Effects of Exposure to Decabromodiphenyl Ether on the Development of the Immune System in Rats

Reiko Teshima,* Ryosuke Nakamura, Rika Nakamura, Akiko Hachisuka, Jun-ichi Sawada, and Makoto Shibutani[1]

Division of Biochemistry and Immunochemistry, National Institute of Health Sciences, 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan

(Received September 30, 2007; Accepted May 1, 2008)

To evaluate the developmental toxicity of decabromodiphenylether (DBDE) after exposure during the period from late gestation to after lactation, maternal Sprague-Dawley rats were given DBDE at dietary concentrations of 0, 10, 100, and 1000 ppm from gestational day 10 (GD 10) to postnatal day (PND) 21. On PND 21 and 77, lymphocytes (Lymph) in the spleen, thymus, and peripheral blood of male pups were subjected to flow cytometric analyses for expression of surface markers [CD3, CD4, CD8a, CD25, CD45RA, CD71, and CD161(NKRP1A)]. On PND 21, the proportions of splenic CD4+ T cells in the 10-ppm group, activated B (CD45RA+CD71+) cells in the 100- to 1000-ppm groups, and activated T cells (CD3+CD71+) in the 1000-ppm group were significantly decreased, and the population of peripheral CD161+ natural kiler cells on PND 21 and 77 had decreased in the 100- to 1000-ppm groups. In the 1000-ppm group, the serum T3 level was significantly decreased on PND 21 and the serum T4 level was decreased on PND 77. The body, spleen, and thymus weights were not significantly decreased, but liver weight was significantly increased on PND 21 in the 10- to 1000-ppm groups. These results suggest that on PND 21, developmental exposure to the highest dose of DBDE had a weak immunomodulatory effect. Although the most of the immunomodulatory effect had recovered to normal levels on PND 77, a decreasing effect on the natural killer (NK) cell population remained.

Key words —— decabromodiphenyl ether, immune system, developmental exposure, natural killer cells

INTRODUCTION

Brominated flame retardants (BFRs) have routinely been added to consumer products for several decades in a successful effort to reduce fire-related injury and property damage.[1)] The five major BFRs in use are tetrabromo bisphenol A (TBBPA), hexabromocyclododecane (HBCD), and three polybrominated diphenylether (PBDEs) known as decabromodiphenylether (DBDE), octabromodiphenylether (OBDE), and pentabromodiphenylether (pentaBDE). More than 200000 metric tons of BFRs are produced worldwide each year. BFR production has increased dramatically over the past 20 years. Of the 117950 tons of BFRs consumed in Asia in 2001, approximately 76% was TBBPA, 21% PBDEs, and 3% HBCD.[2)]

DBDE is the major PBDE product in all markets, and accounts for approximately 80% of total PBDE production worldwide.[3)] DBDE is used as an additive flame retardant primarily in electrical and electronic equipment, as well as in textiles, where it is applied as a polymer backcoat to fabric. Limited evaluations of the ecologic effects of PBDEs have been conducted. In general, the lower brominated mixtures are more toxic than the higher congeners. As PBDEs are lipophilic and easily bioaccumlated in organisms through the food chain, PBDEs have recently been detected in many marine products (average concentration of PBDEs in raw fish, 0.22 ng/g),[4)] and exponential increases in the concentration of PBDE in human milk (0.07 ng/g lipid in 1972 and 4.02 ng/g lipid in 1997) have been reported.[5)]

Mammalian toxicity studies have been conducted in both rats and mice. The most extensive

*[1] Present address: Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3–5–8 Saiwai-cho, Fuchu, Tokyo 183–8509, Japan

*To whom correspondence should be addressed: Division of Biochemistry and Immunochemistry, National Institute of Health Sciences, 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan. Tel.: +81-3-3700-1141 (Ext. 243); Fax: +81-3-3707-6950; E-mail: rteshima@nihs.go.jp

data set exists for DBDE, with studies ranging from acute to chronic laboratory studies. The US National Toxicology Program (NTP) conducted 2-year feeding studies of DBDE[6] and showed that high doses up to 50000 ppm in the diet resulted in neoplastic nodules in the liver in both male and female rats. DBDE had similar effects on male mice but did not induce neoplastic nodules in female mice. Few effects other than the low incidence of tumors were seen. A developmental neurotoxic effect of pentaBDE (BDE99) in mice[7] and a thyroid system-impairing effect of commercial tetra and penta BDE mixtures in several experimental models[8,9] have been reported. However, little information exists on the reproductive effects of DBDE. Furthermore, regulation of the developing immune system by thyroid hormone[10,11] and the effects of experimentally induced hypothyroidism in adult mice on the immune response have been reported,[12] although the developmental effects of DBDE on the immune system have not yet been examined. We therefore investigated the effects of DBDE on the developing immune system in rats to determine the safe dose of the compound.

MATERIALS AND METHODS

Chemicals and Animals —— DBDE was purchased from Wako Pure Chemical Industries (Osaka, Japan). Pregnant Sprague-Dawley: IGS female rats were purchased from Charles River Japan, Inc. (Kanagawa, Japan) on gestational day 3 (GD3). The animals were housed individually in polycarbonate cages (SK-Clean, 41.5 cm × 26 cm × 17.5 cm; CLEA Japan Inc., Tokyo, Japan) on wood chip bedding (Soft Chip; Sankyo Lab Service Corp., Tokyo, Japan) and maintained in an air-conditioned animal room (temperature 24 ± 1°C, relative humidity 55 ± 5%) on a 12-hr light/dark cycle. They were given *ad libitum* access to food and tap water. CRF-1, a regular rodent diet, obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan) was used as the basic diet for the offspring, while dams from GD10 to PND 21 received a soy-free diet (Oriental Yeast Co. Ltd.), prepared based on the NIH-07 open-formula rodent diet and with nutritional standards the same as those of the the CRF-1 (supplier's analysis).[13]

Experimental Protocol —— Immediately after arrival at the test facility, dams were provided with a powdered soy-free diet. On GD10, they were randomized into four groups (6–8 dams/group) and provided with a soy-free diet that contained DBDE at a concentrations of 0, 10, 100, or 1000 ppm until PND 21. Based on the results of a preliminary study, the highest dose was selected as the level to maintain pregnancy, delivery, and lactation (data not shown). On PND 2, the number, weight, and anogenital distance (AGD) of the neonates were recorded. On PND 21, dosing was terminated, the offspring were weaned, and 10 males and 10 females (at least 1 male and 1 female per litter) per group were selcted for prepubertal necropsy, and 10 males and 10 females (at least 1 male and 1 female per litter) per group for necropsy on PND 77. The diet was changed to CRF-1 at weaning to eliminate possible modifications due to the long-term use of a soy-free diet on development after weaning.[14]

The prepubertal necropsy of the male rats was performed on PND 21 to evaluate the weights and histopathology of immune organs (see details below). On PND 77, offspring were subjected to organ weight measurements and histopathologic examination of immune-related organs.

All animals were sacrificed by exsanguination from the abdominal aorta under deep anesthesia. The animal protocols were reviewed in terms of animal welfare and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Cell Phenotyping —— Cell phenotypes were determined using flow cytometric (FCM) analysis with monoclonal murine antibodies. Two-color or three-color analysis of spleen, thymus, and peripheral blood subsets was performed. The antibodies used for FCM were phycoerythrin (PE)-labeled anti-rat CD8a (OX-8; BD Pharmingen), PE-Cy5-labeled anti-rat CD4 (OX-35; BD Pharmingen), fluorescein isothiocyanate (FITC)-labeled anti-rat CD3 (1F4; BD Pharmingen), PE-labeled anti-rat CD25 (IL2Rα chain) (OX-39; BD Pharmingen), PE-Cy5-labeled anti-rat CD45RA (OX33; BD Pharmingen), PE-labeled anti-rat CD71 (transferrin receptor) (OX26; BD Pharmingen), and FITC-labeled anti-rat CD161a (NKRP1A) (10/78; BD Pharmingen) antibodies. All incubations were performed in the dark. A single-cell suspension of lymphocytes in PharMingen Stain Buffer containing 2% Fetal Bovine serum (FBS) was incubated with 50 µl of properly diluted monoclonal antibody at 4°C for 30 min. The cells were washed by centrifugation in phosphate buffered saline (PBS) containing 2% FBS, and after staining, a total of at least 10000 cells

was analyzed with a FACS Calibur (Becton Dickinson, Sunnyvale, CA, U.S.A.). The data were analyzed with Cellquest software.

Antibody Production —— Female offspring exposed perinatally to DBDE until weaning (PND 21) were immunized with keyhole limpet hemocyanin (KLH) (Calbiochem, 25 μg) plus 1 mg of alum three times, on PND 23, 33, and 43. Serum was obtained on PND 50. The serum titers (reciprocal of serum dilution with colorimetric intensity at 50% of the maximum level) of KLH-specific IgG and IgM were determined. A 50-μl volume of KLH (40 μg/ml) in sodium carbonate buffer 50 mM, pH 9.6, was added to each well of a 96-well microtiter plate, and the plate was incubated overnight at 4°C. The solutions were discarded, and each well was washed 4 times with PBS 200 μl containing 0.05% Tween 20 (PBS/Tween). To minimize the nonspecific binding of serum proteins to unoccupied solid-phase sites, 200 μl of 0.1% casein in PBS was added, and the plates were incubated for 1 hr at room temperature. The casein solution was removed, and each well was washed in the same manner as above. Fifty microliters of the diluted serum containing KLH-specific antibodies was added to each well, and the plates were incubated for 20 hr at 4°C. The solution was removed, and each well was washed. Fifty microliters of rabbit anti-rat IgM or IgG [10^{-3} dilution in PBS containing 0.1% casein (Nordic Immunology, Tilburg, the Netherlands)] was added to each well, and the plates were incubated for 1 hr at room temperature. The solution in each well was removed and washed. Fifty microliter peroxidase-conjugated donkey anti-rabbit Ig antibodies (1 : 1000, Amersham) in 0.1% casein-PBS was added and incubated for 1 hr at room temperature and then reacted with a colorimetric substrate solution (TMB reagent, Cat. No. 555214; BD Biosciences, San Diego, CA, U.S.A.). Colorimetric intensity (OD_{450}) was measured according to the manufacturer's protocol.

Hematological Examination —— The red blood cell (RBC) count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and white blood cell (WBC) count were determined with an autohematology analyzer (M-2000, Sysmex Corp., Kobe, Japan). The percentage of segmented neutrophils (Seg Neut), banded neutrophils (Band Neut), eosinophils (Eosino), basophils (Baso), lymphocytes (Lymph), monocytes (Mono), and erythroblasts (Ebl) among WBCs were determined with an automated blood cell analyzer (Microx Heg-120A, Tateishi Electronic Corp., Kyoto, Japan). T3, T4, and thyroid stimulating hormone (TSH) in the sera were measured at the SRL Corporation (Tokyo, Japan).

Histopathological Examination —— Immune-related organs (spleen and thymus) obtained from each animal and fixed in neutralized formalin as described above were embedded in paraffin, processed, and stained with hematoxylin and eosin (HE). The HE-stained sections were examined under a light microscope and evaluated histopathologically.

Statistical Analysis —— All values are expressed as mean ± standard deviation. The group data were first tested for homogeneity of variance. Groups were then compared using one-way analysis of variance (ANOVA) and Dunnett's test. For histopathologic analysis, Fisher's exact probability test was used to compare the differences between groups.

RESULTS

Body, Thymus, Spleen and Liver Weights and Hematologic Examination

The indirect DBDE exposure method, *i.e.*, via the placenta or maternal milk, was utilized in this study. On PND 21, no significant differences in absolute body weight were found between male pups exposed to DBDE and the control male pups, and 8 weeks after the conclusion of DBDE exposure (PND 77), the 10- to100-ppm DBDE-treated groups were examined for increases in body weight. No significant differences in thymus or spleen weight were found between the DBDE groups and control group, but on PND 21 liver weight was significantly increased in the 10- to1000-ppm DBDE groups, but their weight had returned to the control level at 8 weeks after DBDE was removed from the diet (PND 77) (Table 1). An increase in the liver weight of female rats was also observed on PND 21 (data not shown). No significant differences in leukocyte numbers were found in the thymus or spleen (data not shown). No significant differences in RBC, MCV, MCH, MCHC, or WBC and the percentage of neutrophils (Seg and Band Neut), Eosino, Baso, Lympho, or Mono were found between the male DBDE groups and the control group. A tendency toward an increase in the percentage of Ebl was observed in the DBDE group on PND 21 (data not shown).

Table 1. Body and Organ Weights of Offspring Exposed to DBDE during the Period from Mid-Gestation to Lactation

	DBDE in diet (ppm)			
	0	10	100	1000
PND 21				
No. of offspring examined	10	10	10	10
BW (g)	51.6 ± 6.2[a)]	55.8 ± 4.0	52.7 ± 6.0	54.0 ± 3.0
Liver (g/ 100 g BW)	3.62 ± 0.26	3.98 ± 0.12[*]	3.90 ± 0.29[*]	4.39 ± 0.27[**]
Spleen (g/ 100 g BW)	0.392 ± 0.06	0.417 ± 0.074	0.381 ± 0.088	0.382 ± 0.044
Thymus (g/ 100 g BW)	0.442 ± 0.066	0.440 ± 0.055	0.411 ± 0.056	0.414 ± 0.064
PND 77				
No. of offspring examined	10	10	10	10
BW (g)	414.4 ± 22.3[a)]	447.8 ± 24.1[*]	455.1 ± 22.9[**]	423.2 ± 34.5
Liver (g/ 100 g BW)	3.66 ± 0.18	3.65 ± 0.12	3.62 ± 0.10	3.42 ± 0.29
Spleen (g/ 100 g BW)	0.191 ± 0.023	0.197 ± 0.042	0.191 ± 0.035	0.189 ± 0.021
Thymus (g/ 100 g BW)	0.131 ± 0.017	0.121 ± 0.021	0.122 ± 0.014	0.126 ± 0.031

a) Mean ± S.D. DBDE, decabromodiphenylether; PND, postnatal day. [*, **]Significantly different from the controls by Dunnett's test ([*] $p < 0.05$, [**] $p < 0.01$).

Table 2. Serum Levels of Thyroid-Related Hormones of the Offspring Exposed to DBDE during the Period from Mid-Gestation to Lactation

	DBDE in diet (ppm)			
	0	10	100	1000
PND 21				
No. of offspring examined	10	10	10	10
T3 (ng/ml)	1.39 ± 0.11[a)]	1.35 ± 0.15	1.33 ± 0.18	1.17 ± 0.10[**]
T4 (μg/dl)	5.19 ± 0.74	4.89 ± 0.84	5.66 ± 0.71	4.89 ± 0.54
TSH (ng/ml)	5.38 ± 0.89	5.12 ± 0.71	5.85 ± 1.22	4.74 ± 0.69
PND 77				
No. of offspring examined	10	10	10	10
T3 (ng/ml)	0.99 ± 0.09	1.01 ± 0.08	1.01 ± 0.11	1.02 ± 0.11
T4 (μg/dl)	6.02 ± 0.70	6.00 ± 0.66	5.98 ± 0.94	5.17 ± 0.57[*]
TSH (ng/ml)	8.30 ± 3.40	8.81 ± 1.63	9.71 ± 3.45	10.47 ± 2.35

a) Mean ± S.D. DBDE, decabromodiphenylether; PND, postnatal day. [*, **]Significantly different from the controls by Dunnett's test ([*] $p < 0.05$, [**] $p < 0.01$).

Serum Levels of Thyroid-Related Hormones

As shown in Table 2, on PND 21 the serum level of the thyroid-related hormone T3 was significantly decreased in the 1000-ppm DBDE group. On PND 77, the serum T4 level was significantly decreased in the 1000-ppm DBDE group, and a tendency toward an increase in TSH was also found.

Splenocyte, Thymocyte, and Peripheral Blood Lymph Subpopulations

The effect of DBDE on the surface phenotype of Lymph is shown in Table 3. Significant differences were observed in some Lymph populations in the spleen and peripheral blood cells. On PND 21, the proportions of splenic CD4+ T cells in the 10-ppm group, activated B (CD45RA+CD71+) cells in 100- to 1000-ppm groups, and activated T cells (CD3+CD71+) in the 1000-ppm group were significantly decreased, and on PND 21 and 77 the population of peripheral CD161+ natural killer (NK) cells was decreased in the 100- to 1000-ppm group. On PND 77, about 40% of peripheral blood CD161+ NK cells were CD161 and CD4 double-positive cells. On PND 77, a tendency for the population of splenic CD161+NK cells to be reduced was also observed (data not shown).

The CD161 and CD4 double-positive cells seemed to be CD4+NKT cells,[11, 15)] and the CD161+CD4-cells seemed to be classic NK cells. Figure 1 shows the typical pattern of expression of CD161 on the peripheral blood Lymph derived from the DBDE group on PND 77. M1 shows the CD161-positive portion of the Lymph. Two populations of CD161 positive-Lymph are shown. The

Table 3. Flow Cytometric Analysis of Lymphocytes of Offspring Perinatally Exposed to DBDE

T cell subpopulations				
DBDE:	Spleen PND 21			
	0 ppm	10 ppm	100 ppm	1000 ppm
CD8a(−) CD4(+)	14.98 ± 2.43	12.25 ± 1.61*	12.59 ± 3.08	11.34 ± 2.41**
CD8a(+) CD4(−)	6.99 ± 1.49	5.28 ± 0.92*	6.91 ± 1.62	6.73 ± 1.41
CD3(+) CD4(+)	6.28 ± 1.57	4.68 ± 0.86*	6.27 ± 2.53	4.67 ± 1.53
DBDE:	Spleen PND 77			
	0 ppm	10 ppm	100 ppm	1000 ppm
CD8a(−) CD4(+)	27.99 ± 11.13	21.62 ± 3.01	23.33 ± 3.07	21.9 ± 3.66
CD8a(+) CD4(−)	18.99 ± 3.73	18.02 ± 2.05	18.27 ± 3.29	17.74 ± 1.63
CD3(+) CD4(+)	20.26 ± 4.12	19.1 ± 3.13	20.94 ± 2.98	19.02 ± 3.92
Activation of T/B cells				
DBDE:	Spleen PND 21			
	0 ppm	10 ppm	100 ppm	1000 ppm
CD3(+) CD71(+)	0.51 ± 0.1	0.47 ± 0.14	0.42 ± 0.13	0.38 ± 0.09*
CD71(+) CD45RA(+)	3.92 ± 1.12	3.16 ± 0.73	2.69 ± 0.84*	2.42 ± 0.72**
CD3(−) CD45RA(+)	50.67 ± 10.11	49.05 ± 7.13	51.82 ± 7.32	51.54 ± 8.79
CD3(+) CD45RA(−)	15.21 ± 3.65	11.88 ± 2.21*	14.83 ± 4.41	12.24 ± 2.23
DBDE:	Spleen PND 77			
	0 ppm	10 ppm	100 ppm	1000 ppm
CD3(+) CD71(+)	0.36 ± 0.12	0.31 ± 0.09	0.39 ± 0.19	0.34 ± 0.13
CD71(+) CD45RA(+)	1.13 ± 0.41	1.09 ± 0.4	1.29 ± 0.46	1.15 ± 0.38
CD3(−) CD45RA(+)	31.13 ± 5.79	34.46 ± 2.97	33.83 ± 7.5	36.62 ± 3.12*
CD3(+) CD45RA(−)	50.35 ± 7.72	46.42 ± 4.09	49.51 ± 6.3	46.32 ± 4.11
Treg, NK, NKT cells				
DBDE:	Peripheral blood PND 21			
	0 ppm	10 ppm	100 ppm	1000 ppm
CD25(+) CD4(+)	2.33 ± 0.66	2.58 ± 0.79	2.32 ± 0.74	1.91 ± 0.68
NKRP1A(+) CD4(+)	17.04 ± 3.84	12.14 ± 5.9	10.92 ± 4.62*	13.66 ± 4.16
NKRP1A(+) CD4(−)	6.07 ± 1.37	5.48 ± 1.69	5.07 ± 1.21	5.51 ± 1.06
DBDE:	Peripheral blood PND 77			
	0 ppm	10 ppm	100 ppm	1000 ppm
CD25(+) CD4(+)	3.11 ± 0.65	2.9 ± 0.6	2.44 ± 0.52*	2.28 ± 0.52*
NKRP1A(+) CD4(+)	9.95 ± 2.61	7.37 ± 2.59	7.86 ± 2.38	7.02 ± 1.38*
NKRP1A(+) CD4(−)	12.75 ± 2.55	12.1 ± 3.03	12.62 ± 2.62	9.12 ± 0.85**

Values are mean ± S.D. [% (gated)] $n = 10$. *, **Significant difference from control at $p < 0.05$ and $p < 0.01$, respectively.

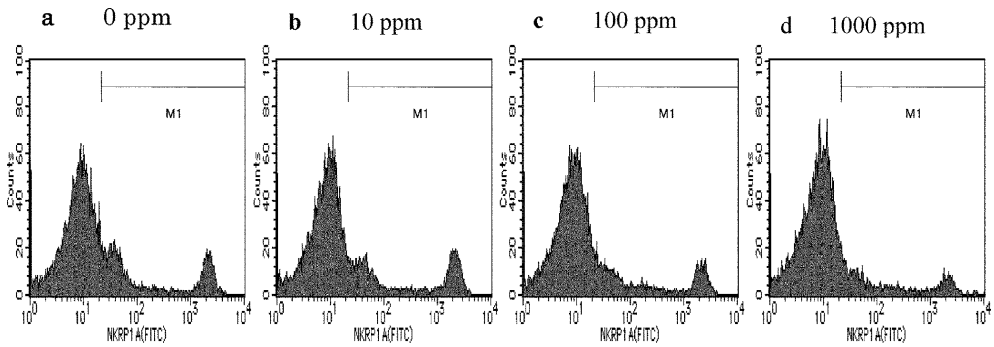

Fig. 1. Expression of CD161 (NKRPIA) on the Peripheral Blood Lymphocytes on PND 77 from DBDE-Exposed Pups

On PND 77, the peripheral blood lymphocytes of DBDE-exposed pups were examined for the presence of CD161-positive cells by flowcytometry as described in the Materials and Methods section. The ordinate shows the relative cell number of cells, and the abscissa shows the log of their fluorescence intensity.

CD161-high expression population was CD4 negative, and the CD161-moderate expression population was CD4 positive (data not shown). A dose-dependent decrease in the percentage of both populations of peripheral CD161-positive Lymph (both NK cells and CD4+NKT cells) was observed. The DBDE group examined on PND 77 showed a decrease in CD4+CD25+ regulatory T (Treg) cells among peripheral blood cells.

Production of Antibody to KLH

Figure 2 shows the serum KLH-specific IgG titers. ELISA titers of specific IgG to KLH tended to be enhanced and reduced in the 10-ppm and 1000-ppm DBDE group, respectively, but the difference was not statistically significant. No tendency for a decrease was observed in the production of KLH-specific IgM.

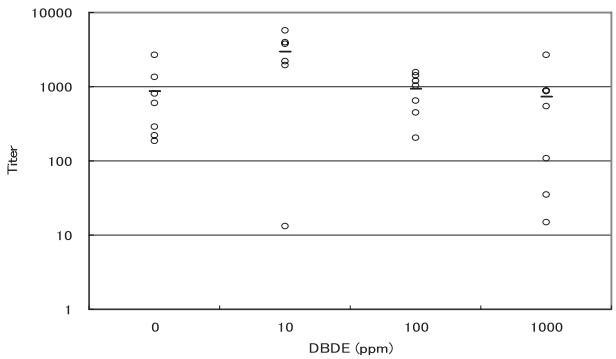

Fig. 2. Effects of DBDE on Production of IgG to KLH

Perinatally DBDE-exposed female pups were immunized with KLH three times over a 4- to 7-week period. Serum was obtained 1 week after final immunization, and the IgG titer to KLH was measured using ELISA as described in the Materials and Methods section. Open circles represent individual values, and dashes interconnected by a line indicate mean values.

Histopathology of the Spleen and Thymus

Table 4 shows the histopathology of the thymus and spleen of SD rats exposed to DBDE perinatally. On PND 21, a slight reduction in the area of red pulp and erythropoiesis was observed in some pups in the 100-ppm group. On PND 77, a slight reduction in the cortical area of the thymus and a reduction in the marginal region of white pulp and the area of red pulp in the spleen was observed in some pups in the 1000-ppm group. However, none of the pathologic changes observed in the DBDE group were significant when compared with the control group.

DISCUSSION

There have been few reports on the effects of BFRs on the immune system. Although an inhibitory effect of TBBPA on Lymph proliferation and expression of CD25 *in vitro* has been reported, [16] there have been few reports on the effects of other BFRs on the immune system. In this study, we first examined the effects of a typical BFR, DBDE, on the developing immune system *in vivo*.

A developmental neurotoxic effect of pentaBDE in mice[7] and a thyroid system-impairing effect of commercial tetra and penta BDE mixtures in rats and mice[8, 9] have been reported. Our own exper-

Table 4. Histopathology of the Thymus and Spleen of Male Rats Perinatally Exposed to DBDE

	DBDE in diet (ppm)			
	0	10	100	1000
PND 21				
No. of animals examined	10	10	10	10
Thymus				
Abnormalities detected	0	0	0	0
Spleen				
Reduction of the area of red pulp (±)[a)]	0[b)]	0	3	0
Reduction of erythropoiesis (±)	0	0	3	0
PND 77				
No. of animals examined	10	10	10	10
Thymus				
Reduction of cortical area (±)	1	0	0	2
Spleen				
Reduction of the mar ginal region of white pulp (±)	0	0	0	1
Reduction of the area of red pulp (±)	0	0	1	2

a) Grade of change: ±, minimal. *b*) Total no. of animals with each finding.

iments revealed a decrease in the serum level of the thyroid hormones T3 (1000 ppm at PND 21) and T4 (1000 ppm at PND 77) in DBDE-exposed pups (Table 2). Therefore indirect exposure of the pups to DBDE through the dams confirmed a thyroid system-impairing effect on pups.

The results in regard to the developing immune system showed that on PND 21 the proportions of splenic CD4+ T cells in the 10-ppm group, activated B (CD45RA+CD71+) cells in the 100- to 1000-ppm groups, and activated T cells (CD3+CD71+) in the 1000-ppm group were significantly decreased, and on PND 21 and 77 the population of peripheral CD161+NK cells had decreased in the 100- and 1000-ppm groups (Table 3). A tendency toward a decrease in anti-KLH IgG antibody production was observed in the 1000-ppm group that was immunized with KLH 3 times (Fig. 2). Since KLH is a T-cell-dependent antigen,[17] the reduction in antibody production seems to be related to the decrease in the percentage of CD4+ helper T cells or the decrease in activated B and T cells. These results suggest that developmental exposure to DBDE had a weak immunomodulatory effect at the highest dosage during the exposure period, which seems to be correlated with the perturbation of thyroid hormone homeostasis. Although most of the modulatory effect had recovered to normal levels after growth, a decreasing effect on the proportion of NK cells remained.

We showed the existence of two populations of NK cells, CD161 single-positive and CD4/CD161 double-positive cells, in peripheral blood (Fig. 1). The CD161+CD4 cells seemed to be classic NK cells, which are a form of cytotoxic Lymph constituting a major component of the innate immune system for the host rejection of both tumors and virally infected cells. The CD4/CD161 double-positive cells seemed to be CD4-positive NKT cells, known as T cells expressing NK receptors and capable of secreting IL-4 and IFN-γ upon stimulation.[18] Collabolation of NKT cell and Treg (CD4+CD25+) cells in the prevention of autoimmunity has also been reported.[19] Therefore the decrease in the population of NK cells might have a major influence on the balance of a variety of immune responses, both innate and aquired.

Induction of UDP-glucuronide transferase (UDPGT), the key phase II metabolizing enzyme involved in the conjugation of T4, has been reported in commercial PBDE mixture-exposed weanling rats[9] and developmentally exposed (GD6-PND 21) rats.[20] Therefore induction of UDPGT presumably also occurred in our DBDE-exposed pups.

DBDE has historically been shown to be poorly absorbed after oral exposure.[21] However, recent studies in the rat have shown that DBDE can be absorbed (> 10% of the dose) when administered orally, and that higher concentrations are found in the plasma and highly perfused tissues such as the liver, heart, adrenal gland, and kidney, whereas adipose tissue has lower concentrations.[22] The same group of investigators also showed that approximately 10% of the dose was eliminated in the bile as hydoxy/methoxy metabolites containing five to seven bromide atoms. It seems to be reasonable that lipophilic DBDE could pass through dam rat placenta or be secreted in maternal milk and exert its immunomodulative activities in the offspring, although the possibility that the drug indirectly affects the offspring through decreases in the maternal thyroid hormones is not completely excluded. Shinohara *et al.* reported that a 4-hydroxy group is necessary for estrogen and thyroid hormone-disrupting activities, and 3,5-dibromo substitution is also necessary for thyroid hormone-disrupting activity.[23] Since the concentrations of these metabolites of DBDE in orally fed rats seems to be low, their thyroid hormone-disrupting effect would also seem to be slight.

In conclusion, developmental exposure to DBDE seems to have a weak immunomodulatory effect at the highest dosage, resulting in a decrease in NK cell populations, a decrease in the helper T cell population, and a decrease in activated B and T cells, which seems to be correlated with the perturbation of thyroid hormone homeostasis.

Acknowledgement This study was supported by a grant from the Ministry of Health, Labor and Welfare of Japan.

REFERENCES

1) Birnbaum, L. S. and Staskal, D. F. (2004) Brominated flame retardants: Cause for concern? *Environ. Health Perspect.*, **112**, 9–17.
2) BSEF (2001) Major Brominated Flame Retardants Volume Estimates: Total Market demand by Regions. Brussels: Bromine Science and Environmental Forum. Available: http://www.bsef.com/docs/BFR_vols_2001.doc [accessed 30 December 2006].
3) BSEF (2000) An Introduction to Bromine Brussels: Bromine Science and Environmental Forum.

Available: http://www.bsef.com/docs/bromine.pdf [accessed 30 December 2006].
4) Nakagawa, R., Ashizuka, Y., Hori, T., Tobiishi, K., Yasutake, D. and Sasaki, K. (2005) Determination of brominated retardants in fish and market basket food samples of Japan. *Organohalogen Compounds*, **67**, 498–501.
5) Meironyt, D., Noren, K. and Bergan, A. (1999) Analysis of polybrominated diphenyl ethers in Swedish human milk. A time-related trend study, 1972–1997. *J. Toxicol. Environ. Health A*, **58**, 329–341.
6) NTP (1986) *Toxicology and Carcinogenesis Studies of Decabromodiphenyl Oxide* (*CAS No. 1163-19-5*) *in F344/N Rats and B6C3F1 Mice* (*Feed Studies*). TR-309. Research Triangle Park, NC: National Toxicology Program.
7) Eriksson, P., Jakobsson, E. and Fredriksson, A. (2001) Brominated flame retardants: a novel class of developmental neurotoxicants in our environment? *Environ. Health Perspect*., **109**, 903–908.
8) Hallgren, S., Sinjari, T., Hakansson, H. and Darnerud, P. O. (2001) Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls(PCBs) on thyroid hormone and vitamin A levels in rats and mice. *Arch. Toxicol.*, **75**, 200–208.
9) Zhou, T., Taylor, M. M., DeVito, M. J. and Crofton, K. M. (2002) Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. *Toxicol. Sci.*, **66**, 105–116.
10) Rooney, A. A., Fournier, M., Bernier, J. and Cyr, D. G. (2003) Neonatal exposure to propylthiouracil induces a shift in lymphoid cell sub-populations in the developing postnatal male rat spleen and thymus. *Cell. Immunol.*, **223**, 91–102.
11) Nakamura, R., Teshima, R., Hachisuka, A., Sato, Y., Takagi, K., Nakamura, R., Woo, G.-H., Shibutani, M. and Sawada, J. (2007) Effects of developmental hypothyroidism induced by maternal administration of methimazole or propylthiouracil on the immune system of rats. *Int. Immunopharmacol.*, **7**, 1630–1638.
12) Teshima, R., Ikebuchi, H. and Terao, T. (1982) Effects of ethylenethiourea on the functions of mouse lymphocytes, *Bull. Natl. Inst. Hyg. Sci.*, **100**, 44–48.
13) Masutomi, N., Shibutani, M., Takagi, H., Uneyama, C. and Hirose, M. (2004) Dietary influence on the impact of ethinylestradiol-induced alterations in the endocrine/ reproductive system with perinatal maternal exposure. *Reprod. Toxicol.*, **18**, 23–33.
14) Masutomi, N., Shibutani, M., Takagi, H., Uneyama, C., Takahashi, N. and Hirose, M. (2003) Impact of dietary exposure to methoxychlor, genistein, or diisononyl phthalate during the perinatal period on the development of the rat endocrine/reproductive systems in later life. *Toxicology*, **192**, 149–170.
15) Nakamura, T., Sonoda, K. H., Faunce, D. E., Gumperz, J., Yamamura, T., Miyake, S. and Stein-Streilein, J. (2003) CD4+ NKT cells, but not conventional CD4+ T cells, are required to generate efferent CD8+ T regulatory cells following antigen inoculation in an immune-privileged site. *J. Immunol.*, **171**, 1266–1271.
16) Pullen, S., Boecker, R. and Tiegs, G. (2003) The flame retardants retardants tetrabromo- bisphenol A and tetrabromobisphenol A-bisallylether suppress the induction of interleukin-2 receptor alpha chain (CD25) in murine splenocytes. *Toxicology*, **184**, 11–22.
17) Ulrich, P., Paul, G., Perentes, A. and Roman, M. D. (2004) Validation of immune function testing during a 4-week oral toxicity study with FK 506. *Toxicol. Lett.*, **149**, 123–131.
18) Ahmad, A. and Alvarez, F. (2004) Role of NK and NKT cells in the immunopathogenesis of HCV-induced hepatitis. *J. Leukoc. Biol.*, **76**, 743–759.
19) Liu, R., La Cava, A., Bai, V. F., Jee, Y., Price, M., Campagnolo, D. I., Christadoss, P., Vollmer, T. L., Van Kaer, L. and Shi, F. D. (2005) Cooperation of invariant NKT cells and CD4+CD25+ T regulatory cells in the prevention of autoimmune myasthenia. *J. Immunol.*, **175**, 7898–7904.
20) Zhou, T., Taylor, M. M., DeVito, M. J. and Crofton, K. M. (2001) Effects of short-term *in vivo* exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicol. Sci.*, **61**, 76–82.
21) El Dareer, S. M., Kalin, J. R., Tillery, K. F. and Hill, D. L. (1987) Disposition of decabromobiphenyl ether in rats dosed intravenously or by feeding. *J. Toxicol. Environ. Health*, **22**, 405–415.
22) Morck, A., Hakk, H., Oru, U. and Klasson-Wehler, E. (2003) Decabromodiphenyl ether in the rats—absorption, distribution, metabolism and excretion. *Drug Metab. Dispos.*, **31**, 900–908.
23) Shinohara, S., Tange, S., Kitamura, S., Sugihara, K., Fujimoto, N. and Ohta, S. (2006) Estrogen and thyroid hormone-disrupting activites of a flame retardant, brominated diphenyl ether. *J. Health Sci.*, **52**, s-133.