Mechanisms of Hexachlorobenzene-induced Adverse Immune Effects

Mechanismen van Hexachloorbenzeen-geïnduceerde Negatieve Immuuneffecten

(met een samenvatting in het Nederlands)

Proefschrift

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door

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Contents

List of abbreviations

AEC	3-amino-9-ethyl carbazole
Ah-receptor	aryl hydrocarbon receptor
ALA	aminolevulinate
ALN	auricular lymph node
ANOVA	analysis of variance
AP	alkaline phosphatase
APC	antigen presenting cell
APP	acute phase protein
APR	acute phase response
ASC	antibody-secreting cells
BCIP	5-bromo-4-chloro-3-indolylphosphate toluidine salt
BN	Brown Norway
BSA	bovine serum albumin
CIS	cytokine-inducible SH2 containing proteins
CsA	cyclosporin A
CYP	cytochrome P450
CYP450	cytochrome P450
DC	dendritic cell
ELISA	enzyme-linked immunosorbent assay
EST	expressed sequence tag
FBS	fetal bovine serum
FITC	fluorescein-isothiocyanate
GC	germinal center
GlyCAM	glycosylation-dependent cell adhesion molecule
GST	glutathione-S-transferase
HCB	hexachlorobenzene
HEV	high endothelial venule
HE	hematoxylin eosin
$HgCl_2$	mercuric chloride
HSP	heat shock protein
IFN-γ	interferon–γ
Ig	immunoglobulin
IL	interleukin
LIX	lipopolysaccharide-induced CXC chemokine
LMWC	low molecular weight compound
LN	lymph node
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase

MHC	major histocompatibility complex
MLN	mesenteric lymph node
MMP	matrix metalloproteinase
MPO	myeloperoxidase
MZ	marginal zone
NBT	nitroblue tetrazolium
NO	nitric oxide
NOAEL	no observed adverse effect level
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
PAMP	pathogen-associated molecular patterns
PCA	principal component analysis
PCB	pentachlorobenzene
PCDD	polychlorinated dibenzodioxins
PCDF	polychlorinated dibenzofurans
PCP	pentachlorophenol
PCP-NAC	n-acetyl-S-(pentachlorophenyl)-cysteine
PBS	phosphate buffered saline
PE	phycoerythrin
PLN	popliteal lymph node
PO	peroxidase
PRR	pattern recognition receptors
RA-PLNA	reporter antigen-popliteal lymph node assay
ROS	reactive oxygen species
SI	stimulation index
STAT	signal transducer and activator of transcription
TCBQ	tetrachlorobenzoquinone
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCHQ	tetrachlorohydroquinone
TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumour necrosis factor-α
TNP	2,4,6-trinitrophenyl
TLR	Toll-like receptor

Chapter 1

General Introduction

Hexachlorobenzene

Occurrence in the environment

Hexachlorobenzene (HCB; C_6Cl_6) is a low molecular weight compound (LMWC; molecular weight: 285) that has been used in the past as a fungicide for seed grains. In the 1970s such a use was prohibited in most countries, but HCB is still generated as a by-product of several industrial processes, for instance during the manufacturing of chlorinated solvents, aluminium and rubber. Furthermore, trace amounts of HCB are present as contaminants in several chlorine-containing pesticides. Global emission of HCB is estimated to be in the range of 14,000-73,000 kg per year *(3)*. Although emission of HCB has decreased substantially compared to 1970s, residues can still be found throughout the environment due to its chemical stability, high persistence and re-emissions of HCB accumulated in soil and water *(41,71)*. HCB can be detected in water *(7)*, soil, and sediment *(71)*, but also in tissues of farm animals *(34)*, fish *(91)* and in human milk, serum and adipose tissue *(37,79,92)*.

Accidental poisoning in Turkey

The toxic effects of HCB on humans were first noted in the eastern part of Turkey in the period of 1950-1959. Seed grain treated with HCB was unfortunately used as food, resulting in a poisoning of approximately 3000-5000 people. Victims developed a syndrome that has been called Porphyria Turcica, characterized by hepatic porphyria *(10)*. Other clinical features were skin lesions in sun-exposed areas, caused by photochemically activation of accumulated porphyrins *(6)*, hyperpigmentation, hirsutism, painless arthritis, small hands, enlarged liver, spleen, lymph nodes and thyroid and neurological symptoms *(27,57)*. Breast-fed infants born from mothers exposed to HCB developed a syndrome that was different from Porphyria Turcica. Since these children developed skin lesions on arms and legs that were rose-red this disease was called Pembe Yara (pink sore). Histology of skin biopsies showed hyperkeratosis and infiltrations of lymphocytes and macrophages. Other clinical symptoms were fever, diarrhea, and hepatomegaly. Thorax radiography revealed the presence of pulmonary infiltrates of unknown etiology. These infants did not develop porphyria and most victims (>95%) died due to heart-lung failure *(11)*. Follow-up studies among 204 victims, 20-30 years after the poisoning have shown that arthritis, enlarged thyroid, neurological and dermatological symptoms still persisted *(15)*.

Toxic effects in laboratory animals

Experiments in laboratory animals confirmed several of the toxic effects observed in humans (Table 1). HCB-induced toxicity has been extensively reviewed *(14,38,52,78,86)*.

Table 1*:* Main toxic effects induced by HCB in laboratory animals

Toxic effect	Reference
• Hepatic porphyria	*17,22,24*
• Cytochrome P450 induction	*24,42,77*
• Immunotoxicity	*52,88*
• Neurotoxic effects	*12,14,35,38*
• Effects on reproductive system	*1,23,63*
• Liver cell and thyroid tumors (group 2B: possible carcinogenic to humans)	*8,9,31,76*

Absorption, distribution and biotransformation

After oral exposure most of the ingested HCB is excreted unchanged in the faeces *(14)*, but a small amount is also absorbed via the small intestines and further taken up via the cisterna chyli, entering the lymphatic system *(32)*. In this way HCB is quickly absorbed

into the systemic circulation and distributed throughout the body. HCB passively diffuses from blood to tissues and accumulates in highly perfused and lipid-rich tissues, for instance adipose tissue, lung and liver *(33,43)*.

Metabolism of absorbed HCB occurs via two metabolic pathways. The first involves cytochrome P450-catalyzed oxidative dehalogenation. Van Ommen *et al. (84)* have shown that in rat liver microsomes HCB is metabolized into pentachlorophenol (PCP), which is further converted into tetrachlorohydroquinone (TCHQ) that is in redox equilibrium with tetrachlorobenzoquinone (TCBQ). HCB is also metabolized via the mercapturic acid pathway resulting in sulphur-containing metabolites. Glutathione-S-transferase catalyzes conjugation to glutathione yielding s-(pentachlorophenyl)-glutathione. This conjugate is transported to the kidney where it is further metabolized into s-(pentachlorophenyl)-cysteine and into n-acetyl-S-(pentachlorophenyl)-cysteine, which is excreted via the urine *(64)*.

Adverse immune effects of HCB

HCB induces adverse immune effects in different species. In humans immunotoxic effects are seen among the victims of the poisoning in Turkey and also in workers exposed occupationally to HCB in a chemical plant in Brazil. In these workers impaired functions of neutrophilic granulocytes and increased serum IgM and IgG levels are observed *(61,62)*.

Studies in laboratory animals revealed that HCB has opposite immunotoxic effects in mice and rats. HCB exposure in mice suppresses the immune system *(5,40)*, whereas in rats stimulation is observed *(53,68,87,89,90)*. Oral exposure of Wistar rats to HCB results in a dose-dependent increase of the number of peripheral neutrophilic and basophilic granulocytes and monocytes and of spleen and lymph node (LN) weights. Histopathology shows increased marginal zones and follicles and extramedullary hemopoiesis in the spleen and increased numbers of high endothelial venules (HEVs) in mesenteric lymph nodes (MLN) and popliteal lymph nodes (PLN). The primary and secondary IgM and IgG responses to tetanus toxoid, a thymus-dependent antigen, are elevated in HCB-exposed rats *(89)*. Schielen *et al. (68)* have shown that HCB increases the number of ED3+ macrophages in the spleens of Wistar rats. These macrophages are associated with experimentally induced autoimmune diseases such as rheumatoid arthritis *(18,19)* and are thought to be capable of activating B-1 cells *(16)*. B-1 cells are known to produce natural antibodies, such as anti-DNA antibodies and both serum IgM levels against such autoantigens as the number of splenic B-1 cells are increased after HCB exposure *(68,69)*.

These results may be indicatory of an autoimmune component in HCB-induced immunostimulation.

A study comparing three different rat strains (Wistar, Brown Norway (BN) and Lewis) has shown that the BN rat appears to be the most susceptible strain for HCB-induced immunotoxicity *(53)*. BN rats are known to be more prone to develop type-2 dependent autoimmunity whereas Lewis rats are more susceptible to develop type-1 mediated autoimmune diseases *(20)*. Drugs known to induce adverse immune effects such as D-penicillamine *(20)* and nevirapine *(72)* induce autoimmunity in BN rats and tolerance in Lewis rats. Effects induced by HCB are less polarized, since adverse immune effects are induced in both strains *(53)*. Table 2 summarizes the immunotoxic effects of HCB in the BN rat.

Table 2: Summary of immunotoxic effects of HCB in the Brown Norway rat[a]

Parameter	Dose[b]	References
• Increased spleen weight	150, 450	*53*
• Increased popliteal, axillary and mandibular LN weight	450	*50,53*
• Increased number of HEVs in PLN	150, 450	*53*
• Granuloma formation in the mesenteric LN	450	*53*
• Inflammatory skin lesions: hyperplasia epidermis, activated dermal vessels, infiltrates of neutrophils, macrophages and eosinophils	150, 450	*51,53*
• Inflammatory lung lesions: focal accumulations of macrophages, granuloma formation, perivascular eosinophilic infiltrates, HEV-like venules	150, 450	*53*
• Increased total serum IgM and IgE levels	450	*53*
• Increased total serum IgG levels	150, 450	*53*
• Increased serum IgM levels against ssDNA	150, 450	*53*
• Increased *in vitro* and *in vivo* airway hyperresponsiveness	450	*50,54*
• Decreased IL-2 mRNA expression	150	*85*[c]
• Increased IL-10 mRNA expression	50	*85*[c]

[a]BN rats were exposed to HCB via the diet for 3 or 4 weeks. The table contains only significant changes. [b]Dietary HCB concentration in mg/kg diet. [c]In this study rats were exposed to HCB for 6 weeks.

Mechanisms of HCB-induced adverse immune effects

Many LMWCs, such as drugs, metals and environmental pollutants, are able to induce systemic allergy or autoimmune-like phenomena. A lot of research has been done to elucidate the mechanisms of LMWC-induced adverse immune effects (for reviews on this topic see refs.: *26,30,58,59,70)*. In the remainder of this chapter possible mechanisms of LMWC-induced autoimmunity or allergy are discussed in context of HCB-induced immunostimulation.

Neoantigen formation and T cell activation

Since LMWC themselves are too small to be recognized by T cells, one possible way in which a LMWC can activate the immune system is by generating neoantigens. T cells can only recognize peptides that are presented in a major histocompatibility (MHC)-restricted manner. Thus, when chemicals are capable of binding to a protein, a hapten-carrier complex is formed. After degradation in the antigen presenting cell (APC) parts of this neoantigen are presented by the MHCII and can be recognized by the T cell receptor (TCR). This is called signal 1, but T cells need a second signal for complete activation. Signal 2 comprises a broad range of factors like costimulatory molecules and pro-inflammatory cytokines *(44)*. Hence, chemicals that are capable of forming neoantigens and simultaneously generate costimulatory signals can cause specific T cell sensitization.

HCB itself is not able to bind to proteins, but its oxidative metabolite TCBQ is protein-reactive *(83,84)*. The CYP450 catalyzed conversion of HCB seems not to be involved in the observed immunostimulation. Schielen *et al. (67)* have shown that after inhibition of cytochrome P450IIIA adverse immune effects still occurred. Furthermore, pentachlorobenzene, a compound that is metabolized into the same oxidative intermediates as HCB, failed to induce any immunostimulating effects in Wistar rats. Involvement of reactive intermediates formed in the mercapturic acid pathway are also not implicated, since pentachloronitrobenzene, a compound that is converted into the same mercapturic acid metabolites as HCB, did not induce any inflammatory effects in BN rats *(49)*.

In the last decade it has become evident that chemicals implicated in adverse immune reactions can be converted in their reactive intermediates by enzymes present in granulocytes (for reviews see refs.: *80,82)*. Neutrophils and monocytes contain enzymes, such as myeloperoxidase (MPO) that can oxidize chemicals. MPO has been shown to convert several chemicals into reactive and potential immunogenic intermediates, for instance gold salts *(28)*, diclofenac *(55)*, procainamide *(81)* and aniline *(93)*. After oral

exposure to HCB phagocytes are attracted to spleen, skin and lung and may be involved in oxidation of HCB into reactive oxidative metabolites that eventually may sensitize hapten-specific T cells.

Adaptive versus innate immunity

The presence of macrophages and granulocytes in skin, lung and spleen of HCB-exposed rats *(53,68)* indicates that the innate immune system may play an important role in the observed immunostimulation. For a long time immunotoxicological research has focused on adaptive immunity and its role in recognition of (neo)antigens. The two main characteristics of the adaptive immune system are clonal specificity and memory, features mediated by T and B cells. In contrast, innate immune recognition is mediated by germline encoded non-clonal receptors, pattern recognition receptors (PRRs), that recognize conserved molecular patterns associated with pathogens, the so-called pathogen-associated molecular patterns (PAMPs). PRRs, for example Toll-like receptors (TLRs) *(48)* and CD14 *(60)*, are present on the surface of innate immune cells such as macrophages, dendritic cells and granulocytes *(47)*. After engagement of the PRR innate immune cells are activated and a cascade of reactions is initiated to induce an optimal immune response. These include increased expression of pro-inflammatory and effector cytokines and chemokines, recruitment of leukocytes, induction of costimulatory molecules on APCs and regulation of T cell differentiation *(46)*.

Some inert chemicals are known to specifically activate innate immune cells, for instance silica. In rats, inhalation of silica dust can lead to granulomatous inflammation and fibrosis in the lung. After deposition in the lung, silica chronically attracts and activates alveolar macrophages leading to the release of pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α and interleukin (IL)-1 and reactive oxygen species (ROS) *(21,29)*. Release of these pro-inflammatory mediators can elicit a cascade of events, such as expression of cell adhesion molecules and release of chemokines leading to the recruitment of inflammatory cells. Thus, silica can be considered as an adjuvant and exposure can eventually lead to autoimmune-like effects. In workers occupationally exposed to silica dust the incidence of scleroderma *(36)* and systemic lupus erythematosus *(13)* was higher. Thus, adjuvant signals can lead to autoimmune-like effects, a phenomenon that can be explained by the so-called danger hypothesis, proposed by Matzinger *(44)*.

The danger model

The danger hypothesis is based on the assumption that the immune system does not discriminate between self and non-self, but rather between harmless and dangerous. According to this hypothesis, danger signals can activate APCs that subsequently can deliver costimulatory signals to T cells and danger signals can be derived from damaged cells, but also heat shock proteins or cytokines are considered as inducible danger signals *(2)*.

Evidence for this theory has been provided by several studies. *In vitro* experiments have shown that immature dendritic cells (DCs) can both phagocytose apoptotic and necrotic cells. Necrotic cells provided maturation signals to the DC, resulting in upregulation of costimulatory molecules. On the contrary, apoptotic cells were not able to activate DCs *(25,66)*. Uptake of necrotic cells, but not apoptotic cells, by macrophages increased CD40 expression and enhanced T cell proliferation *(4)*. Macrophages play an essential role in the clearance of cellular debris and might be responsible for discriminating between danger signals, e.g. necrosis, or harmless signals, e.g. apoptosis. Other *in vitro* studies showed that necrotic cells induced NF-κB and chemokine gene expression in DCs, macrophages and fibroblasts. Notably, activation of NF-κB required TLR2 signaling. This could mean that besides exogenous (microbial) signals for TLR activation, endogenous signals also mediate immune responses via TLR activation *(39)*. This links Janeway's model of pattern recognition of exogenous non-self to Matzinger's danger hypothesis.

Research done by Shi and colleagues suggests that cells contain molecules with adjuvant properties that are released when the cell membrane integrity is lost. In contrast to the above mentioned studies, these adjuvant signals are released either during necrosis or apoptosis *(74,75)*. Recently, evidence for the existence of endogenous adjuvants has been found, i.e. uric acid, a degradation product of nucleotides. Uric acid is produced by injured cells and was capable of DC activation and enhancement of cytotoxic T cell priming *(73)*.

T cell responses can also be augmented by pro-inflammatory cytokines, a feature explained by the ability of cytokines to upregulate costimulatory molecules on APCs and in this way delivering a signal 2 *(45)*. Furthermore, pro-inflammatory cytokines promote clonal expansion and differentiation of helper T cells in an antigen-dependent manner, probably by providing growth and survival signals *(56)***.**

A consequence of the generation of danger signals could be that a signal 2 is delivered to autoreactive T cells. These T cells are present in healthy individuals and are controlled by regulatory T cell populations to prevent immune responses to self *(65)*.

Endogenous danger signals may break this peripheral tolerance and in this way induce an adverse immune response against self. All together these studies show that innate and adaptive immunity are not two separate entities. Innate immune cells are important effector cells in the sense that they detect danger signals during pathological situations and are activated to mount an efficient adaptive immune response *(46,48)*.

The role of innate immunity in HCB-induced immunotoxicity is not completely clear. The presence of granulocytes and macrophages could indicate that innate immunity plays an important role in the induction of an immune response. Figure 1 summarizes schematically our hypotheses on how HCB can stimulate the immune system. We hypothesize that HCB, after storage in tissues, causes cell stress or necrosis, leading to attraction and activation of macrophages. Subsequently a non-specific inflammatory response is induced. Furthermore necrosis or inflammatory cytokines can be sensed as danger signals by innate immune cells, leading to upregulation of costimulatory molecules and enhancement of T cell responses. Our second hypothesis involves biotransformation of HCB in reactive metabolites, by for instance myeloperoxidase. After covalent binding of the metabolites to proteins neoantigens are formed that are presented to T cells leading to signal 1. T cell activation and maturation occurs when a signal 2 is present. Together, this induces an adaptive immune response against the formed neoantigens, leading to sensitization and formation of memory T cells.

Scope of this thesis

The aim of this thesis was to investigate the above proposed hypotheses. We assessed the sensitizing potential of two oxidative metabolites of HCB, TCHQ and TCBQ. Therefore, these reactive metabolites were tested in the mouse reporter-antigen popliteal lymph node assay using TNP-Ficoll as a reporter-antigen (**Chapter 2**). To obtain more insight in the effects of HCB on a molecular level, a toxicogenomics experiment was performed after subchronic exposure of BN rats to HCB (**Chapter 3**). Microarrays containing thousands of genes were used to assess gene expression profiles. In **Chapter 4** the role of T cells was investigated. For this purpose BN rats were exposed orally to HCB and treated simultaneously with Cyclosporin A (CsA) a compound known to reduce peripheral T cell numbers and inhibit antigen-induced T cell activation. In **Chapter 5** experiments were performed to investigate if oral exposure to HCB leads to sensitization and formation of memory T cells. This chapter also describes data from a kinetic study, performed to obtain information on the initiation phase of HCB-induced immunostimulation. Because macrophages are already present in an early stage, their involvement was also investigated

in this chapter. For these experiments the macrophage 'suicide' technique with clodronate liposomes was used. Treatment with clodronate liposomes depletes macrophages in spleen and liver after intraperitoneal injection. Finally, **Chapter 6** summarizes and discusses data described in this thesis.

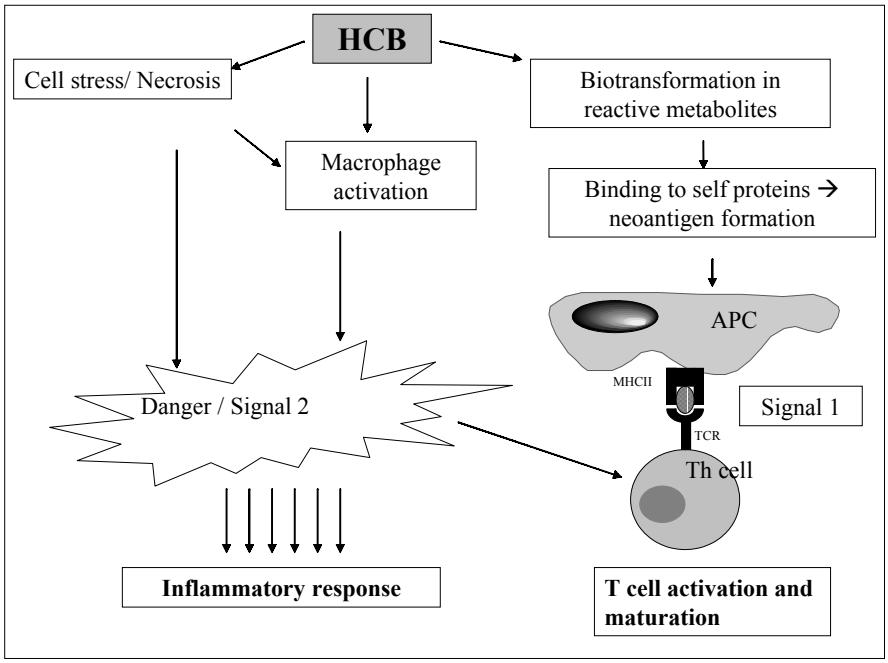

Figure 1: Simplified scheme of the hypothetical pathways of HCB-induced immune stimulation

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Chapter 2

Immunomodulatory effects of tetrachlorobenzoquinone, a reactive metabolite of hexachlorobenzene

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Abstract

Hexachlorobenzene (HCB) is an environmental pollutant that causes autoimmune-like effects in humans and rats. It is not completely clear whether T cells are involved and, if so, how they are stimulated after oral exposure to HCB. HCB as a rather inert chemical is not likely to bind covalently to macromolecules. The oxidative metabolite of HCB, tetrachlorobenzoquinone (TCBQ) which is in redox equilibrium with tetrachloro-hydroquinone (TCHQ) can bind to macromolecules, hence may form hapten-carrier complexes in vivo. We have assessed in the reporter antigen-popliteal lymph node assay (RA-PLNA) whether HCB or TCHQ and TCBQ are able to induce a TNP-specific IgG1 response to the T cell independent antigen 2,4,6-trinitrophenyl (TNP)-Ficoll, which is indicative of neoantigen specific T cell help. To this end, these compounds and silica were injected into the footpad of Balb/c mice. Silica was included as an inert model compound, which causes autoimmune-like effects by activating macrophages. Seven days later cell number and TNP-specific antibody secreting cells (ASC) in the popliteal lymph node (PLN) were determined. Furthermore, a secondary PLNA was performed to find out if TCHQ was capable of eliciting a memory response. Silica, TCHQ and TCBQ, but not HCB, increased PLN cellularity and the number of IgM producing ASC by ELISPOT. Both oxidative metabolites were able to induce the formation of germinal centers as assessed by immunohistochemistry and an IgG1 response to TNP-Ficoll. In the secondary PLNA only mice primed with TCHQ and challenged with TCHQ together with TNP-Ficoll showed a significant increase in TNP-specific IgG1 ASC. Present data show that TCHQ and TCBQ are capable of inducing neoantigen specific T cell help and that TCHQ can induce a compound specific memory response.

Introduction

Hexachlorobenzene (HCB) is a highly persistent environmental pollutant with pronounced toxic and immunotoxic effects. Humans involved in the intoxication incident with HCB in Turkey in the 1950s suffered from a syndrome characterized by hepatic porphyria, skin lesions caused by porphyrins, splenomegaly, enlarged lymph nodes and arthritis *(4)*. Breast-fed infants born to mothers exposed to HCB did not develop porphyria but a syndrome characterized by high mortality, hepatomegaly and inflammatory skin infiltrates predominantly consisting of lymphocytes and macrophages. Also in the lungs infiltrates were present *(5)*.

In several rat studies, oral exposure to HCB induced similar adverse (immune) effects as observed in man *(16,36,37)*. Remarkably, porphyrins appeared not to be involved in the development of skin lesions in the rat *(26)* and several studies have shown that these skin effects could have an (auto)immune-like etiology *(19,27)*. The mechanism of HCB-induced immunopathology is still unclear. HCB is a rather inert, highly hydrophobic low molecular weight compound (LMWC) and only a small portion of ingested HCB is metabolized whereas the remainder is stored in tissues with a high lipid content or excreted in unchanged form via the faeces *(14,15)*. HCB is metabolized via two different pathways. Conjugation to glutathione by glutathion-*s*-transferase eventually leads to the formation of N-acetyl-S-(pentachlorophenyl)-cysteine (PCP-NAC), which is excreted via the urine *(23)*. HCB can also be degraded by P450-catalyzed oxidative dehalogenation to pentachlorophenol (PCP), tetrachlorohydroquinone (TCHQ) and tetrachlorobenzoquinone (TCBQ) *(33)*. Under physiological conditions TCHQ autoxidizes into its semiquinone radical and further into TCBQ *(41)*. Neither of these two metabolic pathways seem involved in the observed immunopathology *(18,26)*. However, conversion of HCB into oxidative metabolites might occur in a non-P450 catalyzed reaction.

It has been shown that autoimmunogenic xenobiotics such as procainamide *(10)*, gold (I) disodium thiomalate *(9)* and propylthiouracil *(35)* (reviewed in *30)* can be metabolized also extrahepatically by mononuclear phagocytes in a non-P450 dependent manner. These reactive metabolites might conjugate to macromolecules and thus act as haptens and be recognized by T cells. The same may apply to benzoquinones and semiquinones, which are highly electrophilic compounds. Notably, van Ommen *et al. (31,32,34)* have shown that TCBQ can bind to macromolecules like proteins and DNA. Recently it has been shown that the oxidative metabolite of benzene, *p*-benzoquinone is able to induce T-cell dependent immune reactions in mice *(8)*. From this we hypothesize that following HCB exposure metabolic enzymes, like for instance myeloperoxidase present in granulocytes, convert HCB into reactive oxidative metabolites that eventually may sensitize hapten-specific T cells. HCB as an inert chemical might also directly activate phagocytes and consequently act as an adjuvant like for instance silica. Silica is a chemical known as a non haptenizing inert compound with intrinsic adjuvant activity that is able to activate macrophages, but has previously been shown not to induce specific T cell activation. Silica-induced macrophage activation can lead to a non-specific stimulation of the immune system and chronic inflammation *(39)*.

To investigate this, we have studied these chemicals in the reporter antigen-popliteal lymph node assay (RA-PLNA) using the T cell-independent antigen TNP-Ficoll *(3,21)*. Important to note is that TNP-Ficoll as a sugar molecule is not recognized by T cells, but

can activate B cells to produce IgM antibodies. For switching to other isotypes, e.g. IgG1, B cells require additional help such as from T cells. Since these T cells cannot be activated by TNP-Ficoll, the help can only come from T cells activated by the injected chemical. In this model an isotype switch to IgG1 indicates that the chemical injected is able to form neoantigens that can stimulate T cells *(1)*. The aim of the present experiments was to assess whether HCB and its metabolites TCHQ and TCBQ can induce hapten specific T cell help. Silica is also included in this assay to serve as a inert model compound. Furthermore, to assess whether help for the TNP-specific isotype switch to IgG1 was TCHQ specific and thus possibly dependent on TCHQ-specific T cells we performed a secondary RA-PLNA protocol with challenge doses of TCHQ together with TNP-Ficoll in mice presensitized to TCHQ.

Materials and Methods

Animals

Female specific pathogen-free BALB/c mice (6-8 weeks of age) (Harlan, Netherlands BV, Zeist, The Netherlands) were housed under hygienic barrier conditions in filter-topped macrolon cages with bedding of wood chips, a temperature of 23±2°C, 50-55% relative humidity and a 12 h light/dark cycle. Mice were assigned randomly to specific treatment groups and were allowed to settle for a week before the start of the experiment. They received standard lab chow and acidified tap water *ad libitum*. All experiments were approved by an ethical committee and conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology.

Chemicals and reagents

HCB (purity>99%) was obtained from Fluka Chemika (Buchs, Switzerland), TCBQ (p-chloranil; purity>98%) from Merck-Schuchardt (Hohenbrunn, Germany), silica (SiO_2) and TCHQ (purity>99%) were obtained from Sigma Chemical Co. (St. Louis, MO). The antigen TNP-Ficoll was prepared as described by Albers *et al. (1)*. Immobilon-P membranes were purchased from Millipore (Etten-Leur, The Netherlands) and alkaline phosphatase (AP)-conjugated goat-anti-mouse human adsorbed IgM and IgG1 from Southern Biotechnology Associates (Birmingham, AL). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate toluidine salt (BCIP) were both from Sigma Chemical Co. (St. Louis, MO) and as solvent for stock solutions of NBT and BCIP we used dimethylformamide (BDH Laboratory Supplies, Poole, UK). For immunohistochemistry, acetone and H_2O_2 were acquired from Merck (Darmstadt,

Germany), 3-amino-9-ethyl carbazole (AEC) from Janssen (Beerse, Belgium) and rabbit-anti-rat-peroxidase (PO) from DAKO (Glostrup, Denmark). Rat anti-mouse B220 (RA3-6B2) was used for detection of B cells. Monoclonal antibodies for staining of surface molecules for flow cytometry were obtained from BD Pharmingen (San Diego, CA): CD4 fluorescein-isothiocyanate (FITC) (RM4-5), CD8α phycoerythrin (PE) (53-6.7), CD19 PE (1D3). Chemicals not specifically mentioned here were from Sigma Chemical Co.

Caution: *HCB, TCHQ, TCBQ and silica are hazardous agents and should be handled accordingly. HCB is very persistent in the environment and possibly a carcinogen, TCHQ and TCBQ are genotoxic compounds. Silica can be harmful by inhalation.*

Treatment of mice

The RA-PLNA was essentially performed as described previously *(1)*. Briefly, all chemicals used in the RA-PLNA were ground to a fine suspension (20 mg/ml) in a glass potter in a solution of 10% (w/v) dextrose and 2% (v/v) Tween-80 in saline. This solution was used to facilitate the suspension of these hydrophobic chemicals *(28)*. All suspensions were placed in an ultrasonic bath for at least one hour. For the primary RA-PLNA a total volume of 50 µl of each suspension together with 10 µg of the reporter antigen TNP-Ficoll was injected sc into the right hind footpad of naive BALB/c mice, hence the dose per animal was 1 mg/mouse. Control mice received the vehicle plus 10 µg TNP-Ficoll. Seven days later, mice were killed by cervical dislocation, and draining PLNs were isolated. For the kinetic study with HCB and silica, mice were killed 21 days after the injection. In the secondary PLNA, mice were primed with either 50 µl solvent or with 1 mg TCHQ in 50 µl solvent. After 4 weeks, mice were challenged sc in the same footpad with a suboptimal dose of 20 µg TCHQ together with 10 µg TNP-Ficoll in 50 µl. This suboptimal dose was predetermined as a dose that is too low to induce a primary PLN response in unprimed mice. Five days after injection of the challenge dose, mice were killed by cervical dislocation and draining PLNs were isolated. For both the primary and the secondary RA-PLNA single cell suspensions were prepared in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA), washed once by centrifugation, resuspended in PBS/1%BSA, counted using a Coulter Counter (Coulter Electronics, Luton, UK) and then adjusted to 1 x 10^6 cells per ml.

ELISPOT assay

This assay was essentially performed as described previously by Schielen *et al. (29)*. Briefly, from each lymph node 0.5 x 10^6 cells in 500 µl PBS/1%BSA were incubated

(37°C, 4 h) in wells containing TNP-BSA-coated (overnight 4°C) Immobilon-P membranes as bottom. Thereafter, membranes were washed and incubated with optimal dilutions of isotype specific AP-conjugated anti-mouse Ig antibodies in PBS/1% BSA (o.n. 4°C). After they were washed, spots were developed by using a freshly prepared solution containing BCIP and NBT in Tris buffer (100 mM Trizma base, 100 mM NaCl, 5 mM $MgCl_2 \cdot 6H_2O$, pH 9.5). Specific ASCs per 10^6 cells were calculated from spot numbers counted with the aid of a stereomicroscope by two observers.

Flow cytometry

For analysis of surface markers single cell suspensions (1 x 10^5 cells) in PBS/1%BSA were centrifuged, resuspended and incubated with combinations of optimal dilutions of FITC- and PE-conjugated mAbs in 96-wells plates (30 min, in the dark, 4°C). Samples were analyzed on a FACScan® flow cytometer with standard FACSflow using CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

Immunohistochemistry

For the visualization of germinal centers acetone-fixed cryostat sections (7 µm) of PLN were incubated with predetermined optimal dilutions of the B cell monoclonal B220 in PBS/1%BSA (overnight 4°C). After three washes in PBS/0.05%Tween, sections were incubated with polyclonal PO-conjugated rabbit-anti-rat-Ig diluted in PBS/5% normal mouse serum (1 h, room temperature). After final washes peroxidase activity was visualized with AEC and sections were counterstained with Mayer's hematoxylin. Acetone-fixed cryostat sections (7 µm) of paws were stained with acid phosphatase to visualize macrophages. Sections were incubated with a solution of 4 mg Naphthol AS-BI phosphate in 0.25 ml dimethyl formamide and 25 ml acetate buffer. To this solution 35 mg diazonium salt was added at 37°C for 45 min. After washing in running water sections were counterstained with Mayer's hematoxylin.

Statistics

Values deviating more than 2 standard deviations from group means were considered as outliers and not included in statistical analyses. Preceding statistical analysis, all data were transformed to log 10 values to homogenize variance. Differences between group means were analyzed using one-way ANOVA with Bonferroni's post hoc test for contrasts ($p<0.05$, $p< 0.01$ and $p<0.001$).

Results

Primary RA-PLNA

Cell number

Female BALB/c mice received a sc injection of 1 mg of the different chemicals together with 10 µg TNP-Ficoll into the right hind footpad. After 7 days, PLNs were removed and cell number and TNP-specific IgM and IgG1 ASC were assessed. TCHQ and TCBQ, but also silica, induced a significant increase in PLN cell number. HCB, however, caused only a slight but not significant ($p<0.077$) increase of cell number (Figure 1). The PLN stimulation indices (SI) from each group were calculated by dividing cell number of the treatment group by the cell number of the control group ($1.3 \pm 0.1 \times 10^6$ cells), resulting in the following SI: 1.9 for HCB ($2.4 \pm 0.6 \times 10^6$ cells), 3.5 for silica ($4.5 \pm 1.6 \times 10^6$ cells), 4.1 for TCBQ ($5.2 \pm 2.2 \times 10^6$ cells) and 5.7 for TCHQ ($7.2 \pm 3.2 \times 10^6$ cells).

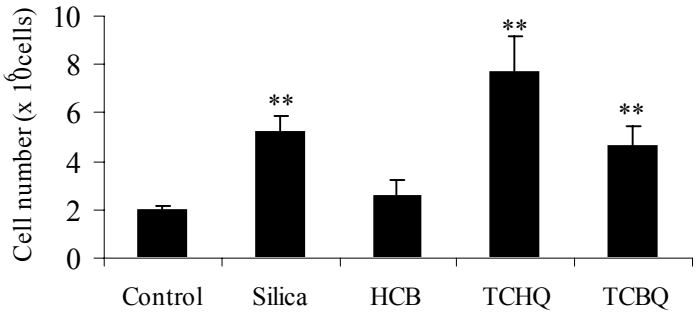

Figure 1: PLN cell number 7 days after injection of different compounds together with TNP-Ficoll. Data are obtained from 2 individual experiments. Bars represent mean ± SE of 5-10 mice per group. ** Significantly different from control group with $p<0.01$. All data were determined by ANOVA followed by Bonferroni's post hoc-test.

TNP-specific antibody-secreting cells

Results of the ELISPOT are presented in Figure 2 as the number of ASC per 10^6 cells. HCB was not able to increase either IgM or IgG1 ASC. TCHQ and TCBQ significantly enhanced the number of IgM ASC as well as of IgG1 ASC. Results show a 6-fold increase in IgM ASC for both metabolites, whereas IgG1 ASC were increased 20-fold and 30-fold for TCHQ and TCBQ, respectively. In case of silica the number of IgM ASC, but not of IgG1 ASC was significantly enhanced.

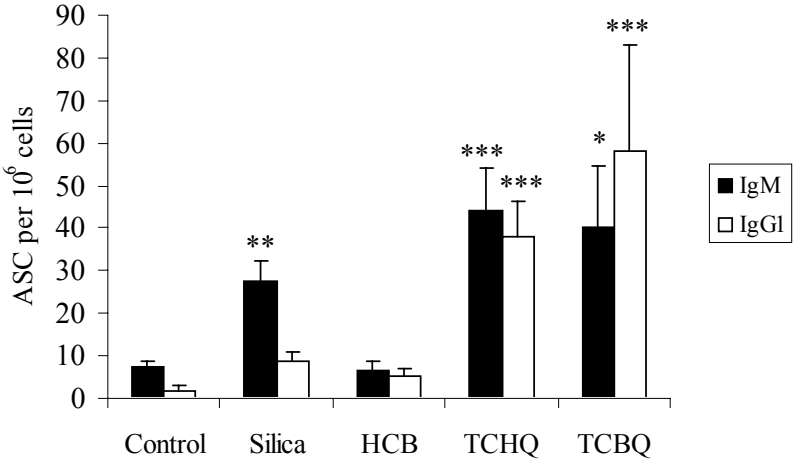

Figure 2: Number of TNP-specific antibody-secreting cells (ASC) per 10^6 cells as determined by ELISPOT at day 7 after injection of the different compounds together with TNP-Ficoll. Data are obtained from 2 individual experiments. Bars represent mean ± SE of 6-9 mice. Significantly different from the control group * $p<0.05$; ** $p<0.01$, *** $p<0.001$.

Immunohistochemistry

To establish whether the different chemicals were capable of inducing germinal center formation all compounds were injected in the footpad without TNP-Ficoll. Seven days later PLNs were removed and immunohistochemistry was performed. B cell staining clearly showed that both TCHQ and TCBQ were capable of inducing germinal center formation (Figure 3), whereas HCB and silica did not induce the development of germinal centers.

Flow cytometry

Table 1 shows relative and absolute amount of B (CD19+) cells, helper T cells (CD4+, Th cells) and cytotoxic T cells (CD8+, Tc cells) in the PLN. Silica induced a significant increase of the absolute number of Th and Tc cells. Injection of HCB caused a significant increase of the absolute number of Tc cells. In the case of TCHQ and TCBQ, the absolute numbers of B, Th and Tc cells were all significantly increased. The relative numbers of lymphocyte subsets give an indication of which cells contribute the most to the PLN enlargement. Data show that the PLN enlargement that is seen after injection of TCHQ and TCBQ is predominantly due to an increase in B cells, since relative B cell numbers

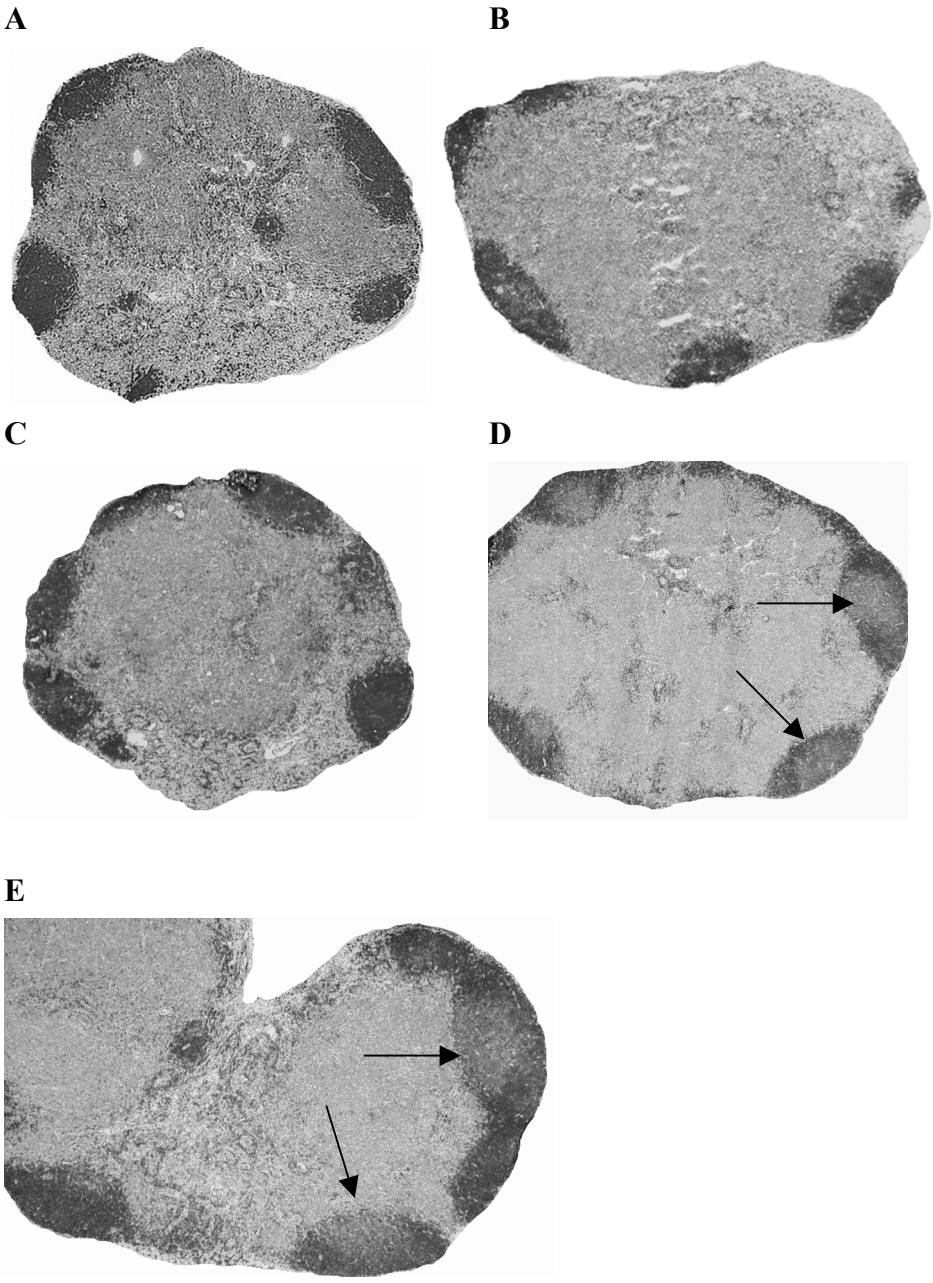

Figure 3: B220 (B cell monoclonal) stained sections of PLN isolated on day 7 from mice injected with 50 µl vehicle (A), 1 mg HCB (B), 1 mg silica (C), 1 mg TCHQ (D) and 1 mg TCBQ (E). TCHQ and TCBQ show germinal centers (arrows).

Table 1*:* Absolute and relative lymphocyte subsets per PLN[1]

	B cells	**Th cells**	**Tc cells**
Control	30 ± 2.3	76 ± 5.8	26 ± 2.2
	(24 ± 2.4)	(59 ± 1.5)	(20 ± 0.5)
Silica	117 ± 19	183 ± 42**	60 ± 11**
	(34 ± 2.2)	(52 ± 2.4)	(18 ± 0.8)
HCB	62 ± 7.5	129 ± 15	50 ± 6.5*
	(26 ± 2.2)	(54 ± 2.0)	(20 ± 1.5)
TCHQ	243 ± 40***	256 ± 20***	90 ± 6.8***
	(39 ± 3.4*)	(45 ± 3.9**)	(16 ± 3.4)
TCBQ	259 ± 61***	229 ± 45***	77 ± 14***
	(45 ± 2.9**)	(42 ± 2.2***)	(15 ± 1.2*)

[1]Absolute (x 10^5) and relative (%; between brackets) numbers of Th cells (CD4+), Tc cells (CD8+), and B cells (CD19+) cells at day 7 after injection of the different chemicals and TNP-Ficoll as determined by flow cytometry. Data are expressed as mean ± SE (4-6 mice per group). Cells were triple stained for combinations of CD4, CD8 and CD19. Significantly different from the control group *p<0.05; **p<0.01, ***p<0.001 determined by ANOVA followed by Bonferroni's post-hoc test.

were significantly elevated from 24% in controls to 39% and 45% for TCHQ and TCBQ, respectively, whereas the relative numbers of T cells were decreased.

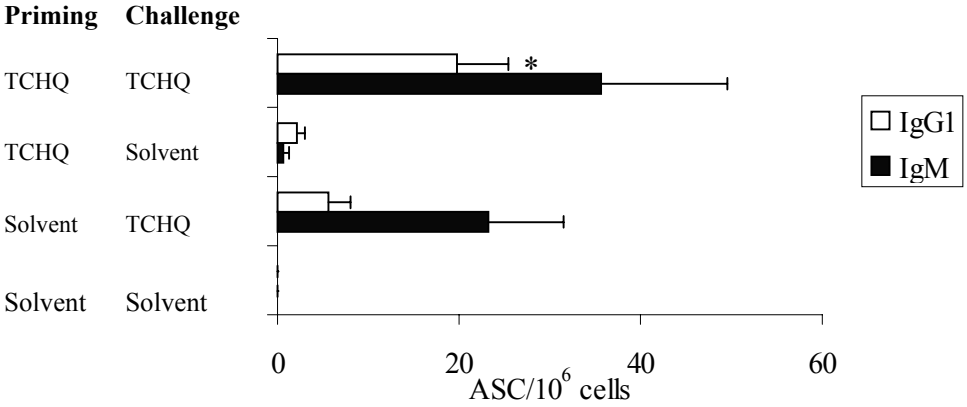

Figure 4: Number of TNP-specific ASC per 10^6 cells. Mice were primed with a sc injection of 1 mg TCHQ or the solvent. After 4 weeks mice were challenged by a sc injection in the same footpad with 20 µg TCHQ or the solvent together with 10 µg TNP-Ficoll. Five days later ASC were assessed. Bars represent mean ± SE of 4 mice. Significantly different from the control group (priming and challenge with solvent) *p<0.05; **p<0.01.

Secondary RA-PLNA with TCHQ

Mice were primed with a sc injection of 1 mg TCHQ or treated with the vehicle. After 4 weeks mice were challenged in the same footpad with 20 µg TCHQ together with 10 µg TNP-Ficoll. Appropriate control groups receiving TCHQ only at priming or only as challenge and a control group only treated with solvent were included. Cell number of all TCHQ-treated groups was significantly increased compared to the mice that were injected with solvent during priming and challenge and there was no significant difference between the groups (data not shown).

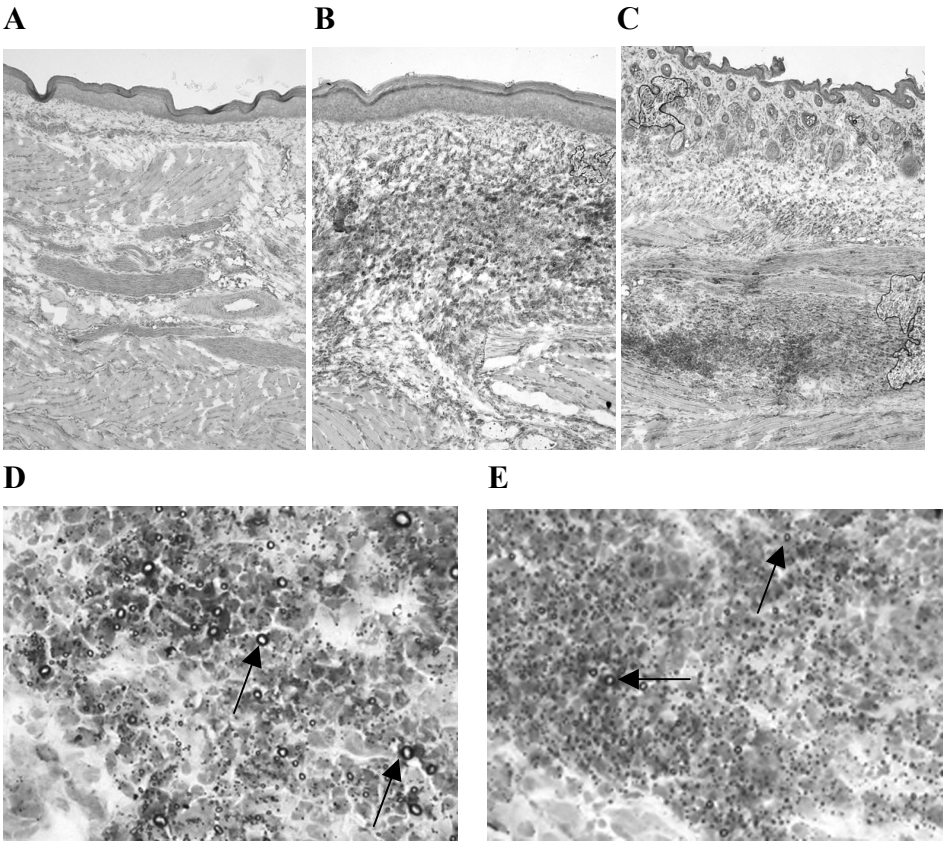

Figure 5: Sections of paws injected with vehicle (A), 1 mg HCB (B), or 1 mg silica (C) stained for acid phosphatase, staining macrophages (in black). With a higher magnification large particles (arrows) can be seen in HCB treated animals (D) and a large number of smaller particles (arrows) in the silica treated mice (E). Both HCB and silica attract macrophages to the paw (B-E).

ASC against TNP-Ficoll were measured in the secondary response (Figure 4). Mice treated with vehicle at priming and challenge had no ASC. Also, mice that were only primed with TCHQ had hardly detectable IgM and IgG1 ASC. Challenging naive mice with 20 µg TCHQ resulted in an increase of predominantly IgM ASC to 23 ± 8 per 10^6 cells (not significant) and no increase in IgG1 ASC. A recall with TCHQ in primed mice resulted in an increase of IgM ASC to 36 ± 14 per 10^6 cells (not significant) and a significant enhancement of IgG1 ASC to 20 ± 6 per 10^6 cells.

Deposit formation and clearance

Because we did not measure an elevation in cell number 7 days after HCB injection, a kinetic study was performed. Deposits were visible after injection of both silica and HCB. HCB deposits were macroscopically completely cleared after 21 days whereas silica deposits were still visible at that time point. Figure 5 shows histology of the paw at day 7 after injection of HCB and silica showing clearly the presence of HCB and silica particles. Staining the paw sections with acid phosphatase revealed an infiltration of macrophages, surrounding the silica and HCB particles. Figure 6 shows the primary PLN response 21 days after injection of 1 mg of HCB or silica. Control animals were injected with the vehicle only. Silica induced a chronic PLN response that was even higher 21 days after injection than after 7 days (Figure 1). The slight increase in PLN cell number 7 days after HCB injection (Figure 1) had disappeared after three weeks.

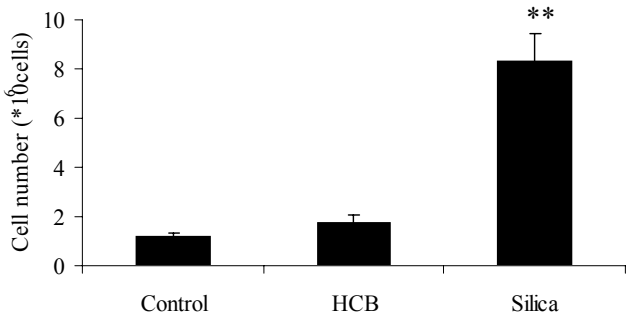

Figure 6: Cell number of PLN 21 days after injection of 1 mg HCB or 1 mg silica. Bars represent mean ± SE of 3 mice per group. ** Significantly different from control group with $p<0.01$. All data were determined by ANOVA followed by Bonferroni's post hoc test.

Discussion

The aim of the present study was to assess whether metabolites of HCB can activate T cells. To this end, we used the RA-PLNA with TNP-Ficoll as a RA and the secondary RA-PLNA to measure a memory response against TCHQ. Silica was included in the primary RA-PLNA as an inert model compound.

We show that silica as well as TCHQ and TCBQ caused a significant increase in PLN cellularity whereas HCB caused only a slight but not significant increase. Both metabolites induced the formation of germinal centers and also an IgG1 response to TNP-Ficoll. A switch to IgG1 in the RA-PLNA with TNP-Ficoll is indicatory for neoantigen specific T cell help, since TNP-Ficoll as a sugar moiety cannot be recognized by T cells *(1)*. So, accordingly, both TCHQ and TCBQ are likely to form neoantigens and stimulate T cells. Silica and HCB were unable to do so and only silica treatment caused an enhancement of the IgM response to TNP-Ficoll. To confirm that the results of the primary response were compound specific, we primed mice with TCHQ and challenged them 4 weeks later with a suboptimal dose of TCHQ together with TNP-Ficoll. A significant increase in TNP-specific IgG1 ASC was only found in mice that were primed and challenged with TCHQ.

Together, we show here that TCHQ and TCBQ, but not HCB itself, are capable of inducing neoantigen specific T cell help and that TCHQ can induce a compound specific memory response. TCBQ is a quinone that can react with macromolecules to form adducts. Importantly, studies on the microsomal oxidation of HCB showed that the major metabolites formed were PCP and TCHQ and revealed covalent binding to protein and DNA *(34)*. Since ascorbic acid prevented covalent binding, it was assumed that TCHQ needs to be oxidized in its semiquinone form or in TCBQ and that the latter two are the ultimate covalent binding compounds *(32)*. TCHQ readily autoxidizes at physiological pH into its semiquinone form and further into TCBQ *(22)* implicating that the ultimate haptenating metabolites can be formed spontaneously in the paw.

By using a secondary RA-PLNA, we show that injection of a suboptimal dose (20 μg) of TCHQ in primed mice resulted in a significant increase of TNP-specific IgG1 ASC. Remarkably, all treatment groups showed an increase in cell number (data not shown). In preliminary experiments, a suboptimal dose was determined as the dose that does not induce a PLN reaction. However, in the memory study mice were injected twice in the same paw, which could lead to irritation and subsequently to an increase in PLN cell number, but not to isotype switching. Nevertheless, in primed mice challenged with the vehicle, no ASC cells were detected, but naive mice challenged with TCHQ showed

only increased IgM but no IgG1 ASC. So, besides eliciting an isotype switch to TNP-Ficoll in the primary RA-PLNA, TCHQ was also able to induce a secondary PLN response. This response follows the pattern of a memory response since it is induced by a much lower dose and in a shorter time period than the primary response. Previously *p*-benzoquinone, a metabolite of benzene, has also been shown to elicit a secondary response in the PLNA *(8)*.

Both HCB and silica were not able to elicit an isotype switch in the RA-PLNA with TNP-Ficoll, as was expected based on their chemical structure. After injection of both chemicals deposits were visible in the footpad, but HCB deposits disappeared completely after 21 days, whereas silica deposits were still present at that time point. The PLN response to silica increased in time, as has already been shown before *(39)*, but HCB was not able to induce an increase in PLN cellularity at any of the assessed time points. Present data showing that HCB induced only moderate PLN responses seem to contradict with our previous work *(28)* in which we showed that different doses of HCB caused a clear and significant elevation of PLN cellularity in the primary PLNA in Balb/c mice. We performed a series of additional unpublished studies to compare different sources of HCB (Fluka vs. BDH derived) as they had a different crystalline appearance. The only difference that seems to remain is the source of BALB/c mice as the previous experiments were done with mice bred in former in-house facilities, whereas the present BALB/c mice were from Harlan.

Data shown here on silica, however, were similar to those observed previously *(1)*. Silica is known to induce a nonspecific immunostimulation, because the undegradable particles cannot be cleared by macrophages. This causes a continuously activation of macrophages which leads to the production of pro-inflammatory cytokines as TNF-α and IL-1β *(7)* and the formation of reactive oxygen species *(11)* leading to immunostimulation and secondary cell damage. Injection of silica into the footpad induced an even higher response 21 days after injection than after 7 days, and previous work revealed that this reaction could last even up to 180 days *(39)*. Apparently, our data show a clear difference between the effects of silica and HCB in the PLNA although they are both insoluble and injected as suspension. Silica and HCB particles are visible as particles and attract macrophages into the paw. The slight elevation in PLN cell number after injection of HCB is probably caused by the presence of immunostimulating macrophages in the paw. The reason for the difference in magnitude of the PLN response may be that in contrast to silica HCB can be processed by macrophages, since HCB deposits eventually disappear. It is known that in the setting of the PLNA, metabolism in the paw is not sufficient to convert chemicals that need biotransformation to form reactive intermediates *(13,20)*.

Upon oral treatment of BN rats as the most susceptible strain of rats for HCB induced immunopathology HCB will probably reach the organs (spleen, skin, lungs) where extrahepatic metabolic conversion can take place and reactive intermediates, like TCBQ can be formed.

Both TCBQ and *p*-benzoquinone *(8)* display typical characteristics of haptens, namely, a low molecular weight and the ability to react with protein. Benzene and HCB can be considered as prohaptens. As already noted, P450 metabolism is not involved in HCB-induced immunopathology *(26)*. Possibly, HCB is metabolized extrahepatically by peroxidases in leukocytes, including granulocytes. Whether peroxidase-rich cell types are capable of converting HCB is as of yet unknown. PCP, however, can be converted by a H_2O_2-dependent horseradish peroxidase-catalyzed oxidation yielding TCBQ as a major product *(25)*. Besides the reported genotoxic activity, that is caused by its reactive metabolite TCBQ *(6,12,40)*, PCP induced immunological aberrations in man and rats. Human exposure to PCP induced T cell activation and autoimmunity, immunosuppression and B cell dysregulation *(17)*. A study in rats showed suppressed antibody responses and enhanced lymphocyte blastogenesis after PCP exposure *(2)*. *In vivo* administration of PCP to rats showed the ability of TCBQ to bind covalently to albumin and hemoglobin and the formation of adducts *(38)* supporting the protein reactivity of this metabolite *in vivo*. So, PCP induced adverse immune effects as well but those are not comparable to the HCB induced immunopathology. Notably, distribution patterns and excretion of HCB and PCP after oral exposure differ, since HCB is a more lipophilic and persistent chemical than PCP and more likely to accumulate in the body. The same might be true for another chemical, pentachlorobenzene (PCB) that has the same oxidative metabolites as HCB, but a shorter half-life. Feeding studies in rats showed that PCB, besides a slight elevation in IgA, does not cause the same immunopathology as HCB *(26)*. To our knowledge, PCB does not induce infiltration of inflammatory cells after oral exposure to rats. Since inflammatory cells like macrophages and granulocytes probably play an important role in HCB-induced immunopathology, either by inducing inflammation or by extrahepatic formation of reactive metabolites, it is quite logical that PCB does not induce the same immunopathology. Another important difference between the metabolic pathways of PCB and HCB is the primary oxidation step. Conversion of PCB into PCP is a simple hydroxylation where no reactive intermediate is formed. The first step in the HCB oxidation is an oxidative dehalogenation that generates a highly reactive intermediate ("benzohaloquinone") *(24)*. This intermediate might also contribute to the observed immunopathology. Difference in kinetics as well as intermediates formed could explain the discrepancy between these compounds that generate the same metabolites, but do not

induce the same immunopathology. Since oral exposure of rats to HCB resulted in infiltrations of peroxidase-rich cell types like granulocytes in spleen, skin and lungs *(19)* these cells may be involved in the conversion of HCB into its haptenating metabolites. Human data from Turkey revealed infiltrations of lymphocytes and macrophages in the skin of the breast-fed infants born to mothers exposed to HCB *(5)*.

So, present data seem to support our hypothesis that both T cells as well as macrophages and granulocytes play an important role in HCB-induced immunopathology. We are currently investigating whether TCBQ-specific T cells are present in BN rats exposed to HCB and if HCB can be metabolized extrahepatically into reactive metabolites by myeloperoxidase or phagocytes. In that case, HCB can be considered as a prohapten that needs to be metabolized first to create haptens which could attribute to the observed immunopathology.

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Chapter 3

Toxicogenomics of subchronic hexachlorobenzene exposure in Brown Norway rats

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Abstract

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 by 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 as 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 the increased transcript levels of markers for granulocytes and macrophages. New findings include the upregulation of genes encoding pro-inflammatory cytokines, antioxidants, acute phase proteins, mast cell markers, complement, 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.

Introduction

Hexachlorobenzene (HCB) was used as a fungicide until the 1970s, when such use was prohibited. Considerable amounts are still generated as waste-byproducts 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 3000-5000 people ingested HCB-treated seed grain and developed a disease called Porphyria Turcica *(14)*, characterized by hepatic porphyria and cutaneous skin lesions caused by a disturbed porphyrin metabolism *(2)*. Other clinical symptoms included enlarged liver, spleen, lymph nodes (LN) 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 *(5)*. 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 *(34,35)*.

In rats, HCB induces hepatic porphyria, neurotoxic effects *(8)* and toxic effects on the reproductive system *(19)*, thyroid function *(22)* and immune system *(30,46)*. 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 *(45)*, but also via the mercapturic acid pathway *(36)*.

Brown Norway (BN) rats are very susceptible to HCB-induced adverse immune effects. Exposure causes 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 *(30,31)*. Although both T cells and macrophages seem to play an important role in HCB-induced immunotoxicity in BN rats *(11)*, 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 by 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 *(11,31)*, and gene expression levels were assessed in spleen, mesenteric lymph node (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) rats were purchased from Harlan (Blackthorn, UK). Rats were acclimatized for 1 week before starting 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 per kg. Body weight (bwt) 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 regimes. Rats were killed by a lethal dose of pentobarbital (Euthesate® Ceva Sante Animale B.V. Maassluis, The Netherlands, 0.3 g/kg bwt, i.p.). 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 and 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; ref. *7*) and purified on an affinity resin (RNeasy, Qiagen, Hilden, Germany) according to manufacturer's instructions. DNA microarray experiments were conducted as recommended by the manufacturer of the GeneChip system (GeneChip Expression Analysis Technical Manual) and as previously described *(25)*. Rat specific RG U34A gene expression probe arrays (Affymetrix, Santa Clara, CA, USA) were used containing 8799 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). 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 diffentially 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 group, compared with the control group. The annotation of the genes was determined by using NetAffx (http://www.affymetrix.com; ref. *24*). Further information on probe sets was found in 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 and 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 HCB sample

To analyze HCB for contaminating polychlorinated dibenzodioxins and polychlorinated dibenzofurans (PCDDs/PCDFs) a solution of acetone containing 13C12-labeled internal quantitation standards (Cambridge Isotope Laboratories, Woburn, MA, USA) of the PCDDs and PCDFs was added to dichloromethane. The solution was brought on a Carbosphere column, then purified on Al_2O_3, evaporated to dryness and redissolved in toluene. 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; J&W Scientific, Folsom, CA, USA; 0.25 mm ID, 0.10 µm film thickness). Ionisation 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 (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 *(31)*. 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 *(31)*. In the high dose group kidney weight was increased significantly as

were observed before in Wistar rats treated with HCB for 25 days *(21)*, but not in BN rats treated with HCB for 21 days *(28)*. 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 *(23)* 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 *(31)*.

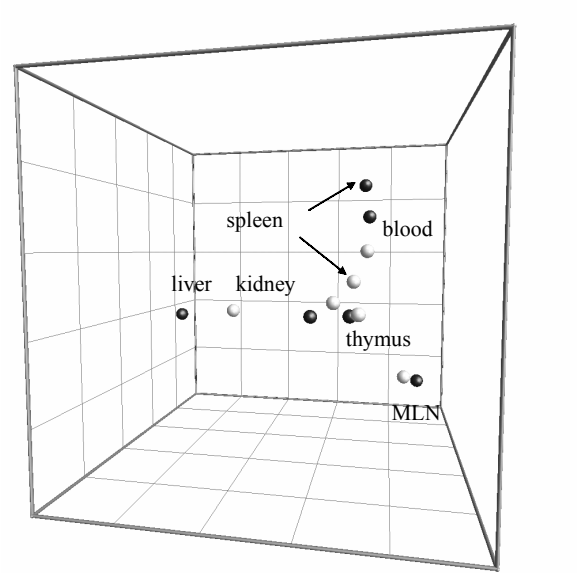

Figure 1: Principal component analysis (PCA) plot of the ratios of low dose versus control (white dots) or high dose versus control (black dots).

DNA microarray analysis

The PCA plot (Figure 1) of the ratios of the low and high dose group 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 paper, 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 discuss the results in a logical order. The complete list of significantly changed probe sets can be found on http://www.ebi.ac.uk/arrayexpress.

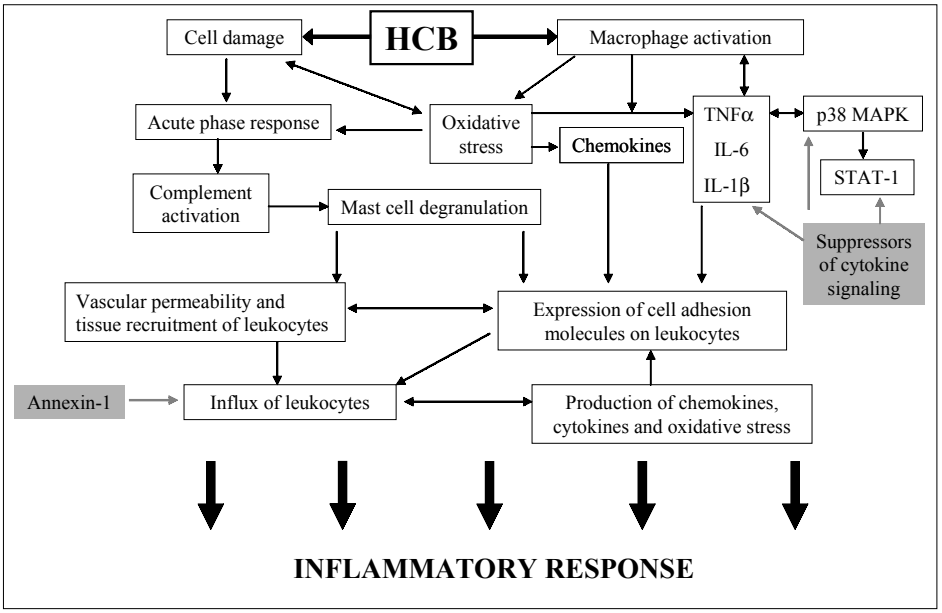

Figure 2: Hypothetical overview of cells and factors involved in the inflammatory response initiated by HCB. Assuming that HCB attracts and activates macrophages this would lead to a cascade of reactions, activating immune cells and pro- and anti-inflammatory (in grey) mediators, eventually leading to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

Table 1: Spleen: representative genes that changed significantly ($p<0.001$) after HCB treatment: immune system[1]

Accession number	Gene name	Fold change low dose	Fold change 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 (CD23)	-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 A3 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 genes to complement-32	-1.3	20
AA818025	CD59 antigen	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	Suppressor of cytokine signaling-2	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	mitogen activated protein kinase kinase kinase 1	1.0	-1.8
U17035	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	Anti-acetylcholine receptor antibody rearranged immunoglobulin gamma-2a chain, VDJC region	1.6	3.2
L07398	Immunoglobulin rearranged gamma-chain V region	1.0	2.4
M18526	Immunoglobulin germlinę 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

[1]Table contains the 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 vs control. Fold changes are calculated with data from 5-6 rats per group. One-way ANOVA was used to determine significance; only probe sets that changed significantly with $p<0.001$ are shown.

Inflammatory response

Macrophages

In HCB-exposed rats macrophage infiltrations were observed in skin, lung *(31)*, spleen *(11,31)* and liver *(8)*. 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.

Pro-inflammatory cytokines

Gene expression of the receptor for tumor necrosis factor (TNF)-α and TNF-β (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 *(32)*. 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 was elevated in liver, a cytokine produced mainly by Kupffer cells.

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 (SOCS). 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 *(6)*.

Oxidative stress and antioxidants

Previous studies have shown that HCB exposure induced oxidative stress *(4)* and increased expression of antioxidants in the liver *(41)*. 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 (not shown). Excessive presence of ROS can activate NFκB, an important factor in regulating the inflammatory response *(39)*. 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.

Table 2: MLN: representative genes that changed significantly ($p<0.001$) after HCB treatment: immune system[2]

Accession number	Gene name	Fold change low dose	Fold change 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 γ 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 molecules			
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-3	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

[2]Table contains the 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 vs control. Fold changes are calculated with data from 5-6 rats per group. One-way ANOVA was used to determine significance; only probe sets that changed significantly with $p<0.001$ are shown.

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 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 APPs, however, is also dependent on iron metabolism. HCB induces iron accumulation in the liver *(41)*. The upregulation of transferrin gene expression in spleen and kidney suggests that this is also the case in these organs.

Table 3*:* Thymus: representative genes that changed significantly ($p<0.001$) after HCB treatment: immune system[3]

Accession number	Gene name	Fold change low dose	Fold change high dose
Granulocytes and macrophages			
L18948	S100 calcium binding protein A9	1.1	2.0
X14323	IgG receptor FcRn	1.2	1.2
Mast cells			
U67911	Mast cell protease 8 precursor	1.5	2.0
Chemokines and cytokines			
AF075383	Cytokine inducible SH2-containing protein	1.2	1.7
Genes associated with B cells			
L22654	Anti-acetylcholine receptor antibody rearranged Ig gamma-2a chain, VDJC region	1.6	3.7
M18526	Ig germline kappa-chain	2.0	3.2

[3]Table contains the 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 vs control. Fold changes are calculated with data from 5-6 rats per group. One-way ANOVA was used to determine significance; only probe sets that changed significantly with $p<0.001$ are shown.

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 *(31)*. 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 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 that is 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 *(27)*.

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 LN *(31)*, 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 *(29)*. In all analyzed organs and blood gene expressions for S100 calcium binding protein A8 and A9 were upregulated. These proteins are abundantly present in the cytoplasm of neutrophils, monocytes and macrophages *(37)*. Other markers associated with granulocytes and macrophages that were affected by HCB were defensin (neutrophils and macrophages), lipocalin (granulocytes) and CD24 (granulocytes, monocytes and

Table 4: Blood: representative genes that changed significantly ($p<0.001$) after HCB treatment: immune system[4]

Accession number	Gene name	Fold change low dose	Fold change 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			
Y67913	Mast cell protease 10	17	16
U67911	Mast cell protease 8 precursor	3.9	4.6
X61654	AD1 (mast cell antigen; CD63)	1.7	2.0
Pattern recognition molecule			
AA875213	Peptidoglycan recognition protein	4.3	7.7
Complement			
AA818025	CD59 protein precursor	1.6	2.6
Cell adhesion molecules			
AF074211	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) (Lipocortin 1)	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	MHCII associated invariant chain gamma	-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

[4]Table contains the 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 vs control. Fold changes are calculated with data from 5-6 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[5]

Accession number	Gene name	Fold change low dose	Fold change high dose
Granulocytes and macrophages			
AA946503	Lipocalin 2	4.3	210
L18948	S100 calcium binding protein A9	3.4	28
AA957003	S100 calcium binding protein A8	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 cells			
M18335	Mast cell protease 8 precursor	2.2	2.8
Complement			
Z50051	Complement component 4 binding protein, alpha	1.3	2.3
Cell adhesion molecules			
D00913	Intercellular adhesion molecule 1	1.2	2.3
Chemokines			
D11445	Gro	1.6	11.5
Cytokines			
AA892553	Signal transducer and activator of transcription 1	1.1	3.3
U77777	Interleukin 18	1.3	1.9
L25785	Transforming growth factor beta stimulated clone	-1.5	-1.5
Genes associated with T and B cells and MHCII expression			
L22654	Anti-acetylcholine receptor antibody, rearranged immunoglobulin gamma-2a chain, VDJC region	-1.0	8.8
U39609	Anti-nerve growth factor-30 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

[5]Table contains the GenBank accession numbers 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 vs control. Fold changes are calculated with data from 5-6 rats per group. One-way ANOVA was used to determine significance; only probe sets that changed significantly with $p<0.001$ are shown.

lymphocytes). HCB also induced gene expression of 12-lipoxygenase and arachidonate 5-lipoxygenase activating protein, both involved in leukotriene activation, which takes place in myeloid cells *(3)*. 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 *(33)*.

T and B cells and MHCII 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 *(40,48)*. In kidney, we observed an increased expression of OX-45, 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 *(31)*. MHCII 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 *(9)*. HCB-induced neurological effects, however, are not the same as symptoms described for MG. Additional experiments performed to detect anti-acetylcholine 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

Table 6: Kidney: representative genes that changed significantly ($p<0.001$) after HCB treatment: immune system[6]

Accession number	Gene name	Fold change low dose	Fold change 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 molecules			
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
AI17646	Selectin, endothelial cell, ligand	1.3	-1.5
Chemokines			
U17035	Chemokine (CXC motif) ligand 10	1.2	2.0
Cytokines and cytokine-associated genes			
M63122	Tumor necrosis factor receptor	1.1	1.9
U48596	Mitogen-activated protein kinase kinase kinase 1	1.2	1.9
M92340	Interleukin 6 signal transducer	1.0	1.5
S79676	Interleukin 1beta converting enzyme	-1.2	1.4
U73142	p38 mitogen activated protein kinase	-1.1	1.3
Genes associated with T and B cells and MHCII expression			
L22654	Anti-acetylcholine receptor antibody, rearranged Ig gamma-2a chain, VDJC region	2.6	5.3
AJ223184	DORA protein (immunoglobulin superfamily member 6)	-1.4	2.6
U75411	Anti-idiotype immunoglobulin 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

[6]Table contains the 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 vs control. Fold changes are calculated with data from 5-6 rats per group. One-way ANOVA was used to determine significance; only probe sets that changed significantly with $p<0.001$ are shown.

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

Accession number	Gene name	Fold change low dose	Fold change 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 heat shock protein 84	-1.1	-1.4
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 1	-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

[5]Table contains the GenBank accession numbers 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 vs control. Fold changes are calculated with data from 5-6 rats per group. One-way ANOVA was used to determine significance; only probe sets that changed significantly with $p<0.001$ are shown.

autoantibodies and are elevated in inflammatory diseases *(10)*. The exact role of these autoantibodies is not yet known. Previously it was shown that HCB increased IgM antibodies against autoantigens, such as ssDNA *(31,38)*. 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 found in several autoimmune diseases *(18)*.

Drug metabolizing enzymes

Cytochrome P450

CYP enzymes are involved in the oxidative dehalogenation of HCB *(45)* 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 *(13)*. 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 *(43)* 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 *(47)* 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 *(15)*. 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 to 4 ng TCDD/kg bwt/day *(44)*. In our study rats were exposed to approximately 2 ng/kg bwt/day (low dose) and 6 ng/kg bwt/day (high dose). So, exposure to dioxins and furans is of the same order of magnitude than 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 *(13,26)*. This discrepancy may be explained by strain differences or by the difference in detection of CYP1A1 (7-ethoxyresorufin-*O*-deethylase induction versus gene expression).

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[8]

Accession number	Gene name	Fold change low dose	Fold change high dose
SPLEEN			
AA800745	Aminolevulinate, delta-, dehydratase	-1.4	10.7
X06827	Porphobilinogen deaminase	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-P450 oxidoreductase	-1.0	-1.3
X04229	Glutathione-S-transferase, Y(b) subunit	-1.1	-1.5
D00680	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-β-hydroxylase (CYP3A1)	5.9	6.9
J02722	Heme oxygenase	1.8	5.2
E01184	P450 MC substituted the C terminal region containing HR2 region for the same region of cytochrome P450d	3.0	5.1
D86297	Aminolevulinate synthase 2, delta	2.1	4.4
S82820	Glutathione-S-transferase, Yc2 subunit	3.5	3.4
M26125	Epoxide hydrolase 1	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 (P4501b1)	1.1	2.9
M37828	Cytochrome P450 (P4504a10)	1.2	2.7
L19998	Minoxidil sulfotransferase	1.1	2.3
M20131	Cytochrome P450IIE1	-1.4	-1.9

[8]Table contains the 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 vs control. Fold changes are calculated with data from 5-6 rats per group. One-way ANOVA was used to determine significance; only probe sets that changed significantly with $p<0.001$ are shown.

Mercapturic acid pathway

The BN rat degrades HCB also via the mercapturic acid pathway that involves glutathione-conjugation catalyzed by glutathione-S-transferase (GST) *(36)*. As expected, gene expression of several GSTs was upregulated in liver. Other phase II enzymes that were induced were mercaptopyruvate sulfurtransferase, uridine diphophate (UDP)-glucuronosyltransferase, and the sulfotransferase family.

Porphyria

One of the main toxic characteristics of HCB is the induction of porphyria in humans *(14)* and experimental animals *(8)*, caused by a disturbance in heme biosynthesis. In the present study gene expression of enzymes involved in heme synthesis were induced, such as aminolevulinate (ALA) dehydratase, porphobilinogen deaminase 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 *(19)* and the proportion of male births was reduced in the group of women that had HCB-induced porphyria *(20)*. In monkeys HCB decreased estrogen levels *(12)* and in Wistar rats HCB exposure reduced serum levels of estrogen and decreased levels of uterine estrogen receptors *(1)*. 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 17–β 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. 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 pro-inflammatory 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 like 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 costimulatory or danger signals are generated that can 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 *(11,30)*.

Data obtained in an extensive study like this can be used to create a database with gene expression profiles of known toxicants, as has been suggested previously *(42)*. 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 *(16,17)* and genomics may be an additional tool in hazard identification.

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Chapter 4

Hexachlorobenzene-induced immunopathology in Brown Norway rats is partly mediated by T cells

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Abstract

Hexachlorobenzene (HCB) is a persistent environmental pollutant with (auto)immune effects in man and rat. The Brown Norway (BN) rat is very susceptible to HCB-induced immunopathology, and oral exposure causes inflammatory skin and lung lesions, splenomegaly, lymph node (LN) enlargement and increased serum levels of IgE and anti-ssDNA IgM. The role of T cells in HCB-induced immunopathology is unclear and to elucidate this Cyclosporin A (CsA) was used. BN rats were exposed to either a control diet or a diet supplemented with 450 mg/kg HCB for 21 days. CsA treatment started 2 days prior to HCB exposure and rats were injected daily with 20 mg/kg body weight CsA. Treatment with CsA prevented the HCB-induced immunopathology significantly. The onset of skin lesions was delayed and the severity was also strongly decreased. Furthermore, CsA prevented the HCB-induced increase in spleen weight partly and the increase in auricular LN weight completely. The increase in serum IgE and IgM against ssDNA levels was prevented completely. Macrophage infiltrations into the spleen and lung still occurred but infiltrations of eosinophilic granulocytes into the lung were prevented. Restimulation of spleen cells with the T cell mitogen ConA and the macrophage activator LPS clearly showed that CsA inhibited T cell activation, but not macrophage activation. Together, our results show that both T cells and macrophages play a prominent role in HCB-induced immunopathology.

Introduction

Hexachlorobenzene (HCB) is an environmental pollutant with pronounced adverse effects on the immune system of humans and rats *(14)*. An accidental poisoning in Turkey in the 1950's revealed several immunopathological effects of HCB in humans, like splenomegaly, enlarged lymph nodes (LN) and painless arthritis *(1,18)*. Breast-fed infants born to mothers exposed to HCB developed inflammatory lung and skin lesions, the latter characterized by infiltrations of lymphocytes and macrophages *(2)*. Data of humans exposed occupationally to HCB indicated that HCB induced significant elevated levels of IgM and IgG, as well as reduced neutrophil functions *(19,20)*.

HCB induced comparable immunostimulatory effects in rats *(24,28)*. The BN rat is a very susceptible strain and oral exposure to HCB caused splenomegaly, enlarged LN, inflammatory skin lesions characterized by an infiltration of eosinophilic granulocytes, inflammatory lung lesions characterized by macrophage infiltration, granuloma formation

and perivascular eosinophilic infiltrates and increased levels of total serum IgM, IgG, IgE and IgM against ssDNA *(15)*.

The mechanism of HCB-induced immunopathology in humans and rats is poorly understood. The involvement of T cells is still controversial and if T cells are involved it is not known how they become activated. Studies in Wistar rats have shown that HCB treatment enhanced thymus-dependent parameters like primary and secondary IgM and IgG against tetanus toxoid and mitogenic responses to T cell mitogens PHA and ConA *(29)*. To investigate the role of thymus-dependent T cells Michielsen *et al. (13)* exposed BN rats depleted of T cells by adult thymectomy, lethal irradiation, and bone marrow reconstitution to HCB and found that skin lesions appeared later in HCB-exposed T cell-depleted-rats than in T cell-competent BN rats. The development of spleen and lung effects by HCB was not influenced by this T cell depletion, so it seemed that HCB-induced inflammatory effects were not thymus-dependent, except for the skin lesions. A drawback of this study was that there were still functional T cells present in the spleen as demonstrated by flow cytometry. In thymectomized rats 4 % T cells were present in the spleen as compared to 15 % in control rats. Since Sado *et al. (21)* have shown that T cells present in the spleen of thymectomized mice were able to proliferate after stimulation with ConA, these residual T cells may be responsible for the observed immune effects elicited by HCB in these thymectomized rats.

Since our previous work did not elucidate the precise role of T cells in HCB-induced immunopathology, we decided to investigate this more thoroughly by using Cyclosporin A (CsA) as CsA not only decreases peripheral T cell number but also inhibits antigen-induced T cell activation *(9,11)*.

Materials and methods

Rats and maintenance

Three-week old specific pathogen-free female inbred Brown Norway (BN/SsNOlaHsD, termed BN) rats were purchased from Harlan (Blackthorn, UK). Rats were allowed to acclimatize one week before starting the experiment. All rats were housed at the animal facilities of the Utrecht University. They were kept two by two in filter-topped macrolon cages on bedding of chips and wood, under standard conditions (50-60% relative humidity, 12-hr dark/12-hr light cycle) with food and acidified drinking water *ad libitum*. The diet consisted of a semisynthetic diet (SSP/TOX, Hope Farms, Woerden, The Netherlands) either or not supplemented with crystalline HCB (99% purity, Aldrich Chemie, Bornem, Belgium) by mixing of homogeneity. The experiments were conducted

according to the guidelines of the animal experiments committee of the Faculty of Veterinary Medicine of the Utrecht University.

Experimental protocol

Rats were randomly assigned to the different experimental groups, either receiving control diet or the diet supplemented with 450 mg HCB per kg. One of the control groups and one of the HCB-fed groups received a daily injection of CsA (a gift from Novartis Pharma Inc, Basel, Switzerland). CsA was dissolved in olive oil in a concentration of 20 mg/ml. Rats treated with CsA received a daily injection of 20 mg/kg/day (=1 ml/kg) via a sc injection in the flank, untreated rats received a daily injection of 1 ml olive oil per kg also in the flank. CsA treatment started 2 days prior to the beginning of HCB exposure. Rats were weighed three times per week and the development of skin lesions was evaluated daily. The severity of the lesional sites was rated as described before *(15)*, irrespective of size, as 1 = minimal (some redness), 2 = moderate (redness), 3 = marked (dry desquamation and crusts) and 4 = severe (exudative lesions). A semiquantitative group score of the macroscopic skin lesions on day 21 was calculated by multiplying the fraction of rats showing skin lesions, the average severity of the skin lesions and the relative lesional area.

After 21 days of exposure to HCB rats were killed by a lethal dose of sodium pentobarbital (Euthesate, 0.3 g/kg bodyweight, intraperitoneally). Blood was drawn from the vena cava and serum was collected after clotting and centrifugation. Liver, spleen, thymus (freed from adjacent lymph nodes), mesenteric LN (MLN) and auricular LN (ALN) were collected and weighed. LN and parts of the liver and spleen were fixed in phosphate-buffered 4% paraformaldehyde and lung was inflated and fixed with phosphate-buffered 4% paraformaldehyde to optimize morphology. Formaldehyde-fixed tissues were embedded in Paraplast and sections (5 μm) were stained with hematoxylin and eosin (HE). Part of the spleen was snap-frozen in liquid nitrogen and used for cryostat sections. To avoid bias at examination, sections were scored under code.

Single cell suspensions were prepared from part of spleen, part of thymus, ALN and MLN in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). LN and thymus were minced between two slides and spleens were cut and gently pressed through a 70 μm pore sieve to prepare single cell suspensions. Suspensions were washed, resuspended in PBS/1%BSA, counted using a Coulter Counter (Coulter Electronics, Luton, UK) and then adjusted to 1 or 2.5 x 10^6 cells per ml.

IgE ELISA

Total serum IgE levels were determined by an isotype-specific sandwich ELISA as described before *(15)*. Individual test serum samples were measured in duplicate. The concentration of IgE in test serum samples was calculated using a standard curve with known amounts of monoclonal rat IgE (Serotec, Oxford, UK) and results were expressed in total serum IgE concentrations (ng/ml).

ssDNA ELISA

ELISA procedures to determine IgM levels against ssDNA were performed essentially as described previously with some modifications *(24)*. In short, serial dilutions of the individual test sera were added in duplicate to wells coated with ssDNA (50 µg/ml; Sigma, St. Louis, MO, USA). Each plate contained serial dilutions of NRS to obtain a reference curve. After incubation alkaline phosphatase labeled goat-anti-rat IgM (µ) (Brunswich, Amsterdam, The Netherlands) was added to the wells. For the color reaction wells were incubated with a solution of 0.01% 4-nitrophenylphospate in diethanolaminobuffer (pH 9.8). The reaction was stopped by adding 50 µl 10% (w/v) EDTA in distilled water. Absorbance was measured at 405 nm. To calculate IgM serum levels against ssDNA calibration curves of the reference serum (NRS) were used. Therefore, absorbance values of serial dilutions of the reference serum were plotted against the 2log of the dilution. The dilution of the test serum that would result in the same absorbance as NRS was calculated by linear regression and was used to obtain the ELISA index, which is the ratio of the test dilution used and the calculated dilution of NRS at the same absorbance. Mean ELISA index of the control group was transformed to 1 and data of the treatment groups was expressed relative to the index of the control group.

Immunohistology

Cryostat sections (6 µm) of spleen were stained with ED3 (monoclonal antibody that recognizes a subpopulation of macrophages). ED3 was a kind gift from Dr. C. Dijkstra (Free University, Amsterdam, The Netherlands). Peroxidase–conjugated rabbit anti-mouse Ig (Dako AS, Glostrup, Denmark) was used as a second step agent. As a substrate 3-amino-9-ethylcarbazole (AEC; Janssen, Beerse, Belgium) was used. Slides were counterstained with hematoxylin.

Flow cytometry

All antibodies for flow cytometry were obtained from Pharmingen (San Diego, St. Louis, MO, USA). The following conjugated mAbs were used in duplo- or triplostaining to phenotype lymphocytes: fluorescein-isothiocyanate (FITC)-conjugated OX-33 (anti-rat CD45RA), phycoerythrin (PE)-conjugated R73 (anti-rat TCRαβ), OX39 (anti-rat CD25), OX-8 (anti-rat CD8α) and biotinylated OX-35 (anti-rat CD4). Single cell suspensions (2 x 10^5 cells/well) in PBS with 1% BSA, 0.1% NaN_3, 4% normal rat serum were centrifuged in a 96-well microtitre plate, resuspended and incubated with combinations of optimal dilutions of FITC, -PE- and biotin-conjugated mAbs (30 min, in the dark, 4°C). Cells were washed twice and the biotin-conjugated antibodies were stained in a second step with streptavidin-CyChrome (30 min, in the dark, 4°C). Samples were analyzed on a FACScan® flow cytometer with standard FACSflow using CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

Ex vivo restimulation of spleen cells

For cytokine measurements spleen cells were restimulated *ex vivo* with the mitogens LPS and ConA or with medium alone. LPS (2 µg/ml), ConA (5 µg/ml) and complete medium (RPMI 1640 with glutamax (Invitrogen Life Technologies, Paisley, Schotland) supplemented with 10% fetal bovine serum (FBS; ICN Biomedicals INC, Costa Mesa, CA), 2% penicillin-streptomycin (Invitrogen Lifetechnologies, Paisley, Schotland) were plated in a 96 well microtitre plate. Spleen cells in complete medium were added to the different stimuli (3.75 x 10^5 cells per well). Cells were incubated for 48 hours at 37°C, 5% CO_2. After incubation supernatants were collected and stored at -70°C until cytokine measurement.

IL-2 measurement

The IL-2-dependent cell line CTLL-2 was kindly provided by Dr. Janssen (Immunotherapy, Utrecht Medical Center, Utrecht, The Netherlands). CTLL-2 cells were grown in Iscove's medium supplemented with 10% FBS, 2% penicillin-streptomycin, L-glutamin, 1% mercapto-ethanol and 20 units/ml of IL-2. For the IL-2 bioassay cells were washed in medium without IL-2 and plated in 96 wells plates in a concentration of 5000 cells per well. Diluted samples and a standard curve were added to the wells and incubated for 24 hours at 37°C in 5% CO_2. Thereafter, cells were labeled for 18 h with 0.5 µCi/well [^{3}H]-thymidine. Plates were harvested with a cell harvester onto filter paper and dried. The filter paper was placed into a bag containing scintillation fluid and [^{3}H]-

Thymidine incorporation was measured with a beta-plate liquid scintillation counter. The standard curve was used to calculate IL-2 (in units/ml) in the individual samples.

TNF-α and nitric oxide measurement

The TNF-α-sensitive murine fibroblast cell line L929 was kindly provided by Dr. Wulferink (PharmaAware, Utrecht University, Utrecht, The Netherlands). L929 cells were seeded in 96-wells flat-bottom microtitre plates (4 x 10^4 cells per well) in complete RPMI and allowed to adhere o.n. at 37°C in a 5% CO_2 atmosphere. After removal of supernatant cells were pretreated with 2 μg/ml Actinomycin-D (Acros Organics, Geel, Belgium). Thereafter, diluted samples were added to the wells. A standard curve of recombinant rat TNF-α (Biosource, Camarillo, CA, USA) was included on each plate. Plates were incubated for 18 h at 37°C in a 5% CO_2 atmosphere. Cell viability was determined by measuring the decrease in mitochondrial activity using the MTT (3-4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma Chemical Co., St. Louis, MO) reduction assay. The standard curve was used to calculate TNF-α production (ng/ml). Nitric oxide (NO) production after LPS restimulation of splenocytes was measured by the Griess reaction that measures the accumulation of nitrite in the culture medium as described before *(8)*. A standard curve of sodium nitrite was used to calculate nitrite production (μM).

Statistical analysis

Significant differences of treatment groups with the control group or between the HCB treated groups with or without CsA treatment were determined by analysis of variance (ANOVA) with Bonferroni post hoc test for contrasts by using SPSS software. Values deviating more than 2 standard deviations from group means were considered as outliers and not included in statistical analyses. Preceding statistical analysis, all data were transformed to log 10 values to homogenize variance.

Results

Macroscopic skin lesions

As described previously *(15)*, oral treatment with HCB caused skin lesions in the head and neck region of BN rats. Table 1 shows that rats treated with HCB alone developed skin lesions after 9 or 10 days of treatment, which is according to previous reports. Skin lesions increased in size and severity resulting in hemorrhagic lesions often with

exudative crusts. CsA caused a delay of the development of skin lesions with 9-11 days as the first lesions were visible on day 19, 20 or 21. As can be seen in Table 1 all rats displayed skin lesions on day 21, but rats treated with CsA had less severe lesions and the affected area was much smaller.

Table 1: Effect of CsA on the onset, incidence, severity and lesional area of skin lesions induced by HCB[1]

			Day 21	
Group	Animal	**Onset**	**Severity**	**Relative lesional area**
HCB	1	day 10	4	2.8
	2	day 10	4	3.9
	3	day 9	4	2.9
	4	day 10	4	1.7
HCB + CsA	1	day 20	2	0.04
	2	day 19	2	0.31
	3	day 21	2	0.16
	4	day 20	2	0.12

[1] Table shows the onset of the skin lesions, e.g. the day the first lesions were visible, the severity (see Materials and Methods for ranking) on day 21 and the relative lesional area of all rats. This was calculated by dividing the lesional area with the total area.

Body weight gain and relative organ weights

Neither HCB nor CsA treatment influenced the gain in body weight during the course of the experiment (data not shown). As shown in Figure 1, relative spleen and ALN weights were significantly increased in both groups treated with HCB as compared to control rats. CsA treatment inhibited this increase in relative spleen weight moderately but spleen weights were still increased when compared to the control group. In contrast, CsA treatment prevented the HCB-induced increase in ALN weight completely. ALN weight of HCB-fed rats treated with CsA was not significantly different from control rats, but was significantly higher than control rats treated with CsA.

Total serum IgE levels and IgM levels against ssDNA

As has been described previously, HCB caused a significant increase in total serum IgE levels in BN rats *(15)*. Table 2 shows that treatment with CsA prevented this increase in IgE levels completely. The BN rat is a strain with high basal IgE levels and in control rats the total serum IgE levels were 195 ng/ml. After CsA treatment total serum IgE levels in both control rats and HCB-fed rats were low and in both groups only 1 out 4 rats had are

given in table 2. Serum levels of IgM against ssDNA are also shown in table 2. Oral exposure to HCB increased anti-ssDNA IgM levels with a factor of 1.7 and treatment with CsA suppressed this completely to control levels.

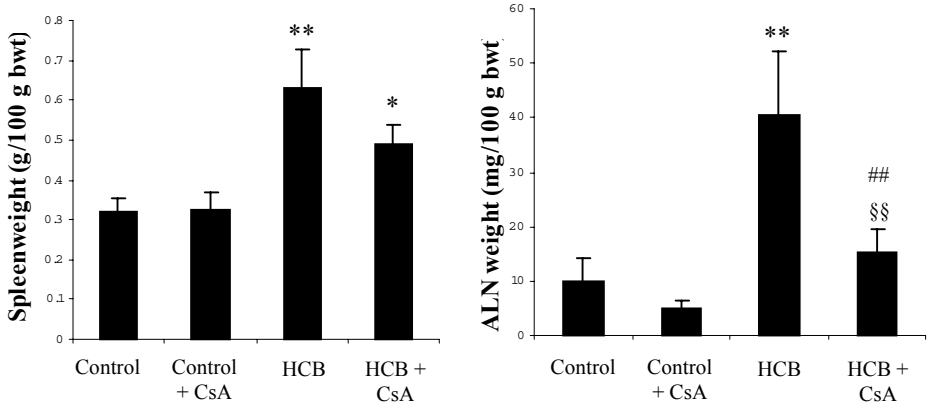

Figure 1: Relative spleen (A) and ALN (B) weights after 21 days of exposure of female BN rats to control diet or HCB diet with or without CsA treatment. Data represent mean ± SE of 4 animals per group. The asterisks above the bars indicate significance from control group (* $p<0.05$; ** $p<0.01$), significance from HCB treated group is depicted with $^{\#\#}$ $p<0.01$. §§ indicates significance from the control group treated with CsA (§§ $p<0.01$).

Histopathology

Lung HE stained sections of formalin-fixed lung show that the morphology of rats fed the control diet either treated with CsA or not appeared normal (Figure 2) except for some scattered microgranulomas consisting of eosinophilic granulocytes. The presence of these microgranulomas in the lung of untreated BN rats have been described before (16). Treatment with HCB caused an infiltration of eosinophilic granulocytes around small blood vessels, granuloma formation and also intra-alveolar accumulation of macrophages. These HCB-induced effects are according to those described before (15). Rats fed with HCB and treated with CsA still had focal accumulations of macrophages comparable to HCB treated rats, but the perivascular infiltrate of eosinophilic granulocytes did not occur.

Spleen The most prominent alterations induced by HCB in the spleen were comparable with effects observed previously in rats *(13,29)*. HCB induced hyperplasia of the B cell compartments (marginal zone (MZ) and follicles) and increased extramedullary hemopoiesis. Morphology of spleens of HCB-fed rats treated with CsA showed depleted T cell areas (PALS), while some but not all of the B cell compartments were hyperplastic.

This hyperplasia was less when compared to the hyperplasia observed in HCB-fed rats not treated with CsA. HCB-induced hemopoiesis was inhibited by CsA (data not shown). Staining of cryostat sections with the MAb ED3, which stains a subpopulation of macrophages, revealed an increase of the amount of ED3+ macrophages (Figure 3) leading to broadening of the rim these macrophages in the MZ (MZ) and scattered infiltrates of ED3+ in the white pulp, as described previously in Wistar rats *(24)*. This infiltration of ED3+ macrophages still occurred after CsA treatment.

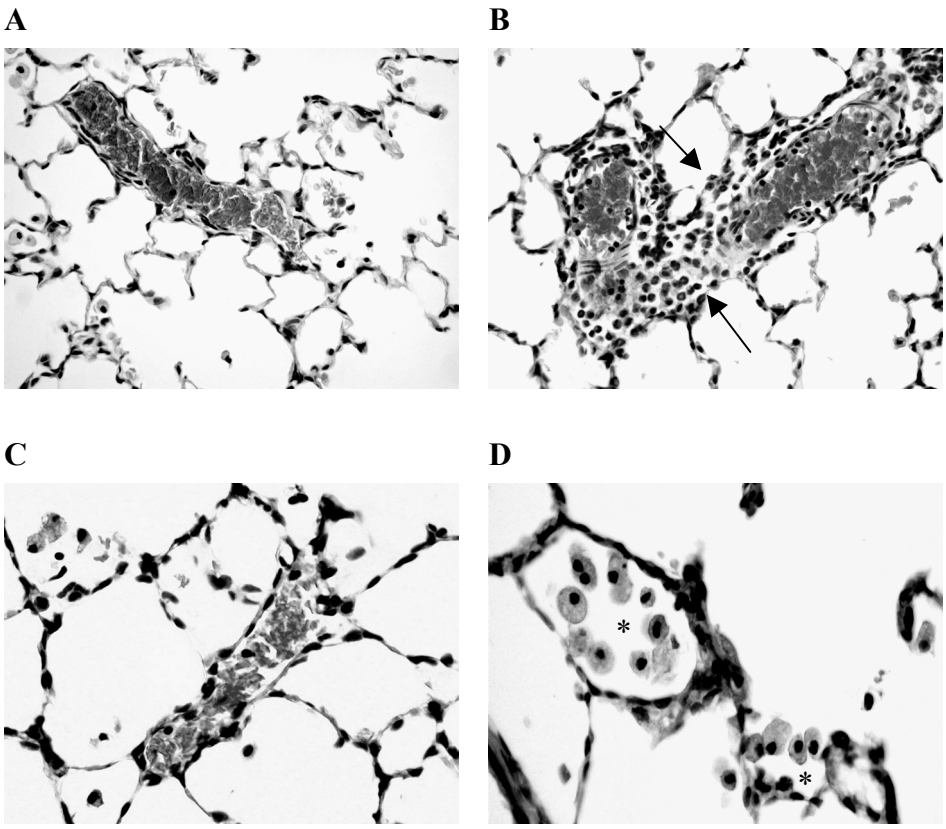

Figure 2: Representative HE stained lung sections of female BN rats exposed to the control diet (A), 450 mg HCB per kg diet (B) or 450 mg HCB per kg diet plus CsA treatment (C and D). Feeding rats with HCB induced perivascular eosinophilic infiltrates in the lung (arrows in B). CsA treatment prevented this infiltration of eosinophils (C) but HCB-induced accumulations of focal macrophages (asterisks) did still occur (D).

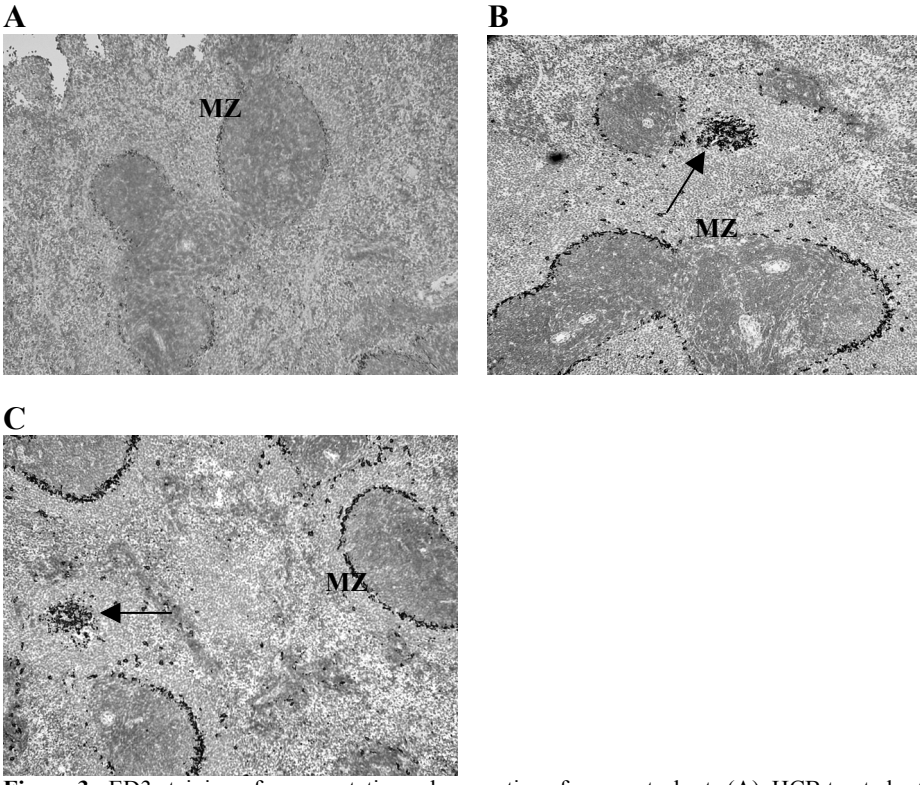

Figure 3: ED3 staining of representative spleen sections from control rats (A), HCB treated rats (B) and HCB and CsA treated rats (C). HCB induced an increase of ED3+ macrophages leading to broadening of the rim of these macrophages in the marginal zone (MZ) and scattered infiltrates of ED3+ in the white pulp (arrows in B and C). This increase was not influenced by CsA treatment.

Table 2: Total serum IgE and IgM levels against ssDNA[2]

Group	**Total serum IgE levels**	**Serum IgM levels against ssDNA**
Control	195 ± 32	1.0 ± 0.1
Control + CsA	48	0.8 ± 0.1
HCB	727 ± 49***	1.7 ±0.2**
HCB + CsA	52	1.0 ± 0.1$^{\#\#}$

[2] Total IgE levels are expressed as ng/ml serum. In both groups treated with CsA only one out of four rats had detectable IgE levels. Only this positive value is depicted in the table. The detection limit of the ELISA was 40 ng/ml. Serum IgM levels against ssDNA are expressed as an ELISA index. Calibration curves of the reference sera were used to calculated the dilution of the test serum that would result in the same absorbance as that of the reference serum as calculated by linear regression. The ELISA index is the ratio of the test dilution used and the calculated dilution. Mean ELISA index of the control group has been transformed to 1 and ELISA indices of the treatment groups have been expressed relative to this index. Data are of 4 animals per group ± SE. Asterisks denote significance from the control group (**p<0.01, *** p<0.001) and from HCB group ($^{\#\#}$ p<0.01).

CD25 expression in the MLN

Figure 4 shows the expression of CD25 in the MLN. HCB treatment induced a stong increase in CD25 expression from 5.6% in control rats to 13%, which was prevented completely by CsA treatment. In control rats CsA treatment decreased CD25 expression slightly to 3.3% (not significant).

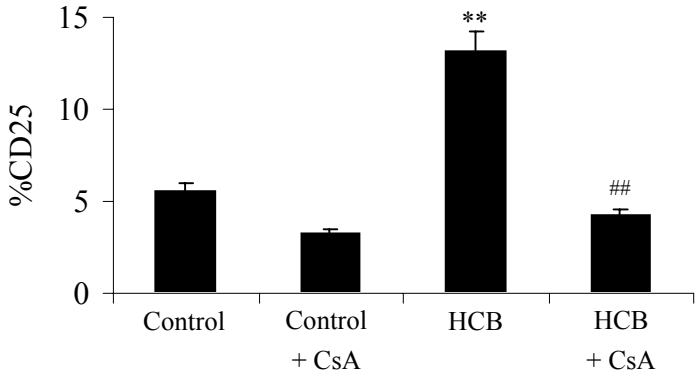

Figure 4: CD25 expression in MLN expressed as percentage cells staining positive. The asterisks above the bar indicate significance from control group (**$p<0.01$) and significance from the HCB group is indicated with $^{\#\#}$ $p<0.01$. Data represent mean ± SE from 4 animals per group.

Effects of CsA on thymic and peripheral T cells

The effects of CsA on thymocyte development have been studied intensively *(11,12)*. In the present study CsA treatment inhibited the development of mature single-positive thymocytes from their double-positive precursors, resulting in a reduction of mature TCR$\alpha\beta$ single-positive $CD4^+$ and to a lesser extent a reduction of mature TCR$\alpha\beta$ single-positive $CD8^+$ thymocytes (data not shown). Table 3 shows the relative and absolute numbers of T and B cells and the CD4/CD8 ratio in the spleen and ALN. CsA treatment caused a decrease of relative and absolute T cell number in spleen and ALN in both the control and the HCB-treated group. The decreased CD4/CD8 ratio shows that predominantly $CD4^+$ T cells were affected by CsA treatment which is in line with previous work *(6)*. In both spleen and ALN absolute T cell and B cell numbers were increased in the HCB treated group, whereas the relative T and B cell numbers in this group were not changed indicating that both cell types contributed equally to the observed enlargement of spleen and ALN.

Table 3: T and B cell number and CD4/CD8 ratio in spleen and ALN[3]

	T cells	B cells	CD4/CD8 ratio
Spleen			
Control	336 ± 16	836 ± 73	2.1
	(13 ± 0.5)	(32 ± 1.3)	
Control + CsA	149 ± 4.9***$^{\#\#\#}$	783 ± 52	1.4**$^{\#\#}$
	(6.6 ± 0.5***)	(34 ± 3)	
HCB	520 ± 31***	1185 ± 149	2.3
	(14 ± 0.2)	(32 ± 2.6)	
HCB + CsA	177 ± 20***$^{\#\#}$	1133 ± 91	1.6*$^{\#\#}$
	(5.7 ± 0.3***$^{\#\#}$)	(37 ± 0.6)	
ALN			
Control	511 ± 59	348 ± 14	3.5
	(48 ± 1.3)	(34 ± 2.4)	
Control + CsA	194 ± 28$^{\#\#}$	228 ± 40$^{\#\#}$	2.7
	(35 ± 2.2)	(41 ± 2.1)	
HCB	1530 ± 512	1413 ± 37**	3.2
	(43 ± 2.4)	(42 ± 3.2)	
HCB + CsA	373 ± 212$^{\#}$	271 ± 42$^{\#\#}$	2.4*
	(36 ± 7.5)	(40 ± 6.6)	

[3]Absolute (x 10^4 cells) and relative (%; between brackets) number of T and B cells and CD4/CD8 ratio in the spleen and ALN. Data represent mean ± SE of 4 animals per group. Asterisks denote significance from the control group (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Significance from HCB group: $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$

Cytokine and NO production by spleen cells after mitogen restimulation

To establish the effect of CsA on T cell activation, spleen cells were restimulated with ConA and subsequently IL-2 levels in the supernatant were measured. Table 4 shows that HCB treatment alone caused a significant increase of IL-2 levels and that after CsA treatment IL-2 levels in both the control group and the HCB-fed group were lower than the untreated control group. To investigate whether CsA treatment affected macrophage function spleen cells were also restimulated with LPS and TNF-α and NO production were measured. TNF-α levels were twice as high after HCB treatment and this increase was not influenced by CsA treatment (Table 4). HCB also induced an increase in NO production and this increase was also not affected by CsA treatment.

Table 4: IL-2, TNF-α and NO production after restimulation of splenocytes with the mitogens ConA and LPS[4]

Group	**IL-2**	**TNF-α**	**NO**
Control	9.2 ± 1.6	3.2 ± 1.3	2.3 ± 1.2
Control + CsA	3.6 ± 1.3*	3.4 ± 0.7	6.1 ± 1.4
HCB	28 ± 8.4**	7.5 ± 1.8**	16 ± 1.7**
HCB + CsA	4.9 ± 3.1$^{\#\#}$	7.2 ± 0.4**	19 ± 3.4**

[4]IL-2 levels were measured in supernatants of ConA-stimulated spleen cells and levels of TNF-α and NO in supernatants of LPS-stimulated spleen cells. Data represent mean ± SE of 4 animals per group. Asterisks denote significant differences from the control group (* $p<0.05$, ** $p<0.01$). Significantly different from the HCB treated group: $^{\#\#}$ $p<0.01$

Discussion

The present study showed that CsA treatment delayed the onset of HCB-induced skin lesions and decreased the severity of these skin effects, but did not affect the incidence. CsA prevented increase in spleen weight, but not to control values. In contrast, the increase in ALN weight was prevented completely and infiltration of eosinophils and increases in serum IgE and anti-ssDNA IgM were absent or at control level, respectively, in HCB-fed animals treated with CsA. The T cell-specific nature of the CsA inhibitory effect was confirmed by the reduction of ConA-induced IL-2 production and the unaffected LPS-induced TNF-α and NO production by splenocytes. In line with this, macrophage infiltrations into lung and spleen were still present regardless of the CsA treatment. Together, these data show that the immunopathological effects of HCB are to a large extent mediated by T cells.

The observed upregulation of CD25, or IL-2 receptor, in the MLN supports the relevance of T cells in HCB-induced immunopathology. The finding that CD25 expression in control rats and HCB-fed rats treated with CsA was not altered is in accordance with knowledge that CsA does not act at the level of the IL-2 receptor, but downregulates IL-2 mRNA *(7)* and production of IL-2 (see above).

Present observations are partly in line with studies investigating the role of T cells in HCB-induced immunopathology by using thymectomized, lethal irradiated, and bone marrow reconstituted BN rats *(13)*. These results already suggested that thymus-dependent T cells were not required for induction of inflammatory skin effects by HCB, but that T cells accelerated the occurrence of these skin lesions. In contrast to our results splenomegaly and eosinophilia in the lung did still occur in these T cell-depleted rats. This

previous study, however, was not completely conclusive since after the T cell-depleting procedure (thymectomy, lethal irradiation plus bone marrow reconstitution) these BN rats still had functional T cells left in the periphery, which could account for some T cell-dependent processes. By using CsA we circumvented this, since CsA not only reduces the number of T cells, but also inhibits antigen-induced T cell activation by interfering with the cytokine production *(7)*. Furthermore, we show that HCB-induced lung eosinophilia is not dependent on mediators derived from other cell types than T cells as was suggested previously *(13)*.

The increase in IgM levels against ssDNA has previously been related to the ability of HCB to activate B1-cells, a subset of B cells present in the MZ of the spleen *(25)* and known to produce low-affinity IgM antibodies against autoantigens. This increase was absent in HCB-fed rats treated with CsA. Remarkably, in these rats in particular the MZ was still hyperplastic, although less than in rats treated only with HCB. The HCB-induced influx of ED3+ macrophages, which constitute an important cell population in the MZ and thought to be capable of activating MZ B cells *(3)*, was not affected by CsA treatment. Interestingly, our data suggest that ED3+ macrophages are responsible for the expansion of the MZ, but that T cells are required for the augmentation of the production of ssDNA-IgM by B-1 cells.

Remarkably, CsA suppressed the severity and delayed the onset of skin lesions but did not influence the incidence, since all rats eventually developed skin lesions. This might be due to HCB-induced macrophage activation, leading to the production of inflammatory cytokines, NO and oxidative stress which was not inhibited by CsA. CD8α macrophages have been observed in the dermis of HCB treated rats before *(13)* and these macrophages might become activated by uptake of the inert HCB and initiate inflammatory responses and lesions in the skin. Nevertheless T cells are indispensable in further acceleration of skin pathology.

Together our results indicate that T cells play a prominent role in HCB-induced eosinophilia and humoral responses but that macrophages do account for at least a part of the observed adverse immune effects. Importantly, we show that macrophage activation and T cell activation by HCB are separate entities.

How macrophage and T cells activation leads to pathology is not completely clear as yet. But one can envisage that the HCB is first taken up by macrophages and that responses similar to those observed after silica exposure take place. Silica is an inert chemical that induces autoimmune-like phenomena due to chronic activation of macrophages, leading to the production of pro-inflammatory cytokines like TNF-α and reactive oxygen species which results in chronic inflammation and tissue damage *(4,10)*.

Subsequently, these adjuvant signals could polyclonally activate the available T cell-pool, including autoreactive T cells. In addition, inflammatory cells, in particular polymorphonuclear cells attracted by the presence of HCB, might induce myeloperoxidase-dependent formation of reactive metabolites of HCB. It is known that pentachlorophenol, an oxidative metabolite of HCB, can be oxidized into tetrachlorohydroquinone (TCHQ) and TCBQ by peroxidases *(22)*. Recently, we have shown by using the mouse popliteal lymph node assay that TCBQ is capable of inducing neoantigen specific T cell hapten *(5)*. Once formed *in vivo*, these reactive intermediates would be able to haptenize to proteins to form neoantigens and then trigger hapten-specific T cells as well. Since cytochrome P450 catalyzed oxidation is not involved in HCB-induced immunopathology *(23)*, extrahepatic metabolism by phagocytes might be implicated, as has been shown before for other allergenic or autoimmunogenic chemicals (reviewed in ref. *27)*.

Our future research will focus on the specificity of T cells and the role of macrophages in HCB-induced immunopathology. Interestingly, the present data stresses the suitability of the HCB-Brown Norway model to separately evaluate cellular and molecular interactions between innate and adaptive immune phenomena in relation to autoimmune-like syndromes. As such it adds to already existing Brown Norway models using metals or pharmaceuticals *(17,26)*.

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Chapter 5

Macrophages play a critical role in hexachlorobenzene-induced adverse immune effects

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Submitted

Abstract

Hexachlorobenzene (HCB) is a persistent environmental pollutant that causes adverse immune effects in man and rat. The Brown Norway (BN) rat is very susceptible to HCB-induced immunopathology and oral exposure causes inflammatory skin and lung lesions, splenomegaly, lymph node (LN) enlargement and increased serum levels of IgE and anti-ssDNA IgM. T cells play an important role, but do not account for all adverse effects induced by HCB. Macrophages are probably also important and the relationship between macrophages and T cells was further investigated. To eliminate macrophages clodronate-liposomes were used. Furthermore, a kinetic study was performed to obtain insight in the early phase of the HCB-induced immune response. Also, experiments were performed to detect specific memory T cells. Therefore, rats were challenged with the reactive metabolite tetrachlorobenzoquinone (TCBQ) and additionally, an adoptive transfer study was performed. Our results indicate that macrophages were indeed involved in HCB-induced skin lesions, lung eosinophilia and elevation of ssDNA-IgM. Kinetics showed that both skin and lung lesions occurred early after exposure. Moreover, no evidence for the presence of specific memory T cells was found and immune effects could not be adoptively transferred. Together, our results indicate that both macrophages and T cells play an important role in HCB-induced immune effects but that HCB exposure does not lead to specific T cell sensitization. Presumably, HCB exposure induces macrophage activation, thereby generating adjuvant signals that polyclonally stimulate T cells. Together, these events may lead to the observed immunopathology in BN rats.

Introduction

Hexachlorobenzene (HCB) is a highly persistent environmental pollutant that is produced as a by-product of many industrial processes. The toxic effects induced by HCB were first noticed after an accidental poisoning in Turkey in the 1950s. The main characteristic clinical sign was hepatic porphyria. Adverse immune effects, such as enlarged spleen and lymph nodes (LN) and arthritis were also observed *(9,19)*. Furthermore, infants born from HCB-exposed mothers developed inflammatory skin and lung lesions *(2)*.

The immunotoxic effects of HCB could be reproduced in rats (for reviews see refs. *(16,31)*. In particular Brown Norway (BN) rats are very susceptible to HCB-induced immunotoxic effects and oral exposure induces inflammatory skin and lung effects, splenomegaly, increased LN weight, and elevated serum levels of total IgM, IgG and IgE, and IgM against ssDNA *(17)*. Recently, we have shown that T cells play an important role

in HCB-induced skin lesions, lung eosinophilia, increase in auricular LN (ALN) cell number, and elevation of humoral responses. Splenomegaly appeared to be only slightly dependent on T cells and macrophage infiltrations in spleen and lung were T cell-independent. The presence of macrophages in spleen and lung could be indicatory for their involvement in some of the HCB-induced immune effects *(5)*.

The exact role of macrophages and T cells in HCB-induced immunostimulation is unknown. First, it is unclear if macrophages are involved in T cell activation. Mediators produced by activated macrophages, such as inflammatory cytokines, are able to increase costimulatory molecules on dendritic cells, thereby enhancing signal 2 that is necessary for T cell activation *(13)*. Furthermore, pro-inflammatory cytokines can directly activate naive T cells *(3,18)*. Second, the potential of HCB to sensitize BN rats after oral exposure and generate memory T cells is not assessed yet. Low molecular weight chemicals (LMWC) can become immunogenic after binding to self proteins, thereby generating hapten-carrier complexes *(32)*. Previously, we have shown that the oxidative metabolite of HCB, tetrachlorobenzoquinone (TCBQ), can be considered as a hapten in the mouse popliteal lymph node assay (PLNA) *(7)*. Alternatively, certain LMWC are known to interfere with antigen presentation, leading to presentation of cryptic epitopes *(25)*. Importantly, sensitization of T cells can only occur when sufficient adjuvant signals are present, e.g. costimulatory molecules or danger signals *(1,14)*.

In this study we investigated the involvement of macrophages in relation to T cell activation and observed immunopathology. Macrophages were eliminated with the so-called macrophage 'suicide' technique using clodronate-liposomes *(28)*. Furthermore, a kinetic study was performed to obtain information on the early phase of HCB-induced immune effects. In addition, we examined whether HCB induced specific T cell activation, either or not specific for TCBQ. For this purpose several experiments were performed, including an adoptive transfer study.

Materials and methods

Chemicals and reagents

The semisynthetic diet SSP/TOX was obtained from Hope Farms (Woerden, The Netherlands). HCB and TCHQ were purchased from Aldrich Chemie (Bornem, Belgium) and TCBQ (p-chloranil; purity >98%) from Merck-Schuchardt (Hohenbrunn, Germany). Euthesate® was obtained from Ceva Sante Animale B.V. (Maassluis, The Netherlands). Myeloperoxidase (MPO), RPMI 1640 with glutamax and penicillin-streptomycin were purchased from Invitrogen Life Technologies (Paisley, Scotland) and fetal bovine serum

(FBS) from ICN Biomedicals (INC, Costa Mesa, CA). All antibodies for flow cytometry were obtained from Pharmingen (San Diego, St. Louis, MO, USA). The BCA protein assay kit was from Pierce (Rockford, Ireland). Clodronate was a kind gift of Roche Diagnostics GmbH (Mannheim, Germany). Alkaline-phosphatase labeled goat-anti-rat IgM (μ) was from Brunswich (Amsterdam, The Netherlands). Capture and detection antibodies for the TNF-α ELISA were obtained from R&D Systems (Oxon, UK) and recombinant rat TNF-α from Biosource (Camarillo, CA, USA). Streptavidine-horseradish-peroxidase was obtained from CLB (Amsterdam). All other chemicals and reagents were obtained from Sigma (St. Louis, MO, USA).

Rats and maintenance

Three- to 4 week old specific pathogen-free female inbred Brown Norway (BN/SsNOlaHsD, termed BN) rats were purchased from Harlan (Blackthorn, UK). Rats were allowed to acclimatize one week before starting the experiments. All rats were housed at the animal facilities of the Utrecht University and were kept two by two in filter-topped macrolon cages on bedding of chips and wood, under standard conditions (50-60% relative humidity, 12-hr dark/12-hr light cycle) with food and acidified drinking water *ad libitum*. The diet consisted of the SSP/TOX diet supplemented with or without crystalline HCB by mixing of homogeneity (450 mg HCB/kg diet). The experiments were conducted according to the guidelines of the animal experiments committee of the Faculty of Veterinary Medicine of the Utrecht University. In all experiments body weight was recorded twice a week and development of skin lesions was examined daily. The severity of the lesional sites was rated as described before *(17)*, irrespective of size, as 1 = minimal (some redness), 2 = moderate (redness), 3 = marked (dry desquamation and crusts) and 4 = severe (exudative lesions).

Dissection protocol

Rats were sacrificed by a lethal injection of sodium pentobarbital (Euthesate®, 0.3 g/kg bodyweight (bwt); i.p.). Blood was drawn from the vena cava and serum was collected after clotting and centrifugation. Organs were collected and weighed. For morphology, portions of organs were fixed in phosphate-buffered 4% paraformaldehyde and lungs were inflated and fixed with phosphate-buffered 4% paraformaldehyde to optimize morphology. Formaldehyde-fixed tissues were embedded in Paraplast and sections (5 μm) stained with hematoxylin and eosin (HE). For cryostat sections, portions of the organs were snap-frozen in liquid nitrogen. To avoid bias at examination, sections were scored under code. Single cell suspensions from spleen and LNs were prepared for flow

cytometry and *ex vivo* restimulation with mitogens in complete medium (RPMI 1640 with glutamax supplemented with 10% FBS and 2% penicillin-streptomycin) and counted using a Coulter Counter (Coulter Electronics, Luton, UK) and then adjusted to 2.5 x 10^6 cells per ml.

Macrophage depletion with clodronate-liposomes

Clodronate-liposomes were prepared as described previously *(30)*. Rats were divided randomly in the following experimental groups: two control groups treated with either PBS (n=4) or clodronate-liposomes (n=5) and three groups exposed to the HCB diet treated with either PBS (n=5), clodronate-liposomes (n=6) or PBS-liposomes (n=4). Sham injections of PBS were included to mimic depletion treatments without affecting macrophages and this group will be used for to compare the effects of clodronate-liposomes on HCB-induced pathology. PBS-liposomes were included as control groups to establish that liposomes themselves do not alter observed effects *(30)*. The first injection with liposomes was i.v. in the tail vein and immediately after this injection rats were placed on the appropriate diet. Rats received 1 ml clodronate-liposomes per 100 g bwt and control groups received a sham injection of 1 ml PBS or PBS-liposomes per 100 g bwt. To maintain depletion rats were injected i.p. on days 5, 9, 13 and 17. This regimen was chosen based on experiments in SCID mice *(8)*, in order to prevent repopulation of macrophages and deplete circulating monocytes. Blood monocytes are only depleted for a short period after i.v. injection of clodronate-liposomes, since macrophage depletion in blood, liver en spleen generates signals to the bone marrow leading to enhanced release of monocytes, thereby increasing the number of circulating monocytes *(10)*. Rats were sacrificed on day 20. Blood, spleen, MLN and ALN were collected and spleen was weighed. Portions of liver and spleen were collected for morphology (formaldehyde-fixation or snap-frozen in liquid nitrogen) and MLN and ALN were also snap-frozen in liquid nitrogen and used for cryostat sections. Single cell suspensions of spleen, MLN and ALN were used for flow cytometry and *ex vivo* restimulation with ConA and LPS.

Time study of HCB-induced immunotoxicity

In the kinetic study rats were exposed to HCB for 0, 4, 7, 10, 14, 18 and 21 days. All rats were sacrificed on the same day. Spleen and lungs were collected for morphology. Splenocytes were *ex vivo* stimulated with LPS.

Ex vivo restimulation of splenocytes with TCHQ and TCBQ conjugates

Conjugates of TCHQ and TCBQ were prepared by incubation of these chemicals with freshly isolated hemoglobin as described previously *(33)*. Hemoglobin was isolated from blood obtained from untreated BN rats. The protein concentration of the conjugates was determined with a BCA protein assay kit. An experiment was performed to examine the ability of the TCHQ- and TCBQ-conjugates to restimulate splenocytes *ex vivo*. Rats were exposed to the control diet (n=1) or the HCB diet (n=2). After 21 days rats were sacrificed and spleen, ALN and MLN were collected and single cell suspensions were prepared. TCHQ-hemoglobin, TCBQ-hemoglobin and uncoupled hemoglobin (as a control for the carrier) were plated in a 96 well microtitre plate in a concentration of 50 or 100 µg/ml. As a negative control, complete medium was included. Cells were added to the different stimuli (3.75×10^5 cells per well) and incubated for 72 hours at 37˚C, 5% CO_2, the last 8 hours labeled with 0.5 µCi/well [^{3}H]-Thymidine. Plates were harvested with a cell harvester onto filter paper and dried. The filter paper was placed into a bag containing scintillation fluid and [^{3}H]-Thymidine incorporation was measured with a beta-plate liquid scintillation counter.

Topical application of TCBQ on the ears of HCB-exposed rats

Rats were randomly assigned to the different experimental groups (n=2), either receiving control diet or the HCB diet. On day 10 rats were challenged on the ears, and rats exposed to HCB received from this time point on the control diet. Ear thickness was measured prior to TCBQ application. Rats received a topical application on the dorsum of both ears of 50 µl vehicle or 50 µl TCBQ (0.01, 0.1, 1 and 2%) in acetone/olive oil 4:1. In order to determine delayed type hypersensitivity reaction ear thickness was measured 24 and 48 hours after challenge. Four days after challenge rats were sacrificed and ALNs were collected and single cells suspensions were prepared in phosphate buffered saline (PBS) containing 1% bovine serum albumin (PBS/1%BSA) and after washing and resuspension in PBS/1%BSA, cells were counted using a Coulter Counter (Coulter Electronics, Luton, UK) and then adjusted to 2.5×10^6 cells per ml. Phenotype analysis was performed by using flow cytometry.

Adoptive transfer study

The protocol for adoptive transfer was adapted from Shenton *et al. (26)*. BN rats exposed to the HCB diet for 21 days were used as splenocyte donors (n=8). Spleens were collected from donor rats and single cell suspensions were prepared under sterile conditions. Erythrocytes were lysed in PBS containing 0.83% $NaHCO_3$ and 0.83% NH_4Cl. After

washing, cell suspensions were pooled in sterile PBS. Naive recipients (n=8) received 6 x 10^8 splenocytes, which equals an entire spleen, via the tail vein and were placed on the diet containing HCB (n=4) or on the control diet (n=4). Control groups were untreated rats that received either the control (n=4) or the HCB diet (n=4). After 21 days rats were sacrificed. Blood, spleen and lungs were collected and used for morphology and splenocytes were used for *ex vivo* restimulation with LPS.

Flow cytometry

The following conjugated monoclonal antibodies were used in duplo- or triplostaining to phenotype lymphocytes: fluorescein-isothiocyanate (FITC)-conjugated OX-33 (anti-rat CD45RA), phycoerythrin (PE)-conjugated R73 (anti-rat TCR$\alpha\beta$), OX-8 (anti-rat CD8α) and biotinylated OX-35 (anti-rat CD4). Incubations and measurements were performed as described previously *(5)*.

Ex vivo restimulation of spleen or lymph node cells

For measurement of pro-inflammatory cytokines cells were stimulated *ex vivo* with the mitogen LPS and for T cell proliferation with the mitogen ConA. LPS (2 μg/ml) and ConA (5 μg/ml) in complete medium were plated in a 96 well microtitre plate and cells (3.75 x 10^5 cells per well) were added. After 24 hours of incubation with LPS supernatants were collected for cytokine measurement. Cells were incubated with ConA for 48 hours and the last 8 hours cells were labeled with 0.5 μCi/well [^{3}H]-Thymidine. Plates were harvested with a cell harvester onto filter paper and dried. The filter paper was placed into a bag containing scintillation fluid and [^{3}H]-Thymidine incorporation was measured with a beta-plate liquid scintillation counter.

Incubation of HCB with MPO and HPLC determinations

To investigate if the enzyme MPO can metabolize HCB in reactive metabolites, incubations of HCB with commercially available MPO were performed. HCB (100 and 200 μM) was incubated with MPO (1, 2 and 5 units) and hydrogen peroxide (100 μM) in phosphate buffer (100 mM KH_2PO_4, 100 mM K_2HPO_4, pH6) for 2 hours. To detect metabolites in the incubation mixture, extraction with ether was performed and the extract was analyzed by HPLC as described previously *(27)*.

TNF-α ELISA

TNF-α was measured in supernatants of cells stimulated with LPS. Procedures were performed according to the description of the manufacturer with some minor modifications. Briefly, 96-well microtitre plates were coated overnight (o.n.) at room temperature (RT) with 2 µg/ml monoclonal anti-rat TNF-α. They were blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN3 for 2 h at RT. After washing, samples and standard curve were added to the plate and incubated o.n. at 4°C. Plates were washed and 200 ng/ml biotinylated anti-rat TNF-α in PBS containing 0.5% Tween-20, 1% BSA and 2% goat serum was added to the plates and incubated for 1 hour at RT. After washing plates were incubated with streptavidin-horesradish-peroxidase for 1 hour at RT and developed with tetramethylbenzidine for 15 min. The color reaction was stopped by adding H_2SO_4 and absorbance was measured at 450 nm. The standard curve of recombinant TNF-α was used to calculate the amount of TNF-α in the samples (pg/ml).

ELISA to detect serum levels of IgM against ssDNA

ELISA procedures to determine IgM levels against ssDNA were performed as described previously *(5,23)*. To calculate IgM serum levels against ssDNA all treatment groups were compared to the control group. Absorbance values of serial dilutions of the control serum were plotted against the 2log of the dilution. The dilution of the individual test sera that would result in the same absorbance was calculated by linear regression and was used to obtain the ELISA index. This index is the ratio of the test dilution used and the calculated dilution of control serum at the same absorbance.

Immunohistochemistry

To visualize macrophages cryostat sections (6 µm) of spleen and LNs were stained with acid phosphatase. Slides were counterstained with hematoxylin. Staining sections was carried out as described before *(7)*. For determination of calcium deposits formaldehyde-fixed sections were stained with von Kossa.

Statistical analysis

Statistically significant differences of treatment groups with the control group or between the HCB-exposed groups treated with PBS, clodronate-liposomes or PBS-liposomes were determined by analysis of variance (ANOVA) with Bonferroni post hoc test for contrasts by using SPSS software. Values deviating more than 2 standard deviations from group

means were considered as outliers and not included in statistical analyses. Preceding statistical analysis, all data were transformed to log 10 values to homogenize variance.

Results

Effects of clodronate-liposomes on HCB-induced adverse immune effects

Body weight gain and macroscopic skin lesions

Body weight gain was not affected by treatment with HCB, clodronate-liposomes or PBS-liposomes. In one out of six HCB-exposed rats treated with clodronate-liposomes skin lesions were completely absent. In four of these six rats the initial development was not affected by clodronate-liposomes, but exacerbation was clearly inhibited, resulting in smaller and less severe lesions after 20 days of exposure. In one of the HCB-exposed rats clodronate-liposomes did not at all prevent onset or aggravation of skin lesions and lesional area and severity was similar as in HCB-exposed rats treated with PBS. Because of these different effects, data on skin pathology of HCB-exposed rats treated with clodronate-liposomes are displayed in Table 1 in three separate groups. Table 1 shows also that administration of PBS-liposomes had no effect on HCB-induced skin lesions.

Table 1: Effects of clodronate-liposomes on the onset, relative lesional area and severity of skin lesions induced by HCB[1]

Group	No.	**Onset**	No.	**Lesional area**	**Severity**
HCB + PBS	3	8 days	6	2.2 ± 0.45	4
	2	9 days			
HCB + clodronate-liposomes	1	No lesions	1	0	0
	3	9 days	4	0.71 ± 0.24	3
	2	10 days	1	3.1	4
HCB + PBS-liposomes	4	9 days	4	3.2 ± 0.67	4

[1]Table shows onset of skin lesions in days of exposure (including number of rats per time-point). The relative lesional area (expressed relative to the total area) and the severity of skin lesions (for ranking see Materials and Methods) after 20 days of exposure to HCB are also shown. Lesional area and severity of HCB-exposed rats treated with clodronate-liposomes were divided in three groups to grade the differences between the rats.

Lung morphology

Administration of clodronate-liposomes prevented HCB-induced lung pathology completely in one rat. Notably, this was a different rat than the rat that did not develop

skin lesions. In the other 5 rats of that group, clodronate-liposomes inhibited the HCB-induced perivascular eosinophilic infiltrate moderately, but could not prevent focal accumulations of macrophages and granuloma formation. In one of the control rats treated with clodronate-liposomes a few microgranulomas and scattered eosinophilic granulocytes were found. Lung morphology of HCB-exposed rats treated with PBS or PBS-liposomes was similar as described before *(17)*. A summary of morphological changes in the lung is given in Table 2.

Table 2: Effects of clodronate-liposomes on the incidence of histopathological effects in lungs of BN rats exposed to the control or HCB diet[2]

	No.	**Accumulation of macrophages**		**Perivascular infiltrate**		**Granuloma formation**	
		slight	moderate	slight	moderate	slight	moderate
Control + clod-lip.	5					1	
HCB + PBS	5	1	4		5	1	4
HCB + clod-lip.	6	3	2	5		3	2
HCB + PBS-lip.	4	2	2		4	2	2

[2]Sections were scored under code. In control rats scattered microgranulomas were observed. The perivascular infiltrate consisted mainly of eosinophils. Granulomas were formed by monocytes, macrophages, Langhans type giant cells and eosinophilic granulocytes.

Spleen: organ weight, number of B and T cells and histopathology

Treatment with clodronate-liposomes caused a nearly complete elimination of splenic macrophages and decreased spleen weight in both control as well as in HCB-exposed rats. In addition absolute numbers of B and T cells (Table 3) were drastically reduced. As a result tissue architecture of the spleen was disturbed (Figure 1). However, the degree of the effect of clodronate-liposomes differed between the different HCB-exposed rats. In 4 out of 6 animals and the control rats treated with clodronate-liposomes a comparable picture was observed (Figure 1E and F), with depleted and necrotic areas, predominantly in the red pulp. The necrotic areas were located subcapsular and contained calcium deposits, as judged by Von Kossa staining (not shown). In two of the HCB-exposed rats, clodronate-liposomes induced less drastic derangements of the splenic architecture, with absence of necrotic areas (Figure 1G) and a lesser degree of macrophage depletion (Figure 1H). HCB exposure induced comparable histopathological changes as those described before *(5,17,23)*, and treatment with PBS liposomes did not alter these HCB-induced splenic changes (not shown).

Table 3: Spleen weight and number of B and T cells[3]

Treatment	**Spleen weight**	**B cells**	**T cells**
Control + PBS	0.33 ± 0.13	100	100
	(0.29 ± 0.02)	(46 ± 2.3)	(17 ± 0.97)
Control + clodronate-lip.	0.066 ± 0.087***	11 ± 1.8*** ###	8.9 ± 3.6* ###
	(0.060 ± 0.008***)	(27 ± 3.9*)	(12 ± 3.0)
HCB + PBS	0.70 ± 0.44***	232 ± 16***	248 ± 28*
	(0.56 ± 0.05***)	(50 ± 1.2)	(17 ± 1.5)
HCB + clodronate-lip.	0.11 ± 0.11*** ###	30 ± 5.9***	30 ± 4.9 ***
	(0.082 ± 0.007*** ###)	(43 ± 4.8*** #)	(16 ± 2.8)
HCB + PBS-liposomes	0.75 ± 0.44***	223 ± 6.3***	205 ± 13**
	(0.57 ± 0.012***)	(46 ± 1.8)	(15 ± 0.71)

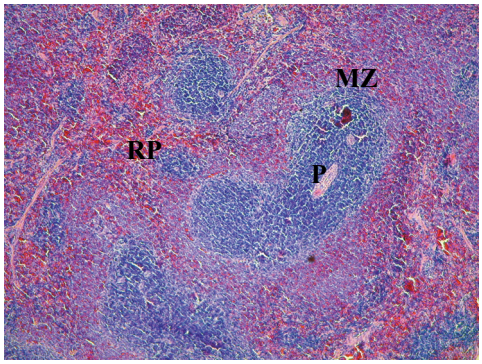
[3]Spleen weight is expressed as absolute (in g) and relative (in g/100 g bwt) weight after 20 days of exposure of female BN rats to control diet or HCB diet with or without treatment with liposomes. Number of B and T cells are expressed as absolute (expressed relative to control group in %) and relative (%; between brackets) numbers. Data represent mean ± SE of 4-6 animals per group. The asterisks above the bars indicate significance from control group (*$p<0.05$; **$p<0.01$, *** $p<0.001$), significance from HCB-exposed group treated with PBS is depicted with #$p<0.05$, ##$p<0.01$, ###$p<0.001$.

Lymph nodes: cell number, number of macrophages, B and T cells

Clodronate-liposomes did not result in depletion of macrophages in the ALN, as judged by histopathology (not shown), nor did it affect the HCB-induced increase in ALN cell number (Table 4) or induction of germinal center (GC) formation (not shown). The HCB-induced increase of ALN cell number is caused by an expansion of both T and B cell areas. Furthermore, relative B cell numbers in the ALN increased significantly as a result of HCB treatment (Table 4).

Administration of clodronate-liposomes did not alter the number of macrophages, or of B and T cells in the MLN. But also HCB-exposure itself appeared not to affect cell number or lymphocyte subsets in these lymph nodes, and GCs were present in both control and HCB-exposed rats (not shown).

A



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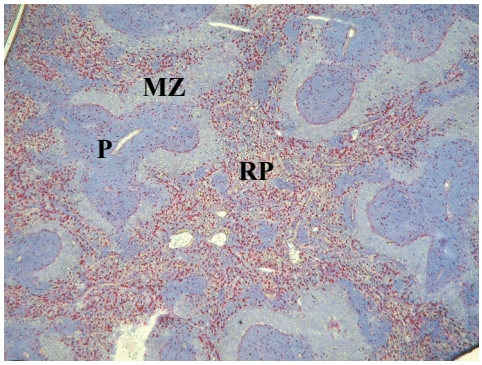

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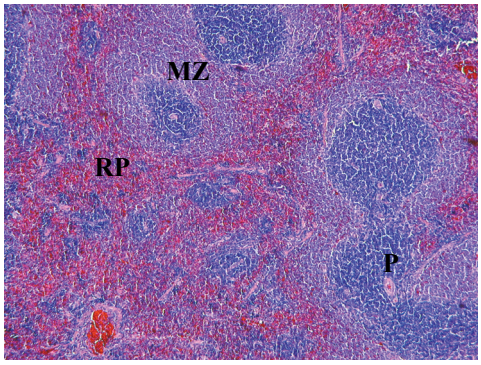

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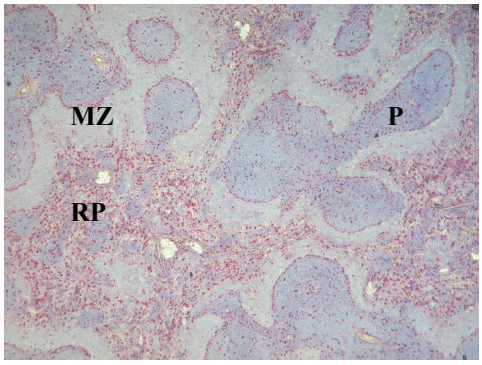

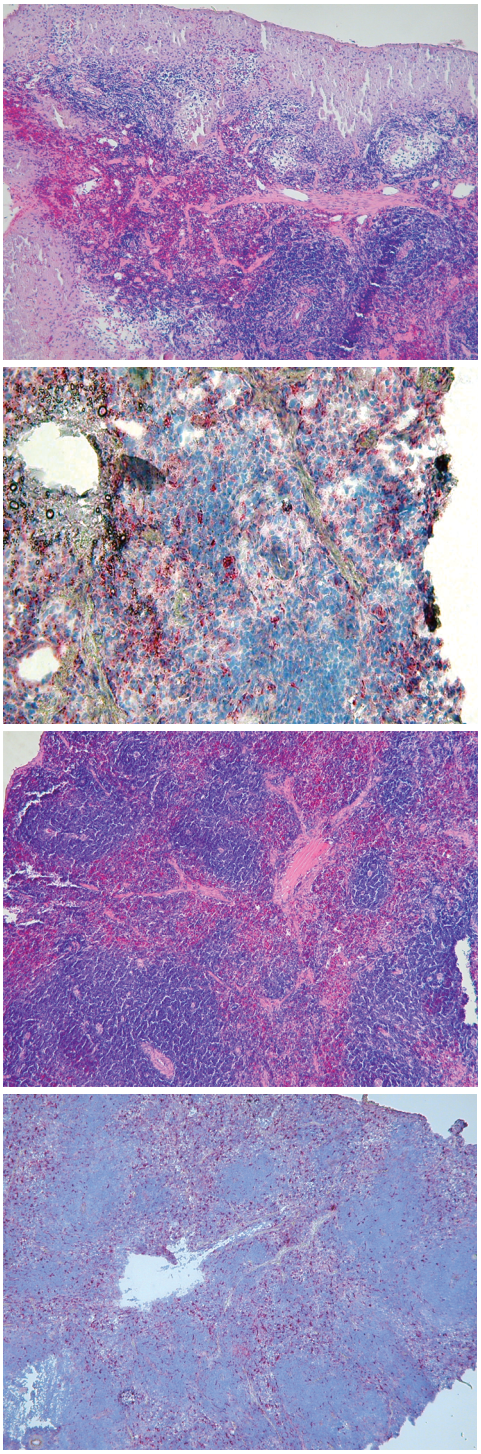

Figure 1: Representative spleen sections from control rats (A-B), HCB-exposed rats (C-D), control rats treated with clodronate-liposomes (E) and HCB-exposed rats treated with clodronate-liposomes (F-H). Sections are stained with either HE (A-C-E-G) or acid phosphatase (B-D-F-H) to reveal macrophages (in red). MZ: marginal zone; P: PALS; RP: red pulp.

Table 4: ALN cell number and number of B and T cells[4]

Treatment	**ALN cell number**	**B cells**	**T cells**
Control + PBS	17 ± 0.84	6.1 ± 0.51 (36 ± 1.7)	9.4 ± 0.39 (56 ± 1.5)
Control + clodronate-liposomes	14 ± 3.4	4.9 ± 1.1 (35 ± 0.79)	8.2 ± 2.0 (57 ± 1.0)
HCB + PBS	53 ± 5.3***	24 ± 2.9*** (44 ± 1.5**)	25 ± 2.3*** (48 ± 1.7*)
HCB + clodronate-liposomes	47 ± 3.5***	19 ± 1.3*** (42 ± 0.74*)	23 ± 1.9*** (50 ± 1.1)
HCB + PBS-liposomes	54 ± 0.85***	25 ± 0.91*** (47 ± 1.4***)	25 ± 1.4*** (46 ± 2.3**)

[4]ALN cell number and number of B and T cells that are expressed as absolute (x 10^6 cells) and relative (%; between brackets) numbers. Data represent mean ± SE of 4-6 animals per group. Asterisks denote significance from the control group (*p<0.05, **p<0.01, ***p<0.001).

TNF-α production of spleen and LN cells after incubation with LPS

Figure 2 shows LPS-induced TNF-α production of cell suspensions from spleen, MLN and ALN. Treatment with clodronate-liposomes significantly prevented the HCB-induced increase of TNF-α production in spleen, MLN and ALN. Furthermore, splenocytes from control rats injected with clodronate-liposomes produced less TNF-α compared to those from control rats injected with PBS. Administration of PBS-liposomes did not affect HCB-induced elevation of TNF-α.

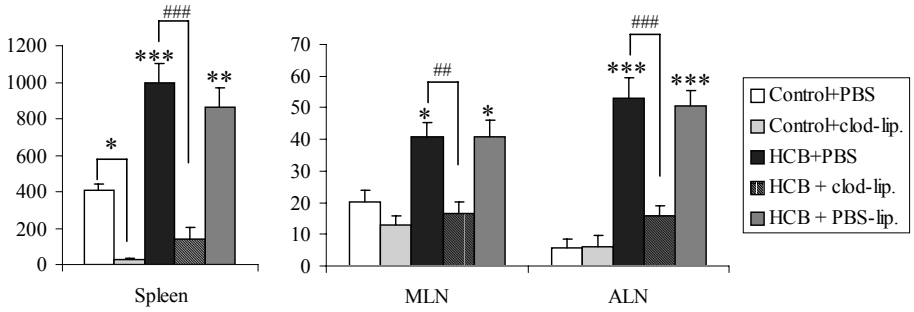

Figure 2: Effect of HCB either or not combined with macrophage depletion on LPS-induced TNF-α production (in pg/ml) of spleen, MLN and ALN cells. Data represent mean ± SE of 4-6 animals per group. Significantly different from the control group: * p<0.05, ** p<0.01, *** p<0.001 and significantly different from the HCB+PBS group: ## p <0.01, ### p<0.001.

ConA-induced proliferation of spleen and LN cells

Table 5 shows the proliferative responses of cell suspensions from spleen, MLN and ALN cells after ConA incubation. In the HCB-exposed group the effect of treatment with clodronate-liposomes on ConA-induced proliferation of splenocytes was not clear, due to high variance. Two rats responded to ConA with higher proliferation (average 25215 cpm) than the HCB-exposed group treated with PBS but lower than the PBS-treated control group. Splenocytes from the other 4 rats hardly responded to the mitogenic response (average 117 cpm), similar to control rats treated with clodronate-liposomes. When proliferation was compared with spleen morphology, high proliferative responses were observed in the 2 rats in which spleen morphology was less disturbed and macrophage depletion was not as pronounced (Figure 1G-H). Remarkably, HCB-exposure itself decreased the susceptibility of splenocytes to proliferate in response to ConA, resulting in decreased proliferation as compared to the PBS-treated control group.

Treatment with clodronate-liposomes of HCB-exposed rats resulted in a decreased mitogen-induced proliferation of MLN cells compared to HCB-exposed rats as well as compared to clodronate-liposome-treated control animals (not significant). HCB exposure itself did not alter ConA-induced proliferation in this organ. Treatment with clodronate-liposomes did also prevent the HCB-induced increase in the mitogenic response of ALN cells, although this was again not significant (due to high variance). Mitogenic responses after HCB-exposure were not significantly affected by administration of PBS-liposomes in any of the assessed organs, although in cell cultures of MLN and ALN higher mean proliferation levels were measured as compared with the HCB-exposed group treated with PBS.

Table 5: Effects of clodronate-liposomes on ConA-induced proliferation[5]

Treatment	**Spleen**	**MLN**	**ALN**
Control + PBS	46592 ± 11747	10491 ± 1369	823 ± 389
Control + clod-lip.	82 ± 9***	16122 ± 3337	3869 ± 1619
HCB + PBS	6754 ± 459***	10301 ± 3762	26420 ± 10284*
HCB + clod-lip.	8483 ± 6683***	4507 ± 944	9366 ± 2401
HCB + PBS-lip.	6800 ± 449***	20889 ± 5483	40619 ± 5357**

[5]Table shows ConA-induced proliferation of spleen, MLN and ALN cells (expressed in cpm). Cells were incubated with ConA (5 µg/ml) for 48 hours and incorporation of [^{3}H]-Thymidine was used to determine proliferation. Each sample was measured in quadriplate and data represent mean ± SE of 4-6 rats per group. Asterisks denote significance from the control group (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Serum levels of IgM against ssDNA

Treatment with clodronate-liposomes prevented the HCB-induced increase of serum levels of ssDNA-specific IgM, and even reduced the level compared to controls (Figure 3). In the control group treated with clodronate-liposomes the ELISA-index was slightly decreased.

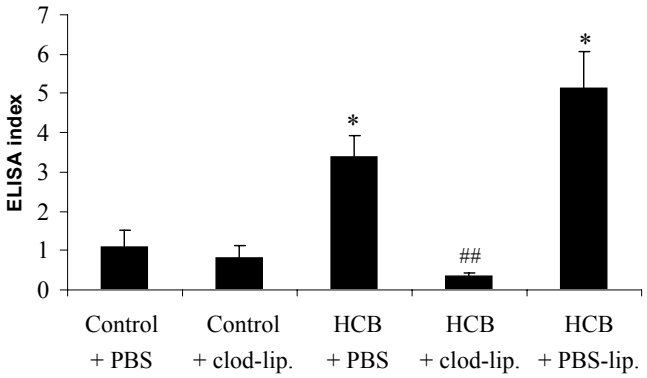

Figure 3: Serum IgM levels against ssDNA are expressed as an ELISA index, calculated by plotting absorbance values of serial dilutions of the control serum against the 2log of the dilution. The dilution of the individual test sera that would result in the same absorbance was calculated by linear regression and was used to obtain the ELISA index. This index is the ratio of the test dilution used and the calculated dilution of the control serum at the same absorbance. Data are of 4-6 rats per group ± SE. Asterisks denote significance from the control group (*$p<0.05$; **$p<0.01$) and from the HCB-exposed group ($^{\#\#}p<0.01$)

Kinetics of HCB-induced immunotoxicity

To get more insight in the early phase of HCB-induced immunostimulation a kinetic study was performed with a focus on occurrence of skin and lung pathology and activation of splenic macrophages, as evidenced by LPS-induced TNF-α production. The time-point at which the first macroscopic skin lesions were visible varied between 6 and 12 days. Most rats, however, displayed skin lesions after 10 days.

Table 6 summarizes kinetics of lung pathology, increases of spleen weight and LPS-induced TNF-α production of splenocytes. After 4 days of exposure infiltrates of macrophages in the lung were observed in all four rats, 3 out of 4 rats displayed slight perivascular eosinophilic infiltrates and granuloma formation was seen in one rat. The eosinophilic infiltrate was present in all rats after 7 days of exposure, whereas granuloma formation was observed in 3 out of 4 rats. After continuous exposure to HCB lung effects became more pronounced. Spleen weight and LPS-induced TNF-α production increased

time-dependently from day 4 onward. However, increases in spleen weight were not significant until day 14 and the elevation of the TNF-α response to LPS was not significant until day 21 of exposure.

Table 6: Kinetics of HCB-induced lung pathology and spleen effects[6]

HCB exposure	**Lung pathology**								**Spleen effects**	
	Macrophages		Eosinophils			Granuloma			Organ weight	TNF-α production
	+	++	±	+	++	±	+	++		
0 days									0.28±0.01	1407±105
4 days	2	2	3					1	0.31±0.01	1680±80
7 days	3	1		4			2	1	0.33±0.02	2302±192
10 days	3	1	1	3		2	1	1	0.36±0.02	2240±201
14 days	2	2		2	2		1	3	0.42±0.02***	2617±242
18 days		4			4		2	2	0.44±0.02***	2871±425
21 days		4		1	3		3	1	0.53±0.03***	4592±954***

[6]Table shows the severity of HCB-induced lung pathology and spleen effects. Lung pathology is ranked by presence of foamy macrophages, perivascular eosinophilic infiltrates and granuloma. Number of rats investigated per time-point was 4. Relative spleen weight is expressed as g/100 g bwt) and TNF-α was measured in supernatants of LPS-stimulated spleen cells and is expressed in pg/ml. Data are expressed as mean ± SE of 4 animals per group. Asterisks denote significance from the control group (0 days), *** $p<0.001$.

Involvement of memory T cells in HCB-induced immunotoxicity

Our data suggest that macrophages are important in HCB-induced immunostimulation, but previous studies indicated that T cells were involved as well. This raised the question whether HCB treatment results in T cell sensitization. Several experiments were performed to investigate this.

To assess whether T cells derived from HCB-treated rats could recognize the reactive metabolite TCBQ, conjugates of both TCHQ and TCBQ coupled to hemoglobin were incubated with total cell suspensions of spleen, ALN and MLN from control or HCB-exposed rats. However, cellular proliferation in the presence of the conjugates was not different from proliferation in the presence of hemoglobin or the medium only. In additional experiments, topical application of different concentrations of TCBQ (0.01, 0.1, 1 and 2% w/v) on the ears of control or HCB-exposed rats did not induce any ear swelling on the assessed time points (24 and 48 hours after application). Only application of 2% TCBQ caused in a moderate increase of ALN cell number, but this was similar in control

and HCB-exposed rats, with respective stimulation indices of 1.5 and 1.4. The challenge with TCBQ had no effect on relative T and B cell numbers in the ALN. Important to note, HCB itself already increased ALN cell numbers as compared to the control rats.

An adoptive transfer study was performed to assess if HCB-induced immune effects could be transferred to naive recipients. Transfer of splenocytes from HCB-exposed donor rats to recipients that were also exposed to HCB did not accelerate development, severity or area of skin lesions. Furthermore, adoptive transfer had no effect on other disease parameters (lung pathology, splenomegaly, LPS-induced TNF-α production, serum levels of IgM against ssDNA).

We also tested whether the enzyme MPO was able to metabolize HCB, but HPLC analyses of the incubation-mixture of HCB with the enzyme MPO could not detect any of the HCB metabolites.

Discussion

We previously demonstrated that T cells play an essential role in some of the HCB-induced adverse immune effects. Macrophages, however, appeared to be important as well *(5)*. Here we investigated the importance of macrophages in comparison to T cells in HCB-induced immunopathology. The present results show that macrophages are indeed important in HCB-induced skin lesions, lung eosinophilia and elevation of anti-ssDNA IgM. Data obtained in the kinetic study also suggest that macrophages are activated in lung and skin early after HCB exposure and that they play a role in HCB-induced clonal expansion of splenic B and T cells. Furthermore, no evidence was found for the induction of (specific) T cell sensitization and immunological memory after HCB exposure.

Skin lesions were not completely prevented in all HCB-exposed rats treated with clodronate-liposomes, but this is very likely due to incomplete macrophage depletion in some animals with the protocol we have used. Macrophage depletion was also not equally apparent in all organs, for instance macrophage infiltrations and granulomas were still noticed in the lung and the amount of macrophages in ALN and MLN was not diminished. The organ-specific depleting effect is in line with earlier findings showing that clodronate-liposomes initially eliminate liver and spleen macrophages without depleting circulating monocytes. However, it has been shown that a single injection of clodronate-liposomes slightly reduced macrophages in some LNs, such as the MLN *(29)*, indicating that the liposomes reach other organs too and repeated administration probably will only increase these effects. This is in accordance with our observation that clodronate-liposomes affect the function of macrophages before they are actually eliminated as illustrated by the

complete inhibition of LPS-induced TNF-α production in ALN and MLN of rats treated with clodronate-liposomes. An alternative explanation for the reduced macrophage activation in the ALN might be the relationship between ALN effects and skin lesions. These skin effects were less severe and as a consequence macrophage activation in ALN could be less pronounced.

Considering the observed differences in efficacy of macrophage depletion, we conclude that skin and lung pathology depend on macrophage activation. Together with the assumption that skin infiltrations of macrophages and neutrophils precede the actual macroscopically visible lesions kinetic data suggest that skin pathology results from an early non-specific inflammation. Previously, we have shown, by treating HCB-exposed rats with CsA, that T cells are also important in both the development of skin lesions and lung eosinophilia *(5)*. But in rats treated with CsA macrophages were still apparent in the lung infiltrates. Combined with present work it is concluded that these T cell-dependent phenomena are secondary to macrophage activation. A similar conclusion can be deduced from data of the kinetic study. These data show that macrophage activation precedes the increase of spleen weight. This seems to be in accordance with our previous data, showing that both HCB-induced macrophage influxes in the spleen as well as splenomegaly were T cell-independent *(5)*.

How T and B cells become activated after HCB exposure is not known, but cytokines, such as IL-6, produced by activated macrophages can induce polyclonal proliferation and activation of both T and B cells *(21)*. Unpublished *in vitro* data show that HCB is indeed able to activate rat alveolar macrophages, thereby inducing IL-6 and TNF-α production. After polyclonal expansion of B cells antibody-secreting cells will be generated. These will also include B-1 cells that produce antibodies against autoantigens, such as ssDNA. These splenic B-1 cells reside in the marginal zone of the spleen and activation of these B-1 cells seems to be under control of ED3+ macrophages *(4)*. Both ED3+ macrophages as well as B-1 cells were found to be activated by HCB *(23)*.

In order to elucidate the effects of macrophage depletion on T cell proliferation, cell suspensions were incubated with the T cell mitogen ConA, as it is well established that ConA-induced T cell proliferation requires interaction with macrophages *(11,12)*. Regarding the severe depletion of macrophages in the spleen of these animals the complete absence of a mitogenic response of splenocytes is not surprising. In accordance, the impairment of macrophage functions (i.e. lower TNF-α production after LPS stimulation) in the ALN and MLN of these animals may also explain the reduced mitogen-induced proliferation in comparison with HCB-exposed rats treated with PBS.

The results of the proliferative responses observed with splenocytes from HCB-exposed rats, however, are not that straightforward. Remarkably, proliferative responses in HCB-exposed animals were lower than in PBS-treated control animals and these responses appeared not affected by the clodronate-liposomes. The latter may be explained by the high variance and less efficient macrophage depletion correlated with higher proliferation rates. The lower responses of splenocytes of HCB-exposed animals are in accordance with earlier findings in Wistar rats *(24)* but they seem to be in contrast with our previous work that showed increased IL-2 production after HCB-exposure *(5)*. However, increased IL-2 production does not always result in increased cellular proliferation, as has been shown before *(20)*. In addition, the decreased mitogenic response of splenic T cells may be due to a decreased susceptibility to a mitogenic trigger of these T cells that have already proliferated vigorously *in vivo*.

The lack of evidence for specific T cell sensitization supports our view that after HCB exposure macrophage activation induced polyclonal T cell stimulation. T cells specific for TCBQ could not be detected. Furthermore, the fact that MPO was not able to metabolize HCB makes the involvement of reactive metabolites in HCB-induced immune effects not very likely. Especially, since previous work has shown that both cytochrome P450 oxidation and the mercapturic acid pathway were not involved in HCB-induced immune effects *(15,22)*. In addition, susceptibility for the HCB-induced immune effects could not be adoptively transferred. Notably, this experiment was performed only once and for adoptive transfer total spleens were used instead of purified T cells. However, nevirapine-induced skin rash in BN rats could be adoptively transferred by splenocytes using the same protocol *(26)*.

In conclusion, present work emphasizes the importance of macrophages as initiators of the HCB-induced adverse immune response. Presumably, HCB activates macrophages to produce pro-inflammatory mediators (i.e. IL-6, TNF-α) and reactive oxygen species. This is in accordance with gene expression profiles assessed with DNA microarrays *(6)*. Additionally, these, pro-inflammatory mediators will provoke a cascade of events, attracting granulocytes and macrophages, and inducing an inflammatory response. In agreement with this, transcriptome profiles have shown that after HCB exposure upregulation of gene expression for inflammatory markers was not confided to immunological organs, but involved also liver and kidney, suggesting a systemic inflammatory response. All these events may not only cause secondary cell damage and hence raise additional danger signals but also induce polyclonal T and B cell activation. Together, this will result in the observed HCB-induced immunopathology.

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Chapter 6

Summary and general discussion

Introduction

Hexachlorobenzene (HCB) is an environmental pollutant that can induce adverse immune effects in humans and rats (reviewed in refs. *16,26)*. Brown Norway rats (BN) appeared to be very susceptible to HCB-induced immune effects. Oral exposure causes inflammatory skin and lung lesions, enlarged spleen and lymph nodes (LN) and elevated humoral responses *(17)*. Although the immunopathological effects induced by HCB are investigated comprehensively, the exact mechanisms involved are unknown. Experiments described in this thesis were performed to obtain more insight in the mechanisms underlying HCB-induced adverse immune effects, both on a cellular and molecular level. We tried to obtain more information on the involvement of oxidative metabolites and the importance of both (memory) T cells and macrophages. Furthermore, experiments focused

on the early phase of HCB-induced immune effects to reveal which cells are important in the initiation stage.

Summary of results

In **chapter 2** the immunomodulatory effects of the oxidative metabolites tetrachlorohydroquinone (TCHQ) and tetrachlorobenzoquinone (TCBQ) were assessed. Therefore, these metabolites were studied in the mouse reporter antigen popliteal lymph node assay (RA-PLNA) with TNP-Ficoll as a RA. In this assay an isotype switch to IgG1 indicates that the chemical injected is able to form neoantigens that can stimulate T cells. Both TCHQ and TCBQ induced an isotype switch to IgG1 and were thus capable of providing neoantigen-specific T cell help. Furthermore, this response was compound-specific as was shown in a secondary PLNA.

In **chapter 3** we determined gene expression profiles with DNA microarray analysis in BN rats. Transcriptome profiling was done in spleen, mesenteric lymph node (MLN), thymus, blood, liver and kidney. Data 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 the upregulation of genes encoding pro-inflammatory cytokines, antioxidants, acute phase proteins, mast cell markers, complement, chemokines, and cell adhesion molecules. In general, gene expression data provide evidence that HCB induces a systemic inflammatory response, accompanied by oxidative stress and an acute phase response.

Chapter 4 describes experiments performed to investigate the role of T cells in HCB-induced immunotoxic effects. The immunosuppressive drug Cyclosporin A (CsA) was used to decrease peripheral T cell numbers and inhibit T cell activation. CsA treatment delayed the development of HCB-induced skin lesions and prevented the increase in spleen weight slightly. Furthermore, increase in ALN weight, lung eosinophilia and humoral responses were prevented completely by in HCB-exposed rats treated with CsA. Macrophage infiltration was independent of T cells and macrophages seem also important.

Therefore, the relationship between macrophages and T cells was further investigated in **Chapter 5**. Co-administration of clodronate-liposomes was used to eliminate macrophages in HCB-exposed rats. Results indicated that macrophages played an important role in HCB-induced skin lesions, in particular in the aggravation of skin lesions. Also, HCB-induced lung eosinophilia and elevation of anti-ssDNA IgM antibodies were less pronounced after depletion of macrophages. To obtain information on events that occur in the initiation phase a kinetic study was performed. This revealed that

both skin and lung pathology were early inflammatory events. Furthermore, macrophage infiltrations in the spleen seemed to precede the increase in spleen weight. Remarkably, studies performed to further assess the functional role and specificity of T cells induced by HCB did not provide any evidence for the presence of T cells specific for TCBQ. Moreover, HCB-induced immune effects could not be adoptively transferred to naive recipients. An overview of results obtained in Chapter 4 and 5 can be found in Table 1.

Table 1: Summary of T cell- and macrophage-dependent immune effects induced by HCB in the BN rat[1]

Immune effects		CsA study (**T cells**)	Clodronate-liposome study (**macrophages**)
Skin pathology	Initial development	- Delayed	- Prevented in 1 out of 6 rats
	Severity and lesional area	- Decreased	- Decreased in 4 out of 6 rats
Lung pathology	Macrophages and granulomas	- Not affected	- Prevented in 1 out of 6 rats
	Eosinophilic infiltration	- Prevented	- Moderately prevented in 5 out of 6 rats
Humoral responses	Increase in IgM against ssDNA	- Prevented	- Prevented
	Increased IgE levels	- Prevented	- Not investigated
Spleen effects	Enlargement	- Slightly prevented	- Depleted and necrotic
	Increased TNF-α production	- Prevented	- Prevented
ALN	Enlargement	- Prevented	- Not affected
	Increased TNF-α production	- Not investigated	- Prevented

[1]Results are derived from Chapter 4 (CsA study) and Chapter 5 (clodronate-liposome study)

Association between macrophages and T cells in HCB-induced immunopathology

One of the main findings in this thesis is that macrophages are important in HCB-induced adverse immune effects *(Chapter 5)*. But we have also demonstrated that the metabolite TCBQ can stimulate IgG1 responses to TNP-Ficoll in the mouse RA-PLNA, which indicates that TCBQ can induce non-cognate T cell help and thus form hapten-carrier complexes or neo-epitopes *(Chapter 2; ref. 10)*. However, after oral exposure to HCB no

hapten-specific T cells could be detected in BN rats and moreover HCB-induced immune effects could not be transferred to naive recipients *(Chapter 5)*. Hence, although HCB pathology could be partly prevented by inhibition of T cells with CsA *(Chapter 4 ref. 8)* HCB exposure does not lead to (specific) T cell sensitization and immunological memory *(Chapter 5)*. Furthermore, no evidence was found for the capability of MPO to convert HCB into reactive intermediates *(Chapter 5)*. In addition, previous studies have shown that reactive metabolites formed by cytochrome P450-catalyzed conversion of HCB *(19)* or generated in the mercapturic acid pathway *(15)* were not involved in the immune effects. Together, these data argue against involvement of reactive metabolites as initiators of HCB-induced immune effects.

Our present findings provide interesting additional information on the mechanism of HCB-induced immune derangements. We postulate that initially HCB activates macrophage in various organs, in particular the lung and skin. Notably, a previous toxicokinetic study showed that as soon as 3 hrs after a single oral dose of HCB peak concentrations of HCB were detected in the lungs (personal communication; D. Overstreet). This means that HCB is rapidly absorbed into the systemic circulation and distributed to the lung. Thus, early deposition of HCB may indeed be associated with the early presence of macrophages in the lungs. This accumulation of macrophages and also the formation of granulomas in the lung occurred independently of T cells *(Chapter 4; ref. 8)*.

Also in the spleen, macrophage infiltration and activation occurred early after exposure and appeared to precede the increase in spleen weight. Moreover, both HCB-induced splenomegaly and splenic macrophage influxes were T cell-independent *(Chapter 4; ref. 8)*. Based on these findings we suggest that after HCB exposure T cell and B cell are polyclonally activated by macrophages. The polyclonal nature of the B cell response is also illustrated previously by the increase of total IgM, IgG and IgE and anti-ssDNA IgM *(17,20)*. In addition, in this thesis we show that HCB increased gene expression of antibodies against the acetylcholine receptor and nerve growth factor *(Chapter 3; ref. 9)*. Notably, antibodies against ssDNA are produced by B-1 cells that are presumably activated by MZ macrophages *(3)*, and these B-1 cells have been found in increased numbers after HCB exposure *(20)*. The absence of IgG against ssDNA in HCB treated rats *(17)* indicates that specific T cell help is not sufficiently available and hence also supports the idea of polyclonal T cell activation.

Comparison with other models of chemical-induced adverse immune effects

Adverse immune effects induced by mercuric chloride and D-penicillamine

In the BN rat other immunostimulating compounds have been tested extensively, such as D-penicillamine and mercuric chloride ($HgCl_2$). Table 2 summarizes immune effects elicited by these compounds and illustrates the similarities and differences with HCB-induced immune effects *(5,7,16,22)*. All chemicals are capable of inducing skin lesions, splenomegaly, enlargement of LNs and polyclonal B (including rise in IgE levels) and T cell responses in BN rats. The main difference between HCB and the other chemicals is that the disease can be adoptively transferred to naive recipients in case of D-penicillamine and $HgCl_2$, but not in case of HCB. Furthermore, both D-penicillamine and $HgCl_2$ generate immune complexes and induce an isotype-switch to IgG against nuclear antibodies. Also, pronounced strain differences with respect to adverse immune reactions have been observed between BN and Lewis rats in response to D-penicillamine and $HgCl_2$ exposure. Both $HgCl_2$ and D-penicillamine induce autoimmunity in BN rats, but resistance to disease in Lewis rats. These two strains are different with respect to the polarization of their immune responses. After stimulation, T cells from BN rats preferentially produce type-2 cytokines, whereas T cells from Lewis rats predominantly secrete type-1 cytokines. After exposing BN rats to $HgCl_2$ and D-penicillamine self-MHC class II-reactive Th2 cells expand and induce development of clinical signs. These autoreactive T cells are also found in Lewis rats but in these rats they produce Th1 cytokines inducing immunosuppression (reviewed in refs: *11,13)*. HCB exposure induces more or less equal immune manifestations in both BN and Lewis rats as well as in outbred Wistar rats. However, BN rats are more susceptible to the induction of skin lesions. Furthermore, splenomegaly is more pronounced in this strain and the increase in total serum IgG and IgE is confined to this strain *(17)*.

Similar to HCB, some of the D-penicillamine- and $HgCl_2$-induced adverse immune effects are T cell-independent. For instance oxidative stress plays a role in the early vasculitis induced by $HgCl_2$ *(28)* and is thought to modulate mast cell function and IL-4 production *(27)*. Here we found that HCB also induces oxidative stress as shown by upregulation of genes coding for antioxidants and by the increased levels of hydroperoxides in the serum of HCB-exposed BN rats. However, the antioxidant N-acetylcysteine did not affect HCB-induced immune effects in BN rats (unpublished data).

Table 2: Immune effects induced in BN rats after exposure to HCB, D-penicillamine and $HgCl_2$

	HCB	D-penicillamine	$HgCl_2$
Skin lesions	+	+	+
Splenomegaly	+	+	+
Increase in serum IgE	+	+	+
IgG antinuclear antibodies	-	+	+
Lymphadenopathy	+	+	+
Immune complexes	-	+	+
Polyclonal B and T cell activation	+	+	+
Adoptive transfer of disease	-	+	+

Mast cells are thought to be essential for the production of IL-4 early after exposure to $HgCl_2$ *(27,28)*. Mast cells may also be activated in HCB-induced immune effects because transcriptome profiles revealed upregulation of several mast cell proteins. HCB exposure, however, does not result in increased IL-4 gene expression in Wistar rats *(25)* nor in the Th2-prone BN rats *(Chapter 3; ref. 9)*. However, gene expression of IL-25 is upregulated in the MLN of these HCB-exposed BN rats. This cytokine is produced by an unidentified non-T cell population and stimulates mast cells and highly polarized Th2 cells to produce Th2 cytokines that are essential for the expansion of eosinophils and elevation of IgE *(14)*.

Development of clinical effects after exposure to D-penicillamine can be inhibited by the iNOS inhibiting compound aminoguanidine, indicating the importance of nitric oxide (NO) *(18)*. NO has a suppressive effect on IFN-γ production, as has been shown previously in the BN model for $HgCl_2$ *(24)*. By inhibiting NO production the cytokine balance will be skewed to Th1, thereby preventing the development of Th2 autoimmunity. However, co-administration of aminoguanidine did not affect HCB-induced immune effects in BN rats (data not published).

Importantly, the immunostimulation induced after HCB exposure seems different from the autoimmune effects induced by D-penicillamine and $HgCl_2$.

Autoimmune-like effects induced by silica

Silica is a chemical that has adjuvant effects and is frequently associated with several autoimmune diseases, such as scleroderma and systemic lupus erythematosus *(1,4)*. Mechanisms are not completely clear, but the adjuvant activity of silica most likely plays

an important role. *In vitro* data provide evidence that this adjuvant activity could lead to polyclonal T cell activation *(23)*. The ability of silica to adjuvate immune responses is caused by a chronic activation of macrophages leading to secretion of pro-inflammatory mediators and cell death *(6)*.

Although both HCB and silica may act by causing macrophage-induced polyclonal lymphocyte activation the stimulatory effects on macrophages seem to be different between the two substances. In the RA-PLNA, for instance, silica induces a progressive stimulation, leading to an ongoing increase in PLN cell number. This is probably due to undegradable silica deposits in the footpad that continuously attract macrophages. Deposits and macrophage infiltrations were also observed after injection of HCB, but in contrast, HCB-deposits disappeared after 3 weeks. PLN cell number was only slightly increased by HCB, but in time cell number restored to control values *(10)*. An important difference between silica and HCB is that silica is completely resistant to degradation by macrophages, whereas HCB, although very inert, is probably eventually taken up by macrophages or due to its lipophilic nature dissolved in adipose tissue or cell membranes. However, *in vitro* data suggest that HCB (added to the cells as a suspension) activates macrophages similar to silica, inducing TNF-α and IL-6 production (unpublished data). Figure 1 shows that similar doses of HCB and silica can induce an equal increase of TNF-α production by rat alveolar macrophages.

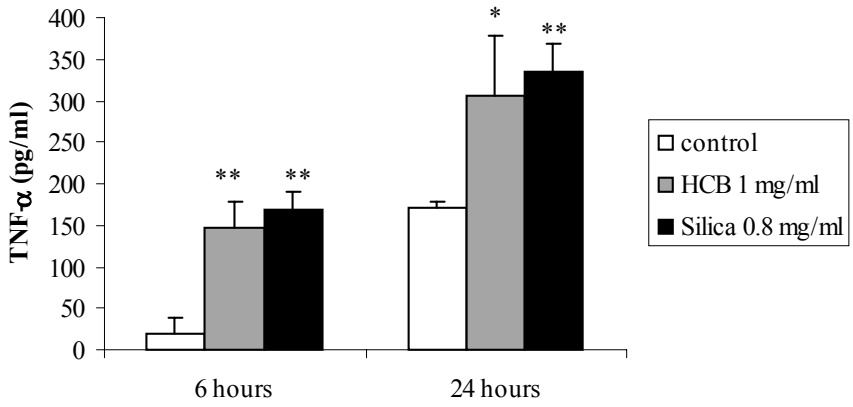

Figure 1: TNF-α release (pg/ml) after exposure of rat alveolar macrophages (NR8383 cell line) for 6 and 24 hours with HCB (1 mg/ml) and silica (0.8 mg/ml). Significantly different from the control group * $p<0.05$ and ** $p<0.01$.

General conclusions

In conclusion, we propose that HCB deposition initially attracts and activates macrophages, thereby generating pro-inflammatory adjuvant signals that induce a systemic inflammatory response with influxes of neutrophils and more macrophages in various non-immune and immune organs. Subsequently, this leads to polyclonal activation of T and B cells, eosinophilia and eventually to visible clinical effects.

What exactly elicits the attraction of macrophages is quite speculative, but HCB deposition can directly induce cell damage, or elicit damage by interfering with the integrity of cell membranes due its lipophilic nature. Cell damage or products released by dying cells can activate macrophages. Figure 2 shows that if macrophages become activated they may release pro-inflammatory mediators, such as TNF-α, IL-1β, IL-6, reactive oxygen species (ROS) and chemokines. These pro-inflammatory mediators are known to be capable of inducing a cascade of events, including upregulation of cell adhesion molecules, thereby facilitating recruitment of other inflammatory cells. Furthermore, pro-inflammatory cytokines may also stimulate T cell responses *(2)*.

Immunostimulation of autoreactive T cells by adjuvant or danger signals is explained by the so-called 'bystander' hypothesis. This hypothesis postulates that dysregulated macrophage activation can lead to expansion of pre-existing autoreactive T cells and B cells (reviewed in ref. *21)*. Evidently in such a situation regulatory mechanisms (i.e. regulatory T cells) are not sufficient to control these pre-existing autoreactive T cells and B cells *(12)*.

In conclusion, the HCB-induced immunopathology supports the notice that the etiology of immune derangements induced by xenobiotics is very complex. It also demonstrates that different compounds can have apparently similar adverse effects that are elicited by a different mode of action.

In general, the BN rat-model for HCB-exposure is an interesting addition to other existing BN rat-models, and illustrates that this strain can be very useful as a candidate for predictive testing of possible immunostimulatory xenobiotics.

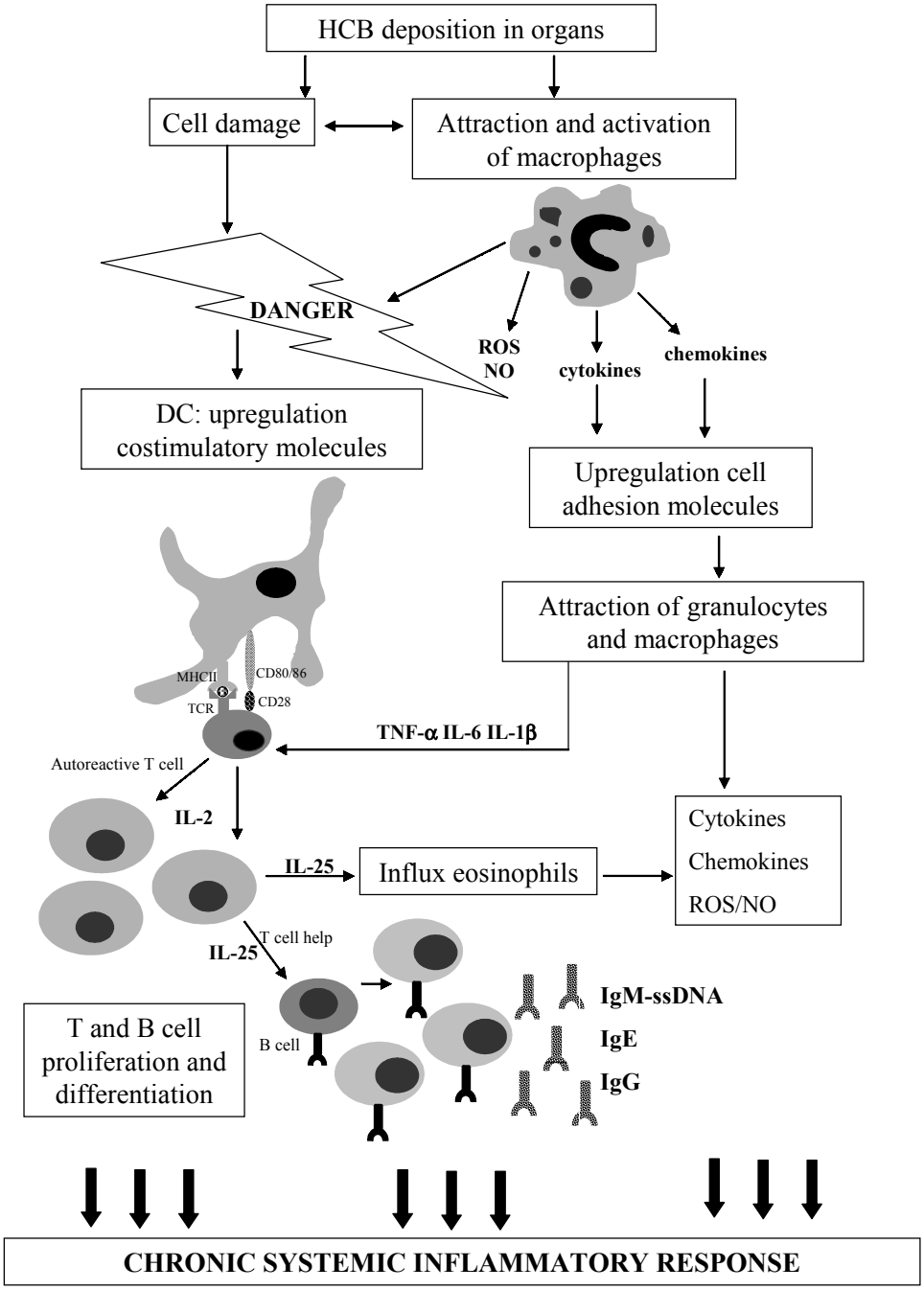

Figure 2: Proposed mode of action of HCB-induced adverse immune effects

Future directions

A lot of additional information on HCB-induced immune effects was obtained with DNA microarrays. However, these were performed after 28 days of exposure, when pathology was already full-blown. In this stage it is very difficult to distinguish between primary and secondary effects. Therefore, it would be very useful to study gene expression profiles early after exposure. This will provide information on which events are important for the initiation of the immune response. DNA microanalysis combined with pathology should be performed in particular in the lung and skin but also in spleen, various lymph nodes (including lymph nodes draining the skin, lung and intestines). This knowledge on early mechanisms may help to clarify unsolved questions on HCB-induced effects, such as the order of events that take place after HCB exposure. This may elucidate that the immune response is initiated locally in the skin and lung. Furthermore, additional information on markers for cytotoxicity may be detected and this could strengthen our hypothesis that the immune response may be elicited by cell damage.

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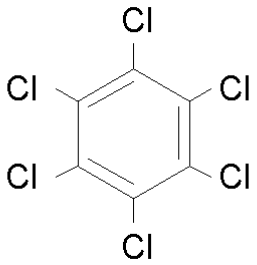
Nederlandse samenvatting

Hexachloorbenzeen

Hexachloorbenzeen (HCB; C_6Cl_6) is een organochloorverbinding (Figuur 1) die in het verleden werd gebruikt als bestrijdingsmiddel tegen schimmels. Mede door de schadelijke effecten in zowel mens als dier is het gebruik van HCB in de jaren ’70 in de meeste landen verboden. Tegenwoordig komt HCB nog steeds vrij in het milieu, omdat het wordt gevormd als tussenproduct tijdens verschillende industriële processen. HCB is een chemisch erg stabiele stof, die moeilijk wordt afgebroken in het milieu, waardoor het accumuleert in water, bodem, en sediment. HCB kan ook teruggevonden worden in weefsels van onder andere vissen en vogels, maar ook in menselijk vetweefsel en moedermelk.



Figuur 1: Chemische structuur van hexachloorbenzeen

Al decennia lang wordt er onderzoek verricht naar de giftige effecten veroorzaakt door HCB. Aanleiding voor dit onderzoek was een vergiftiging die plaats vond in Turkije in de jaren ’50. Tussen de 3000 en 5000 mensen werden ziek, omdat ze brood hadden gegeten dat abusievelijk was gebakken van zaaigraan dat was behandeld met HCB. De meeste slachtoffers ontwikkelden een aandoening aan de lever (porfyrie) met als gevolg dat de lever vergroot was en blaarvormige huideffecten ontstonden op plekken die blootgesteld waren aan de zon. Andere symptomen waren schildklier en milt vergroting, neurologische verschijnselen en gewrichtsontstekingen. Jonge kinderen, die via de moedermelk waren blootgesteld aan HCB, ontwikkelden een zeer ernstige aandoening. Symptomen waren diarree, braken, vermagering, ontsteking van de huid, lever vergroting en infiltraten in de longen. Meer dan 95% van deze kinderen overleefden de vergiftiging niet en overleden aan hart-long falen.

In vervolgonderzoek in proefdieren konden veel van de effecten die werden waargenomen in mensen, ook worden gevonden in proefdieren, zoals porfyrie, lever en milt vergroting en ontstekingen van de huid en longen. Uit deze studies bleek dat HCB ook schadelijke effecten heeft op het immuunsysteem. Dit is in overeenstemming met waarnemingen Turkije, maar ook in Brazilië werden effecten op het immuunsysteem waargenomen bij werknemers die in een fabriek werden blootgesteld aan HCB.

Schadelijke effecten van HCB op het immuunsysteem

HCB is een stof met een laag moleculair gewicht en van verscheidene andere chemicaliën met een laag moleculair gewicht, zoals bepaalde geneesmiddelen, is bekend dat ze het immuunsysteem kunnen verstoren. In het intermezzo is getracht uit te leggen hoe het immuunsysteem werkt en hoe chemische stoffen dit systeem kunnen verstoren.

De effecten van HCB op het immuunsysteem zijn voornamelijk bestudeerd in de rat. Uit eerder onderzoek is gebleken dat vooral de Brown Norway (BN) rat erg gevoelig is

voor immuuneffecten veroorzaakt door HCB. Deze rattenstam wordt vaker gebruikt om de effecten van chemische stoffen op het immuunsysteem te onderzoeken, omdat het immuunsysteem al iets actiever is dan in andere rattenstammen. Zo veroorzaken sommige geneesmiddelen autoimmuniteit alleen in BN ratten. Als BN ratten via het voedsel worden blootgesteld aan HCB ontwikkelen ze ontstekingsreacties in de huid en longen. Deze ontstekingen worden gekenmerkt door de aanwezigheid van allerlei ontstekingscellen zoals macrofagen en granulocyten. Daarnaast zijn de milt en lymfknopen vergroot en geactiveerd. In het serum zijn antistoffen verhoogd, hetgeen ook een indicatie is voor een stimulatie van het immuunsysteem.

Ondanks het onderzoek dat in het verleden is uitgevoerd is het nog steeds onduidelijk hoe HCB het immuunsysteem activeert. Het doel van de experimenten beschreven in dit proefschrift is om hier meer inzicht in te krijgen.

Intermezzo

Het immuunsysteem speelt een zeer belangrijke rol bij het herkennen en bestrijden van ziekteverwekkers, zoals bacteriën, virussen en parasieten. In wezen kan het immuunsysteem worden opgedeeld in twee categorieën: het ‘innate’ of aangeboren immuunsysteem en het adaptieve immuunsysteem. Het ‘innate’ immuunsysteem, is erg belangrijk voor de eerstelijnsafweer tegen allerlei ziekteverwekkers. De ‘innate’ immuuncellen herkennen patronen die aanwezig zijn op bijvoorbeeld bacteriën en worden hierdoor gewaarschuwd over de ‘indringers’. Vervolgens zullen ze proberen deze te vernietigen. ‘Innate’ immuuncellen zijn bijvoorbeeld macrofagen of granulocyten. Na activatie scheiden deze cellen producten uit waarmee ze de ‘indringers’ willen uitschakelen. Tevens produceren ze stoffen waarmee ze andere cellen waarschuwen om ook in actie te komen. Een voorbeeld hiervan is het uitscheiden van cytokines, waardoor bijvoorbeeld T cellen kunnen worden geactiveerd. T cellen horen bij het adaptieve immuunsysteem. De kenmerken hiervan zijn een hoge mate van specificiteit voor de vreemde structuur (=antigeen) en de ontwikkeling van geheugen. Na stimulatie van het adaptieve immuunsysteem ontstaat er een immunologisch geheugen voor het antigeen dat de T cellen hebben herkend. Als gevolg hiervan zal bij een tweede blootstelling dit antigeen veel sneller worden herkend en beter worden bestreden, hetgeen de basis is voor vaccinaties tegen infectieziektes.

Behalve ziekteverwekkers kunnen ook bepaalde chemische stoffen met een laag moleculair gewicht het immuunsysteem stimuleren, zoals bepaalde geneesmiddelen. Dit leidt meestal tot ongewenste reacties, zoals allergie of auto-immune afwijkingen. Een allergische reactie is gericht tegen de chemische stof zelf. Auto-immune afwijkingen

kunnen ontstaan als er onder invloed van de chemische stof een afweerreactie wordt opgewekt tegen lichaamseigen structuren. Er bestaat nog steeds veel onduidelijkheid over hoe chemische stoffen dit soort ongewenste reacties opwekken.

Het immuunsysteem onderscheidt indringers (vreemd) van het eigen lichaam (zelf) en reageert onder normale omstandigheden alleen maar op vreemd. Voor het herkennen van een antigeen worden delen hiervan gepresenteerd aan T cellen door bijvoorbeeld macrofagen. De T cel is zo geprogrammeerd dat hij kan onderscheiden of iets vreemd of zelf is. Als een T cel iets vreemds herkent zal deze geactiveerd worden. Voor complete activatie zijn echter ook extra activeringssignalen nodig, dit wordt co-stimulatie genoemd. Allerlei signalen kunnen co-stimulatoire signalen geven aan de T cel, bijvoorbeeld schade aan lichaamseigen structuren door infectieuze agentia of chemische stoffen. Dit worden gevaarsignalen genoemd. Als er geen co-stimulatie aanwezig is, zal de T cel niet geactiveerd worden.

Een chemische stof kan niet worden gepresenteerd aan een T cel. Echter, als een stof in staat is om in het lichaam aan een eiwit te binden zullen delen van deze nieuwe combinatie gepresenteerd worden aan een T cel. Deze nieuwe combinatie kan als een vreemd antigeen worden herkend. Als er dan ook voldoende gevaarsignalen aanwezig zijn, kan er een immuunreactie ontstaan. De T cellen zullen hierdoor geactiveerd worden en er zullen ook ‘geheugen’ cellen worden gevormd. Er bestaan ook chemische stoffen die zonder de hulp van T cellen een negatieve immuunreactie kunnen opwekken. Meestal activeren deze stoffen macrofagen, bijvoorbeeld omdat ze schade veroorzaken. Door activatie van macrofagen zullen allerlei producten, zoals cytokines, worden uitgescheiden die kunnen leiden tot activatie van andere cellen. Meestal ontstaat hierdoor een ontstekingsreactie. De symptomen die hierdoor worden veroorzaakt kunnen wel lijken op allergie of auto-immuniteit.

In **hoofdstuk 1** wordt een algemeen overzicht gegeven van de nadelige effecten van HCB op het immuunsysteem. Daarnaast wordt in dit hoofdstuk besproken op welke manieren chemische stoffen het immuunsysteem kunnen ontregelen. In **hoofdstuk 2** is onderzocht of HCB of stoffen die ontstaan als HCB wordt omgezet in het lichaam (metabolieten) immuunstimulatie kunnen veroorzaken. Hiervoor is een muizenmodel gebruikt dat veelvuldig wordt toegepast om op een snelle en eenvoudige manier te onderzoeken of een stof het immuunsysteem stimuleert. HCB en zijn metabolieten, tetrachlorohydrochinone (TCHQ) en tetrachlorobenzochinone (TCBQ), werden ingespoten in de voetzool van een muis. Na 7 dagen werd de reactie gemeten in de lymfknoop die in de knieholte zit. Stoffen die immuunstimulerend zijn en T cellen kunnen activeren zullen een positieve reactie

veroorzaken in de lymfknoop. Dit leidt dan tot een vergroting van de lymfknoop en daarnaast zullen de cellen in de lymfknoop antistoffen gaan produceren. HCB zelf had geen effect op de lymfknoop. Dit lag ook in de lijn der verwachting, omdat HCB zelf niet herkend wordt door het immuunsysteem en het ook niet in staat is om te binden aan eiwitten. De metabolieten TCHQ en TCBQ gaven wel een positieve reactie. Uit voorgaand onderzoek was al gebleken dat deze stoffen aan eiwitten kunnen binden. Uit dit onderzoek blijkt dat ze dus ook in staat zijn om nieuwe antigenen te vormen, die in dit muizenmodel T cellen activeren. Het is wel belangrijk om te beseffen dat de route van toediening van deze snelle test niet gelijk is aan de wijze waarop mensen en ratten worden blootgesteld (via de voeding). In de vervolgstudies werd dan ook de BN rat gebruikt om de effecten van HCB verder te onderzoeken, omdat in dit model de dieren werden blootgesteld via het voedsel.

In **hoofdstuk 3** werden BN ratten blootgesteld aan HCB in twee verschillende doseringen, een lage en een hoge dosis. Na 4 weken werden milt, thymus (zwezerik), mesenteriale lymfknoop, bloed, lever en nier verzameld en werd hieruit het mRNA (=genetisch materiaal) gehaald. Met behulp van een recent ontwikkelde techniek is gemeten welke genen geactiveerd werden door HCB. Deze techniek wordt genomics genoemd. Met behulp van zogenaamd DNA microarrays kunnen tegelijkertijd ongeveer 8000 genen gemeten worden. Dit betekent dat er buitengewoon veel informatie kan worden verkregen over de effecten van een toxische stof op verschillende organen. Met deze nieuwe techniek konden we een aantal bekende effecten van HCB aantonen, zoals stimulatie van het immuunsysteem en effecten op de lever (porfyrie). Verder zijn er verscheidene nieuwe bevindingen gedaan, onder andere dat verscheidene cytokines die door macrofagen worden geproduceerd in verhoogde mate gemeten konden worden na blootstelling aan HCB. In het geheel genomen verschaft deze studie inzicht in het effect van HCB op verschillende organen en geeft informatie over welke factoren hierbij een belangrijke rol spelen. De algehele conclusie is dat HCB een systemische (dus in het hele lichaam) ontstekingsreactie veroorzaakt waarbij door macrofagen geproduceerde factoren een belangrijke rol spelen.

In **hoofdstuk 4** is gekeken naar de rol van T cellen in de immuuntoxiciteit veroorzaakt door HCB. Ook hiervoor werden BN ratten gebruikt, die 3 weken werden blootgesteld aan HCB. Om T cellen te blokkeren werd Cyclosporine A (CsA) gebruikt, dit is een immunosuppressief (=remt het immuunsysteem) geneesmiddel dat in de kliniek wordt gebruikt om afstootreacties na transplantaties te voorkomen. CsA heeft als effect dat er minder T cellen aanwezig zijn in het lichaam en remt tevens de activatie van de nog aanwezige T cellen. Uit deze experimenten bleek dat T cellen belangrijk zijn voor de

ontwikkelen van de huideffecten en sommige longeffecten en voor de verhoogde antistof productie in het serum. Echter de effecten van HCB op de milt konden maar voor een deel worden geremd en de aantrekking van macrofagen naar zowel milt als longen werd ook niet beïnvloed.

Om het verband tussen macrofagen en T cellen verder te onderzoeken werden er in **hoofdstuk 5** verschillende experimenten uitgevoerd. Om macrofagen te verwijderen is er gebruik gemaakt van clodronaat liposomen. Dit zijn vetbolletjes met hierin een giftige stof (clodronaat). Macrofagen ruimen deze bolletjes op, maar gaan dood door het clodronaat. Hiermee konden we aantonen dat ook macrofagen een belangrijke rol spelen bij het verergeren van de huideffecten. Daarnaast spelen macrofagen een rol bij de longontstekingen en bij de verhoogde gehaltes van antistoffen in het serum. Om meer inzicht te verkrijgen in de vroege fase van de immuunreactie werd er een experiment uitgevoerd waarbij is gekeken naar de effecten van HCB vroeg na blootstelling. Hieruit bleek dat de door HCB veroorzaakte ontstekingen in de huid en longen al vroeg na HCB toediening optraden en dat macrofagen al snel aanwezig waren in deze organen. Dit duidt erop dat deze cellen een belangrijke rol spelen bij de effecten die worden veroorzaakt door HCB. In dit hoofdstuk is ook nog onderzocht of er na blootstelling aan HCB een ‘geheugen” is ontstaan voor de metabolieten van HCB. Van deze metabolieten is namelijk bekend dat ze kunnen worden herkend door T cellen (**hoofdstuk 2**). Echter, er werd geen bewijs gevonden voor de aanwezigheid van een ‘geheugen’ na blootstelling van BN ratten aan HCB.

In **hoofdstuk 6** worden de in dit proefschrift gevonden resultaten samengevat en bediscussieerd. De conclusie is dat HCB nadat het is opgenomen door het lichaam waarschijnlijk macrofagen aantrekt, waarschijnlijk omdat HCB schade veroorzaakt aan het lichaamseigen structuren en er op deze manier gevaarsignalen ontstaan (zie Figuur 2, **hoofdstuk 6**). Dit zal leiden tot de productie en uitscheiding van verscheidene producten, zoals cytokines. Hierdoor kunnen T cellen worden geactiveerd, maar ook andere immuuncellen. Hierdoor zal een chronische ontstekingsreactie ontstaan in meerdere orgaansystemen.

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Curriculum Vitae

Janine Ezendam werd geboren op 14 januari 1975 te Rijssen. In 1993 behaalde zij haar vwo diploma op College Noetsele, te Nijverdal. In datzelfde jaar begon zij aan haar studie Biomedische Gezondheidswetenschappen aan de Universiteit van Nijmegen. Tijdens de doctoraalfase specialiseerde zij zich in de Toxicologie en liep stage bij 3 vakgroepen. Haar eerste stage vond plaats bij de afdeling Reumatologie van het academisch ziekenhuis in Nijmegen onder begeleiding van Dr. Peter van Lent. Vervolgens liep zij stage bij de afdeling Dermatologie van het academisch ziekenhuis in Nijmegen onder begeleiding van Dr. Piet van Erp. Haar laatste stage vond plaats in Madrid, Spanje, waar zij werkte op het Laboratorio de Inmunologia van de Fundación Jiménez Diaz, onder begeleiding van Prof. Dr. Carlos Lahoz en Dr. Blanca Cardaba. Aansluitend op het behalen van haar doctoraal diploma heeft zij als AIO gewerkt op de afdeling Farmacologie-Toxicologie van de Universiteit Nijmegen. Na anderhalf jaar besloot zij een andere richting in te slaan en is als AIO begonnen aan het onderzoek beschreven in dit proefschrift. Dit onderzoek was een samenwerking van het Institute for Risk Assessment Sciences te Utrecht, het Rijksinstituut voor Volksgezondheid en Milieu, te Bilthoven en de afdeling Pathobiologie van de faculteit Diergeneeskunde, Universiteit Utrecht en werd begeleid door Dr. Raymond Pieters en Prof. Dr. Sjef Vos.