The Role of the Immune System in Hexachlorobenzene-Induced Toxicity

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Hexachlorobenzene (HCB) is a persistent environmental pollutant. The toxicity of HCB has been extensively studied after an accidental human poisoning in Turkey and more recently it has been shown that HCB has immunotoxic properties in laboratory animals and probably also in man. Oral exposure of rats to HCB showed stimulatory effects on spleen and lymph node weights and histology, increased serum IgM levels, and an enhancement of several parameters of immune function. Moreover, more recent studies indicate that HCB-induced effects in the rat may be related to autoimmunity. In Wistar rats exposed to HCB, IgM antibodies against several autoantigens were elevated; in the Lewis rat, HCB differently modulated two experimental models of autoimmune disease. Oral exposure of rats to HCB induces skin and lung pathology in the rat. Recently several studies have been conducted to investigate whether these skin and lung lesions can be related to HCB-induced immunomodulation, and these studies will be discussed in this review. HCB-induced skin and lung lesions probably have a different etiology; pronounced strain differences and correlation of skin lesions with immune parameters suggest a specific involvement of the immune system in HCB-induced skin lesions. The induction of lung lesions by HCB was thymus independent. Thymus-dependent T cells were not likely to be required for the induction of skin lesions, although T cells enhanced the rate of induction and the progression of the skin lesions. No deposition of autoantibodies was observed in nonlesional or lesional skin of HCB-treated rats. Therefore, we concluded that it is unlikely that the mechanism by which most allergic or autoimmunogenic chemicals work, i.e., by binding to macromolecules of the body and subsequent T- and B-cell activation, is involved in the HCB-induced immunopathology in the rat. Such a thymus-independent immunopathology is remarkable, as HCB strongly modulates T-cell–mediated immune parameters. This points at a very complex mechanism and possible involvement of multiple factors in the immunopathology of HCB. *Key words*: autoantibodies, hexachlorobenzene, immunopathology, immunotoxicity, lung, rat, skin. — *Environ Health Perspect* 107(suppl 5):783–792 (1999).
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In this review, following a short introduction on the general properties and general toxicity of hexachlorobenzene (HCB), the role of the immune system in HCB-induced toxicity is discussed. The immunotoxic properties of HCB were observed in several animal studies performed after an accidental poisoning in Turkey (described in detail in this article). The presence of autoantibodies in rats exposed to HCB, as well as clinical features such as enlarged thyroid and rheumatoid arthritis of the hands observed in victims involved in the poisoning incident, prompted the hypothesis that the immune system, including autoimmunity, plays a major role in HCB-induced toxicity.

General Properties and Metabolism of HCB

HCB (C_6Cl_6) is a chlorinated organic compound used extensively as a fungicide for the treatment of seed grain but is prohibited for such use in most countries since 1970. HCB has been used directly and as a chemical intermediate in many industrial processes, including applications as a fluxing agent in aluminum smelting, as a peptizing agent in the rubber industry, and in the manufacture of dye (*1*). Currently, considerable amounts of HCB are generated as waste byproducts of several industrial processes and subsequently emitted into the environment. Jacoff and Scarberry (*2*) estimated that in the United States over 4,000 tons of HCB are generated each year as a waste byproduct mainly from the manufacture of chlorinated solvents. HCB can be easily distributed through the environment because of its volatility and resistance to degradation. The long-distance distribution of HCB via the troposphere is an especially important route of transportation (*3*). Because of its chemical stability and high persistence, HCB readily accumulates in food chains (*4,5*). For example, a significant biomagnification has been reported in field studies in natural aquatic ecosystems (*6*) and in predatory birds (*7*). HCB is present in human adipose tissue, breast milk, and blood (*8–10*). The major source of HCB exposure of the general population today is as a contaminant in the diet (*11,12*).

There is no published information on the elimination half-life of HCB in humans (*12*). Studies in experimental animals have shown that excretion of HCB occurs mainly via the feces (*13*) and half-lives of 1 month in rats and rabbits and 2.5–3 years in rhesus monkeys have been reported (*14*). Studies in a number of animal species showed that a small portion of ingested HCB is metabolized and the remainder is stored in adipose tissue or excreted via the feces (*15–17*). Two major metabolic pathways of HCB in the liver are responsible for the metabolism of HCB. In the mercapturic acid pathway, conjugation of HCB to glutathione leads to formation of the urinary end product *N*-acetyl-*S*-(pentachlorophenyl)-cysteine (PCP–NAC) (*18,19*). In addition, HCB is degraded by cytochrome P450-catalyzed oxidative dehalogenation to the end products pentachlorophenol (PCP) and 1,4-tetrachlorohydroquinone (TCHQ) (*15,20*).

General Toxicity of HCB

Porphyria is regarded as the major potential toxic manifestation of HCB in experimental animals and man. An outbreak of HCB-induced porphyria occurred in Turkey in the 1950s and will be described in detail in the next section. HCB-induced hepatic porphyria is characterized by a deficiency of the enzyme uroporphyrinogen decarboxylase resulting in the accumulation of porphyrins in the liver and increased urinary excretion of highly carboxylated porphyrins (*21,22*). Since the accidental poisoning in Turkey, many attempts have been made to induce hepatic porphyria by administration of HCB to laboratory animals. The rat has been used in several studies and many similarities have been observed between clinical disease in humans and in the rat (*23,24*). HCB-induced porphyria in birds is also comparable to the disease in mammals (*25,26*).

Laboratory animal studies revealed that chronic exposure to HCB could induce liver-cell tumors in rats and mice (*27–29*), renal adenomas in rats (*30*), and liver-cell tumors, haemangioendotheliomas, and thyroid

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adenomas in hamsters (*31*). HCB acts as a promoter of cancer in several studies (*32,33*). Investigation of the cancer incidence among people living in a small village in Spain that was located near a chlorinated solvents factory revealed increased incidence of thyroid neoplasms and soft-tissue carcinomas in males (*34*). On the basis of evidence for carcinogenicity of HCB to laboratory animals and the limited information for carcinogenicity to humans, the International Agency for Research on Cancer has classified HCB as a group 2B carcinogen (possibly carcinogenic to humans) (*35*).

The reproductive effects of HCB have been studied in several animal models. Iatropolous et al. (*36*) demonstrated severe changes in ovarian structure, consisting of follicular degeneration in primordial germ cells and stratification of the epithelium, in rhesus monkeys orally administered HCB. Similar effects have been observed in cynomolgus monkeys exposed to HCB via feed. However, despite the changes in ovarian tissue, the latter monkeys were capable of producing embryos after induction of superovulation, oocyte recovery, and maturation followed by *in vitro* fertilization (*37*). Studies in superovulated female Sprague-Dawley rats fed HCB showed significant elevated serum progesterone levels, whereas there was no effect on serum estradiol levels and uterine weight (*38*). In a subsequent study in adult ovariectomized Sprague-Dawley rats fed the same doses of HCB, it was demonstrated that HCB induces alterations in adrenal steroidogenesis of the rat (*39*). The male reproduction system was affected only by repeated exposure to very high doses of HCB (*40,41*).

In several animal studies, lactational transfer of HCB has been shown to be important in affecting fetus and nursing offspring. A four-generation study in Sprague-Dawley rats showed maternal deaths and a reduced fertility index at the two highest dose levels of HCB, whereas lower doses of HCB caused increased stillbirths and decreased pup survival (*42*). Mink appeared to be particularly sensitive to the toxicity of prenatal HCB exposure; a low dose of HCB resulted in reduced birth weights and increased mortality (*43,44*). A recent study among Turkish women exposed or not exposed to HCB during the accidental poisoning in the 1950s showed an increased risk of spontaneous abortion related to high serum HCB levels but not restricted to women with identifiable exposure (*45*).

Neurologic effects of HCB have been reported in victims of the poisoning incident in Turkey and in short-term as well as in chronic exposure studies in various animal species including rats, rabbits, and guinea pigs (*46*). In adult Japanese quails fed diets containing HCB, tremors have been reported, but histology showed no pathology of the central nervous system (*25*). Reported neurotoxic effects in rats exposed to HCB are hyper-excitability, tremors, weak legs, and paresis (*23,47*). However, no indications for histopathologic changes in the brain, spinal cord, motor and sensory nerves, and skeletal muscles have been found (*48,49*).

Accidental Human Poisoning in Turkey

From 1955 to 1959, approximately 3,000–5,000 people in southeastern Turkey who ingested HCB-treated seed grain developed a disease characterized by hepatic porphyria called porphyria turcica (*50,51*). Porphyria turcica resembled porphyria cutanea tarda, a disease of disturbed porphyrin metabolism manifested by cutaneous lesions; patients showed bullous skin lesions, mainly in sun-exposed skin areas that healed with severe scars. The skin lesions were attributed to the toxicity of photochemically activated cutaneous porphyrins (*52*). Porphyria turcica primarily affected children 6–16 years of age, with only 10% of the patients over 16 years of age (*53,54*). In addition to the disturbance in porphyrin metabolism and dermatologic changes, other reported clinical manifestations included hepatomegaly, enlarged thyroid, splenomegaly, hyperpigmentation, hirsutism, and enlarged lymph nodes. Victims also showed neurologic symptoms such as paresthesia, sensory shading, "cogwheeling," and myotonia. Painless arthritic changes of the hands were observed in 36% of the children 6–16 years of age, and a follow-up study 3–5 years later revealed a further increased incidence of 55% of these children showing such changes (*54,55*).

Porphyria turcica was rare in victims less than 4 years of age. In these infants a condition called Pembe Yara has been described, characterized by rose-red skin lesions on arms and legs, enlarged livers, diarrhea, and fever. There was a high mortality (> 95%) among young children who were exposed to HCB via the placenta or maternal milk (*51,53,54*). These young children developed skin lesions in the absence of porphyria. The skin lesions apparently did not resemble the porphyria-related etiology of the bullous skin lesions observed in the older victims of the poisoning incident. In follow-up studies among 204 victims 25–30 years after the poisoning incident, dermatologic abnormalities, neurologic symptoms, enlarged thyroid, and painless arthritis of the hands still persisted (*56,57*). For these clinical features an immune etiology is conceivable, and such an etiology could also be involved in the porphyria-independent skin lesions of the children with Pembe Yara. Therefore, several studies in laboratory animals have been performed to elucidate the immunotoxic properties of HCB.

Immunomodulation of HCB

Several experimental studies have been performed to investigate the immune effects of HCB, with special emphasis on the functional immune effects in mice and rats. Whereas in the rat most assessed parameters of immune function were enhanced after oral exposure to HCB, in mice the reverse was true; most assessed parameters of immune function were suppressed after oral HCB exposure. Tables 1 and 2 give a summary of the reported immunotoxic effects of HCB in rats and mice. Our animal studies were approved by an ethical committee of the institute and conducted in accordance with the *Guiding Principles in the Use of Animals in Toxicology* (*58*). Briefly, rats were housed at the Utrecht University animal facilities and kept in pairs in filter-topped macrolon cages on wood-chip bedding under standard conditions (50–60% relative humidity, 12-hr dark/12-hr light cycle). The animals had free access to food and acidified water.

HCB-Induced Immunomodulation in the Rat

Reported dose-related immune effects of HCB in male and female Wistar rats are increased spleen and lymph node weights; increased total serum IgM, IgG, and IgA levels; and increased peripheral blood neutrophilic and basophilic granulocytes and monocytes (*59–62*). Histopathologic examination of the spleen and lymph nodes showed increased extramedullary hemopoiesis in the red pulp and hyperplasia of B lymphocytes in marginal zones and follicles of the spleen as well as an increase in high endothelial venules in the lymph nodes (*49,50,59*). Functional assessment of the immune system showed no significant effect of HCB on the phagocytizing and killing capacity of macrophages as shown by *Listeria monocytogenes* mortality assay and clearance of colloidal carbon. In addition, no effect of HCB on cell-mediated immunity, as measured by delayed-type hypersensitivity and skin graft rejection, has been observed. However, HCB induced a stimulation of humoral immunity, as measured by increased primary and secondary IgM and IgG responses to the thymus-dependent antigen tetanus toxoid. In contrast, the thymus-independent IgM response to lipopolysaccharide (LPS) remained unchanged (*59,60,63*).

Slight changes in humoral and pulmonary cellular defenses have been observed in a study that investigated the effect of single or multiple inhalation exposures to HCB (*64*) in male Sprague-Dawley rats. Recently it was shown that exposure of Wistar rats to

Table 1. Summary of the immunotoxic effects of hexachlorobenzene in rats.

Parameter	Study	Dose[a]	Effect[b]	Reference
Peripheral neutrophilic and basophilic granulocytes, and monocytes	Wistar	1000	↑	(59)
Serum IgM levels	Wistar, BN,[c] Lewis,[c]	300[d], 450, 900, 1000	↑	(59,61,62,72)
Serum IgG and IgE levels	BN	450	↑	(72)
Serum IgA levels	Wistar	300[d]	↑	(62)
Serum IgM against ssDNA, dsDNA, rat IgG, phosphatidylcholine	Wistar	500, 1000	↑	(61)
Serum IgM against ssDNA	BN, Lewis	150[e], 450	↑	(72)
Spleen and lymph node weights	Wistar, BN, Lewis	150–2000[f]	↑	(59,61,62,72)
Marginal zones and follicles of spleen	Wistar	500, 1000	↑	(59,61)
Extramedullary hemopoiesis	Wistar	500, 1000	↑	(60,63)
High endothelial venules in lymph nodes	Wistar, BN,[c] Lewis[c]	150, 450, 900, 1000	↑	(59,72)
IL-2 and IFN-γ mRNA of spleen cells	Wistar	150, 450	↑	(65)
IL-2R mRNA of spleen cells	Wistar	450	↑	(65)
Primary and secondary IgM and IgG against tetanus toxoid	Wistar	1000	↑	(59)
Mitogenic response of spleen cells to ConA, PHA, and LPS	Wistar	1000	↑	(59)
Susceptibility to endotoxin (*Escherichia coli*)	Wistar	1000	↑	(59)
Natural killer cell activity in the lung	Wistar	150, 450	↓	(66)
Effect on the induction of AA[g]	Lewis	450	↓	(68)
Effect on severity and spontaneous regression of EAE[g]	Lewis	450	↑	(68)
Prenatal and postnatal exposure				
Peripheral eosinophilic and basophilic granulocytes	Wistar	150	↑	(60)
Serum IgM levels	Wistar	4, 50, 100, 150	↑	(60,63)
Serum IgG levels	Wistar	50, 150	↑	(60)
Popliteal lymph node weight	Wistar	20, 100	↑	(63)
High endothelial venules in lymph nodes	Wistar	4–150	↑	(60,63)
Delayed-type hypersensitivity against ovalbumin	Wistar	4, 100	↑	(63)
Primary and secondary IgM and IgG against tetanus toxoid	Wistar	4, 20, 50, 150	↑	(60,63)
Resistance to *Trichinella spiralis*[h] and *Listeria monocytogenes* infection	Wistar	150	↓	(60)

Abbreviations: AA, adjuvant arthritis; BN, Brown Norway; ConA, concanavalin A; dsDNA, double-stranded DNA; EAE, experimental allergic encephalomyelitis; HCB, hexachlorobenzene; IFN, interferon; IL, interleukin, LPS, lipopolysaccharide; PHA, phytohemagglutin; ssDNA, single-stranded DNA. [a]Dietary HCB concentration (mg/kg). Most experiments with adult rats were short-term exposure studies of 3–4 weeks of HCB exposure. In the prenatal and postnatal studies, exposure started at days 1–3 of pregnancy and was continued until pups were 5 weeks of age. [b]Significantly increased (↑) or decreased (↓) as compared to control rats. [c]Lewis and BN were exposed to 150 and 450 mg/kg HCB. [d]Wistar rats were exposed to HCB for 13 weeks. [e]Significant increase in Lewis exposed to 450 mg/kg HCB and BN exposed to 150 and 450 mg/kg. [f]In Wistar rats that received 450, 500, 900, 1,000, and 2,000 mg/kg HCB, and Lewis and BN that received 450 mg/kg, these effects were observed. [g]Lewis rats were exposed to 450 mg/kg HCB for 6 weeks before investigation of the development of AA or EAE. [h]The IgG response against *T. spiralis* was increased.

150 and 450 mg/kg HCB dose dependently increased the ability of concanavalin A (ConA) to increase interleukin (IL)-2 and interferon-γ mRNA levels of spleen cells, whereas IL-2R mRNA was increased at 450 mg/kg HCB (*65*).

Prenatal and postnatal HCB exposure. The developing immune system seems to be particularly vulnerable to the immunotoxic effects of HCB, as observed in two studies investigating the prenatal and postnatal toxicity of HCB. In the first study, rats received 0, 50, or 150 mg/kg HCB starting at days 1–3 of pregnancy, which was continued during lactation and after weaning until pups were 5 weeks of age (*60*). In the 150-mg/kg group, increased serum IgM and IgG levels and increased numbers of blood basophilic and eosinophilic granulocytes were observed. Histopathologically, focal accumulation of alveolar macrophages in the lung and proliferation of high endothelial venules in lymph nodes were observed. Immune function tests showed decreased resistance to *Trichinella spiralis*, as measured by a higher yield of muscle larvae, and to *Listeria monocytogenes* infection at the high-dose group only. In addition, increased primary and secondary IgM and IgG responses to tetanus toxoid in both dose

Table 2. Summary of the immunotoxic effects of hexachlorobenzene in mice.

Parameter	Study	Dose[a]	Effect[b]	Reference
Primary and secondary plaque-forming response of spleen cells to sheep red blood cells	BALB/c	167	↓	(74)
Serum IgA levels	BALB/c	167	↓	(74)
Susceptibility to endotoxin (*Salmonella typhosa*)	BALB/c	167	↑	(74)
Resistance to *Malaria* infection	BALB/c	100, 167	↓	(74,75)
Resistance to *Leishmania* infection	BALB/c	5,100	↓	(75)
Resistance to mKSA tumor cells	BALB/c	100	↓	(76)
Resistance to EL-4 tumor cells	C57BL/6	100	↓	(76)
Resistance to P388 tumor cells	DBA/2	5, 100	↓	(76)
Resistance to L1210 tumor cells	DBA/2	5, 100	↓	(76)
Natural killer cell activity of spleen	BALB/c	100	↓	(76)
Cytotoxic macrophage activity of the spleen	BALB/c	5, 100	↓	(76)
Graft-versus-host activity of the spleen	C57BL/6	167[c]	↓	(78)
Resistance to mouse hepatitis virus	BALB/c	167	↓	(77)
Resistance to encephalomyocarditis infection	C57BL/6	150	↑	(79)
Resistance to MCA sarcoma cells	C57BL/6	150	↑	(79)
Cytotoxic T-lymphocyte activity of spleen	C57BL/6	15	↑	(79)
Resistance to mouse hepatitis virus	Athymic mice	167[d]	↓	(77)
Prenatal exposure:				
Delayed-type hypersensitivity response to oxazolone	BALB/c mice	0.5, 5	↓	(79)
Mixed-lymphocyte response of spleen cells	BALB/c mice	5	↓	(79)
Number of splenic T cells	BALB/c mice	0.5, 5	↑	(79)
Number of splenic B cells	BALB/c mice	0.5, 5	↓	(79)

HCB, hexachlorobenzene. [a]Dietary HCB concentration (mg/kg). Most experiments with adult mice were short-term exposure studies of 3–10 weeks of HCB exposure. In the prenatal study, 0, 0.5, or 5 mg HCB/kg maternal body weight was given daily in 0.3 g peanut butter and selected immune functions were measured in 45-day-old offspring. [b]Significantly increased (↑) or decreased (↓) as compared to control animals. [c]This parameter remained unaffected after 3, 6, or 13 weeks of exposure and was only depressed after 37 weeks of HCB exposure. [d]In athymic mice this parameter was severely depressed.

groups as well as a significantly increased IgG response to *T. spiralis* infection in the high-dose group were observed. There were no effects of HCB on splenic clearance of *Listeria* and colloidal carbon, skin graft rejection, mitogenic responses of spleen and thymus cells, and IgM responses to LPS. In the second prenatal and postnatal toxicity study, exposure to 0, 4, 20, or 100 mg/kg HCB showed increased serum IgM levels, increased numbers of basophilic peripheral granulocytes in the high-dose group, and increased popliteal lymph node weights in the 20- and 100-mg/kg group. Histopathologic changes in lymph nodes and lung were similar to those described in the first study. In contrast to the first study, there were no effects of 4 and 20 mg/kg HCB on the resistance to *T. spiralis*. There was no significant increase of the IgM and IgG response to ovalbumin, whereas dietary levels as low as 4 mg HCB/kg feed significantly increased delayed-type hypersensitivity reactions to ovalbumin. Primary and secondary IgM and IgG antibody responses to tetanus toxoid were increased in the 4- and 20-mg/kg dose groups (*63*). Moreover, a high mortality of suckling pups of mothers exposed to 100 mg HCB/kg diet has been observed in the same study. No effects were observed of natural killer cell activity in spleens of rats prenatally and postnatally exposed to 4 and 20 mg/kg HCB were observed. More recent findings, however, showed that oral exposure of adult Wistar rats to 150 and 450 mg/kg HCB for 6 weeks dose dependently suppressed natural killer cell activity in the lung (*66*).

Thymus Dependence of the Immune Effects of HCB

The involvement of thymus-dependent T cells in HCB-induced immune effects has been investigated in male athymic (*rnu/rnu*) and euthymic (+/*rnu*) Wistar rats 3–4 weeks of age exposed for 6 weeks to control diets or diets containing 450 mg/kg HCB (*67*). It was observed in this study that HCB-induced toxicity, as judged by effects on body weight and liver effects, was more pronounced in athymic rats than in euthymic rats exposed to the same dose (Table 3). The effect of HCB on spleen weight was also higher in athymic rats than in euthymic rats exposed to the same dose of HCB (Table 3). Morphometric analysis of spleen sections of control and HCB-exposed athymic and euthymic rats was performed to determine the effect of HCB on different spleen compartments. Relative areas of the white pulp and periarteriolar lymphatic sheath (PALS) were measured with an automated image analyzer. These measurements were used to estimate the absolute total weight of red pulp, PALS, marginal zones, and follicles (Table 3). HCB induced a significant increase in absolute red pulp weight in both athymic and euthymic rats. A significant increase of absolute weight of follicles and marginal zones was observed only in euthymic rats exposed to HCB, leading to the conclusion that the effects of HCB on splenic white pulp are thymus dependent (*67*). This all-or-none conclusion may be questioned, as athymic rats showed a significant decrease in body weight and a more marked increase of liver weight compared to euthymic rats exposed to the same dose of HCB. This points at a higher toxicity of HCB in athymic rats compared to euthymic rats and thus the possibility of stress-induced effects on the splenic white pulp in athymic rats. Therefore, we also compared the effects of HCB on the estimated relative (organ-to-body) weight of splenic compartments (Table 3). Then a significant increase of estimated relative weight of marginal zones and follicles was also observed in athymic rats exposed to HCB. This effect on splenic white pulp in athymic rats, however, was disproportional to the more marked relative liver weight increase and increase of red pulp observed in athymic rats compared to euthymic rats. On the basis of this study, we concluded that thymus-dependent T cells are not required for HCB-induced hyperplasia of marginal zones and follicles, but that T cells may enhance the effect of HCB on splenic white pulp in euthymic rats. Recently, we further investigated the effects of HCB on splenic red pulp of athymic and euthymic WAG/Rij rats. Histology showed increased extramedullary erythropoiesis and myelopoiesis; presence of activated cells, mainly macrophages and fibroblasts, in the red pulp; and increased numbers of granulocytes in the venous sinusoidal network bordering the marginal zones (Figure 1).

Table 3. Effects of 6-week oral exposure to 450 mg/kg hexachlorobenzene on body weight; absolute and relative liver and spleen weight; absolute and relative estimated red pulp; PALS; and follicles and marginal zones weight in euthymic and athymic Wistar rats.

	Body weight[a]	Liver weight[a]	Spleen weight[b]	Red pulp[c]	PALS[c]	Follicles and marginal zones[c]
			Absolute weight			
Euthymic						
Control	242 ± 10	9.52 ± 0.41	421 ± 22	277 ± 30	42 ± 6	102 ± 23
450 mg/kg HCB	202 ± 22**	13.26 ± 1.32***	584 ± 75***	405 ± 64***	44 ± 13	134 ± 23*
Athymic						
Control	175 ± 21	6.47 ± 0.94	444 ± 49	319 ± 34	11 ± 3	114 ± 18
450 mg/kg HCB	122 ± 7***	10.77 ± 0.82***	573 ± 89*	452 ± 101**	9 ± 4	112 ± 38
			Organ-to-body weight ratios[d]			
Euthymic						
Control		3.94 ± 0.28	174 ± 11	115 ± 13	17 ± 3	42 ± 9
450 mg/kg HCB		6.59 ± 0.47***	289 ± 19***	200 ± 14***	22 ± 6	67 ± 13**
Athymic						
Control		3.68 ± 0.21	253 ± 6	182 ± 17	6 ± 2	65 ± 5
450 mg/kg HCB		8.88 ± 0.88***	473 ± 80***	374 ± 90***	8 ± 3	91 ± 27*

Abbreviations: HCB, hexachlorobenzene; PALS, periarteriolar lymphatic sheath; SD, standard deviation. [a]Body weight and liver weight ± SD are given in grams. [b]Spleen weight ± SD is given in milligrams. [c]Relative areas of the white pulp and PALS were measured by using an automated image analyzer and these measurements were used to calculate the weight of the red pulp, PALS, follicles, and marginal zones. [d](Milli)gram per 100 g body weight. Asterisks denote significance from the corresponding control group (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, respectively), $n = 6$ per treatment group.

The effect of HCB on two models of autoimmune disease in the Lewis rat. To study the effect of HCB on thymus-dependent autoimmune diseases in the rat, two experimental models of autoimmune disease in the rat, adjuvant arthritis (AA) and experimental allergic encephalomyelitis (EAE), were used (*68*). Male Lewis rats 3–4 weeks of age were orally exposed to diets containing 0, 50, 150, or 450 mg HCB/kg diet. After 6 weeks of HCB exposure, rats were injected either *a*) intradermally via the tail base with Freund's complete adjuvant ([FCA], a mixture of mineral oil, a detergent, and dead *Mycobacterium tuberculosis* H37Ra) to induce AA, or *b*) subcutaneously in the left hind footpad with guinea pig myelin with the same FCA to induce EAE. The development of EAE was investigated daily and the degree of paralysis was rated 0 (no paralysis), 1 (paralysis of one hind limb), 2 (complete hind limb paralysis), 3 (paraplegia), and 4 (death) per rat. The onset of arthritic lesions in the joints was investigated every other day and the severity was rated 0 (no observable lesion or swelling) to 4 (severe swelling and redness) per paw, yielding a maximal possible score of 16.

Figure 2 shows that HCB dose dependently suppressed the induction of AA. Rats exposed to the high dose of 450 mg/kg HCB failed to develop AA, although one rat showed inflammation of the joint of one leg at the end of the study. In contrast, oral exposure to HCB dose dependently enhanced the severity of EAE. Whereas rats that received 0, 50, and 150 mg/kg HCB recovered spontaneously from the active disease, those that received 450 mg/kg HCB developed chronic progressive EAE and died. The mechanism underlying this contradictory effect of HCB on these

two models of autoimmune disease in the Lewis rat is unknown, as both models require specific T-helper 1 cell involvement. A possible explanation may lie in an effect of HCB on other cells or processes involved in these autoimmune models. For example, macrophages are important effector cells involved in the development of clinical signs of EAE (*69*), and recently it has been shown that macrophage-derived IL-12 may contribute to exacerbation and relapse of EAE (*70*). The pathogenesis of AA involves many cell types. Recently it was shown that infiltration of activated neutrophilic granulocytes into the joint is an important step in the development of rheumatoid arthritis (*71*).

HCB-induced autoantibodies. According to recent studies, the immunostimulatory effects of HCB in the rat may be related to autoimmunity. Wistar rats orally exposed to HCB showed increased IgM but not IgG antibodies to several autoantigens such as single-stranded DNA, native DNA, rheumatoid factor, and phosphatidylcholine (*61*). In another study, Brown Norway, Lewis, and Wistar rats orally exposed to HCB showed IgM autoantibodies against single-stranded DNA (*72*). Although the autoantibodies are naturally occurring IgM autoantibodies of low affinity and known to have little pathogenicity (*73*), further studies have investigated whether these autoantibodies are involved in the induction of inflammatory skin and lung lesions in the rat.

HCB-Induced Immunomodulation in Mice

Loose et al. (*74*) showed that male BALB/c mice that received 167 mg HCB per kg diet for over 6 weeks displayed no effects on lung, thymus, and spleen weights and histology, whereas liver cell hypertrophy was present. In the same study HCB induced a suppression of the thymus-dependent humoral immunity, as measured by the response to sheep red blood cells, in the absence of alterations of total serum IgM and IgG values. In another study, Loose and co-workers (*75*) showed impaired host resistance in BALB/c mice exposed to different concentrations of HCB over 3–10 weeks. Increased susceptibility to endotoxin (*Salmonella typhosa*) and significantly suppressed resistance to infection with malaria (*Plasmodium berghei*) and *Leishmania* were noted. In tumor susceptibility studies, different strains of mice orally exposed to HCB showed a dose-related decrease of their resistance to challenges with syngeneic tumor cells, as measured by decreased survival times, probably due to a significant reduction of tumoricidal activity of cytotoxic macrophages in the spleen (*76*). More recently, decreased resistance to mouse hepatitis virus was demonstrated in BALB/c mice exposed to HCB,

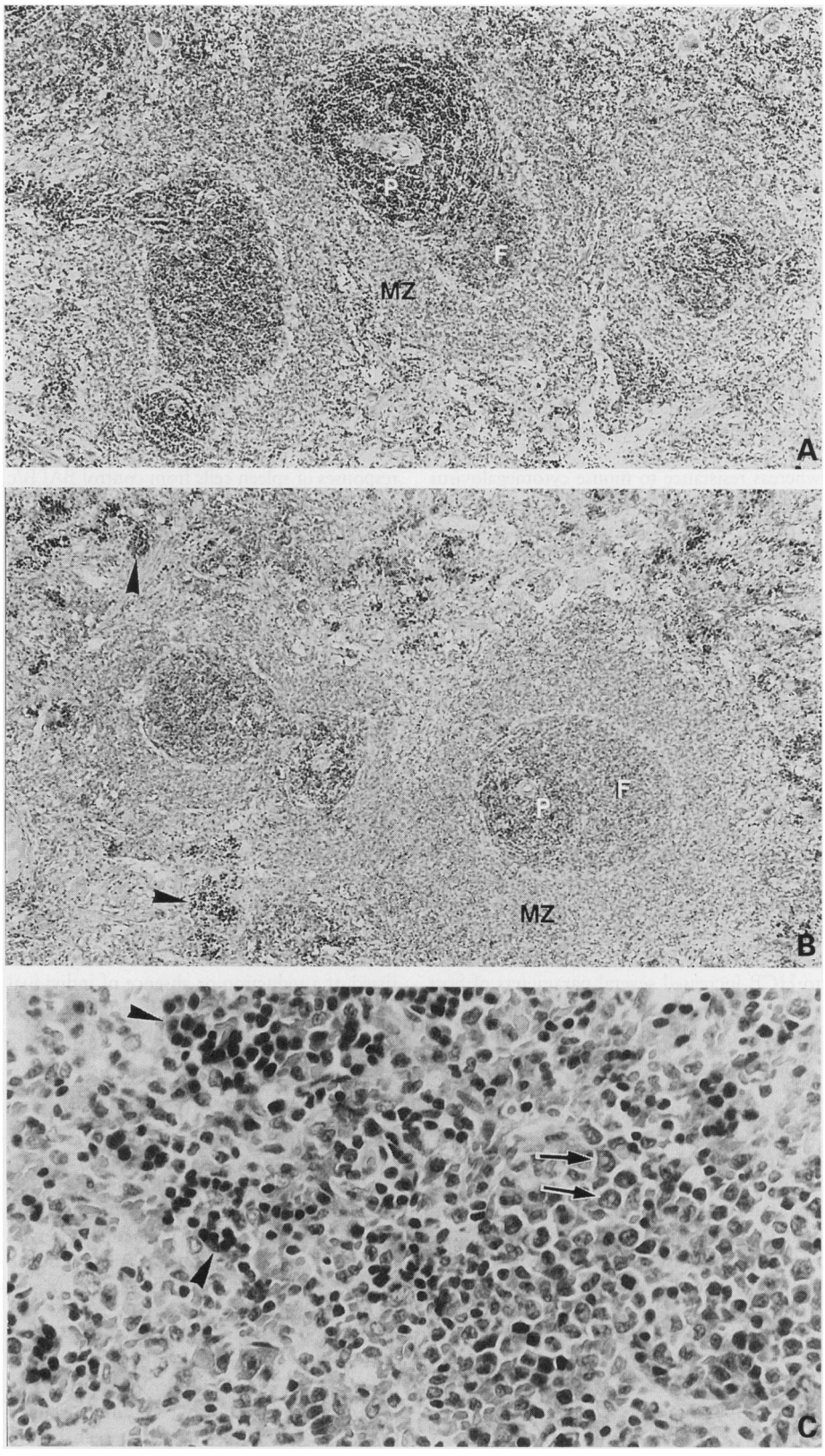

Figure 1. Representative spleen sections from a control rat (*A*) and from rats fed 450 mg hexachlorobenzene (HCB)/kg diet for 4 weeks (*B,C*). The different compartments of the white pulp are indicated by P, periarteriolar lymphatic sheaths; F, follicles; and MZ, marginal zones. Note the hyperplasia of the white pulp, especially of the marginal zone, and the extramedullary hemopoiesis (arrowheads) in the red pulp of HCB-treated rats. Panel *C* shows a higher magnification of the splenic red pulp from an HCB-treated rat. Note the abundant presence of polymorphonuclear granulocytes (arrows) and normoblasts and myeloblasts (arrowheads). Hematoxylin and eosin, magnification *A, B* × 100; *C* × 400.

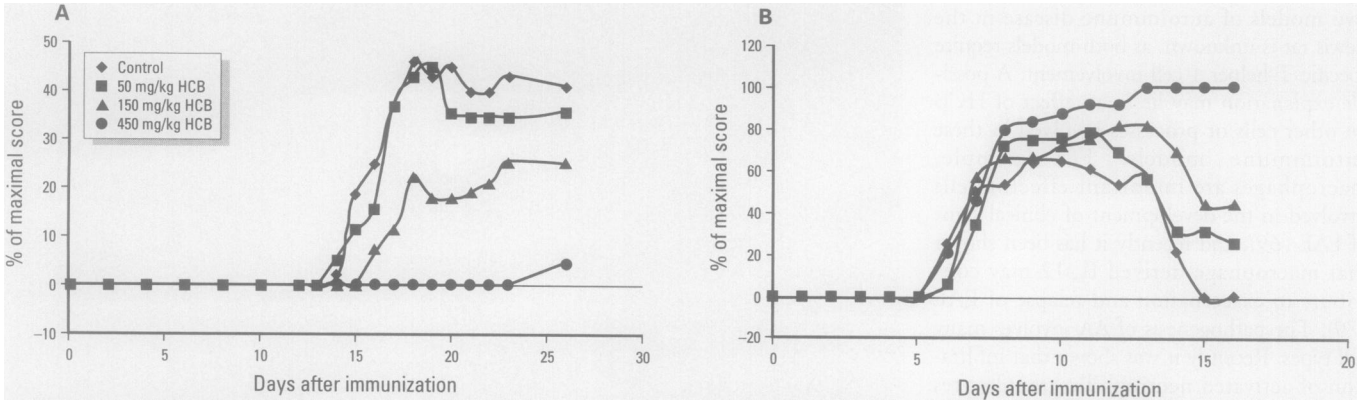

Figure 2. The effect of hexachlorobenzene (HCB) on two experimental models of autoimmune disease in the Lewis rat. Male Lewis rats 3–4 weeks of age were orally exposed to diets containing 0, 50, 150, or 450 mg HCB/kg diet. After 6 weeks of exposure, the effect of HCB on the development of adjuvant arthritis (*A*) and experimental allergic encephalomyelitis (*B*) was investigated.

whereas resistance to mouse cytomegalovirus and a pneumonia-causing virus was not impaired. This has been explained by the HCB-induced decrease of phagocytic and detoxifying capacity of Kupffer cells in the liver, resulting in an enhanced infection of hepatocytes by mouse hepatitis virus (*77*). Decreased phagocytic and detoxifying capacity of Kupffer cells could also explain the HCB-induced increased susceptibility to endotoxin (*77*). Exposure of C57BL/6 mice to 167 mg HCB/kg body weight for 3, 6, or 13 weeks showed no effect on the graft-versus-host activity when spleen cells of these HCB-exposed mice were inoculated into neonatal BDF_1 mice. On the contrary, graft-versus-host activity was significantly depressed after 37 weeks of exposure to HCB (*78*). In another study [quoted in IARC (*79*)] in male C57BL/6 mice, oral exposure to HCB increased several parameters of host resistance. Oral exposure to 150 mg/kg HCB resulted in an increased resistance to encephalomyocarditis infection and increased resistance to progressive growth of methylcholanthracene-induced sarcoma, whereas there was no significant effect of HCB on resistance to Moloney sarcoma virus-induced tumor cells. In the same study, 15 mg/kg HCB increased cytotoxic lymphocyte activity generated *in vivo* by inoculation of C57Bl/6 mice with DBA/2 mastocytoma, increased P815 tumor cells and was assessed *in vitro* in a ^{51}Cr-release cytotoxicity assay. No effect of HCB was observed on the phagocytic activity of peritoneal macrophages (*79*). Barnett and co-workers (*80*) studied the effect of *in utero* HCB exposure on the developing immune response of BALB/c mice. Mice were exposed daily to 0, 0.5, or 5 mg HCB/kg maternal body weight; at 45 days of age selected immune functions were tested in the offspring. A severely decreased delayed-type hypersensitivity response to oxazolone was observed in animals exposed to 0.5 and 5 mg/kg HCB. The mixed lymphocyte responses of spleen cells from control BALB/c mice and BALB/c mice that received HCB was measured by culturing the cells in the presence of mitomycin C-treated allogenic C57BL/6 spleen cells for 72 hr. The mixed lymphocyte response of mice that received 5 mg/kg HCB was significantly decreased, whereas blastogenic responses of isolated spleen cells to ConA, phytohemagglutin, and LPS were unchanged. In the same study, increased numbers of T cells and decreased numbers of B cells were present in spleens of HCB-treated mice.

HCB-Induced Immunomodulation in Other Species Including Man

Female rhesus monkeys that received graded doses of HCB (8, 32, 64, or 128 mg/kg per day) via gastric instillation showed histologic changes in the thymus. These changes consisted of a reduction or absence of individual lobules and hyperplasia of reticular cells, macrophages, and plasma cells in the medulla (*36*).

In beagle dogs administered different doses of 99% pure HCB (1, 10, 100, or 1,000 mg/day in a gelatin capsule) during 1 year, significant neutrophilia was observed after several weeks in most dogs receiving 100 and 1,000 mg/day. Hyperplasia of the gastric lymphoid tissue was also frequently observed in dogs of all dose groups. Moreover, in the high-dose group, 33% of the dogs displayed arteritis–periarteritis of small arteries and arterioles affecting multiple organ sites (*81*). This severe arteritis resembled the earlier described vasculitis in livers of rats exposed to HCB of unknown purity (*48*) and had many characteristics suggesting hypersensitivity angitis or polyarteritis; however, there were no indications for the presence of elevated serum antibody levels.

Recently, a study in 51–66 workers occupationally exposed to HCB showed impaired functions of neutrophilic granulocytes compared to neutrophilic granulocytes of a control group of 48 nonexposed age- and sex-related individuals. Neutrophils from HCB-exposed individuals showed a significantly reduced chemotaxis as well as a significant reduced respiratory burst activity, as measured by nitroblue tetrazolium dye reduction. However, in the HCB-exposed workers, there was no correlation between the length of HCB exposure or HCB concentrations in blood and the changes in the neutrophil functions (*82*). In the same group of workers, increased serum IgM and IgG levels were observed, whereas serum IgA levels were normal (*83*). In a subsequent study phagocytosis and killing of *Candida albicans* and *Candida pseudotropicalis* by polymorphonuclear granulocytes from the HCB-exposed workers or nonexposed control individuals were compared. HCB showed no effect on phagocytosis, whereas lysis of *C. albicans* and *C. pseudotropicalis* was significantly decreased in the HCB-exposed group compared to the control group. As observed in the previous study, there was no correlation between the length of HCB exposure, blood levels of HCB, and the changes of polymorphonuclear cell function (*84*).

The Role of HCB-Induced Immunotoxicity in the Induction of Skin and Lung Lesions in the Rat

The role of metabolism. In the Wistar rat, HCB feeding induces inflammatory skin and lung changes (*59–62*). Several studies investigated whether the parent compound HCB itself or its reactive metabolites are involved in the induction of immune effects and skin and lung lesions. One of the first studies investigated the involvement of porphyrins in the induction of skin lesions by HCB (*62,85*), as these skin lesions have been attributed to dermal accumulation and subsequent photochemical activation of porphyrins (*52*). Coadministration of the P450IIIA1/2 inhibitor triacetyloleandomycin to HCB-treated Wistar rats resulted in a

strong reduction of hepatic porphyria and the formation of the oxidative metabolites PCP and TCHQ. In contrast, autoantibody levels and induction of skin lesions remained unaffected (Table 4). It was concluded from this study that the oxidative metabolites of HCB (i.e., PCP, TCHQ) and HCB-induced porphyria are not involved in the immunomodulating effects and induction of skin lesions by HCB (*62,85*). The suggestion that skin lesions in the rat are not due to phototoxicity of dermal accumulated porphyrins was confirmed by the absence of porphyrin fluorescence in lesional and nonlesional skin of HCB-treated rats. Therefore it is possible that rats develop the juvenile, porphyria-independent form of HCB-induced skin lesions as observed in children under 4 years of age in the Turkish HCB poisoning accident (*72*).

In another study the role of the mercapturic acid biotransformation pathway in the HCB-induced inflammatory skin and lung lesions was investigated (*86*). This biotransformation pathway, resulting in the formation of the urinary end product PCP–NAC, is the major route of metabolism in female Wistar rats exposed to HCB (*85*). Moreover, cumulative urinary levels of PCP–NAC from Wistar rats orally exposed to HCB correlated significantly with serum IgM, IgA, and IgM anti–bromelain-treated red blood cells (*62*). Brown Norway rats exposed to pentachloronitrobenzene, a compound metabolized via the same (mercapturic acid) biotransformation pathway as HCB (*18,19*), showed no effects on spleen, skin, or lung. This indicates that this route of metabolism of HCB is not involved in the induction of splenomegaly and inflammatory skin and lung lesions. Therefore, it is concluded that either the parent compound HCB or yet unidentified metabolites are involved in the inflammatory effects of HCB (*86*).

Strain dependency of HCB-induced skin and lung pathology. In a recent study we investigated the involvement of the immune system in the induction of skin and lung lesions by HCB (*72*). We fed three rat strains (i.e., Brown Norway, Lewis, and Wistar, known to react very diversely to immunomodulating compounds) diets containing different doses of HCB for 4 weeks.

Table 4. The role of metabolism in the target organ toxicity of hexachlorobenzene in the rat.

	Hepatic porphyria	Immune effects	Skin lesions
HCB	+	+	+
HCB + TAO[a]	-	+	+

Abbreviations: HCB, hexachlorobenzene; TAO, triacetyloleandomycin. [a]TAO is a selective inhibitor of P450IIIa1/2. Data from Van Ommen et al. (*20*), Den Besten et al. (*85*), and Schielen et al. (*62*).

Skin lesions were scored during the exposure according to time of onset, incidence, and severity. After 4 weeks of exposure, histopathology of skin and lung as well as various parameters of immunomodulation were examined. There was a marked strain-dependent induction of skin lesions far more prominent in Brown Norway rats than in Lewis and Wistar rats. Skin lesions became macroscopically manifest in the head and neck region of Brown Norway, Lewis, and Wistar rats after 15, 17, and 24 days of exposure, respectively. Skin lesions ranged in severity from slight redness to large hemorrhagic lesions with exudative crusts and were histologically characterized by loss or hyperplasia of the epidermis and deep dermal activation of vessels. An inflammatory infiltrate of mainly eosinophilic granulocytes in Brown Norway and mononuclear cells in Lewis and Wistar rats was observed in the deep dermis. Hyperplasia of the epidermis, activation of deep dermal vessels, and inflammatory infiltrates were also observed in macroscopically intact skin of HCB-exposed rats, although they appeared less severe compared to changes in lesional skin. In the Brown Norway rat, skin lesions correlated with all measured parameters of immunomodulation (Table 5) such as increased lymph node weights; activation of high endothelial venules; increased serum IgM, IgG, and IgE levels; and increased single-stranded DNA-specific IgM. In the Lewis rat, skin lesions correlated only with serum IgE and single-stranded DNA-specific IgM, whereas in Wistar rats no significant correlation between skin lesions and parameters of immunomodulation was observed. This is in contrast with earlier findings of Schielen et al. (*62*). They demonstrated a significant correlation between serum IgM levels and severity of skin lesions in female Wistar rats fed 300 mg HCB/kg diet, but this may be related to the longer exposure period of 13 weeks. HCB induced inflammatory lung lesions in all rat strains that consisted of focal accumulations of alveolar macrophages and proliferation of the endothelium of lung vessels, which were attended by a perivascular infiltrate (*60,63,87,88*). The induction of inflammatory lung lesions by HCB appeared hardly strain dependent and was slightly stronger in Lewis rats compared to Brown Norway and Wistar rats. The perivascular infiltrate varied strain dependently and consisted of mainly eosinophilic granulocytes in Brown Norway rats and mononuclear cells in Lewis and Wistar rats. In contrast to skin lesions, no correlation was found between inflammatory lung lesions and the assessed parameters of immunomodulation. From this study we concluded that the HCB-induced skin and lung lesions probably have different etiology. Pronounced strain differences in skin lesions as well as the positive correlation with several immune parameters indicate a specific involvement of the immune system in the development of skin lesions.

The role of autoantibodies. To investigate whether deposition of autoantibodies in the skin is involved in the induction of skin lesions, skin of control and HCB-exposed rats was incubated with a fluorescein isothiocyanate (FITC)-labeled mouse-anti-rat kappa-chain antibody (MARK-1) (MARK-1–FITC). In addition, to detect serum autoantibodies directed to skin proteins, skin of control Brown Norway rats, as well as nonlesional and lesional skin of HCB-treated Brown Norway rats, was incubated with either control serum or serum of Brown Norway rats exposed to HCB. To detect binding of autoantibodies, sections were subsequently incubated with MARK-1–FITC. As a positive control, kidney sections of a rat

Table 5. Summary and gradation of the effects of hexachlorobenzene in Brown Norway, Lewis, and Wistar rats.[a,b]

	Brown Norway (450 mg/kg)	Lewis (450 mg/kg)	Wistar (900 mg/kg)
Parameters of general toxicity			
Body weight increase	+	±	+
Liver effects	++	++	+±
Parameters of immunomodulation			
Increase in			
Spleen weight	++	+	+
PLN weight	++	+	±
Lymph node	+±	+±	+±
Serum IgM levels	+±	+±	++
Serum IgG levels	+±	–	–
Serum IgE levels	+++	–	–
Serum IgM anti–single-stranded DNA	+±	+±	±
Inflammatory lesions			
Gross skin lesions	+++	+±	±
Lung effects	+±	++	+

Abbreviations: HEV, high endothelial venules; PLN, popliteal lymph node; ssDNA, single-stranded DNA. [a]Gradation scale: –, no increase; ±, minimal; +, slight; +±, moderate; ++, marked; +++, very severe. [b]Reprinted from Michielsen et al. (*72*), with permission of Academic Press.

with experimentally induced immunocomplex-mediated Heymann nephritis (kindly supplied by E. de Heer, Leiden, The Netherlands) were incubated with MARK-1-FITC. No fluorescence above background was observed in the skin of control or HCB-treated Brown Norway rats (either incubated or not incubated with serum of control or HCB-treated rats), indicating that there were no serum autoantibodies to skin proteins and no deposited immune complexes in skin. This contradicts our earlier results obtained with the enzyme-linked immunosorbent assay (ELISA) that showed presence of IgM antibodies against single-stranded DNA and double-stranded DNA in sera of HCB-exposed rats (*72*) but can be explained by the weak affinity of these IgM autoantibodies. Whereas ELISA is a very sensitive method and detects antibodies with high and low affinities for the antigen tested, immunohistochemistry detects only the antibodies, which have a high affinity and therefore are pathogenic. Binding of these autoantibodies was not observed, indicating that autoantibodies are probably not involved in the induction of skin lesions by HCB.

Thymus dependency of HCB-induced skin and lung pathology. In another study we investigated the role of thymus-dependent T cells in HCB-induced inflammatory skin and lung lesions. Brown Norway rats were depleted of T cells by adult thymectomy followed by lethal irradiation and bone marrow reconstitution (*89*). The resulting T-cell depletion was analyzed by fluorescence-activated cell sorter analysis and immunohistochemistry and appeared strong. Skin lesions appeared slower and at a lower incidence in T-cell–depleted Brown Norway rats orally exposed to 450 mg/kg HCB in the diet than in normal Brown Norway rats exposed to the same dose. At the end of the 4-week exposure, however, incidence and severity of skin lesions were comparable as well as the histopathologic changes in lesional and nonlesional skin of HCB-treated normal and T-cell–depleted Brown Norway rats. HCB induced quantitatively and qualitatively comparable inflammatory lung lesions in normal and T-cell–depleted Brown Norway rats as well as in athymic and euthymic WAG/Rij rats that were exposed to HCB. An earlier study with male athymic and euthymic Wistar rats exposed to 450 mg/kg HCB over 6 weeks demonstrated that the induction of inflammatory lung lesions by HCB is thymus independent (*67*). Thus, studies with T-cell–depleted Brown Norway rats confirmed earlier conclusions that the induction of lung lesions by HCB is thymus independent. In addition, thymus-dependent T cells are not likely to be required for the induction of skin lesions by HCB in the rat, although T cells enhance the rate of induction and progression of skin lesions. Because the T-cell depletion appeared strong and resulted only in a slight difference in the rate of induction and progression of skin lesions, we concluded that this immunopathology is probably not due to binding of HCB or its reactive metabolites to macromolecules of the body. Binding of low molecular weight chemicals to macromolecules of the body followed by subsequent T- and B-cell stimulation is considered to be the mechanism involved in most allergic and autoimmunogenic low molecular weight chemicals (*90,91*). A thymus-independent etiology of skin and lung lesions is remarkable, as HCB strongly modulates T-cell–mediated immune parameters but is confirmed by studies described earlier in this section that failed to demonstrate the presence of autoantibodies in nonlesional and lesional skin of HCB-treated Brown Norway rats. However, several mainly inert chemicals, e.g., crystalline silica, are able to induce autoimmunelike effects by the nonspecific generation of cytokines and release of reactive oxygen and nitrogen species by granulocytes and macrophages (*92*). Recently we demonstrated the presence of large numbers of activated $CD8^+$ macrophages in nonlesional and especially lesional skin of HCB-treated Brown Norway rats (*89*). These CD8-expressing macrophages in the rat are able to produce and release nitric oxide upon stimulation (*93,94*). Stimulation of these macrophages to release nitric oxide or other potent mediators could account for the observed pathology and lead to chronic inflammation. In addition, eosinophilic granulocytes that are frequently observed in the lung of HCB-treated highly susceptible Brown Norway rats are also very potent effector cells. Besides their beneficial properties in host defense, eosinophil degranulation or cytolysis of eosinophils can become detrimental to the host and contribute to local pathology at the site of inflammation (*95*). Therefore, involvement of these granulocytes and macrophages in the induction of skin and lung lesions by HCB needs further investigation.

Conclusion

It is clear from many studies in laboratory animals that HCB is an environmental chemical with immunotoxic properties (*59–61,63*). Recently, immune effects such as increased serum IgM and IgG levels and impaired functions of neutrophilic granulocytes have been reported in workers occupationally exposed to HCB (*82–84*). In the rat, HCB mainly induces stimulation of parameters of immune function, whereas in the mouse, most assessed immune function parameters are suppressed by HCB (*63,74–80*). Oral exposure of rats to HCB elevates serum IgM antibodies to several autoantigens (*61,72*) and differently modulates two experimental models of autoimmune disease, AA and EAE (*68*). Moreover, an (auto)immune etiology is also conceivable for the enlarged thyroid, arthritic lesions of the hands, and dermatologic effects in patients of the accidental poisoning in Turkey, which persisted 25 years later (*58*). The mechanism by which HCB affects skin, lung, and immune system is still unclear. HCB-induced lung lesions are strain- and thymus-independent and do not correlate with parameters of immunomodulation (*72,89*). Therefore, an autoimmune or allergic etiology resulting from binding of HCB or metabolites thereof to macromolecules of the body is probably not involved in HCB-induced lung lesions. Induction of skin lesions in the rat is highly strain dependent and correlates with several parameters of immunomodulation (*72*). Thymus-dependent T cells are not likely to be required for the induction of skin lesions but enhance the rate of induction and progression of the lesions in the Brown Norway rat. Moreover, in the skin of HCB-treated rats there was no deposition of immune complexes, and no autoreactive antibodies to skin proteins could be detected in the serum of HCB-treated rats. Therefore, we concluded that the induction of skin and lung lesions by HCB is probably not due to binding of HCB or its reactive metabolites to macromolecules of the body. Such a thymus-independent etiology of the skin and lung lesions is remarkable, as HCB strongly modulates T-cell–mediated immune parameters (*59,60,68*). This points at a very complex mechanism and possible involvement of multiple factors in HCB-induced immunopathology. The presence of large numbers of activated macrophages in the skin and large numbers of macrophages and polymorphonuclear cells in the lung of HCB-exposed patients suggests that these cells may be involved in the induction of skin and lung lesions. Whether the HCB-induced immunopathology is associated with effects of HCB on (eosinophilic) granulocytes and macrophages needs further investigation.

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