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Macrophages are involved in hexachlorobenzene-induced adverse immune effects

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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, an adoptive transfer study was performed. Our results indicate that macrophages are indeed involved in HCB-induced skin lesions, lung eosinophilia, and elevation of IgM against ssDNA. Kinetics showed that both skin and lung lesions appeared early after exposure. Moreover, immune effects could not be adaptively transferred. Thus, both macrophages and T cells are involved in HCB-induced immune effects but 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.

Keywords: Hexachlorobenzene; Macrophages; Clodronate-liposomes; T cells; Kinetics; Immunopathology; Brown Norway rat

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 (Gocmen et al., 1986; Peters, 1976). Furthermore, infants born from HCB-exposed mothers developed inflammatory skin and lung lesions (Cam, 1960).

The immunotoxic effects of HCB could be reproduced in rats (for reviews, see Michielsen et al., 1999; Vos, 1986). 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 (Michielsen et al., 1997). 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. Thus, macrophages could also be involved in some of the HCB-induced immune effects (Ezendam et al., 2004a).

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Furthermore, DNA microarray experiments performed after subchronic exposure to HCB have provided additional evidence for the significance of innate immune cells such as macrophages and granulocytes. HCB upregulated genes encoding macrophages, granulocytes, mast cells, pro-inflammatory cytokines, antioxidants, and acute phase proteins. This inflammatory response was not only confined to the immune organs, but also occurred in the liver and kidney. Thus transcriptome profiles show that HCB induced a systemic inflammation (Ezendam et al., 2004b).

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 (Larsen et al., 1994). Furthermore, pro-inflammatory cytokines can directly activate naive T cells (Curtsinger et al., 1999; Pape et al., 1997). Second, the potential of HCB to sensitize BN rats after oral exposure and generate memory T cells is not known. Low molecular weight chemicals (LMWC) can become immunogenic after binding to self-proteins, thereby generating hapten-carrier complexes (Weltzien et al., 1996). 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) (Ezendam et al., 2003). Alternatively, certain LMWC are known to interfere with antigen presentation, leading to presentation of cryptic epitopes (Sercarz et al., 1993). Importantly, sensitization of T cells can only occur when sufficient adjuvant signals are present, e.g., costimulatory molecules or danger signals (Bretscher, 1992; Matzinger, 1994).

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 (Van Rooijen, 1989). Furthermore, a kinetic study was performed to obtain information on the early phase of HCB-induced immune effects. In addition, an adoptive transfer study was performed to find out if HCB triggers specific T cell activation thereby eliciting immunological memory.

Animals, 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). 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 and recombinant rat IL-6 and capture and detection antibodies for the IL-6 ELISA were obtained from R&D Systems (Oxon, UK) and recombinant rat TNF-α from Biosource (Camarillo, CA, USA). Streptavidin-conjugated horseradish-peroxidase (HRP) was obtained from Sanquin (Amsterdam, The Netherlands), tetramethylbenidine (TMB) from Biosource Europe SA (Nivellid, Belgium) and H_2SO_4 from Merck KgaA (Darmstadt, Germany).

Cell culture and exposure to HCB. The NR8383 cell line, a rat alveolar macrophage cell line, was a kind gift of Prof. Dr. Bast and Dr. van Haaften from the University of Maastricht in the Netherlands. The cells were cultured in 75 cm^2 culture flasks (Cellstar 658.170, Greiner labotechnik, Germany) in 15 ml nutrient mixture Ham's F12 medium containing 15% FBS and 5% penicillin/streptomycin. The cells were subcultured twice a week. Cells were plated in a 24-well plate (3×10^5 cells/well). Cells were incubated with several concentrations of silica and HCB in order to find a non-toxic dose. Cell viability was measured with a MTT reduction assay. For HCB and silica two similar concentrations were chosen after these preliminary experiments. Cells were exposed to 1 mg/ml HCB (250 μl/well, added to the cells as a suspension) or 0.8 mg/ml silica (250 μl/well, added to the cells as a suspension). Silica was included because it is an inert model compound that can activate macrophages. Each stimulus was performed in triplo. After 6 and 24 h supernatants were collected for cytokine measurements.

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 1 week before starting the experiments. All rats were housed at the animal facilities of the Utrecht University and were kept two per cage in filter-topped macrolon cages on bedding of chips and wood, under standard conditions (50–60% relative humidity, 12:12-h dark–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 (Michielsen et al., 1997), 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 bwt ip). 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 2.5×10^6 cells per ml.

Macrophage depletion with clodronate-liposomes. Clodronate-liposomes were prepared as described previously (Van Rooijen and Sanders, 1994). 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 was used to compare the effects of clodronate-liposomes on HCB-induced pathology. PBS-liposomes were included as a control group, to establish that empty liposomes did not have any immunostimulatory effects (Van Rooijen and Sanders, 1994). The first injection with liposomes was iv 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 ip on days 5, 9, 13, and 17. This regimen was chosen based on experiments in SCID mice (Fraser et al., 1995) in order to deplete circulating monocytes and prevent repopulation of macrophages. After iv injection blood monocytes are only depleted for a short period, because macrophage depletion in blood, liver, and spleen generates signals to the bone marrow leading to enhanced release of monocytes, thereby increasing the number of circulating monocytes (Huitinga et al., 1992). Rats were sacrificed on day 20. Blood, spleen, ALN, and mesenteric lymph nodes (MLN) 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 (n = 4 per group). All rats were sacrificed on the same day. Spleen and lungs were collected for morphology. Splenocytes were ex vivo stimulated with LPS.

Adoptive transfer study. The protocol for adoptive transfer was adapted from Shenton et al. (2003). 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 aseptic 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 6×10^8 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 TCRαβ), OX-8 (anti-rat CD8α), and biotinylated OX-35 (anti-rat CD4). Incubations and measurements were performed as described previously (Ezendam et al., 2004a).

Ex vivo restimulation of spleen or lymph node cells. For measurement of pro-inflammatory cytokines cells (3.75×10^5 cells per well) were stimulated ex vivo with LPS (2 μg/ml in complete medium). After 24 h of incubation supernatants were collected for cytokine measurement.

TNF-α and IL-6 ELISA. TNF-α or IL-6 were measured in supernatants of spleen cells stimulated ex vivo with LPS and NR8383 cells incubated with HCB or silica. Procedures were performed according to the description of the manufacturer with some minor modifications. Briefly, 96-well microtitre plates were coated overnight at room temperature (RT) with 2 μg/ml monoclonal anti-rat TNF-α or IL-6. They were blocked with PBS containing 1% BSA, 5% sucrose, and 0.05% NaN_3 for 2 h at RT. After washing, samples and standard curve were added to the plate and incubated overnight at 4 °C. Plates were washed and 200 ng/ml biotinylated anti-rat TNF-α or 100 ng/ml anti-rat IL-6 in PBS containing 0.5% Tween-20, 1% BSA, and 2% goat serum was added to the plates and incubated for 1 h at RT.

After washing plates were incubated with streptavidin–horseradish peroxidase for 1 h 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 or IL-6 was used to calculate the amount of TNF-α or IL-6 in the samples (pg/ml supernatant).

ELISA to detect serum levels of IgM against ssDNA. ELISA procedures to determine IgM levels against ssDNA were performed as described previously (Ezendam et al., 2004a; Schielen et al., 1993). 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.

Histochemical procedures. 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 (Ezendam et al., 2003). For determination of calcium deposits formaldehyde-fixed sections were stained with von Kossa.

Statistical analysis. Significant differences in the in vitro study with macrophages were assessed by analysis of variance (ANOVA) with Bonferroni post hoc test. In the experiment with clodronate-liposomes statistically significant differences between all groups were determined by ANOVA with Scheffe post hoc test, because of unequal group sizes by using SPSS software. In the kinetic study statistical significance was determined by using a one-way ANOVA with two-sided Dunnett post hoc test to compare all time points with the control group. To establish if there was a significant trend in the kinetic study a one-way ANOVA with a post test for trend was performed Graphpad Prism. Statistically significant differences in the adoptive transfer study were assessed with a one-way ANOVA with a Bonferroni post hoc test by using SPSS software.

Power calculations. Power analysis was performed by using ExpDesign software, a freely available tutorial program (Van Wilgenburg et al., 2003).

Results

TNF-α production induced by HCB in rat alveolar macrophages

In vitro studies with the rat alveolar macrophage cell line NR8383 were performed to investigate if HCB can activate these cells similar to silica. Preliminary experiments were performed to assess non-toxic concentrations of HCB and silica by measuring viability with MTT assay (not shown). Comparable non-toxic doses of HCB (1 mg/ml) and silica (0.8 mg/ml) were chosen and added to NR8383 cells. Both silica and HCB were taken up rapidly by macrophages and induced a time-dependent significant increase of TNF-α in the supernatant (Fig. 1).

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

Body weight gain

Body weight gain was not affected by treatment with HCB, clodronate-liposomes, or PBS-liposomes (not shown).

Lung morphology

Effects of clodronate-liposomes on HCB-induced lung pathology varied between the rats. Administration of clodronate-liposomes prevented HCB-induced lung pathology completely in one out of six rats. In the other five rats of that group, the perivascular eosinophilic infiltrate was less than in case of treatment with only HCB, but clodronate-liposomes could not prevent focal accumulations of macrophages and granuloma formation. In one of the control rats treated with clodronate-liposomes a few microgranulomas and a scattered pattern of eosinophilic granulocytes were found, phenomena that have been observed previously in BN rats (Ohtsuka et al., 1997). Lung morphology of HCB-exposed rats treated with PBS or PBS-liposomes was similar as described before (Michielsen et al., 1997). A summary of morphological changes in the lung is given in Table 1.

Macroscopic skin lesions

There was a considerable inhibitory action of clodronate-liposomes on HCB-induced inflammatory skin lesions (Table 2). In one out of six HCB-exposed rats treated with clodronate-liposomes skin lesions were completely absent. Remarkably, this was not the same rat in which lung pathology was prevented. In the other five rats skin lesions developed after 8–10 days of exposure, thus the onset of

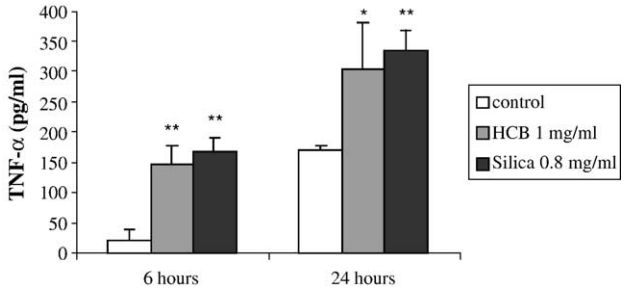

Fig. 1. TNF-α production (pg/ml supernatant) of NR8383 cells after incubation with non-toxic doses of HCB (1 mg/ml) or silica (0.8 ml) for 6 and 24 h. Data represent measurements in triplo. Significantly different from the control (cells incubated with medium) $*P < 0.05$ and $**P < 0.01$.

Table 1
Effects of clodronate-liposomes on the incidence of histopathological effects in lungs of BN rats exposed to the control or HCB diet[a]

Group	Number investigated	Accumulation of macrophages		Perivascular infiltrate		Granuloma formation	
		Slight	Moderate	Slight	Moderate	Slight	Moderate
Control + PBS	4	0	0	0	0	0	0
Control + clod-lip.	5	0	0	0	0	1	0
HCB + PBS	5	1	4	0	5	1	4
HCB + clod-lip.	6	3	2	5	0	3	2
HCB + PBS-lip.	4	2	2	0	4	2	2

[a] Sections were scored under code. The perivascular infiltrate consisted mainly of eosinophils. Granulomas were formed by monocytes, macrophages, Langhans type giant cells and eosinophilic granulocytes.

skin lesions was not different from the HCB-fed animals injected with PBS. However, in four of these five rats exacerbation was clearly inhibited resulting in smaller and less severe lesions after 20 days of exposure. Remarkably, clodronate-liposomes did not affect severity of skin lesions in one of the HCB-exposed rats. Table 2 shows relative skin lesions per individual animal to underscore the differences in this group.

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

Treatment with clodronate-liposomes decreased spleen weight in both control rats as well as in HCB-exposed rats (Table 3) and eliminated splenic macrophages considerably (Fig. 2C). From previous work it is known that clodronate-liposomes predominantly eliminate red pulp macrophages whereas white pulp macrophages are less affected (Biewenga et al., 1995). Furthermore, absolute numbers of B and T cells, which are increased more than twofold by HCB treatment, were drastically reduced as a result of treatment with clodronate-liposomes (Table 3). As a result of these changes tissue architecture of the spleen was completely disturbed. Histology showed depleted and necrotic areas predominantly in the red pulp. The necrotic areas were located mainly subcapsular and contained calcium deposits, as judged by Von Kossa staining (not shown). However, the degree of the effect of clodronate-liposomes differed between the different HCB-exposed rats. In all control rats and 4 out of 6 HCB-exposed rats treated with clodronate-liposomes abovementioned marked splenic effects were observed. However, in the other two HCB-exposed rats, clodronate-liposomes induced less drastic disturbances of the splenic architecture, i.e., no necrotic areas but still considerable macrophage depletion (Fig. 2C). HCB exposure induced comparable histopathological changes as those described before (Ezendam et al., 2004a; Michielsen et al., 1997; Schielen et al., 1993).

Auricular lymph node: cell number, number of macrophages, B and T cells

Clodronate-liposome treatment did not result in depletion of macrophages in the ALN, as judged microscopically by histopathology, nor did it affect the HCB-induced increase in ALN cell number, increased B/T ratio, or induction of germinal center formation (not shown).

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

Fig. 3 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 these organs. Furthermore, splenocytes from control rats injected with clodronate-liposomes produced less TNF-α compared to

Table 2
Summary of effects of clodronate-liposome treatment in individual animals[a]

	Lesional skin area	Lung pathology	Spleen weight	Splenic depletion	TNF-α spleen (pg/ml)
HCB + PBS	2.2 ± 0.45	++	0.56 ± 0.045	–	998 ± 104
HCB + PBS-lip.	3.2 ± 0.68	++	0.58 ± 0.012	–	864 ± 102
HCB + clod-lip					
no. 1	3.1	–	0.08	+	270
no. 2	0.59	+	0.15	+	390
no. 3	0.43	+	0.09	+++	57
no. 4	1.41	+	0.11	+++	47
no. 5	0.43	±	0.12	+++	37
no. 6	no lesions	±	0.08	+++	39

[a] Table summarizes effects of clodronate-liposomes in individual animals. Mean (±SE) of HCB-exposed rats treated with PBS or PBS-liposomes are also included in this table. Lesional skin area is expressed relative to total area and severity of lung pathology is a combination of macrophage accumulation, perivascular infiltrate and granuloma formation (Table 2). Spleen weight is expressed relative to body weight (g/100 g bwt) and splenic depletion refers to the depletion of macrophages, T and B cells judged microscopically. Splenic TNF-α production after LPS stimulation is expressed as pg/ml supernatant.

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

Treatment	Spleen weight	B cells	T cells
Control + PBS	0.33 ± 0.13	100	100
Control + clod-lip.	0.07 ± 0.09***,###	11 ± 1.8***,###	8.9 ± 3.6*,###
HCB + PBS	0.70 ± 0.44***	232 ± 16***	248 ± 28*
HCB + clod-lip.	0.11 ± 0.011***,###	30 ± 5.9***,###	30 ± 4.9***,###
HCB + PBS-lip.	0.75 ± 0.44***	223 ± 6.3***	205 ± 13**

[a] Spleen weight is expressed as absolute (in g) spleen weight after 20 days of exposure of female BN rats to control diet or HCB diet with or without treatment with clodronate-liposomes. Numbers of B and T cells are expressed as absolute (expressed relative to control group in %) numbers. Data represent mean ± SE of 4–6 animals per group. The asterisks indicate significance from control group, and significance from HCB-exposed group treated with PBS is also depicted.

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.
$P < 0.001$.

those from control rats injected with PBS. Administration of PBS-liposomes did not affect HCB-induced elevation of TNF-α.

Table 2 shows an overview of HCB-induced effects in individual clodronate-liposome-treated rats compared to the mean values of HCB-exposed rats treated with PBS. This table illustrates that clodronate-liposomes differentially affect the spleen and that animals with severe splenic depletion (of macrophages and T and B cell areas) produced less TNF-α after LPS stimulation. Spleens that were less depleted had clearly lower TNF-α levels compared to the HCB+PBS group, indicating that in these two animals clodronate-liposomes also depleted macrophages. This table also shows that in one animal lung pathology is completely absent, whereas in another skin lesions did not develop.

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 (Fig. 4).

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.

Table 4 summarizes kinetics of lung pathology, increases of relative 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, three out of four 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 three out of four rats. HCB lung effects became more pronounced from day 7 onwards.

Spleen weight and LPS-induced TNF-α production increased time-dependently from day 7 onwards, but increases in spleen weight were not significant until day 10 and the elevation of the TNF-α response to LPS was not significant until 18 days of exposure. Due to the high variance in the TNF-α data it is difficult to reach statistical

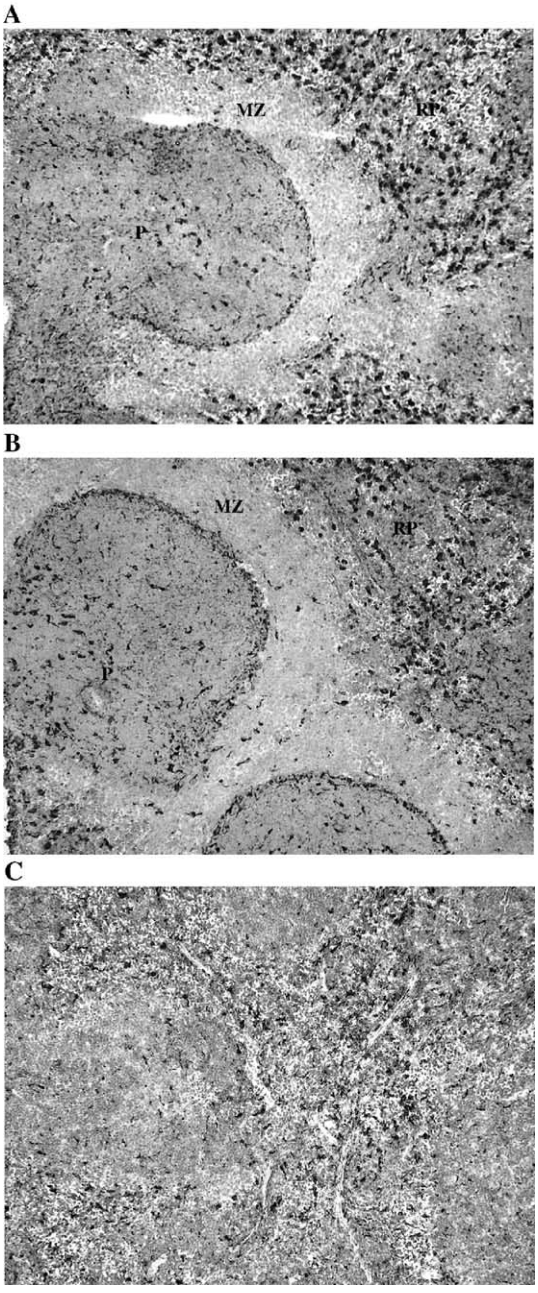

Fig. 2. Representative spleen sections from control rats (A), HCB-exposed rats (B), and HCB-exposed rats treated with clodronate-liposomes (C). Sections are stained with acid phosphatase to reveal macrophages (black dots). MZ: marginal zone; P: PALS; RP: red pulp. Oral exposure to HCB induced hyperplasia of MZ and follicles (B). Clodronate-liposomes not only eliminated macrophages in the spleen of both control and HCB-exposed rats, but also disturbed spleen morphology drastically (C).

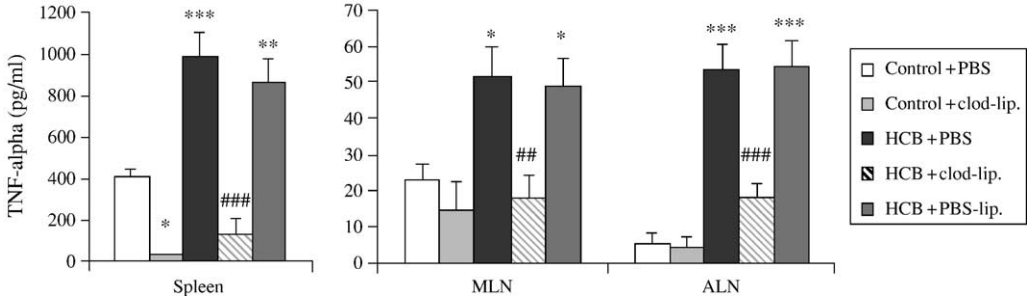

Fig. 3. Effect of HCB with or without macrophage depletion on LPS-induced TNF-α production (in pg/ml medium) of spleen, MLN and ALN cells. Data represent mean ± SE of 4–6 animals per group. Significantly different from the control group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and significantly different from the HCB+PBS group: $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

significance, although TNF-α production was increased after 7 days. However, power analysis of the kinetic data showed that for spleen weight a group size of 4 rats is sufficient from day 7 forward (power > 0.95). Also, this sample size is sufficient for cytokine data, because from day 7 power is higher than 0.95. Additional analysis has shown that the observed trend (e.g., time-dependent increase in spleen weight and TNF-α production) was significant for both parameters.

Adoptive transfer study

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. To investigate this, an adoptive transfer study was performed. No significant differences were observed between recipient HCB-exposed rats that received an adoptive transfer of splenocytes from HCB-exposed donor rats and normal BN rats that were exposed to HCB with respect to skin and lung lesions (not shown). Furthermore, adoptive transfer had no effect HCB-induced splenomegaly, LPS-induced IL-6 production and serum levels of IgM against ssDNA (Table 5). LPS-induced IL-6 production was only significantly increased in HCB-treated rats that received an adoptive transfer and not in HCB-treated rats without adoptive transfer. However, this could be due to the high variance between the groups. Furthermore, IL-6 production was not significantly different between the two HCB-treated groups. Power analysis showed that the power to detect differences between controls and HCB exposed rats was sufficient ($P > 0.98$) with a group size of 4 rats.

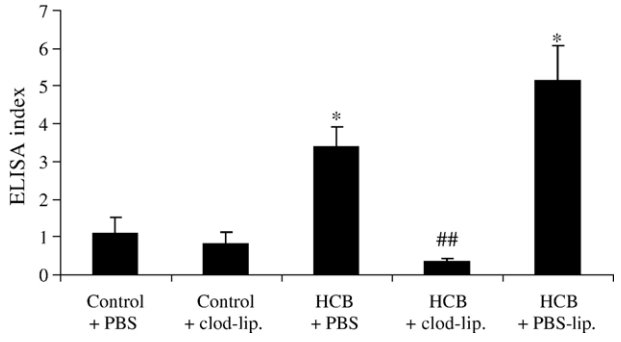

Fig. 4. Serum IgM levels against ssDNA are expressed as an ELISA index, calculated by plotting absorbance values of serial 2log dilutions of the control serum. The dilution of the individual test sera that would result in the same absorbance was calculated by linear regression. The ELISA index expresses 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 ($^{\#\#}P < 0.01$).

Discussion

In the present study we investigated the role of macrophages in HCB-induced immunopathology and data shows that macrophages are indeed important in HCB-induced skin lesions, lung eosinophilia and elevation of anti-ssDNA IgM. Kinetics of HCB-induced immune effects also suggest that macrophages are activated in lung and skin early after HCB exposure and that they are involved in HCB-induced clonal expansion of splenic B and T cells. Furthermore, no evidence was found for the induction of T cell sensitization and immunological memory after HCB exposure.

Skin lesions were completely prevented in only one of the HCB-exposed rats treated with clodronate-liposomes, whereas progression of the skin lesions was clearly prevented in 4 other rats. Remarkably, lung pathology was also completely prevented in one of the HCB-exposed rats, but this was not the same rat as the one without skin effects. It is difficult to explain the variability in effects. From previous work it is know that lung and skin lesions have a different etiology and that these lesions are not causally related (Michielsen et al., 1997). In addition, this variation in results might very likely be due to the fact that it is impossible to completely deplete all macrophages in an animal, because of the continuous release of monocytes from the bone marrow. Variations in effects induced by clodronate-liposomes have also been observed in a previous study in which clodronate-liposomes were administered several times to BN rats in which autoimmunity was induced with D-Penicillamine. In this model fewer rats

Table 4
Kinetics of HCB-induced lung pathology and spleen effects[a]

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***

[a] 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 formation. 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).

* $P < 0.05$.

*** $P < 0.001$.

treated with clodronate-liposomes got sick (60% incidence) than control rats (100% incidence) (Masson et al., 2004). Thus, macrophage depletion is not equally effective in different rats. Furthermore, clodronate-liposomes predominantly depleted macrophage in the spleen and could not prevent macrophage infiltrations in the lung. 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 and only slightly reducing macrophages in some LNs, such as the MLN (Van Rooijen et al., 1990). In HCB-treated rats, TNF-α production of ALN and MLN cell suspensions stimulated with LPS were not increased after administration of clodronate-liposomes. It is not clear if this is a direct inhibitory effect of clodronate-liposomes on macrophages, or an indirect effect as a consequence of the prevention of immunopathology, e.g., skin pathology, in clodronate-liposome treated animals resulting in no activation of macrophages at all in these LNs. The latter would be in line with our previous suggestion that activation of ALN might be secondary to skin lesions (Ezendam et al., 2004a).

From the data that were obtained after macrophage depletion, it can be concluded that skin and lung pathology depend on macrophages. Additionally, the kinetic data indicate that skin and lung pathology are induced early after exposure and are the result of an early non-specific inflammation. Together with the lack of evidence for specific T cell sensitization, these data support our present view that macrophages are involved in HCB-induced T cell stimulation. The specificity of the activated T cells is not known. Initially we proposed that T cells may recognize metabolites of HCB such as TCBQ as this metabolite of HCB was clearly immunogenic in the PLNA (Ezendam et al., 2003). However, the disease could not be adoptively transferred and this suggests that HCB does not induce specific T cell sensitization.

Although results obtained in present work suggest that macrophages are indeed involved in T and B cells activation after HCB exposure, exact mechanisms are still unclear. The in vitro experiments with alveolar macrophages show that HCB is able to activate rat alveolar macrophages similar to silica. One can envisage that cytokines, such as IL-6, produced by activated macrophages, are involved in polyclonal proliferation and activation of both T and B cells (Roitt et al., 1993). Moreover, pro-inflammatory cytokines are known to directly activate naive T cells (Curtsinger et al., 1999; Pape et al., 1997). Additionally, these cytokines can also increase costimulatory molecules on dendritic cells, and in this way provide signal 2 that is needed for T cell activation.

In conclusion, present work emphasizes the importance of macrophages as possible initiators of the HCB-induced adverse immune response. Presumably, HCB activates macrophages to produce pro-inflammatory mediators and reactive oxygen species. This is in accordance with gene

Table 5
Effects of adoptive transfer of splenocytes on HCB-induced immune parameters[a]

Group	Spleen weight	IL-6 production	ssDNA-IgM
Control	0.31 ± 0.02	8.3 ± 2.8	0.9 ± 0.2
Control + adoptive transfer	0.30 ± 0.02	8.1 ± 0.9	1.1 ± 0.3
HCB	0.51 ± 0.05**,##	16 ± 1.0	2.9 ± 0.5*,#
HCB + adoptive transfer	0.52 ±0.03**,##	20 ± 3.8*,#	3.6 ± 0.7*,#

[a] Table shows the results of adoptive transfer of splenocytes from donor HCB-exposed rat to naive BN rats that were exposed to a control or a HCB supplemented diet for 21 days after transfer ($n = 4$ per group). Effects of transfer on relative spleen weight (in g/100 g bwt), LPS-induced IL-6 production (in ng/ml supernatant) and serum IgM levels against ssDNA (see footnote of Table 5 for calculation the ELISA index) are shown. The asterisks indicate significance from control group or from control group + adoptive transfer ($^{###}P < 0.001$).

* $P < 0.05$.

** $P < 0.01$.

\# $P < 0.05$.

\## $P < 0.01$.

expression profiles assessed with DNA microarrays (Ezendam et al., 2004b). Additionally, in this pro-inflammatory environment a cascade of events will be provoked, 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 confined to immunological organs, but involved also liver and kidney, suggesting a systemic inflammatory response (Ezendam et al., 2004b). 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 may result in the observed HCB-induced immunopathology.

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