Cytochrome P450 1A1 and 1B1 in Human Blood Lymphocytes Are Not Suitable as Biomarkers of Exposure to Dioxin-like Compounds: Polymorphisms and Interindividual Variation in Expression and Inducibility

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Cytochrome P450 1A1 (CYP1A1) and 1B1 (CYP1B1) are phase I enzymes, the expression of which can be affected by many environmental compounds, including dioxins and dioxin-like compounds. Because CYP1A1 and CYP1B1 expression can easily be determined in peripheral blood lymphocytes, it is often suggested as biomarker of exposure to these compounds. In this study we investigated the interindividual differences in constitutive expression and induced CYP1A1-catalyzed ethoxyresorufin-O-deethylase (EROD) activity and CYP1A1 and CYP1B1 gene expression in human blood lymphocytes in a group of ten non-smoking females. Freshly isolated lymphocytes were cultured in medium containing the mitogen PHA and were exposed to the most potent dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or the less potent dioxin-like polychlorinated biphenyl 126 (PCB126). In addition, we determined the occurrence of the CYP1A1 MspI and CYP1B1 Leu432Val polymorphisms. All individuals showed a concentration-dependent increase of EROD activity by TCDD, which was significantly correlated with an increase in CYP1A1, but not CYP1B1 expression. The maximum induced EROD activity by TCDD was very different among the individuals, but the EC50 values were about the same. PCB126 also caused a concentration-dependent increase of EROD activity, but was a factor 100–1000 less potent than TCDD among the individuals. The allele frequencies for CYP1A1 MspI and CYP1B1 Leu432Val reflected a normal Caucasian population and in this study the polymorphisms had no apparent effect on the expression and activity of these enzymes. Our study shows a large interindividual variability in constitutive and induced EROD activity, and CYP1A1 and CYP1B1 expression in human lymphocytes. In addition, dioxin concentrations at which effects were observed in our in vitro study are about 10-fold higher than the human blood levels found in vivo, indicating that EROD activity and CYP1A1 and CYP1B1 expression in human lymphocytes might not be applicable as biomarkers of exposure to dioxin and dioxin-like compounds.

Key Words: human lymphocytes; CYP1A1; CYP1B1; dioxin-like compounds; biomarker.

Cytochrome P450 1A1 (CYP1A1) and 1B1 (CYP1B1) are phase I enzymes that can be found in many tissues including peripheral blood lymphocytes (Furukawa et al., 2004). They are involved in the metabolic activation of polycyclic aromatic hydrocarbons (PAHs), but also in the oxidative metabolism of estrogens to potentially genotoxic catechol estrogens (Martucci and Fishman, 1993). CYP1A1 and CYP1B1 expression is regulated through the aryl hydrocarbon receptor (AhR)-mediated pathway. Several environmental contaminants including PAHs and persistent organochlorine pollutants, such as polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-p-dioxins (PCDDs), are AhR agonists and can affect the expression of CYP1A1 and CYP1B1. CYP1A1 and CYP1B1 expression can be readily determined in peripheral blood lymphocytes (Furukawa et al., 2004), making them potential candidates for use as non-invasive biomarkers of exposure to environmental compounds with dioxin-like properties. In order to use CYP1A1 and CYP1B1 expression for this purpose, several possible confounding factors must be considered, including age, gender, and cigarette smoking (Garte et al., 2003; George et al., 1995; Kim et al., 2004; Parkinson et al., 2004). In addition, several genetic polymorphisms have been identified in the CYP1A1 and CYP1B1 genes that might affect gene expression or catalytic activity, thereby complicating the use of these genes as biomarkers of exposure. The CYP1A1 MspI polymorphism, a T to C transition in the 3′-untranslated region (UTR) of the CYP1A1 gene (CYP1A1*2A), is often studied in relation to CYP1A1 inducibility, but the studies are inconclusive about its effect (Crofts et al., 1994; Garte et al., 2003; Goth-Goldstein et al., 2000; Kiyohara et al., 1996). An amino acid substitution in codon 432 (Leu to Val) of the CYP1B1 gene is associated with a higher catalytic activity of the enzyme (Akliyu et al., 2002; Li et al., 2000). In addition, some studies report an association between the Leu/Leu genotype and higher expression...

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2 Parameters used for calculation: plasma weight = 1.02 kg/L, plasma fat = 2%, total blood volume in female = 4.7 l (of which 2.8 l is plasma), MW TCDD = 322.
levels (Hanaoka et al., 2002), while others find no effect of this polymorphism on expression levels (Li et al., 2000).

Although CYP1A1 and CYP1B1 expression are often investigated as biomarkers of exposure, the polymorphisms of CYP1A1 and CYP1B1 are seldom considered. To complicate matters further, polymorphisms are ethnically related (Garte et al., 2001), which makes comparison of the various studies difficult. Landi et al. (2003a) found no differences in CYP1B1 mRNA expression in lymphocytes obtained from control (n = 59) and accidentally TCDD-exposed (n = 62) subjects in the Seveso population (Italy) and no effect of the CYP1B1 Leu432Val polymorphism on CYP1B1 expression levels. In a study of Hungarian workers (n = 161) from an aluminium production plant exposed to polycyclic aromatic hydrocarbons, the number of PAH-DNA adducts in lymphocytes was not different among the CYP1A1 and CYP1B1 genotypes (Schoket et al., 2001). Hanaoka et al. (2002) found a correlation between the presence of at least one CYP1B1 432Leu allele and higher expression of CYP1B1 in the lymphocytes from 37 Chinese coke oven workers. Lin et al. (2003) studied CYP1A1 and CYP1B1 expression in human lymphocytes of 32 Taiwanese male and female subjects including both smokers and non-smokers. They showed a large interindividual variation in induction of CYP1A1 and CYP1B1 upon in vitro exposure of the lymphocytes to PAHs. Spencer et al. (1998) showed that CYP1B1 mRNA levels in human lymphocytes of ten North Carolina volunteers (male and female, both smokers and non-smokers), concentration-dependently increased upon in vitro exposure to 2,3,7,8-tetrachloro-p-dibenzodioxin (TCDD).

Interindividual variations in mRNA expression patterns, catalytic activity, and polymorphisms are important factors to consider when using CYP1A1 and CYP1B1 as biomarkers of exposure, yet little is known about these factors. The objective of this study was to investigate the interindividual differences in concentration-response curves for EROD activity and CYP1A1 and CYP1B1 gene expression in human lymphocytes upon induction with TCDD or the less potent dioxin-like polychlorinated biphenyl 126 (PCB126). We studied the possible influence of the CYP1A1 MspI and CYP1B1 Leu432Val polymorphisms on EROD activity and CYP1A1 and CYP1B1 expression levels. To minimize the role of confounding factors, we recruited ten healthy female Caucasian volunteers who were all non-smokers. Furthermore, the relevance of CYP1A1 and CYP1B1 in human lymphocytes as biomarkers of exposure to environmental factors was addressed by comparing the concentrations at which we found effects on EROD activity and mRNA levels in vitro, with the concentrations of dioxins and dioxin-like compounds that can be found in human blood.

MATERIALS AND METHODS

Study subjects. Venous blood was collected from 10 healthy female individuals in two 7-ml blood collection tubes containing EDTA (BD Vacutainer, Franklin Lakes, NJ). All individuals were Caucasian female non-smokers currently living in Utrecht, The Netherlands, with an average age of 26.4 years (range 24.1–28.7 yr.). This study was approved (number P02.115) by the medical ethical committee of the Leiden University Medical Center (Leiden, The Netherlands) and an informed consent was obtained from all volunteers.

Lymphocyte isolation. One ml of whole blood was stored at −70°C for DNA analysis. Mononuclear cells were isolated from fresh blood samples within 1 h of collection using Ficoll-Paque isolation according to the manufacturer’s instructions (Amersham Biosciences, Piscataway, NJ). Typically, the mononuclear cell preparations predominantly contain lymphocytes yet also contain monocytes. Hereafter the mononuclear cell preparations will be referred to as (peripheral blood) lymphocytes. The lymphocytes were washed twice with warm PBS and then suspended in culture medium consisting of phenol red-free RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1.5% phytohaemagglutinin (PHA), and 10% (v/v) fetal bovine serum (FBS). The lymphocyte concentration was determined using a coulter counter and the cell number was adjusted to 5 × 10⁵ cells/ml. A 300 µl aliquot of cell suspension was centrifuged to remove the medium and the pellet was resuspended in 250 µl RNA Instapure (Eurogentec, Maastricht, The Netherlands) and stored at −70°C (non-mitogen stimulated cells). Of the remaining cell suspension, 1-ml aliquots were added to two 12-well plates. Then, 1 ml of culture medium was added containing twice the desired concentration of TCDD or the solvent vehicle DMSO at a final concentration of 0.1% (v/v). If sufficient lymphocytes were obtained during isolation, a second 12-well plate was incubated with various concentrations of PCB126. Cells were cultured in a humidified atmosphere with 5% CO₂ at 37°C. After 72 h, the culture medium of one plate was replaced with 500 µl RNA Instapure and stored at −70°C and the medium of the other plate was replaced with Tris-buffer for ethoxyresorufin-O-deethylase (EROD) activity determination.

Ethoxyresorufin-O-deethylase (EROD) activity. Cells were exposed to 0.5 ml of a 50 mM Tris buffer (pH 7.8) containing 0.9% w/v NaCl, 6.25 mM MgCl₂, 5 µM 7-ethoxyresorufin, and 10 µM dicumercro. Plates were placed in a 37°C pre-heated Fluostar plate reader (BMG Labtechnologies GmbH, Offenburg, Germany) and fluorescence was measured at an excitation wavelength of 530 nm and emission wavelength of 590 nm every 3 min for half an hour. A calibration curve of resorufin was used for quantification of the activity. After determination of EROD activity, the plates were stored at −20°C until protein determination. Protein contents were measured by the method of Lowry et al. using BSA as protein standard (Lowry et al., 1951).

RNA isolation and quantitation of yield. Total RNA was isolated by using phenol-chloroform extraction. Purity and concentration of the isolated RNA was determined spectrophotometrically at an absorbance wavelength of 260 and 280 nm. RNA samples were stored at −70°C until analysis. Each RNA sample was quantified twice. An RT-PCR was performed with 100 ng RNA in a 25-µl reaction volume using the Access PCR system of Promega (Promega, Madison, WI). Specific RT-PCR conditions, primers, and amplification parameters are described elsewhere (Sanderson et al., 2001). The RT-PCR reaction showed linearity up to 500 ng RNA per reaction. RT-PCR products were separated on a 2% agarose gel and bands were stained with ethidium bromide. Intensity of the ethidium bromide staining was quantified using a FluorImager (Amersham Biosciences, Piscataway, NJ). CYP1A1 and CYP1B1 expression, normalized using β-actin, were calculated relative to the expression in the solvent vehicle-treated cultured lymphocytes of the same individual.

PCR and Restriction Fragment Length Polymorphism (RFLP) analysis. Genomic DNA was isolated from 300 µl whole blood with a DNA isolation kit (Promega, Madison, WI) according to the manufacturer’s instructions. Methods and primers for genotype analysis for CYP1A1 MspI and CYP1B1 Leu432Val were adapted from Kawajiri et al. (1996) and Tang et al. (2000). First, a PCR was carried out with 500 ng DNA using the Access RT-PCR System by Promega (Promega, Madison, WI, USA) in a 25 µl reaction.
volume containing 1× PCR buffer, Taq polymerase (5 U/µl), MgCl₂ (2 mM for CYP1A1 and 1.3 mM for CYP1B1), 200 µM dNTPs and 1 pmole of the reverse primer and 1 pmole of the forward primer. The primers were obtained from Invitrogen (Invitrogen Co., Carlsbad, CA). The PCR reactions started with a denaturing step at 94°C for 3 min followed by 30 cycles of 94°C for 1 min (denaturing), annealing for 1 min (at 66°C and 59°C for CYP1A1 and CYP1B1, respectively) and 72°C for 1 min (extension) and ended with a final extension step at 72°C for 7 min. Subsequently, 10 µl of PCR product was digested for 3 h at 37°C with 10 units of MspI or Eco57I (Fermentas Inc., Hanover, MD) for detection of the CYP1A1 and CYP1B1 polymorphisms, respectively. As positive control for endonuclease restriction 4.5 µg lambda DNA (Fermentas Inc., Hanover, MD) was used. A volume of 10 µl unrestricted PCR product and 10 µl of restricted PCR product were loaded on a 2.5% non-denaturing agarose gel containing ethidium bromide (0.05 µg/ml). The bands were visualized with UV light and genotypes were determined by visual inspection of the restriction fragments. For CYP1A1, endonuclease digestion of the PCR product (340 bp) resulted in two DNA fragments (200 and 140 bp) when the mutated allele (3801T → C transition) was present. For CYP1B1, digestion of the PCR product (271 bp) with the Leu allele (1294C) yielded two fragments of 166 and 105 bp.

Data analysis. The study was performed twice in duplicate and the data points shown are calculated as means of four determinations unless stated otherwise. Deviations from the Hardy-Weinberg equilibrium were calculated using the two-sided χ² test. Statistical significance of differences of the means and variances were determined by one-way ANOVA analysis followed by a Tukey-Kramer multiple comparisons test. Concentration-response curves and EC50 values (50% of the maximum activity, calculated using the fitted concentration-response curve) were obtained using sigmoidal dose-response nonlinear regression curve fit (GraphPad Prism 3.0, GraphPad Software Inc., San Diego, CA). Comparison of the concentration-response curves was performed using a F test. Differences were considered statistically significant if p < 0.05. Statistical calculations were performed using GraphPad InStat 3.06 (GraphPad Software Inc., San Diego, CA).

RESULTS

EROD Activity in Cultured Lymphocytes

The catalytic activity of CYP1A1 in the cultured lymphocytes was determined by measuring EROD activity. Constitutive EROD activity was low (0.23 ± 0.11 pmol resorufin/min/mg protein), but detectable in all individuals and the activity was concentration-dependently increased by TCDD (Fig. 1). Maximal induction of EROD activity by 10 nM TCDD was between 10 and 125-fold above constitutive levels in the ten individuals. Statistically significant differences among the minimum and maximum EROD activities were observed among the individuals (Table 1). However, the relative responses to TCDD were very similar; when the EROD activity was calculated as % of the maximum activity, the concentration-response curves overlapped. Relatively little variation was found in the EC50 values for induction of EROD activity, which varied between 0.52 and 3.88 nM. Only the EC50 value in individual 8 was significantly higher than in all the other subjects. This indicates that although wide variation was observed in the maximal response elicited by TCDD (efficacy), the concentration of TCDD necessary to produce 50% of the maximal EROD activity (potency) did not vary greatly among this relatively small and homogeneous group of women.

From five individuals sufficient lymphocytes were obtained and cultured to determine a concentration-response curve for PCB126, the most toxic PCB. In all individuals, PCB126 concentration-dependently induced EROD activity, but only in the lymphocytes of three individuals (numbers 1, 4, and 8) a maximum response was reached at the highest concentration tested (0.3 µM). In all three individuals, the maximum level of EROD induction reached by PCB126 was about 1.5-fold lower than the maximum induction caused by 10 nM TCDD (data not shown).

Where possible, the PCB126 concentrations at half maximum induction (EC50 values) were calculated and compared with the potency of TCDD in the same individual. These EC50 values were 59.8, 38.4, and 37.4 nM PCB126, for individuals 1, 4, and 8, respectively, resulting in relative potencies (RPs) of 0.010, 0.054, and 0.104. However, because a maximum in induction of EROD activity by PCB126 was not reached in every individual, the EROD activity at 25% induction by TCDD was calculated (EC25) and subsequently, the PCB126
TABLE 1
Summary of EROD Activity, Gene Expression and Genotype of CYP1A1 and CYP1B1 in Cultured Lymphocytes of Ten Healthy Female Volunteers

<table>
<thead>
<tr>
<th>Individual</th>
<th>Control (0.1% v/v DMSO)*</th>
<th>Maximal induced*</th>
<th>EC50 (nM)</th>
<th>EC25 (nM)</th>
<th>EC50 (nM)</th>
<th>EC25† (nM)</th>
<th>EC50 (nM)</th>
<th>EC25† (nM)</th>
<th>CYP1A1 (fold induction)§</th>
<th>CYP1B1 (fold induction)§</th>
<th>CYP1A1 MspI</th>
<th>CYP1B1 Leu432Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.29 ± 0.02</td>
<td>2.95 ± 0.16</td>
<td>0.64 ± 0.39</td>
<td>0.31</td>
<td>63.1</td>
<td>59.8</td>
<td>0.005</td>
<td>0.011</td>
<td>2.2</td>
<td>1.3</td>
<td>*1/1</td>
<td>Val/Val</td>
</tr>
<tr>
<td>2</td>
<td>0.07 ± 0.04</td>
<td>8.73 ± 0.82</td>
<td>0.65 ± 0.21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.0</td>
<td>1.8</td>
<td>*1/1</td>
<td>Val/Leu</td>
</tr>
<tr>
<td>3</td>
<td>0.34 ± 0.03</td>
<td>9.38 ± 0.94</td>
<td>0.82 ± 0.14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.1</td>
<td>2.5</td>
<td>*1/1</td>
<td>Val/Leu</td>
</tr>
<tr>
<td>4</td>
<td>0.22 ± 0.02</td>
<td>6.51 ± 0.04†</td>
<td>2.07 ± 0.20</td>
<td>0.85</td>
<td>151.4</td>
<td>38.4</td>
<td>0.006</td>
<td>0.054</td>
<td>3.2</td>
<td>2.7</td>
<td>*1/1</td>
<td>Val/Leu</td>
</tr>
<tr>
<td>5</td>
<td>0.12 ± 0.07</td>
<td>8.22 ± 0.87</td>
<td>0.98 ± 0.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.7</td>
<td>1.5</td>
<td>*1/1</td>
<td>Val/Leu</td>
</tr>
<tr>
<td>6</td>
<td>0.28 ± 0.09</td>
<td>10.0 ± 1.31</td>
<td>0.59 ± 0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.5</td>
<td>1.7</td>
<td>*1/2A</td>
<td>Val/Leu</td>
</tr>
<tr>
<td>7</td>
<td>0.31 ± 0.15</td>
<td>8.00 ± 0.24</td>
<td>0.50 ± 0.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.6</td>
<td>1.8</td>
<td>*1/1</td>
<td>Leu/Leu</td>
</tr>
<tr>
<td>8</td>
<td>0.06 ± 0.04‡</td>
<td>4.64 ± 0.21†</td>
<td>3.88 ± 1.31</td>
<td>1.23</td>
<td>91.2</td>
<td>37.2</td>
<td>0.014</td>
<td>0.104</td>
<td>2.0</td>
<td>1.7</td>
<td>*1/1</td>
<td>Leu/Leu</td>
</tr>
<tr>
<td>9</td>
<td>0.39 ± 0.10</td>
<td>5.60 ± 0.06</td>
<td>0.52 ± 0.07</td>
<td>0.22</td>
<td>112.2</td>
<td>138.5</td>
<td>0.002</td>
<td></td>
<td>n.d.</td>
<td>1.3</td>
<td>*1/1</td>
<td>Leu/Leu</td>
</tr>
<tr>
<td>10</td>
<td>0.25 ± 0.06</td>
<td>7.23 ± 0.99</td>
<td>0.74 ± 0.09</td>
<td>0.29</td>
<td>173.8</td>
<td>138.5</td>
<td>0.002</td>
<td></td>
<td>2.1</td>
<td>1.4</td>
<td>*1/1</td>
<td>Leu/Leu</td>
</tr>
<tr>
<td>Average</td>
<td>0.23 ± 0.11</td>
<td>7.06 ± 2.71</td>
<td>0.80 ± 0.07</td>
<td>0.58 ± 0.44</td>
<td>118.3 ± 44.7</td>
<td>45.1 ± 12.7</td>
<td>0.006 ± 0.005</td>
<td>0.056 ± 0.047</td>
<td>2.6 ± 1.1</td>
<td>1.8 ± 0.5</td>
<td>CYP1A1*1 0.95 432Val 0.35</td>
<td></td>
</tr>
</tbody>
</table>

*Data are represented as average EROD activity (pmole resorufin/min/mg protein) ± SD of two duplicate measurements.
†C25 is the PCB126 concentration necessary to obtain the EROD activity that was reached at 25% induction by TCDD.
‡Relative potency (RP) of PCB126 to induce EROD activity. RPs are calculated using the (E)C25 or EC50 values of TCDD and PCB126.
§Expression after incubation with 10 nM TCDD for 72 h is calculated relative to CYP1A1 or CYP1B1 expression in lymphocytes cultured for 72 h in media containing the mitogen PHA and 0.1% v/v DMSO.

Statistically significant differences between 1) individual 2 and 9; 2) individual 8 and 3, 9; 3) individual 4 and 6; 4) individual 8 and 2, 3, 5, 6, 7; 5) individual 9 and 2, 3, 6; 6) individual 8 and 1–7, 9, 10.
concentration necessary to obtain the same level of EROD activity (C25) was determined. In this way, a RP could be calculated for the individuals where the concentration-response curves for PCB126 did not reach a maximum. The RPs for PCB126 calculated using the C25 values varied between 0.002 and 0.014 among the individuals (Table 1).

CYP1A1 and CYP1B1 Expression in Uncultured and Cultured Lymphocytes

The expression levels of CYP1A1 and CYP1B1 in the lymphocytes were calculated relative to the expression in the solvent vehicle-treated cultured lymphocytes of the same individual.

In the uncultured (non-mitogen stimulated) lymphocytes, CYP1A1 transcript levels were too low to be detected. CYP1B1 expression on the other hand, was detectable. After 72 h of culture in media containing the mitogen PHA, CYP1A1 expression was still lower than CYP1B1, but both were detectable. Only in individual 9, CYP1A1 expression in cultured solvent-vehicle treated lymphocytes was too low to be detected. Mitogen-stimulation increased the expression of CYP1B1 by about 60% (Fig. 2). Induction by TCDD increased the expression of both genes even further. The induction of CYP1A1 expression by 10 nM TCDD (2.6 ± 1.1 fold) was statistically significantly higher than that of CYP1B1 (1.8 ± 0.5 fold). The differences in maximally induced EROD activity among the individuals was reflected by the CYP1A1 mRNA levels. The level of induction of EROD activity was positively correlated with the level of CYP1A1 expression (r = 0.67, p = 0.04, n = 9; Fig. 3). For CYP1B1 this correlation was not significant (r = 0.02, p = 0.95, n = 10).

For the individuals 1 and 3, who displayed the lowest and the highest induction of EROD activity at 10 nM TCDD, respectively (Fig. 1), an entire concentration-response curve was determined for CYP1A1 and CYP1B1 mRNA expression. When this curve was compared with the concentration-response curve for EROD induction in the same individual, a shift of the curve to the left was seen (Fig. 4) indicating that increased

FIG. 2. CYP1A1 (A) and CYP1B1 (B) mRNA expression in lymphocytes of ten individuals. Expression is calculated relative to CYP1A1 or CYP1B1 expression in lymphocytes cultured for 72 h in media containing the mitogen PHA and 0.1% v/v DMSO. All data points represent the average expression of two duplicate determinations in one individual. The line indicates the mean expression.

FIG. 3. Correlation between induction of EROD activity and induction of CYP1A1 (A; r = 0.67, p = 0.04, n = 9) or CYP1B1 (B; r = 0.02, p = 0.95, n = 10) mRNA levels. Data are represented as fold induction at 10 nM TCDD compared with the vehicle control-treated cultured lymphocytes. Every data point represents one individual (mean of two duplicate measurements).
mRNA expression occurred at lower concentrations of TCDD than EROD induction. For individual 1, the EC50 value was 0.10 nM TCDD for CYP1A1 mRNA induction and 0.63 nM for EROD induction. For individual 3, these values were 0.13 nM and 0.82 nM, respectively. This shift in concentration-response curve was also seen for CYP1B1 expression and the concentration-response curves for CYP1A1 and CYP1B1 induction completely overlapped (data not shown).

**CYP1A1 MspI and CYP1B1 Leu432Val Polymorphisms**

The *CYP1A1* MspI and *CYP1B1* Leu432Val genotypes of each individual were determined in DNA isolated from whole blood (Table 1). The allele frequencies were 0.95 for *CYP1A1*/*1* and 0.35 for *CYP1B1* 432Val. These frequencies did not deviate significantly from the Hardy Weinberg-equilibrium (*p* = 0.99 and *p* = 0.95 for *CYP1A1*/*1* and *CYP1B1* 432Val, respectively). The individuals shown in Figure 1 all had the *CYP1A1*/*1*/1 genotype and for *CYP1B1* these genotypes were Val/Val (individual 1), Val/Leu (individual 3), Leu/Leu (individual 7), and Leu/Leu (individual 9).

Only one individual (number 6) had a mutant *CYP1A1* allele (*CYP1A1*/*1*/2A), which made it impossible to detect an effect of this polymorphism in this study. To study the effect of the *CYP1B1* Leu432Val polymorphism, the individuals were divided into two groups with the same *CYP1B1* genotype, excluding the one individual with the *CYP1A1*/*1*/2A genotype and the one individual with the *CYP1B1* Val/Val genotype. No effects were found of the *CYP1B1* Leu432Val polymorphism on the maximum fold induction of CYP1B1 mRNA, constitutive EROD activity and EC50 for EROD induction by TCDD. The only statistically significant difference observed (*n* = 4 per group, power 93.21%) was the maximum level of TCDD-induced EROD activity, which was higher in the Val/Leu-group (9.5 ± 0.2 pmole RSF/min/mg protein) than in the Leu/Leu-group (6.7 ± 0.8 pmole RSF/min/mg protein).

**DISCUSSION**

The purpose of this study was to obtain a better insight into the possibility of using human peripheral blood lymphocytes as biomarkers of exposure to environmental compounds such as dioxin and dioxin-like compounds. Peripheral blood lymphocytes provide an easily obtainable source of CYP1A1 and CYP1B1, the expression of which is affected by dioxin and dioxin-like compounds, including certain PCBs. However, little is known about the interindividual variability in constitutive expression and catalytic activity of CYP1A1 and CYP1B1, or their responses toward environmental contaminants. Therefore, we investigated in vitro responses toward TCDD and PCB126 in freshly isolated lymphocytes of healthy volunteers. The cell preparations used in this study, although referred to as peripheral blood lymphocytes, do not solely contain lymphocytes but also contain some monocytes and other cell types. However, this is also the case in epidemiological studies using peripheral blood lymphocytes as biomarkers of exposure. Therefore, we believe that the mRNA expression levels in the non-mitogen stimulated cells described in this study can be regarded as representative for biomarker studies. In order to minimize the variability within the population in this study, we recruited a group of young female volunteers with little variation in factors which possibly affect CYP1A1 and CYP1B1 expression, such as smoking, domicile, ethnicity, age, and gender.

**Influence of the CYP1A1 MspI and CYP1B1 Leu432Val Polymorphisms**

With the limited number of individuals in our study, it is not possible to make a conclusive statement about a potential effect of the studied genetic polymorphisms on EROD induction or CYP1A1 and CYP1B1 gene expression levels. The individuals in this study did appear to reflect a normal Caucasian population. The allele frequencies for the studied genotypes...
did not deviate from the Hardy-Weinberg equilibrium and allele frequencies were similar to other published frequencies (Bailey et al., 1998; Garte et al., 2001; Miyoshi et al., 2003; Tang et al., 2000). Although, we did not observe a clear-cut effect of the CYP1A1 MspI and CYP1B1 Leu432Val polymorphisms, it should be noted that in the literature the opinions are not conclusive either. With regard to induction of EROD activity, some studies describe no effect of the CYP1A1 MspI polymorphism on EROD activity in lung microsomes of smokers and non-smokers (Smith et al., 2001) or in induced lymphocytes of Caucasian volunteers (Smart and Daly, 2000). Another study describes a higher basal and induced EROD activity in lymphocytes of individuals with at least one mutated MspI allele (Landi et al., 1994). An effect of the CYP1B1 Leu432Val polymorphism on EROD activity is not expected because of the limited contribution of CYP1B1 to EROD activity (Doostdar et al., 2000). This was also shown by two other studies that described no effect of the CYP1B1 Leu432Val polymorphism on basal EROD activity (Landi et al., 2003a; Li et al., 2000). Yet, the CYP1B1 432Val variant is believed to be the high-activity variant of CYP1B1 (Aklillu et al., 2002). However, the apparent association in this study between higher maximally induced EROD activity and the CYP1B1 Val/Leu-genotype should be considered with care. A more specific marker for CYP1B1 activity, such as estrogen 4-hydroxylation (Hayes et al., 1996), would be more appropriate to study effects of the CYP1B1 Val/Leu-genotype on catalytic activity. However, we did not detect estrogen 4-hydroxylation activity in the cultured lymphocytes, which was probably due to the low activity and the low number of cells in our experimental design (5 × 10^5 cells/well, 12 wells plate) (data not shown).

With regard to the CYP1A1 MspI or CYP1B1 Leu432Val polymorphisms, most studies report no effect on expression level (Crofts et al., 1994; Goth-Goldstein et al., 2000; Landi et al., 1994; Li et al., 2000). Garte et al. (2003) on the other hand described a 3-fold decrease in expression associated with homozygous CYP1A1 MspI genotype in lymphocytes of 177 healthy volunteers. Hanaoka et al. (2002) showed an association between the CYP1B1 Leu/Leu genotype and a higher expression level in lymphocytes of workers exposed to PAHs. Landi et al. (2003a) describe greater induction by TCDD of CYP1B1 among individuals reached an apparent maximum. It is possible that upon incubation with higher PCB126 concentrations the maximum level of EROD induction would appear to be somewhat higher, as would be expected in the case of the two individuals for whom a maximum was not reached. The WHO established the TEF value of PCB126 to be 0.1 (van den Berg et al., 1998). In our study, the relative potency of PCB126 to induce EROD activity in human lymphocytes depended greatly on the method of calculation (see Table 1). However, the RPs for PCB126 that were calculated using the EC50 values, which is the most common method for RP calculation, were in the same order of magnitude of the TEF established by the WHO (van den Berg et al., 1998).

CYP1A1 and CYP1B1 mRNA Expression

In contrast to CYP1B1 expression, extrahepatic CYP1A1 expression is low (Furukawa et al., 2004; Werck-Reichhart and Feyereisen, 2000), which was also seen in this study. CYP1B1 mRNA could be detected readily in uncultured, non-mitogen stimulated lymphocytes, whereas CYP1A1 mRNA was undetectable, although our RT-PCR method may not have been sensitive enough to detect low levels of expression. In addition, the mononuclear cell preparations prepared using Ficoll-Paque, although consisting primarily of lymphocytes, also contain monocytes. Monocytes express relatively high levels of CYP1B1 mRNA (Baron et al., 1998), which might substantially contribute to the CYP1B1 expression levels found in non-mitogen stimulated cells. Incubation with mitogens, such as PHA, causes lymphocytes to proliferate (Baum et al., 1997) a process which occurs concomitantly with the activation of several cell signaling pathways and increases in gene transcription, such as the

EROD Activity in Cultured Human Lymphocytes

EROD activity is commonly used as a marker for CYP1A1-mediated catalytic activity, although CYP1B1 also exerts some EROD activity (Doostdar et al., 2000). The constitutive EROD activity in the cultured lymphocytes was very low, but detectable (average of 0.20 ± 0.14 pmole/min/mg protein) and varied substantially between the individuals in this study. EROD activity in the cultured lymphocytes was readily induced by TCDD, with maximum levels of induction by TCDD between 10 and 150-fold above basal activity. The interindividual variations in efficacy were probably not due to experimental variability since the study was performed twice finding the same (relative) efficacies for each of the individuals. The interindividual differences in responsiveness toward dioxin and dioxin-like compounds might be attributed to differences in AhR expression in the lymphocytes, variation in vivo exposure to AhR agonists or genetic factors that might affect gene expression.

To estimate the potency of a dioxin-like compound, the TEF (toxic equivalent factor) concept was developed (Safe, 1990) and evaluated by the World Health Organization (WHO) (van den Berg et al., 1998). TEF values are derived by evaluating the potency of a compound relative to that of TCDD, assuming that the efficacies are equal. We found a slight difference in EROD induction efficacy between TCDD and PCB126 in our study. However, at the highest concentration of PCB126 tested (0.3 μM), the concentration-response curves of only three individuals reached an apparent maximum. It is possible that upon incubation with higher PCB126 concentrations the maximum level of EROD induction would appear to be somewhat higher, as would be expected in the case of the two individuals for whom a maximum was not reached. The WHO established the TEF value of PCB126 to be 0.1 (van den Berg et al., 1998). In our study, the relative potency of PCB126 to induce EROD activity in human lymphocytes depended greatly on the method of calculation (see Table 1). However, the RPs for PCB126 that were calculated using the EC50 values, which is the most common method for RP calculation, were in the same order of magnitude of the TEF established by the WHO (van den Berg et al., 1998).
expression of CYP1A1 and CYP1B1. And even though β-actin expression also increased, normalized CYP1A1 and CYP1B1 expression levels were higher in mitogen stimulated cells than in non-mitogen stimulated cells. Furthermore, PHA stimulation has been shown to increase the AhR expression level in lymphocytes (Whitlock et al., 1972), which would make them more susceptible to induction of gene expression by dioxin-like compounds. This suggests that the effects on mRNA expression in lymphocytes elicited by dioxin-like compounds in this in vitro study, might be larger than the effects expected upon in vivo exposure.

The mRNA levels appeared to be a more sensitive indicator of exposure to TCDD than EROD activity, because the effects on mRNA levels were detected at lower concentrations. This effect might be explained by the fact that upon binding of dioxin or dioxin-like compounds to the AhR, the CYP1A1 and CYP1B1 genes are first transcribed into mRNA before translation to the functional enzymes occurs. Therefore, it can be expected to detect increased mRNA levels before increased catalytic activity.

**CYP1A1 and CYP1B1 as Biomarkers of Exposure**

To make a statement based on this study concerning the use of CYP1A1 and CYP1B1 in human lymphocytes as biomarkers of exposure to dioxin and dioxin-like compounds, it is important to compare the concentration-response curves in vitro with human blood levels found in vivo. Human blood levels of a mixture of dioxin and dioxin-like compounds are expressed as toxic equivalents (TEQs), compound concentrations normalized with their WHO-TEF values. Background TEQs that are found in human plasma in industrialized countries are about 20 ng TEQ/kg fat (IARC, 1997). This TEQ can be translated to a TCDD concentration in whole blood of about 0.8 pM TCDD. In our study, the concentration that caused a significant induction of EROD activity in human lymphocytes was about 10–15 pM TCDD, but induction of CYP1A1 and CYP1B1 gene expression was detected at lower concentrations. This suggests that the concentration of TCDD necessary to evoke a statistically significant induction of CYP1A1 and/or CYP1B1 expression in human lymphocytes is about a factor 10 higher than the background levels that are currently present in human blood. The average TEQs in plasma of human populations accidentally exposed to high concentrations of dioxin and dioxin-like compounds can be as high as about 100 ng/kg fat which corresponds to about 4 pM TCDD in whole blood (IARC, 1997). This is a concentration at which minor effects on CYP1A1 and/or CYP1B1 expression could occur. However, the constitutive variation in levels of CYP1A1 and CYP1B1 expression among individuals is very large, which makes it very difficult to filter out potential subtle effects on expression by environmental exposure. Furthermore, this study was performed with mitogen-stimulated lymphocytes, which might increase the sensitivity of the cultured lymphocytes toward dioxin and dioxin-like compounds (Whitlock et al., 1972), resulting in an overestimation of the effects elicited by dioxin and dioxin-like compounds. Taken together, we conclude that it is unlikely that dioxin and dioxin-like compounds in blood of either background or highly exposed human populations are significantly affecting CYP1A1 and CYP1B1 activity or expression. This conclusion is in agreement with the findings of Landi et al. (2003b) who described the lack of association between TCDD plasma levels and CYP1A1 and CYP1B1 mRNA expression and EROD activity in cultured lymphocytes obtained from subjects from Seveso, Italy who were accidentally exposed to TCDD. However, it should be noted that in the above calculations, we assumed that all TEQs are bioavailable. Yet, the free concentration of the TEQs available to the lymphocytes may be considerably lower, since these highly lipophilic dioxins and dioxin-like compounds are dissolved in blood fat and bind to proteins. Further, the amount of TCDD or PCB126 added to the lymphocyte cultures in vitro or measured in blood samples in vivo may be substantially different from the amount available to cause an effect in lymphocytes. For example, compounds in vitro can adsorb to the plastic walls of the well plates, differences in serum content in blood and medium can cause differences in dissociation from serum proteins, and partitioning of the compounds into cell membranes is strongly dependent on the amount of cell membrane present (cell concentration) (Heringa et al., 2004).

Although the population in our study was limited, these findings provide some insight into interindividual differences in basal CYP1A1 and CYP1B1 expression and induction upon exposure to dioxin and dioxin-like compounds. We showed a large interindividual variation in constitutive and induced EROD activity and mRNA levels of CYP1A1 and CYP1B1 in cultured human peripheral blood lymphocytes. Furthermore, the concentration of dioxin or dioxin-like compounds necessary to evoke an in vitro effect on EROD activity or CYP1A1 or CYP1B1 mRNA levels are much higher than the human blood levels found in vivo. Therefore, we feel that EROD activity or CYP1A1 or CYP1B1 gene expression levels in peripheral blood lymphocytes are not suitable to use as biomarkers of exposure to dioxin-like compounds. However, epidemiological studies investigating the correlation between exposure to dioxin-like compounds and CYP1A1 and CYP1B1 gene expression and catalytic activity should be performed to support or reject this statement. In addition, the role of polymorphisms in the CYP1A1 and CYP1B1 genes should be explored, because these might influence the interindividual sensitivity and responses to dioxin-like compounds.

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