Toxicology Letters 213 (2012) 392–401

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Role of regulatory T cells in the induction of atopic dermatitis by immunosuppressive chemicals

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HIGHLIGHTS

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

ARTICLE INFO

Article history:
Received 24 June 2012
Received in revised form 18 July 2012
Accepted 19 July 2012
Available online xxx

Keywords:
Atopic dermatitis
NC/Nga mice
Picryl chloride
Parathion
Methoxychlor
Piperonyl butoxide

ABSTRACT

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

1. Introduction

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

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

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http://dx.doi.org/10.1016/j.toxlet.2012.07.018

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

2. Materials and methods

2.1. Reagents

Methoxychlor standard ($C_{16}H_{15}Cl_3O_2$, >97% pure), parathion standard ($C_{10}H_{14}NO_5PS$, 99.5% pure), piperonyl butoxide ($C_{19}H_{30}O_5$, >98% pure) standard, dexamethasone ($C_{22}H_{29}FO_5$, 98–102% pure), 0.5% methylcellulose solution, and olive oil were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cyclophosphamide monohydrate ($C_7H_{15}C_{l2}N_2O_2P{\cdot}H_2O$, 100.6% pure) was purchased from Sigma–Aldrich Japan (Tokyo, Japan). Picryl chloride (wetted with ca. 15% water; $C_6H_2ClN_3O_6$; 100.2% pure) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Corn oil was purchased from Hayashi Chemicals (Tokyo, Japan). Methoxychlor, parathion, and piperonyl butoxide were diluted in corn oil and dexamethasone and cyclophosphamide were diluted in 0.5% methylcellulose solution. After recrystallization with ethanol, picryl chloride was dissolved in acetone–olive oil (v/v, 4:1) solution to 0.5% or 1%.

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

2.2. Animals

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

2.3. Experimental protocol

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

2.4. Enzyme immunoassay for serum substance P

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

2.5. Enzyme-linked immunosorbent assay for total serum immunoglobulin

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

2.6. IgE production by B cells in auricular LNs

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

2.7. Flow cytometry of auricular LNs

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

2.8. Cytokine production from T cells in auricular LNs

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

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

2.9. Cytokine gene expression in pinnae

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

Table 1
Primers used.

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

F, forward; R, reverse.

2.10. Regulatory T-cell analysis of spleen

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

2.11. Statistical analysis

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

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

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

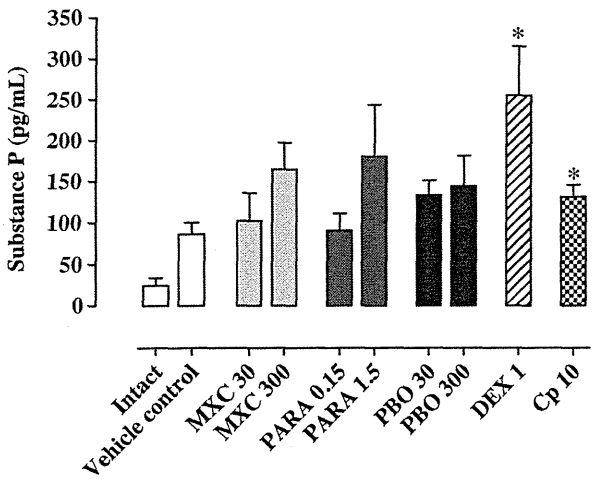

Fig. 1. Serum levels of substance P. Serum levels of substance P in mice sensitized and challenged with picryl chloride after oral exposure to nothing (intact), vehicle only, or each test substance. Substance P levels (pg/mL) are expressed as mean ± 1 SD ($n = 8$ per group). *$P < 0.05$ (Dunnett's multiple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

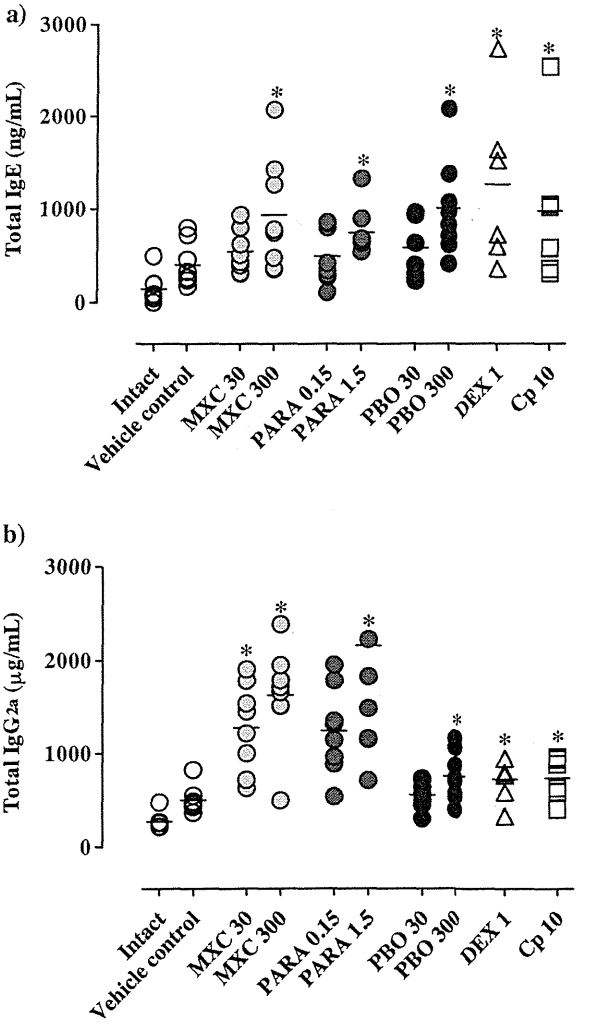

Fig. 2. Total serum immunoglobulins. Total (a) IgE and (b) IgG_{2a} in the serum of mice orally exposed to nothing (intact), vehicle only, or each test substance. Total IgE levels (ng/mL) are expressed as mean ± 1 SD ($n = 8$ per group); total IgG_{2a} levels (μg/mL) are expressed as mean ± 1 SD ($n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

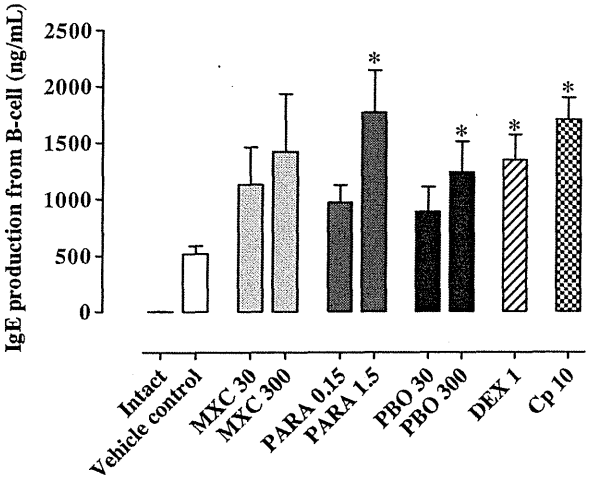

Fig. 3. Total IgE production by B cells from auricular lymph nodes. IgE production by B-cells in auricular lymph nodes from mice orally exposed to nothing (intact), vehicle only, or each test substance. IgE levels (ng/mL) are expressed as mean ± 1 SD ($n = 8$ per group). *$P < 0.05$ (Dunnett's multiple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

3. Results

3.1. Clinical observations

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

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

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

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

3.3. IgE production by B-cells in auricular LNs

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

Table 2
Ear thickness (mm).

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

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

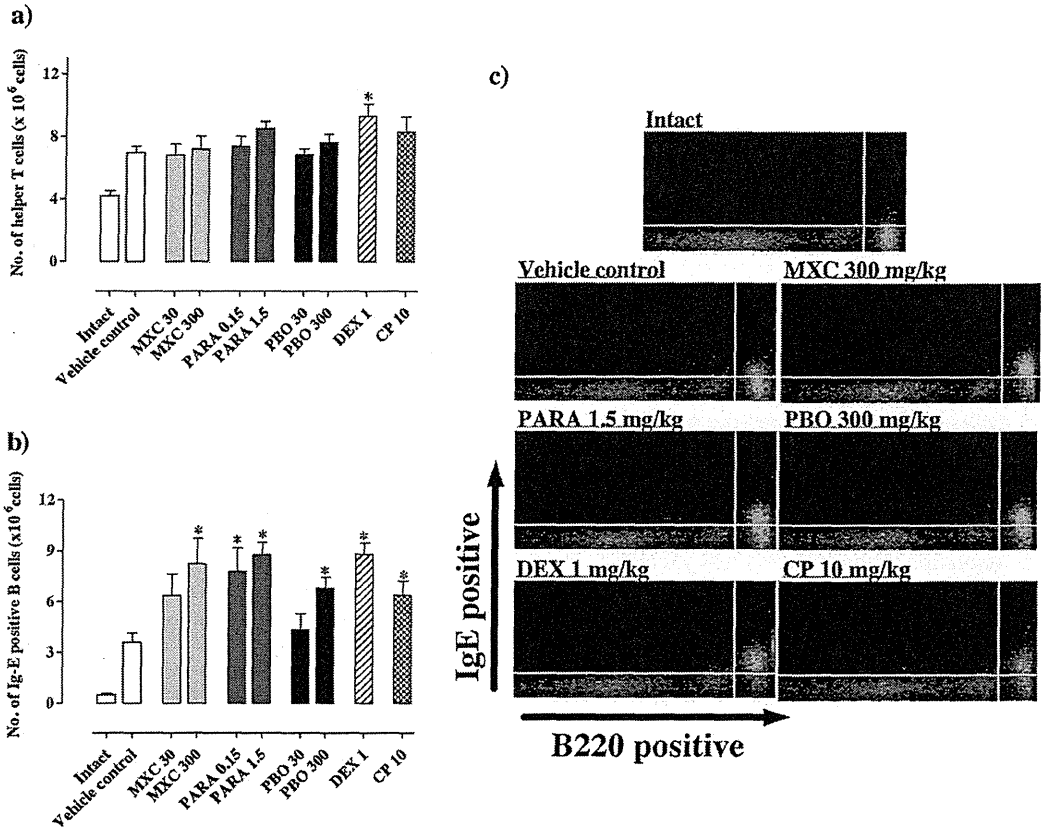

Fig. 4. Helper ($CD4^+CD8^-$) T lymphocyte and IgE-positive (IgE^+B220^+) B-lymphocyte counts in auricular lymph nodes (LNs). Lymphocytes from auricular LNs were stained with anti-CD3, anti-CD4, anti-CD8, anti-B220, and anti-IgE antibodies, and (a) helper T lymphocytes and (b) IgE-positive B lymphocytes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

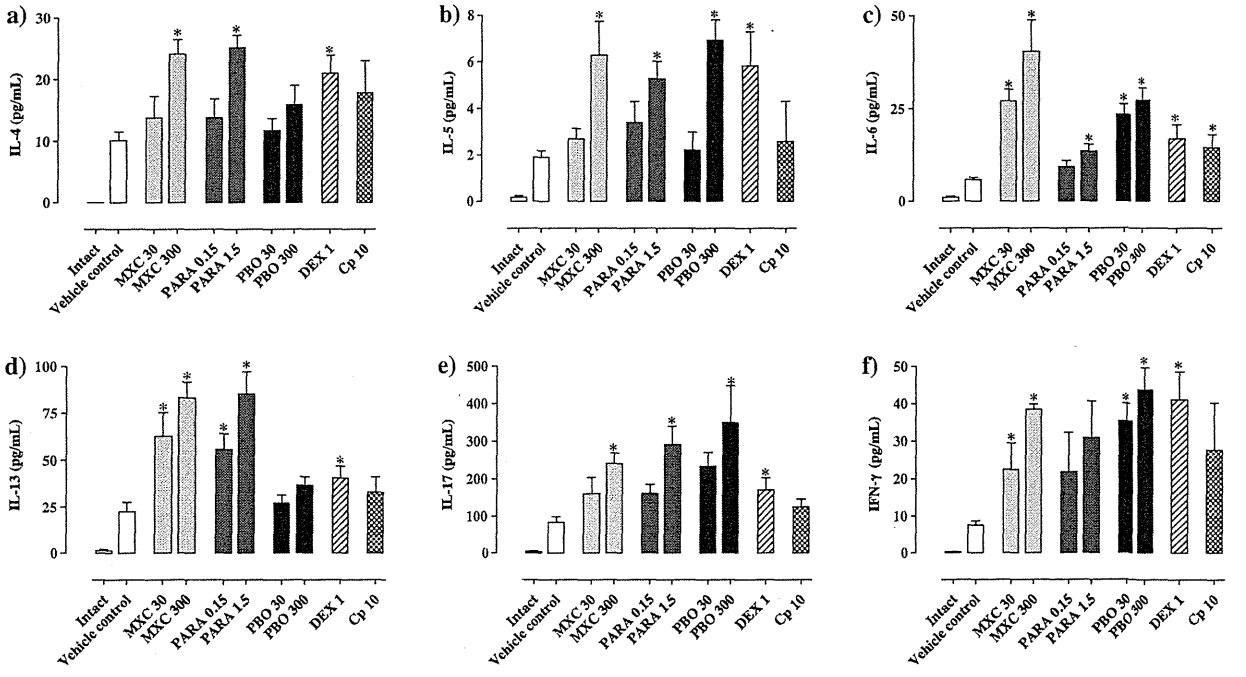

Fig. 5. Cytokine production from auricular lymph nodes from mice. (a) IL-4, (b) IL-5, (c) IL-6, (d) IL-13, (e) IL-17A, and (f) IFN-γ production in lymphocytes from auricular lymph nodes of mice orally exposed to nothing (intact), vehicle only, or each test substance. Cytokine production (pg/mL) is expressed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

Table 3
Clinical score.

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

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

3.4. Flow cytometry of auricular LNs

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

3.5. Cytokine production in auricular LNs

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

3.6. Cytokine gene expression in pinnae

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

3.7. Treg cell counts in spleen

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

4. Discussion

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

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

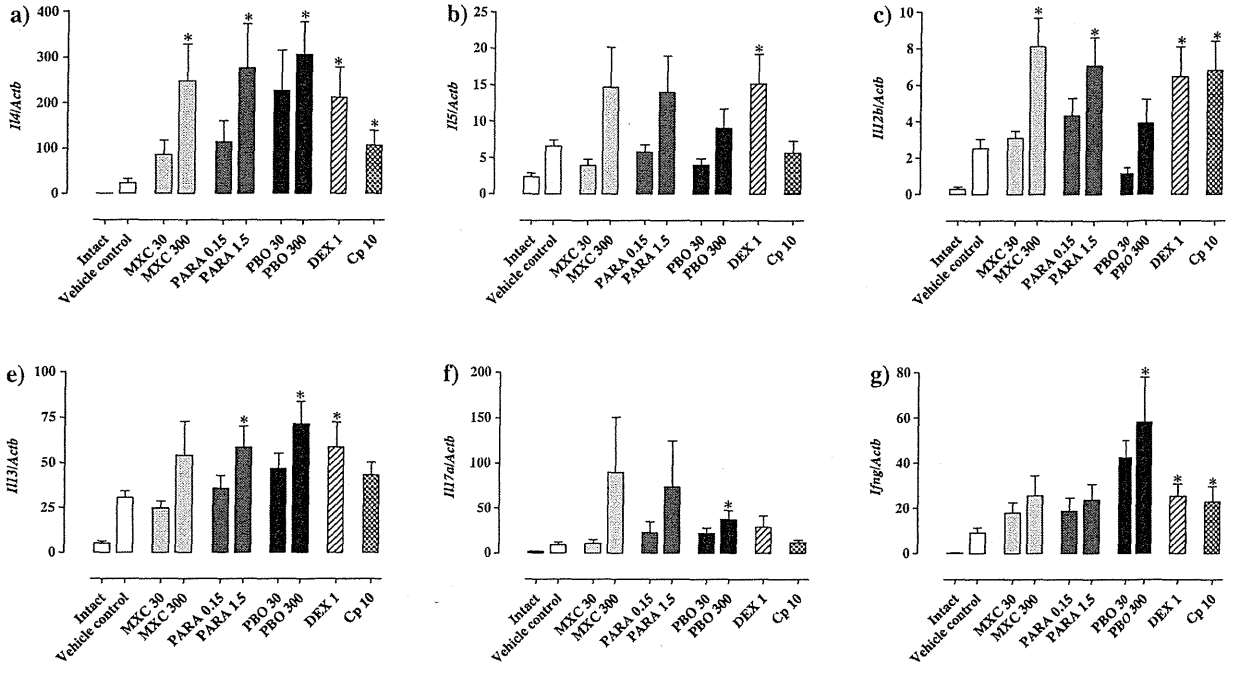

Fig. 6. Cytokine gene expression in ear auricle from mice. Gene expression of (a) *Il4*, (b) *Il5*, (c) *Il12b*, (d) *Il13*, (e) *Il17a*, and (f) *Ifng* in pinnae from mice orally exposed to nothing (intact), vehicle only, or each test substance. Gene expression values are presented as the increase in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

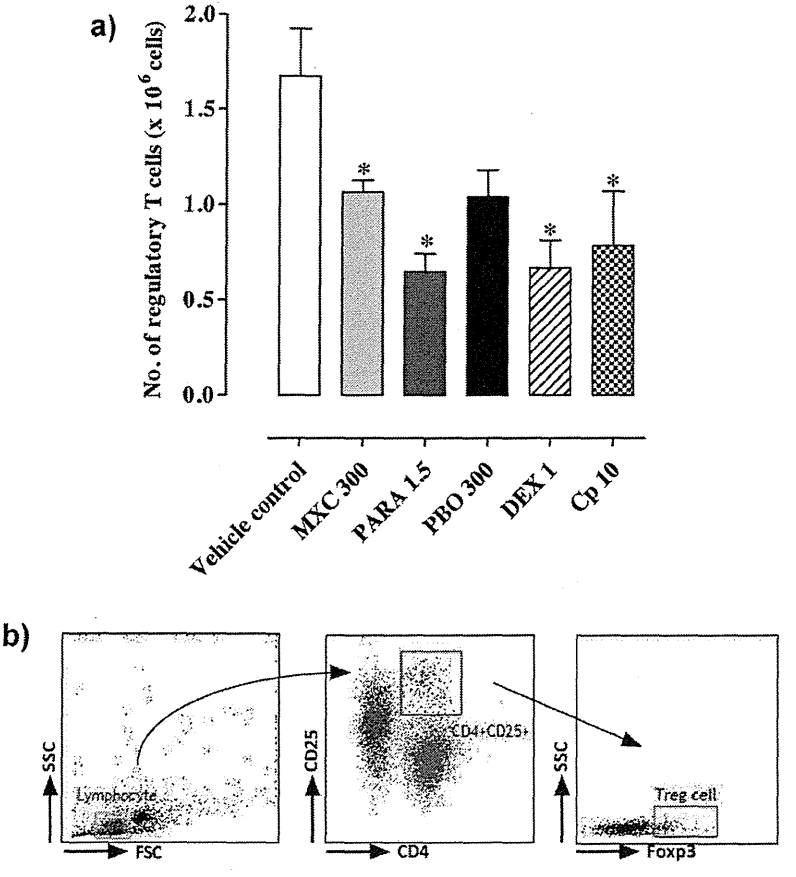

Fig. 7. Splenic regulatory ($CD4^+CD25^+Foxp3^+$) T-lymphocyte counts. (a) Suspensions of splenic lymphocytes were stained with anti-CD4, anti-CD25, and anti-FoxP3 antibodies. Cell counts ($\times 10^6$ cells) are expressed as mean ± 1 SD ($n = 8$ per group). *$P < 0.05$ (Dunnett's multiple comparison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

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

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

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

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

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

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

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

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

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

Funding

This work was supported by a research Grant in Aid from the Ministry of Health, Labor, and Welfare of Japan.

Conflict of interest

The author states that they have no conflict of interest.

Acknowledgments

We thank Drs. Y. Shutoh, H. Fujie, A. Motomura, and Y. Komatsu (Institute of Environmental Toxicology, Ibaraki, Japan) for their technical assistance.

Appendix A. Supplementary data

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

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Journal of Immunotoxicology, 2012; Early Online: 1–10

ISSN 1547-691X print/ISSN 1547-6901 online
DOI: 10.3109/1547691X.2012.703252

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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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(Received 05 March 2012; revised 25 April 2012; accepted 12 June 2012)

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

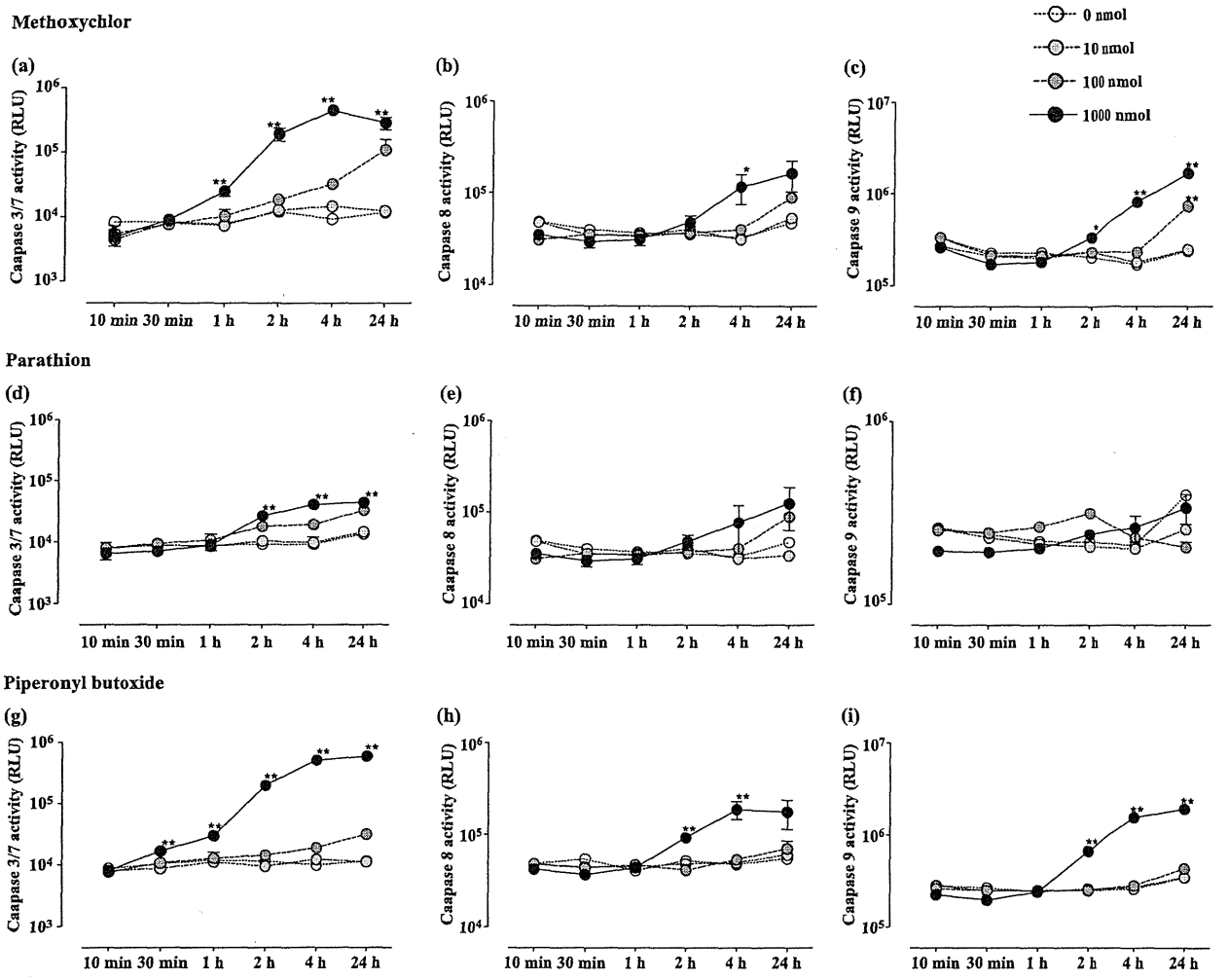

Figure 1. Caspase-3/7, -8, and -9 activities in Jurkat T-cells treated with test chemicals for 10 min to 24 h. Caspase-3/7 (a: Methoxychlor; d: Parathion; g: Piperonyl butoxide), Caspase-8 (b: Methoxychlor; e: Parathion; h: Piperonyl butoxide), and Caspase-9 (c: Methoxychlor; f: Parathion; i: Piperonyl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

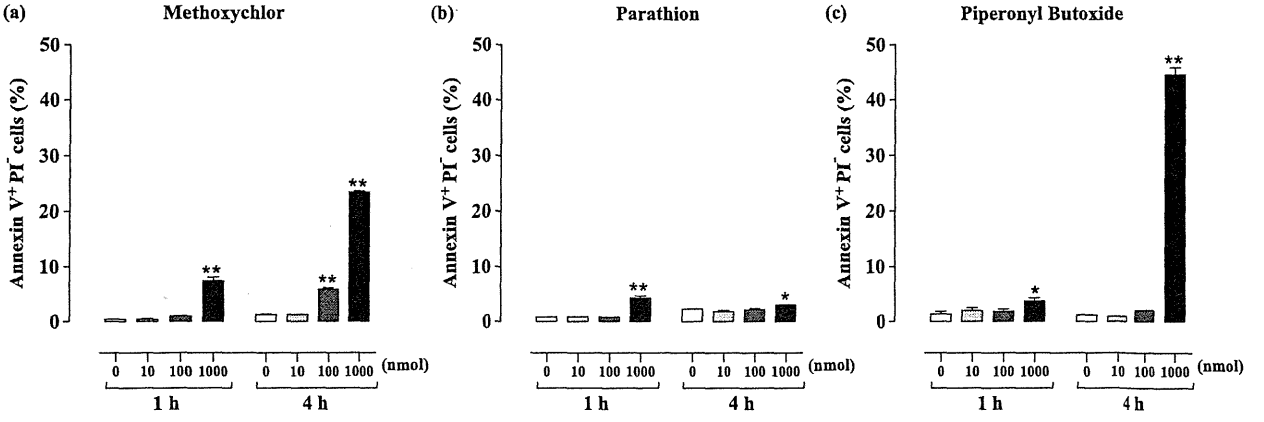

Figure 2. Annexin V-FITC positive cells among Jurkat T-cells treated with test chemicals for 1 or 4 h. (a) Methoxychlor; (b) Parathion; and (c) Piperonyl butoxide. Annexin V+-FITC+ cells are expressed as the mean (proportion of total, %) ± SD. Value significantly differs from control at * $p < 0.05$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

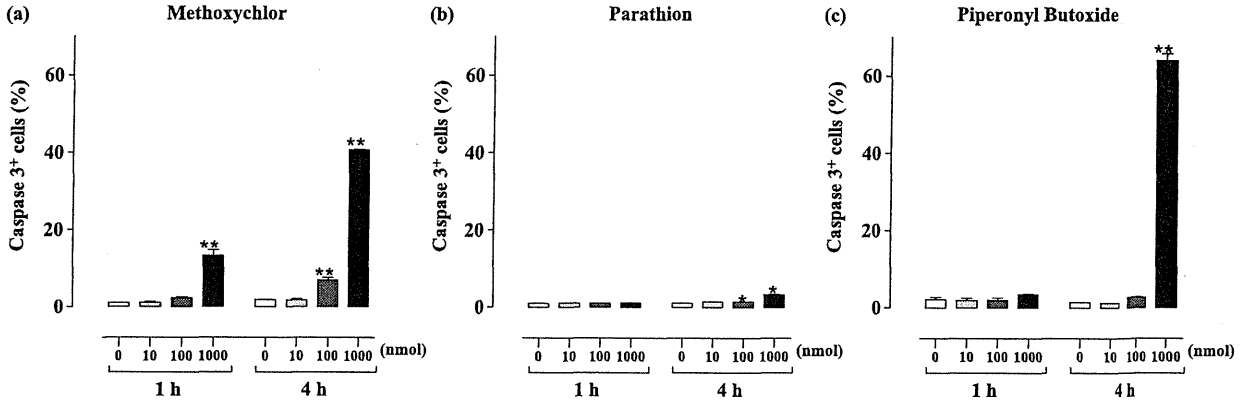

Figure 3. Caspase-3+ cells among Jurkat T-cells treated with test chemicals for 1 or 4 h. (a) Methoxychlor; (b) Parathion; and (c) Piperonyl butoxide. Caspase-3+ cells are expressed as the mean (proportion of total, %) ± SD. Value significantly differs from control at * $p < 0.05$ and ** $p < 0.01$.

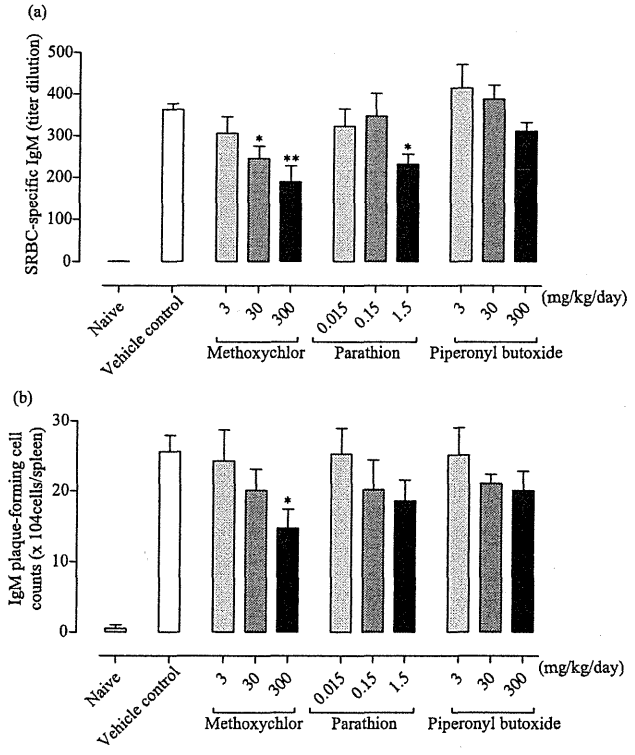

Figure 4. Anti-SRBC IgM response in C3H/He female mice treated with test chemicals. (a) SRBC-specific IgM response in serum. (b) Splenocyte IgM plaque-forming cell responses to SRBC. Values (titer dilution and cell counts) are expressed as mean ± SD. Value significantly differs from control at * $p 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

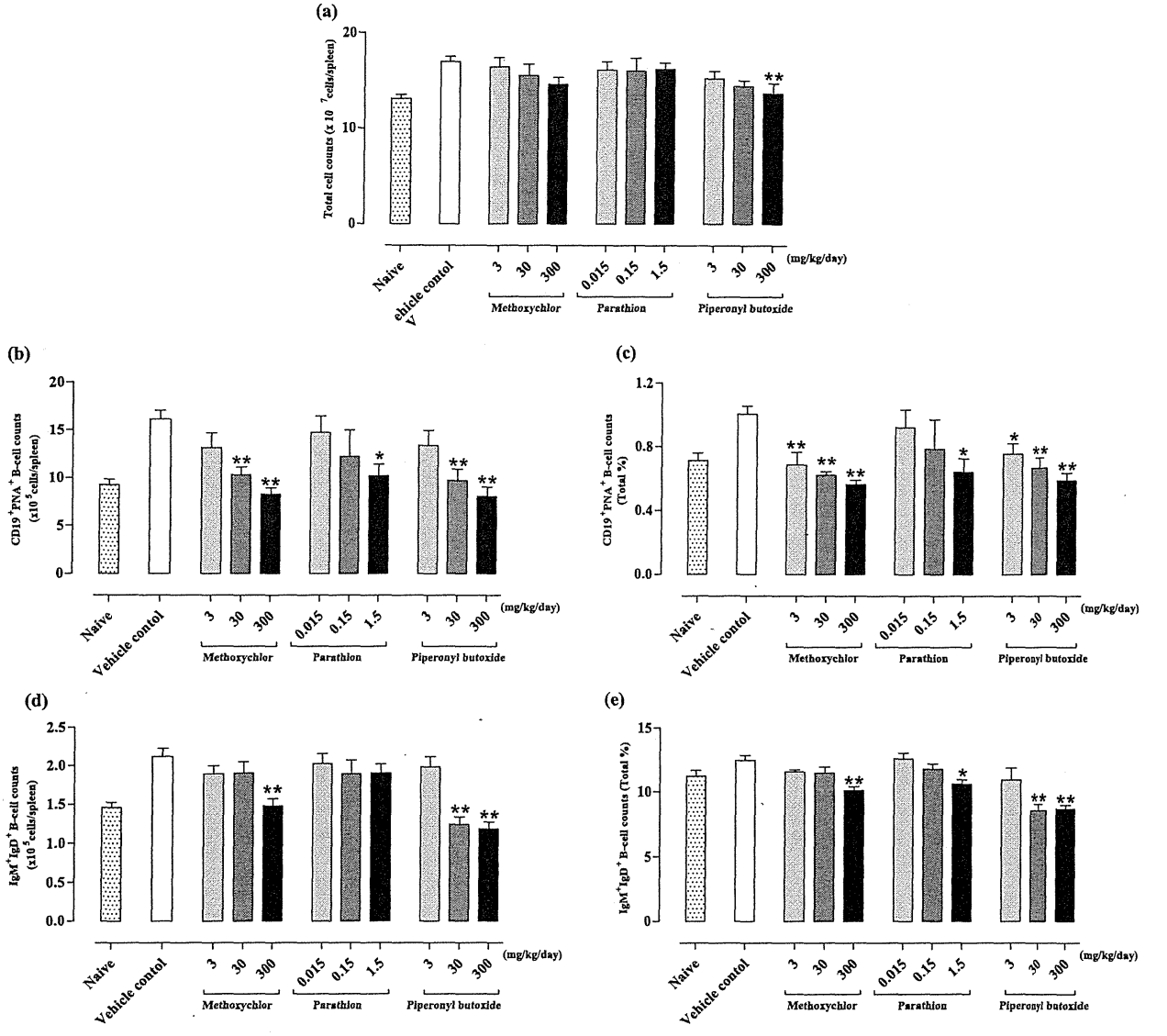

Figure 5. Flow cytometric analyses of splenocytes from C3H/He female mice treated with test chemicals. (a) Total cell counts; (b) germinal center-positive B-lymphocyte counts (CD19+PNA+); (c) germinal center-positive B-lymphocyte counts (CD19+PNA+, proportion of total, %); (d) IgM+ B-lymphocyte counts (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.

Acknowledgments

The authors wish to thank Dr Y. Shutoh, Dr H. Fujie, Dr A. Motomura, and Dr Y. Komatsu of the Institute of Environmental Toxicology (Ibaraki, Japan) for their technical assistance. This work was supported by a research Grant-in-Aid from the Ministry of Health, Labor and Welfare of Japan.

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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