Toxicogenomics of Subchronic Hexachlorobenzene Exposure in Brown Norway Rats

Janine Ezendam,[1,2,3] Frank Staedtler,[4] Jeroen Pennings,[2] Rob J. Vandebriel,[2] Raymond Pieters,[1] Johannes H. Harleman,[5] and Joseph G. Vos[2,3]

[1]Institute for Risk Assessment Sciences (IRAS), Immunotoxicology, Utrecht University, Utrecht, the Netherlands; [2]Laboratory for Toxicology, Pathology and Genetics, National Institute for Public Health and the Environment, Bilthoven, the Netherlands; [3]Faculty of Veterinary Medicine, Department of Pathobiology, Utrecht University, Utrecht, the Netherlands; [4]Biomarker Development and [5]Preclinical Safety, Novartis Pharma AG, Basel, Switzerland

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. *Key words:* Brown Norway rat, DNA microarray analysis, drug metabolism, estrogen metabolism, genomics, hexachlorobenzene, immunotoxicity, inflammation, oxidative stress, porphyria. *Environ Health Perspect* 112:782–791 (2004). doi:10.1289/txg.6861 available via *http://dx.doi.org/* [Online 7 April 2004]

Hexachlorobenzene (HCB) was used as a fungicide until the 1970s, when such use was prohibited. Considerable amounts are still generated as waste by-products of industrial processes and emitted into the environment. Because of its chemical stability, persistence, and long-range transport, HCB can be found throughout the environment and is detectable in human milk, blood, and adipose tissue.

In the 1950s, an accidental poisoning in Turkey revealed several toxic effects of HCB in humans. Approximately 3,000–5,000 people ingested HCB-treated seed grain and developed a disease called porphyria turcica (Gocmen et al. 1986), characterized by hepatic porphyria and cutaneous skin lesions caused by a disturbed porphyrin metabolism (Bickers 1987). Other clinical symptoms include enlarged liver, spleen, lymph nodes (LNs), and thyroid, neurological symptoms, and arthritis. Infants born to mothers exposed to HCB developed a different syndrome called pembe yara, characterized by high mortality, diarrhea, fever, hepatomegaly, and skin lesions in the absence of porphyria, but with infiltrations of macrophages and lymphocytes and infiltrates in the lung (Cam 1960). Immunotoxic effects were reported in the Turkish poisoning victims, but also in occupationally exposed workers in Brazil. Increased levels of IgM and IgG were observed, as well as impaired function of neutrophil granulocytes (Queiroz et al. 1998a, 1998b).

In rats HCB induced hepatic porphyria and neurotoxic effects (Courtney 1979), and toxic effects on the reproductive system (Jarrell et al. 1998), thyroid function (Kleiman de Pisarev et al. 1990), and immune system (Michielsen et al. 1999; Vos 1986). Because HCB is a lipophilic xenobiotic, exposure leads to accumulation in adipose tissue, whereas only a small part of ingested HCB is metabolized. HCB can be converted in a cytochrome P450 (CYP)-dependent manner (Van Ommen and Van Bladeren 1989) and also via the mercapturic acid pathway (Renner 1981).

Brown Norway (BN) rats are very susceptible to HCB-induced adverse immune effects. Exposure caused a dose-dependent immunostimulation characterized by enlarged spleen and LNs and increased serum levels of total IgM, IgG, IgE, and IgM against single-stranded (ss)DNA. Furthermore, rats developed inflammatory skin and lung effects characterized by infiltrates of eosinophilic granulocytes and macrophages (Michielsen et al. 1997, 1999). Although both T cells and macrophages seem to play an important role in HCB-induced immunotoxicity in BN rats (Ezendam et al. 2004), exact mechanisms are unknown.

In this study we used DNA microarray analysis to assess changes associated with HCB exposure at the gene expression level. Transcript levels were measured using the Affymetrix RG U34A GeneChip. BN rats were exposed to 0, 150, or 450 mg HCB per kg diet, doses used also in earlier studies (Ezendam et al. 2004; Michielsen et al. 1997), and gene expression levels were assessed in spleen, mesenteric lymph nodes (MLN), thymus, blood, liver, and kidney. This approach revealed several changes in line with the known toxic effects but also revealed novel ones, which may suggest additional (immuno)toxic effects of HCB exposure and/or provide more insight into the mechanisms of HCB-induced adverse effects.

Materials and Methods

Rats and Maintenance

Three-week-old SPF female inbred Brown Norway (BN/SsNOlaHsD, termed BN)

Address correspondence to J. Ezendam, IRAS, Yalelaan 2, 3584 CM, Utrecht, the Netherlands. Telephone: 0031 302535328. Fax: 0031 302535077. E-mail: j.ezendam@iras.uu.nl

We thank S. Bongiovanni, M. Goetschy, S. Laurent, and N. Hartmann (Novartis, Basel, Switzerland) for performing the DNA microarray experiments. We thank M. de Baets (Department of Neurology, Academic Hospital of Maastricht, Maastricht, the Netherlands) for performing the radioimmunoassay to determine antiacetylcholine receptor antibodies, and we thank B. Baumann (RIVM, Bilthoven, the Netherlands) for measuring the dioxin-like contamination of HCB.

The authors declare they have no competing financial interests.

Received 13 November 2003; accepted 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_2/O_2. 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 Sante 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 ^{13}C12-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_2O_3, 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 dependently changed, 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 immunology (Tables 1–6), acute phase responses (APRs) and oxidative stress

(Table 7), and enzymes involved in drug metabolism, porphyria, and estrogen metabolism (Table 8).

Figure 2 shows a deduced scheme of immune cells and mediators involved in the inflammatory response. This scheme is used to simplify the cascade of reactions that occur during inflammation and to present the results in a logical order. The complete list of significantly changed probe sets can be found on the ArrayExpress website (http://www.ebi.ac.uk/arrayexpress).

Inflammatory Response

Macrophages. In HCB-exposed rats, macrophage infiltrations were observed in skin, lung (Michielsen et al. 1997), spleen (Ezendam et al. 2004; Schielen et al. 1993), and liver (Courtney 1979). As expected, HCB increased gene expression of macrophage markers in spleen and MLN and Kupffer cell markers in liver, supporting the significance of macrophages in HCB-induced immunotoxicity.

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 Catabbi 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.[a]

Accession number	Gene name	Fold change	
		HCB low dose	HCB high dose
Granulocytes and macrophages			
AA957003	S100 calcium binding protein A8	2.8	34
U50353	Defensin 3a	2.5	32
AA946503	Lipocalin 2	1.7	24
L18948	S100 calcium binding protein A9	3.2	19
L06040	12-lipoxygenase	1.9	5.7
M32062	Fc receptor, IgG, low affinity III	1.4	2.0
AA894004	ESTs, 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
U67888	Mast cell protease 3	3.4	20
U67907	Mast cell protease 4 precursor	1.5	8.7
M21622	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
M38759	Histidine decarboxylase	3.7	4.3
Pattern recognition molecules			
AF087943	CD14 antigen	1.1	1.7
Complement			
AF036548	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
M98820	Interleukin 1 beta	1.5	–1.2
M55050	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
U03491	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 immunoglobulin gamma-2a chain	3.2	1.6
L07398	Immunoglobulin rearranged gamma-chain V region	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
D13555	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	–1.4
D10728	Lymphocyte antigen CD5	–1.2	–1.6
M85193	RT6.2	–1.3	–1.6
U24652	Linker 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.

[a]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 infiltrations 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.[a]

Accession number	Gene name	Fold change	
		HCB low dose	HCB high dose
Granulocytes and macrophages			
L18948	S100 calcium binding protein A9	2.2	22
AA957003	S100 calcium binding protein A8	2.6	19
M32062	Fc gamma receptor	2.0	2.8
AJ223184	DORA protein (immunoglobulin superfamily, member 6)	1.1	2.6
Pattern recognition molecules			
U44129	Mannose-binding lectin 1	1.5	2.6
AF087943	CD14 antigen	1.8	2.5
Cell adhesion			
L08100	Glycam 1	3.1	2.5
Chemokines			
U92803	CC-chemokine-binding receptor JAB61	1.9	2.6
AF053312	CC chemokine ST38 precursor	2.4	16
Cytokines			
M26744	Interleukin 6	2.3	4.3
AF075383	Suppressor of cytokine signaling	1.9	2.5
M63122	Tumor necrosis factor receptor	1.2	1.8
AA891209	ESTs, highly similar to interleukin 25	1.2	1.5
Genes associated with T and B cells and MHCII expression			
M28671	Rearranged IgG-2b	1.5	3.2
X07189	Immunoglobulin heavy chain constant region	2.5	3.1
M18526	Immunoglobulin germline kappa-chain	1.4	1.8

[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.[a]

Accession number	Gene name	Fold change	
		HCB low dose	HCB high dose
Granulocytes and macrophages			
L18948	S100 calcium binding protein A9 (MRP-14)	1.1	2.0
X14323	IgG receptor FcRn	1.2	1.2
Mast cell			
U67911	Mast cell protease 8 precursor	1.5	2.0
Cytokine			
AF065161	Cytokine inducible SH2-containing protein	1.2	1.7
Genes associated with B cells			
L22654	Antiacetylcholine receptor antibody rearranged immunoglobulin gamma-2a chain, VDJC region	1.6	3.7
M18526	Ig germline kappa-chain	2.0	3.2

[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	Fold change HCB low dose	Fold change HCB high dose
Granulocytes and macrophages			
AA957003	S100 calcium binding protein A8	4.7	34
L18948	S100 calcium binding protein A9	4.7	19
L06040	12-lipoxygenase	1.6	3.6
U49062	Heat stable antigen CD24	–1.2	–3.0
Mast cells			
U67913	Mast cell protease 10	17	16
U67911	Mast cell protease 8 precursor	3.9	4.6
X61654	CD63	1.7	2.0
Pattern recognition molecule			
AA875213	Peptidoglycan recognition molecule	4.3	7.7
Complement			
AA818025	CD59 protein precursor	1.6	2.6
Cell adhesion			
AF072411	Acid translocase/CD36 antigen	2.4	3.6
AJ009698	Embigin	1.9	2.0
D00913	Intercellular adhesion molecule 1	2.2	1.8
Chemokines			
E13732	CC chemokine receptor	1.3	2.4
U90610	CXC chemokine receptor (CXCR4)	2.2	1.1
Anti-inflammatory response			
AI171962	Annexin 1 (p35)	2.1	4.1
Genes associated with T and B cells and MHCII expression			
X76697	CD52/B7 antigen	1.6	2.1
X53517	CD37 antigen	–1.2	–1.8
Z49761	RT1.Ma	–1.4	–1.8
D13555	T-cell receptor CD3, subunit zeta	–1.6	–2.0
X53430	CD3d antigen (T3 delta)	–1.5	–2.0
X53054	RT1.D beta chain	–1.4	–2.1
X13044	MHC-associated invariant chain γ	–1.5	–2.3
M15562	MHC class II RT1.u-D-alpha chain	–1.3	–2.5
U39609	Anti-nerve growth factor 30 antibody light-chain, variable and constant regions	–1.5	–2.7

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

Accession number	Gene name	Fold change HCB low dose	Fold change HCB high dose
Granulocytes and macrophages			
AA946503	Lipocalin 2	4.3	210
L18948	S100 calcium binding protein A9 (MRP-14)	3.4	28
AA957003	S100 calcium binding protein A8 (MRP-8)	1.1	8.5
X76489	CD9 for cell surface glycoprotein	1.4	3.6
AI104781	Arachidonate 5-lipoxygenase activating protein	–1.1	2.3
AA893191	ESTs: phosphatidic acid phosphatase type 2c	1.2	2.0
M55532	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
Mast cell			
U67911	Mast cell protease 8 precursor	2.2	2.8
Complement			
Z50051	Complement component 4 binding protein, alpha	1.3	2.3
Cell adhesion			
D00913	Intercellular adhesion molecule 1	1.2	2.3
Chemokine			
D11445	Gro	1.6	11.5
Cytokines			
AA892553	STAT-1	1.1	3.3
U77777	Interleukin 18	1.3	1.9
L25785	Transforming growth factor beta stimulated clone 22	–1.5	–1.5
Genes associated with T and B cells and MHCII expression			
L22654	Antiacetylcholine receptor antibody, rearranged immunoglobulin gamma-2a chain, VDJC region	–1.0	8.8
U39609	Anti-NGF30 antibody light-chain mRNA, variable and constant regions	1.9	8.7
X68782	Immunoglobulin heavy chain VDJ-region CH1-CH2	1.4	4.6
X53054	RT1.D beta chain	1.5	2.0

[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 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.

arachidonate 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.[a]

Accession number	Gene name	Fold change	
		HCB low dose	HCB high dose
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	Allograft inflammatory factor	–1.1	2.5
AA946503	Lipocalin 2	1.1	2.0
U49062	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			
M84488	Vascular cell adhesion molecule 1	1.0	3.0
D00913	Intercellular adhesion molecule 1	1.0	2.0
U82612	Fibronectin 1	1.0	1.6
AI176461	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
U48596	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	DORA protein (immunoglobulin superfamily member 6)	–1.4	2.6
U75411	Antiidiotype Ig M light chain	–1.0	2.0
X13016	MRC OX-45 surface antigen	1.1	1.6
AF029240	MHC class Ib RT1.S3	1.0	1.4
S59893	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.u-D-alpha chain	–1.3	–2.5

[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.

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 Ommen 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 aminolevulinate (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

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

Accession number	Gene name	Fold change HCB low dose	Fold change HCB high dose
Spleen			
U24441	Matrix metalloproteinase-9 (gelatinase B)	1.1	7.4
M58040	Transferrin receptor	–1.1	7.1
AI233261	Glutamate-cysteine ligase	1.2	5.0
K01933	Haptoglobin	1.3	4.2
U06099	Thiol-specific antioxidant (peroxiredoxin 2)	1.2	3.0
D38380	Transferrin	1.0	2.1
M11794	Metallothionein-1 and -2	1.1	2.0
L33869	Ceruloplasmin	1.0	1.9
AA944397	Heat shock protein 86	1.2	1.8
X07365	Glutathione peroxidase	1.4	1.7
Y00497	Manganese-containing superoxide dismutase	–1.0	1.6
AI170613	Heat shock 10 kD protein 1	1.1	1.3
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
AA817854	Ceruloplasmin	1.0	2.2
S72594	Tissue inhibitor of metalloproteinase-2	1.5	2.0
Blood			
AA926149	Catalase	1.7	2.8
AI236795	ESTs, similar to mouse HSP 84	–1.1	–1.6
M11942	70 kd heat-shock-like protein	–1.1	–1.9
Liver			
L32132	Lipopolysaccharide binding protein	1.7	8.3
AI169327	Tissue inhibitor of metalloproteinase-1	1.0	6.9
V01216	Orosomucoid 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
X12367	Glutathione peroxidase I	–1.3	–1.8
Kidney			
L33869	Ceruloplasmin	1.3	4.2
D38380	Transferrin	1.3	2.7
X68041	Epididymal secretory superoxide dismutase	1.4	–1.6

[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.

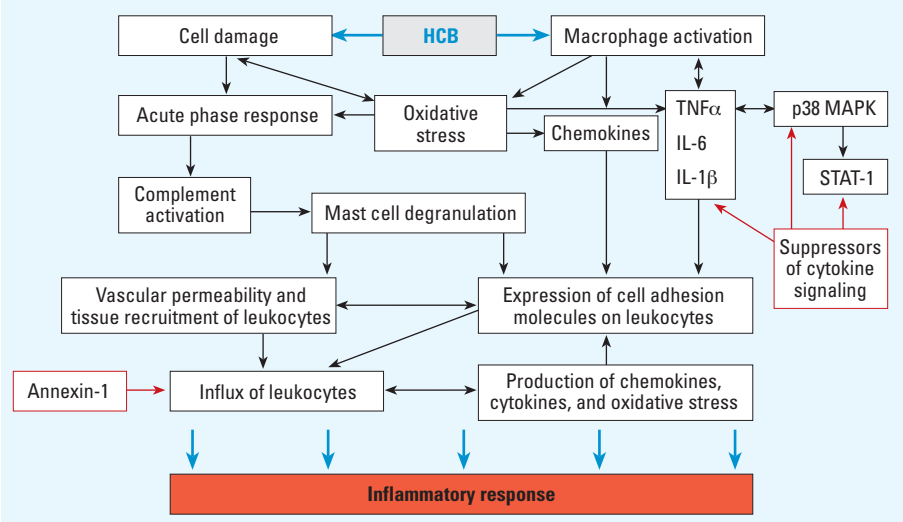

Figure 2. Hypothetical overview of cells and factors involved in the inflammatory response initiated by HCB. Assuming that HCB activates macrophages, this would lead to a cascade of reactions, activating immune cells and pro- and anti-inflammatory (in red) mediators, eventually leading to inflammation.

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.[a]

Accession number	Gene name	Fold change HCB low dose	Fold change HCB high dose
Spleen			
AA800745	Aminolevulinate, delta-, dehydratase	–1.4	10.7
X06827	Porphobilinogen deaminase (hydroxymethylbilane synthase)	1.2	8.9
Y00350	Uroporphyrinogen decarboxylase	–1.0	4.0
D50564	Mercaptopyruvate sulfurtransferase	1.1	2.8
AA859700	ESTs, highly similar to ppox, mouse protoporphyrinogen oxidase	–1.1	2.5
AI176856	Cytochrome P450 1b1	1.5	1.9
M10068	NADPH-cytochrome P-450 oxidoreductase	–1.0	–1.3
X04229	Glutathione *S*-transferase Y(b) subunit	–1.1	–1.5
S82820	Glutathione *S*-transferase Yc2 subunit	–1.0	–1.7
MLN			
U36992	Cytochrome P450 7b1	1.4	2.6
Blood			
AI228110	UDP-glucuronosyltransferase 8	1.8	3.8
D50564	Mercaptopyruvate sulfurtransferase	1.7	2.4
Liver			
E00778	Cytochrome P450, family 1, subfamily a, polypeptide 1	65	125
J02852	Cytochrome P450 IIA3	6.4	46
S76489	Estrogen sulfotransferase isoform 3	20	43
K00996	Cytochrome P450e (phenobarbital-induced)	11	13
M13646	Pregnenolone 16-alpha-carbonitrile-inducible cytochrome P450	3.2	12
L24207	Testosterone 6-beta-hydroxylase (CYP3A1)	5.9	6.9
J02722	Heme oxygenase	1.8	5.2
E01184	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	2.8	2.7
S72505	Glutathione *S*-transferase Yc1 subunit	1.7	1.6
J03914	Glutathione *S*-transferase Yb subunit	1.9	1.8
X60328	Cytosolic epoxide hydrolase	–1.7	–3.1
X91234	17-Beta hydroxysteroid dehydrogenase type 2	–1.9	–18
Kidney			
AI176856	Cytochrome P450, subfamily 1B, polypeptide 1	1.1	2.9
M37828	Cytochrome P450 4a10	1.2	2.7
L19998	Minoxidil sulfotransferase	1.1	2.3
M20131	Cytochrome P450 IIE1	–1.4	–1.9

[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.

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.

References

Affymetrix, Inc. 2002. GeneChip Expression Analysis: Data Analysis Fundamentals. Santa Clara, CA:Affymetrix, Inc. Available: http://www.affymetrix.com/support/downloads/manuals.pdf [accessed: 25 April 2004].

Alvarez L, Randi A, Alvarez P, Piroli G, Chamson-Reig A, Lux-Lantos V, et al. 2000. Reproductive effects of hexachlorobenzene in female rats. J Appl Toxicol 20:81–87.

Bickers DR. 1987. The dermatologic manifestations of human porphyria. Ann NY Acad Sci 514:261–267.

Bigby TD. 2002. The yin and the yang of 5-lipoxygenase pathway activation. Mol Pharmacol 62:200–202.

Billi de Catabbi S, Sterin-Speziale N, Fernandez MC, Minutolo C, Aldonatti C, San Martin de Viale L. 1997. Time course of hexachlorobenzene-induced alterations of lipid metabolism and their relation to porphyria. Int J Biochem Cell Biol 29:335–344.

Cam C. 1960. Une nouvelle dermatose épidémique des enfants. Anales de Dermatologie 87:393–397.

Chen S, Anderson PO, Li L, Sjogren HO, Wang P, Li SL. 2003. Functional association of cytokine-induced SH2 protein and protein kinase C in activated T cells. Int Immunol 15:403–409.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Courtney KD. 1979. Hexachlorobenzene (HCB): a review. Environ Res 20:225–266.

De Baets M, Stassen MH. 2002. The role of antibodies in myasthenia gravis. J Neurol Sci 202:5–11.

Dicou E, Perrot S, Menkes CJ, Masson C, Nerriere V. 1996. Nerve growth factor (NGF) autoantibodies and NGF in the synovial fluid: implications in spondylarthropathies. Autoimmunity 24:1–9.

Ezendam J, Hassing I, Bleumink R, Vos JG, Pieters R. 2004. Hexachlorobenzene-induced immunopathology in Brown Norway rats is partly mediated by T cells. Toxicol Sci 78:88–95.

Foster WG, McMahon A, Younglai EV, Jarrell JF, Lecavalier P. 1995. Alterations in circulating ovarian steroids in hexachlorobenzene-exposed monkeys. Reprod Toxicol 9:541–548.

Franklin MR, Phillips JD, Kushner JP. 1997. Cytochrome P450 induction, uroporphyrinogen decarboxylase depression, porphyrin accumulation and excretion, and gender influence in a 3-week rat model of porphyria cutanea tarda. Toxicol Appl Pharmacol 147:289–299.

Gocmen A, Peters HA, Cripps DJ, Morris CR, Dogramaci I. 1986. Porphyria turcica: hexachlorobenzene-induced porphyria. In: Hexachlorobenzene: Proceedings of an International Symposium (Morris C, Cabral JR, eds). IARC Sci Publ 77:567–573.

Hahn ME, Goldstein JA, Linko P, Gasiewicz TA. 1989. Interaction of hexachlorobenzene with the receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin *in vitro* and *in vivo*. Evidence that hexachlorobenzene is a weak Ah receptor agonist. Arch Biochem Biophys 270:344–355.

Hamadeh HK, Bushel PR, Jayadev S, DiSorbo O, Bennett L, Li L, et al. 2002a. Prediction of compound signature using high density gene expression profiling. Toxicol Sci 67:232–240.

Hamadeh HK, Bushel PR, Jayadev S, Martin K, DiSorbo O, Sieber S, et al. 2002b. Gene expression analysis reveals chemical-specific profiles. Toxicol Sci 67:219–231.

Huhn P, Pruijn GJ, van Venrooij WJ, Bachmann M. 1997. Characterization of the autoantigen La (SS-B) as a dsRNA unwinding enzyme. Nucleic Acids Res 25:410–416.

Jarrell JF, Gocmen A, Akyol D, Brant R. 2002. Hexachlorobenzene exposure and the proportion of male births in Turkey 1935–1990. Reprod Toxicol 16:65–70.

Jarrell J, Gocmen A, Foster W, Brant R, Chan S, Sevcik M. 1998. Evaluation of reproductive outcomes in women inadvertently exposed to hexachlorobenzene in southeastern Turkey in the 1950s. Reprod Toxicol 12:469–476.

Kennedy SW, Wigfield DC. 1990. Dose-response relationships in hexachlorobenzene-induced porphyria. Biochem Pharmacol 40:1381–1388.

Kleiman de Pisarev DL, Rios de Molina MC, San Martin de Viale LC. 1990. Thyroid function and thyroxine metabolism in hexachlorobenzene-induced porphyria. Biochem Pharmacol 39:817–825.

Kuper FC, De Heer E, Van Loveren H, Vos JG. 2002. Immune system. In: Handbook of Toxicological Pathology Vol 2. (Haschek WM, Rousseaux CG, Wallig MA, eds). New York:Academic Press, 585–646.

Liu G, Loraine AE, Shigeta R, Cline M, Cheng J, Valmeekam V, et al. 2003. Netaffx: Affymetrix probesets and annotations. Nucleic Acids Res 31:82–86.

Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, et al. 1996. Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat Biotechnol 14:1675–1680.

Machala M, Matlova L, Svoboda I, Nezveda K. 1996. Induction effects of polychlorinated biphenyls, polycyclic aromatic hydrocarbons and other widespread aromatic environmental pollutants on microsomal monooxygenase activities in chick embryo liver. Arch Toxicol 70:362–367.

Mantovani A, Allavena P, Vecchi A, Sozzani S. 1998. Chemokines and chemokine receptors during activation and deactivation of monocytes and dendritic cells and in amplification of Th1 versus Th2 responses. Int J Clin Lab Res 28:77–82.

Michielsen CP, Bloksma N, Ultee A, van Mil F, Vos JG. 1997. Hexachlorobenzene-induced immunomodulation and skin and lung lesions: a comparison between Brown Norway, Lewis, and Wistar rats. Toxicol Appl Pharmacol 144:12–26.

Michielsen CC, van Loveren H, Vos JG. 1999. The role of the immune system in hexachlorobenzene-induced toxicity. Environ Health Perspect 107:783–792.

Michielsen C, Zeamari S, Leusink-Muis A, Vos J, Bloksma N. 2002. The environmental pollutant hexachlorobenzene causes eosinophilic and granulomatous inflammation and *in vitro* airways hyperreactivity in the Brown Norway rat. Arch Toxicol 76:236–247.

Naka T, Nishimoto N, Kishimoto T. 2002. The paradigm of IL-6: from basic science to medicine. Arthritis Res 4(suppl 3):S233–242.

Perretti M, Gavins FN. 2003. Annexin 1: an endogenous anti-inflammatory protein. News Physiol Sci 18:60–64.

Queiroz ML, Bincoletto C, Perlingeiro RC, Quadros MR, Souza CA. 1998a. Immunoglobulin levels in workers exposed to hexachlorobenzene. Hum Exp Toxicol 17:172–175.

Queiroz ML, Quadros MR, Valadares MC, Silveira JP. 1998b. Polymorphonuclear phagocytosis and killing in workers occupationally exposed to hexachlorobenzene. Immunopharmacol Immunotoxicol 20:447–454.

Renner G. 1981. Biotransformation of the fungicides hexachlorobenzene and pentachloronitrobenzene. Xenobiotica 11:435–446.

Roth J, Vogl T, Sorg C, Sunderkotter C. 2003. Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. Trends Immunol 24:155–158.

Schielen P, Schoo W, Tekstra J, Oostermeijer HH, Seinen W, Bloksma N. 1993. Autoimmune effects of hexachlorobenzene in the rat. Toxicol Appl Pharmacol 122:233–243.

Schreck R, Albermann K, Baeuerle PA. 1992. Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). Free Radic Res Commun 17:221–237.

Schulte A, Althoff J, Ewe S, Richter-Reichhelm HB. 2002. Two immunotoxicity ring studies according to OECD TG 407-comparison of data on cyclosporin A and hexachlorobenzene. Regul Toxicol Pharmacol 36:12–21.

Stonard MD, Poli G, De Matteis F. 1998. Stimulation of liver heme oxygenase in hexachlorobenzene-induced hepatic porphyria. Arch Toxicol 72:355–361.

Thomas RS, Rank DR, Penn SG, Zastrow GM, Hayes KR, Hu T, et al. 2002. Application of genomics to toxicology research. Environ Health Perspect 110(suppl 6):919–923.

Van Birgelen AP. 1998. Hexachlorobenzene as a possible major contributor to the dioxin activity of human milk. Environ Health Perspect 106:683–688.

Van Birgelen AP, Van der Kolk J, Fase KM, Bol I, Poiger H, Brouwer A, et al. 1995. Subchronic dose-response study of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in female Sprague-Dawley rats. Toxicol Appl Pharmacol 132:1–13.

Van Ommen B, Van Bladeren PJ. 1989. Possible reactive intermediates in the oxidative biotransformation of hexachlorobenzene. Drug Metab Drug Interact 7:213–243.

Vos JG. 1986. Immunotoxicity of hexachlorobenzene. In: Hexachlorobenzene: Proceedings of an international symposium Symposium (Morris CR, Cabral JR, eds). IARC Sci Publ 77:347–356.

Vos JG. 2000. Health effects of hexachlorobenzene and the TEF approach. Environ Health Perspect 108:A58.

Vos JG, Van Logten MJ, Kreeftenberg JG, Kruizinga W. 1979. Hexachlorobenzene-induced stimulation of the humoral response in rats. Ann NY Acad Science 320:535–550.