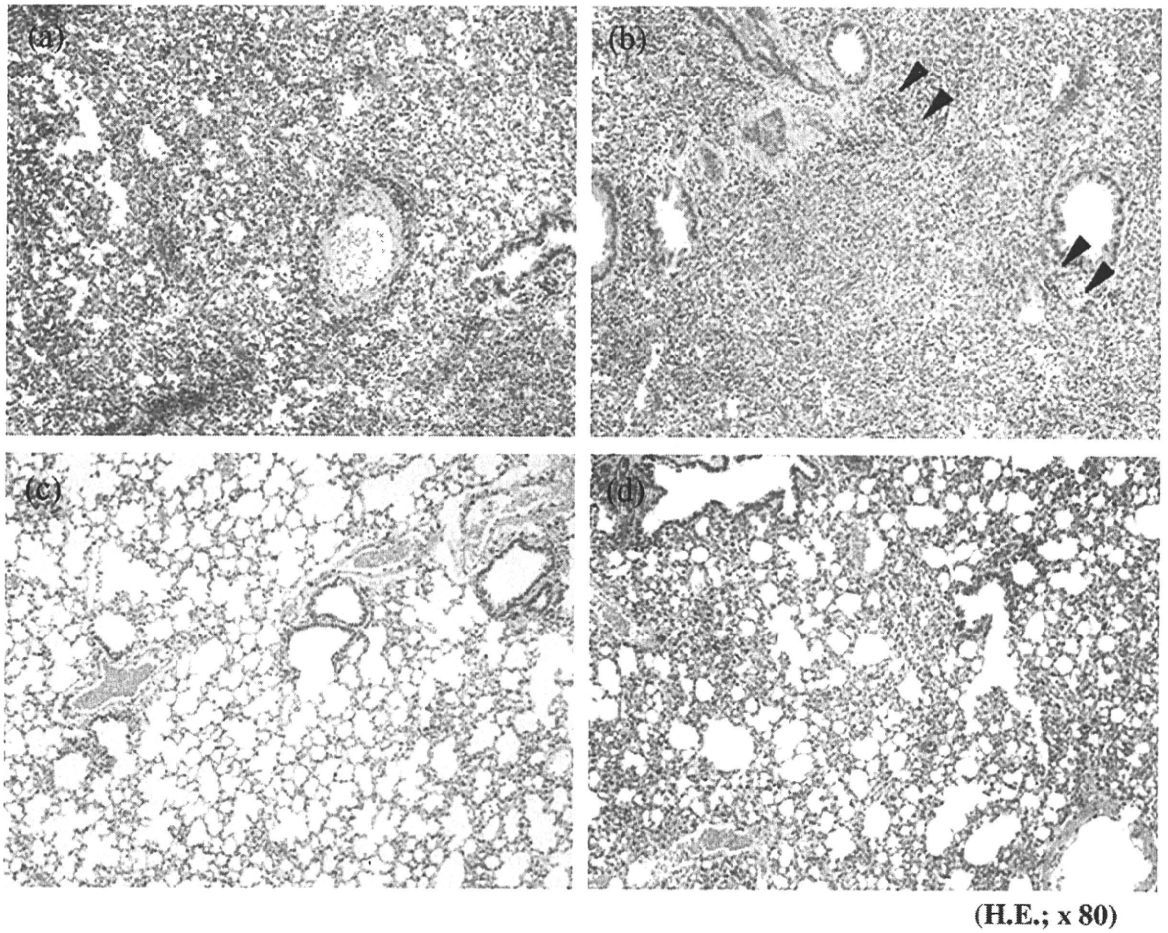

Fig. 1. Lungs of mice 5 days after mock or RSV infection. In this experiment, 4–5 mice per group were used, and representative data are shown. Arrowheads indicate macrophage hyperplasia. (a) Control mouse with RSV infection. (b) TBBPA-treated mouse with RSV infection. (c) Control mouse with mock infection. (d) TBBPA-treated mouse with mock infection. Hematoxylin and eosin stain.

that TBBPA caused disorder of the immune system in RSV-infected mice. Because the MAP kinases and protein kinase C were reported to be activated by TBBPA [20,21], disorder of the signal transduction system may be involved in the unusual production of the cytokines. On day 7 post-infection, IL-4 and IL-10, Th2 cytokines, were decreased in TBBPA-treated mice (Table 3). Studies for the development of a vaccine demonstrated that an imbalance of Th1/2 cytokines in the immune system is involved in the severity of RSV-induced disease [22]. Because TBBPA treatment strongly enhanced the production of

Table 3
Effects of TBBPA on cytokine concentration in BALF from RSV-infected mice.[a]

	Day 1 pi		Day 3 pi		Day 5 pi		Day 7 pi	
Cytokine	Control	TBBPA	Control	TBBPA	Control	TBBPA	Control	TBBPA
TNF-α	0.25 (0.17)	1.44* (0.74)	0.11 (0.02)	0.14 (0.03)	ND	ND	ND	ND
IL-1β	<0.04	0.05 (0.01)	ND	ND	ND	ND	ND	ND
IL-6	0.40 (0.14)	1.05** (0.06)	<0.02	<0.02	ND	ND	ND	ND
IL-12	0.11 (0.01)	0.15 (0.05)	0.15 (0.01)	0.18 (0.04)	ND	ND	ND	ND
IFN-γ	0.12 (0.02)	0.22 (0.07)	0.16 (0.02)	0.21 (0.07)	1.08 (0.86)	5.57** (2.82)	3.16 (2.66)	2.38 (0.99)
IL-4	ND	ND	<0.02	<0.02	0.04 (0.01)	0.03 (0.01)	0.06 (0.01)	0.04** (0.01)
IL-10	ND	ND	<0.08	<0.08	0.09 (0.02)	0.07 (0.02)	0.16 (0.02)	0.13* (0.01)

*Statistically different from control at $P<0.05$ (Student's t-test). **Statistically different from control at $P<0.01$ (Student's t-test).

[a] Concentration (ng/ml) of each cytokine in BALF from RSV-infected mice treated with or without 1% TBBPA was measured by ELISA for each specific cytokine. Data represents mean values of 5–6 mice. Numbers in parentheses indicate standard deviation. ND, not determined.

Table 4
Effects of TBBPA on subpopulations in BAL and spleen cells of RSV-infected mice.

	% of total cell populations[a]					
	BAL cells				Spleen cells	
	Day 1 pi		Day 3 pi		Day 3 pi	
Subpopulation	Control	TBBPA	Control	TBBPA	Control	TBBPA
RSV-infected						
CD11b	91.2	92.5	24.1	24.1	10.5	6.7
CD49b	23.1	30.5	25.9	23.7	ND	ND
CD4CD8	5.3	4.0	16.0	29.1	0.1	0.1
CD3CD25	2.8	0.8	2.8	2.2	ND	ND
Mock-infected						
CD11b	ND	ND	ND	ND	6.8	7.5
CD4CD8	ND	ND	23.5	19.6	ND	ND

Spleen cells were obtained from a representative mouse in each group. ND, not determined; pi, post-infection.

[a] BAL cells were collected from 6 mice in each group on day 1 or day 3 post-infection and pooled by group.

Th1 cytokines such as IFN-γ, Th2 cytokines might be suppressed reciprocally in TBBPA-treated mice with RSV infection.

To clarify the disorder in immune cell populations due to TBBPA administration, FACS analysis was performed on BAL cells (Table 4). Initially, it was speculated that macrophages and/or dendritic cells were affected in TBBPA-treated mice, because they mainly work in the early phase of RSV infection [19] and produce IL-6 and TNF-α (Table 3). However, the percentages of CD11b+ cells did not differ in control and TBBPA-treated mice. The numbers of CD11b molecules per cell as monitored by the intensity of fluorescence did not differ in the groups either (data not shown). TBBPA might have induced a qualitative change in immune cells that worked in the early phase of RSV infection. Pullen et al. reported that activated T cells, CD3+CD25+ cells, were damaged by TBBPA in murine spleen cell cultures in vitro [12]. However, the percentages of CD3+CD25+ cell populations in BAL cells were not clearly changed by TBBPA treatment in this study. NK cells (CD49b+), which work to eliminate RSV as an early induced immune response [23], were not affected either. Only the percentages of double-positive T (CD4+CD8+) cell populations were affected by TBBPA treatment in this study. Partial inhibition of the maturation of T cells due to TBBPA may result in an increase of viral titers (Table 2). Thus, TBBPA affected not only the quality of immune cells but also the maturation of T cells in local immunity in mice.

RSV infection is an important infectious disease in the field of pediatrics because clinically severe RSV infection has been seen primarily in young children with naïve immune systems and/or genetic dispositions [3]. It was reported that RSV infection in early life was related to the risk of asthma in childhood [24,25]. Therefore, the exacerbation of RSV infection demonstrated in this study needs to be avoided in children. Moreover, developmental exposure to BFRs threatens to cause more severe disorder of the host immunity in RSV-infected mouse pups because DBDE was reported to show immunotoxicity in RSV-infected offspring mice after perinatal exposure [9], although the compound did not have that affect in this study. From the view point of the health of mothers and children, it is important to reduce contamination of the environment by TBBPA.

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Functional Disorder of Primary Immunity Responding to Respiratory Syncytial Virus Infection in Offspring Mice Exposed to a Flame Retardant, Decabrominated Diphenyl Ether, Perinatally

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Perinatal exposure to a representative flame retardant, decabrominated diphenyl ether (DBDE), was shown previously to increase viral titers in the lungs of respiratory syncytial virus (RSV)-infected offspring on day 5 post-infection, resulting in exacerbation of pneumonia. In this study, the significant increase of pulmonary viral titers was confirmed even on day 1 post-infection and the effect on the primary immune response to RSV infection were examined to assess a mode of DBDE action on developmental immunotoxicity. On day 1 after infection, the secretion of both TNF-α and IL-6 decreased significantly in the bronchoalveolar lavage fluid prepared from RSV-infected offspring exposed to DBDE perinatally, but IL-1β increased. However, in ex vivo lipopolysaccharide stimulation test, the productivity of TNF-α in the bronchoalveolar lavage cells, which are mainly primary immune cells responding to RSV infection, prepared from offspring mice exposed to DBDE perinatally was not lower than that in the control. The primary immune cells retained normally the ability of cytokine production after the DBDE exposure. Gene expressions of innate pattern recognition receptors (Toll-like receptor 3 and 4, melanoma differentiation-associated gene-5, and retinoic acid-inducible gene I) in lung tissues were not affected by DBDE exposure. Because the levels of TNF-α, IL-6, and IL-1β are known to be elevated in the lungs of RSV-infected mice, these irregular productions due to perinatal DBDE exposure indicate a disorder of the primary immune response to RSV infection. Thus, perinatal exposure to DBDE was suggested to cause a functional disorder of primary immunity responding to RSV infection. *J. Med. Virol. 82:1075–1082, 2010.*

KEY WORDS: decabrominated diphenyl ether; respiratory syncytial virus; TNF-α; macrophages; innate pattern recognition receptors

INTRODUCTION

Human respiratory syncytial virus (RSV), a member of the family *Paramyxoviridae*, is the most prevalent infectious agent of acute lower respiratory illness in infants and young children [MacDonald et al., 1982]. Infection and reinfection with RSV are frequent during the first few years of life and most of children are infected by age 24 months [Collins et al., 2001]. Clinically severe RSV infection has been seen primarily in young children with naïve immune systems and/or genetic predispositions [Holberg et al., 1991], patients with suppressed T-cell immunity [MacDonald et al., 1982], and the elderly [Morales et al., 1983].

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Brominated flame retardants (BFRs) including polybrominated diphenyl ethers (PBDEs) are used as additive flame retardants at concentrations of 5–30% in many different polymers, resins and substrates, and common plastics, including acrylonitrile butadiene styrene and high impact polystyrene [Hedemalm et al., 1995]. As they are leached and escape from the finished polymer product, they exist ubiquitously in the environment and are suspected of being toxic to children [Fischer et al., 2006].

Decabrominated diphenyl ether (DBDE) accounts for more than 85% of PBDEs used commercially, mainly pentabrominated diphenyl ether, was reported using animal models as follows: effects on liver enzymes [Zhou et al., 2001; Lundgren et al., 2007], endocrine disruption [Ceccatelli et al., 2006; Kuriyama et al., 2007], neurotoxicity [Eriksson et al., 2002; Viberg et al., 2003], reproductive damage [McDonald, 2005; Lilienthal et al., 2006], and immunotoxicity [Fowles et al., 1994; Reistad and Mariussen, 2005; Martin et al., 2007]. It was reported that DBDE could be absorbed (>10% of the dose) orally, and the highest concentrations were found in the plasma and highly perfused tissues of rats [Mörck et al., 2003]. DBDE has also shown to be the debrominated compounds by metabolizing [Birnbaum and Staskal, 2004].

A novel assay system for evaluation of the developmental immunotoxicity of BFRs using a mouse model of RSV infection has been established and reported previously [Watanabe et al., 2008a]. Using this model, it was shown that perinatal exposure to DBDE elevated the levels of interferon-γ (IFN-γ) in the bronchoalveolar lavage fluid (BALF) of RSV-infected offspring mice with an increase of pulmonary viral titers and exacerbated pneumonia [Watanabe et al., 2008b], indicating that DBDE is a risk factor for RSV infection across the human generations. However, in cyclophosphamide-treated offspring mice, pulmonary viral titers increased, but the levels of IFN-γ decreased. Mechanisms or sites of action of cyclophosphamide, a representative immunosuppressive agent, and DBDE in RSV-infected offspring mice are different, and DBDE was suggested to affect specific site(s) of the immune system in offspring mice.

In this report, to assess a mode of action of DBDE on developmental immunotoxicity, when the significant increase of pulmonary viral titers occurs in offspring mice exposed to DBDE perinatally after RSV infection was determined and a significant increase on day 1 post-infection was observed. On day 1 after infection, to clarify effects of perinatal exposure to DBDE on the specific site(s) or the immune cells on innate immunity, the levels of several cytokines and gene expressions of innate pattern recognition receptors were compared in offspring mice exposed or unexposed to DBDE perinatally. Also, to reveal whether DBDE exposure damages the function of immune cells, the productivity of TNF-α in the bronchoalveolar lavage cells as primary immune cells responding to RSV infection were examined.

MATERIALS AND METHODS

Mice

Female (6 weeks old) and male (8 weeks old) BALB/c mice were purchased from Kyudo Animal Laboratory (Kumamoto, Japan) and housed at 25 ± 2°C. The mice were allowed free access to a conventional solid diet CRF-1 (Oriental Yeast Co., Chiba, Japan) and water and used in this experiment after 7 days acclimation. The animal experimentation guideline of the Kyushu University of Health and Welfare was followed in the animal studies.

Cells and Virus

Human epidermoid carcinoma (HEp-2) cells (American Type Culture Collection CCL-23) were purchased from Dainippon Pharmaceutical (Osaka, Japan) and maintained in Eagle's minimum essential medium supplemented with heat-inactivated 10% fetal calf serum (FCS). The A2 strain of RSV was obtained from American Type Culture Collection (Rockville, MD) and grown in HEp-2 cell cultures. Viral titers were measured using HEp-2 cells by the plaque method, and expressed as plaque-forming units per milliliter (PFU/ml) [Watanabe et al., 2008a].

Reagent

DBDE (purity: ≥98.0%) was purchased from Wako Pure Chemicals (Osaka, Japan) and mixed into a soy-free powder diet, based on the formulation of the NIH-07 open-formula rodent diet [Masutomi et al., 2004], produced by Oriental Yeast Co. (Chiba, Japan).

Perinatal Exposure to DBDE

Perinatal exposure to DBDE was performed as described in the previous report [Watanabe et al., 2008b]. Briefly, 7-week-old female mice and 9-week-old male mice were paired and fed the CRF-1 diet for 3 days. At 3 days after conception, the CRF-1 diet was replaced with a soy-free diet to avoid the estrogen-like effect of soybeans. The female mice were divided randomly into three groups for DBDE exposure at 0, 1,000, or 10,000 ppm. These mice were exposed to DBDE mixed with the soy-free diet from 10 days after conception to weaning on postnatal day 21. After weaning, offspring mice were fed the CRF-1 diet. Finally, on postnatal day 28, offspring mice in each group were used for the following RSV infection test. Throughout the experiments, both chows and drinking water were given ad libitum.

RSV Infection

A RSV infection test was performed according to the previous report [Watanabe et al., 2008b]. Briefly, 4-week-old offspring mice (control: 12 pups, DBDE exposure at 1,000 ppm: 14 pups, and 10,000 ppm: 12 pups) were infected intranasally with 5×10^6 PFU of the A2 strain of RSV under anesthesia in experiment 1

TABLE I. Effects of Perinatal Exposure to DBDE on Pulmonary Viral Titers 1 or 5 Days After RSV Infection in Offspring Mice

Group/DBDE exposure (ppm)	Pulmonary viral titers (PFU/ml)[a]			
	Experiment 1		Experiment 2	Experiment 3
	Day 1 pi	Day 5 pi	Day 1 pi	Day 1 pi
Control	1,723 ± 220 (6)	12,778 ± 3,202 (6)	11 ± 10 (3)	13 ± 5 (7)
1,000	1,879 ± 195 (7)	12,917 ± 4,853 (6)	ND	ND
10,000	2,350 ± 343* (6)	17,889 ± 6,313* (6)	272 ± 134 (3)	142 ± 112* (8)

ND, not done; pi, post-infection.
Offspring mice were infected intranasally with 5×10^6 PFU of RSV in experiment 1, and with 5×10^5 PFU in experiments 2 and 3.
[a]Values represent mean ± standard deviation. Numbers in parenthesis indicate numbers of mice used in each group.
*Statistically different from control at $P < 0.05$ (Student's t-test).

(Table I). In experiments 2 and 3 (Table I), lower titers of RSV (5×10^5 PFU) were used also to observe the apparent net increase of virus titer. On days 1 and 5 after infection, BALF was obtained from the mice under anesthesia by instilling of 1.0 ml of cold phosphate-buffered saline (PBS) into the lungs and aspirating it from the trachea using a tracheal cannula [Kurokawa et al., 2002]. Following the acquisition of BALF, the lungs were removed, frozen immediately in liquid N_2, and stored at −80°C until virus titration. Ice-cold BALF was centrifuged at 100*g* at 4°C for 10 min. After centrifugation, the supernatant was stored at −80°C until to use. The cell pellet was suspended in 0.5 ml of cellbanker-1 (Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Japan) as bronchoalveolar lavage cells, and then stored at −80°C prior to use. Cellbanker-1 is used widely for freeze preservation of mammalian tissues or cells. It was reported that the cells or tissues preserved in it retained intact functions when they were thawed by appropriate procedure [Shinohara et al., 2002; Nagano et al., 2007]. Previously, it was confirmed that the levels of TNF-α from bronchoalveolar lavage cells were almost equivalent between the isolated freshly and the preserved in the reagent. Frozen lung tissue was homogenized with cold quartz sand in a homogenizer, and viral titers in the supernatants of the homogenates were measured by a plaque assay [Watanabe et al., 2008a].

Assay of TNF-α Production From Bronchoalveolar Lavage Cells

Frozen bronchoalveolar lavage cells were thawed quickly at 37°C, and suspended subsequently in RPMI1640 medium supplemented with 10% heat-inactivated FCS, 100 units/ml of penicillin G, 100 μg/ml of streptomycin, and 50 μM of 2-mercaptoethanol (RPMI medium). The bronchoalveolar lavage cell suspension from each mouse was collected and pooled in each treatment group. The viable cells were counted under a phase contrast microscope (magnification 100×). Two hundred microliters of bronchoalveolar lavage cell suspension (2.5×10^5 cells/ml) was seeded on each well in a 96-well microtiter plate and incubated at 37°C for 24 hr in a humidified air with 5% CO_2. After incubation, the culture medium was removed by aspiration and replaced in fresh RPMI medium with or without 100 ng/ml of lipopolysaccharide (*W E. coli* O127: B8, Difco, Detroit, MI; LPS) [Kurokawa et al., 2003]. Following 24 hr further incubation, the culture supernatant was harvested from each well and the amount of TNF-α was measured by ELISA.

ELISA

IL-1β and IL-6 levels in BALF were measured using specific ELISA kits (Ready-set-go, eBioscience, Inc., San Diego, CA) for IL-1β and IL-6, respectively, according to the manufacturer's instructions. IL-12 levels in BALF were measured using a specific kit (Ready-set-go, eBioscience, Inc.) for IL-12 p70, without interference by p40 monomer or the related protein IL-23, according to the manufacturer's instructions. Amounts of TNF-α in BALF and the culture supernatant of bronchoalveolar lavage cells were measured by the TNF-α ELISA kit (Ready-set-go, eBioscience, Inc.). These products were tested and found to conform to all eBioscience, Inc. Quality control release specifications. The lower limits of detection sensitivity in the kit are IL-1β, 8 (pg/ml); IL-6, 4 (pg/ml); IL-12 p70, 15 (pg/ml); and TNF-α, 8 (pg/ml). The intra- and inter-assay coefficients of variation for these ELISA were less than 10%.

Real-Time RT-PCR

Gene expressions of innate pattern recognition receptors in the lung tissues were measured by real-time RT-PCR, and the data were evaluated by normalizing results to those of mouse β-actin. Briefly, RNA was isolated from the frozen lung tissues using trizol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The isolated RNA was transcribed into cDNA by ReverTra Ace α (Toyobo Co. Ltd, Osaka, Japan) using an oligo-dT(20) primer according to the manufacturer's instructions. The Toll-like receptor 4 cDNA was amplified and analyzed on a Roche LightCycler P2000 real-time PCR system using a LightCycler FastStart DNA Master HybProbe kit (Roche Diagnostics, Indianapolis, IN) with a mouse Toll-like receptor 4 Hybprobe primer-probe kit (Nihon Gene Research Laboratories, Inc., Sendai, Japan) according to the manufacturer's instructions. Amplification and analysis of the cDNAs of Toll-like receptor 3, melanoma

differentiation-associated gene-5, and retinoic acid-inducible gene I were performed using a Roche LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics) with the specific primers according to the manufacturer's instructions. Pairs of specific primers were as follows: Toll-like receptor 3, forward: 5′-GATACAGGGATTGCACCCATA-3′, reverse: 5′-TCCCCCAAAGGAGTACATTAGA-3′; melanoma differentiation-associated gene-5, forward: 5′-AACACAGATGGTGCGTGCT-3′, reverse: 5′-GCCCAGCACATTTTTATGGT-3′; retinoic acid-inducible gene I, forward: 5′-GACCCCACCTACATCCTCAG-3′, reverse: 5′-GGCCCTTGTTGTTCTTCTCA-3′. The amounts of Toll-like receptor 4 cDNA (number of copies) were determined according to the manufacture's instructions. The amounts of cDNAs of Toll-like receptor 3, melanoma differentiation-associated gene-5, and retinoic acid-inducible gene I were determined by comparing the crossing point values of the cDNA samples to those of the TA vectors harboring parts of the murine genes of Toll-like receptor 3 (nt. 148–480), melanoma differentiation-associated gene-5 (nt. 944–1213) and retinoic acid-inducible gene I (nt. 83–345), respectively. Amplification and analysis of the β-actin cDNA were performed using a Roche LightCycler FastStart DNA Master SYBR Green I kit with a pair of β-actin-specific primers (forward: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, reverse: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′) The amounts of β-actin cDNA were determined also by comparing the crossing point value of the cDNA sample to those of plasmid, a TA vector harboring part of the murine β-actin gene (nt. 728–1076).

Statistical Analysis

Comparisons of pulmonary viral titers and the levels of cytokines between the control and experimental groups were carried out using Student's *t*-test. A *P*-value of 0.05 or less was considered to be significant.

RESULTS

Effect of Perinatal Exposure to DBDE on RSV Infection in Offspring

To clarify effects of perinatal exposure to DBDE on immune system in offspring mice, dam mice were exposed to DBDE dietary up to 10,000 ppm from day 10 after gestation to weaning on postnatal day 21. Although mean body weight was suppressed approximately 10% in dam mice exposed to DBDE at 10,000 ppm, there was no significant difference in body weights of offspring mice between control and DBDE-exposed group (10.6 ± 1.2 and 10.6 ± 1.2 g, respectively). No significant differences between control and DBDE-exposed group were detected in food consumption of dams (51.8 ± 4.2 and 50.4 ± 7.7 g/week, respectively), in the numbers of litter (6.8 ± 2.4 and 7.2 ± 2.1 per dam, respectively), and in the survival rates of pups (70.4 ± 12.2 and 65.2 ± 14.3%, respectively) by postnatal day 21, either [Watanabe et al., 2008b]. No particular toxicological sign or abnormal behavior was observed in dams and offspring mice. Then, offspring mice were infected intranasally with the A2 strain of RSV at 5×10^6 PFU (Table I). No change of body weights was observed in the tested offspring mice after RSV infection. However, on day 1 post-infection, the viral titers of offspring mice treated with DBDE at 10,000 ppm were increased significantly ($P < 0.05$) compared with those of controls (experiment 1, Table I). A significant increase of the viral titers on day 5 post-infection was shown as reported previously [Watanabe et al., 2008b]. When the lower virus titer (5×10^5 PFU/mouse) was used to clarify the net increase of virus yields, pulmonary viral titers of DBDE-exposed mice perinatally were markedly higher (approximately 25-fold) than those of control mice (experiment 2, Table I). The significant increase of pulmonary viral titers by perinatal DBDE exposure was confirmed by a repeated experiment (experiment 3, Table I). These results indicated strongly that the significant increase of pulmonary viral titers in offspring mice due to perinatal exposure to DBDE had occurred even on day 1 post-infection.

Effect of Perinatal Exposure to DBDE on Primary Cytokine Production in Offspring

The findings that the viral titers were enhanced already on day 1 after infection due to perinatal DBDE exposure (Table I) suggested that DBDE affected the early immune response in RSV-infected offspring mice. Then, the amounts of major cytokines (TNF-α, IL-6, IL-1β, and IL-12) induced by RSV infection in BALF of offspring mice exposed to DBDE perinatally were compared (Table II). In mock-infected offspring mice, the levels of the cytokines in BALF were under limit of detection. TNF-α, which inhibits the progress of RSV infection both directly and indirectly [Neuzil et al., 1996], was decreased dose-dependently, and the levels at 10,000 ppm were significantly ($P < 0.05$) lower than those of the control in RSV-infected offspring. The levels of IL-6 were decreased by DBDE exposure at 10,000 ppm. In contrast to these results, IL-1β which contributes to T cell activation, was increased significantly ($P < 0.05$) at 10,000 ppm compared with the control. The levels of IL-12, one of the potent immune cytokines that activates natural killer (NK) cells, were under limit of detection in this study. Thus, perinatal exposure to DBDE suppressed production of TNF-α and IL-6 but enhanced production of IL-1β in RSV-infected offspring mice.

Effects of Perinatal Exposure to DBDE on TNF-α Production From Bronchoalveolar Lavage Cells

Bronchoalveolar lavage cells were collected from RSV-infected offspring mouse on day 1 post-infection, and pooled by treatment group, and then tested for their ability on TNF-α production in vitro. Microscopic observation showed that BALF contained mainly (91–93%) the macrophage/monocyte-like cells. There was no

TABLE II. Effects of Perinatal Exposure to DBDE on Levels of TNF-α, IL-6, IL-1β, and IL-12 in Bronchoalveolar Lavage Fluid From Mock- or RSV-Infected Offspring on Day 1 Post-Infection

Group/ DBDE exposure (ppm)	Concentration (pg/ml)[a]							
	RSV-infected				Mock-infected			
	TNF-α	IL-6	IL-1β	IL-12	TNF-α	IL-6	IL-1β	IL-12
Control	136 ± 62	268 ± 97	35 ± 9	<15	<8	<4	<8	<15
1,000	79 ± 18	177 ± 58	74 ± 47	<15	<8	<4	<8	<15
10,000	51 ± 7*	119 ± 59*	78 ± 38*	<15	<8	<4	<8	<15

[a]Data represent mean ± standard deviation of 6–7 mice.
*Statistically different from control at $P < 0.05$ (Student's t-test).

significant difference in the yields of these cells between the control and DBDE-exposed group (Table III). Bronchoalveolar lavage cells were incubated 24 hr with or without LPS (100 ng/ml). After incubation, the culture supernatant was harvested and the amounts of TNF-α were measured by ELISA (Table III). In bronchoalveolar lavage cells from RSV-infected offspring exposed to DBDE at 10,000 ppm, LPS stimulation doubled approximately the amount of TNF-α compared with the control. Thus, these results suggested that the capacity of bronchoalveolar lavage cells to produce TNF-α was not damaged due to perinatal exposure to DBDE in offspring mice.

Effect of Perinatal Exposure to DBDE on Gene Expressions of Innate Pattern Recognition Receptors in Lungs of Offspring

The levels of gene expression of innate pattern recognition receptors were measured in lung tissues of offspring on day 1 post-infection (Fig. 1). Amounts of mRNA of Toll-like receptor 3 and 4, melanoma differentiation-associated gene-5, and retinoic acid-inducible gene I were measured by real-time RT-PCR and the results were normalized to the amount of β-actin mRNA. Perinatal exposure to DBDE did not affect significantly the gene expressions of these innate pattern recognition receptors in lungs of offspring mice.

DISCUSSION

To investigate the mechanism of action of DBDE on developmental immunotoxicity, pulmonary viral titers on day 1 post-infection were measured and it was revealed that those increased significantly in RSV-infected offspring mice exposed to DBDE perinatally at 10,000 ppm (Table I). As reported previously [Watanabe et al., 2008b], both the viral titers and levels of IFN-γ in BALF were elevated significantly on day 5 post-infection after perinatal exposure to DBDE compared with those of the controls. However, in RSV-infected offspring mice treated with cyclophosphamide, a non-specific immunosuppressive agent, pulmonary viral titers increased, but the levels of IFN-γ in BALF decreased. These results suggested that immunosuppressive manner of DBDE was not non-specific. This was consisted with the report that exposure to PBDE mixture suppressed induction of the selective cytokines in coxsackievirus B3-infected mice [Lundgren et al., 2009]. To clarify the effects of perinatal exposure to DBDE on the specific site(s) or cells of the immune system, the early phase, day 1, after RSV infection was focused on.

Effects of perinatal exposure to DBDE at 1,000 or 10,000 ppm on primary cytokine productions in BALF of offspring mice were examined (Table II). It was exhibited already that perinatal exposure to DBDE at 10,000 ppm exacerbated pneumonia, such as hypertrophy and/or hyperplasia of the bronchial epithelium, in RSV-infected offspring by histopathological analysis [Watanabe et al., 2008b]. Throughout this study, perinatal exposure to DBDE at 10,000 ppm did not show any toxicological sign in offspring mice. The dosage of DBDE based on the average food consumption and body weight was calculated at approximately 3,300 mg/kg/day. The dosage sounds very high, but it has reported

TABLE III. Effects of Perinatal Exposure to DBDE on TNF-α Production From Bronchoalveolar Lavage Cells and on the Yields of the Cells on Day 1 Post-Infection in RSV-Infected Offspring Mice*

DBDE exposure (ppm)	TNF-α (pg/ml)[a]		Yields of bronchoalveolar lavage cells (×10^5)[b]
	−LPS	+LPS	
Control	<35	298.0 (265.6–330.4)	2.5 (2.2–2.7)
1,000	<35	294.3 (245.7–342.8)	3.1 (1.2–5.0)
10,000	<35	590.0 (434.6–745.4)	2.5 (2.3–2.6)

*Bronchoalveolar lavage cells were collected from control, DBDE-treated at 1,000 or 10,000 ppm offspring mice on day 1 post-infection and pooled by treatment group.
[a]Data represent mean of values of two separate experiments. Numbers in parentheses indicate the range of values.
[b]Viable cells were counted under a phase contrast microscope (magnification 100×). Data represent mean of values of two separate experiments. Numbers in parentheses indicate the range of values.

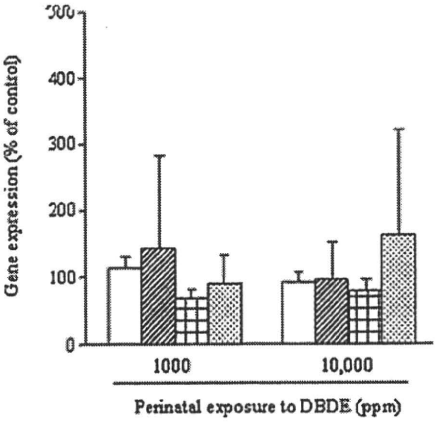

Fig. 1. Effects of perinatal exposure to DBDE on gene expressions of mouse innate pattern recognition receptors in lung tissues of RSV-infected offspring on day 1 post-infection. Amounts of mRNA of innate pattern recognition receptors were measured by real-time RT-PCR and normalized to the amount of mouse β-actin mRNA. The levels of gene expression of each molecule in DBDE-treated offspring mice were expressed as the percentages of mean of those in the Control group (n = 6). The data represent mean ± standard deviation of values of DBDE-treated at 1,000 ppm (n = 7), or 10,000 ppm (n = 6) offspring mice. Open bar, Toll-like receptor 3; shaded bar, Toll-like receptor 4; hatched bar, melanoma differentiation-associated gene-5; dotted bar, retinoic acid-inducible gene I.

that the US National Toxicology Program [1986] completed the carcinogenesis study using B6C3F1 mice exposed to DBDE at 50,000 ppm for 104 weeks. Teshima et al. [2008] reported that perinatal exposure to DBDE at 1,000 ppm, corresponded to the dose in this study, showed a weak immunomodulatory effect in offspring rat. Therefore, to evaluate a potential of DBDE as a developmental immunotoxicant, the much higher dosage, 10,000 ppm was used.

In the early phase of RSV infection in mice, the levels of TNF-α, IL-6, and IL-1β have been shown to increase locally in the lungs, which contribute to immunological host defenses against RSV infection, such as the reduction of pulmonary viral titers [Gordon and Read, 2002]. In this study, however, perinatal exposure to DBDE reduced significantly the levels of TNF-α and IL-6 in BALF on day 1 after infection, but increased significantly the level of IL-1β (Table II). Because viral titers were increased significantly by perinatal DBDE exposure on day 1 after infection (Table I), the perinatal exposure was probably responsible for the disorder of cytokine secretion against RSV infection in the early phase. Consequently, the primary immune response to RSV infection was disturbed, and viral titers increased in the lungs of offspring mice exposed to DBDE perinatally. It was shown already that perinatal DBDE exposure elevated significantly the level of IFN-γ on day 5 after infection [Watanabe et al., 2008b]. In this study, perinatal DBDE exposure increased significantly the level of IL-1β on day 1 after infection, suggesting that the increased IL-1β might activate T cells and affect the subsequent elevation of IFN-γ secretion.

Perinatal exposure to DBDE reduced significantly the levels of TNF-α and IL-6 in BALF on day 1 after infection, but the secretion of IL-1β increased significantly after DBDE exposure. The time point of 1 day after RSV infection corresponds to the phase from innate immunity to early induced immune response [Janeway et al., 2001]. In the early phase of RSV infection, TNF-α is produced mainly by macrophages and inhibits the progress of RSV infection both directly and indirectly [Neuzil et al., 1996]. IL-6 and IL-1β are produced from macrophages and activate T cells [Janeway et al., 2001]. Because production of IL-1β was reported to be regulated by caspase-1 and the nucleotide-oligomerization domain family [Franchi et al., 2009; Ichinohe et al., 2009] and the regulatory mechanism of IL-1β is different from those of TNF-α and IL-6, disorder of the signal transduction system for IL-1β may be involved in the unusual production of the cytokines. Although IL-12 is induced from macrophages [Janeway et al., 2001] and contributes to activation of NK cells, the levels of IL-12 were low on day 1 after infection in this study (Table II). NK cells are one of the key effectors of virus clearance, but it takes few days after RSV infection for NK cell to eliminate the virus [Hussell and Openshaw, 1998]. RSV infection does not induce significant levels of type I interferon [Hall et al., 1981]. Because macrophages are major effector cells for the clearance of initial infection of RSV [Gordon and Read, 2002], it is probable that DBDE exposure disordered the cytokine production by macrophages.

To reveal whether DBDE exposure damaged the function of TNF-α production in immune cells in lung tissues, ex vivo LPS stimulation tests were performed on bronchoalveolar lavage cells that were mainly the macrophage/monocyte-like cells (Table III). In DBDE-exposed offspring mice, the amount of LPS-induced TNF-α was increased rather than suppressed. These results suggest that DBDE does not damage cytokine production. Perinatal DBDE exposure caused probably some functional disorder of primary immune cells, like macrophages, responding to RSV infection. Because RSV infects predominantly epithelial cells in the respiratory tract, it is still possible that DBDE affected those cells remaining in lung tissues of offspring mice.

To investigate whether DBDE affected innate immunity, the levels of gene expression of innate pattern recognition receptors, which recognizes RSV and/or LPS [Haynes et al., 2001], were measured (Fig. 1). While no obvious change was observed in the gene expression of these molecules after DBDE exposure, involvement of DBDE in the processing of viral antigen in macrophages was not denied in innate immunity. It was reported that pentabrominated diphenyl ether (DE-71) induces respiratory burst, which is associated with the processing of viral antigen, in human granulocytes [Reistad and Mariussen, 2005]. DBDE may affect the processing of viral antigen and induce subsequently disorder of cytokine production in macrophages.

This study revealed that perinatal exposure to DBDE-induced disorder of the primary immune response to RSV infection in offspring mice. It is obvious that the disorder triggered the developmental immunotoxicity of

DBDE. However, we did not examine the effects of perinatal exposure to DBDE on cytotoxic T lymphocytes, which are the major effector cells in adaptive immune response and contribute to RSV clearance. Recent study in the rat has shown that perinatal exposure to DBDE decreased the populations of peripheral NK cells, and the proportions of splenic activated T and B cells in offspring [Teshima et al., 2008]. In the report, association between anti-thyroid activity and immune suppressive effects due to perinatal exposure to DBDE was discussed. However, DBDE might affect immune system directly in this study, because mouse was shown to be resistant to anti-thyroid effect [Watanabe et al., 2008a]. It is doubtful that DBDE affects only the primary immune response. Therefore, further studies on adaptive immune responses, including humoral immune response, are needed to illuminate the mechanism of action of DBDE on the developmental immunotoxicity.

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