Perinatal exposure to tetrabromobisphenol A (TBBPA), a brominated flame retardant, exacerbated the pneumonia in respiratory syncytial virus (RSV)-infected offspring mice

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ABSTRACT — To investigate the effects of perinatal exposure to tetrabromobisphenol A (TBBPA), a brominated flame retardant, on the immune system, a respiratory syncytial virus (RSV) infection mouse model was utilized. Female mice were exposed to TBBPA mixed with the diet from 10 days after conception to weaning on postnatal day 21. Offspring mice were infected intranasally with A2 strain of RSV. Although no general toxicological sign was observed, the pulmonary viral titers of offspring mice exposed to 0.1% TBBPA were significantly increased compared with the control on day 5 post-infection. TBBPA did not affect RSV growth in vitro. Histopathological analysis confirmed that the exacerbation of interstitial pneumonia was due to TBBPA exposure in the lung tissues in RSV-infected offspring. Moreover, gene expression of interleukin (IL)-24 was shown to be elevated typically in the lung tissues of TBBPA-treated offspring by a DNA microarray and was also confirmed by immunohistopathological analysis using an anti-IL-24 antibody. Thus, developmental exposure to TBBPA affected the immune response to RSV infection, resulting in the exacerbation of pneumonia. Thus, IL-24 should be a key molecule to understand the mechanism of action of TBBPA.

Key words: TBBPA, RSV, Pneumonia, IL-24

INTRODUCTION

Brominated flame retardants (BFRs) have been used for reduction of fire-related injury and property damage worldwide (Birnbaum and Staskal, 2004). TBBPA is a representative of major BFRs, and more than 140,000 tons were produced as an additive of epoxy resin for electronic circuit boards in 2004 (ECB, 2006). Because TBBPA may be easily released into the environment due to deterioration or abrasion of the material, human health, particularly of mothers and children, may be threatened. TBBPA was found in human breast milk (Shi et al., 2009) and umbilical cord serum (Cariou et al., 2008). TBBPA is considered to be a chemical of high concern in children by the Washington State Department of Health in the Children’s Safe Product Act (Saegusa et al., 2012). Although the toxicity of TBBPA has been reported using several administration routes in animal models, and further, low acute toxicity has been shown (Colnot et al., 2014), its effects on the developmental immune system have not been studied definitively (Teshima et al., 2008).

RSV, a member of the Paramyxoviridae family, is a prevalent infectious agent of acute lower respiratory illness in infants and young children (MacDonald et al.,...
Infection with RSV may be severe in infants and young children with immune deficiency and patients with suppressed T-cell immunity (Holberg et al., 1991). To evaluate the immunotoxicity of environmental chemicals including BFRs, we established a novel assay system using a murine model of RSV infection because the severity of RSV infection reflects the condition of the host immunity (Watanabe et al., 2008a). We subsequently demonstrated that decabrominated diphenyl ether (DBDE), a BFR, and methamidophos, a representative organophosphate insecticide, caused developmental immunotoxicity (Watanabe et al., 2008b) and suppressed the production of proinflammatory cytokines using this model (Watanabe et al., 2013), respectively. In addition, we also revealed that titanium dioxide caused immune disorder and then exacerbated pneumonia in RSV-infected mice (Hashiguchi et al., 2015).

To clarify whether TBBPA causes developmental immunotoxicity, the effects of perinatal exposure to TBBPA on pneumonia in RSV-infected offspring mice were evaluated in this study.

MATERIALS AND METHODS

Animals
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 d acclimation. The animal experimentation guideline of the Kyushu University of Health and Welfare were followed in the animal studies (approval number: 27111).

Cell 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 (MEM) supplemented with heat-inactivated 10% fetal calf serum (FCS). The A2 strain of RSV was obtained from American Type Culture Collection (Rockville, MD, USA) and grown in HEp-2 cell cultures. The viral titers were measured by the plaque method, and expressed as plaque-forming units per milliliter (PFU/mL).

Chemical compound
TBBPA (purity: ≥ 98.0%) was purchased from Tokyo Kasei (lot. T556F, Tokyo, Japan). The compound was mixed at 0, 0.01, 0.1 or 1.0% (w/w) into a powdered diet, which was soy-free to avoid the estrogen-like effect of soybeans and produced by Oriental Yeast Co.

Perinatal exposure of female mice to TBBPA
Seven-week-old female and 9-week-old male mice were paired and fed CRF-1 diet for 3 d. The conception day was determined by recognition of a vaginal plug in the female. At 3 d 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 randomly divided into four groups for TBBPA- exposure (0, 0.01, 0.1, or 1.0% TBBPA in the diet). The control group (0%) and each TBBPA-exposed group were composed of 10 and 8 mice, respectively. These mice were exposed to TBBPA mixed with the diet from 10 d after conception to weaning on postnatal day 21. Seven of 10 mice in the control group and 6 of 8 mice in each TBBPA-exposed group produced litters of 5 to 8 young. After weaning, offspring mice were fed the CRF-1 diet. On postnatal day 28, offspring mice in each group were randomly selected and used for following RSV infection test. Throughout these experiments, both chows and drinking water were given ad libitum, and the consumption was checked weekly.

RSV infection test
An RSV infection test was performed according to our previous report (Watanabe et al., 2008a). Two or three female offspring mice were selected randomly from each dam and collected in an each TBBPA-exposed group, and then used for following test. Briefly, four-week-old offspring mice were infected intranasally with 5 × 10^6 PFU of the A2 strain of RSV under anesthesia. In a mock-infected group, offspring mice were given phosphate-buffered saline (PBS) intranasally. On day 1 or 5 after infection, mock- or RSV-infected mice were sacrificed by cervical dislocation under anesthesia. Blood samples were also prepared from offspring mice under anesthesia on day 5 post-infection.

For virus titration of lung tissues, the lungs were removed on day 5 post-infection and immediately frozen in liquid N₂, and stored at -80°C until use. Frozen lung tissue was homogenized with cold quartz sand in a homogenizer. After centrifugation at 480 × g at 4°C for 15 min, the supernatants of the homogenates were used for measuring viral titers by a plaque assay as reported previously (Watanabe et al., 2008a). Viral titers in lungs of mice were also expressed as PFU/mL.

For histological examination of the lungs, the lungs were removed on day 5 post-infection and placed in 10% buffered formalin for a minimum of 24 hr. The tissue was then embedded in low-melting point paraffin, sectioned at
a thickness of 5 μm, and stained with hematoxylin and eosin.

For a DNA microarray test of the lung tissues, the lungs were removed on day 1 post-infection and immediately placed in RNAlater reagent (Qiagen, Hilden, Germany) and stored at 4°C until use.

DNA microarray test

 DNA microarray analysis of the lungs of mock- or RSV-infected mice was performed by Hokkaido System Science Co., Ltd. (Hokkaido, Japan). Briefly, RNA was isolated from each lung tissue from 6 control or 5 TBBPA-exposed mice with or without RSV infection using an RNeasy kit according to the manufacturer’s instructions. The isolated RNA was quantified by optical density (OD260) and mixed in equal amount, and then pooled in each group. After a quality check of the isolated RNA, cDNA was synthesized and amplified from the RNA sample using a Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA). Exhaustive analysis of the gene expression of the sample was performed using a SurePrint G3 Mouse GE 8 x 60K 1 color system (Agilent Technologies). Approximately 2-fold changes in gene expression were discovered and analyzed using the software program GeneSpring (Agilent Technologies).

Immunohistochemical evaluation

The lung tissue sections were deparaffinized and hydrated through xylenes and graded alcohols. After washing with water, they were incubated in an unmasking solution (Vector Laboratories, Inc., Burlingame, CA, USA) at 90°C for 30 min. Then the sections were incubated in 0.3% H2O2 in PBS for 30 min to quench the endogenous peroxidase activity and treated with blocking serum (Vector Laboratories, Inc.) for 30 min. The lung tissues were stained with a goat polyclonal antibody against mouse IL-24 (1:100, R&D Systems, Inc., Minneapolis, MN, USA) for 90 min. Then, IL-24 proteins were detected using a VECTASTAIN ABC kit (Vector Laboratories, Inc.) according to the manufacturer’s instructions. The sections were faintly counterstained with hematoxylin.

Statistical analysis

Comparison of the viral titers of lung tissues between the controls and experimental groups was carried out using the Mann-Whitney U-test. A P value of 0.05 or less was considered to be significant.

RESULTS

Effects of perinatal exposure to TBBPA on RSV infection in offspring mice

To reveal whether TBBPA has developmental immunotoxicity, female mice were exposed perinatally to TBBPA mixed with the diet from 10 days after conception to weaning on postnatal day 21. On postnatal day 28, body weights of offspring mice were not suppressed due to perinatal exposure to TBBPA. In this study, from 5 to 8 offspring were born to each dam in control and TBBPA-treated groups. After weaning, not only were no significant differences in the size of litters, survival rates, or food consumption of pups observed between control and TBBPA-treated groups but also no abnormal behavior or particular toxicological sign (data not shown). Changes in general toxicological signs such as suppression of body weight gain and food consumption in dams were also not observed (data not shown). These results demonstrated that perinatal exposure to TBBPA did not have developmental toxicity.

To investigate the effects of perinatal exposure to TBBPA on the immune system of RSV-infected offspring, the pulmonary viral titers were measured by a plaque method on day 5 post-infection. Viral titers of offspring exposed to TBBPA (0.1%) were significantly (P < 0.01) increased and reached approximately 20-fold of those of controls (Fig. 1). Conversely, the viral titers of the TBBPA (1.0%)-treated group were significantly (P < 0.01) suppressed. In the cell culture assay, TBBPA did not affect the viral growth in vitro (data not shown). Thus, these
results suggest that perinatal exposure to TBBPA affected the immune system of offspring mice and promoted the growth of RSV in the lung tissues.

**Effects of perinatal exposure to TBBPA on severity of pneumonia in RSV infection in offspring mice**

Because RSV-induced pneumonia was expected to be exacerbated by perinatal exposure to TBBPA, a histopathological analysis of lungs of offspring was performed on day 5 post-infection. Typical data are shown in Fig. 2A. A slight peri-bronchiolar inflammation was observed in the RSV-infected offspring mouse compared with mock-infected offspring (Figs. 2A-a, -b). In RSV-infected offspring mouse treated with 0.1% TBBPA, moderate peri-bronchiolar inflammation with infiltration of mononuclear cells was found (Figs. 2A-c), and the phenomenon was enhanced with edema in the offspring treated with 1.0% TBBPA (Fig. 2A-d). These results strongly suggest that perinatal exposure to TBBPA affected development of immune system defenses against infectious agents such as RSV, and finally exacerbated the pneumonia in the RSV-infected offspring mice.

**Gene expression in lung tissues of offspring exposed perinatally to TBBPA**

To clarify the mechanism of action of TBBPA on RSV infection, we tried to find a change in gene expression in lung tissues of offspring by an exhaustive analysis using a DNA microarray. The offspring mice treated perinatally with or without 1.0% of TBBPA were infected with or without RSV. On day 1 post-infection, the mice were sacrificed, and the lung tissues were used in this assay. We integrated the data of gene expressions that changed during RSV infection compared with mock-infection and then changed due to TBBPA treatment compared with the control. Data with a more than approximately 2-fold change of gene expression were selected, and 204 genes were finally determined. Five genes related to the immune and/or inflammatory system were found among them (Table 1). Due to perinatal exposure to TBBPA, expressions of four genes were increased. Particularly, the gene expression of interleukin (IL)-24 was enhanced up to 3.6-fold of that of RSV-infected control offspring.

**Protein expression of IL-24 in lung tissues of offspring exposed perinatally to TBBPA**

To investigate whether the protein expression of IL-24 due to perinatal exposure to TBBPA in the lung tissues was involved in the exacerbation of pneumonia, sections of the lung tissues of RSV-infected mice were stained immunohistochemically with a goat polyclonal antibody against IL-24 protein (Fig. 2B). The IL-24-positive cell was not detected in lung tissue of mock-infected mouse (data not shown). Positive expressions of IL-24 were observed in alveolar macrophages and epithelium of bronchi in RSV-infected mouse (Fig. 2B-a), and IL-24-positive cells were increased due to 0.01% of TBBPA in the diet (Fig. 2B-b). On the contrary, the counts of IL-24-positive cells were reduced in the lung tissue of TBBPA (0.1%)-treated offspring and, particularly, there were no positive cells in the region of severe inflammation (Fig. 2B-c). Finally, although the gene expression of IL-24 was elevated clearly due to TBBPA exposure (1.0%) on day 1 post-infection (Table 1), no IL-24-positive cell was observed in the lung tissues of TBBPA (1.0%)-treated offspring on day 5 post-infection (data not shown). Thus, these results suggested that perinatal exposure to TBBPA affects the expression of specific cytokine genes in lung tissues of RSV-infected offspring mice in the early phase of RSV infection, resulting in the exacerbation of pneumonia.

**DISCUSSION**

We demonstrated that perinatal exposure to TBBPA exacerbated pneumonia due to changes in the immune response to RSV infection in offspring mice. Using the murine RSV infection model, we previously determined that DBDE caused developmental immunotoxicity and exacerbated the pneumonia in offspring mice (Watanabe et al., 2008b). To characterize the developmental immunotoxicity of TBBPA, the effect of perinatal exposure to TBBPA on humoral immunity was evaluated in offspring rats, but the antibody response against keyhole limpet hemocyanin (KLH) was not clearly affected by TBBPA treatment (Teshima et al., 2008). Although TBBPA did not affect the growth of RSV in vitro, pulmonary RSV titers were significantly ($P < 0.01$) increased compared with the control in TBBPA (0.1%)-treated offspring mice (Fig. 1). These results demonstrated that perinatal exposure to TBBPA caused disorder of the immune system in RSV-infected offspring mice. Then, to clarify the immunotoxic effects of TBBPA on RSV-infected offspring mice, a histopathological analysis of lung tissues was performed (Fig. 2A). RSV-induced interstitial pneumonia progressed dose-dependently after TBBPA exposure. Particularly, although a markedly strong inflammation with edema was observed in the TBBPA (1.0%)-treated mice (Fig. 2A-d), the viral titers were strongly suppressed (Fig. 1). These results suggest that the formation of pneumonia might be hastened and enhanced by TBBPA- expo-
Fig. 2. Lungs of mice 5 days after RSV-infection. A) Hematoxylin and eosin staining (× 100). (a) Control mouse with mock infection. (b) Control mouse with RSV infection. (c) TBBPA-treated (0.1%) mouse with RSV infection. (d) TBBPA-treated (1.0%) mouse with RSV infection. B) Immunostained with anti-IL-24 antibodies (1:100) and counterstained with hematoxylin (× 200). (a) Control mouse with RSV infection. (b) TBBPA-treated (0.01%) mouse with RSV infection. (c) TBBPA-treated (0.1%) mouse with RSV infection. Open arrows indicate IL-24-positive cells.
sure, resulting in the termination of viral replication in lung tissues on day 5 post-infection (Fig. 1). Discovering the optimal time of RSV replication in TBBPA-treated offspring may make it possible to resolve understanding of the discrepancy.

To reveal the mechanism of action of perinatal exposure to TBBPA on RSV infection, an exhaustive search for a change of gene expression in the lung tissues was performed using a DNA microarray at day 1 post-infection (Table 1). Predominant gene expression of IL-24, which is a member of the IL-10 superfamily and categorized as an IL-20 receptor- related cytokine (Wang and Liang, 2005), was found in the lung tissues. In our previous study, we demonstrated that oral administration of TBBPA enhanced the production of proinflammatory cytokines in the lung tissues on day 1 post-infection and exacerbated pneumonia in RSV-infected adult mice (Watanabe et al., 2010). But, in the present study, elevation of the genes of proinflammatory cytokines was not determined (Table 1). These results suggested that mechanism of action of TBBPA on immunity between oral administration and perinatal exposure should be different. Elevation of IL-24 expression was also confirmed by immunohistochemical analysis using an anti-IL-24 antibody (Fig. 2B). Although expression of IL-24 has been reported to lyse specific ovarian tumors (Zerbini et al., 2011), the effects of IL-24 on immune response against viral infection have not been known. Concerning inflammation, upregulation of the gene expression of IL-24 was associated with activation of signal transducer and activator of transcription 3 (STAT3), resulting in psoriasis-like skin inflammation (Kumari et al., 2013). Predominance of the gene expression of IL-24 may be a key factor in pneumonia in RSV-infected offspring mice. Interestingly, enhancement of the expression of IL-24 protein was shown in macrophages and bronchial epithelial cells in the lung tissues of offspring at the low-dose (0.01%) TBBPA treatment, and the expression conversely decreased with the high-dose (0.1%) TBBPA treatment. To clarify the correlation of the gene expression of IL-24 with the protein expression of it during progression of RSV infection, further DNA microarray tests need to be done using the lung tissues of 0.01 or 0.1% TBBPA-exposed offspring. IL-24 should be a trigger, not an effector, of exacerbation of pneumonia and work during the initial period of RSV-infection (Fig. 2B).

Because electrical materials and/or epoxy resin containing TBBPA have been used worldwide, leaching of TBBPA into the environment is predicted on their deterioration. Our data should be useful in the health sciences to manage risk in both mothers and children and to identify the mechanism of action of developmental immunotoxicity of TBBPA.

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**Conflict of interest**---- The authors declare that there is no conflict of interest.

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