of decreasing amounts of recombinant IS RNA. The left lane had CYP1B1 but no IS, and the right lane had IS but no CYP1B1 RNA in the reaction. A, one-fifth of each reaction was separated and stained by electrophoresis in 1× TAE buffer through a 2% NuSieve 3:1 agarose gel containing 0.5 μg/ml ethidium bromide. DNA product sizes were estimated relative to a 100-bp DNA ladder (Promega). B, the log ratios of intensities of CYP1B1:IS and log of the number of copies of IS were fit by linear regression. The number of molecules of CYP1B1 in the total RNA sample is equal to the antilog of the x-intercept, where the ratio of CYP1B1:IS is equal to 1.

Quantitative RT-PCR. Quantitative RT-PCR was carried out using a modified competitive titration assay which uses a heterologous IS recombinant RNA (Fig. 1; Refs. 27 and 28). As shown in Table 1, the amplicon consisted of RNA target-specific forward and reverse primers separated by a heterologous spacer molecule derived by amplification using primers specific to the human GSTM1 gene. The forward and reverse primer set span the location of a known human CYP1B1 intron (29), ensuring that the amplified PCR product was derived from RNA and not from any genomic DNA that may have been present. The IS amplicon was designed to be smaller than the CYP1B1 mRNA target by 118 bp and was thus easily discernible when analyzed using gel electrophoresis. In addition, levels of β-actin were measured for the RNA samples in this study; the specific primer sequences used are given in Table 1. All primers were purchased from Bioserve Biotechnologies, Ltd. (Laurel, MD). The IS RNA was prepared by PCR amplification of a plasmid containing a portion of the human GSTM1 gene with the primers shown in Table 1. The PCR products were purified using a Microcentrifug 100 DNA purification cartridge (Amicon, Beverly, MA), transcribed into RNA using the RiboMAX large-scale production system-T7 (Promega, Madison, WI) followed by an RNase-free DNase treatment to remove the DNA template. The IS RNA was subsequently purified using TRI REAGENT as described previously. RNA concentrations were determined by measuring absorbance at 260 nm, diluted to 4 × 10^8 copies/μl in DEPC-treated water, and stored at −70°C. Competitive reverse transcription was performed in a final volume of 20 μl, containing 0.1 μg of total RNA, one of six IS RNA concentrations, 5 mM MgCl₂, PCR buffer (16.6 mM NH₄SO₄, 5 mM 2-mercaptoethanol, 6.8 μM EDTA, 67 μM Tris-Cl, pH 8.8 (all from Sigma), and 0.1 mg/ml BSA (Calbiochem, San Diego, CA)), 1 μM each deoxyribo-nucleoside triphosphate, 15 units of recombinant RNasin RNase Inhibitor, 80 units of Moloney murine leukemia virus reverse transcriptase, and 0.1 μg of oligo(dT)₃₅(CYP1B1) or 1.25 μM RTP (β-actin). The samples were heated to 37°C for 15 min and then at 99°C for 5 min, and then cooled to 4°C. Following reverse transcription, 20 μl of PCR mixture was added to each tube. The PCR mixture contained PCR buffer, 0.5 μM each of the forward and reverse primers (Table 1), and 1 unit of Taq DNA polymerase. The final MgCl₂ concentration in the PCR was 2.5 mM (CYP1B1) or 2.0 mM (β-actin). The reactions were heated to 94°C for 4 min and then cycled at 94°C for 30 s, 60°C (CYP1B1) or 54°C (β-actin) for 30 s and 75°C for 30 s. Following 35 cycles (CYP1B1) or 25 cycles (β-actin), the reactions were held at 75°C for 4 min and then stored at 4°C. All reverse transcription and PCR reagents, excluding PCR buffer and primers, were purchased from Promega.

For each sample of RNA, an initial RT-PCR was performed using a 10-fold dilution series of IS RNA. It is important to note that RNA samples were not quantitated against a set of IS dilutions that were amplified on the same plate. Rather, each RNA sample was co-amplified in six different tubes in the presence of a different IS concentration of RNA (see Fig. 1). The measured values are derived from at least six independent reactions. Using the results from the 10-fold dilution series, the procedure was then repeated at least once using a 2-fold IS dilution series for each RNA. In essence, all RNA samples were quantitated at least twice, once with a 10-fold IS dilution series and again with a 2-fold series. When a 2-fold IS serial dilution assay was repeated, the average of the two values was used.

RT-PCR Quantitation. The resultant PCR products were mixed with 5× Orange G loading dye (0.4% in 10% Ficoll, 10 mM Tris-Cl, pH 7.5, 50 mM EDTA; all from Sigma). One-fifth of each mixture was separated by electrophoresis at 100 V for 3 h through a 2% NuSieve 3:1 (FMC Bio-Products, Rockland, ME) agarose gel in TAE (40 mM Tris-acetate, 1 mM EDTA) buffer and stained with 0.5 μg/ml ethidium bromide. Digitized images of stained cDNA products were captured as 8-bit digital TIFF files using an Eagle Eye II still video camera system and EagleSight software, version 3.2 (Stratagene Cloning Systems, La Jolla, CA). The density of each specific band was measured using the public domain program: NIH Image, version 1.61 (developed at the NIH and available on the Internet at http://rsb.info.nih.gov/nih-image/). The log₁₀ of the number of copies of IS RNA in each reaction was plotted against the log of the ratio (target RNA amplicon:IS RNA amplicon) and fit by linear regression. The number of copies of target amplicon was determined by interpolation using a log₁₀ ratio = 0. This point corresponds to where the number of molecules of CYP1B1 in
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Results

Quantitative RT-PCR Assay Development. In this study, we measured the expression of the CYP1B1 gene in human peripheral blood lymphocytes using a quantitative RT-PCR titration assay. The assay used an IS system that allowed the quantitation of CYP1B1 in RNA isolates by setting up a RT-PCR competition between the RNA sample being evaluated and the IS RNA (see Fig. 1). The CYP1B1 level in each RNA sample was determined, and the quantitation was repeated for each sample at least once using a six-reaction, 2-fold serial dilution of the IS RNA.

To ensure that the RT-PCR amplified products were derived from RNA and not from potentially contaminating genomic DNA, both the IS RNA (10^7 copies) and the RNA samples being evaluated (0.1 μg) were amplified by PCR with and without reverse transcriptase. As shown in Fig. 2, there were no observable products in reactions lacking reverse transcriptase.

Table 1  RT-PCR and IS primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>RT-PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1B1^a</td>
<td>Forward</td>
<td>accgcgaacttgaagaactc</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>gttggtgcaggtgcagatg</td>
</tr>
<tr>
<td></td>
<td>RTP</td>
<td>0.18</td>
</tr>
<tr>
<td>β-Actin^b</td>
<td>Forward</td>
<td>aaactcttcaactctcactac</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>atgatgcgtgcttgtg</td>
</tr>
<tr>
<td></td>
<td>RTP</td>
<td>tgtggggtggc</td>
</tr>
<tr>
<td>T7</td>
<td>Forward^c</td>
<td>T7-ccgcgaactctgaactctcagaagtctggaggaactc</td>
</tr>
<tr>
<td></td>
<td>Reverse^c</td>
<td>(0.18-tpgcagtgctgcagagtgaactgtcagctcagattg</td>
</tr>
<tr>
<td>IS-CYP1B1'</td>
<td>Forward</td>
<td>T7-acctcttcaactctcagcggcagtgtgctttgcaggaacgt</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>tggagaatggcagatgtctgtgctattgtgatagagtcttcagattg</td>
</tr>
</tbody>
</table>

^a PCR product corresponds to the human CYP1B1 cDNA (residues 1140–1566), GenBank accession number U03688.
^b PCR product corresponds to the human actin cDNA (residues 878–1040), GenBank accession number M10278.
^c Primers consist of the forward or reverse CYP1B1-specific primers sequences (underlined) flanking primer sequences specific for the human glutathione S-transferase gene (GSTM1; exon 6-intron 6-exon 7), GenBank accession number S62935.
^d The forward primer is flanked by a T7 site for use in an in vitro RNA transcription.
^e The reverse primer is flanked by an oligo(dT)_18 site to incorporate a poly(dA)_18 tail into the internal standard RNA.

0.1 μg of lymphocyte total RNA is equivalent to the number of copies of IS RNA in the reaction (see Fig. 1).

Dose-response Analysis. Curve fits were carried out using KaleidaGraph version 3.05. Dose-dependent changes in RNA levels of CYP1B1 were modeled with the sigmoid-E_{max} function (30) Y = E_{0} + [E_{max} * X^{n}/(E_{max} + X^{n})], where Y = response, X = concentration, E_{0} = effect in controls, E_{max} = the maximum response, E_{50} = concentration at half of the maximum response, and n = the shaping parameter (Hill coefficient). Independent parameters were selected using an iterative process using the Marquardt-Levenberg algorithm to calculate the least sum of squared differences between the observed and predicted values to select those that give the best fit of the observed values to the equation.

Statistical Analysis. ANOVA procedures were used to assess the significance of inter-subject variability, TCDD effects, and time-related trends. The variance-stabilizing logarithmic transformation was used in these analyses. Pairwise comparisons were made by Fisher’s least significant difference test.

CYP1B1 Induction in Mitogen-stimulated Lymphocytes. To determine the time course of CYP1B1 expression, mitogen-stimulated lymphocytes were treated with 10 nm TCDD, and expression was measured in lymphocytes harvested daily for 5 days. A concentration of 10 nm TCDD was used because this dose has previously been shown to near maximally induce murine and human lymphocyte CYP1A1 enzyme activity (10, 24).

A time-dependent increase in the expression of CYP1B1 mRNA in mitogen-stimulated lymphocytes due to TCDD induction was observed (Fig. 3). The data shown in Fig. 3A are the average CYP1B1 induction for three individuals for mitogen-stimulated lymphocytes, both without (control) and with TCDD treatment. The absolute difference in CYP1B1 levels from the two females and one male included in this experiment are shown in Fig. 3B. ANOVA procedures for all three individuals at all time points revealed a highly significant (P < 0.01) TCDD effect, a significant (P < 0.01) difference among the 5 days, but the subject-to-subject variability was not significant. Pairwise comparisons indicated that the mean TCDD-induced level of CYP1B1 was significantly (P < 0.05) elevated over controls after 3 and 4 days of culture only. The maximal induction of CYP1B1 was achieved after 3 days of culture, so these conditions were used in subsequent analyses.

Fig. 2. RNA-specific amplification of IS and CYP1B1. 0.1 μg of total RNA and 10^7 copies of IS RNA were amplified by PCR, both with and without reverse transcriptase. The products were separated and stained by electrophoresis in 1% TAE buffer through a 2% NuSieve 3:1 agarose gel containing 0.5 μg/ml ethidium bromide. DNA product sizes were approximated using a 100-bp DNA ladder (Promega).
To determine the response of lymphocytes to varying doses of TCDD, lymphocytes from another three individuals were cultured in the presence of both mitogens and TCDD concentrations of 0, 0.1, 0.5, 2.5, 10, and 25 nM for 3 days, and CYP1B1 mRNA levels were measured. CYP1B1 expression was inducible in a dose-dependent fashion by TCDD as shown in Fig. 4. For the three individuals evaluated, the maximal response for induction of CYP1B1 mRNA was achieved at doses above 2.5 nM. The dose-response data are also presented as a percent of the maximum value (Fig. 4B), showing that although these individuals had a different magnitude of response, the shape of the dose-response curve for the three individuals was similar. Subsequent experiments used a 10 nM treatment to ensure that the potential near maximum induction was attained.

CYP1B1 Induction in Human Cell Lines. To compare the results obtained with human lymphocytes, we also measured the response to TCDD treatment in two human cell lines: the MCF-7 human breast cancer cell line and the HepG2 human hepatoblastoma cell line (Fig. 5). Interestingly, although CYP1B1 has previously been undetectable in HepG2 cells using RNA hybridization analysis (31), it showed a clear dose-dependent induction by TCDD in these cells when quantitated using RT-PCR. The level of expression of CYP1B1 was highest in MCF-7 cells and lowest in HepG2 cells (Table 2). The expression of CYP1B1 in mitogen-stimulated human lymphocytes was intermediate to the level of expression in these cell lines. Dose-dependent induction of CYP1B1 by TCDD has previously been shown in MCF-7 cells by Northern blot analysis (19). By comparison, β-actin levels were not affected by TCDD treatment in either cell line or in mitogen-stimulated lymphocytes (Table 2). To compare the shape of the dose-response curves between lymphocytes and these cell lines, the dose-response data for CYP1B1 RNA levels and TCDD concentration treatment were fit using the sigmoid E_max function. The calculated EC_{50} values, which are the concentrations of TCDD treatment required to obtain half-maximal induction of CYP1B1, were similar for all three cell types (Table 2).

CYP1B1 Expression in Uncultured and Mitogen-stimulated Lymphocytes. The constitutive expression of CYP1B1 in the uncultured peripheral blood lymphocytes of ten North Carolina volunteers was quantitated using competitive RT-PCR. The levels of expression of CYP1B1 ranged from 16,300 to 491,760 copies of CYP1B1 per μg of RNA, corresponding to a 30-fold difference between the lowest and highest values (Fig. 6). When these levels were expressed relative to the corresponding
β-actin levels which ranged 19-fold (data not shown), there was still a 6-fold difference in the range for CYP1B1 levels.

When the lymphocytes were stimulated by PHA and PWM in culture, the observed levels of CYP1B1 mRNA were greater than that of uncultured lymphocytes from the same individual (Fig. 6). The fold increase in absolute CYP1B1 levels ranged from 3-fold to 45-fold following mitogen stimulation. Interestingly, the mitogen-stimulated levels of CYP1B1 measured in the lymphocytes of the six individuals varied only about 2.5-fold from the lowest to highest, whereas the range for the uncultured varied 30-fold. β-actin levels were increased an average of 17-fold following mitogen-stimulation (range, 6–208-fold).

The CYP1B1 levels measured in mitogen-stimulated lymphocytes were used as the control values for the TCDD-treated lymphocytes from the same individual, to calculate the fold induction in gene expression due to TCDD. The inducibility of CYP1B1 in response to TCDD exposure varied widely for the six individuals evaluated. The induced expression (treated/control) was variable among these individuals, displaying a 4–19-fold difference in the magnitude of CYP1B1 induction. The rank order of the TCDD-induced CYP1B1 levels was different from that of the control (mitogen-stimulated) levels, indicating that TCDD inducibility is independent of the control level for a given individual (Fig. 6).

Discussion
Our primary goals in this study were to analyze the expression of CYP1B1 in normal human lymphocytes and to develop a quantitative method by which we could measure the expression of CYP1B1 in ongoing studies of lymphocytes of dioxin-exposed populations. Specifically, these populations include an occupationally exposed cohort who worked at the Boehringer-Ingelheim chemical plant in Hamburg-Moorfleet, Germany and an accidentally exposed cohort of individuals living near the ICMSA chemical plant in Seveso, Italy. The characterization of CYP1B1 in human lymphocytes described in this paper used a cohort of individuals from North Carolina who presumably have only been exposed to background levels of dioxins normally present in the environment.

We characterized CYP1B1 expression in normal, uncultured human lymphocytes and evaluated time course and dose-response relationships for CYP1B1 expression in mitogen-stimulated lymphocytes treated in vitro with TCDD. Using this information, we have determined the appropriate conditions to use to quantitatively measure CYP1B1 in human lymphocytes in our TCDD-exposed cohorts. The general approach we are using in these studies analyzes both gene expression in normal, uncultured human lymphocytes and the effect of TCDD on gene expression in mitogen-stimulated lymphocytes. The use of uncultured lymphocytes will allow us to determine whether interindividual differences in gene expression in vivo are related to prior exposure to dioxin-like compounds. The use of mitogen-stimulated lymphocytes will allow us to determine whether prior exposure to dioxins alters individual response to TCDD in vitro and also whether there are interindividual differences in responsiveness that might be related to differences in sensitivity to the effects of dioxin. Ultimately, this information will provide insights into the factors governing interindividual susceptibility, which may reduce uncertainties in risk assessment for human exposure to dioxins.

Constitutive levels of CYP1B1 mRNA were high compared to those of CYP1A1, which were barely detectable in the same cells (25). The pattern of high CYP1B1 expression and low CYP1A1 expression in uncultured human peripheral mononuclear cells is similar to that observed in human mammary epithelial cells (32, 33). The high constitutive expression of CYP1B1 may be due to both interindividual differences in factors affecting basal CYP1B1 expression as well as exposure to AHR agonists in vivo. The very low basal expression of CYP1A1 suggests that measurable increases in CYP1A1 expression in uncultured lymphocytes may be indicative of exposure to AHR agonists, such as that seen in smokers (10). It is hypothesized that highly responsive individuals may be at greater risk for the development of adverse health effects resulting from dioxin exposure. Therefore, parallel analysis of both CYP1A1 and CYP1B1 may help to identify those who are highly responsive with respect to multiple dioxin-inducible end points.

In uncultured lymphocytes, the range of measured CYP1B1 expression between individuals was variable. The variability observed may be due to a number of factors, including differences in actual exposure to environmental agents that induce CYP1B1 gene expression, interindividual genetic differences in response to this exposure, or experimental variability. Within this context, polymorphisms in the CYP1B1 gene have recently been identified, although it is not known whether there is a relationship between these polymorphisms and CYP1B1 expression (34). In addition, it is important to note that the “uncultured lymphocytes” referred to in this paper, although primarily consisting of lymphocytes, also contain monocytes and other cell types. A recent report suggests that CYP1B1 may be a major P450 isozyme in human blood monocytes (35). Consequently, some of the variability in levels of CYP1B1 observed may be in part due to differences in the relative proportions of monocytes and lymphocytes in the individuals we examined. Although the number of samples (10) analyzed in this study was small, we believe that these values estimate an approximate range of CYP1B1 expression in the normal population. Ongoing studies in our laboratory should confirm this, and the information will be used to establish a reference range for comparisons with study cohorts in which individuals were highly exposed to TCDD-like compounds.

Mitogen stimulation using PHA and PWM increased the level of expression of CYP1B1 in the lymphocytes of the six individuals studied, but with variable differences: 3-fold to 45-fold over that of the uncultured (Fig. 6). However, the final CYP1B1
the lymphocytes in culture. It is important to note that factors in the cell culture medium or possibly to differentiation of expression levels. Alternatively, it may be due to the effect of mitogen stimulation may be due to the effect of elevated AHR (20). The elevated basal expression of CYP1B1 observed following that there are cell-specific factors involved in gene regulation expression varies widely among different human tissues, indicat-

all individuals regardless of the constitutive expression. CYP1B1 stimulation increased the levels of CYP1B1 to a similar level for of 10 nM TCDD.

expressed relative to to a 30-fold difference in the uncultured lymphocytes. When a range of only 2.5-fold between the highest and lowest, compared values measured for these individuals reaches a similar level, with a range of only 2.5-fold between the highest and lowest, compared to a 30-fold difference in the uncultured lymphocytes. When expressed relative to β-actin, the range of CYP1B1 levels was 6-fold. The level of CYP1B1 in mitogen-stimulated lymphocytes appeared to be independent of the constitutive level, i.e., mitogen stimulation increased the levels of CYP1B1 to a similar level for all individuals regardless of the constitutive expression. CYP1B1 expression varies widely among different human tissues, indicating that there are cell-specific factors involved in gene regulation (20). The elevated basal expression of CYP1B1 observed following mitogen stimulation may be due to the effect of elevated AHR expression levels. Alternatively, it may be due to the effect of factors in the cell culture medium or possibly to differentiation of the lymphocytes in culture. It is important to note that β-actin levels were also increased following mitogen stimulation, and therefore the increase in CYP1B1 was likely due in part to general increases in gene transcription. Elevation in CYP1A1 enzyme activity by mitogen stimulation of human lymphocytes has also been observed (7), indicating that this is not a phenomenon unique to CYP1B1.

CYP1B1 is inducible by TCDD in a dose-dependent and time-dependent manner. The maximal response for CYP1B1 was achieved following treatment with 10 nM TCDD and the optimal time for maximum induction was determined to be 3 days. Based on these observations, ongoing studies in our laboratory are using 10 nM TCDD treatments for 3 days to determine CYP1B1 RNA expression in lymphocytes from individuals who have been sub-

jected to high levels of dioxin through environmental accidents or through occupational exposure.

The dose-dependent induction in CYP1B1 in human lymphocytes was intermediate to that observed In the MCF-7 and HepG2 human cell lines. The EC50, which is the concentration of TCDD required to obtain a half-maximal induction of CYP1B1, was calculated from the observed response to TCDD for the human lymphocytes as well as for the MCF-7 and HepG2 cell lines. As shown in Table 2, the EC50 for the human lymphocytes in our study ranged from 0.50 to 1.05 nM TCDD, compared to 0.158 for MCF-7 and 0.63 nM for HepG2. The lymphocyte EC50 measured is comparable to the values determined for the two cells lines, suggesting that although all three human cells responded to TCDD in a similar fashion, lymphocytes may be more comparable to a lower expressing cell line like HepG2. By comparison, the EC50 for CYP1A1 enzyme induction in human and mouse lymphocytes was 1.8 and 1.3 nM TCDD respectively (24). The EC50 for CYP1B1 induction by TCDD in MCF-7 cells, when measured by Northern blot analy-

sis, has previously been shown to be 0.1 nM (19). Interestingly, using RT-PCR, we were able to quantitatively measure the dose-dependent induction of CYP1B1 in HepG2 cells, whereas it was previously undetectable in this cell line by Northern hybridization analysis (RNA blotting; Ref. 31).

Peripheral blood lymphocytes are an easily accessible source of human tissue that can be used for a variety of studies, including the analysis of gene expression. Thus, the use of lymphocytes allows the measurement of the expression of dioxin-inducible genes to evaluate their use as biomarkers with potential application in risk assessment. In this study, levels of CYP1B1 expression have been shown to be quantifiable, and comparisons were made among uncultured, stimulated, and TCDD-induced lymphocytes. In addition, because the EC50 value for the lymphocytes ranges between those of the two established human cell lines, it appears that choice of lymphocytes as a representative tissue to evaluate TCDD-induced expression of CYP1B1 is valid.

In summary, CYP1B1 is a dioxin-inducible gene that is expressed in uncultured human lymphocytes. There was interind-

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Copies CYP1B1/µg total RNA (×10^3)</th>
<th>EC50 (nM TCDD)</th>
<th>β-Actin/µg of total RNA (×10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>10 nM TCDD</td>
<td>Control</td>
</tr>
<tr>
<td>MCF-7</td>
<td>3223</td>
<td>98559</td>
<td>0.158</td>
</tr>
<tr>
<td>Lymphocytes (range)</td>
<td>508–1276</td>
<td>3567–16469</td>
<td>0.50–1.05</td>
</tr>
<tr>
<td>HepG2</td>
<td>1</td>
<td>253</td>
<td>0.63</td>
</tr>
</tbody>
</table>

a The number of CYP1B1 copies was determined by RT-PCR in total RNA, as described in “Patients and Methods,” isolated from cells cultured in the presence or absence of 10 nM TCDD.

b β-Actin mRNA levels were quantitated using a 3.162-fold internal standard dilution series.

c The EC50 is the concentration of TCDD required to obtain half-maximal induction of CYP1B1.

Fig. 6. Interindividual variation of CYP1B1 levels in uncultured, mitogen-

stimulated, and TCDD-induced human peripheral blood lymphocytes. CYP1B1 levels were measured by RT-PCR in total RNA isolated from normal, uncultured lymphocytes of 10 individuals (6 females and 4 males). Each individual is represented by a different symbol: filled symbols, males; open symbols, females. For six of these individuals (four females and two males) CYP1B1 levels were measured after mitogen stimulation and lymphocyte culture for 3 days in the presence or absence (control) of 10 nM TCDD.

vidual variability in constitutive levels of CYP1B1 and induction by TCDD in mitogen-stimulated lymphocytes. This observed variability may be indicative of in vivo exposure to dioxin-like compounds or inherent differences in gene expression. In vitro exposure of mitogen-stimulated human lymphocytes to TCDD resulted in a dose- and time-related increase in CYP1B1 expression. These results indicate that CYP1B1 expression in uncultured lymphocytes may be evaluated as a biomarker of human exposure to dioxin-like compounds. In contrast, the analysis of CYP1B1 in TCDD-treated mitogen-stimulated lymphocytes in vitro may be used to evaluate the relationship between prior dioxin exposure and the responsiveness of a given individual. Furthermore, the measurement of CYP1B1 induction in human lymphocytes should be useful in ongoing and future studies assessing the relationship between exposure and response in dioxin-exposed human populations and comparison with responses in rodent models commonly used in risk assessment.

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References


Quantitative Analysis of Constitutive and 2,3,7,8-Tetrachlorodibenzo-\(p\)-dioxin-induced Cytochrome P450 1B1 Expression in Human Lymphocytes


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