

Applications of Gene Arrays in Environmental Toxicology: Fingerprints of Gene Regulation Associated with Cadmium Chloride, Benzo(a)pyrene, and Trichloroethylene

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Toxicity testing of unknown chemicals currently uses a number of short-term bioassays. These tests are costly and time consuming, require large numbers of animals, and generally focus on a single end point. The recent development of DNA arrays provides a potential mechanism for increasing the efficiency of standard toxicity testing through genome-wide assessments of gene regulation. In this study, we used DNA arrays containing 148 genes for xenobiotic metabolizing enzymes, DNA repair enzymes, heat shock proteins, cytokines, and housekeeping genes to examine gene expression patterns in the liver in response to cadmium chloride, benzo(a)pyrene (BaP), and trichloroethylene (TCE). Dose-response studies were carried out in mice for each chemical; each produced a unique pattern of gene induction. As expected, CdCl₂ markedly up-regulated metallothionein I and II (5- to 10,000-fold at the highest doses) and several of the heat shock/stress response proteins and early response genes. In contrast, administration of BaP up-regulated only *Cyp1a1* and *Cyp1a2* genes and produced no significant increases in any of the stress response genes or any of the DNA repair genes present on the array. Likewise, TCE-induced gene induction was highly selective; only *Hsp 25* and *86* and *Cyp2a* were up-regulated at the highest dose tested. Microarray analysis with a highly focused set of genes is capable of discriminating between different classes of toxicants and has potential for differentiating highly noxious versus more subtle toxic agents. These data suggest that use of microarrays to evaluate the potential hazards of unknown chemicals or chemical mixtures must include multiple doses and time points to provide effective assessments of potential toxicity of these substances. **Key words:** benzo(a)pyrene, cadmium chloride, DNA arrays, gene expression, trichloroethylene. *Environ Health Perspect* 109:71-74 (2001). [Online 15 December 2000] <http://ehpnet1.niehs.nih.gov/docs/2001/109p71-74bartosiewicz/abstract.html>

DNA microarrays are a powerful, high-throughput tool for monitoring the expression of thousands of genes simultaneously (1-3). The application and use of DNA arrays in drug discovery and development (4), in identifying changes in gene expression associated with various disease processes, and in screening populations for allelic variants (single nucleotide polymorphisms) (5-8) has received considerable attention. Recently, there has been interest in using arrays in toxicology to quickly classify toxicants based on characteristic expression profiles and to use these profiles as a means of identifying putative mechanisms of action (9-11), as well as for identifying genes that are under similar regulatory control. Thus, the expression profiles associated with a heavy metal, a polycyclic aromatic hydrocarbon, and an oxidant stressor are likely to differ considerably and could be useful not only in assessing the potential toxicity of new synthetic chemical entities but in monitoring animal populations in the environment for potential point sources of environmental contamination. Finally, the application of this technology to address the potential human health relevance of environmental disasters such as the recent tire fires in California (12) may provide a rapid means of assessing the bioavailability

and potential toxicity of complex mixtures of chemicals released into the air and into groundwater. In this case, changes in gene regulation in tissues from sentinel animals placed at the site could form the basis for these assessments.

In this paper we describe the application of a simple toxicology chip to study gene expression in mice treated with one of three distinct environmental/occupational contaminants: cadmium chloride, benzo(a)pyrene (BaP), and trichloroethylene (TCE). These chemicals were selected to meet the following criteria: each chemical was in a different chemical class [i.e., heavy metal, polycyclic aromatic hydrocarbon (PAH), chlorinated hydrocarbon]; each has high environmental/occupational relevance; each has potential to affect human health (13-16); and data sets for changes in the regulation of selected genes were available in the literature for comparison. CdCl₂ is primarily an industrial by-product that humans are exposed to in a variety of media (13). Contamination of the environment with PAHs such as BaP occurs from natural and anthropogenic sources (i.e., mining, coal burning, oil refineries, etc.), and TCE contamination is a result of its widespread use as an industrial solvent (14-16). A number of genes have been reported to be

up-regulated in response to both CdCl₂ (17) and BaP (18,19) (i.e., metallothionein, cytochrome P-450 *1a1*, *Cyp1a2*, etc.). Although changes in gene expression in response to TCE are not as well documented, the widespread contamination of soil and groundwater with this solvent support the need for a more global approach to examining changes in gene expression associated with both acute and more chronic exposures (16).

In our previous work (20) we focused on the validation of the use of microarrays in an *in vivo* model. These earlier studies used a wide range of doses of β -naphthoflavone, a known inducer of *Cyp1a1* and *Cyp1a2* genes in murine liver, to address issues of sensitivity and dynamic range of microarray analysis in comparison with accepted Northern blot assays as well as to investigate sources of variability in the assay (slide to slide, spot to spot, and animal to animal). The data derived from this work demonstrated that the dynamic range and sensitivity of DNA microarrays is comparable to Northern blotting analysis and that there is far more variability introduced in the data through interanimal differences than through variations related to spotting and hybridization.

The chip used for these studies contained 148 genes coding for both phase I and II metabolizing enzymes, DNA repair enzymes, stress proteins, and cytokines as well as housekeeping genes. Studies examining gene regulation in the liver in response to varying doses of CdCl₂, BaP, and TCE clearly indicate a unique fingerprint of gene-level alterations resulting from exposure to the different chemical classes. Although the arrays used in these studies were limited to a highly focused set of genes, this work provides proof of the concept that patterns of gene regulation assessed by array technology

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are sufficiently unique that they may be useful in whole-animal studies to classify unknowns (i.e., heavy metal, PAH, chlorinated hydrocarbon, alkylating carcinogen, etc.).

Materials and Methods

Animals. Male Swiss Webster mice (20–25 g) were obtained from Charles River Breeding Laboratories (Portage, MI) and were housed in animal care facilities at the University of California, Davis, which are accredited by the American Association of Laboratory Animal Care. Mice received food and water *ad libitum* and were housed in HEPA-filtered racks for at least 1 week before use.

Animal treatment and isolation of mRNA. Mice (four to five per group) were treated intraperitoneally (ip) with either CdCl₂ (Sigma, St. Louis, MO; dissolved in isotonic saline, 10 mL/kg), BaP (Sigma), or TCE (Sigma; dissolved in corn oil such that 10 mL was administered per kilogram body weight). Doses of each toxicant are indicated in Figure 1. Controls received either isotonic saline or corn oil only, as appropriate. We killed mice with an overdose of pentobarbital at 6 hr (CdCl₂ and TCE) or at 6 and 24 hr (BaP) and isolated hepatic RNA from individual animals as described previously (20). Briefly, RNA was isolated as described by Chomczynski and Sacchi (21) using Trizol (GIBCO BRL, Bethesda, MD). We used DNase-treated total RNA (500 µg) to isolate mRNA using Oligotex dt resin (Qiagen, Valencia, CA) following the manufacturer's instructions.

Preparation of DNA chips. Chips containing 148 unique genes involved in phase I and II metabolism, heat shock, DNA repair, inflammation, transcription, and housekeeping were used in the current studies. The methods for preparation and analysis of these, along with applications to whole animal studies, have been presented in a previous publication (20) and are described only briefly here. We purchased cDNAs, corresponding to the 3' region of the respective genes, from the Image Consortium cDNA mouse libraries through suppliers (Research Genetics, Huntsville, AL, or American Type Culture Collection, Bethesda, MD). NCBI BLAST (Basic Local Alignment Tool) was used to determine which fragments, corresponding to desired genes, contained the least homology to other known genes. For the fragments that contained areas of high homology (i.e., *Cyp1a1* and *Cyp1a2*), polymerase chain reaction (PCR) primers were designed to amplify unique regions. A complete list of genes used in these studies is available on our web site (22).

Sequence-verified PCR products, prepared as described in detail earlier (20), varied

in size from approximately 500 bp to 1,200 bp. These were purified according to the manufacturer's protocol by using Qiaquick columns (Qiagen), and the concentrations were determined by absorbance at 260 nm.

Spotting cDNA arrays. We mixed each purified PCR product (10 µL at concentrations > 200 ng/µL) with 10 µL of 8 M NaSCN in 96-well plates and spotted the DNA using the Molecular Dynamics Microarray Spotter Gen II (Molecular Dynamics, Sunnyvale, CA), onto slides treated by vapor deposition of 3-aminopropyltrimethoxysilane (23). Each gene was spotted in eight separate quadrants of the slide. The slides were baked at 80°C for 2 hr and stored at room temperature in a desiccator until use. Before hybridization, the slides were washed in isopropanol for 10 min and then boiled in water for 5 min.

Preparation of cDNA probes labeled with Cy3 or Cy5. Cy3- and Cy5-labeled probes were prepared using 1 µg mRNA from control or treated animals, respectively. Synthesis of the labeled first strand was conducted

using Superscript II (GIBCO), and template RNA was removed by incubation with RNase as described earlier (20). We purified single-stranded cDNA probes using a PCR purification kit (Qiagen) and determined the absorbance of each probe preparation at 260 nm and either 550 nm (Cy3) or 650 nm (Cy5). The total dye content (picomoles), amount of probe (nanograms), and specific activity [(number of Cy molecules incorporated)/(number of bases)] were calculated. Probe mixtures were evaporated in a vacuum centrifuge.

Hybridization. Hybridization solution was filtered through a 0.1-µm filter. Poly A (Amersham Life Sciences) and mouse Cot DNA (GIBCO) were added to the hybridization solution at a final concentration of 16 ng/µL and 40 ng/µL, respectively. Probes were resuspended in hybridization solution containing 50% formamide, 5× standard saline citrate (SSC), and 0.1% SDS. Cy3 (control) and Cy5 (treated) probes were matched based on total dye content (picomoles) and were then mixed. The probe

| | 5,001–10,000 | | 2,501–5,000 | | 1,001–2,500 | | 501–1,000 | | 251–500 | | 126–250 | | 76–125 | | 51–75 | | 25–50 | | |
|--|---------------------------|-------|-------------|------|-------------|------|-------------|-----|---------|-----|---------|-------------|--------|-----|-------|--|-------|--|--|
| | CdCl ₂ (mg/kg) | | | | | | BaP (mg/kg) | | | | | TCE (mg/kg) | | | | | | | |
| Gene | 10 | 2.5 | 1.0 | 0.25 | 0.1 | ctrl | 100 | 10 | 1 | 0.1 | ctrl | 1000 | 100 | 10 | ctrl | | | | |
| Stress genes | | | | | | | | | | | | | | | | | | | |
| Metallothionein I | 2,671 | 1,726 | 254 | 354 | 246 | 126 | | | | | | | | | | | | | |
| Metallothionein II | 6,805 | 2,077 | 368 | 423 | 283 | 115 | | | | | | | | | | | | | |
| Hsp105 | 2,276 | 210 | 120 | 119 | 117 | 107 | | | | | | | | | | | | | |
| Hsp25 | 3,720 | 450 | 80 | 38 | 57 | 98 | | | | | | 232 | 79 | 76 | 98 | | | | |
| Hsp86 | 1,329 | 493 | 113 | 105 | 112 | 119 | | | | | | 237 | 104 | 109 | 103 | | | | |
| GFP | 1,089 | 105 | 141 | 116 | 103 | 101 | | | | | | | | | | | | | |
| chop10 | 387 | 132 | 95 | 90 | 98 | 81 | | | | | | | | | | | | | |
| c-jun | 743 | 95 | 96 | 87 | 99 | 98 | | | | | | | | | | | | | |
| jun-b | 327 | 165 | 115 | 97 | 100 | 61 | | | | | | | | | | | | | |
| Xenobiotic metabolizing enzymes | | | | | | | | | | | | | | | | | | | |
| Cyp2F2 | 24 | 142 | 116 | 140 | 149 | 122 | | | | | | | | | | | | | |
| CYP3A11 | 30 | 125 | 152 | 131 | 161 | 99 | | | | | | | | | | | | | |
| Methyl TX 111 | 34 | 94 | 109 | 104 | 110 | 96 | | | | | | | | | | | | | |
| AcetylTx | 424 | 240 | 116 | 107 | 133 | 117 | | | | | | | | | | | | | |
| Cyp1a2 | | | | | | | 438 | 222 | 102 | 95 | 105 | | | | | | | | |
| Cyp1a1 | | | | | | | 3,596 | 765 | 123 | 90 | 122 | | | | | | | | |
| Cyp2a | | | | | | | | | | | | 281 | 150 | 41 | 108 | | | | |

Figure 1. Patterns of gene expression in livers of mice treated with CdCl₂, BaP, or TCE. Up- or down-regulation of gene expression is denoted with a color pattern (scale at top); the numbers refer to the expression level as a percent control (control = 100%). All colored boxes represent gene expression levels that were significantly different from control. All values are the mean of four to five mice at each dose and time point studied. Standard deviations are omitted for clarity but were generally less than 15% of the mean value.

mixture was placed on a previously prepared array and the slides were hybridized overnight at 42°C in a humid chamber. After hybridization, slides were placed in a wash solution (2× SSC, 0.1% SDS) for 5 min at 37°C with gentle shaking. Cover slips floated off the slides during this initial wash. Slides were then washed once in 0.1× SSC, 0.1% SDS at room temperature for 5 min and twice in 0.1× SSC at room temperature for a total time of 5 min. The slides were then rinsed briefly in water and dried with a gentle stream of nitrogen.

Analysis of fluorescence spots. We scanned slides, prepared as described above, on the Molecular Dynamics MicroArray Scanner (23). Both Cy3 and Cy5 channels were scanned at a photomultiplier tube setting of 750 v. We analyzed the data sets using ArrayVision Software (Imaging Research, St. Catharines, Ontario, Canada). We calculated the fluorescence intensity of each spot using local median background subtraction. The relative fluorescent units (RFUs) were then normalized to the median signal of probe (Cy3 and Cy5) for that slide. The change in gene expression for each spot was calculated as $RFU_{\text{treated}}/RFU_{\text{control}}$. This value was then compared to its duplicate—that is, $RFU_{\text{treated}}/RFU_{\text{control}}$ for spot 1 (left side of the slide) versus spot 5 (right side of the slide). If the ratio of the value for these two spots was between 0.67 and 1.5, the spot and duplicate were accepted, and an average value of induction was calculated. If the value was not within 50% of its replicate, we rejected the spot from the set. This criterion tended to exclude those spots that were contaminated with dust or those slides that had variable and high backgrounds. We rejected 10–20% of the values obtained during this work using these criteria. The data on those genes up- or down-regulated by chemical treatment are presented as the percentage of the Cy-3 controls. Significant differences between control and treatment doses were determined using a two-tailed *t*-test (SigmaStat, SPSS, Chicago, IL). In those cases where data used in the *t*-test were not normally distributed, a Mann-Whitney Rank Sum test was performed. We considered *p*-values < 0.05 statistically significant.

Results

Comparison of genes up-regulated by chemicals. Figure 1 presents a visual comparison for all genes that were influenced significantly at any dose of CdCl₂, BaP, or TCE studied. Even with the highly focused set of genes used in these studies, there are clear differences in the pattern of genes up- or down-regulated by the three chemicals.

Changes in gene expression in the liver by CdCl₂. Ten genes were significantly induced over controls by ip administration of the

highest dose of CdCl₂ tested (10 mg/kg; Figure 1). Induction of metallothionein-I was noted at doses of CdCl₂ as low as 0.25 mg/kg; at 1 mg/kg and higher metallothionein-II was up-regulated significantly. Up-regulation of heat shock proteins (Hsp 105 and 86) occurred at 2.5 mg/kg, and Hsp 25 was elevated significantly only at 10 mg/kg. Several immediate early genes were induced as well. *c-jun* showed significant induction over the control at 1 mg/kg CdCl₂, whereas increases in *jun-b* were significant at the 2.5 mg/kg dose of CdCl₂. Other hepatic genes that were induced by administration of CdCl₂ included growth-factor-induced protein, *chop 10* (*gadd 153*), and acetyltransferase 96. In addition to gene up-regulation noted with CdCl₂ treatment, the expression levels of a number of genes were repressed upon treatment with CdCl₂. Methyltransferase 111, *Cyp2f2*, and *Cyp3a11* were all repressed significantly at the highest dose of CdCl₂ (10 mg/kg). Repression was not observed at lower doses.

Induction of genes by BaP. BaP was administered ip to mice at doses of 0.1–100 mg/kg. Animals treated with 100 mg/kg were killed at either 6 or 24 hr. Two genes, *Cyp1a1* and *Cyp1a2*, showed significant induction over controls, and both genes were up-regulated at the 10 and 100 mg/kg doses of BaP. The pattern of gene expression noted in the 6-hr samples (i.e., significant elevation in mRNA for *Cyp1a1* and *Cyp1a2*) did not change in the 24-hr samples (data not shown). However, none of the other genes previously reported to be up-regulated by TCDD, a ligand with very high affinity for the aryl hydrocarbon (Ah) receptor, were up-regulated by the doses of BaP used in these studies (18,19). Moreover, none of the genes reported to be up- or down-regulated in response to BaP in other systems [*p53* in A549 or 3T3 cells (24), *c-myc* and transforming growth factor (TGF)-β in a placental choriocarcinoma cell line (25), and glutathione-S-transferase (GST)-Ya in HEPG2 cells (26)] responded to BaP at any of the doses or times studied.

Induction of genes by TCE. Groups of four to five mice were treated (ip) with TCE at doses varying from 10 mg/kg to 1,000 mg/kg. Only three genes (*Cyp2a*, *Hsp 25*, and *Hsp 86*) were significantly induced at the highest dose of TCE tested (1,000 mg/kg). *Cyp2a* appeared to be repressed at the 10 mg/kg dose of TCE, but this effect was not statistically significant.

Discussion

The introduction of hundreds of new chemical entities to the market each year presents a substantial challenge for determining the potential adverse health and environmental effects associated with their use. Currently,

toxicologic screening of these chemicals relies heavily on the use of a combination of short-term assays. Separate bioassays are implemented to determine immunotoxicity, neurotoxicity, and genetic toxicity end points. These assays, however, are costly, require many animals, and may take several years and millions of dollars to complete [see Gelbke et al. (27) for review]. Similarly, for chemical contaminants in the environment, the Environmental Protection Agency uses three-species bioassays to determine the relative toxicity in water of specific chemicals or of specific effluents to wildlife (28). Because these tests are limited to lethality, growth, and reproductive end points, considerable attention has focused on optimization of these processes by developing methodologies that are more efficient, informative, and cost effective.

As outlined in a recent symposium (9) and as described previously by Nuwaysir et al. (10), the use of microarrays to measure changes in gene expression in response to chemicals may improve the efficiency of standard toxicity bioassays. In environmental monitoring, microarrays could provide not only a method to quickly categorize chemicals and assign a mode of toxic action but also allow more sensitive end points to be addressed—specifically, gene expression. Moreover, as studies are conducted with larger gene chips, we will gain a more complete understanding of similarities in regulatory sequences, which control the expression of a variety of gene products. Our immediate goals have been to understand the potential limitations of this technology (dynamic range, sensitivity, and sources of variability) (20) and to determine whether microarrays offer a useful alternative approach in toxicity testing and environmental monitoring.

Important questions need to be addressed if microarrays are to be used in toxicity testing and environmental monitoring: *a*) Will microarrays provide distinct patterns of gene expression for separate chemical classes? *b*) How will this chemical signature change with dose and tissue studied, and, in those cases where the chemical species is unknown, will the study of gene regulation in multiple tissues lead to clearer understanding of the class of chemicals being studied? *c*) Are the methods sufficiently robust to provide reliable assessments? and *d*) Will these assays provide an improved method for assessing the potential synergistic effects of chemical mixtures? Although these questions will require substantial long-term efforts to fully address, our current work provides data supporting proof of concept; namely, that three environmental contaminants (CdCl₂, BaP, and TCE) belonging to different chemical classes, elicit unique patterns of gene expression over the

doses tested in an *in vivo* model (Figure 1). Although limited in scope, the data presented here on gene induction by BaP are consistent with the patterns of gene induction noted in earlier studies on β -naphthoflavone (20). There are, however, several additional genes, all of which were present on our chip but were not up-regulated significantly at either of the doses or times studied with BaP but are under control of the Ah receptor (18,19). These include *p53* in A549 or 3T3 cells (24), *c-myc* and TGF- β in a placental choriocarcinoma cell line (25), and GST-Ya in HEPG2 cells (26). At this point, it is not clear whether these differences are primarily a result of the different systems used (i.e., an *in vivo* system with liver tissue compared to cell lines from various sources including liver) or whether these additional genes would be up-regulated in other tissues at different doses and times after BaP administration.

For the doses tested and genes examined, CdCl₂ was the only chemical treatment where the expression pattern changed appreciably with dose. This was readily apparent when the CdCl₂ concentration was increased from noncytotoxic to probable cytotoxic doses (17). At low concentrations of CdCl₂ (0.25–1.0 mg/kg) up-regulation of metallothionein occurred (Figure 1). As the concentration was increased, the magnitude of induction for metallothionein increased, and several other genes were induced or repressed including heat shock proteins. The dose-response data are consistent with earlier studies in which *gadd 153* (*chop 10*) is up-regulated when CdCl₂ is present at cytotoxic concentrations (29). Although gene expression changed with dose, the signature response for CdCl₂ was discernable from BaP and TCE (i.e., metallothionein remained elevated in CdCl₂ treated animals throughout the dose range). Additionally, the graded response of gene induction observed with CdCl₂ treatment illustrates the potential use of microarrays to assess a degree of exposure for specific chemicals or classes of chemicals.

Currently, we are testing the DNA arrays with additional chemicals from the same

chemical classes and are including work on additional tissues to determine whether this provides a further mechanism for distinguishing chemical classes. We will use this as a basis for studying chemical mixtures to assess whether the arrays can provide information about the additive or subtractive effects of mixtures. This information will be helpful in assessing whether DNA arrays can be used to screen effluents and other complex mixtures. Finally, a larger mouse array is under construction to encompass more biochemical pathways and responses. With these larger arrays, it may be possible to begin making associations between pathways that act in concert during periods of chemical stress.

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