**Table 5** Genes extracted from cDNA microarray analysis that were up- or downregulated by FL treatment in the livers of B6C3F$_1$ *gpt* delta mice

| Gene symbol | Gene name | Fold change | GO categorize |
|---|---|---|---|
| Ccna2 | Cyclin A2 | 3.7 | Cell cycle |
| Ccnb1 | Cyclin B1 | 12.7 | Cell cycle |
| Ccnb2 | Cyclin B2 | 4.0 | Cell cycle |
| Ccnd1 | Cyclin D1 | 3.1 | Cell cycle |
| Cdk1 | Cyclin-dependent kinase 1 | 8.6 | Cell cycle |
| Chek1 | Checkpoint kinase 1 homolog (S. pombe) | 3.2 | Cell cycle, DNA damage |
| Fos | FBJ osteosarcoma oncogene | 8.0 | Cell proliferation |
| Jun | Jun oncogene | 4.1 | Cell proliferation, Apoptosis |
| Tnf | Tumor necrosis factor | 4.4 | Cell proliferation, Apoptosis, Immune system |
| Exo1 | Exonuclease 1 | 6.7 | DNA repair |
| Rad18 | RAD18 homolog (S. cerevisiae) | 2.4 | DNA repair |
| Rad51 | RAD51 homolog (S. cerevisiae) | 5.4 | DNA repair |
| Cyp1a1 | Cytochrome P450, family 1, subfamily a, polypeptide 1 | 23.5 | Drug metabolism |
| Cyp2b10 | Cytochrome P450, family 2, subfamily b, polypeptide 10 | 6.2 | Drug metabolism |
| Ccl2 | Chemokine (C–C motif) ligand 2 | 19.6 | Immune system |
| Ccl3 | Chemokine (C–C motif) ligand 3 | 9.3 | Immune system |
| Ccl4 | Chemokine (C–C motif) ligand 4 | 7.6 | Immune system |
| Ccl7 | Chemokine (C–C motif) ligand 7 | 10.2 | Immune system |
| Ccr2 | Chemokine (C–C motif) receptor 2 | 3.7 | Immune system |
| Ccr7 | Chemokine (C–C motif) receptor 7 | 3.7 | Immune system |
| Il1b | Interleukin 1 beta | 2.8 | Immune system |
| Il1f8 | Interleukin 1 family, member 8 | −3.7 | Immune system |
| Nfkb2 | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100 | 1.8 | Immune system |
| Ugt2b1 | UDP glucuronosyltransferase 2 family, polypeptide B1 | −2.5 | Drug metabolism |
| Cyp7b1 | Cytochrome P450, family 7, subfamily b, polypeptide 1 | −3.1 | Drug metabolism |

Listed genes were extracted under the cutoff condition of $p < 0.05$ and exhibiting at least 1.5-fold change in expression when comparing the MeIQx group with the MeIQx + FL group

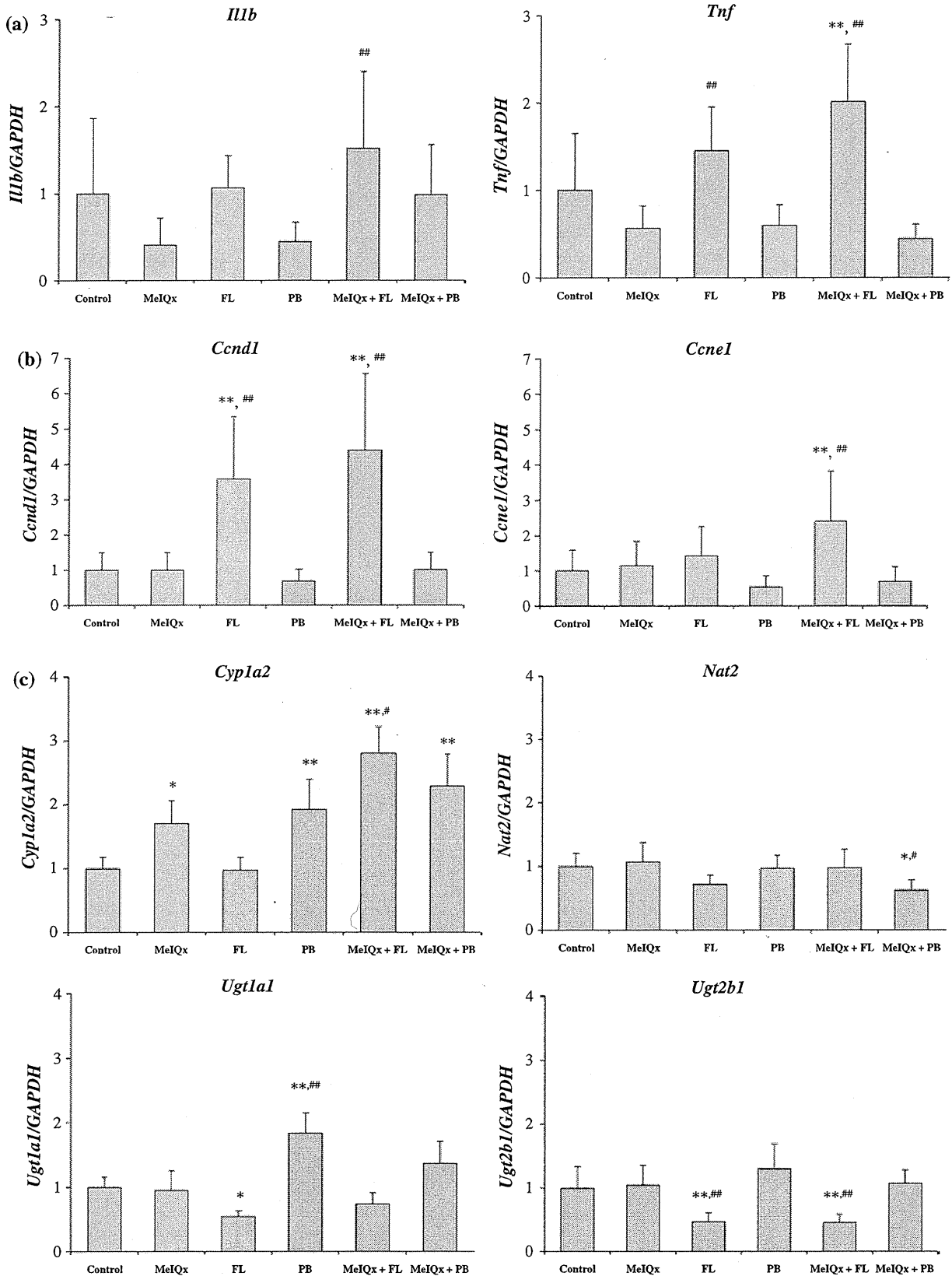
the analysis software GeneSpring (Agilent Technologies), normalization of gene expression data and filtering probe sets by expression levels, flags, and errors were performed. Differences in gene expression between the MeIQx group and the MeIQx + FL group were analyzed by analysis of variance (*t* test; cutoff value: $p < 0.05$; multiple testing corrections: Benjamini-Hochburg false discovery rate [FDR]). Extracted genes were analyzed by a gene ontology approach using GeneSpring software.

## Quantitative real-time PCR for mRNA expression

In all animals, cDNA copies of total RNA were obtained using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems Japan Ltd.). PCR was performed on an Applied Biosystems 7900HT FAST Real-Time PCR System (Applied Biosystems) with TaqMan Gene Expression Assays (Applied Biosystems) and TaqMan Rodent GAPDH control reagents (Applied Biosystems). The primers for assayed genes in TaqMan Gene Expression Assays (Applied Biosystems) were used. Expression levels of target genes were calculated by the relative standard curve method. GAPDH levels were used as an endogenous control, and data were presented as fold-change values of treated samples relative to controls.

## Statistical analysis

The significance of differences for body and liver weights, in vivo mutation assays, and real-time PCR analyses were evaluated using Turkey's multicomparison test. A $p$ value of less than 0.05 was considered significant.


(a)
Il1b
Il1b/GAPDH
Tnf
Tnf/GAPDH
(b)
Ccnd1
Ccnd1/GAPDH
Ccne1
Ccne1/GAPDH
(c)
Cyp1a2
Cyp1a2/GAPDH
Nat2
Nat2/GAPDH
Ugt1a1
Ugt1a1/GAPDH
Ugt2b1
Ugt2b1/GAPDH
Control
MeIQx
FL
PB
MeIQx + FL
MeIQx + PB

◀ **Fig. 4** Changes in mRNA level of cytokines (*Il1b* and *Tnf*) (**a**), cell cycle-related factors (*Ccnd1* and *Ccne1*) (**b**), and enzymes related to MeIQx metabolism (*Cyp1a2*, *Nat2*, *Ugt1a1*, and *Ugt2b1*) (**c**) in the livers of B6C3F$_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQx + PB for 13 weeks. Values are the mean ± SD of data for 5 mice. Values were normalized to the mRNA levels of GAPDH and are presented as fold-change values of treated samples relative to controls. *,**Significantly different from the control at $p < 0.05$ and 0.01, respectively; #, ##Significantly different from the MeIQx group at $p < 0.05$ and 0.01, respectively

## Results

### General signs, body weight, food consumption, and liver weight

No deaths and no remarkable changes in general signs were observed in all of the treated groups. In both the FL- and MeIQx + FL-treatment groups, reduced body weight gain and decreased food consumption were observed (Fig. 1). Data for final body and liver weights are summarized in Table 1. Final body weight was significantly decreased in the MeIQx + FL group, and liver weight was significantly increased in the FL, MeIQx + FL, and MeIQx + PB groups.

### Histopathological examinations and BrdU analysis of the liver

Histopathologically, hypertrophy of centrilobular hepatocytes with vacuolation was observed in all of the FL-treated groups (Fig. 2). Slight cell infiltration was also observed in these groups. In all of the PB-treated groups, only hypertrophy of centrilobular hepatocytes was observed (Fig. 2). The number of BrdU-positive liver cells was increased in the FL and MeIQx + FL groups, and the number of BrdU-positive cells was significantly higher in the MeIQx + FL group than in the MeIQx group (Fig. 3).

### In vivo mutation assays

Data for *gpt* and Spi$^-$ MFs in the liver are summarized in Tables 2, 3, and 4. A clear elevation of *gpt* MFs was observed in the MeIQx group. In the MeIQx + FL group, *gpt* MFs were further increased, and this increase was statistically significant compared with the MeIQx group. There were no significant increases in *gpt* MFs in the FL and PB groups. Cotreatment with PB and MeIQx did not alter the *gpt* MF compared to MeIQx treatment alone (Table 2). In the *gpt* mutation spectra, increases in GC:TA transversions and single base pair deletions were observed in the MeIQx and MeIQx + FL groups (Table 3). In Spi$^-$ assays, Spi$^-$ MFs increased in the MeIQx group and were further elevated in the MeIQx + FL group, exhibiting a statistically significant difference (Table 4).

### cDNA microarray analysis

When comparing the MeIQx group with the MeIQx + FL group, 2,224 genes (upregulated: 1,176; downregulated: 1,068) were modulated by FL treatment under the cutoff condition of $p < 0.05$ and with at least a 1.5-fold change in expression. A gene ontology approach was conducted, and apoptosis-, cell cycle/proliferation-, DNA damage/repair-, immune system-, and drug metabolism-related genes were extracted. Representative data for extracted genes are summarized in Table 5. The molecular functions of genes altered by FL treatment were induction of apoptosis (*Tnf* and *Jun*), cell cycle progression (*Ccnd1*, *Ccne1*, *Cdk1*, *Jun*, and *Fos*), cytokines (*Tnf*, *Il1b*, and *Ccl*), DNA repair (*Rad51*, *Rad18*, and *Exo1*), and drug metabolism (*Cyp1a1*, *Cyp2b10*, *Cyp7b1*, and *Ugt2b1*).

### Quantitative real-time PCR

In order to confirm our cDNA microarray analysis data, representative genes altered by FL treatment were analyzed by quantitative real-time PCR. The expression of cytokines (*Il1b* and *Tnf*) and cell cycle progression factors (*Ccnd1* and *Ccne1*) were increased in the MeIQx + FL group as compared with the control and/or the MeIQx group (Fig. 4). In addition, we also investigated the mRNA expression of several enzymes related to MeIQx metabolism. The expression levels of transcription factors such as *Jun*, *Fos*, and *Nfkb2* did not change among all treated groups (data not shown). In contrast, the expression of *Cyp1a2* was increased in the MeIQx + FL group as compared with the MeIQx group, while the expression of *Ugt2b1* was decreased in the FL and MeIQx + FL groups as compared with the control. The expression of *Nat2* was not altered in any treatments (Fig. 4).

## Discussion

In the present study, 13-week feeding of MeIQx caused clear increases in *gpt* and Spi$^-$ MFs in the livers of *gpt* delta mice. In mutation spectrum analysis of *gpt* mutant colonies, characteristic mutational patterns of MeIQx exposure such as GC:TA transversions and single base pair deletions were observed, in line with previous reports (Masumura et al. 2003; Okamura et al. 2010). Although no changes in *gpt* or Spi$^-$ MFs were observed in mice treated with FL alone, cotreatment with MeIQx and FL significantly increased *gpt* and Spi$^-$ MFs, reaching twofold that of MeIQx treatment alone. In addition, the mutation patterns observed in

*gpt* mutant colonies in the cotreatment group were consistent with those observed in mice treated with MeIQx alone. Considering the lack of increase in the MFs of reporter genes following FL exposure, these results clearly implied that FL enhanced MeIQx-induced in vivo mutagenicity.

MeIQx is known to be metabolically activated by CYP1A2, followed by modification to its proximal carcinogenic form by *N*-acetyltransferases (NATs) and sulfotransferase, allowing for the formation of DNA adducts, such as $N^2$-(deoxyguanosine-8-yl)-MeIQx (Schut and Snyderwine 1999; Snyderwine et al. 1993; Ochiai et al. 1993; Solomon et al. 1996). These bulky adducts on guanine are believed to be responsible for mutations, such as G:C to T:A transversions (Masumura et al. 2003). On the other hand, MeIQx is eliminated by UDP-glucuronyltransferase (UGT), especially UGT1A6 and UGT2B1, in the mouse liver (Mori et al. 2003). In fact, MeIQx-DNA adduct formation was effectively inhibited by purpurin, an inducer of glutathione-s-transferase (GST) and UGT (Takahashi et al. 2007). Thus, the present data demonstrating the enhancing effects of FL on MeIQx-induced mutagenicity allow us to speculate that FL treatment is capable of affecting some metabolizing enzymes responsible for activating and/or eliminating MeIQx. The present real-time PCR data demonstrated that *Cyp1a2* mRNA levels were significantly higher in the MeIQx + FL group than in mice treated with MeIQx alone, in spite of the fact that *Nat2* mRNA levels were not changed. Likewise, in addition to the tendency toward reduction in *Ugt1a1* mRNA levels, *Ugt2b1* mRNA levels were significantly lower in the MeIQx + FL group than in mice treated with MeIQx alone. Thus, it is likely that the effects of FL exposure on specific enzymes associated with MeIQx metabolism may account for the enhancing effects of FL.

DNA lesions are considered to be fixed gene mutations after cell division (Ames et al. 1993), and therefore, cells with damaged DNA under high cell proliferative conditions are prone to becoming mutated cells. In the present study, FL exposure clearly increased BrdU-positive hepatocytes, in line with cDNA microarray and real-time PCR analysis data showing increases in the mRNA expression of cell cycle-related genes, such as *Ccnd1* and *Ccne1*, in FL-treated groups. In addition to histopathological data showing obvious hepatocellular injury following FL exposure, mRNA levels of genes encoding cytokines, such as *Tnf* and *Il1b*, were increased. Since these cytokines are known to be released by Kupffer cells during hepatocellular injury (Salazar-Montes et al. 2000, 2006; Roberts et al. 2007; Iimuro and Fujimoto 2010), it is highly probable that the increased cell proliferation resulting from FL treatment is attributable to the compensatory regenerative response. Thus, regenerative cell proliferation induced by FL may be an additional causal factor for the observed enhancing effects of FL on MeIQx-induced in vivo mutagenicity. Although PB is known to induce a transient increase in hepatocyte proliferation in the early phases (Jones et al. 1993; Counts et al. 1996), cotreatment with PB did not alter *gpt* or $Spi^-$ MFs compared with MeIQx treatment alone. Considering that PB did not increase cell proliferation or hepatic injury in the present study, prolonged injury and a consequent sustained increase in cell proliferation are requisite for enhancement of gene mutation rates.

MeIQx is the most abundant heterocyclic amine in cooked foods, and there is no current way to prevent exposure of humans to this compound (Wakabayashi et al. 1993). On the other hand, FL has been evaluated as a nongenotoxic carcinogen, and its acceptable daily intake (ADI) has been determined based on toxicological studies (JECFA 2004). Here, we show that the combination of MeIQx and FL caused enhancement of MeIQx-induced in vivo mutagenicity. The present results suggest that further investigations of the combined effects of chemicals in food are required to adequately assess risks to human health.

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**Conflict of interest** The authors declare that there are no conflicts of interest.

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RESEARCH ARTICLE

# Effects of prior oral exposure to combinations of environmental immunosuppressive agents on ovalbumin allergen-induced allergic airway inflammation in Balb/c mice

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**Abstract**

Humans are exposed daily to multiple environmental chemicals in the atmosphere, in food, and in commercial products. Therefore, hazard identification and risk management must account for exposure to chemical mixtures. The objective of the study reported here was to investigate the effects of combinations of three well-known environmental immunotoxic chemicals – methoxychlor (MXC), an organochlorine compound; parathion (PARA), an organophosphate compound; and piperonyl butoxide (PBO), an agricultural insecticide synergist – by using a mouse model of ovalbumin (OVA)-induced allergic airway inflammation. Four-week-old Balb/c mice were exposed orally to either one or two of the environmental immunotoxic chemicals for five consecutive days, prior to intraperitoneal sensitization with OVA and an inhalation challenge. We assessed IgE levels in serum, B-cell counts, and cytokine production in hilar lymph nodes, and differential cell counts and levels of related chemokines in bronchoalveolar lavage fluid (BALF). Mice treated with MXC + PARA or PBO + MXC showed marked increases in serum IgE, IgE-positive B-cells and cytokines in lymph nodes, and differential cell counts and related chemokines in BALF compared with mice that received the vehicle control or the corresponding individual test substances. These results suggest that simultaneous exposure to multiple environmental chemicals aggravates allergic airway inflammation more than exposure to individual chemicals. It is expected that the results of this study will help others in their evaluation of immunotoxic combinational effects when conducting assessments of the safety of environmental/occupational chemicals.

**Keywords**

Allergic airway inflammation, environmental chemicals, methoxychlor, parathion, piperonyl butoxide

**History**

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## Introduction

Every day, humans are exposed concurrently or sequentially to a huge number of products that contain environmental agents (e.g. cosmetics, pesticides, drugs, and biotechnology-derived products) and to multiple environmental chemicals in the atmosphere and in food[1–5]. Because of this constant exposure, safety assessments must take into consideration the effects of combined exposure. However, most current toxicity assessments are based on exposure to individual substances and, as such, the effects of combined exposure to environmental chemicals remain unclear. The objective of the studies reported here was to investigate the combined toxicologic effects of multiple chemicals, focusing on their influence on the immune system.

In our previous studies, we demonstrated the combined effects of three well-known environmental immunotoxic chemicals – methoxychlor (MXC), an organochlorine compound; parathion (PARA), an organophosphate compound; and piperonyl butoxide (PBO), an agricultural insecticide synergist – by using a short-term oral exposure method that we developed[6]. Briefly, Balb/c mice exposed to MXC + PARA or PBO + MXC had lower immune responses to subsequent immunization with sheep red blood cells (SRBCs), including lower SRBC-specific IgM production and fewer SRBC-specific T and B cells, than mice exposed to the corresponding individual chemicals. This suggests that environmental chemicals can have additive immunotoxic effects when encountered simultaneously. In the current study, to develop the advanced mechanisms of the combined immunotoxicologic effects of multiple chemicals, we used a mouse model of ovalbumin (OVA)-induced allergic airway inflammation and focused on the mechanisms by which the allergic potential is modulated[7].

Recent reports suggest that prior exposure to environmental immunosuppressive chemicals induces allergic diseases in immature rodents[8,9]. Our laboratory previously demonstrated that prior exposure to immunosuppressive environmental chemicals aggravates T cell-mediated allergic reactions[10],

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mite- and chemical allergen-induced atopic dermatitis-like immunoreactions[11,12]. These data indicate that immunosuppression by environmental chemicals is closely related to the aggravation of allergic reactions.

In the current study, we demonstrate the immunomodulatory effects of exposure to MXC + PARA and PBO + MXC compared to exposure to the individual agents in a mouse model of OVA-sensitization and challenge, one of the most popular allergic airway inflammation models[7]. In this study, to explore the mechanisms of airway inflammation development, we analyzed leukocyte numbers, chemokine production, and immunoglobulin (Ig) E levels in serum and B-cells and cytokine production in hilar lymph node (LN) cells.

## Materials and methods

### Chemicals

Standard MXC ($C_{16}H_{15}Cl_3O_2$, >97% pure), standard PARA ($C_{10}H_{14}NO_5PS$, 99.5% pure), and standard PBO ($C_{19}H_{30}O_5$, >98% pure) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil was purchased from Hayashi Chemicals (Tokyo, Japan). Albumin from chicken egg white (ovalbumin, OVA) was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan) and Sigma-Aldrich Japan K.K. (Tokyo, Japan). Aluminum hydroxide hydrate gel suspension (ALUM) was purchased from Cosmo Bio Co., Ltd. For the intraperitoneal (i.p.) sensitization, OVA (10 μg) was dissolved in PBS (0.2 ml/animal) containing ALUM (2 mg). For the inhalation challenge, OVA was dissolved in PBS to 0.1% (w/v). MXC, PARA, or PBO diluted in corn oil to a fixed final volume was orally administered to mice. Based on the EPA Immunotoxicity Guidelines (1998), which states that doses should "not produce significant stress, malnutrition, or fatalities", doses used in this study were <1/5 the median lethal dose ($LD_{50}$; dose at which ≥50% of animals would be expected to die) and were administered to avoid induction of clear general or immune toxicity (i.e., changes in appearance, posture, behaviour, respiration, consciousness, neurologic status, temperature, excretion, etc.[12]; The single-chemical dosages used in this study were: MXC, 100 mg/kg day; PARA, 1.0 mg/kg day; and PBO, 100 mg/kg day. Combination dosages (MXC + PARA and PBO + MXC) were prepared by mixing each chemical so that the final concentration of each chemical was half that of the single dosage. As the objective in this study was to investigate the relationship between combined exposure to immunosuppressive chemicals and allergic responses in a mouse model, we selected relatively high doses compared with actual human exposures. The examination of the relationship of allergic responses to doses comparable to human exposures is now underway.

### Animals

Balb/cAnN mice (female, 3-wk-old) were purchased from Charles River Laboratories (Atsugi, Kanagawa, Japan) and housed individually in cages under controlled lighting (lights on, 07:00–19:00), temperature (22 ± 3 °C), humidity (55% ± 15%), and ventilation (at least 10 complete fresh-air changes/h). Standard rodent chow (Certified Pellet Diet MF; Oriental Yeast Co., Tokyo, Japan) and filtered water were available *ad libitum*. All aspects of the current study were conducted in accordance with the Animal Care and Use Program of the Institute of Environmental Toxicology, Japan (IET IACUC Approval No. 12103).

### Experimental protocol

The experimental protocol used in this study is depicted in Figure 1. Following a 6-d acclimatization period, healthy Balb/c mice (now 4-wk-old) were subjected to grouping. All animals were weighed individually on the day of initiation of treatment and those with extremely high or low body weights were eliminated. The remaining animals were allocated to each dose group ($n = 8$ mice per group) for dosing, vehicle control, or no treatment (intact group), through the stratified random sampling method on their body weights. After allocation, it was confirmed that all individual body weights were within a range of 80–120% of the mean value. According to our preliminary study, animals receiving only OVA sensitization or challenge showed almost the same patterns of allergic airway inflammation as the intact group. Therefore, data of only OVA sensitization or challenge are not shown in this article. On Days 1–5, mice were given an oral dose (by gavage, without any anaesthesia) of a single or combination test solution (MXC, PARA, PBO, MXC + PARA, PBO + MXC), or vehicle only. For sensitization, OVA/ALUM was injected intraperitoneally into each mouse on days 29, 36, and 42. For the challenge, OVA/PBS was administered by inhalation (see section "Method of inhalation exposure") on days 43, 45, 47, 50, 52. One day after the last challenge (day 53), all animals were anesthetized and sacrificed by pentobarbital sodium injection (75 mg/kg, i.p.). Blood samples were taken from the inferior vena cava, and serum samples were assayed for IgE levels. Bronchoalveolar lavage fluid (BALF) was collected by cannulating the trachea and lavaging the lungs 3 times with 1 mL PBS supplemented with 1% heat-inactivated foetal calf serum (FCS; Life Technologies Co., Ltd., Tokyo, Japan). The first BALF fraction from each animal was centrifuged at 350 × *g* for 5 min, and chemokine levels were measured. The cell pellets of all three fractions were resuspended, pooled, and centrifuged at 350 × *g* for 5 min. The supernatants were removed, and the cell pellets were used for differential cell counts. Hilar LN removed from each mouse were stored in RPMI 1640 medium (Life Technologies Co., Ltd., Carlsbad, CA). Single-cell suspensions were prepared from LNs by passage through a sterile 70-μm nylon cell strainer in 1 mL RPMI 1640 supplemented with 5% FCS. Single-cell suspensions were used to analyze the IgE-positive B-cells and cytokine production.

### Method of inhalation exposure

For the inhalation challenge, animals were exposed to 0.1% OVA/PBS mist continuously for 30 min/d. The appearance of the inhalation exposure system is shown in Figure 1. The animals were individually held in animal holders (Tokiwa Kagakukikai Co., Ltd., Tokyo, Japan) attached to a nose-only exposure chamber (total volume 31.2 L, Tokiwa Kagakukikai Co., Ltd.) so that only their noses were exposed to the chemical mist. The mist was generated by an atomizer

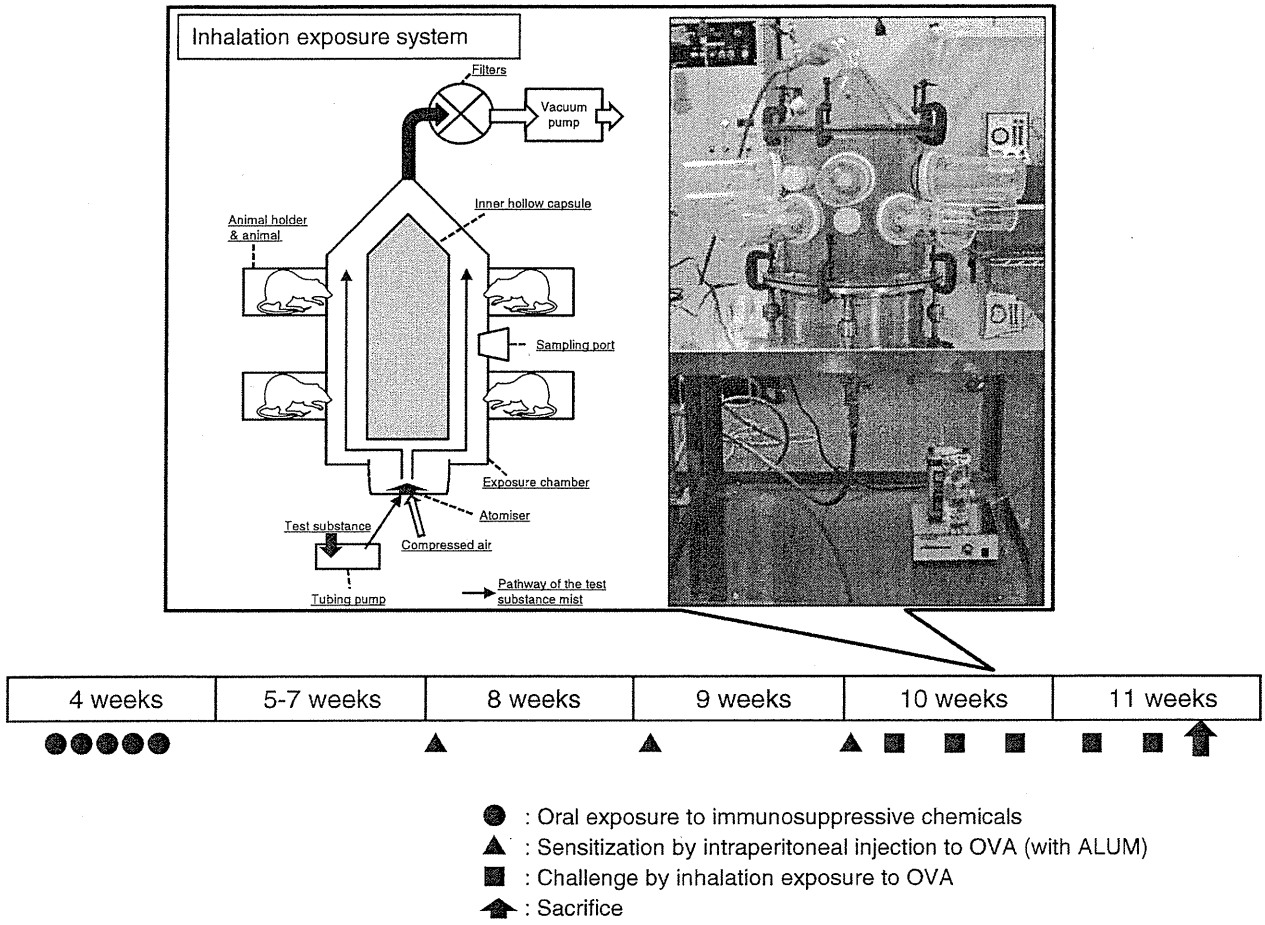


Figure 1. Inhalation exposure system and experimental protocol. See the "Materials and Methods" section for a detailed description. OVA, ovalbumin; ALUM, Aluminum hydroxide hydrate gel suspension.



(Ikeuchi Co., Ltd., Tokyo, Japan) with compressed air (ES4AD-5, Kobelco, Tokyo, Japan) and supplied to the exposure chamber through an air filter (F3000-10-Y, CKD Corporation, Aichi, Japan). Airflow to the chamber was controlled by an area flowmeter (NSPO-4, Nippon Flow Cell, Tokyo, Japan) at a rate of 20 L/min. The chamber air was exhausted through an air filter system consisting of a glass wool filter, a mist trap, and an activated charcoal filter (Tokiwa Kagakukikai Co., Ltd.) and was emitted to the atmosphere by means of a blower (TFO-K4P, Hitachi, Ltd., Tokyo, Japan). The actual concentration, mass median aerodynamic diameter (MMAD), and geometric standard deviation (GSD) were monitored by gravimetric analysis by using an air sampler (MF-200, Oct Science Co., Ltd., Osaka, Japan) and an Andersen-type personal sampler (Model 1312S, Kanomax Japan, Inc., Osaka, Japan). The mean values of the actual concentration of active ingredient, MMAD, and GSD were kept at approximately 5 mg/m$^3$, 4.0 μm, and 2.0, respectively, throughout the inhalation exposure.

### Assay for total and OVA-specific IgE levels in serum

Total (BD OptEIA Mouse IgE ELISA Set, BD Pharmingen, Tokyo, Japan) and OVA-specific (Mouse OVA-IgE ELISA KIT, AKRIE-030, Shibayagi, Gunma, Japan) IgE levels in serum were measured using enzyme-linked immunosorbent assays (ELISAs) in accordance with the manufacturer's protocols.

### Flow cytometric analysis of BALF and hilar LN cells

The following antibodies used for flow cytometric analysis were purchased from BD Pharmingen: fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgE (clone R35-72), FITC-conjugated anti-mouse Gr-1 (RB6-8C5), phycoerythrin (PE)-conjugated hamster anti-mouse CD11c (HL3), and allophycocyanin-cyanin-7-conjugated anti-mouse CD45R/B220 (RA3-6B2). To avoid nonspecific binding, $1 \times 10^6$ cells were incubated with 1 μg Mouse BD Fc BlockTM (BD Pharmingen) for 5 min at 4 °C, followed by incubation with the monoclonal antibodies for 30 min at 4 °C in the dark. Cells were washed twice with 5% FCS in PBS, resuspended at $1 \times 10^6$ cells per tube in 500 μL PBS, and analyzed on a FACSVerse flow cytometer (BD Pharmingen) using FACSuite (BD Pharmingen). For each sample, 20 000 events were collected and analyzed for expression of antigens.

For differential cell counts, half of the cells were used for Cytospins (Shandon Inc., Pittsburgh, PA) and the remaining cells were used for flow cytometric analysis. The Cytospin preparations were stained with Giemsa stain (Wako Pure

Chemical Industries), and 200 cells in each sample were examined to differentiate the numbers of macrophages, eosinophils, and neutrophils present (according to standard leukocyte typing). According to the results, the differential cell counts from the cytospin preparations showed almost similar patterns as those from the flow cytometric analysis, therefore data are not shown.

### Cytokine production in LN cells

To stimulate T-cell receptor signalling, we cultured single-cell suspensions recovered from LNs ($1 \times 10^6$ cells/well) for 24 or 96 h with Dynabeads Mouse T-Activator CD3/CD28 (25 μg/well) antibodies (Life Technologies Co., Ltd., Tokyo, Japan) in 24-well plates at 37 °C in a 5% $CO_2$ atmosphere. The levels of cytokines (interleukin [IL]-4, -5, and -13 in supernatants [cell culture medium]) were measured using a cytometric bead array (BD CBA Mouse Flex Set, BD Pharmingen) in accordance with the manufacturer's protocol.

### Chemokine levels in BALF

The levels of chemokines (KC, MIP-1β, RANTES) were measured using a cytometric bead array (BD CBA Mouse Flex Set, BD Pharmingen) in accordance with the manufacturer's protocol.

### Statistical analysis

All data are expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to evaluate the results. For significant results, differences between vehicle control and treatment groups were then assessed by using Dunnett's multiple comparison test. Statistical significance of differences between single-chemical and combination-treatment groups was determined by using Tukey's multiple comparison test. Statistical significance was defined as $p < 0.05$.

## Results

No abnormal clinical signs (e.g. decreased activity) or changes in body weight were observed in any of the treatment groups (data not shown).

### Total and OVA-specific IgE levels in serum

The MXC + PARA mice displayed significantly higher total IgE levels in serum compared with the values for the vehicle control (2754 versus 1392 pg/ml, $p < 0.01$), MXC-treated mice (1694 pg/ml, $p < 0.01$), and PARA-treated mice (1671 pg/ml, $p < 0.01$) (Figure 2). The PBO + MXC mice had average total IgE levels in mice treated with vehicle control, MXC, and PBO, respectively (2254 versus 1392, 1694 and 1562 pg/ml), but this increase was not significant.

The MXC + PARA mice had significantly higher OVA-specific IgE levels than mice treated with vehicle control (19.5 versus 10.5 U/ml, $p < 0.05$) and PARA (10.8 U/ml, $p < 0.05$). Mice treated with MXC + PARA also had 152% the levels of mice treated with MXC alone (12.8 U/ml); however, this increase was not significant. The PBO + MXC mice had significantly higher OVA-specific IgE values than mice treated with the vehicle control (19.1 versus 10.5 U/ml, $p < 0.05$) or PBO (10.9 U/ml, $p < 0.05$). Mice treated with PBO + MXC also had 149% the levels in mice treated with MXC alone; however, this increase was not significant.

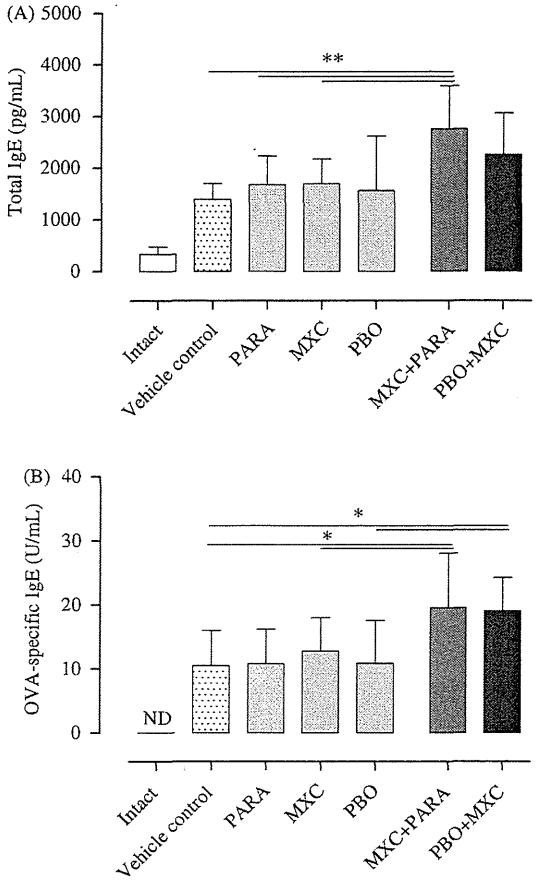


Figure 2. Total and OVA-specific IgE levels in serum. Mice were treated with nothing (intact), vehicle, methoxychlor (MXC), parathion (PARA), piperonyl butoxide (PBO), or the indicated combinations of the agents, and total (A) or OVA-specific (B) IgE levels in serum were measured. IgE levels are expressed as mean ± SD (titre; $n = 8$ per group). $*p < 0.05$ and $**p < 0.01$ (Dunnett's multiple comparison test) versus vehicle control group; $*p < 0.05$ and $**p < 0.01$ (Tukey's multiple comparison test) versus MXC-, PARA- or PBO-treated group.

### BALF analysis

To assess the allergic airway inflammation in the lung, we counted eosinophils, lymphocytes, macrophages, and neutrophils (Figure 3) and measured the levels of related chemokines (KC, MIP-1β, and RANTES; Figure 4) in BALF. In the MXC, PARA, and PBO treatment groups, all responses in BALF were comparable with those of the vehicle control group (Figures 3 and 4).

The MXC + PARA and PBO + MXC mice had significantly higher numbers of eosinophils, lymphocytes, and neutrophils than mice treated with the vehicle control, MXC, or PARA. The change in eosinophil counts was particularly large: the MXC + PARA mice had 447, 305, and 307% the numbers in mice treated with vehicle control, MXC, and PARA (7881 versus 1764, 2566 and 2584 pg/ml), respectively, and the PBO + MXC had eosinophil counts 415, 284, and 310% the counts in mice treated with vehicle

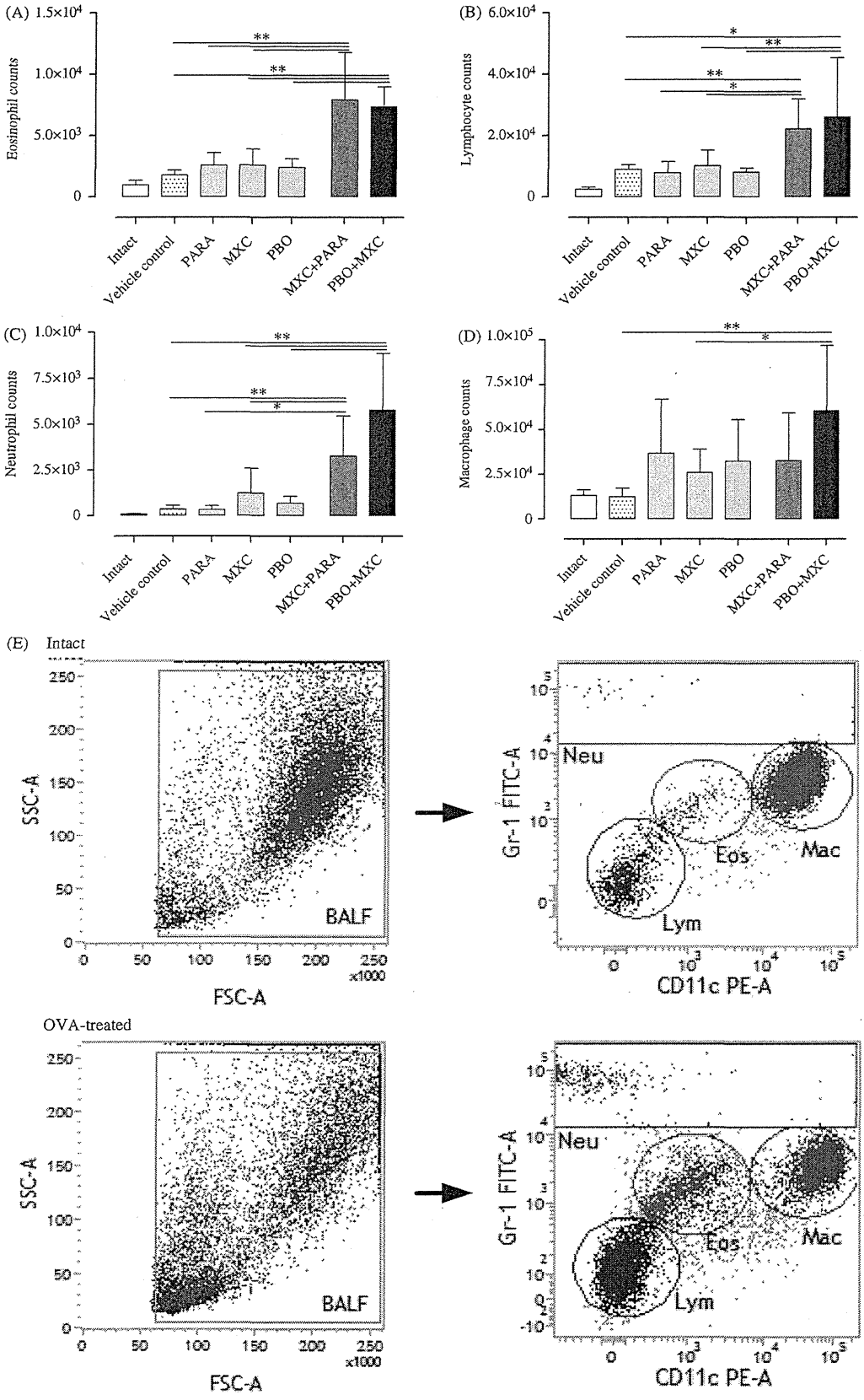


Figure 3. Immune cells counts in BALF. Designations of treatments are as in Figure 2. (A) Eosinophils, (B) lymphocytes, (C) neutrophils, (D) macrophages, (E) representative dot plots from an intact (untreated) mouse and a mouse exposed to OVA only. Cell counts are expressed as mean ± SD ($n = 8$ per group). *$p < 0.05$ and **$p < 0.01$ (Dunnett's multiple comparison test) versus vehicle control group; *$p < 0.05$ and **$p < 0.01$ (Tukey's multiple comparison test) versus MXC-, PARA- or PBO-treated group.

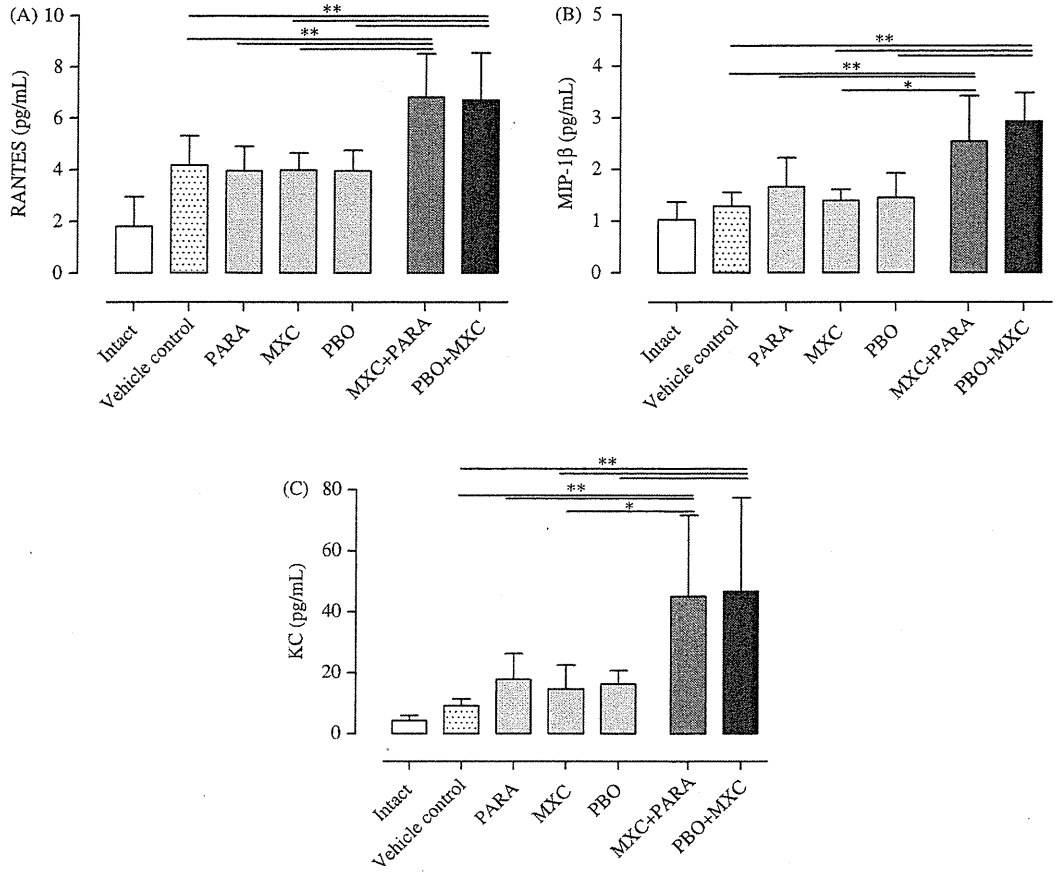


Figure 4. Chemokine levels in BALF. The levels of (A) RANTES, (B) MIP-1β, and (C) KC are expressed as mean ± SD (pg/mL; $n = 8$ per group). Designations of treatments are as in Figure 2. $^{**}p < 0.01$ (Dunnett's multiple comparison test) versus vehicle control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ (Tukey's multiple comparison test) versus MXC-, PARA- or PBO-treated group.

control, MXC, and PBO (7328 versus 1764, 2584 and 2365 pg/ml), respectively. The PBO + MXC mice also had significantly higher numbers of macrophages than mice treated with the vehicle control ($p < 0.01$) or MXC ($p < 0.05$), and also had 188% the number of macrophages in PBO-treated mice, although this increase was not significant. The MXC + PARA treatment group had numbers of macrophages comparable with those of the vehicle control, MXC-only, and PARA-only treatment groups.

The MXC + PARA mice displayed significantly higher levels of RANTES, MIP-1β, and KC than the levels in mice treated with the vehicle control, MXC, or PARA. The MXC + PARA mice had RANTES levels 163, 171, and 172% the levels in mice treated with vehicle control, MXC, and PARA (6.81 versus 4.18, 3.99 and 3.95 pg/ml), respectively. The PBO + MXC mice also had significantly higher levels of these chemokines than mice treated with the vehicle control, MXC, or PBO. The PBO + MXC mice had RANTES levels 161, 168, and 170% the levels in mice treated with vehicle control, MXC, and PBO (6.71 versus 4.18, 3.99 and 3.95 pg/ml), respectively.

## Analysis of hilar LN cells

To evaluate the proliferation of IgE-positive B-cells following the single and combination treatments, isolated lymphocytes were stained with anti-B220 and anti-IgE antibodies. In the MXC, PARA, and PBO treatment groups, IgE-positive B-cell counts were comparable with those of the vehicle controls (Figure 5). The MXC + PARA-treated mice had significantly higher counts than mice treated with the vehicle control (1.11 versus $0.29 \times 10^6$ cells, $p < 0.01$), MXC ($0.43 \times 10^6$ cells, $p < 0.01$), or PARA ($0.59 \times 10^6$ cells, $p < 0.05$). The PBO + MXC-treated mice had significantly higher values than mice treated with the vehicle control (1.35 versus $0.29 \times 10^6$ cells, $p < 0.01$), MXC ($p < 0.01$), or PBO ($0.52 \times 10^6$ cells, $p < 0.05$).

We also examined the production of related cytokines (IL-4, -5, and -13) from T-cells. In the MXC, PARA, and PBO treatment groups, production of all cytokines was comparable with that in the vehicle controls (Figure 6). The MXC + PARA mice had significantly higher levels of IL-4 than mice treated with the vehicle control (39.8 versus 20.4 pg/ml, $p < 0.01$), MXC (22.9 pg/ml, $p < 0.01$), and PARA (19.0 pg/ml, $p < 0.01$). The PBO + MXC mice had, on average the levels of IL-4 of mice treated with the vehicle control, MXC, or PBO (39.8 versus 20.4, 22.9 and 23.5 pg/ml), but this increase was not significant.

For the levels of IL-5, the MXC + PARA mice had, on average the levels in mice treated with the vehicle control, MXC, or PARA (55.7 versus 21.7, 27.9 and 29.5 pg/ml), but this increase was not significant. In contrast, the PBO + MXC

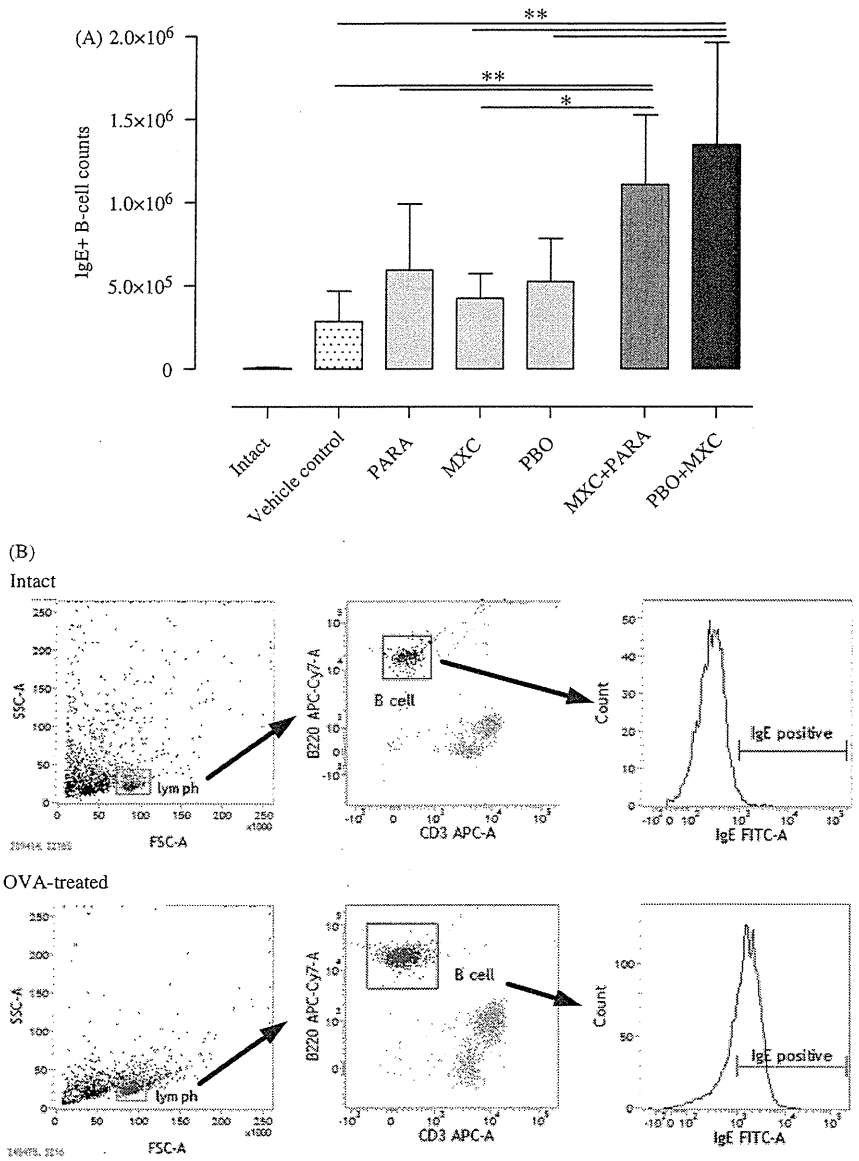


Figure 5. Responses in hilar LN cells. (A) IgE-positive B-cell counts; (B) representative histograms from an intact mouse and a mouse treated only with OVA. Designations of treatments are as in Figure 2. Cell counts and total IgE levels (ng/mL) are expressed as mean ± SD ($n = 8$ per group). **$p < 0.01$ (Dunnett's multiple comparison test) versus vehicle control group; *$p < 0.05$ and **$p < 0.01$ (Tukey's multiple comparison test) versus MXC-, PARA- or PBO-treated group.

mice had significantly higher levels of IL-5 than mice treated with the vehicle control (86.3 versus 21.7 pg/ml, $p < 0.01$), MXC ($p < 0.01$), or PBO (27.4 pg/ml, $p < 0.01$).

For the levels of IL-13, the MXC + PARA mice had significantly higher values than the mice treated with the vehicle control (129.7 versus 46.9 pg/ml, $p < 0.01$), MXC (73.9 pg/ml, $p < 0.01$), or PARA (77.0 pg/ml, $p < 0.05$). The PBO + MXC mice had significantly higher levels of IL-13 than mice treated with the vehicle control (144.7 versus 46.9 pg/ml, $p < 0.01$), MXC ($p < 0.01$), or PBO (78.1 pg/ml, $p < 0.01$).

## Discussion

In the current study, we sought to better understand the immunological mechanisms by which combined exposures to three well-known environmental chemicals – MXC, PARA, and PBO – aggravate allergic airway inflammation. To that end, we examined the allergic effects of these chemicals in Balb/cAnN mice using an OVA-induced allergic airway inflammation model. Changes in the host immune status were assessed by measures of effects on total and OVA-specific IgE levels in serum, differential cell counts and related chemokine levels in BALF, and IgE positive B-cell counts and cytokine production from T-cells in the hilar lymph nodes.

MXC, PARA, and PBO were selected on the basis of previous studies: MXC exposure causes atrophy of $CD4^+CD8^+$ T-cells in the thymus[13]; PARA markedly inhibits antigen-specific IgM production[14]; and PBO depletes T-cells in the spleen and thymus, induces bone marrow hypoplasia, and inhibits T-cell proliferation in lymphoid tissues[15–17]. We also previously showed that exposure to MXC, PARA, or PBO results in increased thymocyte apoptosis, markedly inhibits and SRBC-specific IgM production[18,19]. We selected the doses of MXC (100 mg/kg day), PARA (1.0 mg/kg day) and PBO (100 mg/kg day) based on the results from acute oral LD50 doses (2900 mg/kg, 5 mg/kg and 2600 mg/kg,

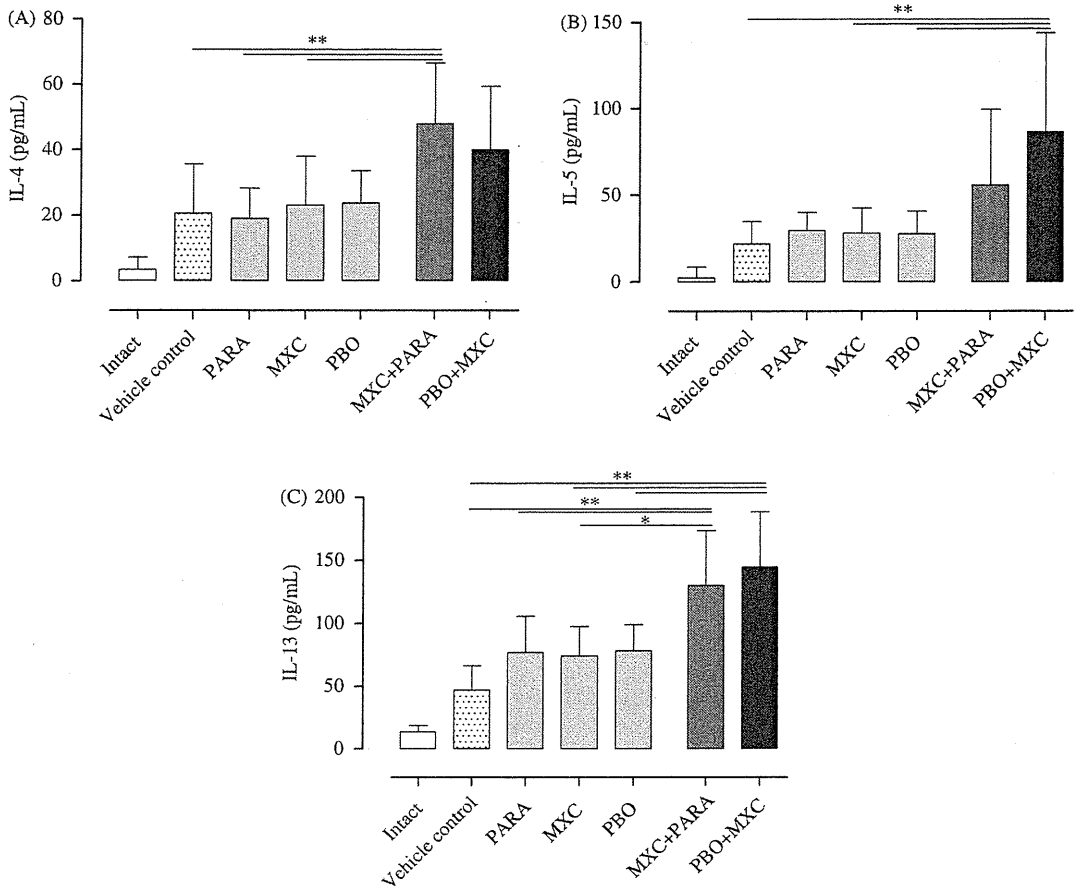


Figure 6. Cytokine production in hilar LN cells. The levels of (A) IL-4, (B) IL-5, and (C) IL-13 are expressed as mean ± SD (pg/mL; $n = 8$ per group). Designations of treatments are as in Figure 2. $^{**}p < 0.01$ (Dunnett's multiple comparison test) versus vehicle control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ (Tukey's multiple comparison test) versus MXC-, PARA- or PBO-treated group.

respectively) and subchronic general or immune toxicity. The objective in this study was to investigate the relationship between combined immunosuppressive chemicals and allergic responses using confirmed environmental immunosuppressive chemicals using a mice model focused on workers safety assessment. Therefore, we selected relatively-high doses (approximately 1/5 of LD50) and short-term exposure compared with estimator of human exposures in actual consumers. The examination focused on the safety assessment for consumers which exposure levels were equivalent to acceptable daily intake (ADI) is now underway.

OVA is a common antigen used to evaluate allergic airway inflammation. After sensitization and challenge with OVA, total and OVA-specific IgE responses in serum can be assessed with ELISAs. IgE antibodies play an important role in the development of allergic asthma, and can be used as a marker of OVA-induced allergic asthma[20]. In addition, in the development of the allergic response, activated B-cells can act as antigen-presenting cells for helper T-cells, resulting in hyperproduction of IgE[21,22]. According to the results of our current study, compared with the vehicle control mice, MXC + PARA and PBO + MXC mice had significant increases in serum IgE responses, whereas mice treated with MXC, PARA, or PBO alone did not. In addition, the total IgE responses of the MXC + PARA mice were significantly higher than those of mice treated with MXC or PARA alone, and the OVA-specific IgE responses with the MXC + PARA and PBO + MXC treatment led to significant increases relative to those seen in PARA and PBO mice. Along with serum IgE responses, the numbers of IgE-positive B-cell, which take a central role in the production of IgE, were significantly higher in the MXC + PARA and PBO + MXC mice than in mice treated with the vehicle control or single chemicals. These results suggested to us that MXC + PARA or PBO + MXC exposures induced more severe allergic airway inflammatory responses than exposure to any of the three chemicals individually.

Allergic airway inflammation, such as asthma, is recognized by the airway infiltration of eosinophils and other inflammatory cells, bronchial hyper-responsiveness, and airway obstruction[23]. In addition, degranulation and release of inflammatory mediators such as KC, RANTES, and MIP-1β by activated neutrophils and eosinophils may cause extensive tissue damage and propagate airway obstruction[22,24,25]. In the present study, the administration of MXC + PARA and PBO + MXC significantly exacerbated airway eosinophilic inflammation and increased the total inflammatory cell number and the production of KC, RANTES, and MIP-1β in the BALF, as compared to the administration of vehicle control or individual chemicals.

The observed changes in pulmonary inflammation possibly resulted from concomitant changes in the levels of KC, RANTES, and MIP-1β in the BALF[26]. These results suggested to us that MXC + PARA or PBO + MXC exposures induced a more severe induction in allergic airway inflammatory responses, including IgE reactions, than exposure to vehicle control or any of the three individual chemicals.

Allergic airway inflammation is orchestrated by Th2 cells, leading to IgE production and eosinophil activation[27]. Th2 cells produce and secrete a large quantity of Th2 cytokines, such as IL-4, IL-5, and IL-13, and these cytokines promote IgE production and eosinophil migration[28]. In the present study, the administration of MXC + PARA and PBO + MXC significantly increased the production of IL-4, -5, and -13 in the hilar LN cells as compared to the vehicle control and individual chemicals. These observations indicate that Th2 cytokines are important in the development of airway inflammation. Therefore, increased Th2 cytokine production in the hilar LN cells may subsequently result in aggravated airway inflammation, which was observed in groups treated with combined chemicals.

The results reported here demonstrate that prior exposure to combinations of immunosuppressive environmental chemicals such as MXC, PARA, and PBO can modulate immune functions and increase the severity of allergic airway inflammation compared to exposure to the vehicle control or any of the individual chemicals. This may occur through prevention of the central tolerance of autoreactive T or B cells, because mice exposed to immunosuppressive environmental chemicals showed significant decreases in Treg cell populations[12]. After the immunosuppressive compounds are withdrawn, recovery from immunosuppression occurs and an immune disorder develops in the mice, including increased production of antibodies and autoreactive T- or B-cells. Then, if antigens are encountered, abnormal hypersensitivity reactions are induced.

Overall, our data show that combined exposure to certain environmental chemicals can aggravate allergic inflammation beyond that caused by exposure to the individual chemicals in the mixtures, as shown by effects on IgE responses, eosinophil counts, and levels of related chemokines and cytokines. These syndromes may result in the activation of mast cell degranulation and aggravation of asthma-like symptoms such as labored respiration, increase airway resistance and wheezing[29]. In addition, the aggravation of allergic airway inflammation is in line with our preliminary study, which found that combined exposure to certain environmental chemicals can suppress acquired immune responses[12]. The combined toxicity may be affected by the age of the animal, chemical structure, receptor binding, and immune pathways involved; further studies are currently in progress. It is expected that the results of this study will help others in their evaluation of combinatorial immunotoxic effects when conducting assessments of the safety of environmental or occupational chemicals.

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## Declaration of interest

The authors have no conflicts of interest to declare. The authors alone are responsible for the content of this manuscript. This work was supported by a research Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan.

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RESEARCH ARTICLE

# Effects of short-term oral combined exposure to environmental immunotoxic chemicals in mice

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**Abstract**

People are constantly exposed to environmental chemicals through contact with the atmosphere or by ingestion of food. Therefore, when conducting safety assessments, the immunotoxic effects of combinations of chemicals in addition to toxicities produced by each chemical alone should be considered. The objective of the studies reported here were to demonstrate the combined effects of three well-known environmental immunotoxic chemicals – methoxychlor (MXC), an organochlorine compound; parathion (PARA), an organophosphate compound; and piperonyl butoxide (PBO), an agricultural insecticide synergist – by using a short-term oral exposure method. Seven-week-old Balb/cAnN mice received daily oral exposure to either one or two of the environmental immunotoxic chemicals for 5 consecutive days. On Day 2, all mice in each group were immunized with sheep red blood cells (SRBC), and their SRBC-specific IgM responses were analyzed by using an enzyme-linked immunosorbent assay and plaque-forming cell assay. T- and B-cell counts in the mouse spleens were also assessed via surface antigen expression. Mice that received MXC + PARA and PBO + MXC treatment showed marked decreases in SRBC-specific IgM production and T- and B-cell counts compared with those in mice that received vehicle control or the corresponding individual test substance. This suggests that simultaneous exposure to multiple environmental chemicals increases the immunotoxic effects of the chemicals compared to individual exposure.

**Keywords**

Acquired immunotoxicity, combined toxicity, methoxychlor, parathion, piperonyl butoxide

**History**

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## Introduction

Humans are exposed daily to a vast range of products that contain environmental agents (e.g. cosmetics, pesticides, drugs, and biotechnology-derived products) and to multiple environmental chemicals in the atmosphere and in food (Gilbert et al., 2011; Groten et al., 1997; Kortenkamp et al., 2007; Teuschler et al., 2002). Because of this constant exposure, when conducting safety assessments one must take into consideration the effects of combined exposure. For example, combined exposure to pesticides and heavy metals is known to enhance overall toxicity compared with that from exposure to the individual agents (Institoris et al., 1999, 2002). Approaches to assess effects from combined exposures have been described (Feron et al., 1995; Groten et al., 2001; Hernandez et al., 2013; Simmons, 1995). However, most toxicity assessments are conducted based on exposure to individual substances and, as such, mechanisms of effects from combined exposure to environmental chemicals remain unclear. Therefore, the objective of the studies reported here were to investigate the combined toxicologic effects of multiple chemicals.

In the study reported here, the toxic effects from combined exposure to three common environmental chemicals were investigated by examining the impact on immune functions. It is well known that exposure to environmental agents can compromise immunologic function (Fukuyama et al., 2010, 2013; Nishino et al., 2013). For example, several animal studies have shown there are alterations of primary humoral responses induced by immunotoxicants like dioxins and pesticides (Flipo et al., 1992; Smialowicz et al., 1997). To avoid these risks, immunotoxicity tests have been developed for evaluating the safety of environmental chemicals and pharmaceuticals (Holsapple, 2003; Luster et al., 1988). Based on those analyses, guidelines have been introduced over the years to regulate exposure to many agents; these include those published by the US Environmental Protection Agency (EPA, 1998), the Food and Drug Administration (FDA, 2002), the European Medicines Agency (Committee for Proprietary Medicinal Products, 2000), and the International Conference on Harmonization (ICH, 2006).

Our laboratories previously developed a short-term oral exposure method for assessment of the immunosuppressive potential of environmental chemicals (Fukuyama et al., 2013). In the current study, using this method, we demonstrate the combined immunotoxic effects of three well-known environmental chemicals, i.e. methoxychlor (MXC) – an organochlorine compound, parathion (PARA) – an organophosphate compound, and piperonyl butoxide (PBO) – an agricultural insecticide synergist. These three chemicals were selected on the basis of previous studies: MXC exposure causes atrophy of $CD4^+CD8^+$ T-cells in the thymus (Takeuchi et al., 2002a,b); PARA markedly inhibits antigen-specific IgM production (Casale et al., 1984); and PBO depletes T-cells in the spleen and thymus, induces bone

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marrow hypoplasia, and inhibits T-cell proliferation in lymphoid tissues (Diel et al., 1999; Battaglia et al., 2010; Mitsumori et al., 1996). We also previously showed that MXC, PARA, and PBO exposure results in increased thymocyte apoptosis, markedly inhibited sheep red blood cell (SRBC)-specific IgM production, and aggravation of immune disorders such as atopic dermatitis and allergic airway inflammation (Fukuyama et al., 2011; Nishino et al., 2013).

## Materials and methods

### Chemicals

Standard MXC ($C_{16}H_{15}Cl_3O_2$, >97% pure), standard PARA ($C_{10}H_{14}NO_5PS$, 99.5% pure), standard PBO ($C_{19}H_{30}O_5$, >98% pure), and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil was purchased from Hayashi Chemicals (Tokyo, Japan). For the *in vivo* portion of this study, MXC, PARA, or PBO diluted in corn oil to a fixed final volume was orally administered to mice. Based on the EPA Immunotoxicity Guidelines (1998) that states doses should 'not produce significant stress, malnutrition, or fatalities', doses used in this study were < 1/5 the median lethal dose ($LD_{50}$; dose at which ≥50% of animals would be expected to die) and administered concurrently to avoid induction of clear general or immune toxicity (i.e. changes in appearance, posture, behavior, respiration, consciousness, neurologic status, temperature, excretion, etc.) (Fukuyama et al., 2013). The single-chemical dosages used in this study were: MXC, 100 mg/kg day; PARA, 1.0 mg/kg day; and PBO, 100 mg/kg day. Combination dosages were prepared by mixing each chemical so that the final concentration of each chemical was half that of the single dosage. Actually, there were no abnormal signs during the examination period. With regard to body weight measurements, treated groups' values were comparable with those of the vehicle control and intact groups (data not shown). Therefore, we selected relatively high doses compared with actual human exposures. Actual doses and preparation of the test substances are presented in Table 1.

### Animals

Balb/cAnN mice (female, 6-weeks-old) were purchased from Charles River Laboratories (Atsugi, Kanagawa, Japan) and housed individually in cages under controlled lighting (lights on, 07:00–19:00), temperature (22 ± 3 °C), humidity (55% ± 15%), and ventilation (at least 10 complete fresh-air changes/h). Standard rodent chow (Certified Pellet Diet MF; Oriental Yeast Co., Tokyo) and filtered water were available *ad libitum*.

Female mice were selected as the model for this study because the EPA Immunotoxicity Guidelines (EPA, 1998) consider the mouse a model species for use in immunotoxicity studies that examine effects of agricultural chemicals (see Casale et al., 1984; Diel et al., 1999; Battaglia et al., 2010). The guideline indicates that either rats or mice may be used. Additionally, if ADME data are similar between species, then either rats or mice may be used. According to our preliminary immunotoxicity study data for MXC, PARA, and PBO, mice were more sensitive than rats. Therefore, we selected mice for the current study. Furthermore, in immunotoxicity studies, only one gender need be evaluated; in general, females are considered to yield more consistent outcomes than male animals when evaluating humoral immune responses. Consequently, Balb/cAnN mice were selected because our laboratory has historical immunotoxicity study data for our selected chemicals on this strain (data not shown). All aspects of the current study were conducted in accordance with the Animal Care and Use Program of the Institute of Environmental Toxicology, Japan (IET IACUC Approval No. 12027).

### Chemical exposure of mice

After a 1-week acclimatization period, mice (now 7-weeks-old) were allocated randomly to two groups ($n = 8$ mice/group): treatment/vehicle control and to a no treatment (intact group). On Days 1–5, mice were given an oral dose (by gavage, without anaesthesia) of a single or combination test solution (MXC, PARA, PBO, MXC + PARA, PARA + PBO, PBO + MXC) or vehicle only. On Day 2, a solution of SRBC ($6 \times 10^7$ cells/animal; Nippon Bio-Supp. Center, Tokyo) was injected via the tail vein into all test and control mice for immunization. One day after the final oral administration (i.e. on Day 6 of study), all mice were anaesthetized with Isoflurane and blood samples taken from the inferior vena cava. Serum samples were assayed for SRBC-specific serum IgM. After exsanguination from the abdominal aorta, the thymus of each animal was carefully removed and weighed. The spleen was removed and placed in phosphate-buffered saline (PBS, pH 7.4; Life Technologies Co., Ltd., Tokyo). Single-cell splenocyte suspensions in 5 ml modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum (FBS, Life Technologies) were prepared by passage through a stainless-steel screen and sterile 70-μm nylon cell strainer (Falcon, Tokyo). Numbers of lymphocytes in each suspension were determined using a Z2 Coulter Counter (Beckman Coulter, Tokyo).

### Determination of serum SRBC-specific IgM response

Levels of SRBC-specific IgM in the serum were determined using a modified version of the method of Temple et al. (1993). In brief, SRBC-membrane antigen was extracted with Tris-HCl and 0.1% sodium dodecyl sulfate in PBS. The samples were then dialyzed for 2 days against PBS. The protein content of each conjugated sample was determined using the method of Lowry et al. (1951). SRBC-specific IgM levels were then measured by means of enzyme-linked immunosorbent assay (ELISA) in flat-bottomed microplates (Nalge Nunc, Tokyo) whose wells had been coated with SRBC-membrane antigen (2 μg/ml coating buffer; BD Pharmingen, Tokyo) during an overnight incubation at 4 °C. Following washing of each well 5-times with wash buffer (BD Pharmingen) and blocking of potential non-specific binding by incubation for 2 h at room temperature (RT) with assay diluent (BD Pharmingen), a dilution of each mouse serum sample (in assay diluent, from 1:4 to 1:16384) was added to each well and the plates incubated a further 2 h at RT. After gentle rinsing with wash buffer to remove all unbound materials, peroxidase-conjugated anti- mouse IgM (secondary antibody, Rockland Inc., Gilbertsville, PA; dilution 1:15 000) was added to each well and

Table 1. Chemicals and dose settings.

| Test substance | Type | $LD_{50}$ (mg/kg) | Dose (mg/kg day) |
|---|---|---|---|
| Methoxychlor ($C_{16}H_{15}C_{13}O_2$) | Organochlorine compound | 2900 | 100 |
| Parathion ($C_{10}H_4NO_5PS$) | Organophosphate pesticide | 5 | 1 |
| Piperonyl butoxide ($C_{19}H_{30}O_5$) | Insecticide synergist | 2600 | 100 |

the plate incubated for 2 h at RT. The wells were then rinsed again to remove non-adherent anti-mouse IgM. Finally, to quantify the amount of bound antibodies in each well, tetramethylbenzidine (100 µl/well) was added to each well and the plate incubated in the dark at RT for 30 min. Optical density was then measured at 450 nm by in a Spectra MAX 190 microplate reader (Molecular Devices, Osaka).

### Assessment of the splenocyte IgM plaque-forming cell response to SRBC

The IgM plaque-forming cell (PFC) response to SRBC was determined using a modified version of the methods of Cunningham (1965) and Jerne & Nordin (1963). Briefly, $\approx 1 \times 10^6$ cells were incubated with 1% SRBC and a 1:30 dilution of guinea pig complement (Denka Seiken Co., Tokyo) for 10 min at 4 °C. The cells were then applied to a Cunningham chamber (Takahashi Giken Glass Co., Tokyo) and incubated for 1.5 h at 37 °C in a 5% $CO_2$ atmosphere. The number of plaques in each sample was then counted using a stereomicroscope.

### Flow cytometric analysis

Isolated splenocytes were stained all at one time with fluorescein isothiocyanate (FITC)- conjugated anti-peanut agglutinin (Vector Laboratories, Inc., Burlingame, CA) and the monoclonal antibodies (MAb) phycoerythrin-cyanine-7-conjugated anti-mouse CD4 (PE-Cy7, clone RM- 4-5), allophycocyanin-cyanine-7-conjugated anti-mouse CD8 (APC-Cy7, clone 53-6.7), APC-conjugated anti-mouse CD3 (clone 145-2C11), and peridinin chlorophyll protein-Cy5.5-conjugated rat anti-mouse CD19 (PerCP-Cy5, clone 1D3 (all BD Pharmingen). To avoid non-specific binding, $10^6$ cells were incubated with 1 µg Mouse $F_c$ BlockTM (BD Pharmingen) for 5 min at RT, followed by incubation with MAb for 30 min at 4 °C in the dark. Cells were then washed twice with 5% FBS in PBS, re-suspended at $10^6$ cells/tube in 500 µl PBS and then analyzed on a FACSVerse flow cytometer (BD Pharmingen) using FACSuite software. A minimum of 20 000 events/sample was collected and analyzed for antigen expression.

### Statistical analysis

All data are expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to evaluate the results. For significant results, differences between vehicle control and treatment groups were then assessed using a Dunnett's multiple comparison test. Statistical significance of differences between single-chemical and combination-treatment groups was determined using a Student's *t*-test. $p$ values $<0.05$ were considered significant in each test.

## Results

### Overall toxicity of the various treatments to the mice

Throughout the studies, there were no abnormal clinical signs (e.g. decreased activity) or changes in body weight or body weight gain due to any of the treatment regimens.

### Thymus weights

Thymus weights are shown in Figure 1. All treatment groups had significantly ($p<0.01$) decreased values compared with those seen with the vehicle control mice. The MXC + PARA mice did display a significant decrease ($\approx 22.2\%$, $p<0.01$) compared against the values for the PARA treatment mice, and also a decrease of 8.1% versus MXC mice; however, this decrease was not significant. The PBO + MXC mice had average decreases of thymus weight of $\approx 13.0\%$ vs the PBO hosts, but this decrease too was not significant. Thymus weights in the PARA + PBO mice were comparable with those of the PARA or PBO hosts.

### Serum SRBC-specific IgM responses

Serum SRBC-specific IgM responses are shown in Figure 2. In the PARA, PBO, and PARA + PBO treatment groups, SRBC-specific IgM responses were comparable with that of the vehicle control group. However, the MXC + PARA and PBO + MXC treatment groups displayed significantly decreased ($p<0.05$) SRBC-specific IgM responses compared with that by the vehicle controls – decreases of $\approx 40.9\%$ and 29.5%, respectively. Furthermore, the MXC + PARA- treated mice had a significantly decreased ($\approx 41.8\%$) response compared with that of the MXC-only mice. Further, while the MXC + PARA mice displayed a decreasing trend in response compared with that by the PARA-only mice, the net difference (a decrease of $\approx 36.3\%$) was not significant. Similarly, the PBO + MXC mice had a significantly decreased response compared with the PBO $\approx 29.2\%$, $p<0.05$) and MXC (30.6%, $p<0.01$) treatment mice.

### Splenocyte IgM PFC response to SRBC

Splenocyte IgM PFC responses to SRBC are shown in Figure 2. All treatment groups had significantly ($p<0.01$) lower IgM PFC responses to SRBC compared with the vehicle control mice. The MXC + PARA mice had decreases of $\approx 22.7\%$ vs the MXC and 29.9% vs the PARA groups, but the decreases were not significant. However, PBO + MXC treatment did cause a significant decrease ($\approx 38.3\%$, $p<0.01$) from PBO mice values; there

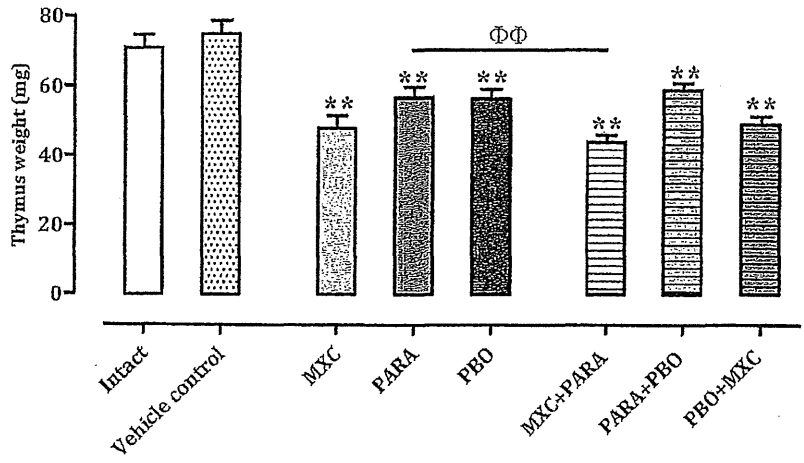


Figure 1. Absolute thymus weights. Mice were treated with nothing (intact naive), vehicle, methoxychlor (MXC), parathion (PARA), piperonyl butoxide (PBO), or combinations of the agents (two at a time). Absolute thymus weights are expressed as mean ± SD (mg; $n = 8$ per group). **$p<0.01$ (Dunnett's multiple comparison test) vs vehicle control group; ΦΦ $p<0.01$ (Student's *t*-test) vs single test substance groups.

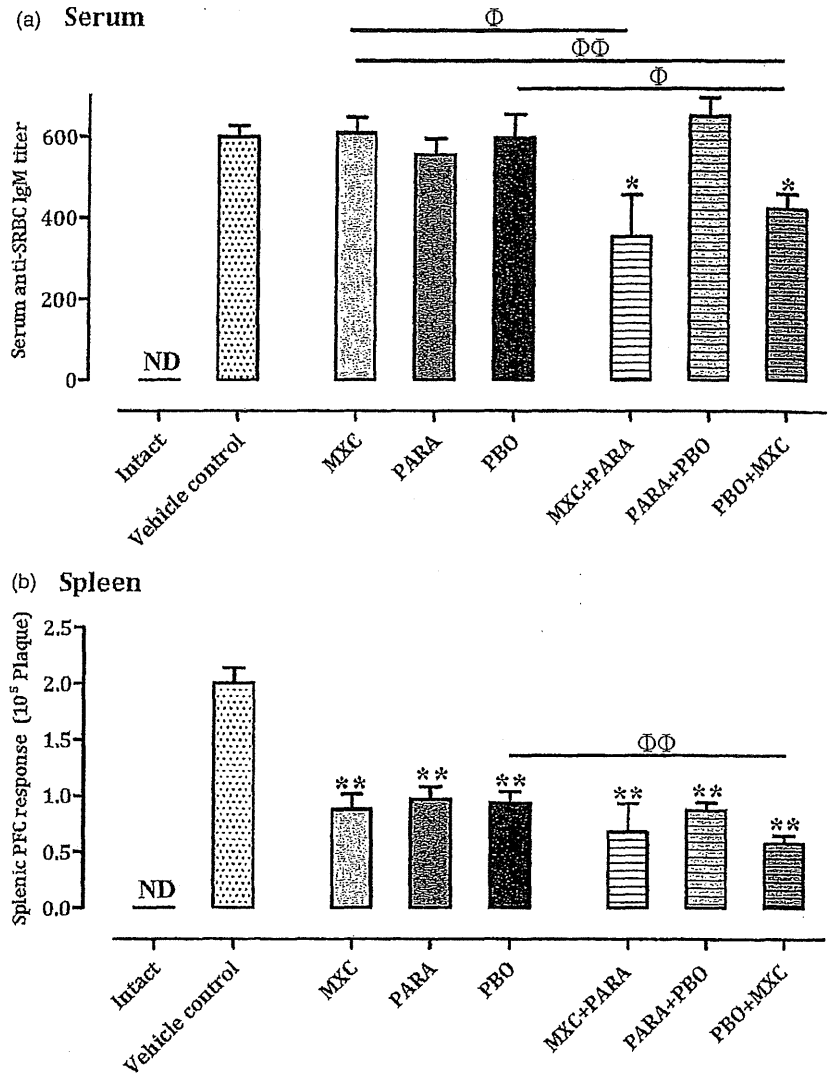


Figure 2. Serum and splenic IgM responses. Mice were treated as described in the Figure 1 legend. (a) Serum and (b) spleen IgM responses are shown. IgM responses are expressed as mean ± SD (titre; $n = 8$ per group). IgM responses in the spleen are expressed as mean ± SD ($n = 8$ per group). $^*p < 0.05$ and $^{**}p < 0.01$ (Dunnett's multiple comparison test) vs vehicle control group; $\Phi p < 0.05$ and $\Phi\Phi p < 0.01$ (Student's *t*-test) vs single test substance groups.

was a decrease of ≈34.1% vs MXC mice values, but this was not significant. Splenocyte PFC responses with PARA + PBO mice were comparable to that seen with their PARA- or PBO-only counterparts.

### Splenocyte T-cell counts

To evaluate the level of T-cell immunosuppression following the single or combination treatments, isolated lymphocytes were stained with anti-CD3, -CD4, and -CD8 antibodies. The numbers of total, helper, and cytotoxic T-cells are shown in Figure 3. In the MXC, PARA, PBO, PARA + PBO, and PBO + MXC treatment groups, all T-cell counts were comparable with those of the vehicle control group. The PBO + MXC group showed a decrease in T-cell counts, but this was not statistically significant. The MXC + PARA group had significantly decreased total ($p < 0.01$), helper ($p < 0.05$), and cytotoxic ($p < 0.01$) T-cell counts compared with those of control, MXC, and PARA mice – decreases of, respectively, ≈52.5, 42.8, and 58.0% vs control; 42.1, 26.1, and 49.9% vs MXC alone; and 33.2, 20.0, and 39.6% vs PARA alone.

### Splenocyte B-cell counts

To evaluate B-cell immunosuppression following the single or combination treatments, isolated lymphocytes were stained with anti-CD19 and anti-peanut agglutinin (PNA) antibodies (Figure 4). In the MXC, PARA, PBO, PARA + PBO, and PBO + MXC treatment groups, total B-cell ($CD19^+$) counts were comparable with that in the vehicle controls. The PBO + MXC group had a decrease in total B-cell counts, but this was not significant. The MXC + PARA group had significantly decreased total B-cell counts ($p < 0.01$) compared with the vehicle control, MXC, and PARA groups – decreases of, respectively, ≈50.9, 36.1, and 33.8%.

In all of the groups given a test substance, germinal center B-cell ($CD19^+PNA^+$) counts were lower than that in the vehicle control mice. In addition, the MXC ($p < 0.05$) PBO ($p < 0.05$), MXC + PARA ($p < 0.05$), and PBO + MXC ($p < 0.01$) groups had significantly decreased germinal center B-cell counts compared with that of the vehicle controls. The PBO + MXC treatment mice also had significantly decreased ($p < 0.05$) germinal center B-cell counts compared with the PBO and MXC treatment group – decreases of ≈42.7 and 49.2%, respectively.

## Discussion

Our objective was to provide new insights into effects of combined exposures to three well-known environmental chemicals: methoxychlor, parathion, and piperonyl butoxide. This study examined immunotoxic effects of these chemicals in Balb/cAnN mice using a short-term oral exposure protocol. Changes in host immune status were assessed by measures of effects on thymus

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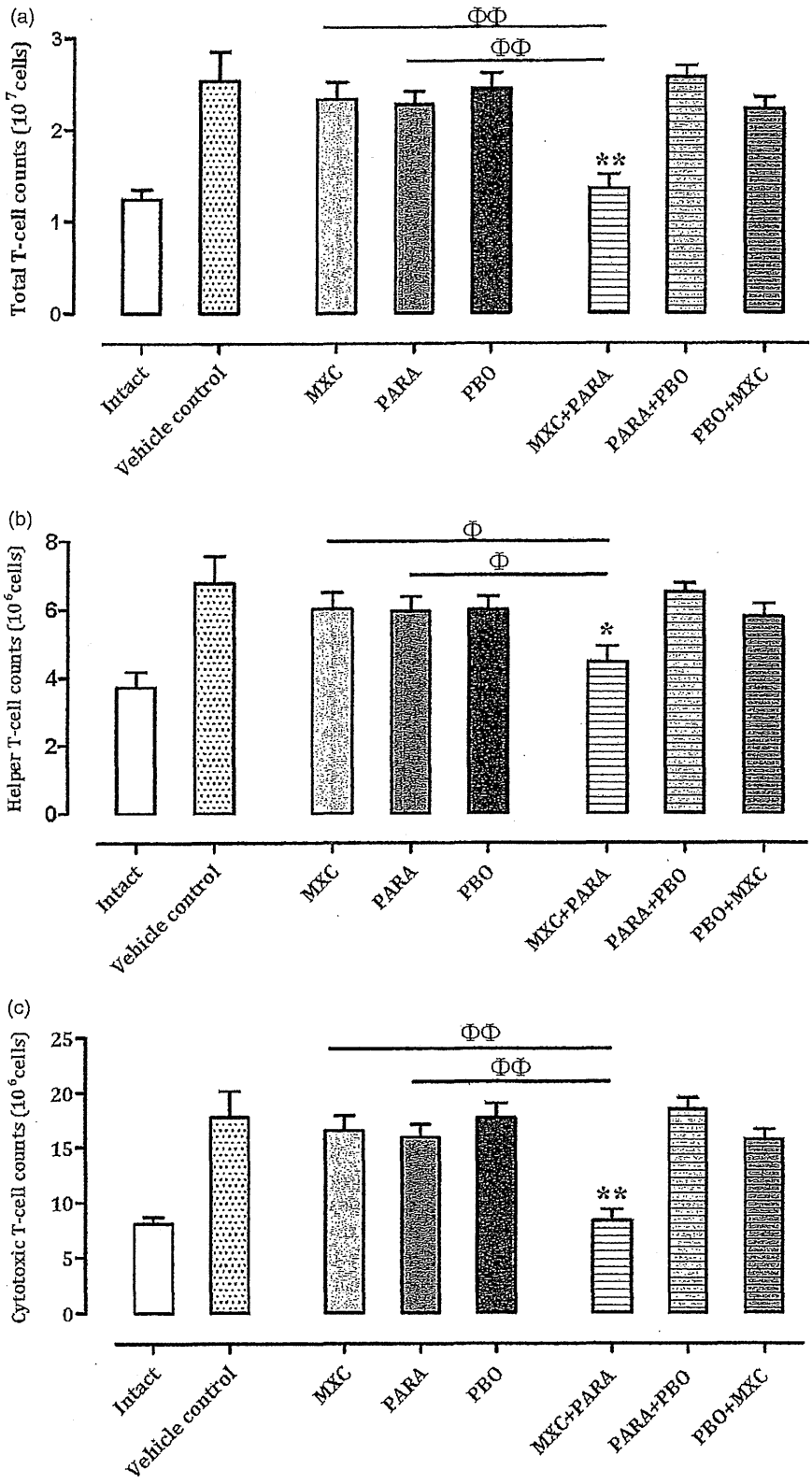


Figure 3. T-cell sub-type counts in spleens. Mice were treated as described in the Figure 1 legend. (a) Total, (b) helper, and (c) cytotoxic T-cell counts are shown. Results for intact, vehicle, and individual agent-treated mice are included in each chart. Cell counts are expressed as mean ± SD ($n = 8$ per group). $*p < 0.05$ and $**p < 0.01$ (Dunnett's multiple comparison test) vs vehicle control group; $\Phi p < 0.05$ and $\Phi\Phi p < 0.01$ (Student's $t$-test) vs single test substance groups.

weight, anti-SRBC IgM responses, and T- and B-cell counts in serum and spleen.

Based on our previous report (Fukuyama et al., 2010), immunosuppressive environmental chemicals induce thymocyte apoptosis and reduced thymus weights. Thus, changes in thymus weights were analyzed as a general measure of *in situ* immunotoxicity from the test agents here as the thymus is a key lymphoid organ, and precursor T-cells migrate there to undergo maturation (Janeway et al., 2004). In our study, compared to what was seen with vehicle control mice, all treatments induced significant decreases in thymus weight. Among the combined exposure groups, the MXC + PARA mice had values significantly decreased compared with those of PARA-only mice (but not vs MXC mice). In contrast, while PBO + MXC mice had a trend toward decreasing values vs the same PBO hosts, the decrease was not significant. PARA + PBO mice had values not altered