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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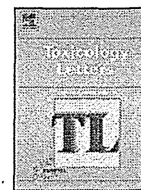

Prior exposure to organophosphorus and organochlorine pesticides increases the allergic potential of environmental chemical allergens in a local lymph node assay

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ABSTRACT

The dysregulation of immune functions by some pesticides leads to various immune disorders, including immunodeficiency, tumorigenesis, allergies, and autoimmunity. This study's primary objective was to examine the relationship between immune disorders and the immunosuppression induced by immunosuppressive pesticides. We focused on the modulation of allergic potential by the organophosphorus pesticide parathion, organochlorine pesticide methoxychlor, phenoxyacetic acid herbicide 2,4-D-butyl, and benzoic acid fungicide eugenol, as detected by a local lymph node assay (LLNA), which was developed initially for hazard identification of skin sensitization. Parathion and methoxychlor are immunosuppressive chemicals, and 2,4-D-butyl and eugenol are contact allergens. After the immunosuppressive characteristics of parathion and methoxychlor were confirmed in a pilot study, 4-week-old mice were orally administered parathion (0, 0.4, 1.2 mg/kg) or methoxychlor (0, 100, 300 mg/kg). Four weeks after the last administration, an LLNA was conducted using 2,4-D-butyl (0%, 2.5%, 5%, and 10%) and eugenol (0%, 5%, 10%, and 25%). In addition, detailed analysis of their auricular lymph nodes for number of surface antigen expression of T cells and local cytokine production were performed using 5% 2,4-D-butyl and 5% eugenol treatment groups. EC3 values (estimated concentration to yield a stimulation index of 3) of 2,4-D-butyl and eugenol decreased markedly in parathion- and methoxychlor-pretreated groups. Parathion- and methoxychlor-pretreated groups induced marked increase in number of surface antigen expression of T cells and levels of Th1 cytokines (IFN-γ, TNF-α, and IL-17) produced by ex vivo restimulated lymph node cells. According to our results, the allergic potentials of 2,4-D-butyl and eugenol are increased by prior exposure to parathion and methoxychlor.

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1. Introduction

Organochlorine (OC) and organophosphorus (OP) pesticides play roles in the dysregulation of immune functions (Hirai and Ichikawa, 1991; Kawashima et al., 1990; Kohler et al., 1987; Kosuda et al., 1996; Li et al., 2007, 2009; Nakadai et al., 2006; Pieters et al., 1989; Staples et al., 1998; Zelikoff et al., 1994). OCs such as methoxychlor were widely used in agriculture and pest control after being introduced in the 1940s. Because of their persistence in the environment, bioaccumulation in the food chain, and possible health effects, the U.S. Environmental Protection Agency restricted and banned the use of most of OCs during the 1970s and 1980s. Although OCs are rarely used today, measurable amounts of OCs or their metabolites can still be found in human tissues. Moreover, OCs continue to be heavily used in some developing countries, primarily for mosquito and malaria control (Turusov et al., 2002; Xu et al., 2010). In a previous study by our group, methoxychlor exposure resulted in atrophy of $CD4^+CD8^+$ double-positive T-lymphocytes in the thymus (Takeuchi et al., 2002, 2004). In addition, Chapin et al. (1997) reported that doses of 150 $mg\,kg^{-1}\,day^{-1}$ of methoxychlor in rats led to markedly decreased thymus weights.

OPs such as parathion were introduced as replacements for OCs led to the legal ban or restriction of their use in the 1970s (Peakall et al., 1975; Ellenhorn and Barceloux, 1988; Wessels et al., 2003; Nakadai et al., 2006; Lopez et al., 2007; OECD, 2008). The widespread exposure of humans to OPs has been demonstrated by the presence of common and specific OPs metabolites in urine samples taken from the general population (Bouvier et al., 2005; Cocker et al., 2002; Bradman et al., 2007; Lacasana et al., 2010). Several previous studies demonstrated that exposure of mice to OPs led

Abbreviations: ANOVA, analysis of variance; DDT, dichlorodiphenyltrichloroethane; FITC, fluorescein isothiocyanate; IL, interleukin; LLNA, local lymph node assay; LN, lymph node; OC, organochlorine; OP, organophosphorus; PBS, phosphate-buffered saline; PE, phycoerythrin; SI, stimulation index; Th, T helper.

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to markedly decreased thymus and spleen weights (Casale et al., 1983; Jeong et al., 1995; Khalaf-Allah, 1999; Handy et al., 2002).

Our previous study demonstrated the immunomodulative action of an OP (parathion) and OC (methoxychlor) following in vitro exposure (Fukuyama et al., 2010a). Parathion and methoxychlor caused elevations in apoptosis-related factors, including annexin-V-expressing T-lymphocyte populations, caspase activities, and DNA fragmentation, in a Jurkat T-cell line and mouse primary thymocytes. Such factors, however, represent a tiny fraction of the immunosuppressive action of OPs and OCs, and the mechanisms are still not fully understood (Fukuyama et al., 2010a,b). In addition, current evidence suggests that the dysregulation of immune functions caused by several types of pesticides leads to a variety of immune disorders, including immunodeficiency, tumorigenesis, allergies, and autoimmunity (Sobel et al., 2005; Zhang et al., 2005; Narita et al., 2007; Wang et al., 2007; Ward et al., 2009; Xu et al., 2010).

The primary objective of this study was to examine the relationship between several types of immune disorders and the immunosuppressive reaction induced by OPs and OCs in vivo. We focused on the modulation of allergic potential and used a local lymph node assay (LLNA) for its detection. The chemicals used in the LLNA were the phenoxyacetic acid herbicide 2,4-D-butyl and the benzoic acid fungicide eugenol. These chemicals were chosen based on previous studies (Cushman and Street, 1982; Fukuyama et al., 2009).

2. Materials and methods

2.1. Chemicals

2,4-D-Butyl standard ($C_{12}H_{14}Cl_2O_3$, 98% pure), eugenol ($C_{10}H_{12}O_2$, >95% pure), methoxychlor standard ($C_{16}H_{15}Cl_3O_2$, >97% pure), parathion standard ($C_{10}H_{14}NO_5PS$, 99.5% pure), acetone, and olive oil were each purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Corn oil was purchased from Hayashi Chemicals (Tokyo, Japan). For the initial oral administration, methoxychlor and parathion were diluted in corn oil. For the dermal sensitization in the LLNA, eugenol and 2,4-D-butyl were dissolved in 4:1 acetone:olive oil. The maximum doses used in this study were selected to avoid systemic toxicity or excessive local sensitization (particularly in the preliminary test; data not shown), while still permitting comparisons of composite toxicological potencies.

2.2. Animals

Female BALB/c mice (age 3 weeks) (for the pilot and detailed study) and CBA/Jn mice (age 3 weeks) (for the LLNA study) were purchased from Charles River Japan Laboratories (Atsugi, Kanagawa, Japan) and housed individually under controlled lighting (lights on from 0700 to 1900 h), temperature (22 ± 3 °C), humidity (55 ± 15%), and ventilation (at least 10 complete fresh-air changes hourly). Food (Certified Pellet Diet MF, Oriental Yeast Co., Tokyo, Japan) and water were available ad libitum.

This study was conducted in accordance with the guidelines for animal experimentation of the Japanese Association for Laboratory Animal Science (1987).

2.3. Pilot study

After a 1-week acclimatization period, BALB/c mice (4 weeks old) were allocated randomly to dose and control groups for each chemical (methoxychlor and parathion, $n = 6$ per group). On days 1–5, each mouse received orally an aliquot of the test solution (methoxychlor or parathion) or solvent (corn oil) alone. On the day after the last administration (day 6), all mice were anesthetized with pentobarbital sodium (75 mg/kg) and sacrificed. Each animal's thymus and spleen were removed, weighed, and pooled by mouse in phosphate-buffered saline (PBS; Gibco, Tokyo, Japan). Single-cell suspensions of thymocytes and splenocytes in 5 ml of PBS supplemented with 5% heat-inactivated fetal calf serum (FCS, Gibco) were prepared by passage through a stainless-steel screen and sterile 70-μm nylon cell strainers (Falcon, Tokyo, Japan). The number of thymocytes and splenocytes was determined with a Coulter counter Z2 (Beckman Coulter, Tokyo, Japan). Flow cytometric analysis was performed by staining thymocytes and splenocytes with fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse CD3 (clone 145-2C11), phycoerythrin (PE)-conjugated rat anti-mouse CD4 (clone RM4-5), PE-Cy5-conjugated rat anti-mouse CD8 (clone 53-6.7), and PE-Cy5-conjugated rat anti-mouse CD45R/B220 (clone RA3-6B2). All antibodies for flow cytometric analysis were purchased from BD Pharmingen (San Diego, CA, USA). To avoid nonspecific binding, 1×10^6 cells were incubated with 20% normal goat serum (Sigma, St. Louis, MO, USA) for 10 min at 4 °C; this was followed by incubation with FITC-, PE-, and PE-Cy5-conjugated monoclonal antibodies for 30 min at 4 °C in the dark. The cells were washed twice with a FCS–PBS, resuspended at 1×10^6 cells per tube in 1 ml of PBS, and then analyzed with a FACSCaliber flow cytometer (BD Pharmingen) using the Cell Quest program (BD Pharmingen). For each sample, 10,000 events were collected and analyzed for the expression of antigens.

2.4. Main study (local lymph node assay)

After a 1-week acclimatization period, CBA/J mice (4 weeks old) were allocated randomly to dose and control groups for each chemical (methoxychlor and parathion, $n = 20$ per group). On days 1–5, each mouse received orally an aliquot of the test solution or solvent (corn oil) alone. About 4 weeks after the last oral administration (day 31), the LLNA was performed as described by Kimber and Weisenberger (1989), with minor modifications. Mice were allocated randomly to dose and control groups for each chemical (2,4-D-butyl and eugenol, $n = 5$ per group). A 25-μl aliquot of test solution or solvent (4:1 acetone:olive oil) only was applied daily to the dorsum of each ear of each mouse for three consecutive days (days 31–33). In the LLNA, on day 36, ^{3}H-methyl thymidine (^{3}H-TdR, 20 μCi/animal; Perkin Elmer Japan, Tokyo, Japan) was injected via the tail vein into all test and control mice. At 5 h after the injection, the mice were euthanized, and the auricular lymph nodes (LNs) on both sides of each mouse were removed, weighed, and pooled by mouse in PBS (Gibco). Single-cell suspensions of LNs in 5 ml of PBS were prepared by passage through sterile 70-μm nylon cell strainers (Falcon). The LN cell suspension was washed twice with an excess of PBS, and the cell pellet was incubated in 3 ml of 5% trichloroacetic acid (Wako Pure Chemical Industries Ltd.) at 4 °C for approximately 18 h. Each cell pellet was resuspended in 1 ml of trichloroacetic acid and transferred to 9 ml of scintillation fluid (AtomLight, Perkin Elmer Japan). Incorporation of ^{3}H-TdR was measured with a β-scintillation counter (LC-5100, Aloka, Tokyo, Japan) as disintegrations per minute for each mouse.

Stimulation indexes (SIs) and EC3 values were calculated from the ^{3}H-TdR incorporation data. The SI was calculated by dividing the mean ^{3}H-TdR incorporation value for each treatment group by that of the control group. The EC3 value is an estimate of the amount of test solution required to induce an SI of 3 (Basketter et al., 1999). In the standard LLNA, the criterion for a positive response is an SI of 3 or greater (Dearman et al., 1999).

2.5. Main study (detailed analysis)

After a 1-week acclimatization period, Balb/c mice (4 weeks old) were allocated randomly to dose and control groups for each chemical ($n = 18$ per group). On days 1–5, each mouse received orally an aliquot of the test solution or solvent (corn oil) alone. About 4 weeks after the last oral administration (day 31), mice were allocated randomly to dose and control groups for each chemical ($n = 6$ per group). A 25-μl aliquot of test solution or solvent only was applied daily to the dorsum of each ear of each mouse for three consecutive days (days 31–33). To confirm the allergenicity of the test chemicals, we adopted a sensitization dose that was less than the EC3 values (see Table 1). On day 36, the mice were euthanized, and the LNs on both sides of each mouse were removed and pooled by mouse in PBS. For cell counts, flow cytometry, RNA analysis and cell culture, single-cell suspensions of LNs in 1 ml RPMI 1640 (Gibco) supplemented with 5% FCS were prepared by passage through sterile 70-μm nylon cell strainers. The number of LN cells was determined with a Coulter Z2 counter.

Flow cytometric analysis was performed by staining lymphocytes with FITC-conjugated hamster anti-mouse CD3 (clone 145-2C11), PE-conjugated rat anti-mouse CD4 (clone RM4-5), and PE-Cy5-conjugated rat anti-mouse CD8 (clone 53-6.7). All antibodies for flow cytometric analysis were purchased from BD Pharmingen. To avoid nonspecific binding, 1×10^6 cells were incubated with 20% normal goat serum for 10 min at 4 °C; this was followed by incubation with FITC-, PE- and PE-Cy5-conjugated monoclonal antibodies for 30 min at 4 °C in the dark. The cells were washed twice with a FCS–PBS, resuspended at 1×10^6 cells per tube in 1 ml of PBS, and then analyzed with a FACSCaliber flow cytometer using the Cell Quest program. For each sample, 10,000 events were collected and analyzed for the expression of antigens.

To stimulate T-cell-receptor signaling, we cultured single-cell suspensions of LN (1×10^6 cells/well) with anti-CD3 (2 μg/ml) (BD Pharmingen) T-cell antibodies for 24 h in 24-well plates (Multidish, Nalge Nunc International K.K., Tokyo, Japan) at 37 °C in a 5% carbon dioxide atmosphere. Tumor necrosis factor-alpha (TNF-α), interferon gamma (IFN-γ) and interleukin-17 (IL-17) in the supernatants were assayed by BD cytometric bead array (BD Pharmingen) in accordance with the manufacturer's protocol.

2.6. Statistical analysis

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

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Table 1
Local lymph node assay results of the 2,4-D-butyl group pretreated with parathion.

Pretreatment at 4 weeks (parathion)	Dose of LLNA (2,4-D-butyl)	Final body weight (g; mean ± SD)	Lymph node weight (mg; mean ± SD)	^{3}H-TdR incorporation	
				DPM (mean ± SD)	SI[a]
0 mg/kg	0%	20.8 ± 1.2	3.85 ± 0.90	134 ± 40	1.0
	2.5%	21.1 ± 3.2	4.48 ± 1.27	222 ± 92	1.7
	5%	21.7 ± 2.6	4.70 ± 0.70	322 ± 49**	2.4
	10%	21.4 ± 1.6	6.26 ± 1.02**	461 ± 42**	3.5
0.4 mg/kg	0%	21.0 ± 1.1	3.20 ± 0.80	130 ± 38	1.0
	2.5%	21.4 ± 1.6	5.58 ± 0.87*	601 ± 252	4.6
	5%	20.9 ± 1.6	6.75 ± 1.55**	1054 ± 214*	8.1
	10%	21.8 ± 1.1	9.66 ± 0.81**	2107 ± 832**	16.1
1.2 mg/kg	0%	20.9 ± 1.6	3.88 ± 0.22	129 ± 52	1.0
	2.5%	21.2 ± 1.1	8.45 ± 1.19**	816 ± 367	6.3
	5%	21.0 ± 1.1	11.56 ± 1.86**	2106 ± 683**	16.3
	10%	21.5 ± 1.6	12.52 ± 2.22**	2957 ± 243**	22.9

DPM: disintegrations per minute.
* Values for treatment and control groups were compared by Dunnett's multiple comparison test: $P < 0.05$.
** Values for treatment and control groups were compared by Dunnett's multiple comparison test: $P < 0.01$.
[a] Stimulation index (SI) is the ratio of the mean of the treatment group to that of the vehicle control group. The criterion for a positive response in the standard LLNA is an SI of 3 or greater.

3. Results

3.1. Pilot study

In the mice treated with parathion, both thymus weights and double-positive T-cell counts decreased in a dose-dependent manner, and in the 1.2-mg/kg treatment group were significantly different than control (Fig. 1A and B). The spleen weights, helper T-cell counts, and B-cell counts decreased in a dose-dependent manner, and significant differences were noted in B-cell counts at a dose of 1.2 mg/kg (Fig. 1C–F).

In the mice treated with methoxychlor, both thymus weights and double-positive T-cell counts decreased in a dose-dependent manner, and statistically significant differences were found in the 100- and 300-mg/kg treatment groups (Fig. 2A,B). The spleen weights for both the 100- and 300-mg/kg treatment groups were similar to that of the control group. Helper and cytotoxic T-cell counts and B-cell counts decreased in a dose-dependent manner, and significant differences were found in the 300-mg/kg treatment group (Fig. 2C–F).

3.2. Main study (LLNA)

The LLNA results of the 2,4-D-butyl group pretreated with parathion are presented in Table 1. Pretreatment with solvent only induced a positive ^{3}H-TdR incorporation response, defined as $SI \geq 3$, in the 10% sensitization group. Parathion 0.4-mg/kg pretreatment induced a positive response in the 2.5%, 5%, and 10% sensitization groups. Parathion 1.2-mg/kg pretreatment induced a positive response in the 2.5%, 5%, and 10% sensitization groups. EC3 values of the 2,4-D-butyl group pretreated with parathion 0-, 0.4- and 1.2-mg/kg are 7.73, 1.39 and 0.94, respectively.

The results of the eugenol group pretreated with parathion are listed in Table 2. Pretreatment with solvent only induced a positive response in the 10% and 25% sensitization groups. Parathion 0.4-mg/kg pretreatment induced a positive response in the 5%, 10%, and 25% sensitization groups. Parathion 1.2-mg/kg pretreatment induced a positive response in the 5%, 10%, and 25% sensitization groups. EC3 values of the eugenol group pretreated with parathion 0-, 0.4- and 1.2-mg/kg are 5.28, 2.66 and 0.83, respectively.

The results of the 2,4-D-butyl group pretreated with methoxychlor are presented in Table 3. Pretreatment with solvent only induced a positive response in the 10% sensitization group. Methoxychlor 100-mg/kg pretreatment induced a positive response in the 5% and 10% groups. Methoxychlor 300-mg/kg pretreatment induced a positive response in the 2.5%, 5%, and 10% sensitization groups. EC3 values of the 2,4-D-butyl group pretreated with methoxychlor 0-, 100- and 300-mg/kg are 6.32, 3.19 and 1.32, respectively.

Table 2
Local lymph node assay results of the eugenol group pretreated with parathion.

Pretreatment at 4 weeks (parathion)	Dose of LLNA (eugenol)	Final body weight (g; mean ± SD)	Lymph node weight (mg; mean ± SD)	^{3}H-TdR incorporation	
				DPM (mean ± SD)	SI[a]
0 mg/kg	0%	20.3 ± 2.5	3.85 ± 1.14	304 ± 208	1.0
	5%	20.7 ± 1.4	5.28 ± 0.77	593 ± 159	2.0
	10%	20.8 ± 1.2	6.15 ± 0.62	2287 ± 705	7.5
	25%	20.5 ± 1.6	10.54 ± 1.93**	8964 ± 2588**	29.5
0.4 mg/kg	0%	20.6 ± 1.3	3.70 ± 0.48	205 ± 133	1.0
	5%	20.2 ± 1.5	4.35 ± 1.41	972 ± 448	4.7
	10%	20.7 ± 1.9	8.03 ± 0.99**	2931 ± 1843	14.3
	25%	20.5 ± 1.5	12.44 ± 1.80**	11,492 ± 2841**	56.1
1.2 mg/kg	0%	20.8 ± 1.8	4.50 ± 1.14	302 ± 170	1.0
	5%	20.7 ± 1.3	7.58 ± 1.72	2648 ± 1392	8.8
	10%	21.2 ± 1.1	11.46 ± 3.20**	6947 ± 3184**	23.0
	25%	20.0 ± 1.2	14.76 ± 2.64**	13,775 ± 2869**	45.6

DPM: disintegrations per minute.
** Values for treatment and control groups were compared by Dunnett's multiple comparison test: $P < 0.01$.
[a] Stimulation index (SI) is the ratio of the mean of the treatment group to that of the vehicle control group. The criterion for a positive response in the standard LLNA is an SI of 3 or greater.

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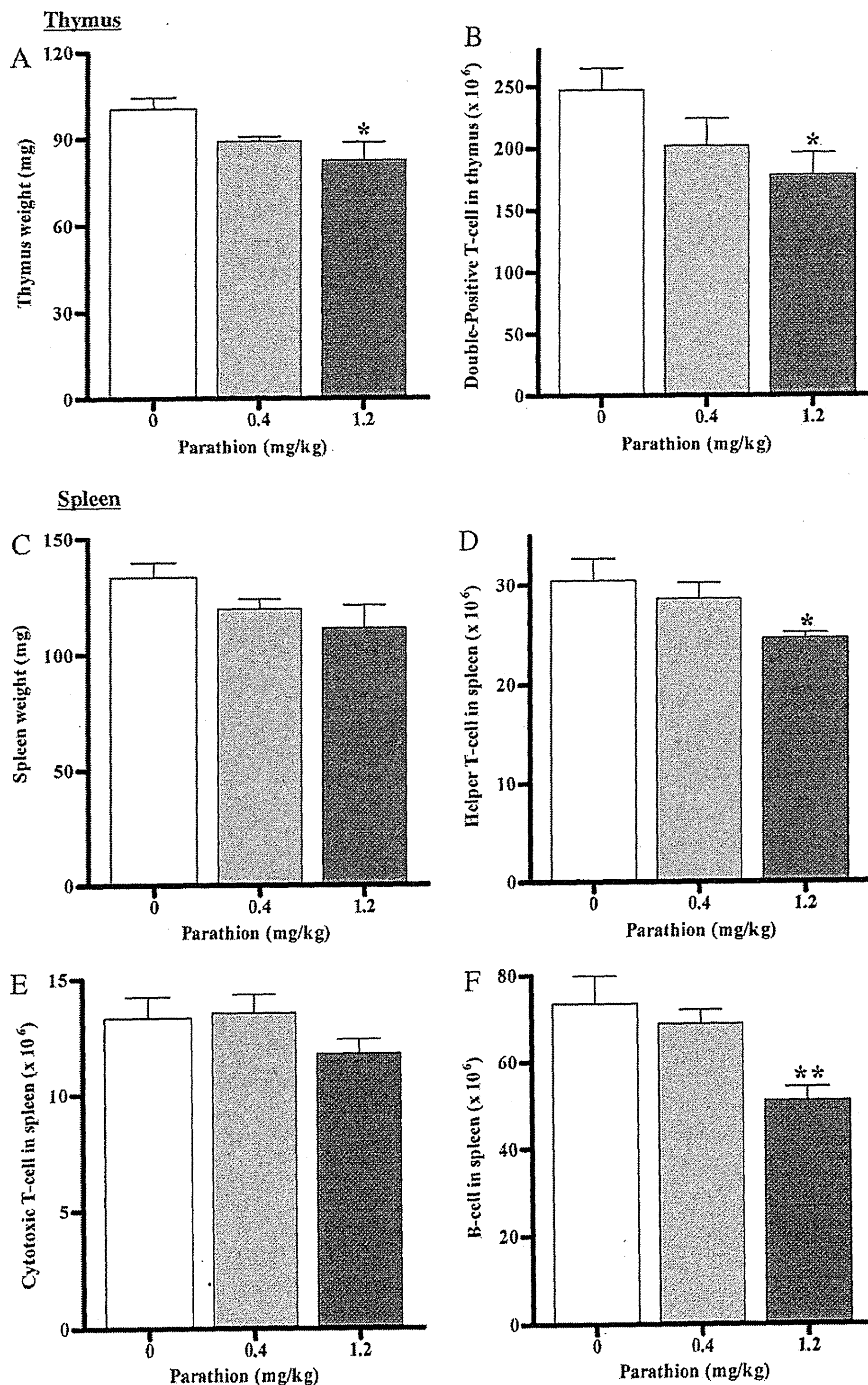

Fig. 1. Thymus weights (A), double-positive ($CD4^+CD8^+$) T-cell counts (B), spleen weights (C), helper ($CD4^+CD8^-$) T-cell counts (D), cytotoxic T-cell ($CD4^-CD8^+$) counts (E), and B-cell (CD45R/B220+) counts (F) of mice treated with parathion. Values are expressed as mean ± SD ($n = 6$ per group). Statistical significance is marked by asterisks: $^*P < 0.05$, $^{**}P < 0.01$ (Dunnett's multiple comparison test).

The results of the eugenol group pretreated with methoxychlor are listed in Table 4. Pretreatment with solvent only induced a positive response in the 10% and 25% sensitization groups. Methoxychlor 100-mg/kg pretreatment induced a positive response in the 5%, 10%, and 25% sensitization groups. Methoxychlor 300-mg/kg pretreatment induced a positive response in the 5%, 10%, and 25% sensitization groups. EC3 values of the eugenol group pretreated with methoxychlor 0-, 100- and 300-mg/kg are 6.45, 3.13 and 2.33, respectively.

3.3. *Main study (detailed analysis)*

Results are presented in Figs. 3 and 4, respectively. All parameters induced by AOO pretreated with parathion or methoxychlor

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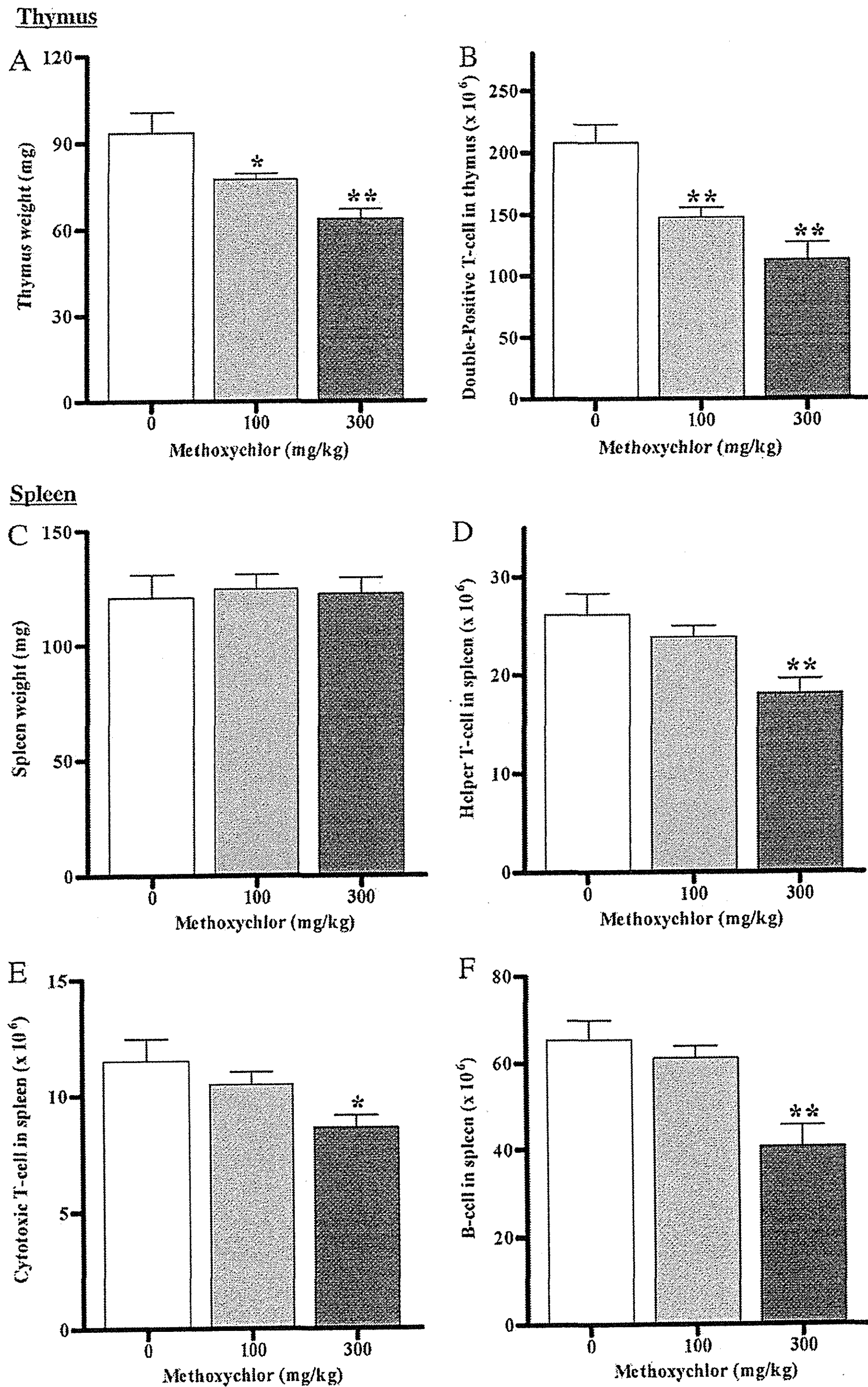

Fig. 2. Thymus weights (A), double-positive ($CD4^+CD8^+$) T-cell counts (B), spleen weights (C), helper ($CD4^+CD8^-$) T-cell counts (D), cytotoxic T-cell ($CD4^-CD8^+$) counts (E), and B-cell (CD45R/B220+) counts (F) of mice treated with methoxychlor. Values are expressed as mean ± SD ($n = 6$ per group). Statistical significance is marked by asterisks: $^*P < 0.05$, $^{**}P < 0.01$ (Dunnett's multiple comparison test).

revealed no increase. All parameters induced by 2,4-D-butyl or eugenol pretreated with parathion or methoxychlor were increased in a dose-dependent manner. All parameters in the parathion 1.2-mg/kg and methoxychlor 300-mg/kg pretreatment groups showed a significant increase relative to the control (0-mg/kg pretreatment) groups. IFN-γ, TNF-α and IL-17 productions in the parathion 0.4-mg/kg treatment groups showed a significant increase relative to the control groups. Cytotoxic T-cell counts, IFN-γ, TNF-α and IL-17 productions in the methoxychlor 100-mg/kg pretreatment group showed a significant increase relative to the control groups.

4. Discussion

The incidence of multiple chemical toxicity is increasing, especially in infants and children (Ban and Hettich, 2005; Hopkin, 1997; Howarth, 1998). This syndrome is characterized by a wide range of

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Table 3
Local lymph node assay results of the 2,4-D-butyl group pretreated with methoxychlor.

Pretreatment at 4 weeks (methoxychlor)	Dose of LLNA (2,4-D-butyl)	Final body weight (g; mean ± SD)	Lymph node weight (mg; mean ± SD)	^{3}H-TdR incorporation	
				DPM (mean ± SD)	SI[a]
0 mg/kg	0%	21.4 ± 1.5	4.63 ± 0.47	134 ± 76	1.0
	2.5%	21.7 ± 1.9	4.98 ± 0.24	254 ± 34	1.9
	5%	21.3 ± 1.8	4.55 ± 0.70	334 ± 26	2.5
	10%	21.2 ± 2.0	6.84 ± 1.23**	592 ± 249**	4.4
100 mg/kg	0%	21.8 ± 1.7	4.08 ± 0.19	122 ± 43	1.0
	2.5%	21.5 ± 2.1	5.80 ± 1.02	230 ± 58	1.9
	5%	21.2 ± 1.5	9.20 ± 3.04**	721 ± 248**	5.9
	10%	21.6 ± 1.4	9.60 ± 1.34**	1705 ± 208**	14.0
300 mg/kg	0%	21.3 ± 2.3	4.18 ± 0.76	129 ± 13	1.0
	2.5%	21.7 ± 2.6	7.80 ± 2.52*	616 ± 360	4.8
	5%	21.3 ± 2.2	9.00 ± 1.41**	843 ± 188*	6.5
	10%	21.2 ± 2.1	12.50 ± 1.35**	1942 ± 555**	15.1

DPM: disintegrations per minute.
* Values for treatment and control groups were compared by Dunnett's multiple comparison test: $P < 0.05$.
** Values for treatment and control groups were compared by Dunnett's multiple comparison test: $P < 0.01$.
[a] Stimulation index (SI) is the ratio of the mean of the treatment group to that of the vehicle control group. The criterion for a positive response in the standard LLNA is an SI of 3 or greater.

signs and symptoms triggered when affected patients come into contact with a number of chemical products (Bartha et al., 1999; Brown and Jason, 2007). Common triggers include pesticides and perfumes (Brown and Jason, 2007; Kreutzer et al., 1999). Although multiple chemical toxicity has emerged as a public health problem, detailed mechanisms underlying this syndrome remain unclear. In this study we focused on the modulation of allergic potential, which is one part of multiple chemical toxicity, and attempted to detect an immune disorder following exposure to several types of agricultural chemicals (parathion, methoxychlor, 2,4-D-butyl, and eugenol) using an LLNA. Parathion and methoxychlor are immunosuppressive chemicals, and 2,4-D-butyl and eugenol are contact allergens. Our findings demonstrated that the allergic potentials of 2,4-D-butyl and eugenol were increased by prior exposure to parathion and methoxychlor in vivo.

In a previous study, we reported elevations in apoptosis-related factors in both human and mouse T lymphocytes following in vitro parathion and methoxychlor exposure (Fukuyama et al., 2010a,b). These findings suggest that parathion and methoxychlor modulate T-cell activation, and these reactions may trigger several types of immune disorder. Therefore, in a pilot study we first evaluated the immunosuppressive response of parathion and methoxychlor in vivo using young (4 weeks old) female Balb/c mice. We found that both parathion and methoxychlor modulated the immune response, including thymus weight and the number of double-positive T cells in the thymus, helper and cytotoxic T cells in the spleen, and B cells in the spleen (see Figs. 1 and 2). These results matched exactly our previous in vitro results and there was a possibility that the breakdown of the central tolerance of autoreactive T or B cells by parathion and methoxychlor. Thus, in the main study, we focused on the immune disorder of allergic responses to investigate further whether parathion and methoxychlor induce immune dysregulation.

In the main study, we used the phenoxyacetic acid herbicide 2,4-D-butyl and the benzoic acid fungicide eugenol. 2,4-D-butyl was found to elicit both contact and respiratory hypersensitivity (Cushman and Street, 1982; Fukuyama et al., 2009), whereas exposure to eugenol causes contact hypersensitivity but not respiratory hypersensitivity reactions (Kimber et al., 2003). The LLNA assesses, in mice, the potential of a chemical to cause a primary T-lymphocyte proliferative response after topical application of the chemical to the skin (Dearman et al., 1999). In the present study, marked decrease in EC3 values of 2,4-D-butyl and eugenol were observed in parathion- and methoxychlor-pretreated groups (see Tables 1–4). In the LLNA, contact allergens can be categorized on the basis of relative skin sensitization potency based on derived

Table 4
Local lymph node assay results of the eugenol group pretreated with methoxychlor.

Pretreatment at 4 weeks (methoxychlor)	Dose of LLNA (eugenol)	Final body weight (g; mean ± SD)	Lymph node weight (mg; mean ± SD)	^{3}H-TdR incorporation	
				DPM (mean ± SD)	SI[a]
0 mg/kg	0%	20.9 ± 1.7	3.65 ± 0.32	392 ± 113	1.0
	5%	21.1 ± 1.3	5.48 ± 1.85	808 ± 536	2.1
	10%	21.0 ± 1.1	7.60 ± 0.53**	2029 ± 1276	5.2
	25%	21.2 ± 1.9	11.42 ± 1.76**	9476 ± 1707**	24.2
100 mg/kg	0%	21.9 ± 2.3	3.38 ± 1.13	289 ± 128	1.0
	5%	21.7 ± 2.5	9.90 ± 0.44**	1209 ± 435	4.2
	10%	21.5 ± 1.2	12.75 ± 3.71**	4087 ± 2839*	14.1
	25%	21.8 ± 1.7	14.92 ± 2.32**	12,157 ± 4083**	42.1
300 mg/kg	0%	20.8 ± 1.6	3.85 ± 0.37	267 ± 113	1.0
	5%	21.0 ± 1.8	8.63 ± 1.85*	1411 ± 596	5.3
	10%	20.8 ± 1.4	11.85 ± 3.93**	5434 ± 3324**	20.4
	25%	21.2 ± 1.8	15.56 ± 1.95**	15,085 ± 2995**	56.5

DPM: disintegrations per minute.
* Values for treatment and control groups were compared by Dunnett's multiple comparison test: $P < 0.05$.
** Values for treatment and control groups were compared by Dunnett's multiple comparison test: $P < 0.01$.
[a] Stimulation index (SI) is the ratio of the mean of the treatment group to that of the vehicle control group. The criterion for a positive response in the standard LLNA is an SI of 3 or greater.

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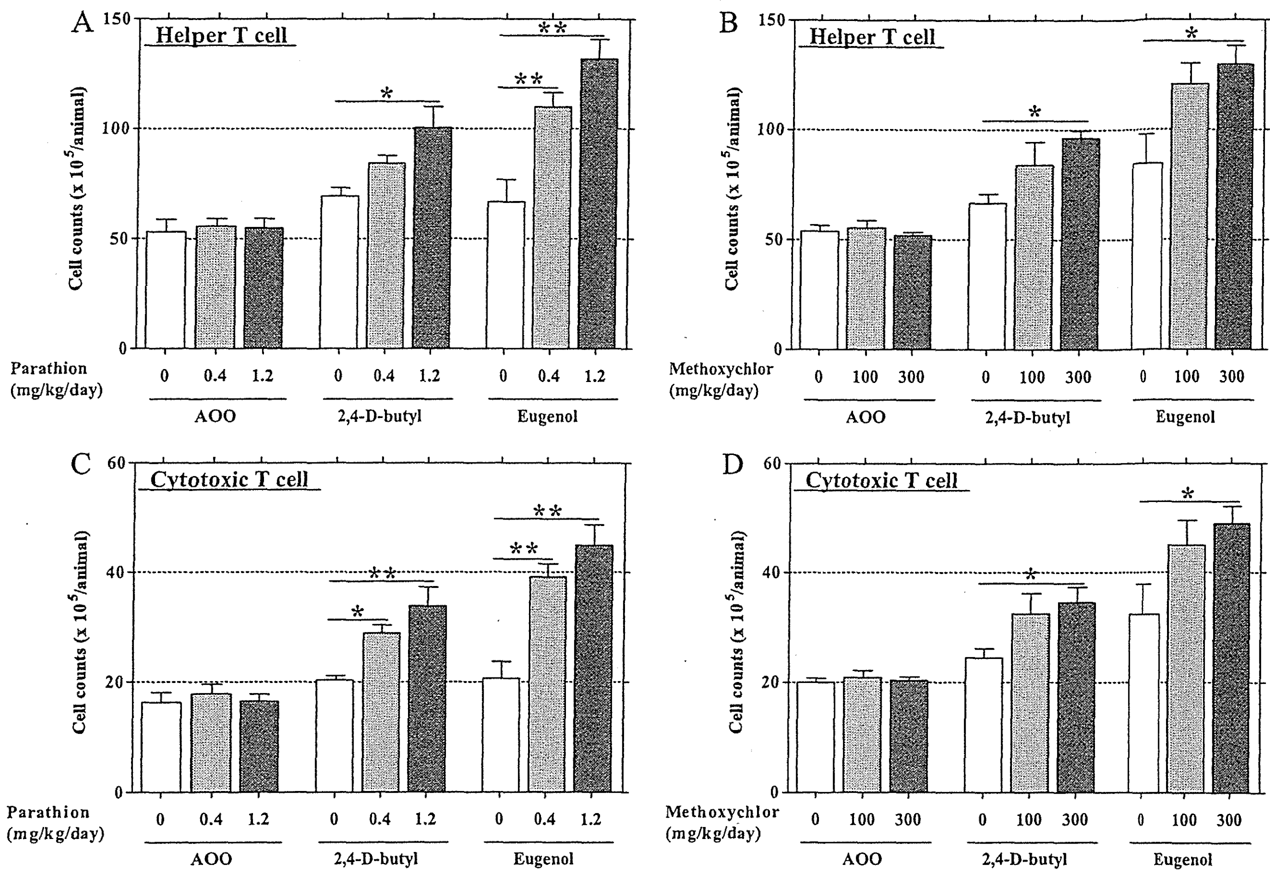

Fig. 3. Helper (CD4$^+$CD8$^-$) (A: Parathion; B: Methoxychlor) and cytotoxic (CD4$^-$CD8$^+$) (C: Parathion; D: Methoxychlor) T-cell counts in auricular lymph nodes of mice treated with test solution of mice. Values are expressed as mean ± SD ($n = 6$ per group). Statistical significance is marked by asterisks: $^*P < 0.05$, $^{**}P < 0.01$ (Dunnett's multiple comparison test).

EC3 values (Kimber et al., 2003). On the basis of the EC3 values, 2,4-D-butyl was categorized as a moderate sensitizer after pretreatment with vehicle and as a strong sensitizer after pretreatment with 1.2 mg/kg parathion. Likewise, eugenol was a moderate sensitizer after pretreatment with vehicle and a strong sensitizer after pretreatment with 1.2 mg/kg parathion. These findings suggest that the failure or breakdown of immunological regulation caused by parathion and methoxychlor enhances the acquired allergy to 2,4-D-butyl and eugenol.

To clarify the mechanisms of increased sensitizing potential by previous oral exposure to parathion and methoxychlor, we performed detailed analysis. Because the LLNA assesses only the T-lymphocyte proliferative response, we focused T cells and T-cell-mediated cytokines. Typical antigens that cause cutaneous hypersensitivity responses are highly reactive small molecules that can easily penetrate intact skin, especially if they cause itching that leads to scratching. These chemicals then react with self proteins, creating protein:hapten complexes that can be processed to hapten:peptide complexes, which can bind to major histocompatibility complex (MHC) molecules that are recognized by CD4$^+$ and CD8$^+$ T cells as foreign antigens (Murray, 1998; Santana and Rosenstein, 2003). There are two phases to a cutaneous hypersensitivity response – sensitization and elicitation. During the sensitization phase, cutaneous Langerhans' cells take up and process antigen, and migrate to local lymph nodes. In the elicitation phases, further exposure to the sensitizing chemical leads to antigen presentation to memory T cells in the dermis, with the release of Th1-cell cytokines such as IFN-γ and TNF-α (Janeway et al., 2004, Mosmann and Coffman, 1989; Yssel and Groux, 2000). In this study, we assessed the membrane expression of T cells (CD4$^+$ and CD8$^+$) and cytokine productions (IFN-γ and TNF-α) in lymph nodes. We found significant increases in T cell (CD4$^+$ and CD8$^+$) populations, and cytokine productions (IFN-γ and TNF-α) in 2,4-D-butyl and eugenol sensitization groups pretreated with parathion or methoxychlor. According to our results, misleading of 2,4-D-butyl and eugenol to antigen presenting T cells were induced by the breakdown of the MHC molecules that are recognized by CD4$^+$ and CD8$^+$ T cells. In consequence, because 2,4-D-butyl and eugenol is a potential contact hypersensitivity reactions (Cushman and Street, 1982; Fukuyama et al., 2009), release of Th1-cell cytokines such as IFN-γ and TNF-α were increased.

Along with Th1-cell cytokines such as IFN-γ and TNF-α, we assessed the IL-17 production in lymph nodes and 2,4-D-butyl and eugenol sensitization groups pretreated with parathion or methoxychlor induced significant increases in IL-17 production. Our data suggested that IL-17 is of clinical importance to the development and severity of sensitizing potential by previous oral exposure to parathion and methoxychlor. Recent clinical analysis suggests that in addition to Th1 factors, IL-17 is increased clinical contact hypersensitivity reactions (Joshi et al., 2009). In 2000, Infante-Duarte et al. first demonstrated that IL-17-producing T cells were a distinct Th population from Th1 and Th2 cells in both mice and humans (Infante-Duarte et al., 2000; Oboki et al., 2008). Subsequently, the importance of IL-17-producing-T cells rather than Th1 cells for the development of contact hypersensitivity was demonstrated in IL-17 deficient mice (Nakae et al., 2002; Oboki et al., 2008).

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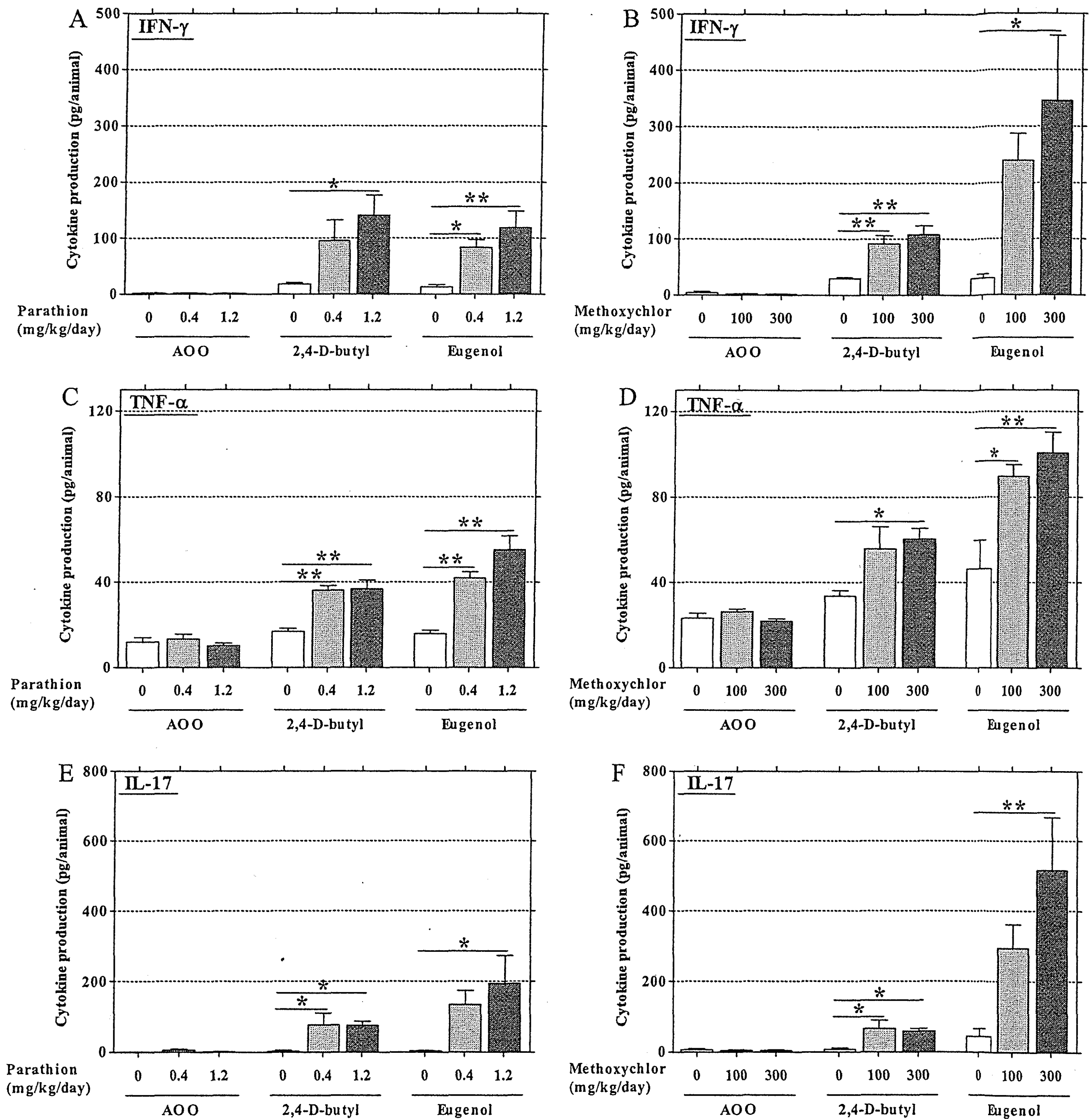

Fig. 4. IFN-γ (A: Parathion; B: Methoxychlor), TNF-α (C: Parathion; D: Methoxychlor) and IL-17 (E: Parathion; F: Methoxychlor) production by auricular lymph nodes of mice treated with test solution of mice. To determine the effects of cytokine production, lymph node cell suspensions were cultured with T-cell antibodies (CD3) for 24 h, and the supernatant was assayed by cytometric bead array. Values are expressed as mean (pg/mouse) ± SD ($n = 6$ per group). Statistical significance is marked by asterisks: *$P < 0.05$, **$P < 0.01$ (Dunnett's multiple comparison test).

Research has shown that some environmental chemicals lead to autoimmune disease and cancer (Schwartz, 2005). Sobel et al. (2005) and Wang et al. (2007) demonstrated that systemic lupus erythematosus in (NZB × NZW) F1 mice was increased by the OC chlordecone. Ward et al. (2009) reported that the incidence of childhood leukemia in industrialized countries was associated with exposure to polychlorinated biphenyls and OCs. Xu et al. (2010) showed that OCs might be associated with an increased risk for breast cancer and prostate cancer. It is important to note, however, that the relationship between allergies and the breakdown of immunological regulation by environmental chemicals is not fully understood. Therefore, we attempted to detect the modulation of allergic potential by prior exposure to immunosuppressive chemicals. We elicited positive reactions using parathion, methoxychlor, 2,4-D-butyl, and eugenol, but whether the same events occur after exposure to other widespread chemicals in the environment remains uncertain. Studies of other endpoints and other types of allergy are currently underway in our laboratories.

Environmental chemicals that lead to immune disorders are thought to fall into three general categories (Rao and Richardson,

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1999; Sobel et al., 2005): (1) the chemical alters the self antigen such that it appears foreign to the immune system; (2) the chemical prevents the central tolerance of autoreactive T or B cells; and (3) the chemical alters gene expression. Hormones, such as estrogens, are thought to belong to this last category (Grimaldi et al., 2002), and several studies linking environmental chemicals with immune disorders have noted the chemicals' estrogenic character (Sobel et al., 2005; Ward et al., 2009; Xu et al., 2010). In this issue, 4 weeks of recovery terms were set between oral exposure and allergen sensitization. Therefore, parathion and methoxychlor could not be the self antigen was denied. In terms of relativity of hormones, we elicited positive reactions using parathion, which is not a hormone disruptor. According to our previous in vitro study and results of in this issue, it suggests that the chemical prevents the central tolerance of autoreactive T or B cells. To our knowledge, this is the first study to demonstrate the relationship between allergies and the breakdown of the central tolerance of autoreactive T or B cells by non-estrogenic environmental chemicals. However, the underlying mechanisms are an important issue to be solved.

Conflict of interest

None.

Acknowledgements

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RESEARCH ARTICLE

Detection of thymocytes apoptosis in mice induced by organochlorine pesticides methoxychlor

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Abstract
The thymus has long been known to be vulnerable to atrophy when exposed to variety of stimuli, including hormones, immunosuppressive pharmaceuticals, and environmental chemicals. The organochlorine pesticide methoxychlor (MXC) is an immunosuppressive agent thought to affect thymic atrophy by inducing apoptosis of thymocyte T cells. We sought to develop an experimental protocol to detect *in vivo* thymocyte apoptosis induced by MXC in Balb/c mice. We treated the mice with 150–400 mg/kg MXC. We then measured thymus weight, cell counts, caspase activity (3/7, 8, and 9), annexin V labeling of phosphatidylserine (PS) and DNA fragmentation. In MXC-treated mice we observed decreases in thymus weight and cell counts and increases in caspase activity (3/7, 8, and 9), annexin V PS labeling and DNA fragmentation. These results suggest that MXC induces thymic atrophy caused by thymocyte apoptosis, and that our protocol may be useful for detecting *in vivo* thymocyte apoptosis induced by environmental chemicals in short-time.

Keywords: *Methoxychlor; apoptosis; thymus; mouse*

Introduction

The immune system is highly sensitive to the toxic effects of several types of chemicals. The thymus in particular has long been known to be vulnerable to atrophy associated with exposure to variety of substances, including hormones, immunosuppressive pharmaceuticals, and environmental chemicals.[1–5] The thymus is a complex organ that is responsible for the maturation and differentiation of most peripheral T cells.[6] Given the complexity of the thymus, compounds that cause thymic atrophy could be acting on a variety of cellular targets and causing atrophy by various mechanisms. Proposed mechanisms include inhibition of thymocyte precursors in the bone marrow or fetal liver and inhibition of intrathymic development of the thymocytes themselves. Such inhibition may result from indirect effects on the supporting stromal elements that produce growth factors and signals or direct mechanisms such as decreased cell proliferation and increased cell death through apoptosis.[5]

Apoptosis is an essential process underlying multicellular organism development and function. In the immune system, apoptosis is required for lymphocyte development and homeostasis. The dysregulation of apoptosis leads to a variety of immune disorders, including immunodeficiency, tumorogenesis, allergies, and autoimmunity.[7] Detection of *in vivo* chemical-induced thymocyte apoptosis is difficult, however, because of the rapid clearance of apoptotic cells by phagocytic cells.[8–12] In addition, it has been taken a long time to detect the apoptosis *in vivo*. Therefore, protocols are needed for the detection of *in vivo* chemical-induced thymocyte apoptosis in short time.

Some recently reported studies have found that organochlorine pesticides such as dichlorodiphenyltrichloroethane (DDT) and methoxychlor (MXC) affect the immune system[13,14] as well as the reproductive system. Developed to replace DDT,[15] MXC has a shorter half-life than DDT, lower toxicity in mammals, and greater biodegradability.[15–17] Because of their nature of persistence in the environment, bioaccumulation in the food chain, and possible health effects, the US Environmental Protection Agency restricted and banned the use of most of organochlorine pesticides during the 1970s and 1980s.

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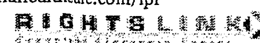

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Although organochlorine pesticides are rarely used today, measurable amounts of organochlorine pesticides or their metabolites can still be found in human tissues. Moreover, organochlorine pesticides continue to be heavily used in some developing countries, primarily for mosquito and malaria control.[18,19] Thus, the health effects of organochlorine pesticides exposure remain an important public health concern. In a previous study, we demonstrated that MXC affects the immune system, leading to atrophy of CD4$^+$CD8$^+$ double-positive (DP) T cells in the thymus.[20–22] In addition, Chapin et al.[13] reported that doses of 150 mg/kg/day in rats led to markedly decreased thymus weights. The mechanisms behind the immunosuppressive action of MXC are not fully understood, however.

In this issue, we attempt to detect thymocyte apoptosis induced by MXC in short time. To that end, we used our original protocol and MXC at rather high doses (about one fourth of the LD50). We sought to determine whether MXC induces thymic atrophy in young mice and whether exposure to MXC triggers *in vivo* apoptosis in thymocytes.

Materials and methods

Chemicals and reagents

MXC (more than 95% pure) was purchased from Sigma-Aldrich (St Louis, MO). Dexamethasone (DEX; more than 99% pure) and ethanol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil was purchased from Hayashi Chemicals (Tokyo, Japan). MXC was dissolved in ethanol and diluted in corn oil. DEX was diluted in saline. DEX treatment group is referred to as the "positive control" group in this study.

Animals and housing conditions

Female BALB/c mice (age, 4 weeks) were purchased from Charles River Japan Laboratories (Kanagawa, Japan). The mice were housed individually under controlled lighting (lights on from 0700 to 1900 h), temperature (22 ± 2°C), humidity (55% ± 15%), and ventilation (at least ten 100% fresh air changes hourly). Food (Certified Pellet Diet MF, Oriental Yeast Co., Tokyo, Japan) and water were available *ad libitum*.

This study was conducted in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science.[23]

Experimental design

After a 1-week acclimatization period, mice (5 weeks old) were allocated randomly to four treatment groups and one control group ($n = 8$ per group). The experimental protocol used in this study is depicted in Figure 1. As a positive control, one treatment group received a single intraperitoneal injection of DEX (10 mg/kg). The other four treatment groups received three oral doses in 8-h intervals of MXC of 0 (negative control), 150, 300, or 400 mg/kg. All dose amounts of MXC were selected to avoid death and systemic toxicity (particularly in the preliminary test) while still permitting comparisons of the immunological potencies of the chemicals. Eight hours after the last treatment, mice were anesthetized with diethyl ether and killed. Each animal's thymus was carefully removed and weighed. Approximately one-half of the thymus tissue from each mouse was used in a single-cell suspension for analysis of annexin V and caspases 3/7, 8, and 9. The remaining thymus tissue was stored at −80°C until use for the detection of DNA ladder formation.

Preparation of cell suspension

Single-cell suspensions of thymocytes in 10 mL PBS (Gibco, Carlsbad, CA) supplemented with 5% heat-inactivated fetal calf serum (Gibco) were prepared by passage through a stainless-steel screen. The number of thymocytes was determined with a Coulter counter Z2 (Beckman Coulter, Tokyo, Japan).

Caspase 3/7, 8, and 9 analysis

From each cell suspension, 100 µL (1×10^5 cells) was seeded in duplicate into opaque-walled 96-well plates (Nulge Nunc International, Tokyo, Japan). Caspases 3/7, 8, and 9 in each well were measured with a luciferin-luciferase system (caspase-Glo 3/7, 8 and 9 assay, Promega, Tokyo, Japan). We used a microplate luminometer (TR717, Berthold Japan, Tokyo, Japan) to measured the activity of each caspase in relative light units (RLU).

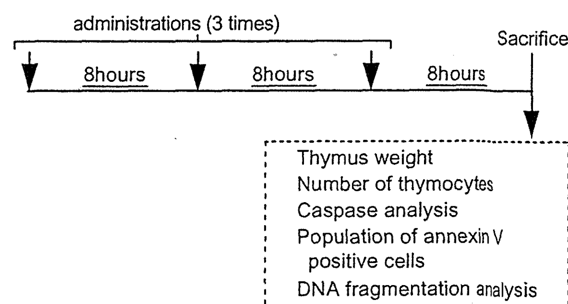

Figure 1. Experimental protocol used to administration BALB/c mice with methoxychlor (MXC). Eight hours after the last treatment, mice were anesthetized with diethyl ether and euthanized. Each animal's thymus was carefully removed and weighed. Approximately one-half of the tissues were pooled per mouse in phosphate-buffered saline (PBS, Gibco, Tokyo, Japan) analyzed for annexinV, caspase-3/7, caspase-8, and caspase-9. The other half of the thymus was kept at -80°C until used for the detection of DNA ladder formation.

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Cell staining and flow cytometric analysis

In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment.[24] Because externalization of PS occurs in the earlier stages of apoptosis, annexin V staining can enable identification of apoptosis at an earlier stage than assays that measure nuclear changes.

Flow cytometric analysis was performed by staining thymocytes with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide. To assay the thymocytes we used an annexin V: FITC Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA) in accordance with the manufacturer's protocol. Briefly, thymocytes (1×10^6 cells/animal) were pelleted by centrifugation and resuspended in 1 mL of annexin V-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$). We then incubated 100 µL of cell suspension (1×10^5 cells) with FITC-conjugated annexin V and propidium ioide for 15 min at room temperature in the dark. We added 400 µL of annexin V-binding buffer and then performed analysis with a FACSCalibur flow cytometer (BD Pharmingen) with the Cell Quest program (BD Pharmingen). For each sample, 10,000 events were collected and analyzed for the expression of antigens to annexin V and propidium ioide.

DNA fragmentation analysis

Isotissue® (Nippon Gene, Tokyo, Japan) DNA extraction kit was used to extract DNA from the frozen thymus samples. Each sample was placed in a microfuge tube with extraction buffer and proteinase K and incubated at 55°C until completely dissolved. We then added 5 M NaCl and centrifuged the mixture for 20 min (12,000*g* at 4°C. The supernatant was placed in a microfuge tube and incubated for 15 min at 55°C with RNase A (0.4 mg/mL) and then incubated for another 15 min at the same temperature with proteinase K (0.4 mg/mL). After being washed with washing buffer and 70% ethanol, DNA was eluted with Tris-EDTA (pH 8.0) buffer. The eluate was used as the DNA sample for agarose gel electrophoresis.

DNA samples were electrophoretically separated on 2% agarose gel (Mercury Heat & Pour Agarose, Pretech Instruments KB, Sollentuna, Sweden) in 90 mM Tris-boric acid-EDTA solution (Nippon Gene) at 8.5 V/cm for 2 h. After electrophoresis, the gels were stained with Vistra Green nucleic acid gel stain (GE Healthcare Bio-Sciences Corp, Piscataway, NJ), and the nucleic acids were visualized with a fluorimager (FluorImager 585, GE Healthcare UK Ltd. Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England).

Statistical analysis

We transformed the data logarithmically to homogenize the variances and used analysis of variance (ANOVA) to evaluate the difference between test (MXC and DEX) and vehicle treatment group. When ANOVA was significant, the differences between groups were assessed by means of ANOVA followed by the Dunnett's multiple comparison test. A value of $P < 0.05$ was considered to be significant. Values are expressed as the mean ± standard deviation (SD).

Results

Thymus weights and numbers of thymocytes

In the MXC treatment groups thymus weights decreased in a dose-dependent manner, and statistically significant differences were found between the group that received a dose of 400 mg/kg and the other groups ($P < 0.01$) (Figure 2A). The total numbers of thymocytes were

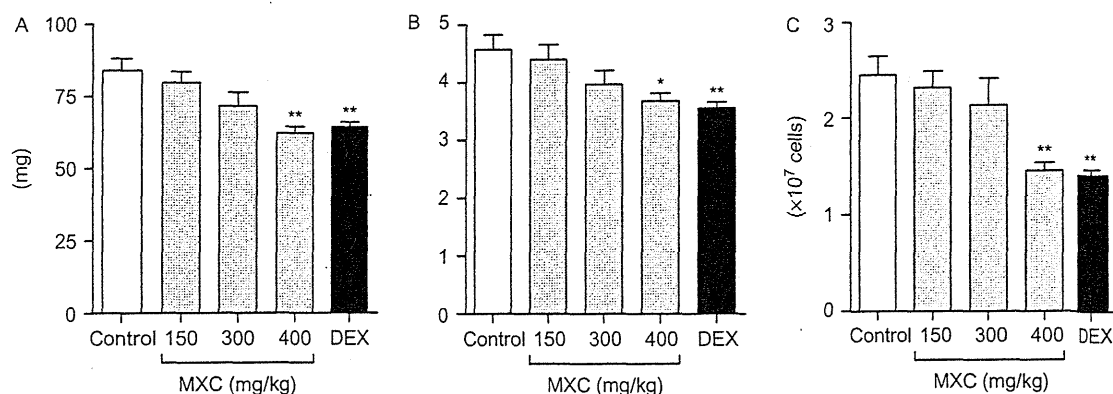

Figure 2. Absolute thymus weight (A) relative thymus weight (B) and total thymocytes count (C) of mice treated with test solution. Absolute thymus weights are expressed as means (mg/mouse) ± SD. Relative thymus weight are expressed as means (absolute thymus weigh, mg/body weight, g) ± SD. Thymocyte counts are expressed as means ($\times 10^7$ cells/mouse) ± SD. Statistical significance is marked by asterisks: $^{**}P < 0.01$ (Dunnet's *t*-test).

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lower in the 400 mg/kg group, but the decrease was not statistically significant (Figure 2B). Absolute thymus weight and number of thymocytes in the MXC 150 mg/kg treatment group were 79.63 ± 10.8 mg and 2.32 ± 0.5 × 10^7 cells, and in the MXC 300 mg/kg treatment group were 71.50 ± 13.8 mg and 2.14 ± 0.8 × 10^7 cells. Results for both of these groups were similar to those in the control group (absolute thymus weight, 83.88 ± 12.0 mg; number of thymocytes; 2.45 ± 0.5 × 10^7 cells). In the MXC 400 mg/kg treatment group, absolute thymus weight was 62.00 ± 6.7 mg, approximately 74% of that in the control group; this is a 26% decrease, and the number of thymocytes was 1.45 ± 0.2 × 10^7 cells, approximately 60% of the cell numbers in the control; this is a 40% decrease. (Figure 2).

In the DEX treatment group, statistically significant decreases were noted in both thymus weight and total number of thymocytes ($P < 0.01$) (Figure 2). The absolute thymus weight and total number of thymocytes were 64.25 ± 4.7 mg and 2.19 ± 0.2 × 10^7 cells, approximately 75% of those for the control group (Figure 2).

Caspase 3/7, 8, and 9 analysis

Caspase 3/7, 8, and 9 activities induced by 400 mg/kg MXC treatment were significantly higher than those of the control group ($P < 0.01$) (Figure 3). Caspase 3/7, 8, and 9 activities in the MXC 150 mg/kg treatment group were 3854 ± 574, 19,603 ± 2599, and 45,325 ± 6659 RLU, and were similar to the control group values of 4241 ± 623, 22,011 ± 2443, and 50,514 ± 5406 RLU, respectively (Figure 3). In the MXC 300 mg/kg treatment group, caspase 3/7, 8, and 9 activity values were 33,378 ± 50,723, 23,285 ± 9147, and 63,915 ± 39,268 RLU, respectively, approximately 800, 105, and 130% of those of the control group (Figure 3). In the MXC 400 mg/kg treatment group, caspase 3/7, 8, and 9 activity values were 155,655 ± 47,397, 46,950 ± 11,576, and 163,973 ± 47,386 RLU, respectively, approximately 3600, 200, and 300% of those of the control group (Figure 3).

In the DEX treatment group, statistically significant increases were noted in the activity of all caspases ($P < 0.01$) (Figure 3). The activity values of caspases 3/7, 8, and 9 were 196,822 ± 13,049, 52,918 ± 3723 and 182,498 ± 14,771 RLU, approximately 4600, 240, and 360% higher, respectively, than those in the control group (Figure 3).

Flow cytometric analysis

To evaluate the state of apoptotic cells after treatment with MXC, flow cytometric analysis was performed with thymocyte staining by FITC-conjugated annexin V and propidium iodide (Figures 4 and 5). The real cell numbers of annexin V-positive thymocytes were represented. The number of annexin V-positive thymocytes

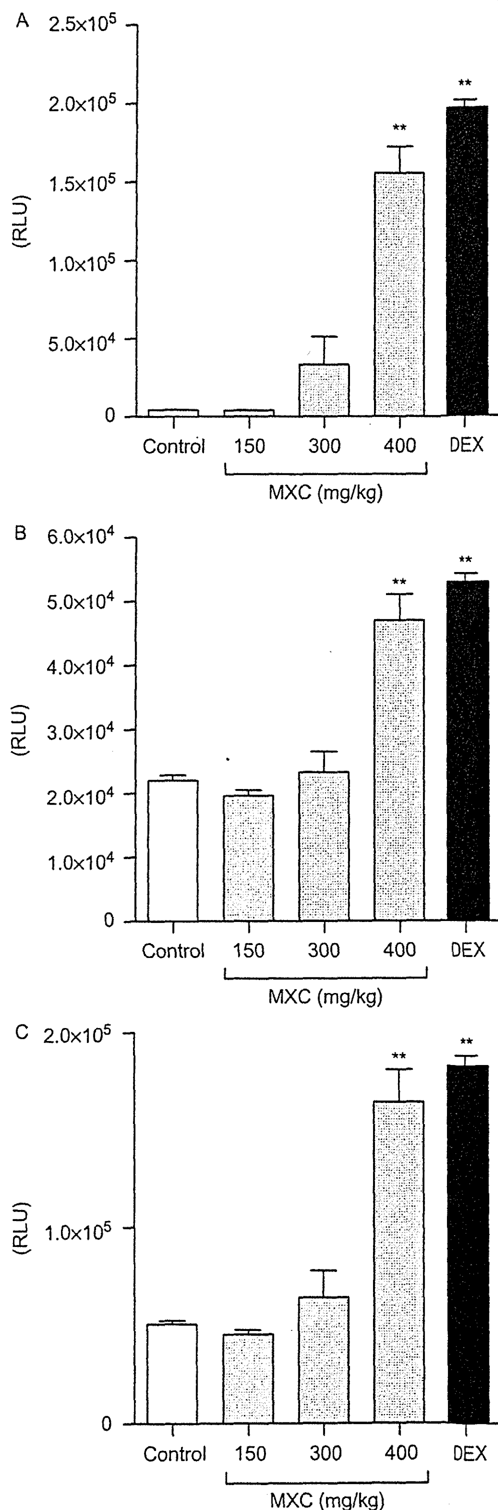

Figure 3. Caspases activities (A: 3/7; B: 8; C: 9) in the thymus of mice treated with test solution. These activities are expressed as means (RLU) ± SD. Statistical significance is marked by asterisks: $^{**}P < 0.01$ (Dunnet's *t*-test).

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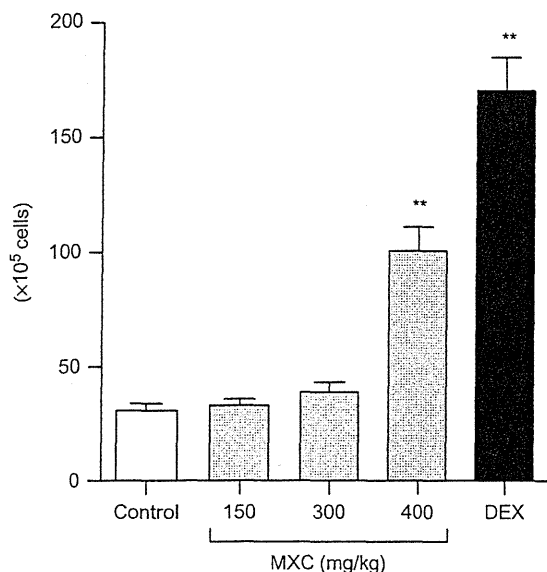

Figure 4. The populations of annexin V-positive cells (A: 3/7; B: 8; C: 9) in the thymocytes of mice treated with test solution. The populations of annexin V-positive cells are expressed as means (×10^5 cells/mouse) ± SD. Statistical significance is marked by asterisks: **$P < 0.01$ (Dunnet's *t*-test).

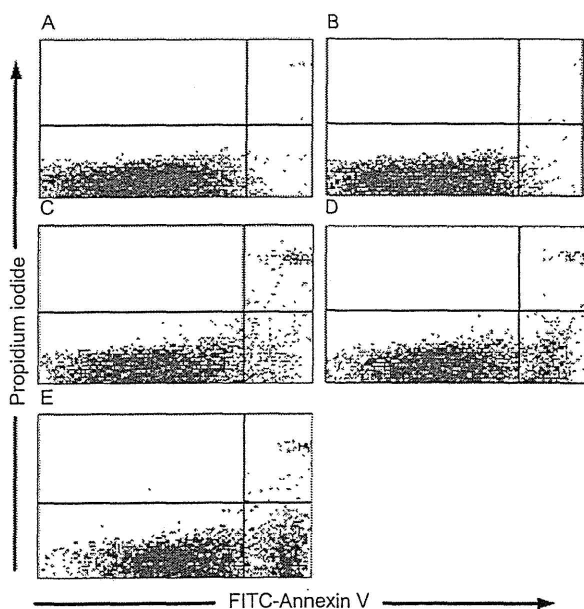

Figure 5. Characteristic exemplification of dot-plots of annexin V-positive cells (A: control; B: MXC 150mg/kg; C: MXC 300mg/kg; D: MXC 400mg/kg; E: DEX) in the thymocytes of mice treated with test solution. (See colour version of this figure online at www.informahealthcare.com/ipi)

was significantly higher in the 400mg/kg MXC-treated group than in control group ($P < 0.01$) (Figure 4). In the MXC 150mg/kg treatment group, the number of annexin V-positive thymocytes was $33.13 \pm 7.9 \times 10^5$ cells, approximately 107% of the control group value of $30.88 \pm 8.7 \times 10^5$ cells (Figures 4, 5A and 5B). In the MXC 300mg/kg treatment group, the number of annexin V-positive thymocytes was $38.89 \pm 12.6 \times 10^5$ cells, approximately 130% that of the control group (Figures 4 and 5C). In the MXC 400mg/kg treatment group, the number of annexin V-positive thymocytes was $100.60 \pm 29.8 \times 10^5$ cells, approximately 325% that of the control group (Figures 4 and 5D). The number of annexin V-positive thymocytes in the DEX treatment group ($170.4 \pm 41.3 \times 10^5$ cells) was approximately 550% that of the control group (Figures 4 and 5E) and this difference was highly significant ($P < 0.01$).

DNA fragmentation analysis

We used agarose gel electrophoresis to assess the degradation pattern of nuclear DNA in MXC-induced cell death because it has been observed that oligonucleosomal cleavage accompanies apoptosis in most systems.[25–27] In the 300 and 400mg/kg MXC treatment groups, we observed increase in fragmented DNA of 200bp (Figure 6C and 6D), whereas essentially no fragmented DNA was detected in the control and MXC 150mg/kg treatment groups (Figure 6A and 6B). In the positive control DEX treatment group, marked DNA fragmentation was noted (Figure 6E).

Discussion

Our results indicate that MXC at doses of 400mg/kg administered three times in 8h by oral gavage modulated the immune response in young female Balb/c mice. The indicators of apoptosis affected were thymus weight, number of thymocytes, caspase activity (3/7, 8, and 9), number of annexin V-positive thymocytes and DNA fragmentation in thymocytes (see Figures 3–6). This study is the first to demonstrate these particular effects of *in vivo* environmental chemical-induced thymocyte apoptosis in short time.

Detection of *in vivo* chemical-induced apoptosis in short time is difficult because of the rapid clearance of apoptotic cells by phagocytic cells.[8–12] To overcome this problem, we used a protocol in which mice were killed 8h after receiving the last of three oral doses of MXC. In addition, we focused on several endpoints such as movement of PS, DNA fragmentation, and caspase activity. By using this procedure we detected more apoptosis in MXC-treated mice than in controls.

A relatively early marker of apoptosis is movement of PS residues to the external leaflet of the plasma membrane.[28] This process is thought to facilitate macrophage recognition, engulfment, and removal of dying cells.[29,30] Our results indicate that MXC increased the

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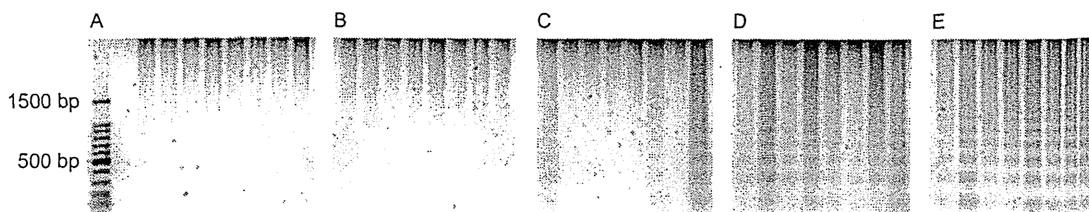

Figure 6. Agarose gel electrophoresis of DNA extracted from the thymus (A: control; B: MXC 150 mg/kg; C: MXC 300 mg/kg; D: MXC 400 mg/kg; E: DEX) of mice treated with test solution. The lanes correspond to each mouse in the experimental group. Molecular weight markers are indicated to the left.

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number of PS-exposed cells in a dose-dependent manner (see Figures 4 and 5). This upregulation of PS movement thus seems to be a useful endpoint for identifying chemical-related thymocyte apoptosis.

A later-stage marker of apoptosis is DNA fragmentation. Apoptosis has been characterized biochemically by the production of internucleosomal DNA fragments of 180–200 bp resulting from the activation of an endonuclease. We observed prominent increases in the DNA fragmentation in the 300 and 400 mg/kg MXC treatment groups (see Figure 6). This upregulation of DNA fragmentation also seems to be a useful endpoint for identifying chemical-related thymocyte apoptosis.

Caspase activation is a step in apoptosis, and measurement of caspase 3/7, 8, and 9 activity is a reliable indicator of caspase-dependent apoptosis.[31] Caspases 8 and 9 are thought to be initiator caspases, and caspases 3 and 7 effector caspases. Caspase 3, the first caspase linked to apoptosis that is upstream of DNA fragmentation, is activated by caspases 8 and 9. Caspase 3 is required for DNA degradation and chromatin condensation. Thus the dose-dependent increase in caspase 3 (see Figure 3A) indicated an increase in MXC-induced thymocyte apoptosis. Caspase 8 is the apical caspase in the tumor necrosis factor–family death-receptor or extrinsic pathway. Caspase 8 activation initiates Fas (Apo-1/CD95), tumor necrosis factor receptor and death receptor-3.[32–36] Active caspase 8 cleaves and activates the downstream effector caspase 3, which cleaves cellular targets and induces apoptosis. Furthermore, caspase 8 cleaves the BH3-only protein Bid and generates a small fragment of Bid, which translocates to the mitochondrial outer membrane and inhibits the function of anti-apoptotic proteins Bcl-2 and Bcl-x_L. This step leads to the activation of the intrinsic death pathway.[7] Caspase 9 is the apical caspase in the intrinsic or mitochondrial pathway. Active caspase 9 further activates downstream effector caspases, including caspases 3 and 7, which in turn cleave hundreds of cellular components and result in irreversible cell death. In the present study, caspase 8 and 9 activity in thymocytes treated with MXC increased significantly. This result suggests that MXC-induced thymocyte apoptosis affects both intrinsic and extrinsic apoptosis pathways.[7]

To detect *in vivo* MXC-induced thymocyte apoptosis in short time, we used our original protocol and MXC at rather high doses (about one fourth of the LD50). In recent years, several reports have suggested that oral MXC exposure suppresses immune function by inducing thymic atrophy.[13,14] Whether this thymic atrophy occurs because MXC triggers apoptosis is not clear, however. Our study is the first to demonstrate severe thymic atrophy with cellular depletion and increased thymocyte apoptosis in MXC-treated mice, findings indicating that MXC induces thymocyte apoptosis. Takeuchi et al.[21] demonstrated that morphometrical analysis revealed a significant decrease in the size of the thymic cortical area in MXC-treated rat pups. In addition, phenotyping of thymic lymphocytes revealed a significant decrease in the DP immature T cells ($CD3^{int}CD4^{+}CD8^{+}$) located in the cortex area. These reports and our results indicate that MXC may cause thymic cortical atrophy via a thymocytes apoptotic process. In general, DP cells represent the majority of thymic lymphocytes located in the cortex area and have low expression of Bcl-2, which is associated with resistance to apoptosis.[37,38] DP cells are sensitive when exposed to chemicals, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin and organotins, and tend to show apoptotic cell death in the *in vitro* and *in vivo* studies.[39–43] This suggests that immunotoxic chemicals that have the potential to cause thymic atrophy may affect DP thymocytes and result in apoptosis, as was observed with MXC.

Numerous environmental toxic chemicals such as pesticides, perfumes, organotins, and heavy metals play roles in the dysregulation of immune functions, including thymocyte apoptosis.[44–52] Such immunosuppression may predispose the highly sensitive immune system to loss of tolerance to self-antigens and subsequent increased risk for autoimmune disease and allergies. Alternatively, altered repertoires of T cells responsive to foreign antigens may cause the neonate to become more susceptible to infections.[44–46,53] From this point of view, we attempted to develop a method for detection of *in vivo* chemical-induced thymocyte apoptosis and we obtain positive reactions using MXC as a typical chemical. Whether the same events occur after administration of other chemicals

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that appear in many places in the environment remains uncertain, however. Experiments are currently under investigation in our laboratories.

In summary, we detected *in vivo* MXC-induced thymocyte apoptosis in short time by measuring thymus weight, number of thymocytes, caspase activity (3/7, 8, and 9), number of annexin V-positive thymocytes and DNA fragmentation in thymocytes.

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Declaration of interest

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