Hexachlorobenzene (HCB) is a persistent environmental pollutant with toxic effects in man and rat. Reported adverse effects are hepatic porphyria, neurotoxicity, and adverse effects on the reproductive and immune system. To obtain more insight into HCB-induced mechanisms of toxicity, we studied gene expression levels using DNA microarrays. For 4 weeks, Brown Norway rats were fed a diet supplemented with 0, 150, or 450 mg HCB/kg. Spleen, mesenteric lymph nodes (MLN), thymus, blood, liver, and kidney were collected and analyzed using the Affymetrix rat RGU-34A GeneChip microarray. Most significant (p < 0.001) changes, compared to the control group, occurred in spleen, followed by liver, kidney, blood, and MLN, but only a few genes were affected in thymus. This was to be expected, as the thymus is not a target organ of HCB. Transcriptome profiles confirmed known effects of HCB such as stimulatory effects on the immune system and induction of enzymes involved in drug metabolism, porphyria, and the reproductive system. In line with previous histopathological findings were increased transcript levels of markers for granulocytes and macrophages. New findings include the upregulation of genes encoding proinflammatory cytokines, antioxidants, acute phase proteins, mast cell markers, complements, chemokines, and cell adhesion molecules. Generally, gene expression data provide evidence that HCB induces a systemic inflammatory response, accompanied by oxidative stress and an acute phase response. In conclusion, this study confirms previously observed (immuno)toxicological effects of HCB but also reveals several new and mechanistically relevant gene products. Thus, transcriptome profiles can be used as markers for several of the processes that occur after HCB exposure. **Keywords:** Brown Norway rat, DNA microarray analysis, drug metabolism, estrogen metabolism, genomics, hexachlorobenzene, immunotoxicology, inflammation, oxidative stress, porphyria. *Environ Health Perspect* 112:782–791 (2004). doi:10.1289/txg.6861 available via [http://dx.doi.org/](http://dx.doi.org/) [Online 7 April 2004]
rats were purchased from Harlan (Blackthorn, UK). Rats were acclimatized for 1 week before the start of the experiment. They were kept two by two under standard conditions with food and acidified drinking water *ad libitum*. The diet consisted of a semisynthetic diet (SSP/TOX; Hope Farms, Woerden, the Netherlands) with or without crystalline HCB (99% purity; Aldrich Chemie, Bornem, Belgium) by mixing of homogeneity. The experiments were approved by the animal experiments committee of the Faculty of Veterinary Medicine of the Utrecht University.

**Experimental Protocol**

Rats were randomly assigned to different experimental groups (*n* = 6) receiving either control diet or the diet supplemented with 150 mg (low dose) or 450 mg (high dose) HCB/kg. Body weight (bw) and skin lesions were recorded twice per week. After 28 days rats were killed by CO<sub>2</sub>O<sub>2</sub>. Blood was collected in tubes containing EDTA to prevent clotting and transferred into Fastubes (Endotell, Allschwill, Switzerland) containing guanidinium isothiocyanate in 0.9% NaCl solution. Tubes were snap-frozen in liquid nitrogen. Spleen, MLN, thymus (freed from adjacent LN), liver, and kidney were collected, weighed, and snap-frozen in liquid nitrogen.

In additional experiments for pathology, blood, and serum analysis, rats were exposed to the same dosing regimens. Rats were killed by a lethal dose of pentobarbital (Euthesate; 0.3 g/kg bw ip; Ceva Santé Animal B.V., Maassluis, the Netherlands). One part of the blood was collected in EDTA tubes for total and differential leukocyte counts; the other part was used for serum analysis. Spleen, MLN, thymus, liver, and kidney were fixed in phosphate-buffered 4% formaldehyde; after embedding in Paraplast, 5-μm sections were stained with hematoxylin and eosin.

**DNA microarray experiment.** Total RNA was obtained by acid guanidinium isothiocyanate–phenol–chloroform extraction (Trizol; Invitrogen Life Technologies, San Diego, CA, USA) (Chomczynski and Sacchi 1987) and purified on an affinity resin (RNeasy; Qiagen, Hilden, Germany) according to manufacturer instructions. DNA microarray experiments were conducted as recommended by the manufacturer of the GeneChip system (Affymetrix, Inc. 2002) and as previously described (Lockhart et al. 1996). Rat specific RG U34A gene expression probe arrays (Affymetrix, Inc., Santa Clara, CA, USA) were used containing 8,799 probe sets interrogating primarily annotated genes. Per tissue and per animal, one chip was used. The resulting image files (.dat files) were processed using the Microarray Analysis Suite 5 (MAS5) software (Affymetrix, Inc.). Tab-delimited files were obtained containing data regarding signal intensity (Signal) and categorical expression level measurement (Absolute Call).

**Data Analysis**

To determine which genes were differentially expressed between the three treatment groups, a one-way analysis of variance (ANOVA) was applied to genes that had a present call in at least one of the samples. Genes with a *p*-value < 0.001 were considered statistically significant. Group average fold changes were calculated by using the average of the low- or high-dose groups compared with the control group. The annotation of the genes was determined by using NetAffx (http://www.affymetrix.com; Liu et al. 2003). Further information on probe sets was found in the literature or in the KEGG database (http://www.genome.ad.jp/kegg/kegg2.html). Additional data analysis by principal component analysis (PCA) was performed using GeneMaths (Applied Maths, Sint-Martens-Latem, Belgium). Averages of gene expression levels in control, low-, and high-dose groups were calculated; low values were cut off using a lower threshold of 10, and the values were log transformed before PCA.

**GC–MS Analysis of Contamination in the Hexachlorobenzene Sample**

To analyze HCB for contaminating polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), a solution of acetone containing 13C<sub>12</sub>-labeled internal quantitation standards (Cambridge Isotope Laboratories, Woburn, MA, USA) of the PCDDs and PCDFs was added to dichloromethane. The solution was brought to a Carbosphere (Alltech B.V., Zaandam, the Netherlands) column, then purified on Al<sub>2</sub>O<sub>3</sub>, evaporated to dryness, and redissolved in toluene. Gas chromatography–mass spectrometry (GC–MS) analyses were performed on a double-focusing mass spectrometer coupled to a gas chromatograph. GC separations were carried out on a nonpolar capillary column (60 m DB-5MS; 0.25 mm ID, 0.10-μm film thickness; J&W Scientific, Folsom, CA, USA). Ionization of the sample was performed in the electron impact mode. Detection was performed by selected ion recording.

**Results and Discussion**

**Body Weight Gain, Macroscopic Skin Lesions, and Organ Weights**

During treatment with the low-dose diet, body weight increased significantly from day 10 onward, whereas rats exposed to the high-dose diet had a significantly higher body weight on days 10 and 20 (data not shown). One of the rats in the high-dose group died after 25 days of exposure to HCB. Time of onset, severity, and size of the skin lesions were similar as described previously (Michielsen et al. 1997). Increased liver and spleen weights in both dosing groups were also in accordance with previous work, as were the observed histopathological changes in these organs (Michielsen et al. 1997). In the high-dose group, kidney weight increased significantly, as observed before in Wistar rats treated with HCB for 25 days (Kennedy and Wigfield 1990) but not in BN rats treated with HCB for 21 days (Michielsen et al. 2002). Histopathological changes were not observed. Thymus weight decreased significantly in the high-dose group. It is likely that this thymus atrophy is caused by stress, as typical stress-induced alterations (Kuper et al. 2002) were observed. No significant differences in MLN weight were found, but histopathology of MLN of the high-dose group showed comparable morphology as reported previously (Michielsen et al. 1997).

**DNA Microarray Analysis**

The PCA plot (Figure 1) of the ratios of the low- and high-dose groups over the control group shows that gene expression in spleen, blood, and liver is dose dependent, whereas this is less clear for MLN, thymus, and kidney. Spleen and blood cluster close together, as do kidney and thymus, but liver and MLN are more distant from those tissues. Most significant changes (*p* < 0.001) in gene expression occurred in spleen (679 probe sets), followed by liver (346), kidney (232), blood (144), MLN (104), and thymus (28). The low number of changes in thymus is not surprising, as the thymus is not a target organ of HCB. Remarkably in kidney, many genes were affected, although this organ has rarely been described to be affected by HCB. Furthermore, although organ weights were increased, no histopathological changes were detected in the present study. Because not all significantly changed genes can be included in this article, we present only genes associated with immunity (Tables 1–6), acute phase responses (APRs) and oxidative stress.
superfamily, member 1) in MLN, spleen, and kidney was increased. In addition, IL-6 gene expression was affected in MLN, just as the IL-6 signal transducer in kidney. IL-6 is a pleiotropic cytokine that plays an important role in B-cell differentiation, growth of T cells, and differentiation of macrophages (Naka et al. 2002). HCB also induced gene expression of IL-1β in spleen (low-dose group) and IL-1β–converting enzyme in kidney, an enzyme that converts IL-1β and IL-18 to their active form. Gene expression of IL-18, a cytokine produced mainly by Kupffer cells, was elevated in liver.

**Proinflammatory cytokines.** Gene expression of the receptor for tumor necrosis factor (TNF)α and TNFβ (TNF receptor superfamily, member 1) in MLN, spleen, and kidney was increased. In addition, IL-6 gene expression was affected in MLN, just as the IL-6 signal transducer in kidney. IL-6 is a pleiotropic cytokine that plays an important role in B-cell differentiation, growth of T cells, and differentiation of macrophages (Naka et al. 2002). HCB also induced gene expression of IL-1β in spleen (low-dose group) and IL-1β–converting enzyme in kidney, an enzyme that converts IL-1β and IL-18 to their active form. Gene expression of IL-18, a cytokine produced mainly by Kupffer cells, was elevated in liver.

**p38 MAPK signaling pathway.** The mitogen-activated protein kinase (MAPK) family consists of signal transduction molecules important during inflammation. HCB induced expression of p38 MAPK and other MAPKs in kidney. Activation of p38 MAPK leads to phosphorylation of several transcription factors, such as signal transducer and activator of transcription-1 (STAT-1). Gene expression of STAT-1 was increased in liver. Both MAPK and STAT-1 are important in cytokine production, and negative regulation of cytokine signaling occurs at the level of transcription of these molecules. Proteins involved in suppression of cytokine production are the so-called suppressors of cytokine signaling (SOCSs). HCB exposure increased gene expression of several of these proteins, probably to counteract the high cytokine levels. In spleen, SOCS-2 was upregulated in the low-dose group, but downregulated in the high-dose group, and SOCS-3 was upregulated in MLN. In the thymus, cytokine inducible SH2-containing protein was upregulated, a protein that plays a critical role in controlling T-cell activation (Chen et al. 2003).

**Oxidative stress and antioxidants.** Previous studies have shown that HCB exposure induced oxidative stress (Billi de Catafbba et al. 1997) and increased expression of antioxidants in the liver (Stonard et al. 1998). The present work confirms these findings, as several antioxidants were induced in liver. Transcriptome profiles show that antioxidants are also increased in spleen, MLN, blood, and kidney. The infiltrated macrophages and granulocytes probably generate these reactive oxygen species (ROS). Additional experiments showed that serum hydroperoxides were significantly increased in HCB-exposed BN rats (data not shown). Excessive presence of ROS can activate nuclear factor kappa B, an important factor in regulating the inflammatory response (Schreck et al. 1992). In addition, ROS can cause cell damage, providing danger signals that can attract inflammatory cells. Therefore, increased oxidative stress induced by HCB may play a pivotal role in the observed immunostimulation.

**Acute phase response.** Acute phase proteins (APPs) are important in inflammatory responses. HCB increased gene expression of several APPs, such as heat shock proteins (HSPs) in spleen and MLN. HSPs protect cells against cellular stress. HCB also increased gene expression of matrix metalloproteinase-9 (MMP-9) in spleen and of the natural inhibitors of MMPs, tissue inhibitor of metalloproteinase-1 (TIMP-1) in liver and TIMP-2 in MLN. MMPs play an important role in the cleavage of membrane components, enabling leukocytes to extravasate the blood. HCB also affected transcript levels of other APPs, such as haptoglobin (a hemoglobin scavenger), lipopolysaccharide-binding protein, orosomucoid (important in immunomodulation), and metallothionein and ceruloplasmin (antioxidants). Negative APPs (transferrin and its receptor) were also induced; these proteins are normally downregulated during an APR. Synthesis of these

**Figure 1.** PCA plot of the ratios of low dose versus control (blue circles) or high dose versus control (red circles).
APPs, however, is also dependent on iron metabolism. HCB induced iron accumulation in the liver (Stonard et al. 1998). The upregulation of transferrin gene expression in spleen and kidney suggests that this is also the case in these organs.

**Complement system.** Complement components are also important in inflammatory responses. HCB increased gene expression of several components of the complement pathway in spleen, blood, kidney, and liver.

**Mast cells.** HCB enhanced gene expression of mast cell enzymes, probably a consequence of complement activation. This finding may also be explained by a characteristic of the BN rat, a strain that tends to respond in a more T helper-2–skewed fashion. Basal levels of serum IgE are high, and HCB increases IgE levels even more (Michielsen et al. 1997). Loading of mast cells with IgE may result in degranulation and release of inflammatory mediators.

**Chemokines and chemokine receptors.** In all analyzed organs, HCB increased gene expression of chemokines, important mediators in the recruitment of leukocytes.

### Table 1. Spleen: representative genes that changed significantly \((p < 0.001)\) after HCB treatment—immune system.

| Accession number | Gene name | HCB low dose | HCB high dose |
|------------------|-----------|--------------|---------------|
| Granulocytes and macrophages | | | |
| AA95703 | S100 calcium binding protein A8 | 2.8 | 34 |
| U50353 | Defensin 3a | 2.5 | 32 |
| AA940503 | Lipocalin 2 | 1.7 | 24 |
| L19894 | S100 calcium binding protein A9 | 3.2 | 19 |
| L08040 | 12-lipoxygenase | 1.9 | 5.7 |
| M22062 | Fc receptor, IgG, low affinity III | 1.4 | 2.0 |
| AA894004 | Ets, highly similar to Capg mouse macrophage capping protein | 1.2 | 1.4 |
| X73579 | Fc receptor, IgE, low affinity II | −1.1 | −2.3 |
| Mast cells | | | |
| U67913 | Mast cell protease 10 | 12 | 42 |
| U67898 | Mast cell protease 3 | 3.4 | 20 |
| U67907 | Mast cell protease 4 precursor | 1.5 | 8.7 |
| M2222 | High-affinity IgE receptor | 3.2 | 7.0 |
| U67914 | Mast cell carboxypeptidase A precursor | 1.8 | 6.8 |
| U67908 | Mast cell protease 5 precursor | 1.2 | 6.0 |
| M38795 | Histidine decarboxylase | 3.7 | 4.3 |
| Pattern recognition molecules | | | |
| AF087943 | CD14 antigen | 1.1 | 1.7 |
| Complement | | | |
| AF038548 | Response gene to complement | −1.3 | 20 |
| AA818025 | CD59 antigen precursor | 1.1 | 1.7 |
| Cell adhesion molecules | | | |
| X05834 | Fibronectin 1 | 1.8 | 3.5 |
| AJ009698 | Embigin | 1.4 | 3.3 |
| Chemokines | | | |
| U90448 | CXC chemokine LIX | 1.0 | 1.9 |
| U17035 | Chemokine (CXC motif) ligand 10 | 1.0 | −2.3 |
| Cytokines and cytokine-associated genes | | | |
| M63122 | Tumor necrosis factor receptor | 1.3 | 1.3 |
| AF075382 | Suppression of cytokine signaling | 1.3 | −1.3 |
| M98920 | Interleukin 1 beta | 1.5 | −1.2 |
| M50506 | Interleukin 2 receptor beta chain | 1.2 | −1.4 |
| L00981 | Lymphotoxin, tumor necrosis factor alpha | −1.1 | −1.4 |
| M34253 | Interferon regulatory factor 1 | −1.1 | −1.6 |
| U14647 | Interleukin 1 beta converting enzyme | 1.1 | −1.6 |
| U69272 | Interleukin 15 | −1.1 | −1.7 |
| U48596 | MAPK kinase kinase 1 | 1.0 | −1.8 |
| U03941 | Transforming growth factor, beta 3 | −2.9 | −3.0 |
| Genes associated with T and B cells and MHCII expression | | | |
| U39609 | Anti-nerve growth factor 30 antibody light-chain | 1.3 | 3.8 |
| L22654 | Antiacetylcholine receptor antibody rearranged | 3.2 | 1.6 |
| L07398 | Immunoglobulin gamma-2a chain | 1.0 | 2.4 |
| M18526 | Immunoglobulin germline kappa-chain | 1.2 | 1.6 |
| X13016 | MRC OX-45 surface antigen | 1.1 | −1.3 |
| U11681 | Rapamycin and FKBP12 target-1 protein | −1.0 | −1.3 |
| D13565 | T-cell receptor CD3, subunit zeta | −1.1 | −1.4 |
| U31599 | MHC class II-like beta chain RT1.Mb | −1.0 | −1.4 |
| L14004 | Polymeric immunoglobulin receptor | 1.0 | −1.6 |
| D10728 | Lymphocyte antigen CD6 | −1.2 | −1.6 |
| M85193 | RT6.2 | −1.3 | −1.6 |
| U24652 | Lineker of T-cell receptor pathways | −1.0 | −1.7 |
| X14319 | T-cell receptor active beta-chain, V region | −1.2 | −2.1 |

EST, expressed sequence tag.

*Table contains GenBank accession numbers (http://www.ncbi.nih.gov/entrez/query.fcgi?db=nucleotide) of the cDNA fragments present on Affymetrix RG U34A gene chips, gene name, and average fold change in expression of both low dose and high dose versus control. Fold changes are calculated with data from five to six rats per group. A one-way ANOVA was used to determine significance; only probe sets that changed significantly with \(p < 0.001\) are shown.*
from the circulation. HCB induced gene expression of several CXC chemokines and their receptors: lipopolysaccharide-induced CXC chemokine (LIX), chemokine (CXC motif) ligand 10, growth-related oncogene (Gro) and the CXC chemokine receptor 2 (CXCR2). LIX is a potent neutrophil chemoattractant, whereas chemokine (CXC motif) ligand 10 plays an important role in chemotaxis of activated T cells and monocytes. Gro is a ligand that binds to CXCR2, a receptor present on neutrophils. HCB induced gene expression of two CC chemokine receptors: CC chemokine–binding receptor JAB61, a receptor that binds monocyte chemoattractant protein-1 and -3, and the receptor for macrophage inflammatory protein-1α that is present on neutrophils and eosinophils (Mantovani et al. 1998).

**Cell adhesion molecules.** Chemokines induce expression of cell adhesion molecules on both endothelial cells and leukocytes. HCB affected gene expression of cell adhesion molecules in all organs except the thymus. Intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and selectin are endothelial cell adhesion molecules that recognize receptors on hemopoietic cells. Other cell adhesion molecules in which gene expression was induced by HCB were fibronectin-1, embigin, CD36, and glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1). The latter is expressed only on high endothelial venules (HEVs) in LNs. Previous reports have shown that HCB increased the development of HEVs in LNs (Michielsen et al. 1997), which probably results in increased GlyCAM-1 mRNA expression.

**Granulocytes.** Upregulation of chemokines and cell adhesion molecules leads to influx of leukocytes. Data obtained in this study confirm increased numbers of monocytes and neutrophilic granulocytes in blood (unpublished data) and cellular infiltrates in spleen of BN rats (Michielsen et al. 1999). In all analyzed organs and blood, gene expressions for S100 calcium-binding protein A8 (MRP-8) and A9 (MRP-14) were upregulated. These proteins are abundantly present in the cytoplasm of neutrophils, monocytes, and macrophages (Roth et al. 2003). Other markers associated with granulocytes and macrophages that were affected by HCB were defensin (neutrophils and macrophages), lipocalin (granulocytes), and CD24 (granulocytes, monocytes, and lymphocytes). HCB also induced gene expression of 12-lipoxygenase- and

### Table 2. MLN: representative genes that changed significantly ($p < 0.001$) after HCB treatment—immune system.$^\text{a}$

| Accession number | Gene name | HCB low dose | HCB high dose |
|------------------|-----------|--------------|---------------|
| L18948           | S100 calcium binding protein A9 | 2.2 | 22 |
| AA057003         | S100 calcium binding protein A8 | 2.6 | 19 |
| M32062           | Fc gamma receptor | 2.0 | 2.8 |
| AJ227184         | DORA protein (immunoglobulin superfamily, member 6) | 1.1 | 2.8 |
| U44129           | Mannose-binding lectin 1 | 1.5 | 2.6 |
| AF087943         | CD14 antigen | 1.8 | 2.5 |
| L98100           | Glycam 1 | 3.1 | 2.5 |
| Chemokines       | CC-chemokine-binding receptor JAB61 | 1.9 | 2.6 |
| AF053312         | CC chemokine ST38 precursor | 2.4 | 16 |
| Interleukin 6    | Suppressor of cytokine signaling | 2.3 | 4.3 |
| M63322           | Tumor necrosis factor receptor | 1.2 | 1.8 |
| AA989129         | ESTs, highly similar to interleukin 25 | 1.2 | 1.5 |
| M23671           | Rearranged IgG-2b | 1.5 | 3.2 |
| X07189           | Immunoglobulin heavy chain constant region | 2.5 | 3.1 |
| M18526           | Immunoglobulin germ line kappa-chain | 1.4 | 1.8 |

$^\text{a}$Table contains GenBank accession numbers (http://www.ncbi.nih.gov/entrez/query.fcgi?db=nucleotide) of cDNA fragments present on Affymetrix RG U34A gene chips, gene name, and average fold change in expression of both low dose and high dose versus control. Fold changes are calculated with data from five to six rats per group. One-way ANOVA was used to determine significance; only probe sets that changed significantly with $p < 0.001$ are shown.

### Table 3. Thymus: representative genes that changed significantly ($p < 0.001$) after HCB treatment—immune system.$^\text{a}$

| Accession number | Gene name | HCB low dose | HCB high dose |
|------------------|-----------|--------------|---------------|
| L18948           | S100 calcium binding protein A9 (MRP-14) | 1.1 | 2.0 |
| X14323           | IgG receptor FcRn | 1.2 | 1.2 |
| Mast cell        | Mast cell protease B precursor | 1.5 | 2.0 |
| U67911           | Cytokine | 1.2 | 1.7 |
| AF057003         | Cytokine inducible SH2-containing protein | 1.2 | 1.7 |
| Genes associated with B cells | Antiacetylcholine receptor antibody | 1.6 | 3.7 |
| L22654           | Rearranged immunoglobulin gamma-2a chain, VDJC region | 2.0 | 3.2 |

$^\text{a}$Table contains GenBank accession numbers (http://www.ncbi.nih.gov/entrez/query.fcgi?db=nucleotide) of cDNA fragments present on Affymetrix RG U34A gene chips, gene name, and average fold change in expression of both low dose and high dose versus control. Fold changes are calculated with data from five to six rats per group. One-way ANOVA was used to determine significance; only probe sets that changed significantly with $p < 0.001$ are shown.
Table 4. Blood: representative genes that changed significantly ($p < 0.001$) after HCB treatment were functionally grouped—immune system.a

| Accession number | Gene name                        | HCB low dose | HCB high dose |
|------------------|----------------------------------|--------------|--------------|
| AA957003         | Granulocytes and macrophages     | S100 calcium binding protein A8 | 4.7 | 34 |
| L18948           |                                  | S100 calcium binding protein A9 | 4.7 | 19 |
| L06040           |                                  | 12-lipoxygenase                | 1.6 | 3.6 |
| U49602           |                                  | Heat stable antigen CD24       | −1.2 | −3.0 |
| U67913           | Mast cells                       | Mast cell protease 10          | 17 | 16 |
| U67911           |                                  | Mast cell protease 8 precursor | 3.9 | 4.6 |
| X61654           |                                  | CD63                         | 1.7 | 2.0 |

Table 5. Liver: representative genes that changed significantly ($p < 0.001$) after HCB treatment—immune system.a

| Accession number | Gene name                        | HCB low dose | HCB high dose |
|------------------|----------------------------------|--------------|--------------|
| AA946503         | Granulocytes and macrophages     | Lipocalin 2  | 4.3 | 210 |
| L18948           |                                  | S100 calcium binding protein A9 (MRP-14) | 3.4 | 28 |
| AA957003         |                                  | S100 calcium binding protein A9 (MRP-8) | 1.1 | 8.5 |
| X76489           |                                  | CD9 for cell surface glycoprotein | 1.4 | 3.6 |
| A104781          |                                  | Arachidonate 5-lipoxygenase activating protein | −1.1 | 2.3 |
| AA693191         |                                  | ESI3: phospholipid acid phosphatase type 2c | 1.2 | 2.0 |
| M65532           |                                  | Carbohydrate binding receptor (Kupffer cell receptor) | 1.1 | 1.6 |
| S79263           |                                  | Interleukin-3 receptor beta subunit (colony stimulating factor 2 receptor beta 1, low affinity (granulocyte-macrophage) | 1.7 | 1.3 |
| U67911           | Mast cell                       | Mast cell protease 8 precursor | 2.2 | 2.8 |
| Z50051           | Complement component 4 binding protein, alpha | 1.3 | 2.3 |
| D00913           | Complement component 4 binding protein, alpha | 1.3 | 2.3 |
| D11445           | Interleukin 18                   | 1.3 | 1.9 |
| L75702           | Transforming growth factor beta stimulated clone 22 | −1.5 | −1.5 |
| L26544           | Anti-acetylcholine receptor antibody, rearranged immunoglobulin gamma-2a chain, VDJC region | −1.0 | 8.8 |
| U99089           | Anti-NGF30 antibody light-chain | 1.9 | 8.7 |
| X68782           | Immunoglobulin heavy chain VDJ-region CH1-CH2 | 1.4 | 4.6 |
| X50534           | RT1.D beta chain                | 1.5 | 2.0 |

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*aTable contains GenBank accession numbers (http://www.ncbi.nih.gov/entrez/query.fcgi?db=nucleotide) of cDNA fragments present on Affymetrix RG U34A gene chips, gene names, and average fold change in expression of both low dose and high dose versus control. Fold changes are calculated with data from five to six rats per group. One-way ANOVA was used to determine significance; only probe sets that changed significantly with $p < 0.001$ are shown.
arachidonic acid 5-lipoxygenase-activating protein, both involved in leukotriene activation, which takes place in myeloid cells (Bigby 2002). Gene expression of Fc receptors was also elevated by HCB, probably because of the increase in the number of cells bearing this receptor. The same is true for the upregulation of gene expression of several pattern recognition molecules, such as CD14, mannose-binding lectin, and peptidoglycan recognition molecules, present on monocytes, macrophages, and neutrophils.

This work indicates that HCB exposure results in a systemic inflammatory response. To counterbalance this response, the immune system produces anti-inflammatory mediators. HCB exposure induced gene expression of one of these mediators, annexin-1, which blocks leukocyte migration and induces apoptosis in inflammatory cells (Perretti and Gavins 2003).

**T and B Cells and Major Histocompatibility Complex II Expression**

Gene expression of T-cell markers such as CD3 a subunit of the T-cell receptor, was decreased in spleen, whereas in blood, HCB decreased gene expression for CD3 and CD37, the latter being a B-cell marker. Furthermore, HCB increased gene expression of CD52 or B7 antigen, a marker present on antigen-presenting cells, such as B cells and monocytes. This is in line with previous studies that have shown a stronger increase of monocytes and granulocytes in blood after HCB exposure, resulting in relatively fewer lymphocytes (Schulte et al. 2002; Vos et al. 1979). In kidney we observed an increased expression of OX 45 (homolog to CD2), a membrane protein involved in the binding to LFA-3, important in adhesion of T cells to other cell types and in T-cell activation. HCB enhanced gene expression of immunoglobulins in spleen, MLN, liver, and kidney. This is in line with the observed increase of serum levels of IgM, IgG, and IgE in BN rats (Michielsen et al. 1997). Major histocompatibility complex (MHC)II gene expression was decreased in spleen and blood and increased in liver and kidney.

**Autoantibodies**

The anti-acetylcholine receptor antibody gene (rearranged Ig γ-2a chain) was upregulated in spleen, thymus, liver, and kidney. These autoantibodies are associated with the autoimmune disease myasthenia gravis (MG), a neurological disease characterized by degeneration of the acetylcholine receptor and resulting in muscle weakness (De Baets and Stassen 2002). HCB-induced neurological effects, however, are not the same as symptoms described for MG. Additional experiments performed to detect antiacetylcholine receptors antibodies (total Ig) in serum did not confirm gene expression data. HCB exposure also increased gene expression of anti–nerve growth factor-30 antibodies in spleen and liver and downregulated expression in blood. These antibodies belong to the naturally occurring autoantibodies and are elevated in inflammatory diseases (Dicou et al. 1996). The exact role of these autoantibodies is not yet known. Previously it was shown that HCB increased IgM antibodies against autoantigens such as ssDNA (Michielsen et al. 1997; Schielen et al. 1993). Expression of La (= autoantigen SS-B/La) was induced in kidney. This protein plays a role in RNA polymerization and is often a target of autoantibodies.

**Table 6. Kidney: representative genes that changed significantly (p < 0.001) after HCB treatment—immune system.**

| Gene name                                      | Accession number | Fold change   |
|------------------------------------------------|------------------|---------------|
| Granulocytes and macrophages                   |                  |               |
| L18948                                         | S100 calcium binding protein A9 | 1.2 | 9.6 |
| AA957003                                        | S100 calcium binding protein A8 | 1.7 | 3.8 |
| M32062                                         | Fc gamma receptor | 1.2 | 2.7 |
| U10894                                         | Allopretant inflammatory factor | 1.1 | 2.5 |
| AA946603                                       | Lipocalin 2      | 1.1 | 2.0 |
| U498602                                        | Heat stable antigen CD24 | 1.1 | 1.8 |
| Complement                                     |                  |               |
| X71127                                         | Complement protein C1q beta chain | 1.3 | 4.0 |
| D88250                                         | Complement component 1, subcomponent | 1.1 | 2.9 |
| Cell adhesion                                  |                  |               |
| MB4498                                         | Vascular cell adhesion molecule 1 | 1.0 | 3.0 |
| D00813                                         | Intercellular adhesion molecule 1 | 1.0 | 2.0 |
| U82612                                         | Fibronectin 1    | 1.0 | 1.6 |
| AI76461                                        | Selectin, endothelial cell, ligand | 1.3 | 1.5 |
| Chemokine                                      |                  |               |
| U17035                                         | Chemokine (CXC motif) ligand 10 | 1.1 | 1.8 |
| Cytokines and cytokine-associated genes         |                  |               |
| M63122                                         | Tumor necrosis factor receptor | 1.1 | 1.9 |
| U48956                                         | MAPK kinase kinase 1 | 1.2 | 1.9 |
| M92340                                         | Interleukin 6 signal transducer | 1.0 | 1.5 |
| S79676                                         | Interleukin 1 beta converting enzyme | 1.2 | 1.4 |
| U73142                                         | p38 MAPK         | 1.1 | 1.3 |
| Genes associated with T and B cells and MHCII expression |                  |               |
| L22654                                         | Anti-acetylcholine receptor antibody rearranged immunoglobulin gamma-2a chain, VDJC region | 2.6 | 5.3 |
| AJ223184                                       | DOER protein (immunoglobulin superfamilly member 6) | 1.4 | 2.6 |
| U75411                                         | Antidiotype Ig M light chain | 1.0 | 2.0 |
| X10316                                         | MHC OX-45 surface antigen | 1.1 | 1.6 |
| AF029240                                       | MHC class II S3 | 1.0 | 1.4 |
| S58983                                         | La-autoantigen SS-B/La | 1.0 | 1.4 |
| X56596                                         | MHC class II antigen RT1.B-1 beta chain | 1.3 | 1.3 |
| X53054                                         | RT1.D beta chain | 1.5 | 1.2 |
| M15562                                         | MHC class II RT1.D-α chain | 1.3 | 2.5 |

*Table contains GenBank accession numbers (http://www.ncbi.nih.gov/entrez/query.fcgi?db=nucleotide) of cDNA fragments present on Affymetrix RG U34A gene chips, gene name, and average fold change in expression of both low dose and high dose versus control. Fold changes are calculated with data from five to six rats per group. One-way ANOVA was used to determine significance; only probe sets that changed significantly with p < 0.001 are shown.*
found in several autoimmune diseases (Huhn et al. 1997).

**Drug-Metabolizing Enzymes**

*Cytochrome P450.* CYP enzymes are involved in the oxidative dehalogenation of HCB (Van Omphen and Van Bladeren 1989). HCB exposure increased gene expression of several CYPs and of epoxide hydrolase, an enzyme involved in detoxification of epoxides in liver (Table 8). In spleen, MLN and kidney expression of CYP enzymes was also induced but to a lesser extent than in liver.

**Role of dioxin-like contamination of HCB.** Surprisingly, gene expression of CYP1A1 was strongly upregulated in liver. This was an unexpected finding, as previous work showed that HCB induced much more CYP2B than CYP1A1 (Franklin et al. 1997). CYP1A1 upregulation is associated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or related compounds that activate the aryl hydrocarbon (Ah) receptor. It is still the subject of debate if HCB is a dioxin-like compound. Van Birgelen (1998) suggested that HCB should be considered as one, as HCB meets the criteria for dioxin-like compounds: the ability to bind to the Ah receptor, induction of dioxin-like effects, and bioaccumulation. Vos (2000) commented, however, that although TCDD and HCB share some target organs, the toxic effects in these systems are quite different. Furthermore the affinity for the Ah receptor is 10,000 times less for HCB than for TCDD (Hahn et al. 1989). HCB was analyzed to investigate whether contamination with dioxin-like compounds was responsible for the observed effects. Indeed, HCB was contaminated with PCDDs and PCDFs, and the toxic equivalent was 187 pg/mg HCB. The calculated no observed adverse effect level (NOAEL) of CYP1A1 induction was 0.7–4 ng TCDD/kg bw/day (Van Birgelen et al. 1995). In our study rats were exposed to approximately 2 ng/kg bw/day (low dose) and 6 ng/kg bw/day (high dose). Therefore, exposure to dioxins and furans is of the same order of magnitude as the calculated NOAEL and therefore not likely to be responsible for the observed strong increase in gene expression for CYP1A1. This is not in accordance with previous work showing that HCB could only moderately or not at all induce CYP1A1 by HCB (Franklin et al. 1997; Machala et al. 1996). This discrepancy may be explained by strain differences or by the difference in detection of CYP1A1 (7-ethoxyresorufin–O-deethylase induction versus gene expression).

**Mercapturic acid pathway.** The BN rat degrades HCB also via the mercapturic acid pathway that involves glutathione conjugation catalyzed by glutathione S-transferase (GST; Renner 1981). As expected, gene expression of several GSTs was upregulated in liver. Other phase II enzymes that were induced are mercaptopyruvate sulfurtransferase, uridine diphosphate (UDP)-glucuronosyltransferase, and the sulfotransferase family.

**Porphyria**

One of the main toxic effects of HCB is the induction of porphyria in humans (Gocmen et al. 1986) and experimental animals (Courtney 1979), caused by a disturbance in heme biosynthesis. In the present study, gene expression of enzymes involved in heme synthesis were induced. These include aminolevulinic acid (ALA) dehydratase, porphobilinogen deaminase (hydroxymethylbilane synthase), and uroporphyrinogen decarboxylase in spleen and ALA synthase in liver.

**Estrogen/Androgen Metabolism**

Several reports have shown that HCB exposure induces effects on the reproductive system. In humans, serum HCB levels from women exposed during the accident in Turkey correlated with spontaneous abortion (Jarrell et al. 1998), and the proportion of male births was reduced in the group of women that had HCB-induced porphyria (Jarrell et al. 2002). In monkeys, HCB decreased estrogen levels (Foster et al. 1995), and in Wistar rats, HCB exposure reduced serum levels of estrogen and decreased levels of uterine estrogen receptors (Alvarez et al. 2000). Gene expression of estrogen sulfotransferase was upregulated in liver. This enzyme is important in the sulfation of estrogen, a pathway that inactivates estrogen. The enzyme 17β-hydroxysteroid dehydrogenase was downregulated in the liver. This enzyme catalyzes the interconversion of testosterone and androstenedione as well as estradiol and estrone. Both can lead to lower estrogen levels in the liver.

**Table 7.** Representative genes that changed significantly (p < 0.001) after HCB treatment were functionally grouped: APR and oxidative stress.*

| Accession number | Gene name | HCB low dose | HCB high dose |
|------------------|-----------|--------------|--------------|
| **Spleen**       |           |              |              |
| U24441           | Matrix metalloproteinase-9 (gelatinase B) | 1.1 | 7.4 |
| M58040           | Transferin receptor | –1.1 | 7.1 |
| A123261          | Glutamate-cysteine ligase | 1.2 | 5.0 |
| K01933           | Haptoglobin | 1.3 | 4.2 |
| U06099           | Thiolic-specific antioxidant (peroxiredoxin 2) | 1.2 | 3.0 |
| D38380           | Transferin | 1.0 | 2.1 |
| M11794           | Metallotrinose-1 and -2 | 1.1 | 2.0 |
| L33869           | Ceruloplasmin | 1.0 | 1.9 |
| AA944397         | Heat shock protein 86 | 1.2 | 1.8 |
| X07385           | Glutathione peroxidase | 1.4 | 1.7 |
| Y00497           | Manganese-containing superoxide dismutase | –1.0 | 1.6 |
| A170813          | Heat shock 10-kD protein 1 | 1.1 | 1.1 |
| M21060           | Copper-zinc containing superoxide dismutase | 1.0 | 1.3 |
| D00680           | Plasma glutathione peroxidase precursor | –1.2 | –3.5 |
| **MLN**          |           |              |              |
| D00680           | Plasma glutathione peroxidase precursor | 2.0 | 4.3 |
| Y00497           | Manganese-containing superoxide dismutase | 1.8 | 2.6 |
| AA917854         | Ceruloplasmin | 1.0 | 2.2 |
| S72534           | Tissue inhibitor of metalloproteinase-2 | 1.5 | 2.0 |
| **Blood**        |           |              |              |
| AA926149         | Catalase | 1.7 | 2.8 |
| A1236795         | EST, similar to mouse HSP 84 | –1.1 | –1.6 |
| M11942           | 70 kD heat-shock-like protein | –1.1 | –1.9 |
| **Liver**        |           |              |              |
| L21312           | Lipopolysaccharide binding protein | 1.7 | 8.3 |
| A1693277         | Tissue inhibitor of metalloproteinase-1 | 1.0 | 6.0 |
| V01216           | Oroxomucoid 1 | 3.1 | 6.1 |
| J02722           | Heme oxygenase | 1.8 | 5.2 |
| L33869           | Ceruloplasmin | 1.4 | 2.0 |
| Y00497           | Manganese-containing superoxide dismutase | 1.4 | 1.6 |
| X12267           | Glutathione peroxidase I | –1.3 | –1.8 |
| **Kidney**       |           |              |              |
| L33869           | Ceruloplasmin | 1.3 | 4.2 |
| D38380           | Transferin | 1.3 | 2.7 |
| X688041          | Epidymal secretory superoxide dismutase | 1.4 | –1.6 |

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levels. Together, these results indicate that HCB interferes with estrogen metabolism.

**Conclusions**

Gene expression profiles confirmed known effects of HCB such as stimulatory effects on the immune system and induction of enzymes involved in drug metabolism, porphyria, and the reproductive system. New findings include upregulation of genes encoding proinflammatory cytokines, antioxidants, APPs, complement, mast cell markers, chemokines, and cell adhesion molecules. Thus, most transcriptome profiles are consistent with and complementary to previous pathological findings and can be used as markers for several processes that occur after HCB exposure.

Presumably, after oral exposure to HCB, macrophages are attracted to organs such as spleen, lung, and skin and become activated by HCB. This leads to a cascade of reactions involving innate immune cells, as depicted in Figure 2. The gene expression profiles provide evidence for the importance of macrophages and granulocytes and mediators released by these cells in the adverse inflammatory response against HCB. In this way, co-stimulatory or danger signals are generated that could polyclonally activate T cells. Thus, DNA microarray analysis revealed the complexity of cells and mediators involved in the immune response elicited by HCB and confirms previous work showing the importance of macrophages and granulocytes (Ezendam et al. 2004; Michielsen et al. 1999).

Data obtained in an extensive study such as this can be used to create a database with gene expression profiles of known toxicants, as has been suggested previously (Thomas et al. 2002). Chemicals can be screened by establishing their gene expression profiles and comparing them with profiles of known toxic chemicals. In this way classes of toxic compounds can be recognized, as has previously been shown for hepatotoxicants (Hamadeh et al. 2002a, 2002b), and genomics may be an additional tool in hazard identification.

**Table 8.** Representative genes that changed significantly ($p < 0.001$) after HCB treatment were functionally grouped: enzymes involved in drug metabolism, porphyria, and estrogen metabolism.*

| Accession number | Gene name                                                                 | Fold change |
|------------------|---------------------------------------------------------------------------|-------------|
|                  |                                                                           | RCB low dose | RCB high dose |
| Spleen           |                                                                           |             |              |
| AA800745         | Aminolevulinate, delta-, dehydratase                                      | −1.4        | 10.7         |
| X0827            | Porphobilinogen deaminase (hydroxymethylbilane synthase)                  | 1.2         | 8.9          |
| Y00350           | Uroporphyrinogen decarboxylase                                            | −1.0        | 4.0          |
| D50564           | Mercaptopyruvate sulfotransferase                                         | 1.1         | 2.8          |
| AA659700         | ESTs, highly similar to ppox, mouse protoporphyrigen oxidase              | −1.1        | 2.5          |
| A1178056         | Cytochrome P450 1b1                                                       | 1.5         | 1.9          |
| M100068          | NAPDH-cytochrome P-450 oxoductase                                         | −1.0        | −1.3         |
| X04229           | Glutathione S-transferase Yb subunit                                      | −1.1        | −1.5         |
| S82820           | Glutathione S-transferase Yc2 subunit                                     | −1.0        | −1.7         |
| MLN              | Cytochrome P450 7b1                                                       | 1.4         | 2.6          |
| Blood            |                                                                           |             |              |
| A0228110         | UDP-glucuronosyltransferase 8                                            | 1.8         | 3.8          |
| D50654           | Mercaptopyruvate sulfotransferase                                         | 1.7         | 2.4          |
| Liver            |                                                                           |             |              |
| E00778           | Cytochrome P450, family 1, subfamily a, polypeptide 1                      | 65          | 125          |
| J025852          | Cytochrome P450 IA3                                                       | 6.4         | 46           |
| S76489           | Estrogen sulfotransferase isom 3                                          | 20          | 43           |
| K02989           | Cytochrome P406e (phenobarbital-induced)                                   | 11          | 13           |
| M13646           | Pregnenolone 16-alpha-carboninitril-inducible cytochrome P450             | 3.2         | 12           |
| L24207           | Testosterone B-beta-hydroxylase (CYP3A1)                                  | 5.9         | 6.9          |
| J02722           | Heme oxygenase                                                            | 1.8         | 5.2          |
| E01194           | P-450 MC substituted the C terminal region cytochrome containing HR2 region for the same region of CYPd | 3.0 | 5.2 |
| D86297           | Aminolevulinate synthase 2, delta                                          | 2.1         | 4.4          |
| S82820           | Glutathione S-transferase Yc2 subunit                                     | 3.5         | 3.4          |
| M26125           | Epoxide hydrolase                                                         | 2.7         | 2.8          |
| M13506           | Liver UDP-glucuronosyltransferase, phenobarbital-inducible form           | 2.9         | 2.7          |
| S72505           | Glutathione S-transferase Yc1 subunit                                     | 1.7         | 1.6          |
| J03914           | Glutathione S-transferase Yb subunit                                      | 1.9         | 1.8          |
| X80328           | Cytosolic epoxide hydrolase                                               | −1.7        | −3.1         |
| X91234           | 17-Beta hydroxysteroid dehydrogenase type 2                               | −1.9        | −18          |
| Kidney           |                                                                           |             |              |
| A1178056         | Cytochrome P450, subfamily 1b, polypeptide 1                              | 1.1         | 2.9          |
| M37028           | Cytochrome P450 4a10                                                     | 1.2         | 2.7          |
| L13899           | Minoxidil sulfotransferase                                                | 1.1         | 2.3          |
| M20131           | Cytochrome P450 IIE                                                       | −1.4        | −1.9         |

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