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GENOTOXICITY AND CARCINOGENICITY

Flumequine enhances the in vivo mutagenicity of MeIQx in the mouse liver

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Abstract The combined effects of various carcinogens found in food products are a concern for human health. In the present study, the effects of flumequine (FL) on the in vivo mutagenicity of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) in the liver were investigated. Additionally, we attempted to clarify the underlying mechanisms through comprehensive gene analysis using a cDNA microarray. Male *gpt* delta mice were fed a diet of 0.03 % MeIQx, 0.4 % FL, or 0.03 % MeIQx + 0.4 % FL for 13 weeks. The effects of cotreatment with phenobarbital (PB) were also examined. Treatment with MeIQx alone increased *gpt* and Spi$^-$ mutant frequencies, and cotreatment with FL, but not with PB, further exacerbated these effects, despite the lack of in vivo genotoxicity in mice treated with FL alone. FL caused an increase in *Cyp1a2* mRNA levels and a decrease in *Ugt1b1* mRNA levels, suggesting that the enhancing effects of FL may be due in part to modification of MeIQx metabolism by FL. Moreover, FL induced an increase in hepatocyte proliferation accompanied by hepatocellular injury. Increases in the mRNA levels of genes encoding cytokines derived from Kupffer cells, such as *Il1b* and *Tnf*, and cell cycle-related genes, such as *Ccnd1* and *Ccne1*, suggested that FL treatment increases compensatory cell proliferation. Thus, the present study clearly demonstrated the combined effects of 2 different types of carcinogens known as contaminants in foods.

Keywords MeIQx · Flumequine · In vivo mutagenicity · *gpt* delta mouse · Combined effects

Introduction

A variety of carcinogens are produced in foods or have been found to accidentally contaminate foods. Many studies have reported the toxicities of individual carcinogens. Although some carcinogens are known to cause either synergistic or antagonistic effects (Takayama et al. 1989; Hasegawa et al. 1991), the effects of many combinations of carcinogens have not yet been determined. 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) is a heterocyclic amine produced in cooked meat and has been shown to induce tumors in the liver, Zymbal glands, clitoral glands, and skin in rats and in the liver, lungs, hematopoietic system, and colon in mice (Ohgaki et al. 1987; Sugimura et al. 2004; Nishikawa et al. 2005). The in vitro genotoxicity of MeIQx has been demonstrated, and MeIQx has also been shown to exhibit in vivo mutagenicity in the livers of transgenic mice (Itoh et al. 2000; Masumura et al. 2003). Recently, we reported that the in vivo mutagenicity of MeIQx in the liver was enhanced by cotreatment with carbon tetrachloride (CCl_4) in *gpt* delta mice (Okamura et al. 2010). These data suggested that the genotoxicity of carcinogens could be enhanced by injury or inflammation in the target organs.

Flumequine (FL) is a fluoroquinolone compound with antimicrobial activity against gram-negative organisms and has been used in the treatment of enteric infections in domestic animals and livestock (Greenwood 1998); FL is

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thought to persist in food products made from such livestock (Choma et al. 1999). FL induces hepatotoxicity, characterized by hepatocyte vacuolation and inflammatory cell infiltration, in both rats and mice and induces liver tumors in mice (JECFA 1998; Pronk 2004). Based on the negative results of genotoxicity tests, JECFA concluded that FL is a nongenotoxic hepatocarcinogen and that hepatocellular necrosis-regeneration cycles caused by hepatotoxicity are mechanistically relevant to its induction of liver tumors in mice (JECFA 2004). FL and MeIQx, both of which possibly exist in foods, could be ingested by humans on a long-term basis. Moreover, given that FL exhibits hepatotoxicity similar to CCl_4, FL indeed may exert additional effects on the genotoxicity of MeIQx.

Therefore, in the present study, we investigated the effects of FL on the in vivo mutagenicity of MeIQx in the mouse liver. *gpt* delta mice were given MeIQx and FL for 13 weeks. To clarify the relationship between changes in reporter gene mutations and molecular mechanisms, mouse livers were used for histopathological examination, bromodeoxyuridine (BrdU) immunostaining, and analysis of reporter gene mutations (*gpt* and Spi^- assays) as well as comprehensive gene expression analysis by cDNA microarray. In addition, cotreatment with phenobarbital (PB), a nonhepatotoxic tumor promoter for hepatocarcinogenesis, was examined as a comparative control.

Materials and methods

Chemicals

FL, a white crystallized powder (purity: 99.3 %), was kindly provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). MeIQx and PB were purchased from Toronto Research Chemicals (North York, Canada) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. BrdU was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and treatments

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Male $B6C3F_1$ *gpt* delta mice carrying 80 tandem copies of the transgene lambda EG10 in a haploid genome were raised by mating C57BL/6 *gpt* delta and nontransgenic C3H/He mice (Japan SLC, Inc., Shizuoka, Japan). Thirty male $B6C3F_1$ *gpt* delta mice were randomized by weight into 6 groups. Animals were housed in a room with a barrier system and maintained under the following constant conditions: temperature of 24 ± 1 °C, relative humidity of 55 ± 5 %, ventilation frequency of 18 times/h, and a 12-h light/dark cycle. The animals were housed in plastic cages (5 mice/cage) on soft chips (Sankyo Labo-Service, Tokyo, Japan). Throughout the experimental period, chips were renewed every 3 or 4 days, and mice were provided tap water ad libitum.

Starting at 6 weeks of age, *gpt* delta transgenic mice were fed a diet containing 0.03 % MeIQx, 0.4 % FL, 0.05 % PB, 0.03 % MeIQx and 0.4 % FL, or 0.03 % MeIQx and 0.05 % PB for 13 weeks. Animals in the control group received the basal diet only. General signs were observed daily, and body weight and food consumption per cage were measured once a week. After 13 weeks, all mice were killed, and a part of the left lateral lobe of the liver was stored at −80 °C for in vivo mutation assays and cDNA microarray analysis. At autopsy, the body and liver weights were measured.

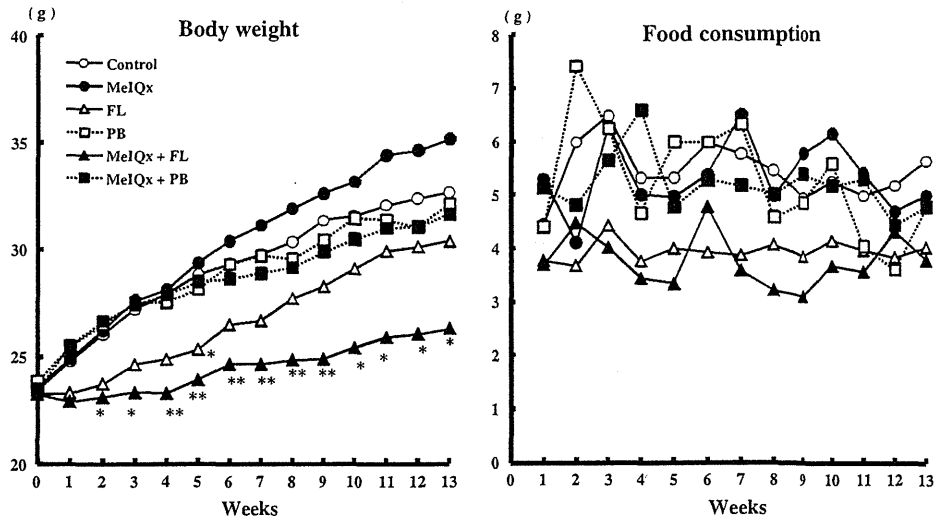


Fig. 1 Body weight and food consumption for $B6C3F_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQX + PB for 13 weeks. *, **Significantly different from control group at $p < 0.05$ and 0.01, respectively

Table 1 Final body and liver weights in B6C3F$_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQx + PB for 13 weeks

Group	Final body weight (g)	Liver weight	
		Absolute (g)	Relative (g/100 g BW)
Control	32.7 ± 3.9[a]	1.40 ± 0.15	4.29 ± 0.23
MeIQx	35.4 ± 4.4	1.63 ± 0.21	4.63 ± 0.40
FL	31.1 ± 1.7	1.60 ± 0.15	5.13 ± 0.23**
PB	32.4 ± 2.9	1.57 ± 0.07	4.88 ± 0.30
MeIQx + FL	26.4 ± 2.1*	1.33 ± 0.10	5.02 ± 0.15*
MeIQx + PB	31.7 ± 2.2	1.64 ± 0.16	5.20 ± 0.47**

*, ** Significantly different from control group at $p < 0.05$, 0.01, respectively

[a] Mean ± SD

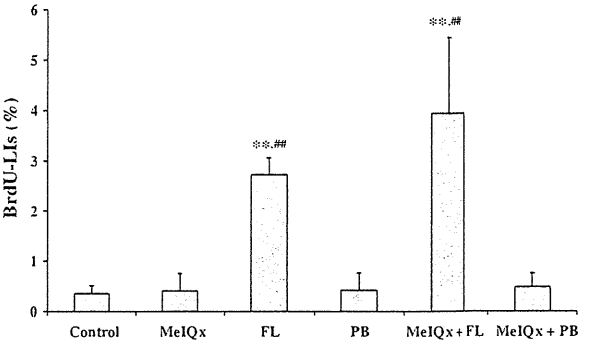


Fig. 3 BrdU-LIs for hepatocytes from B6C3F$_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQx + PB for 13 weeks. Values are the mean ± SD of data for 5 mice. **,## Significantly different from control and MeIQx groups, respectively, at $p < 0.01$

Quantification of hepatocyte proliferation

In order to examine the proliferative activity of hepatocytes, all animals were given BrdU (100 mg/kg) by intraperitoneal (i.p.) injection once a day for the final 2 days before killing and once on the day of killing at 2 h before being euthanatized at autopsy. For immunohistochemical staining of BrdU, after activation of the antigen by autoclaving, tissue sections were treated with rat anti-BrdU (AbD Serotec Inc., NC, USA) diluted by Dako Antibody Diluent (Dako, Glostrup, Denmark), followed by incubation with a high polymer stain (HISTOFINE Simple Stain, NICHIREI, Japan). At least 2,000 hepatocytes in each liver were counted, and labeling indices (LIs) were calculated as the percentage of cells positive for BrdU incorporation.

In vivo mutation assays

The 6-TG and Spi$^-$ (insensitive P2 interference) selections were carried out as previously described (Nohmi et al. 1996, 2000). Briefly, genomic DNA was extracted from the liver tissue, and lambda EG10 DNA (48 kb) was rescued as the lambda phage through in vitro packaging. For 6-TG selection, the packaged phage was incubated with *Escheichia coli* YG6020, expressing Cre recombinase, and converted to a plasmid carrying *gpt* and chloramphenicol

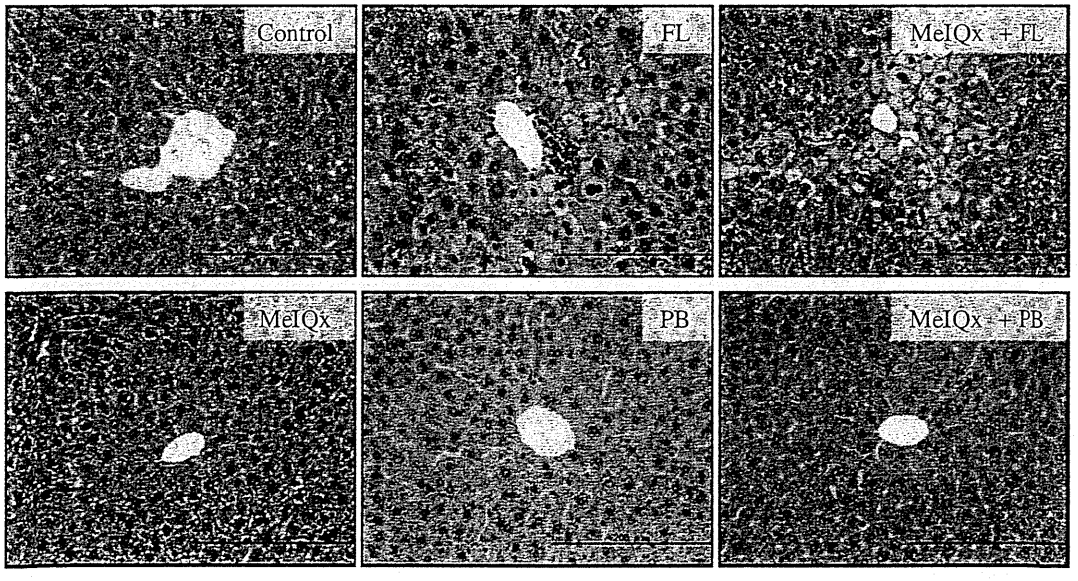


Fig. 2 Histopathological features in the livers of B6C3F$_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQx + PB for 13 weeks. Note that no obvious alterations (MeIQx group), centrilobular hypertrophy of hepatocytes with vacuolation (FL and MeIQx + FL groups), or centrilobular hypertrophy of hepatocytes (PB and MeIQx + PB groups) are evident. *Bar* represents 200 μm

Table 2 *gpt* mutant frequencies (MFs) in the livers of B6C3F$_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQx + PB for 13 weeks

Group	Animal no.	Cm^R colonies ($\cdot 10^5$)	6-TG^R and Cm^R colonies	Mutant frequency ($\cdot 10^{-5}$)	Mean ± SD
Control	1	26.7	4	0.15	0.20 ± 0.12
	2	12.5	3	0.24	
	3	26.7	2	0.07	
	4	1.7	0	0.00[b]	
	5	26.2	9	0.34	
MeIQx[a]	6	0.7	3	4.33	5.47 ± 2.03
	7	3.2	18	5.56	
	8	3.5	10	2.85	
	9	2.3	19	8.18	
	10	3.1	20	6.44	
FL	11	25.6	7	0.27	0.29 ± 0.15
	12	20.2	8	0.40	
	13	20.4	10	0.49	
	14	22.4	4	0.18	
	15	7.9	1	0.13	
PB	16	26.6	4	0.15	0.24 ± 0.12
	17	17.2	7	0.41	
	18	23.9	7	0.29	
	19	18.5	2	0.11	
	20	22.4	5	0.22	
MeIQx + FL[a]	21	3.0	21	7.11	11.92 ± 6.26**,#
	22	3.4	37	10.93	
	23	5.1	28	5.51	
	24	1.9	29	15.06	
	25	1.0	20	20.96	
MeIQx + PB[a]	26	5.7	5	0.88	2.57 ± 2.60
	27	3.2	7	2.17	
	28	5.3	7	1.32	
	29	2.3	3	1.33	
	30	1.3	9	7.14	

** Significantly different from control group at $p < 0.01$

Significantly different from the MeIQx group at $p < 0.05$

[a] For mice treated with MeIQx alone and for cotreatment groups, *gpt* mutants were analyzed only on one selected plate whose colony numbers were closest to the average number of 5 plates, and MFs were calculated using the packaging efficiency values (Cm^R colonies) divided by 5

[b] Data of animal No. 4 were excluded for the calculation of the MF because of the poor packaging efficiency of the transgene

acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. In order to determine the total number of rescued plasmids, infected cells were also poured on plates containing chloramphenicol without 6-TG. The plates were incubated at 37 °C for the selection of 6-TG-resistant colonies, and the *gpt* mutant frequency (MF) was calculated by dividing the number of *gpt* mutants after clonal correction by the number of rescued phages. To characterize *gpt* mutations, a 739-bp DNA fragment containing the 456-bp coding region of the *gpt* gene was amplified by PCR as previously described, and the PCR products were analyzed with an Applied Biosystems 3,730 × 1 DNA Analyzer (Applied Biosystems Japan Ltd.). For the group of mice treated with MeIQx alone and for cotreatment groups, *gpt* mutants were analyzed only on one selected plate based on the assumption that the colony number on the selected plate was closest to the average number of 5 plates, and MFs were calculated by the packaging efficiency value (Cm^R colonies) divided by 5.

For Spi^- selection, the packaged phage was incubated with *E. coli* XL-1 Blue MRA for survival titration and *E. coli* XL-1 Blue MRA P2 for mutant selection. Infected cells were mixed with molten lambda-trypticase agar plates. The next day, plaques (Spi^- candidates) were punched out with sterilized glass pipettes, and the resulting agar plugs were suspended in SM buffer. In order to confirm the Spi^- phenotype of candidates, the suspensions were spotted on 3 types of plates containing XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains and were spread with soft agar. The numbers of mutants that made clear plaques on each plate were counted as confirmed Spi^- mutants. The Spi^- MF was calculated by dividing the number of Spi^- mutants by the number of rescued phages. For the group of mice treated with MeIQx alone and for the cotreatment groups, confirmation of Spi^- phenotype candidates

Table 3 Mutation spectra of *gpt* mutant colonies in the livers of B6C3F$_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQx + PB for 13 weeks

Mutation	Control		MeIQx		FL		PB		MeIQx + FL		MeIQx + PB	
	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)
Transversions												
GC-TA	4[a] (22.2)	0.03 ± 0.05[b]	36 (51.4)	2.85 ± 0.77	6 (20.0)	0.05 ± 0.05	6 (24.0)	0.05 ± 0.06	74 (54.8)	6.90 ± 4.36**,#	16 (51.6)	1.21 ± 1.15
GC-CG	0	0	2 (2.9)	0.15 ± 0.21	1 (3.3)	0.01 ± 0.02	1 (4.0)	0.01 ± 0.02	2 (1.5)	0.14 ± 0.23	1 (3.2)	0.04 ± 0.08
AT-TA	1 (5.6)	0.01 ± 0.02	3 (4.3)	0.19 ± 0.28	0	0	0	0	5 (3.7)	0.48 ± 0.34*	0	0
AT-CG	0	0	1 (1.4)	0.06 ± 0.14	2 (6.7)	0.02 ± 0.03	0	0	0	0	0	0
Transisions												
GC-AT	9 (50.0)	0.08 ± 0.05	2 (2.9)	0.35 ± 0.63	9 (30.0)	0.10 ± 0.06	13 (52.0)	0.13 ± 0.11	11 (8.1)	0.77 ± 0.83	7 (22.6)	0.67 ± 0.98
AT-GC	0	0	0	0	3 (10.0)	0.03 ± 0.03	3 (12.0)	0.03 ± 0.03	0	0	0	0
Deletion												
Single bp	3 (16.7)	0.04 ± 0.07	22 (31.4)	1.55 ± 1.24	6 (20.0)	0.06 ± 0.06	2 (8.0)	0.02 ± 0.03	37 (27.4)	3.14 ± 1.36**,#	5 (16.1)	0.50 ± 0.63
Over 2 bp	0	0	1 (1.4)	0.09 ± 0.19	2 (6.7)	0.02 ± 0.03	0	0	1 (0.7)	0.21 ± 0.47	0	0
Insertion	0	0	2 (2.9)	0.15 ± 0.21	1 (3.3)	0.01 ± 0.02	0	0	2 (1.5)	0.12 ± 0.26	1 (3.2)	0.06 ± 0.14
Complex	1 (5.6)	0.01 ± 0.02	1 (1.4)	0.09 ± 0.19	0	0	0	0	3 (2.2)	0.16 ± 0.26	1 (3.2)	0.09 ± 0.20

*, ** Significantly different from control group at $p < 0.05$ and 0.01, respectively

Significantly different from the MeIQx group at $p < 0.05$

[a] Number of colonies with independent mutations

[b] Mean ± SD

Table 4 Spi^- mutant frequencies (MFs) in the livers of $B6C3F_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQx + PB for 13 weeks

Group	Animal no.	Plaques within XL-1 Blue MRA ($\cdot 10^5$)	Plaques within WL95 (P2)	Mutant frequency ($\cdot 10^{-5}$)	Mean ± SD
Control	1	34.6	8	0.23	0.15 ± 0.07
	2	14.9	1	0.07	
	3	49.4	8	0.16	
	4	6.0	2	0.33	
	5	21.1	3	0.14	
MeIQx[a]	6	3.2	4	1.27	3.53 ± 1.97*
	7	5.7	12	2.12	
	8	7.3	24	3.28	
	9	9.6	58	6.07	
	10	12.0	59	4.91	
FL	11	39.8	6	0.15	0.17 ± 0.03
	12	25.6	5	0.20	
	13	31.3	5	0.16	
	14	41.4	6	0.14	
	15	20.2	4	0.20	
PB	16	46.6	7	0.15	0.20 ± 0.09
	17	45.7	8	0.17	
	18	30.4	4	0.13	
	19	17.3	6	0.35	
	20	32.9	7	0.21	
MeIQx + FL[a]	21	4.9	27	5.48	7.66 ± 3.02**,#
	22	5.5	30	5.49	
	23	8.0	43	5.40	
	24	6.5	70	10.80	
	25	4.0	44	11.11	
MeIQx + PB[a]	26	7.2	3	0.42	1.72 ± 1.74
	27	5.7	4	0.71	
	28	8.3	7	0.84	
	29	6.0	12	1.99	
	30	3.0	14	4.64	

*, ** Significantly different from the control group at $p < 0.05$ and 0.01, respectively

Significantly different from the MeIQx group at $p < 0.05$

[a] For mice treated with MeIQx alone and for cotreatment groups, confirmation of Spi^- phenotype candidates was performed only on the first of 2 plates, and MFs were calculated using the packaging efficiency values (plaques within XL-1 Blue MRA) divided by 2

was performed only on the first of 2 plates, and MFs were calculated using the packaging efficiency value (plaques within XL-1 Blue MRA) divided by 2.

RNA isolation

The livers from all animals were soaked overnight in RNAlater-ICE (Applied Biosystems/Ambion, Austin, TX) at −20 °C, and total RNA was then isolated using RNeasy Mini Kits (Qiagen GmbH, Hilden, Germany). The concentration and quality of total RNA were analyzed using a UV–VIS spectrophotometer (Nanodrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

cDNA microarray

In 3 mice from the control, MeIQx, and MeIQx + FL groups, purified total RNA was labeled with cyanine-3 dye using a Quick Amp Labeling Kit (Agilent Technologies). RNA concentration, dye incorporation, and quality were analyzed using a UV–VIS spectrophotometer and an Agilent 2100 Bioanalyzer. Fluorescently labeled cRNA was hybridized to Agilent 4 × 44 K whole mouse genome microarray gene expression chips following the manufacturer's protocol (Agilent Technologies). Hybridized microarray chips were then scanned using an Agilent Microarray Scanner (Model G2565BA, Agilent Technologies). Feature Extraction software (Agilent Technologies) was employed for imaging analysis and data extraction processes. Using

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

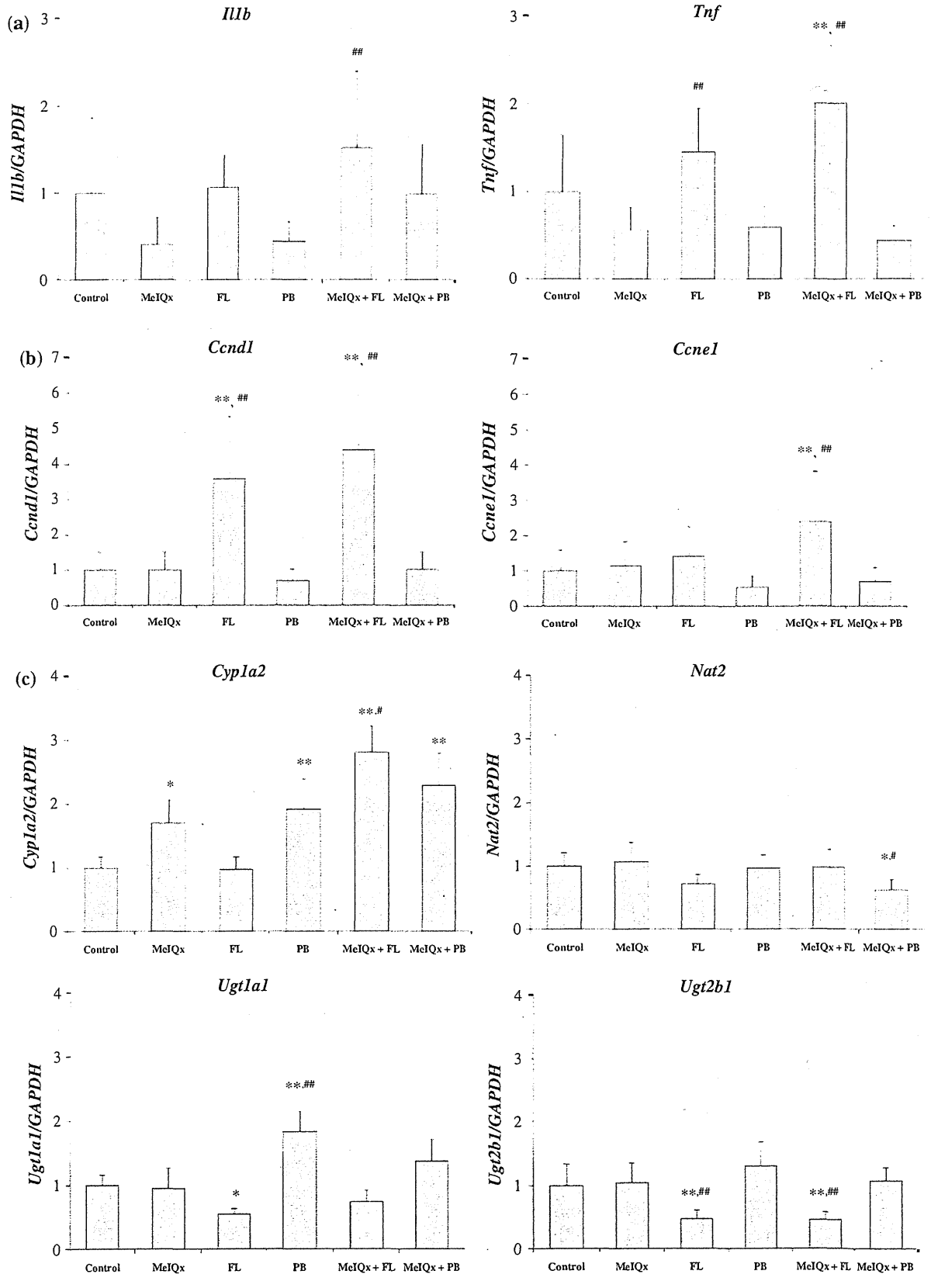
the analysis software GeneSpring (Agilent Technologies), normalization of gene expression data and filtering probe sets by expression levels, flags, and errors were performed. Differences in gene expression between the MeIQx group and the MeIQx + FL group were analyzed by analysis of variance (*t* test; 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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Role of regulatory T cells in the induction of atopic dermatitis by immunosuppressive chemicals

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HIGHLIGHTS

- Prior exposure to immunosuppressive chemicals can aggravate atopic dermatitis.
- Regulatory T cells may be related to the induction of atopic dermatitis.

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ABSTRACT

Immunosuppressive environmental chemicals may exacerbate allergic diseases, including atopic dermatitis (AD). We examined the effects of the immunosuppressive environmental chemicals methoxychlor, parathion, piperonyl butoxide, dexamethasone, and cyclophosphamide on picryl-chloride-induced AD in NC/Nga mice. Mice were orally exposed (age, 5 weeks) to these chemicals; during their sensitization and challenge (age, 8–12 weeks) with picryl chloride, we measured ear thickness and scored skin dryness, erythema, edema, and wounding. After the challenge, we analyzed dermatitis severity and cytokine gene expression in the pinna, serum levels of IgE and IgG2a, T- and B-cell numbers and cytokine production in auricular lymph nodes, and counted splenic regulatory T cells. Exposure to environmental immunosuppressive chemicals markedly increased dermatitis severity and gene expression in the pinna; serum IgE and IgG2a levels; and numbers of helper T cells and IgE-positive B cells, production of Th1 and Th2 cytokines, and production of IgE in auricular lymph-node cells and markedly decreased the numbers of splenic regulatory T cells. Prior exposure to immunosuppressive environmental chemicals aggravates AD; a decrease in the numbers of regulatory T cells may influence this process.

1. Introduction

Current evidence suggests that environmental chemicals increase the potency of allergens and thereby play a role in the development of allergic diseases (Casillas et al., 1999; Peat and Li, 1999; Yanagisawa et al., 2008). Recently, we demonstrated that prior oral exposure to immunosuppressive environmental chemicals aggravates T-cell-mediated allergic reactions as measured by the local lymph node assay, a thymidine-uptake test used to screen chemicals for their potential to cause dermal hypersensitivity (Fukuyama et al., 2010a,b). Although the local lymph node assay is efficient and informative, it is not a good predictor of potential changes in the host's organ-specific functionality. Therefore, more detailed evaluations are needed to clarify the role of immunosuppressive environmental chemicals in the aggravation of allergic reactions. In the current study, we used a mouse model of atopic dermatitis (AD) to explore the mechanisms involved in the aggravation of allergic responses after oral exposure to several environmental chemicals.

AD is characterized by chronic and relapsing inflammatory dermatitis, immunologic disturbances, and pruritic and eczematous skin lesions (Jang et al., 2011; Tanaka and Matsuda, 2011). In recent years, AD has become one of the most common skin diseases: 10–20% of children worldwide are affected, and its incidence is increasing in industrial countries (Leung, 2000). The complex mechanisms of AD include increased numbers of activated circulating CD4+ and CD8+ T cells and marked infiltration of CD4+ T cells into the dermis. In particular, allergen-specific Th2-type T cells bearing the cutaneous lymphocyte antigen are recruited to the skin. The initial phase of AD is dominated by Th2-type T cells that produce IL-4, IL-5, and IL-13. During the subsequent chronic phase, the number of Th1 cells that produce IFN-γ increases (Anthoni et al., 2007; Tanaka and Matsuda, 2011).

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NC/Nga mice are the most extensively studied animal model of AD (Jang et al., 2011; Matsuda et al., 1997). These mice spontaneously develop AD-like eczematous skin lesions when kept in conventional housing but not when maintained under SPF conditions (Shiohara et al., 2004). However, even NC/Nga mice housed under SPF conditions develop AD-like skin lesions after repeated treatment with hapten (Mosmann and Coffman, 1989; Sampson and Albergo, 1984). In the current study, we used picryl chloride (1-chloro-2,4,6-trinitrobenzene), which causes overt dermatitis in 100% of NC/Nga mice (Choi et al., 2012; Shiohara et al., 2004). To explore the mechanisms of AD development in this model, we analyzed dermatitis severity and expression of inflammation-associated genes in the pinna, IgE and IgG_{2a} levels in serum, and T- and B-cell surface-antigen expression and local cytokine production in auricular lymph nodes. In addition, we hypothesized the relation between immunosuppressive environmental chemicals and autoreactive T or B cells, leading to abnormal hypersensitivity. Therefore, we measured the surface antigen expression of splenic regulatory T (Treg) cells.

2. Materials and methods

2.1. Reagents

Methoxychlor standard ($C_{16}H_{15}Cl_3O_2$, >97% pure), parathion standard ($C_{10}H_{14}NO_5PS$, 99.5% pure), piperonyl butoxide ($C_{19}H_{30}O_5$, >98% pure) standard, dexamethasone ($C_{22}H_{29}FO_5$, 98–102% pure), 0.5% methylcellulose solution, and olive oil were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cyclophosphamide monohydrate ($C_7H_{15}Cl_2N_2O_2P{\cdot}H_2O$, 100.6% pure) was purchased from Sigma–Aldrich Japan (Tokyo, Japan). Picryl chloride (wetted with ca. 15% water; $C_6H_2ClN_3O_6$; 100.2% pure) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Corn oil was purchased from Hayashi Chemicals (Tokyo, Japan). Methoxychlor, parathion, and piperonyl butoxide were diluted in corn oil and dexamethasone and cyclophosphamide were diluted in 0.5% methylcellulose solution. After recrystallization with ethanol, picryl chloride was dissolved in acetone–olive oil (v/v, 4:1) solution to 0.5% or 1%.

All antibodies for flow cytometry were purchased from BD Pharmingen (Tokyo, Japan).

2.2. Animals

Female NC/NgaTnd mice (age, 3 weeks) were purchased from Charles River Japan Laboratories (Atsugi, Kanagawa, Japan) and housed individually under controlled lighting (lights on, 07:00 to 19:00 h), temperature (22 ± 3 °C), humidity (50 ± 20%), and ventilation (at least 10 complete fresh-air changes hourly). Food (Certified Pellet Diet MF, Oriental Yeast, Tokyo, Japan) and water were available ad libitum. The current study was conducted in accordance with the Code of Ethics for Animal Experimentation of the Institute of Environmental Toxicology.

2.3. Experimental protocol

After a 1-week acclimation period, NC/Nga mice (age, 4 weeks) were allocated into 5 or 6 groups (n = 8 mice per group) for each chemical (methoxychlor, parathion, piperonyl butoxide, dexamethasone, and cyclophosphamide): intact group (no treatment), vehicle-only control group (oral administration with corn oil or 0.5% methylcellulose solution, followed by sensitization and challenge with picryl chloride), low- and high-dose groups (oral administration of chemical, followed by sensitization and challenge with picryl chloride), and Treg analysis group (oral administration with high-dose chemical only). Chemical doses were: methoxychlor, 30 and 300 mg kg^{-1} day^{-1}; parathion, 0.15 and 1.5 mg kg^{-1} day^{-1}; piperonyl butoxide, 30 and 300 mg kg^{-1} day^{-1}; dexamethasone, 1 mg kg^{-1} day^{-1}; and cyclophosphamide, 10 mg kg^{-1} day^{-1}. Based on the EPA Immunotoxicity Guidelines established in 1998, the highest dose level used in a host should "not produce significant stress, malnutrition, or fatalities". Accordingly, in this study, the maximum doses used were selected to be <1/3 of the LD50 (dose at which ≥50% of animals would be expected to die) and concurrently to avoid the induction of clear systemic toxicity (i.e., changes in appearance, posture, behavior, respiration, consciousness, neurologic status, body temperature, excretion, etc.). On each of days 1–5, mice were given an oral dose of the test solution (methoxychlor, parathion, piperonyl butoxide, dexamethasone or cyclophosphamide) or vehicle. Four weeks after the last oral administration (day 29), 100 μL of 1% picryl chloride was applied to each mouse's abdomen, which had been clipped free of fur 24 h previously, for sensitization. For challenge, a 25-μL aliquot of 0.5% picryl chloride was applied to the dorsum of each ear of each mouse on days 29, 32, 36, 39, 43, 46, 50, 53, 56, and 59. On days 30, 37, 44, 51, and 58, we measured ear thickness by using a gauge and determined clinical scores for skin dryness, erythema, edema, and wounding. Scores were assigned according to the following system: 0, no symptoms; 1, mild; 2, moderate; and 3, severe (Takano et al., 2006). On day 60 (the day after the last challenge), all mice were anesthetized and then euthanized by pentobarbital injection. Blood was collected from the inferior vena cava and serum samples assayed for substance P and total IgE and IgG_{2a} levels. The right pinna was removed from each mouse, pooled, and stored in RNAlater (Applied Biosystems, Tokyo, Japan) until used for RNA analysis. Auricular lymph nodes (LN) and spleens were removed, and pooled by tissue type in RPMI 1640 (Gibco, Tokyo, Japan). Single-cell suspensions from LNs and spleens were prepared by passage of the tissues through sterile 70-μm nylon cell strainers into 1 mL or 10 mL RPMI 1640 supplemented with 5% fetal calf serum (FCS, Gibco), respectively. The cell counts of the resulting suspensions were determined on an automated cell counter (model Z2, Beckman Coulter, Tokyo, Japan).

2.4. Enzyme immunoassay for serum substance P

Serum levels of substance P were measured by using an enzyme immunoassay (Substance P EIA Kit, Cosmo Bio, Tokyo, Japan) according to the manufacturer's protocol. The optical density at 405 nm was read by using a microplate reader (SpectraMax 190, Molecular Devices, Tokyo, Japan).

2.5. Enzyme-linked immunosorbent assay for total serum immunoglobulin

Total IgE and IgG_{2a} levels in serum were measured by using enzyme-linked immunosorbent assays (OptEIA Mouse Kit, BD Pharmingen, San Diego, CA, USA) in accordance with the manufacturer's protocol. The optical density at 405 nm was read by using a microplate reader.

2.6. IgE production by B cells in auricular LNs

B cells were isolated from auricular LNs by a magnetic cell-sorting system (autoMACS Separator, Miltenyi Biotec, Tokyo, Japan) and B220 microbeads (Miltenyi Biotec). To stimulate IgE production, we cultured B cells (1×10^6 cells/well) for 8 days with anti-CD40 ligand antibodies (150 ng/mL; R&D Systems, Tokyo, Japan) and recombinant IL-4 (150 ng/mL; R&D Systems) in 24-well plates at 37 °C in 5% CO_2. Total IgE concentrations in supernatants were measured by using enzyme-linked immunosorbent assays (BD Pharmingen).

2.7. Flow cytometry of auricular LNs

Auricular LNs were stained with fluorescein-isothiocyanate (FITC)-conjugated rat anti-mouse IgE (clone R35-72), FITC-conjugated hamster anti-mouse CD3 (clone 145-2C11), phycoerythrin (PE)–Cy5-conjugated rat anti-mouse CD45R/B220 (clone RA3-6B2), and phycoerythrin–Cy5-conjugated rat anti-mouse CD4 (clone RM4-5; all from BD Pharmingen). To avoid nonspecific binding, 1×10^6 cells were incubated with 20% normal goat serum for 10 min at 4 °C, followed by incubation with FITC- and PE–Cy5-conjugated monoclonal antibodies for 30 min at 4 °C in the dark. Cells were washed twice with 5% fetal calf serum in PBS, resuspended at 1×10^6 cells per tube in 1 mL PBS, and then analyzed on a FACSCaliber flow cytometer (BD Pharmingen) using Cell Quest software (BD Pharmingen). For each sample, 20,000 events were collected and analyzed for expression of antigens.

2.8. Cytokine production from T cells in auricular LNs

To stimulate T-cell receptor signaling, we cultured single-cell suspensions obtained from LNs (1×10^6 cells/well) with either anti-CD3 (2 μg/mL; BD Pharmingen) or anti-CD28 (2 μg/mL; BD Pharmingen) or both antibodies for 24 h or 96 h in 24-well plates at 37 °C in 5% CO_2. The concentrations of IL-4, IL-5, IL-6, IL-13, IL-17A, and interferon gamma (IFN-γ) in the supernatants were assayed by using the BD Cytometric Bead Array (BD Pharmingen) in accordance with the manufacturer's protocol.

IFN-γ in the supernatants was quantified after culture for 24 h in the presence of anti-CD3. IL-6 levels in the supernatants were quantified after culture for 24 h in the combined presence of anti-CD3 and anti-CD28. Amounts of IL-4, IL-5, IL-13, and IL-17A in supernatants were quantified after culture for 96 h in the combined presence of anti-CD3 and anti-CD28.

2.9. Cytokine gene expression in pinnae

Total RNA was extracted from pinnae by using NucleoSpin RNA II (Takara Bio, Tokyo, Japan) according to the manufacturer's protocol. The PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio) was used to reverse-transcribe RNA into cDNA, in accordance with the manufacturer's protocol. PCR primers (Table 1) for genes encoding IL-4 (*Il4*), IL-5 (*Il5*), IL-12/IL23P40 (*Il12b*), IL-13 (*Il13*), IL-17A (*Il17a*), IFN-γ (*Ifng*), and β actin (*Actb*) were purchased from Takara Bio. Resulting cDNAs were amplified by quantitative real-time polymerase chain reaction (PCR) analysis by using the Thermal Cycler Dice system (Takara Bio). The data acquired for each sample were normalized to the expression levels recorded for the housekeeping gene *Actb*.

Table 1
Primers used.

Gene	GenBank accession no.	Primers	Product (bp)
Il4	NM_021283.2	F: 5′-TCTCGAATGTACCAGGAGCCATATC-3′ R: 5′-AGCACCTTGGAAGCCCTACAGA-3′	183
Il5	NM_010558.1	F: 5′-TCAGCTGTGTCTGGGCCACT-3′ R: 5′-TTATGAGTAGGGACAGGAAGCCTCA-3′	133
Il12b	NM_008352.2	F: 5′-GCTCATGGCTGGTGCAAAGA-3′ R: 5′-GAGACGCCATTCCACATGTCA-3′	99
Il13	NM_008355.3	F: 5′-CAATTGCAATGCCATCTACAGGAC-3′ R: 5′-CGAAACAGTTGCTTTGTGTAGCTGA-3′	150
Il17a	NM_010552.3	F: 5′-ACGCGCAAACATGAGTCCAG-3′ R: 5′-AGGCTCAGCAGCAGCAACAG-3′	66
Ifng	NM_008337.3	F: 5′-CGGCACAGTCATTGAAAGCCTA-3′ R: 5′-GTTGCTGATGGCCTGATTGTC-3′	199
Actb	NM_007393.3	F: 5′-CATCCGTAAAGACCTCTATGCCAAC-3′ R: 5′-ATGGAGCCACCGATCCACA-3′	171

F, forward; R, reverse.

2.10. Regulatory T-cell analysis of spleen

All antibodies and buffers for analysis were purchased from BD Pharmingen. Lymphocytes in splenic T-cell suspensions were stained by using the Mouse Foxp3 Buffer Set, FITC-conjugated rat anti-mouse CD25 (clone 3C7), PE-conjugated rat anti-mouse Foxp3 (clone MF23), and PE–Cy5-conjugated rat anti-mouse CD4 (clone RM4-5) in accordance with the manufacturer's protocol. The cells were analyzed on a FACSCaliber flow cytometer using Cell Quest software. For each sample, 10,000 events were collected and analyzed for expression of antigens.

2.11. Statistical analysis

Statistical significance of the difference between the vehicle control and treated groups was estimated at the 5% and 1% levels of probability.

Data from the vehicle-only control and the methoxychlor-, parathion-, and piperonyl butoxide-treated groups were evaluated by Bartlett's test for equality of variance. When group variances were homogeneous, a parametric one-way analysis of variance was conducted to determine statistical differences among groups. When the analysis of variance was significant, Dunnett's multiple comparison test was applied. When group variances were heterogeneous, data were evaluated by Kruskal–Wallis non-parametric analysis of variance. When differences were significant, Dunnett's mean rank-sum test was applied.

Student's *t*-test was applied to data from the vehicle-only control and dexamethasone- or cyclophosphamide-treated groups for analysis of Treg cells (Figs. 1 and 2).

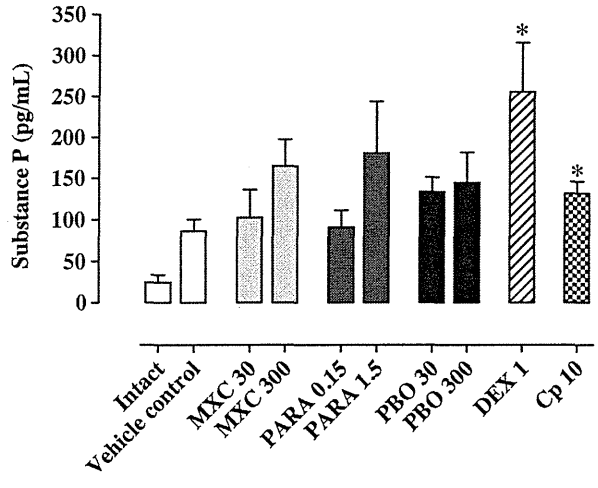

Fig. 1. Serum levels of substance P. Serum levels of substance P in mice sensitized and challenged with picryl chloride after oral exposure to nothing (intact), vehicle only, or each test substance. Substance P levels (pg/mL) are expressed as mean ± 1 SD ($n = 8$ per group). *$P < 0.05$ (Dunnett's multiple comparison test or Student's *t*-test) compared with the value for the vehicle-only control group. MXC, methoxychlor; Cp, cyclophosphamide; DEX, dexamethasone; PARA, parathion; PBO, piperonyl butoxide; numerals refer to dose levels (see Section 2).

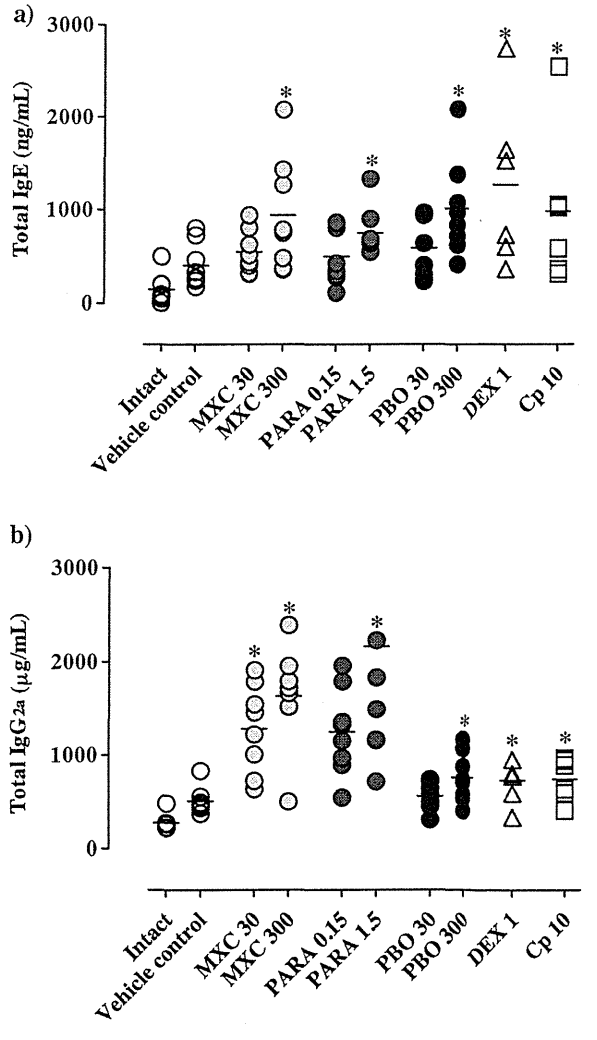

Fig. 2. Total serum immunoglobulins. Total (a) IgE and (b) IgG_{2a} in the serum of mice orally exposed to nothing (intact), vehicle only, or each test substance. Total IgE levels (ng/mL) are expressed as mean ± 1 SD ($n = 8$ per group); total IgG_{2a} levels (μg/mL) are expressed as mean ± 1 SD ($n = 8$ per group). *$P < 0.05$ (Dunnett's multiple comparison test or Student's *t*-test) compared with the value for the vehicle-only control group.

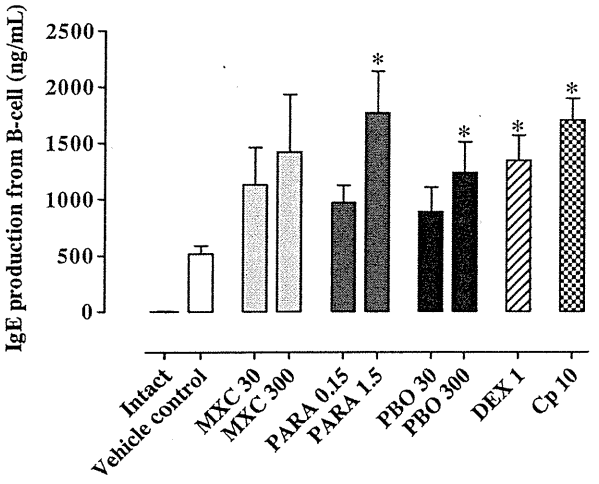

Fig. 3. Total IgE production by B cells from auricular lymph nodes. IgE production by B-cells in auricular lymph nodes from mice orally exposed to nothing (intact), vehicle only, or each test substance. IgE levels (ng/mL) are expressed as mean ± 1 SD ($n = 8$ per group). *$P < 0.05$ (Dunnett's multiple comparison test or Student's t-test) compared with the value for the vehicle-only control group.

3. Results

3.1. Clinical observations

When the mice were 8–12 weeks old, we weekly measured ear thickness (Table 2) and scored the four major clinical symptoms of atopic dermatitis—skin dryness, erythema, edema, and wounding. In mice exposed to the test chemicals, ear thickness and clinical symptoms increased dramatically and in a dose-dependent manner between the ages of 8–12 weeks. Ear thickness was significantly greater in chemical-treated mice compared with vehicle-only control groups (Table 3).

We then measured serum levels of substance P, a strong inducer of the release of chemical mediators from mast cells and a highly pruritogenic peptide in mice and humans (Andoh et al., 1998; Tanaka et al., 2007), in mice treated or not treated with the test chemicals (Fig. 3). Serum levels of substance P correlate closely with the clinical severity of dermatitis in NC/Nga mice (Ohmura et al., 2004; Tanaka et al., 2007). Serum levels of substance P increased dose-dependently among pretreated mice. In particular, the groups orally exposed to dexamethasone and cyclophosphamide showed statistically significant increases in serum concentrations of substance P relative to those in the vehicle-only control groups.

3.2. Total IgE and IgG_{2a} levels in serum

To examine whether exposure to test substance elicits a systemic immune response during AD, we measured total IgE and IgG_{2a} levels in serum (Fig. 4). Groups exposed to high-dose methoxychlor, high-dose parathion, high-dose piperonyl butoxide, dexamethasone, or cyclophosphamide showed significant and dose-dependent increases in total serum IgE and IgG_{2a} relative to levels in the vehicle-only control groups. In addition, total IgG_{2a} in the low-dose (30 mg/kg) methoxychlor group was significantly higher than that in the vehicle-only group.

3.3. IgE production by B-cells in auricular LNs

We harvested B cells from the auricular LNs of intact (no treatment) and picryl-chloride-treated mice, incubated the cells with anti-CD40 ligand and recombinant IL-4, and measured the in vitro

Table 2
Ear thickness (mm).

Age (weeks)	Intact	Vehicle only	Methoxychlor (mg/kg)		Parathion (mg/kg)		Piperonyl butoxide (mg/kg)		Dexamethasone	Cyclophosphamide
			30	300	0.15	1.5	30	300	1 mg/kg	10 mg/kg
8	0.27 ± 0.04	0.23 ± 0.04	0.21 ± 0.04	0.25 ± 0.08	0.23 ± 0.08	0.26 ± 0.04	0.22 ± 0.08	0.23 ± 0.04	0.20 ± 0.08	0.25 ± 0.10
9	0.22 ± 0.04	0.25 ± 0.04	0.64 ± 0.08*	0.71 ± 0.06*	0.73 ± 0.10*	0.67 ± 0.13*	0.68 ± 0.06*	0.75 ± 0.13*	0.60 ± 0.10	0.60 ± 0.05
10	0.24 ± 0.03	0.57 ± 0.10	1.16 ± 0.50*	1.20 ± 0.28*	1.10 ± 0.24*	1.17 ± 0.36*	1.03 ± 0.16*	1.23 ± 0.34*	1.10 ± 0.20	1.06 ± 0.13
11	0.23 ± 0.02	0.78 ± 0.19	1.09 ± 0.20	1.23 ± 0.44*	0.98 ± 0.20	1.20 ± 0.33*	1.24 ± 0.26*	1.38 ± 0.32*	1.28 ± 0.15	1.23 ± 0.05
12	0.23 ± 0.01	0.82 ± 0.10	1.23 ± 0.35*	1.35 ± 0.22*	1.15 ± 0.28*	1.28 ± 0.10*	1.11 ± 0.23*	1.29 ± 0.26*	1.28 ± 0.18	1.39 ± 0.34

* $P < 0.05$ (Dunnett's multiple comparison test or Student's t-test) compared with the value for the vehicle-only control group.

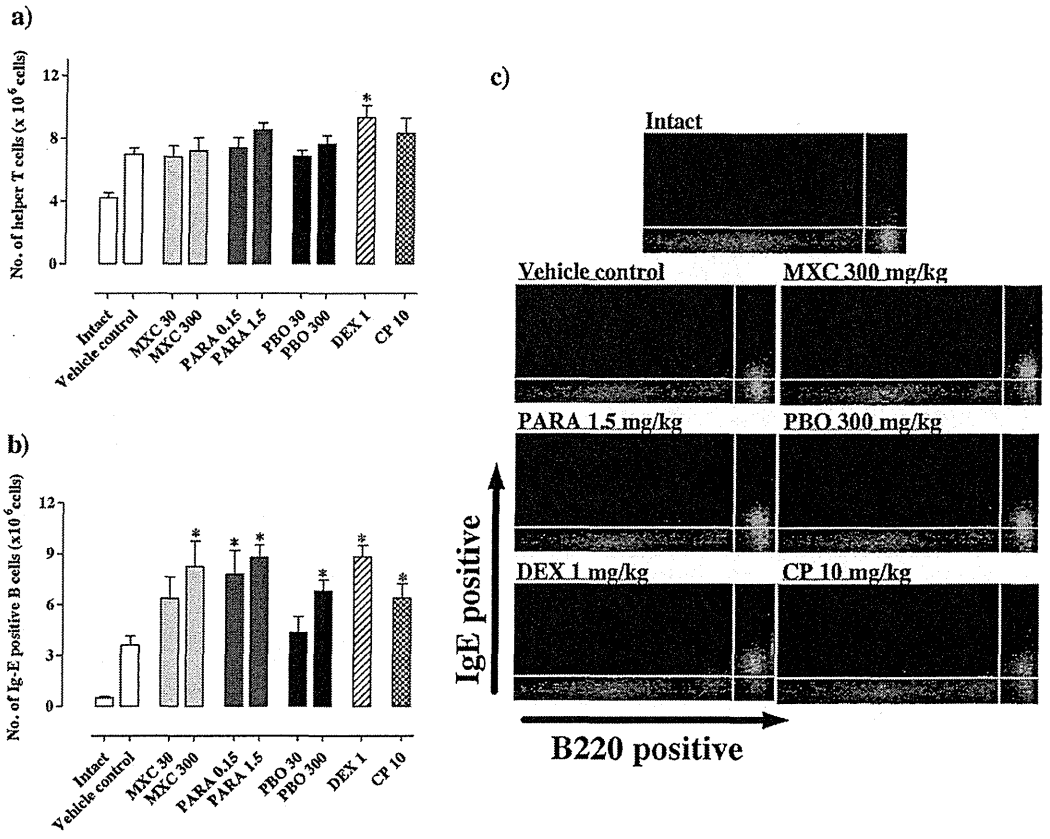

Fig. 4. Helper ($CD4^+CD8^-$) T lymphocyte and IgE-positive (IgE^+B220^+) B-lymphocyte counts in auricular lymph nodes (LNs). Lymphocytes from auricular LNs were stained with anti-CD3, anti-CD4, anti-CD8, anti-B220, and anti-IgE antibodies, and (a) helper T lymphocytes and (b) IgE-positive B lymphocytes of mice orally exposed to nothing (intact), vehicle only, or each test substance were counted. Cell counts ($\times 10^5$ cells) are expressed as mean ± 1 SD ($n = 8$ per group). *$P < 0.05$ (Dunnett's multiple comparison test or Student's t-test) compared with the value for the vehicle-only control group. (c) Representative dot plots of IgE-positive B lymphocytes from the auricular LNs of mice.

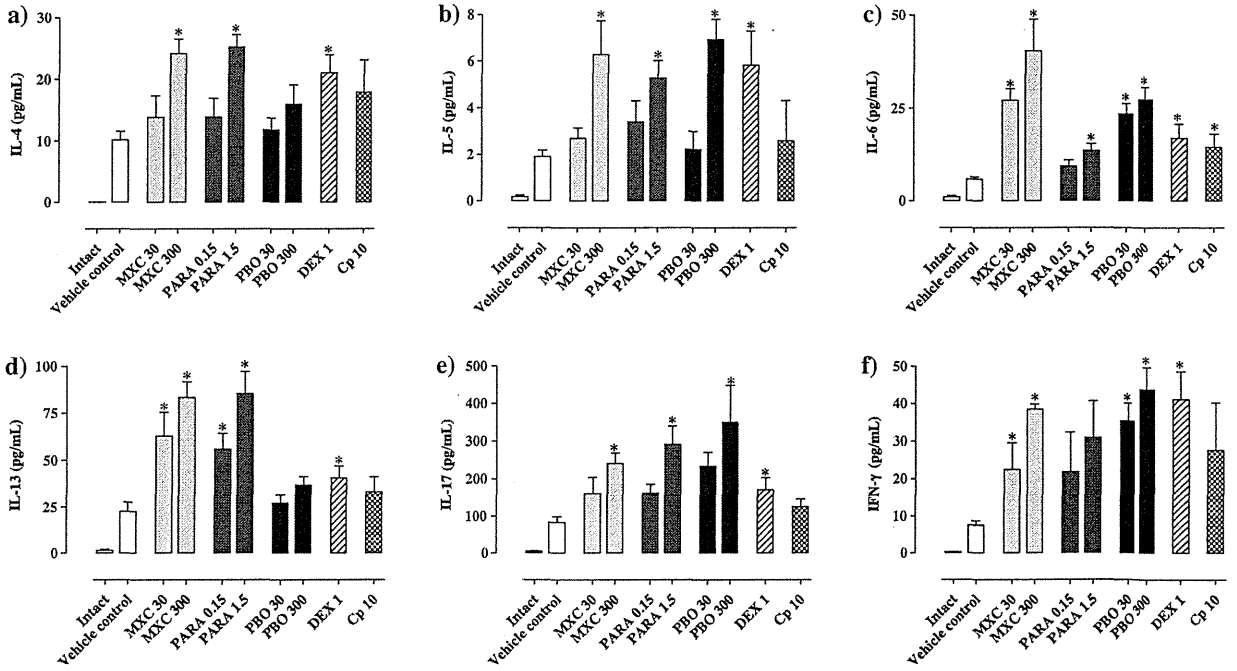

Fig. 5. Cytokine production from auricular lymph nodes from mice. (a) IL-4, (b) IL-5, (c) IL-6, (d) IL-13, (e) IL-17A, and (f) IFN-γ production in lymphocytes from auricular lymph nodes of mice orally exposed to nothing (intact), vehicle only, or each test substance. Cytokine production (pg/mL) is expressed as mean ± 1 SD ($n = 8$ per group). *$P < 0.05$ (Dunnett's multiple comparison test or Student's t-test) compared with the value for the vehicle-only control group.

Table 3
Clinical score.

Age (weeks)	Intact	Control	Methoxychlor (mg/kg)		Parathion (mg/kg)		Piperonyl butoxide (mg/kg)		Dexamethasone 1 mg/kg	Cyclophosphamide 10 mg/kg
			30	300	0.15	1.5	30	300		
8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
9	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.3 ± 0.5	1.3 ± 0.5	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
10	0.0 ± 0.0	1.0 ± 0.0	2.6 ± 0.5	2.8 ± 0.5	2.5 ± 0.5	2.8 ± 0.5	2.4 ± 0.7	2.9 ± 0.4	2.7 ± 0.5	2.2 ± 0.4
11	0.0 ± 0.0	1.4 ± 0.5	2.6 ± 0.5	2.9 ± 0.4	2.0 ± 0.9	2.8 ± 0.5	2.8 ± 0.5	2.9 ± 0.4	3.0 ± 0.0	2.8 ± 0.4
12	0.0 ± 0.0	1.9 ± 0.4	2.8 ± 0.5	3.0 ± 0.0	2.5 ± 0.5	3.0 ± 0.0	2.4 ± 0.9	2.9 ± 0.4	3.0 ± 0.0	3.0 ± 0.0

production of total IgE by these cells. B cells from mice pre-treated with test substances showed dose-dependent increases in secreted total IgE, with statistically significant differences observed for the high-dose parathion, high-dose piperonyl butoxide, dexamethasone, and cyclophosphamide groups compared with the vehicle-only controls (Fig. 5).

3.4. Flow cytometry of auricular LNs

To evaluate the activation states of T and B cells after treatment with test substance and picryl chloride, we used flow cytometry to measure the numbers of helper T cells and IgE-positive B cells in auricular LNs (Fig. 6). All mice given test substance showed dose-dependent increases in these cell populations. Mice exposed to dexamethasone showed significantly increased helper T-cell counts relative to those in the vehicle-only control group. Compared with those in the vehicle-only control group, IgE-positive B-cell counts were increased significantly in mice exposed to high-dose methoxychlor, high-dose parathion, both doses of piperonyl butoxide, dexamethasone, and cyclophosphamide.

3.5. Cytokine production in auricular LNs

To examine whether exposure to test substance elicits the cytokine profile seen for T cells during AD, we cultured auricular LN cell suspensions with antibodies to T-cell markers (CD3, CD28, or both) for 24 h or 96 h and assayed the supernatant by cytometric bead array. All cytokines increased dose-dependently in all groups exposed to test substances (Fig. 7). The increases in IL-4 levels were statistically significant in the groups treated with high-dose methoxychlor, high-dose parathion, or dexamethasone (Fig. 7a). The increases in IL-5 levels were statistically significant in the high-dose methoxychlor, high-dose parathion, high-dose piperonyl butoxide, and dexamethasone treatment groups (Fig. 7b). IL-6 levels increased significantly after exposure to high-dose parathion, both doses of methoxychlor and piperonyl butoxide, dexamethasone, and cyclophosphamide (Fig. 7c). Dexamethasone and both doses of methoxychlor and parathion significantly increased IL-13 levels (Fig. 7d). IL-17 levels increased significantly after exposure to high-dose methoxychlor, high-dose parathion, high-dose piperonyl butoxide, or dexamethasone (Fig. 7e). The increases in IFN-γ levels were statistically significant in both groups treated with methoxychlor, both groups treated with piperonyl butoxide, and the dexamethasone-treated group (Fig. 7f).

3.6. Cytokine gene expression in pinnae

To further explore the effects of test substances on picryl-chloride-induced AD, we examined the expression of genes associated with T helper (Th)-1, -2, and -17 cells in the pinnae of our mice. For all genes evaluated, expression increased dose-dependently in all groups exposed to test chemical (Fig. 6). Compared with that in the vehicle-only group, expression of *Il4* increased significantly in the group pre-exposed to high-dose methoxychlor, high-dose parathion, high-dose piperonyl butoxide, dexamethasone, or cyclophosphamide (Fig. 8a); expression of *Il5* increased significantly after dexamethasone treatment (Fig. 8b); and that of *Il12b* increased in the groups pre-exposed to high-dose methoxychlor, high-dose parathion, dexamethasone, or cyclophosphamide (Fig. 8c). Expression of *Il13* increased in mice treated with high-dose parathion, high-dose piperonyl butoxide, or dexamethasone (Fig. 8d); *Il17a* levels increased after high-dose piperonyl butoxide (Fig. 8e); and *Ifng* levels increased significantly in the groups given dexamethasone and cyclophosphamide (Fig. 8f).

3.7. Treg cell counts in spleen

To evaluate whether exposure to test substance activated Treg cells, we used flow cytometry to measure the number of $CD4^+$, $CD25^+$, and $Foxp3^+$ cells in spleen (Fig. 7). In the groups treated with high-dose methoxychlor, high-dose parathion, dexamethasone, or cyclophosphamide, Treg cell counts decreased significantly compared with those in the vehicle-only controls.

4. Discussion

Our primary objective in the current study was to clarify the mechanism by which immunosuppressive environmental chemicals induce the development of AD. To that end, we used several immunosuppressive chemicals, including the organochloride agent methoxychlor, the organophosphate agent parathion, the agricultural insecticide synergist piperonyl butoxide, dexamethasone, and cyclophosphamide, in a mouse model of AD. We evoked AD in NC/Nga mice through their repeated dermal exposure to picryl chloride and assessed the subsequent immune response by using several detection methods, including scoring of dermatitis severity, measurement of cytokine gene expressing in the pinna, assessment of IgE and IgG_{2a} levels in serum, and evaluation of T- and B-cell surface-antigen expression and local cytokine production in auricular LN. In addition, we measured splenic Treg surface-antigen expression to detect a relation between increases in autoreactive T or B cells and exposure to immunosuppressive environmental chemicals. Our results show that methoxychlor, parathion, piperonyl butoxide, dexamethasone, and cyclophosphamide each, as sole agents, aggravated the allergic response of NC/Nga mice. All five chemicals also reduced Treg surface-antigen expression in spleen, perhaps revealing a role for this process in the exacerbation of AD.

Organochloride agents such as methoxychlor were introduced in the 1940s and were widely used in agriculture and for pest control. Although organochloride agents are rarely used in developed countries currently, measurable amounts of these chemicals or their metabolites can still be found in human tissues (Turusov et al., 2002; Xu et al., 2010). Moreover, organochloride agents continue to be used heavily, primarily for mosquito and malaria control, in some developing countries (Turusov et al., 2002; Xu et al., 2010). Organophosphate compounds such as parathion were introduced as replacements for organochloride agents and led to the ban or

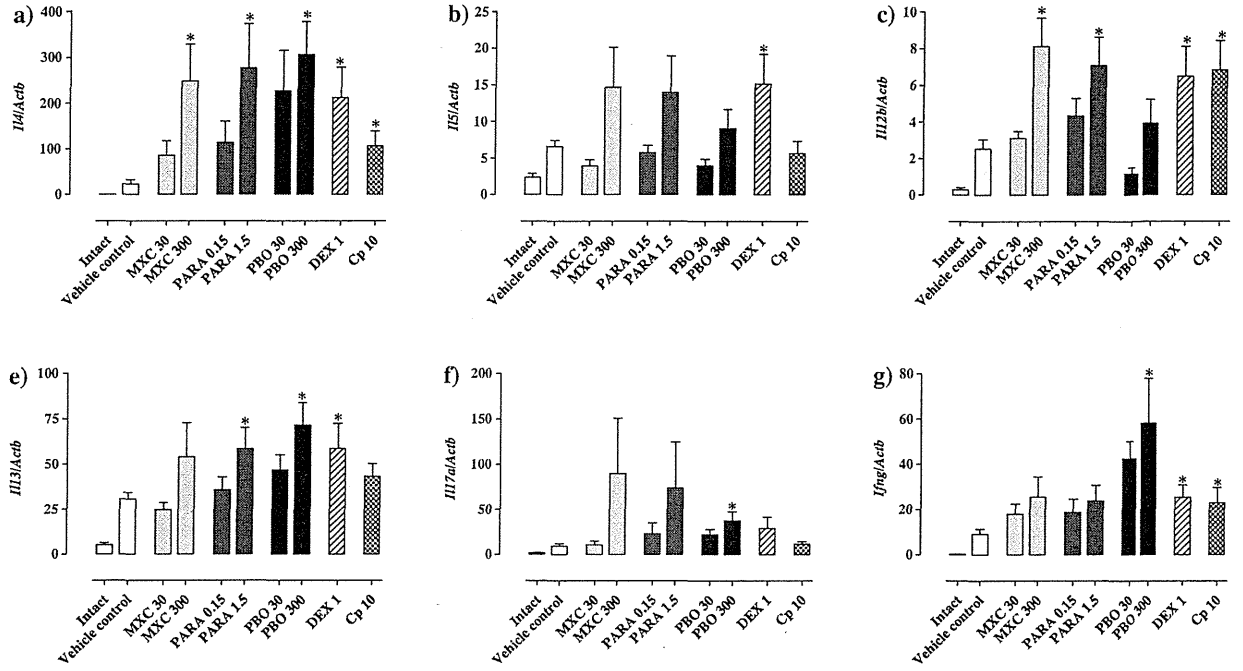

Fig. 6. Cytokine gene expression in ear auricle from mice. Gene expression of (a) *Il4*, (b) *Il5*, (c) *Il12b*, (d) *Il13*, (e) *Il17a*, and (f) *Ifng* in pinnae from mice orally exposed to nothing (intact), vehicle only, or each test substance. Gene expression values are presented as the increase in expression compared with the value for the expression of *Actb* and are expressed as mean ± 1 SD ($n = 8$ per group). *$P < 0.05$ (Dunnett's multiple comparison test or Student's *t*-test) compared with the value for the vehicle-only control group.

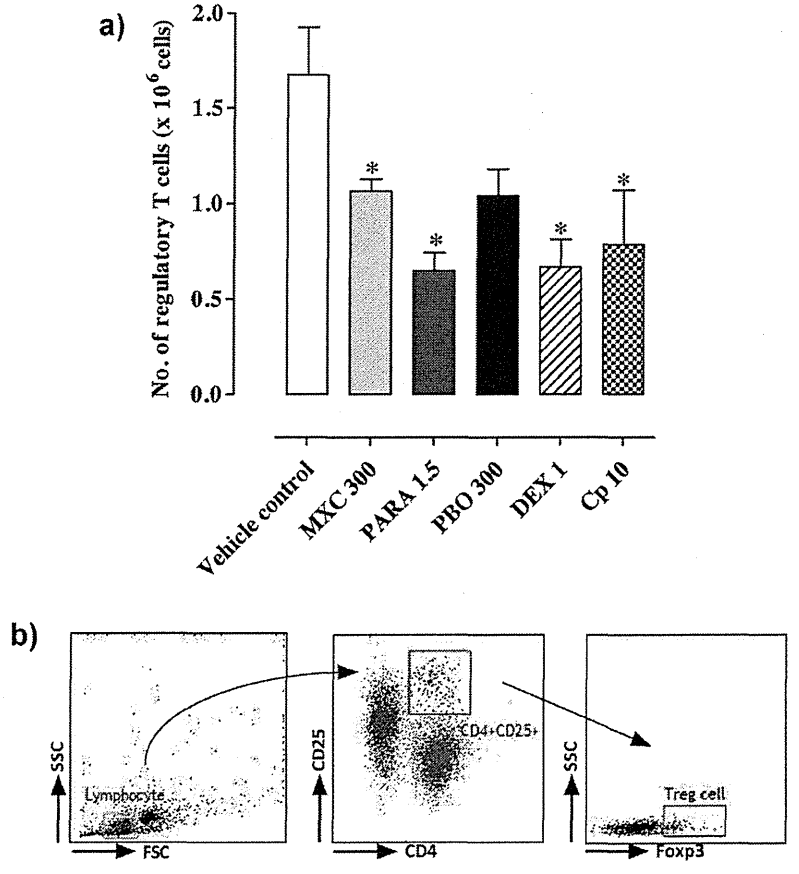

Fig. 7. Splenic regulatory ($CD4^+CD25^+Foxp3^+$) T-lymphocyte counts. (a) Suspensions of splenic lymphocytes were stained with anti-CD4, anti-CD25, and anti-FoxP3 antibodies. Cell counts ($\times 10^6$ cells) are expressed as mean ± 1 SD ($n = 8$ per group). *$P < 0.05$ (Dunnett's multiple comparison test or Student's *t*-test) compared with the value for the vehicle-only control group. (b) Representative strategy of histograms dot plots of Treg cells from the splenic lymphocytes of mice.

restricted use of organochloride agents in the 1970s (Wessels et al., 2003; Lopez et al., 2007). The widespread exposure of humans to organophosphate agents is substantiated through the presence of common and specific metabolites of these chemicals in urine samples obtained from the general population (Bouvier et al., 2005; Lacasana et al., 2010). Piperonyl butoxide is an agricultural insecticide synergist that is used mainly with pyrethroids (Carson et al., 1988; Mitsumori et al., 1996; Emerson et al., 2001). Recent evidence suggests that piperonyl butoxide depletes T lymphocytes in the spleen and thymus, induces hypoplasia of the bone marrow, and inhibits T-lymphocyte proliferation in lymphoid tissues (Mitsumori et al., 1996; Diel et al., 1999; Battaglia et al., 2010). Glucocorticoides, including dexamethasone, are widely used immunosuppressive and anti-inflammatory agents, in part because of their suppression of cytokine gene transcription (Almawi et al., 1996) and induction of leukocyte death through apoptosis (Fukuyama et al., 2010a,b; Planey and Litwack, 2000). Cyclophosphamide is well known to inactivate rapidly cycling cell populations due to its alkylating activity and is widely used as an immunosuppressive drug in recalcitrant collagen disease and prior to bone marrow transplantation (Drossler et al., 1983; Ikezawa et al., 2005).

The environmental immunosuppressive chemicals we evaluated have various roles in the dysregulation of immune function (Kosuda et al., 1996; Zelikoff et al., 1994). Several previous studies demonstrated that exposure of mice to these chemicals led to markedly decreased thymic and splenic function (Battaglia et al., 2010; Casale et al., 1983; Diel et al., 1999; Mitsumori et al., 1996; Takeuchi et al., 2002). We similarly showed that in vivo or in vitro exposure to methoxychlor, parathion, or piperonyl butoxide resulted in elevations in apoptosis-related factors and atrophy of T and B lymphocytes in immune organs (Fukuyama et al., 2010b, 2011a). However, although these chemicals contribute to suppressing immune functions, we have obtained contradictory results from studies in which T-lymphocyte-mediated allergic reactions were exacerbated by prior oral exposure to methoxychlor or parathion (Fukuyama et al., 2010a, 2011b). To resolve this paradox, we undertook the current study using a typical animal model of AD.

NC/Nga mice sensitized with picryl chloride develop AD-like skin lesions and increased IgE and IgG_{2a} production, with a close relationship between serum Ig levels and the development of skin lesions (Matsumoto et al., 1999). In the current study, the exposure of mice to environmental immunosuppressive chemicals before treatment with picryl chloride led to marked amplification of dermatitis severity (ear thickness, clinical symptoms) as well as increases in serum levels of substance P, IgG, and IgE. Consistent with our observations of the effects of these chemicals on dermatitis severity and serum Ig levels, we also noted significant increases in the number of IgE-expressing B cells in and the production of IgE by the auricular LNs of mice exposed to various environmental chemicals.

Most patients with AD acutely manifest strong polarization toward a Th2-type immune response (e.g. IL-4, -5, -13), resulting in hyperproduction of IgE (Beltrani, 2005). However, during the subsequent, chronic phase of AD, the numbers of Th1 cells producing IL-12, IFN-γ, and TNF-α increase (Anthoni et al., 2007). Here, prior exposure to environmental immunosuppressive chemicals substantially increased the production of Th2-type cytokines (IL-4, IL-5, and IL-13) from LN cells and the expression of RNA corresponding to these genes in the pinnae of mice. Furthermore, mice exposed to the test chemicals also mounted a Th1 cytokine profile, as indicated by the increased LN production and ear tissue RNA expression of IL-12, IFN-γ, and TNF-α. These increases were associated with increased numbers of helper T cells and IgE-positive B cells in LN.

Recent clinical data suggest that, in addition to Th1 factors, levels of Th17 are increased during allergic reactions. With an AD patient, it has been reported that the numbers of Th17 cells producing IL-17 (Joshi et al., 2009). Here we observed that exposure to each test chemical induced a marked increase in IL-17 production. These data suggest that modulation of IL-17 by exposure to environmental chemicals may be of clinical importance to the development and severity of AD.

To elucidate the phenomenon through which modulation of IL-17 levels exacerbates AD, we focused on $CD4^+CD25^+FoxP3^+$ Treg cells, which exert their regulatory effects in a cell–cell contact-dependent manner (Ikezawa et al., 2005). In addition, a sufficiency of Treg cells is crucial to prevent inflammation, autoimmunity, and the induction of tumor antigen tolerance (Carson et al., 2008). In previous reports, cyclophosphamide decreased the number, percentage, and function of Treg cells, which otherwise suppress the induction of contact hypersensitivity (Ikezawa et al., 2005). Similarly, our current results show that methoxychlor, parathion, dexamethasone, and cyclophosphamide each statistically decreased the numbers of splenic Treg cells; this decrease contributes to the exacerbation of AD.

In this study, we selected several types of environmental chemicals which have differing mechanisms to immunotoxic reaction; e.g., methoxychlor and parathion induced immunotoxicity via the estrogen receptor signaling and inhibition in cholinesterase activity, respectively. Then the expression pattern in each endpoint differs by each chemical treatment group. Actually, although piperonyl butoxide resulted in less elevations in so-called allergy-related factors (IgE, IL-4, IL-13) than other chemicals, marked increasing in IL-17 production as compared with other chemicals. However, main purpose of this study was to detect an aggravation of allergy by environmental immunotoxic chemicals using AD model and several high sensitive endpoints. In conclusion, an aggravation of allergy can be detected using our protocol and it seems that Treg cells play some roles with these phenomenon. Characterization of mechanisms and causative agent in each chemical is secondary object and next step. It is possible that knocked out models of related genes can be clear up these problems. The experiments are currently under investigation in our laboratories for the next step.

Overall, the results we report here demonstrate that prior exposure to immunosuppressive chemicals such as methoxychlor, parathion, piperonyl butoxide, dexamethasone, and cyclophosphamide can modulate immune functions and increase the severity of AD in mice. Environmental immunosuppressive chemicals associated with immune disorders including AD are thought to act by at least one of three general mechanisms (Rao and Richardson, 1999; Sobel et al., 2005): (1) altering self-antigen such that it appears foreign to the immune system; (2) preventing central tolerance of autoreactive T or B cells; and (3) altering gene expression. Hormones such as estrogens and estrogenic environmental chemicals are thought to alter gene expression (Grimaldi et al., 2002). We allowed a 4-week recovery period between oral exposure to test substance and allergen (i.e., picryl chloride) sensitization so that each chemical would not function as self-antigen. Several studies linking environmental chemicals with immune disorders have noted the estrogenic character of those chemicals (Sobel et al., 2005; Wang et al., 2007; Ward et al., 2009; Xu et al., 2010). In contrast, we noted that exposure to parathion, piperonyl butoxide, dexamethasone, and cyclophosphamide (which is not a hormone disruptor) each increased all of the parameters we measured, thereby suggesting that the resulting increase in AD severity is not due only to hormonal effects. Instead, prevention of the central tolerance of autoreactive T or B cells is implicated because mice exposed to immunosuppressive environmental chemicals showed significant decreases in Treg cell populations. To our knowledge, this study is the first to demonstrate the relationship between allergies and autoreactive T or B cells by non-estrogenic environmental immunosuppressive chemicals. Future studies likely will

further define the relationship between human allergic diseases and the destructive effect of exposure to environmental chemicals on immune regulation.

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Conflict of interest

The author states that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2012.07.018.

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