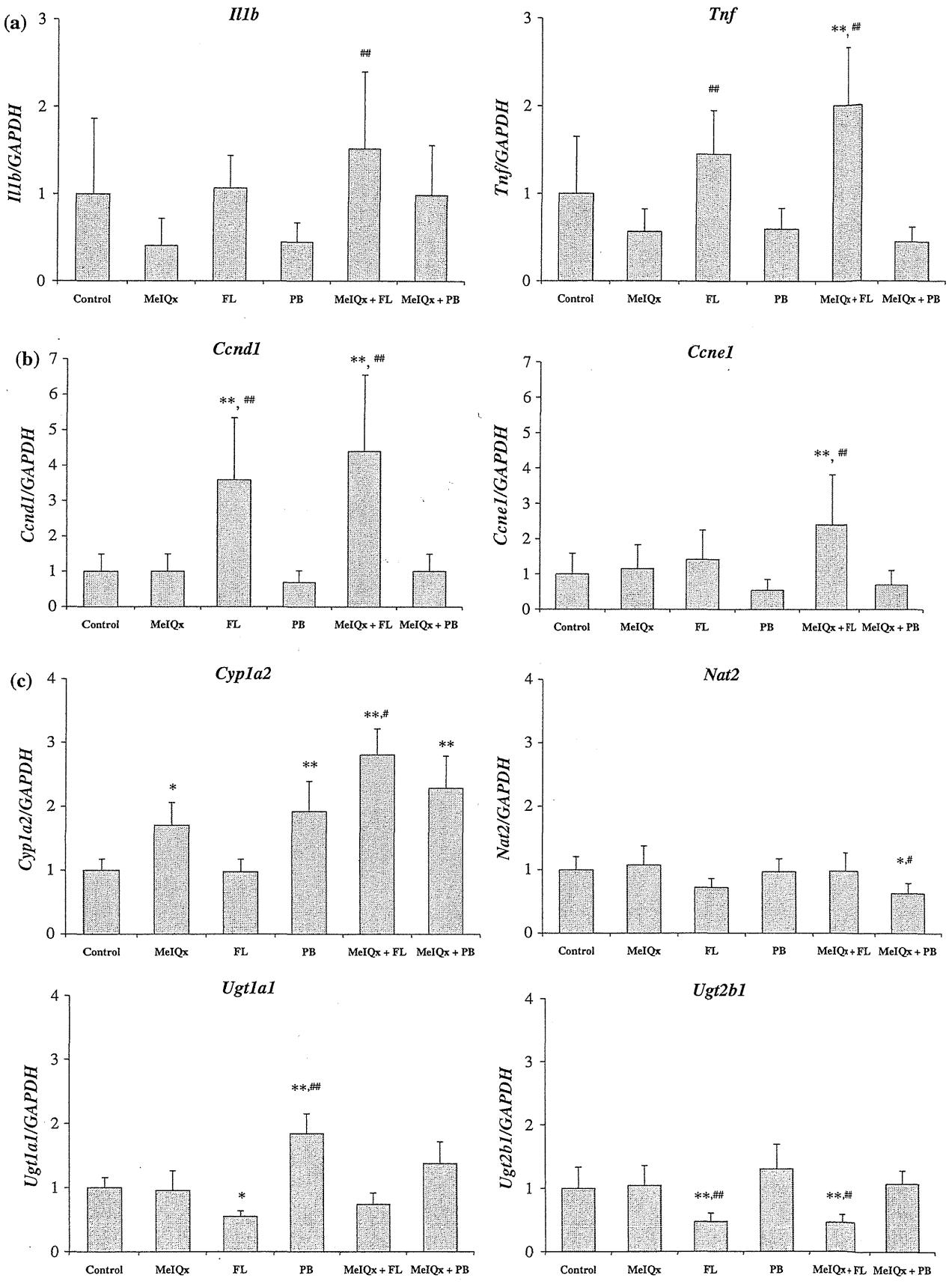
(a)
Il1b
Il1b/GAPDH
Tnf
Tnf/GAPDH
(b)
Ccnd1
Ccnd1/GAPDH
Ccne1
Ccne1/GAPDH
(c)
Cyp1a2
Cyp1a2/GAPDH
Nat2
Nat2/GAPDH
Ugt1a1
Ugt1a1/GAPDH
Ugt2b1
Ugt2b1/GAPDH
Control
MeIQx
FL
PB
MeIQx + FL
MeIQx + PB

◀ **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

Immunotoxicity in mice induced by short-term exposure to methoxychlor, parathion, or piperonyl butoxide

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Abstract

Exposure to environmental agents can compromise numerous immunological functions. Immunotoxicology focuses on the evaluation of the potential adverse effects of xenobiotics on immune mechanisms that can lead to harmful changes in host responses such as: increased susceptibility to infectious diseases and tumorigenesis; the induction of hypersensitivity reactions; or an increased incidence of autoimmune disease. In order to assess the immunosuppressive response to short-term exposure to some commonly used pesticides, the studies here focused on the response of mice after exposures to the organochlorine pesticide methoxychlor, the organophosphorus pesticide parathion, or the agricultural insecticide synergist piperonyl butoxide. In these studies, 7-week-old mice were orally administered (by gavage) methoxychlor, parathion, or piperonyl butoxide daily for five 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 subsequently assessed. In addition, levels of B-cells in the spleen of each mouse were also analyzed via surface antigen expression. The results of these studies indicated that treatments with these various pesticides induced marked decreases in the production of SRBC-specific IgM antibodies as well as in the expression of surface antigens in IgM- and germinal center-positive B-cells. Based on these outcomes, it is concluded that the short-term exposure protocol was able to detect potential immunosuppressive responses to methoxychlor, parathion, and piperonyl butoxide *in situ*, and, as a result, may be useful for detecting other environmental chemical-related immunotoxicities.

Keywords: Parathion, methoxychlor, piperonyl butoxide, Jurkat T-cell, apoptosis, T-dependent antigen response (TDAR)

Introduction

Exposure to environmental agents can compromise numerous immunological functions. In the United States alone, 20,000 pesticide products are on the market, and 1 billion pounds of active ingredients are applied annually for agricultural, industrial, and residential pest control (EPA, 2003). Against this background, studies in animals and humans have indicated that the immune system is a potential target, and that damage to this system can be associated with increased morbidity and even mortality. Immunotoxicologic analyses can evaluate the potential adverse effects of xenobiotics (e.g. chemicals, pesticides, drugs, biotechnology-derived products) on host immune mechanisms. Many of these effects can lead to harmful changes in host responses, including increased susceptibility to infectious diseases and tumorigenesis, the induction of hypersensitivity reactions, or an increased incidence of autoimmune disease (Herzyk and Holsapple, 2007).

Immunotoxicological testing has emerged in recent years as an important adjunct to routine safety evaluations of environmental chemicals and newly-developed pharmaceuticals, and has been incorporated into the guidelines issued by several regulatory authorities, including the Environmental Protection Agency (EPA, 1998), Food and Drug Administration (FDA, 2002), the European Medicines Agency (EMEA) (CPMP, 2000), and

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the International Conference on Harmonization (ICH, 2006). The most recent immunotoxicology guidance documents recommend T-dependent antigen response (TDAR) tests, primarily because this assay represents a comprehensive evaluation of immune function based on an assessment of various components of the immune system (e.g., antigen-presenting cells, T-helper lymphocytes, and B-lymphocytes) involved in an antigen-specific antibody response (White et al., 2010).

Originally this test was configured as an IgM/complement dependent *ex vivo* plaque-forming cell (PFC) assay in mice, with sheep red blood cells (SRBC) as both the immunogen and the target for complement-mediated lysis. Subsequently, an enzyme-linked immunosorbent assay (ELISA)-based format was developed using SRBC as both the immunogen and antigen in the antibody detection system (Temple et al., 1993). SRBC-based TDAR assays (PFC and ELISA) have also been used in rats, and have ultimately gained acceptance as validated tests for detecting the immunosuppressant activity of drugs and chemicals (Ladics et al., 1998).

The above guidelines generally recommend using repeated doses, 28-day exposures, and adult animals to detect immunotoxicity caused by environmental chemicals. The use of long-term exposure offers the advantage of being able to compare results with those obtained from general toxicity tests. However, long-term exposure is time-consuming, costly, and may lead to immunotoxic drug resistance because the immune system is highly sensitive to the toxic effects of several types of chemicals (Fukuyama et al., 2011a); such a change would distort estimates of advanced immunotoxicity. Therefore, new short-term exposure protocols are needed to detect immunotoxicity. Previously, in the first stage of our studies, we developed a short-term method (administration via oral gavage for 3 days) for detecting thymocyte apoptosis induced by a typical immunosuppressant, methoxychlor (Fukuyama et al., 2010, 2011a). Indeed, methoxychlor induced prominent increases in several parameters indicative of induced thymocyte apoptosis, including Annexin V-FITC$^+$ cells, caspase (3/7, 8, and 9) activities, and DNA fragmentation. This type of dysregulation of apoptosis in the thymus is known to lead to various immune disorders, including immunodeficiency, tumorigenesis, allergies, and autoimmunity (Zhang et al., 2005). Our previous results demonstrated that short-term exposure has the potential to detect the immunosuppression caused by chemicals present in the environment.

In light of these previous results, the aim of this study was to develop a new short-term immunotoxicology protocol using several immunologic endpoints that tested short-term exposure to the organochlorine pesticide methoxychlor, the organophosphorus pesticide parathion, and the agricultural insecticide synergist piperonyl butoxide. These three chemicals were chosen on the basis of previous studies; parathion markedly inhibits antigen-specific-IgM production (Casale et al., 1984), and we previously showed that methoxychlor exposure results in atrophy of CD4$^+$CD8$^+$ T-lymphocytes in the thymus (Takeuchi et al., 2002, 2004; Fukuyama et al., 2011b). Piperonyl butoxide is an agricultural insecticide synergist used mainly with pyrethroids (Carson et al., 1988; Mitsumori et al., 1996; Emerson et al., 2001). Recent evidence suggests that piperonyl butoxide administration 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).

Materials and methods

Chemicals

Standard parathion ($C_{10}H_{14}NO_5PS$, 99.5% pure), standard methoxychlor ($C_{16}H_{15}Cl_3O_2$, > 97% pure), standard piperonyl butoxide ($C_{19}H_{30}O_5$, > 98% pure), dimethyl sulfoxide (DMSO), and acetone were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil was purchased from Hayashi Chemicals (Tokyo, Japan). For *in vitro* studies, parathion was dissolved in DMSO to 0.1% (w/v). Methoxychlor and piperonyl butoxide were dissolved in acetone to 0.1% (w/v).

Three concentrations (10, 100, and 1000 nmol/ml) of these pesticides were selected for use in the *in vitro* portions of these studies on the basis of results from preliminary cell viability analyses (data not shown); the exact concentrations chosen were based on the ability of each given agent to substantially inhibit cell viability but not cause 100% lethality.

For the *in vivo* portions of these studies, oral administration was used to introduce parathion, methoxychlor, and piperonyl butoxide (diluted in corn oil to a fixed final volume) into murine hosts. 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 LD_{50} (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.). The actual doses used are presented in Table 1.

Note, there is no overt relationship between the doses used in the *in vivo* and *in vitro* portions of these studies. The endpoints to be measured in each respective series of studies are distinct, and the information gleaned from the *in vitro* studies were only meant to be used for helping to explain outcomes (related to immunomodulation, etc.) that might appear in the agent-treated mice.

Animals

Female C3H/HeN (6-weeks-old) mice were purchased from Charles River Japan Laboratories (Atsugi, Kanagawa, Japan) and housed individually under controlled lighting

Table 1. Chemical doses used.

Chemical	Classification	Oral LD_{50} values (mg/kg)	Doses in this study (mg/kg)
Methoxychlor	organochlorine compound	2900[a]	0, 3, 30, 300
Parathion	organo-phosphorus compound	5[a]	0, 0.015, 0.15, 1.5
Piperonyl butoxide	agricultural insecticide synergist	2600[a]	0, 3, 30, 300

LD_{50}: dose at which 50% of animals died.
[a]Registry of Toxic Effects of Chemical Substances NIOSH CD-ROM (2003).

(lights on from 07:00 to 19:00 h), temperature (22 ± 3°C), humidity (55% ± 15%), and ventilation (at least 10 complete fresh-air changes hourly). Based on the EPA guideline (1998), mice are a model species recommended for use in immunotoxicity studies that test effects of agricultural chemicals (see as was done in Casale et al., 1984; Diel et al., 1999; Battaglia et al., 2010). Furthermore, for the immunotoxicity study, only one gender needed to be evaluated; in general, female animals are considered to yield more consistent outcomes than are male counterparts during evaluation of effects from test articles on host humoral immune responses. Therefore, in this study, female C3H/HeN were used. These particular hosts were also selected as our laboratory has historical immunotoxicity study data on this strain (data not shown). Food (Certified Pellet Diet MF, Oriental Yeast Co., Tokyo, Japan) and water were available *ad libitum*. This study was conducted in accordance with the Code of Ethics for Animal Experimentation of the Institute of Environmental Toxicology.

Cell culture

The human acute T-cell leukemia cell line Jurkat E6.1 was obtained from DS Pharma Biomedical Co., Ltd. (Tokyo, Japan). Jurkat E6.1 cells were cultured in 70-ml EasYFlasks (Nalge Nunc International K.K., Tokyo, Japan) in 5 ml of RPMI 1640 (Gibco, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco) at 37°C in a 5% CO_2 atmosphere. The medium was changed three times per week.

Chemical treatment of the cells

The Jurkat E6.1 cells were seeded into flasks at 8×10^5 cells/ml. Quadruplicate flasks per dose per agent were then treated with 0, 10, 100, and 1000 nmol/ml of each test agent. Control cell cultures received vehicle-containing medium only. In all cases, the flasks were incubated for 30 min to 24 h at 37°C under 5% CO_2. At the end of the exposure time, the culture medium (containing all cells that were originally seeded, i.e. both live and dead) from each flask was transferred to a 5 ml tube and used in apoptosis assays. In general, to assess the extent of apoptosis, caspase-3/7, -8, and -9 activities as well as the proportions of AnnexinV-FITC$^+$ and caspase-3$^+$ cells were quantified.

Cell viability and caspase (3/7, 8, and 9) activity

Cell viability was determined in cultured cells by quantitation of ATP, an endpoint that is indicative of metabolically-active cells. Cell viability, caspase-3/7, -8, and -9 activities were measured at 30 min, 1, 2, 4, and 24 h after initiation of exposure to the chemicals by using a luciferin-luciferase system (CellTiter-Glo™ Luminescent Cell Viability Assay and Caspase-Glo™ assay, Promega, Tokyo, Japan). From each cell suspension, 3×10^4 cells were seeded in duplicate into opaque-walled 96-well plates (Corning Japan K.K., Tokyo, Japan). A microplate luminometer (Spectra MAX L, Molecular Devices, Osaka, Japan) was used to measure the caspase activities in relative light units (RLU).

Annexin-V and caspase-3 cell staining and flow cytometric analysis

Flow cytometric analysis of apoptotic cells was performed at 1 and 4 h after initiation of exposure by staining cells with fluorescein isothiocyanate (FITC)-conjugated Annexin-V, FITC-conjugated caspase-3, and propidium iodide. To assay the cells, an Annexin-V:FITC Apoptosis Detection Kit and an FITC Active Caspase-3 Apoptosis Kit (BD Pharmingen, San Diego, CA) was used, in accordance with manufacturer protocols. All samples were subsequently analyzed using a FACSCaliber flow cytometer (BD Pharmingen) and its accompanying Cell Quest program. For each sample, 10,000 events were collected and analyzed for antigen expression.

Chemical exposure of mice

After a 1-week acclimatization period, C3H/HeN mice (7-weeks-old) were allocated randomly to groups (n = 6 mice/group) for dosing, vehicle control, and no treatment (naïve group). Chemical dosages were as follows: methoxychlor, 3, 30, and 300 mg/kg/day; parathion, 0.015, 0.15, and 1.5 mg/kg/day: or piperonyl butoxide, 3, 30, and 300 mg/kg/day. On Days 1–5, mice were given an oral dose (by gavage, without anesthesia) of the test solution (methoxychlor, parathion, or piperonyl butoxide) or vehicle. On Day 2, a solution of sheep red blood cells (SRBC, 6×10^7 cells/animal; Nippon Bio-Supp. Center, Tokyo, Japan) was injected via the tail vein into all test and control mice for immunization.

One day after the last oral administration (i.e. on Day 6 of study), all mice were anesthetized and sacrificed by over-anesthesization with diethyl ether. Blood samples were taken from the inferior vena cava, and serum samples were assayed for SRBC-specific serum IgM. Each animal's spleen was removed and pooled in phosphate-buffered saline (PBS; Gibco). Single-cell suspensions of splenocytes in 5 ml of modified Eagle medium supplemented (MEM; Gibco) containing 5% heat-inactivated FCS were prepared by passage through a stainless-steel screen and sterile 70-μm nylon cell strainers (Falcon, Tokyo, Japan). The number of lymphocytes in the spleen preparation was then determined using a Coulter counter Z2 (Beckman Coulter, Tokyo, Japan).

SRBC-specific IgM responses in serum

Levels of SRBC-specific serum IgM 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% SDS in PBS. The samples were then extensively dialyzed for 2 days against PBS. The protein content of each conjugated sample was determined by the method of Lowry et al. (1951). Specific serum IgM was then measured by means of an ELISA using flat-bottomed microplates (Nalge Nunc) whose wells had been coated with SRBC-membrane antigen (2 μg/ml in coating buffer; BD Pharmingen) during an overnight incubation at 4°C. Following washing of each well five times with wash buffer (BD Pharmingen), and blocking of potential non-specific binding by incubation with assay diluent (BD Pharmingen) for 2 h at room temperature (RT), dilutions of each mouse serum sample (in assay diluent, from 1:4 to 1:16,384) was added to each well and the plates incubated for 2 h at RT. After gentle rinsing with wash buffer to remove all unbound materials, peroxidase conjugated anti-mouse IgM (secondary antibody; Rochland Inc. PA, dilution 1:15,000) was added to each well and 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 (TMB; 100 μl/well) substrate was added to each well and the plate incubated in the dark at RT for 30 min. Optical density measurements were then made at 450 nm in a Spectra MAX 190 microplate reader (Molecular Devices, Osaka, Japan).

IgM plaque-forming cell response to SRBC in splenocytes

The IgM plaque-forming cell (PFC) response to SRBC was determined by using a modified version of the methods of Cunningham (1965) and Jerne and Nordin (1963). Briefly, 1×10^6 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 applied to a Cunning-ham chamber (Takahashi Giken Glass Co., Ltd, Tokyo, Japan), 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 stereo-microscope.

Flow cytometric analysis

Isolated splenocytes were stained with fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (Vector Laboratories, Inc., Burlingame, CA), FITC-conjugated rat anti-mouse IgM (R6-60.2; BD), phycoerythrin (PE)-conjugated rat anti-mouse IgD (clone 11-26c.2a; BD), PE-cyano dye (Cy)5-conjugated rat anti-mouse CD45R/B220 (clone RA3-6B2; BD), and/or peridinin chlorphyll protein (PerCP)-Cy5.5-conjugated rat anti-mouse CD19 (clone 1D3; BD) to perform the flow analyses. To avoid non-specific binding, 1×10^6 cells were incubated with 20% normal goat serum (Sigma, St. Louis, MO) for 10 min at 4°C; this was followed by incubation with FITC-, PE-, PE-Cy5-, and PerCP-Cy5.5-conjugated monoclonal antibodies for 30 min at 4°C in the dark. The cells were washed twice with FCS-MEM, re-suspended at 1×10^6 cells per tube in 1 ml of PBS, and then analyzed with the FACSCaliber flow cytometer and its Cell Quest program. For each sample, 10,000 events were collected and analyzed for antigen expression.

Statistical analysis

The data were transformed logarithmically to equalize the variance, and analysis of variance (ANOVA) was used to evaluate the results. For statistically significant ANOVA, the differences between groups were assessed by using Dunnett's multiple comparison test. A p-value < 0.05 was considered to indicate statistical significance.

Results

Preliminary in vitro study (confirmation of apoptosis)

In vitro analyses were performed to evaluate the extent of apoptosis resulting from parathion, methoxychlor, or piperonyl butoxide treatment. Caspase-3/7 activities (Figures 1a,d, and g) increased significantly relative to control levels in the methoxychlor (1–24 h: 1000 nmol), parathion (2–24 h: 1000 nmol), and piperonyl butoxide (0.5–24 h: 1000 nmol) treatment groups. Caspase-8 activity (Figures 1b, e, and h) increased significantly relative to control values in the methoxychlor (4 h: 1000 nmol) and piperonyl butoxide (2–4 h: 1000 nmol) treatment groups. Caspase-9 activity (Figures 1c, f, and i) increased significantly relative to control levels in the methoxychlor (2–4 h: 1000 nmol, 24 h: 100 and 1000 nmol) and piperonyl butoxide (2–24 h: 1000 nmol) treatment groups.

Flow cytometric analysis was used to evaluate the extent of apoptosis resulting from methoxychlor, parathion, or piperonyl butoxide treatment. The analyses revealed that the proportion of Annexin-V^+PI^- cells (Figure 2) increased significantly relative to the same values for the controls in the methoxychlor (1 h: 1000 nmol, 4 h: 100 and 1000 nmol), parathion (1 and 4 h: 1000 nmol), and piperonyl butoxide (1 and 4 h: 1000 nmol) treatment groups. The proportion of caspase-3^+ cells (Figure 3) increased significantly relative to those seen in the controls as a result of the methoxychlor (1 h: 1000 nmol, 4 h: 100 and 1000 nmol), parathion (4 h: 100 and 1000 nmol), and piperonyl butoxide (4 h: 1000 nmol) treatments.

In vivo study (confirmation of anti-SRBC IgM responses in mice)

The SRBC-specific IgM responses in serum are shown in Figure 4a The 30 and 300 mg methoxychlor/kg, as well as the 1.5 mg parathion/kg, treatments caused a significant decrease in responses relative to those seen with the vehicle controls. The piperonyl butoxide treatments cause a decreasing trend, but this was mild; no significant differences from control were noted.

The IgM plaque-forming cell (PFC) responses to SRBC in splenocyte are shown in Figure 4b. The 300 mg methoxychlor/kg treatment caused a significant decrease

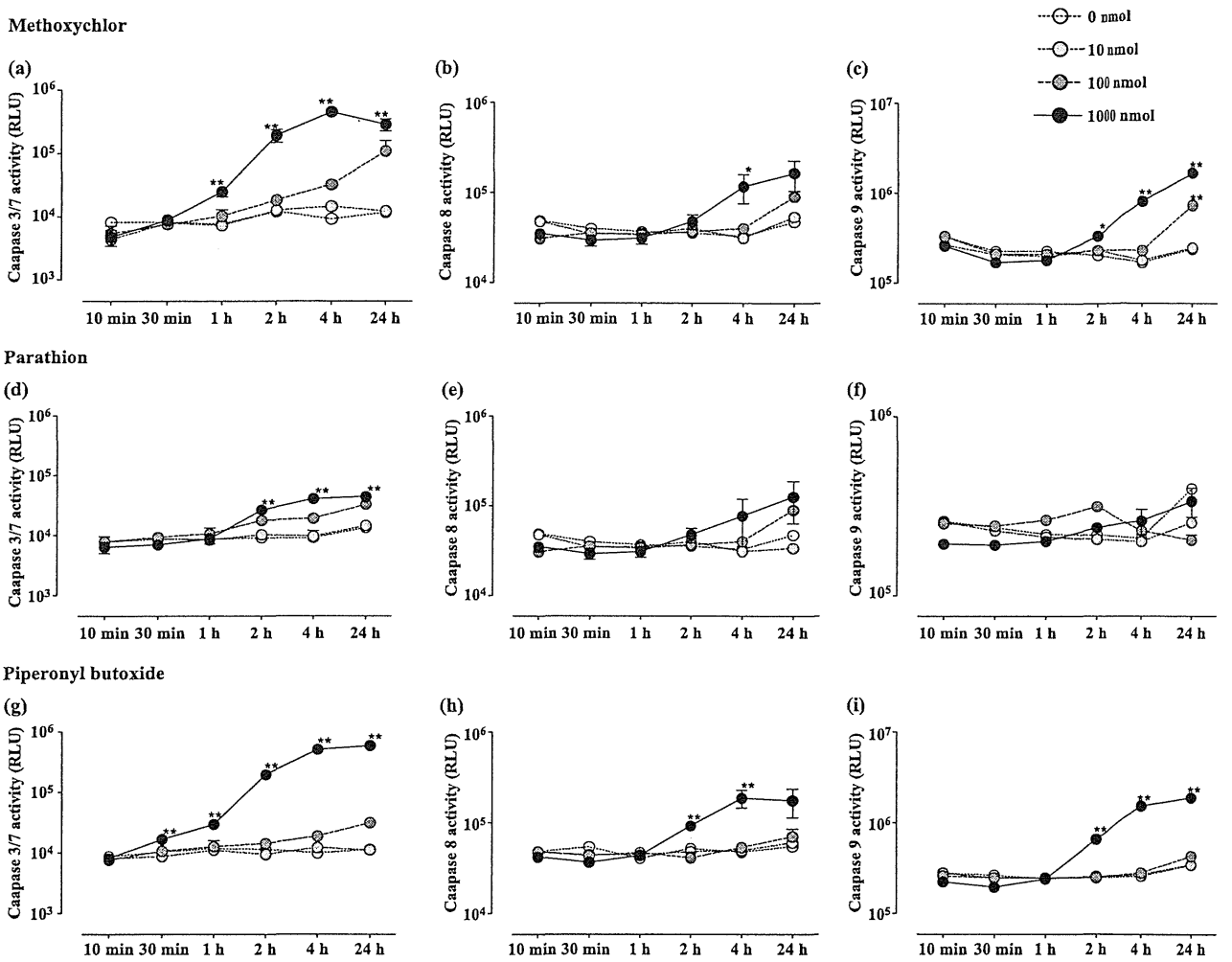

Figure 1. Caspase-3/7, -8, and -9 activities in Jurkat T-cells treated with test chemicals for 10 min to 24 h. Caspase-3/7 (a: Methoxychlor; d: Parathion; g: Piperonyl butoxide), Caspase-8 (b: Methoxychlor; e: Parathion; h: Piperonyl butoxide), and Caspase-9 (c: Methoxychlor; f: Parathion; i: Piperonyl butoxide). All activities are expressed as mean (RLU) ± SD. Value significantly differs from control at * $p < 0.05$ and ** $p < 0.01$.

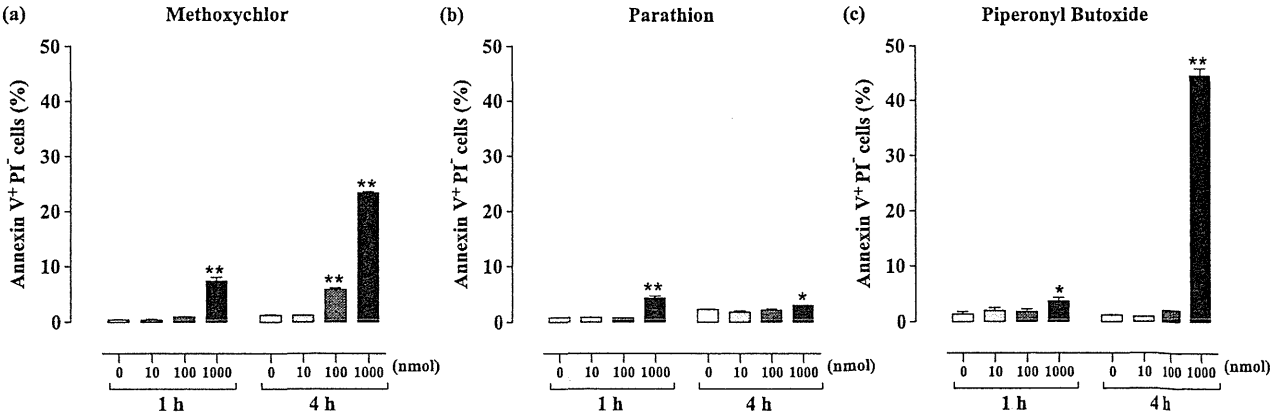

Figure 2. Annexin V-FITC positive cells among Jurkat T-cells treated with test chemicals for 1 or 4 h. (a) Methoxychlor; (b) Parathion; and (c) Piperonyl butoxide. Annexin V+-FITC+ cells are expressed as the mean (proportion of total, %) ± SD. Value significantly differs from control at * $p < 0.05$ and ** $p < 0.01$.

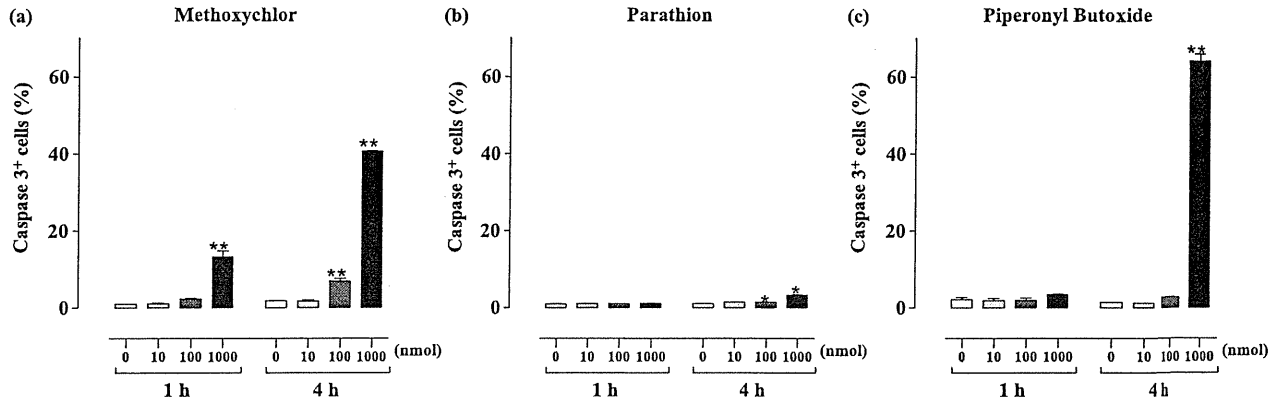

Figure 3. Caspase-3+ cells among Jurkat T-cells treated with test chemicals for 1 or 4 h. (a) Methoxychlor; (b) Parathion; and (c) Piperonyl butoxide. Caspase-3+ cells are expressed as the mean (proportion of total, %) ± SD. Value significantly differs from control at * $p < 0.05$ and ** $p < 0.01$.

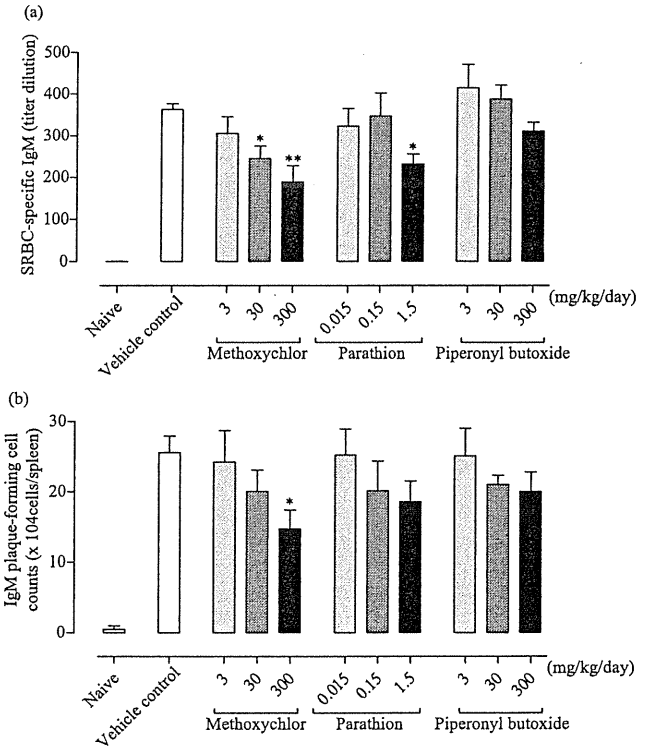

Figure 4. Anti-SRBC IgM response in C3H/He female mice treated with test chemicals. (a) SRBC-specific IgM response in serum. (b) Splenocyte IgM plaque-forming cell responses to SRBC. Values (titer dilution and cell counts) are expressed as mean ± SD. Value significantly differs from control at * $p < 0.05$ and ** $p < 0.01$.

in this parameter relative to the value seen with the vehicle controls. Both the parathion and piperonyl butoxide treatments led to a decreasing trend in this measure, but these decreases were mild and the final values did not significantly differ from the control values.

In vivo study (flow cytometric analysis)

To evaluate the activation of B-lymphocytes in the spleen following methoxychlor, parathion, and piperonyl butoxide treatment, flow cytometric analysis was performed using the lymphocytes stained with anti-CD19, -CD45R/B220, -IgD, -IgM, and -peanut agglutinin antibodies. Total cell counts in the spleen are shown in Figure 5a. The piperonyl butoxide 300 mg/kg treatment caused a significant decrease in this value relative to that in the vehicle controls. The methoxychlor treatments led to a decreasing trend as well, but no significant differences were noted. The numbers and ratio of IgM-positive B-lymphocytes ($B220^+IgD^+IgM^+$) are shown in Figures 5d and e. The 300 mg methoxychlor/kg, 1.5 mg parathion/kg, and the 30 and 300 mg piperonyl butoxide/kg treatments led to a significant decrease relative to values seen in the corresponding

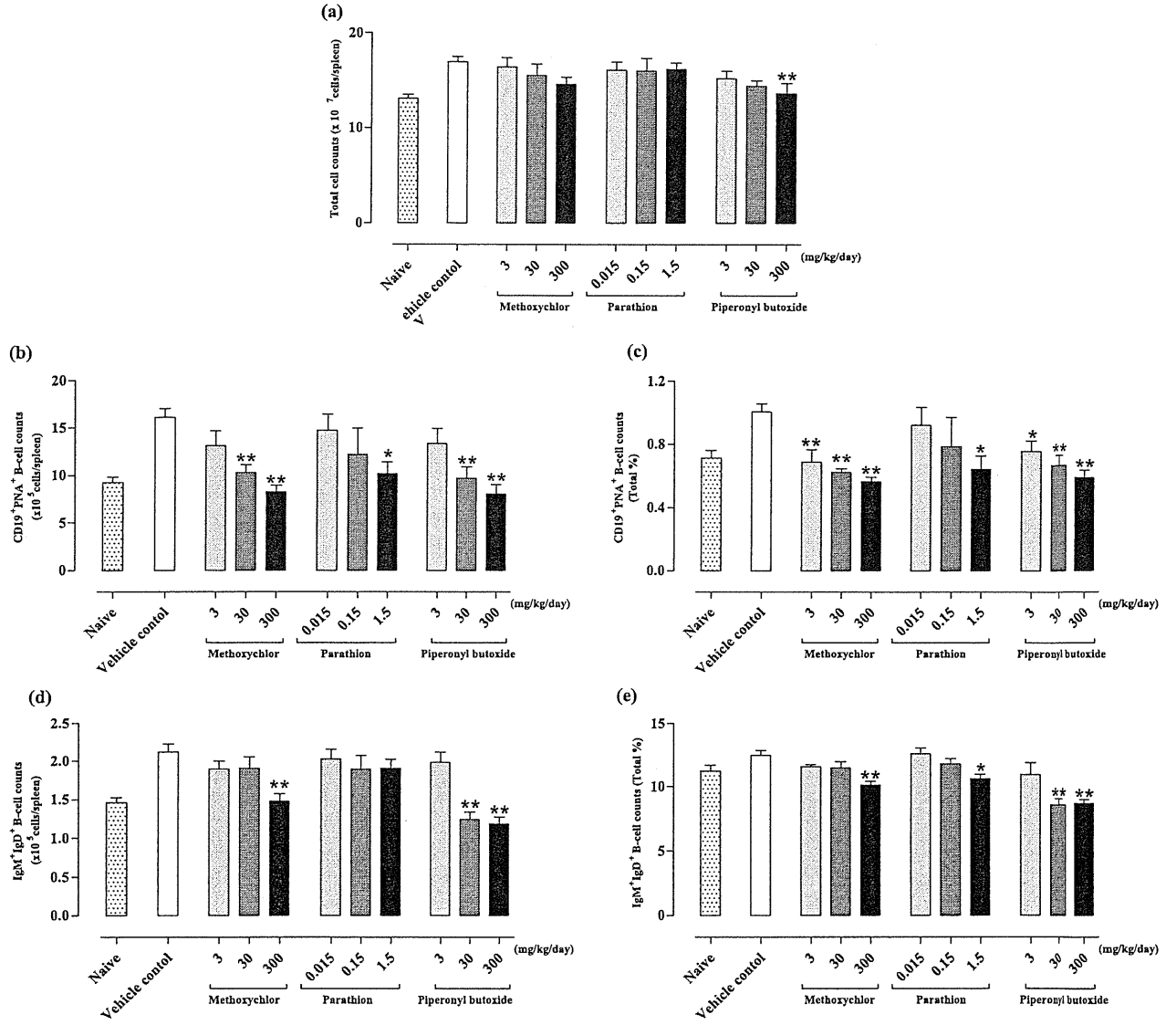

Figure 5. Flow cytometric analyses of splenocytes from C3H/He female mice treated with test chemicals. (a) Total cell counts; (b) germinal center-positive B-lymphocyte counts (CD19+PNA+); (c) germinal center-positive B-lymphocyte counts (CD19+PNA+, proportion of total, %); (d) IgM+ B-lymphocyte counts (B220+IgD+IgM+); and (e) IgM+ B-lymphocyte counts (B220+IgD+IgM+, proportion of total, %) in spleen. Counts are expressed as mean ± SD. Value significantly differs from control at * $p < 0.05$ and ** $p < 0.01$.

cells from vehicle control hosts. The numbers and ratio of germinal center B-lymphocytes ($CD19^+PNA^+$) are shown in Figures 5b and c. The treatments with 3, 30, and 300 mg methoxychlor/kg, 1.5 mg parathion/kg, and 3, 30, and 300 mg piperonyl butoxide/kg caused significant decreases in this parameter relative to the values noted with the vehicle controls.

Discussion

Our primary objective in this study was to improve upon the current method of detecting environmental chemical-related immunotoxicity. To that end, we exposed mice by using a short-term exposure protocol (i.e. 5 days) to commonly used immunosuppressive chemicals, namely the organochlorine agent methoxychlor, the organophosphorus agent parathion, and the agricultural insecticide synergist piperonyl butoxide. We then assessed the effects of this short-term exposure via several types of detection methods, including induction of lymphocyte apoptosis in Jurkat T-cells, anti-SRBC IgM responses in serum and spleen, and numbers of IgM- and germinal center-positive B-lymphocytes in the spleen. Our results showed that methoxychlor, parathion, and piperonyl butoxide each could modulate the apoptosis of Jurkat T-cells *in vitro*. To assess apoptosis, caspase activities (3/7, 8, and 9), and the proportion of Annexin V- and caspase-3^+ cells were examined. In mice, chemical-related immunotoxicity was detected by using our short-term exposure protocol. Indeed, all three chemicals induced

prominent immuno-suppressive responses, including reducing the anti-SRBC IgM response (SRBC-specific IgM levels in serum and the IgM PFC response to SRBC in splenocyte), and the numbers of IgM- and germinal center-positive B-lymphocytes in the spleen.

Apoptosis is an essential process underlying multicellular organism development and function. In the immune system, apoptosis is required for lymphocyte development and homeostasis. Dysregulation of apoptosis leads to a variety of immune disorders, including immunodeficiency, tumorogenesis, allergies, and autoimmunity (Zhang et al., 2005). Detection of chemical-induced thymocyte apoptosis *in vivo* is difficult, however, because of the rapid clearance of apoptotic cells by phagocytes (Savill and Haslett, 1995; Kamath et al., 1997; Pryputniewicz et al., 1998). Therefore, in the current study, we first confirmed the immunosuppressive effects of methoxychlor, parathion, and piperonyl butoxide by assessing changes in the occurrence of apoptosis in a human leukemia cell line, Jurkat E6.1.

We observed a significant increase in caspase activities (3/7, 8, and 9) and in the proportion of Annexin V^+ and caspase-3^+ cells that resulted from exposure to the test chemicals (Figures 1–3). Annexin V^+ staining is reported to detect apoptosis at an early stage, allowing visualization of alterations in the cell membrane that occur when phosphatidylserine residues move to the external leaflet of the plasma membrane (Morris et al., 1984; Vermes et al., 1995). Caspases are required in the apoptotic pathway; therefore, measurement of caspases-3/7, -8, and -9 reliably indicates caspase-dependent apoptosis (Gurtu et al., 1997). Caspase-3 and -7 were the first caspases linked to apoptosis, and, together with caspase-8 and -9, act upstream of DNA fragmentation in the pathway. Our findings suggest that methoxychlor and piperonyl butoxide induced T-lymphocyte apoptosis *in vitro* via several caspase pathways. In contrast, parathion induced T-lymphocyte apoptosis via mainly the caspase-3 pathway. The immunomodulation demonstrated *in vitro* in this study may reflect what could occur *in situ*, i.e. these types of reactions in a growing fetus might predispose the highly sensitive fetal immune system to a loss of tolerance to self-antigens and lead to a subsequent increased risk for autoimmune disease and allergies as the offspring develops after birth.

On the basis of our preliminary *in vitro* data, we then performed our primary study using female C3H/He mice (7-weeks-old). To detect environmental chemical-related immunotoxicity, we focused on short-term exposure (i.e., 5 days) and several immune endpoints. Among them, we used a T-dependent antigen response (TDAR) test primarily and measured the antigen (SRBC)-specific IgM response using an ELISA and a PFC assay. There is no doubt that the antigen-specific IgM antibody plays a key role in all types of immune response to immunotoxic chemicals (Anderson et al., 2006). The SRBC-specific IgM ELISA utilizes solubilized, hemoglobin-free SRBC membranes, and measures SRBC-specific antibodies in serum that are generated by all antibody-producing tissues (i.e. spleen, lymph nodes, bone marrow), reflecting the systemic humoral immune response (Temple et al., 1993). On the other hand, the PFC assay has been well characterized across multiple labs, and is likely the most validated endpoint in immunotoxicology.

Combining the use of these two assays allows for studies to evaluate mechanisms of action of xenobiotic-induced immunotoxicity (Herzyk and Holsapple, 2007). In our study, methoxychlor, parathion, and piperonyl butoxide induced a prominent decrease both in antigen-specific serum IgM levels and IgM PFC counts compared to the vehicle controls. Generally speaking, these chemicals have immunosuppressive effects, and our preliminary study suggests that apoptosis was induced in Jurkat T-cells. Therefore, this down-regulation demonstrates that our protocol and methods were effective for identifying environmental chemical-related immunotoxicity.

In addition to the serum and spleen antigen-specific IgM responses, we analyzed the IgM- and IgD-expressing B-cell populations in spleen by use of flow cytometry. The first antibodies to be produced in a humoral immune response are always IgM, because IgM can be expressed without isotype switching. Surface IgM- and IgD-expressing B-cells are necessary for IgM production (Janeway et al., 2004). According to our results, significant decreases in the IgM-expressing B-lymphocyte population were observed in both the methoxychlor- and piperonyl butoxide-treated groups (Figure 5). This down-regulation of IgM-expressing B-lymphocytes would thus be a useful endpoint for identifying chemical-related immunotoxicity.

Some B-cells are activated at the T-/B-lymphocyte border and migrate to form a germinal center within a primary follicle. Germinal centers are sites of rapid B-lymphocyte proliferation and differentiation (Janeway et al., 2004). Therefore, the germinal center and germinal center B-lymphocyte development represent major responses to exposure to T-lymphocyte-dependent antigen (Vieira and Rajewsky, 1990; Takahashi et al., 1998). We used PNA as a representative germinal center B-lymphocyte surface antigen because all germinal center B-lymphocytes bind PNA, beginning at the earliest stages of germinal center formation (Shinall et al., 2000). We observed dose-dependent and significant decreases in the PNA^+ B-lymphocyte population in each chemical-treated group (Figure 5). These results support our belief that this short-term exposure protocol could be used to detect immune suppression followed by chemical treatment.

Conclusions

Our protocol detected environmental chemical (i.e. methoxychlor, parathion, and piperonyl butoxide)-induced immunotoxic responses, such as increased apoptosis in lymphocytes *in vitro*, decreased antigen-specific

IgM responses, and decreased IgM- and germinal center-positive B-lymphocyte counts. Additional studies to confirm these results should be expanded to include other parallel changes in cellular function that can occur in response to chemical exposure, as well as immunologic or histologic markers. Our ongoing studies continue to focus on the detection of weak immunotoxic reactions using this short-term exposure protocol. However, our results and protocols have yet to be formally validated, which is a pre-requisite for inclusion in guidelines endorsed by regulatory authorities. Validation experiments therefore represent the next important task for us to undertake.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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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 (6×10^7 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 ($C_{16}H_{15}Cl_3O_2$)	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 (≈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 ≈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 ≈40.9% and 29.5%, respectively. Furthermore, the MXC + PARA- treated mice had a significantly decreased (≈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 ≈36.3%) was not significant. Similarly, the PBO + MXC mice had a significantly decreased response compared with the PBO ≈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 ≈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 (≈38.3%, $p<0.01$) from PBO mice values; there

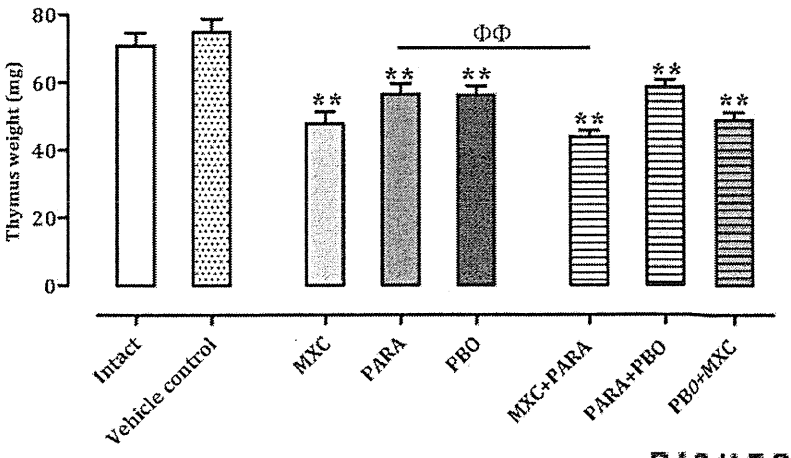

Figure 1. Absolute thymus weights. Mice were treated with nothing (intact naive), vehicle, methoxychlor (MXC), parathion (PARA), piperonyl butoxide (PBO), or combinations of the agents (two at a time). Absolute thymus weights are expressed as mean ± SD (mg; $n=8$ per group). **$p<0.01$ (Dunnett's multiple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

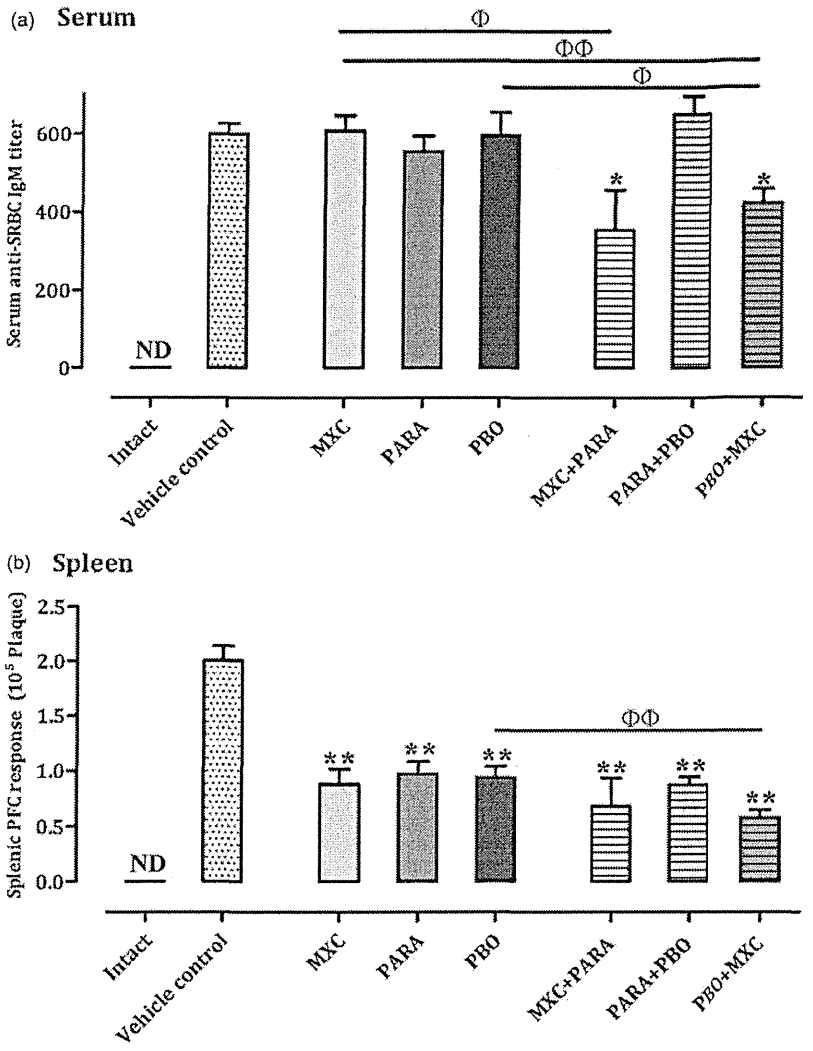

Figure 2. Serum and splenic IgM responses. Mice were treated as described in the Figure 1 legend. (a) Serum and (b) spleen IgM responses are shown. IgM responses are expressed as mean ± SD (titre; $n = 8$ per group). IgM responses in the spleen are expressed as mean ± SD ($n = 8$ per group). $^*p < 0.05$ 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

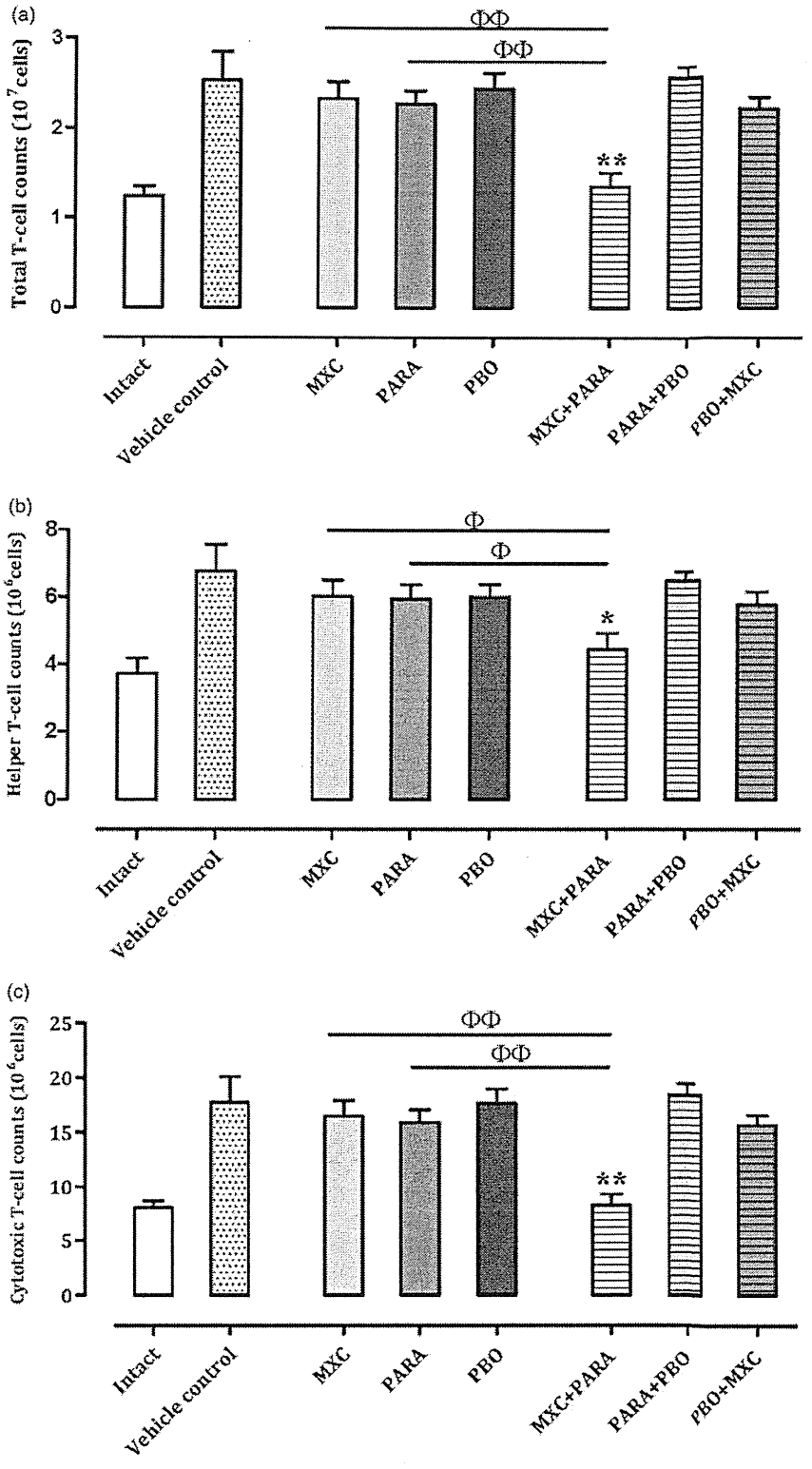

Figure 3. T-cell sub-type counts in spleens. Mice were treated as described in the Figure 1 legend. (a) Total, (b) helper, and (c) cytotoxic T-cell counts are shown. Results for intact, vehicle, and individual agent-treated mice are included in each chart. Cell counts are expressed as mean ± SD ($n = 8$ 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

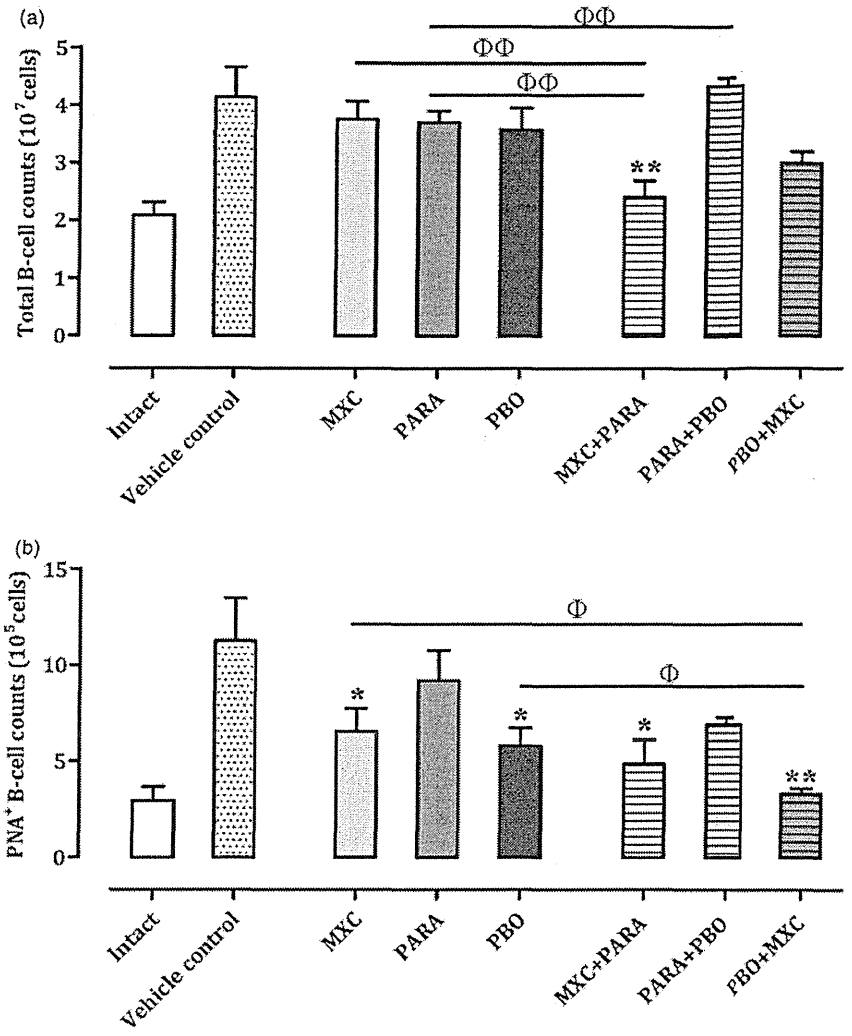

Figure 4. Total B-cell and germinal center B-cell counts in spleens. Mice were treated as described in the Figure 1 legend. (a) Total B-cell and (b) germinal center B-cell counts are shown. Results for intact, vehicle, and individual agent-treated mice are included in each chart. Cell counts are expressed 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.

from their individual agent counterparts. These results suggested to us that, at the level of thymocyte damage (i.e. potential apoptosis), there was little to no interactive effect from the combined exposures to PARA + PBO and PBO + MXC exposures. In contrast, as MXC + PARA exposure appeared to have induced a more severe effect compared with exposure to each individual chemical, it is likely that some interactive effect (most likely synergistic) was occurring *in situ* to amplify the toxicities of each individual test agent. As will become clear below, this *preferentially* strong toxicity by MXC + PARA compared to the other combinational regimens becomes evident in several other aspects of these studies.

SRBC is a common antigen used to evaluate general immune status. After immunization with SRBC, SRBC-specific IgM responses in serum and spleen can be assessed using ELISA and PFC assays, respectively (Temple et al., 1993; White et al., 2010). Use of these two assays allows for evaluation of the mechanisms of action of xenobiotic-induced immunotoxicities (Herzyk & Holsapple, 2007). Compared with the vehicle control mice, MXC + PARA and PBO + MXC mice had significant decreases in serum SRBC-specific IgM responses, whereas MXC, PARA, PBO, and PARA + PBO mice did not. In addition, the IgM responses with the MXC + PARA mice were significantly decreased vs that of MXC mice, and PBO + MXC treatment led to significant decreases relative to those seen with PBO and MXC mice. In contrast, in all groups given a test substance, spleen SRBC-specific IgM PFC responses were significantly decreased relative to those seen with the vehicle control group. In addition, the SRBC-specific IgM responses with spleens from the PBO + MXC mice were significantly decreased compared to that of organs from PBO mice. Based on our historic data (Fukuyama et al., 2013), the peak response to SRBC for the SRBC-specific IgM ELISA occurs ~2 days after the maxima that would be used to optimize results for a PFC. Thus, as we utilized a protocol that was focusing mainly on the PFC assay, it is not a complete surprise that the SRBC-specific IgM ELISA responses were weaker than the PFC ones. Under these conditions, MXC + PARA and PBO + MXC led to significant decreases relative to those seen with PBO and MXC in serum SRBC-specific IgM responses. These results suggested to us that MXC + PARA or PBO + MXC exposures induced a more severe reduction in humoral immune responses compared with exposure to any of the three individual chemicals.

To further clarify mechanisms of MXC + PARA- or PBO + MXC-induced immunosuppression, total, helper, and cytotoxic T-cell counts, as well as total and germinal center B-cell counts in the spleens were analyzed via flow cytometry based on cell-specific surface makers (Janeway et al., 2004). It was clear that the MXC + PARA combined treatment damaged T-cells. Total, helper, and cytotoxic T-cell counts in hosts that received this combined treatment were decreased compared with those in vehicle control, MXC, and PARA mice. In contrast,