の印加電圧は 300 mV に設定した。分離カラムは Inertsil ODS3（150 × 4.6 mm）を使用し、移動相は 20 mM リン酸二水素一ナトリウム：メタノール=97：3 を用いた。

B-6. Griess 法によるフェノール性化合物の RNS 発生の評価

フェノール性化合物を 37°C でインキュベートし、2.5% リン酸に溶かした 3% Sulfanilamide、0.3% *N*-(1-Naphthyl)-ethylene diamine を加え、室温で 5 分間放置した後、プレートリーダーを用いて、測定波長 540 nm にで吸光度を測定した。

C. 研究結果

C-1. 人工胃液中におけるフェノール性化合物と $NaNO_2$ の反応

フェノール性化合物のニトロ化体の標準品が市販されていないことから標準品を確保するために合成を試みた。得られた化合物を LC/MS および NMR で確認したところ、CaA がニトロ化反応を受けた化合物 6,7-dihydroxy-1,2-(4H)-benzoxazin-4-one（CaA-NO-1）、2-(3,4-dihydroxyphenyl)-2-oxoethanaloxime（CaA-NO-2）、2-oxy-3-(3,4-dihydroxyphenyl)-1,2,5-oxadiazole（CaA-NO-3）の生成を確認した（Fig. 1）。また、得られた標準品を用い、LC/UV による測定を行ったところ、良好なクロマトグラムを得ることができた（Fig. 2）。

C-2. 人工胃液中におけるフェノール性化合物と $NaNO_2$ の反応

反応 pH がニトロ化合物の生成に与える影響を調べるために、人工胃液中および 50 mM 酢酸緩衝液（pH 4.0）で反応を行った。その結果、反応 pH によって生成する化合物および濃度が変化することが分かった（Fig. 3）。

また、生体内における各々の化合物の安定性試験を行うために、人工胃液および PBS 中で反応を行い、経時的に測定を行った。その結果、全てのフェノール性化合物は人工胃液条件下において、安定に存在することが確認された。しかし、中性条件下においては、CaA-NO-2 および CaA-NO-3 の顕著な分解が確認された（Fig. 4）。

C-3. DPPH 法による Antioxidant 作用の評価

DPPH 法は簡便な Antioxidant 作用の分析法であり、現在でも汎用性の高い方法として認知されている。この方法は、比較的安定な有機ラジカルである DPPH ラジカルが抗酸化物質と反応し消去されると、可視部の吸光度が低下する特徴を利用して Antioxidant 作用を直接的に評価する。フェノール性化合物およびニトロ化されたフェノール性化合物の Antioxidant 作用を評価した。その結果、ニトロ化を受けることで Antioxidant 作用が増強する結果が得られた（Fig. 5）。

C-4. ESR および HPLC/UV/ECD による Prooxidant 作用の評価

ROS 生成の評価のために ESR を用いた測定を行った。スピントラッピング剤には α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone を用いた。フェノール性化合物（CaA、CaA-NO-1～3、ChA および ChA-NO）と銅と反応させたところ、ヒドロキシラジカルの生成を確認した。また、ニトロ化反応を受けることでヒドロキシラジカルの生成量が減少する

結果が得られた（Fig. 6A）。

また、ESR で検出された ROS 生成について生体内でも同様の結果が得られるかを評価をするために、DNA 酸化の指標である 8-OHdG の測定を試みた。その結果、ESR で得られた結果と同様に、フェノール性化合物と銅を併用することで、8-OHdG 量の増加が認められ、ニトロ化反応を受けることで 8-OHdG 量が減少した（Fig. 6B）。

C-5. Griess 法によるフェノール性化合物の RNS 発生の評価

ChA、CaA およびニトロ化されたフェノール性化合物について、RNS 発生能は Griess 法によって評価した。その結果、全ての化合物について、人工胃液中では NO_2 の産生が認められなかった。しかし、中性条件下では CaA-NO-3 について、NO_2 の産生が経時的に認められた（Fig. 7）。

D. 考察

食品に含まれている ChA および CaA と $NaNO_2$ を併用することにより胃酸酸性条件下でニトロ化反応を引き起こす結果が得られた。ニトロ化反応は反応 pH とフェノール性化合物の化学構造によってベンゼン環が直接ニトロ化反応を受ける場合と、側鎖がニトロ化される場合がある。ベンゼン環がニトロ化反応を受ける場合、電子密度が大きく変化すると考えられ、ヒドロキシラジカルに代表されるような反応性が高い分子種はベンゼン環に結合するものと考えられる。

他方、CaA のように側鎖にニトロ化が起こった場合、不安定な化学構造になることが考えられ、ROS 生成には寄与しないが、RNS の生成を引き起こす可能性がある。RNS は ROS と反応することでペルオキシナイトライトなど反応性の高い分子種に変化することが知られている。今回、Griess 法によって活性が認められた CaA-NO-3 については、構造内にフロキサン骨格を有している。フロキサン骨格は NO のドナーとして使用される構造である。そのため、CaA と $NaNO_2$ を併用して生成された CaA-NO-3 については、NO_2 が産生したものと考えられ、今後、生体影響などを解明する必要があると考えられる。

E. 結論

本研究において、フェノール性化合物と $NaNO_2$ を併用することにより胃酸酸性条件下でニトロ化反応を引き起こし、ROS および RNS の生成に関与しているかを *in vitro* の系で検証した。

コーヒーに含まれているクロロゲン酸、CaA と $NaNO_2$ を人工胃液中で反応させることによって、ニトロ化反応が胃酸条件下で進行し、ニトロクロロゲン酸およびニトロカフェイン酸の生成を確認した。

また、フェノール性化合物と $NaNO_2$ を併用することにより胃酸酸性条件下でニトロ化反応が引き起こされ、ニトロ化反応を受けた化合物は Antioxidant 作用が増強することが示唆された。さらに、Prooxidant 作用はニトロ化反応を受けることで減少した。しかし、一部のニトロ化合物は NO_2 を産生することが明らかとなった。

本研究の結果より、生体内でニトロ化反応を受け、新たに生成される化合物について ROS 生成量は減少することが示唆されたが、RNS の増加が認められたことから、生

体への影響を考える上で、他の毒性評価も行うことが必要と考えられる。

F. 研究発表

1. 論文発表

該当なし

2. 学会発表

1) 食品関連フェノール性化合物と金属の相互作用によるヒドロキシルラジカルの生成

平澤 貴之[1]、丸山 陽介[1]、岩崎 雄介[1]、石井 雄二[2]、梅村 隆志[2]、伊藤 里恵[1]、斉藤 貢一[1]、西川 秋佳[2]、中澤 裕之[1]（[1]星薬大、[2]国立衛研病理部）（第71回分析化学討論会 2010年5月15日－16日 島根）

2) クロロゲン酸およびカフェイン酸と亜硝酸ナトリウム併用投与による活性酸素種産生

岩崎 雄介[1]、平澤 貴之[1]、中野 有紀[1]、石井 雄二[2]、伊藤 里恵[1]、梅村 隆志[2]、斉藤 貢一[1]、西川 秋佳[2]、中澤 裕之[1]（[1]星薬大、[2]国立衛研病理部）（第23回バイオメディカル分析科学シンポジウム 2010年7月21日－23日 宮城）

3) フェノール性化合物とグルコン酸銅の併用によるDNA の酸化的損傷

平澤 貴之[1]、大八木 章仁[1]、岩崎 雄介[1]、石井 雄二[2]、梅村 隆志[2]、伊藤 里恵[1]、斉藤 貢一[1]、西川 秋佳[2]、中澤 裕之[1]（[1]星薬大、[2]国立衛研病理部）（第23回バイオメディカル分析科学シンポジウム 2010年7月21日－23日 宮城）

4) 食品中のフェノール性化合物の複合反応が酸化ストレスに与える影響

平澤 貴之[1]、大八木 章仁[1]、岩崎 雄介[1]、石井 雄二[2]、梅村 隆志[2]、伊藤 里恵[1]、斉藤 貢一[1]、西川 秋佳[2]、中澤 裕之[1]（[1]星薬大、[2]国立衛研病理部）（環境・衛生部会フォーラム2010 環境衛生トキシコロジー 2010年9月9日－10日 東京）

5) カテキン類とグルコン酸銅の併用による活性酸素種生成

平澤 貴之[1]、大八木 章仁[1]、岩崎 雄介[1]、石井 雄二[2]、梅村 隆志[2]、伊藤 里恵[1]、斉藤 貢一[1]、西川 秋佳[2]、中澤 裕之[1]（[1]星薬大、[2]国立衛研病理部）（第100回 日本食品衛生学会学術講演会 2010年9月16日－17日 熊本）

6) 食品関連フェノール性化合物の複合反応によるDNAの酸化的損傷

平澤 貴之[1]、大八木 章仁[1]、丸山 陽介[1]、岩崎 雄介[1]、石井 雄二[2]、梅村 隆志[2]、伊藤 里恵[1]、斉藤 貢一[1]、西川 秋佳[2]、中澤 裕之[1]（[1]星薬大、[2]国立衛研病理部）（第131回 日本薬学会 2011年3月28日－31日 静岡）

G. 知的財産権の出願・登録状況
（予定を含む。）

特許取得

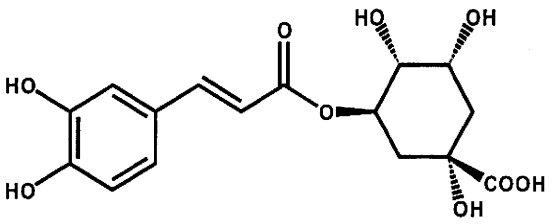
該当なし

実用新案登録

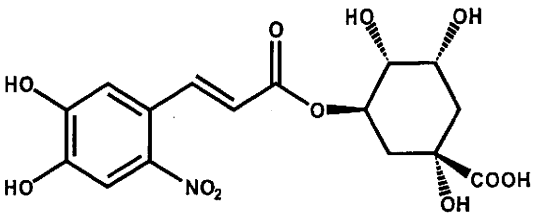
該当なし

その他

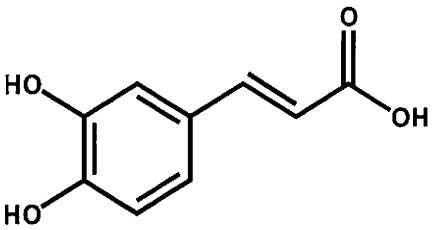
該当なし



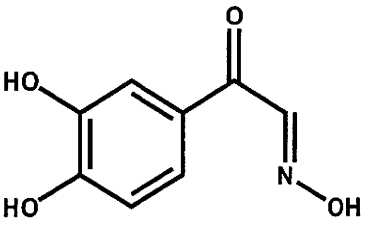
Chlorogenic acid (ChA)



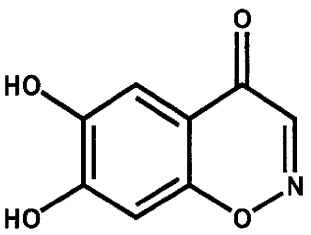
Nitrochlorogenic acid
(ChA-NO)



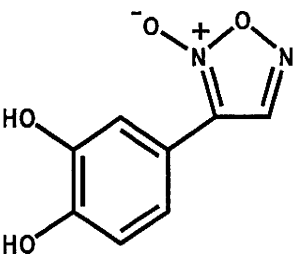
Caffeic acid (CaA)



2-(3,4-Dihydroxyphenyl)-2-oxoethanaloxime
(CaA-NO-1)



6,7-Dihydroxy-1,2-(4*H*)-benzoxazin-4-one
(CaA-NO-2)



2-Oxy-3-(3,4-dihydroxyphenyl)-1,2,5-oxadiazole
(CaA-NO-3)

Figure 1 Structure of ChA, CaA, and nitrated phenolic compounds

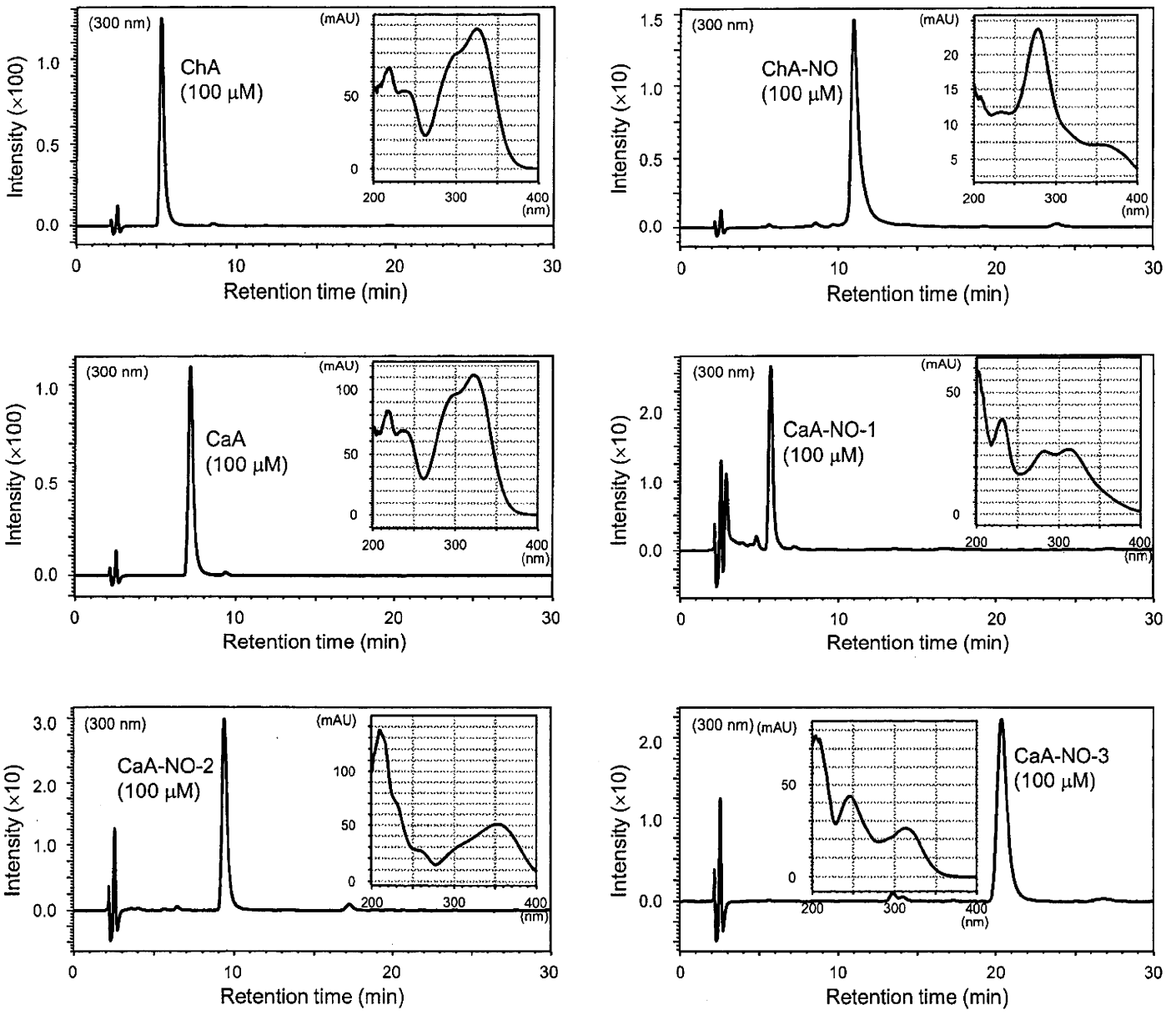


Figure 2 UV chromatograms and spectra of ChA, CaA, and nitrated phenolic compounds

(A)

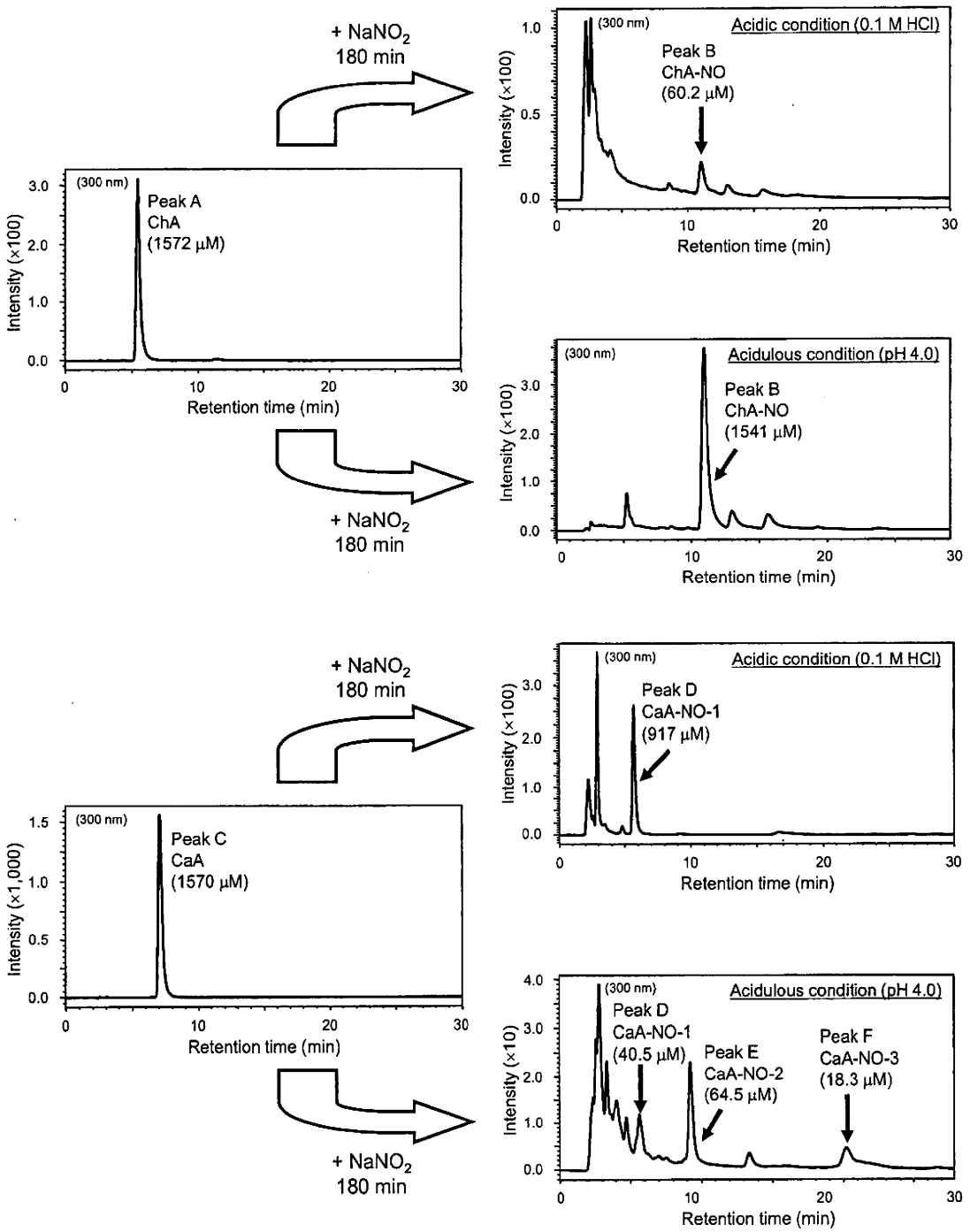


Figure 3 Interaction phenolic compound and sodium nitrate under acidic condition

(A) UV chromatograms of reacted interaction between phenolic compound and sodium nitrate. (B) Time course of generated compounds under acidic or acidulous conditions.

(B)

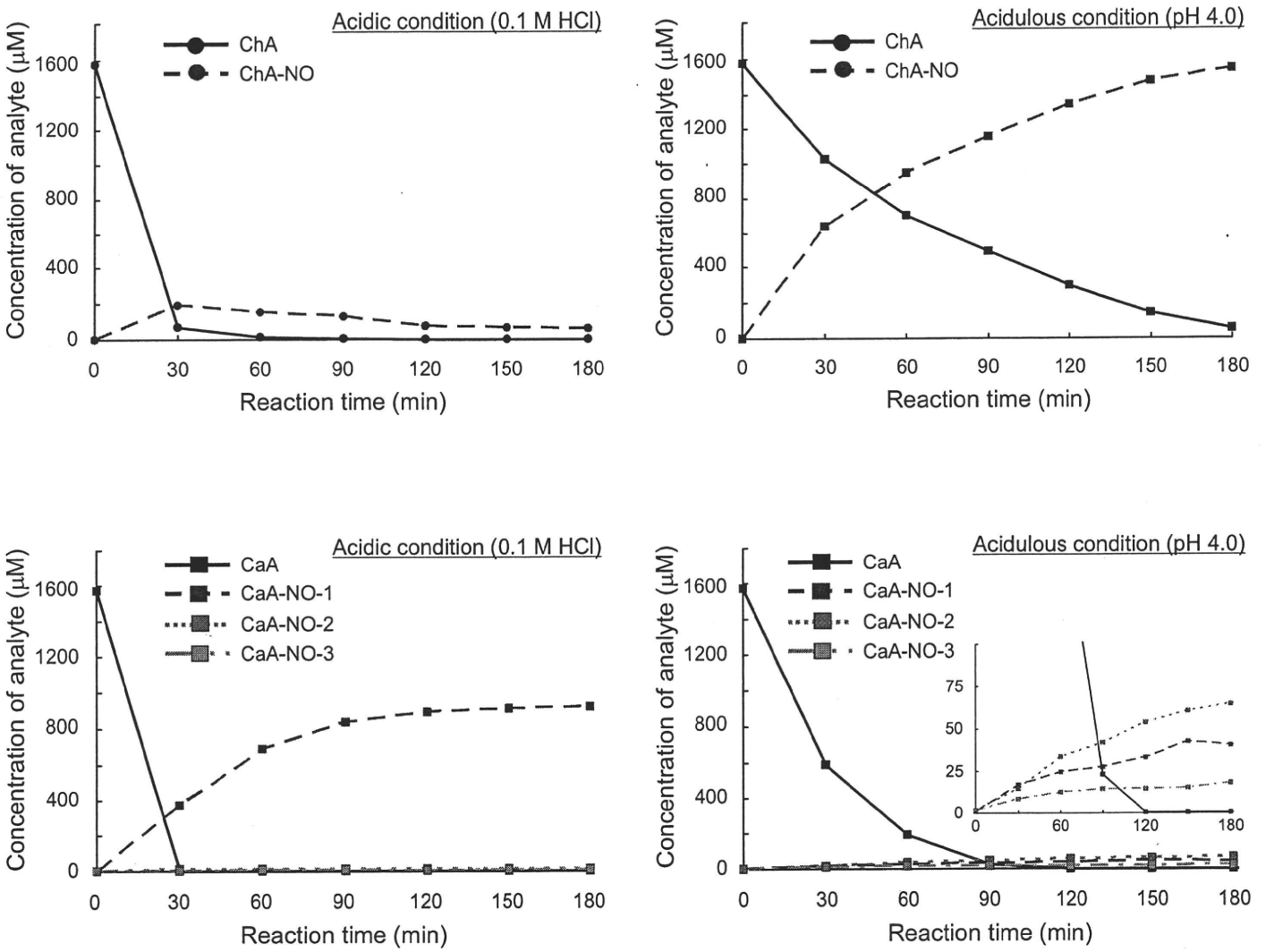


Figure 3 (continued)

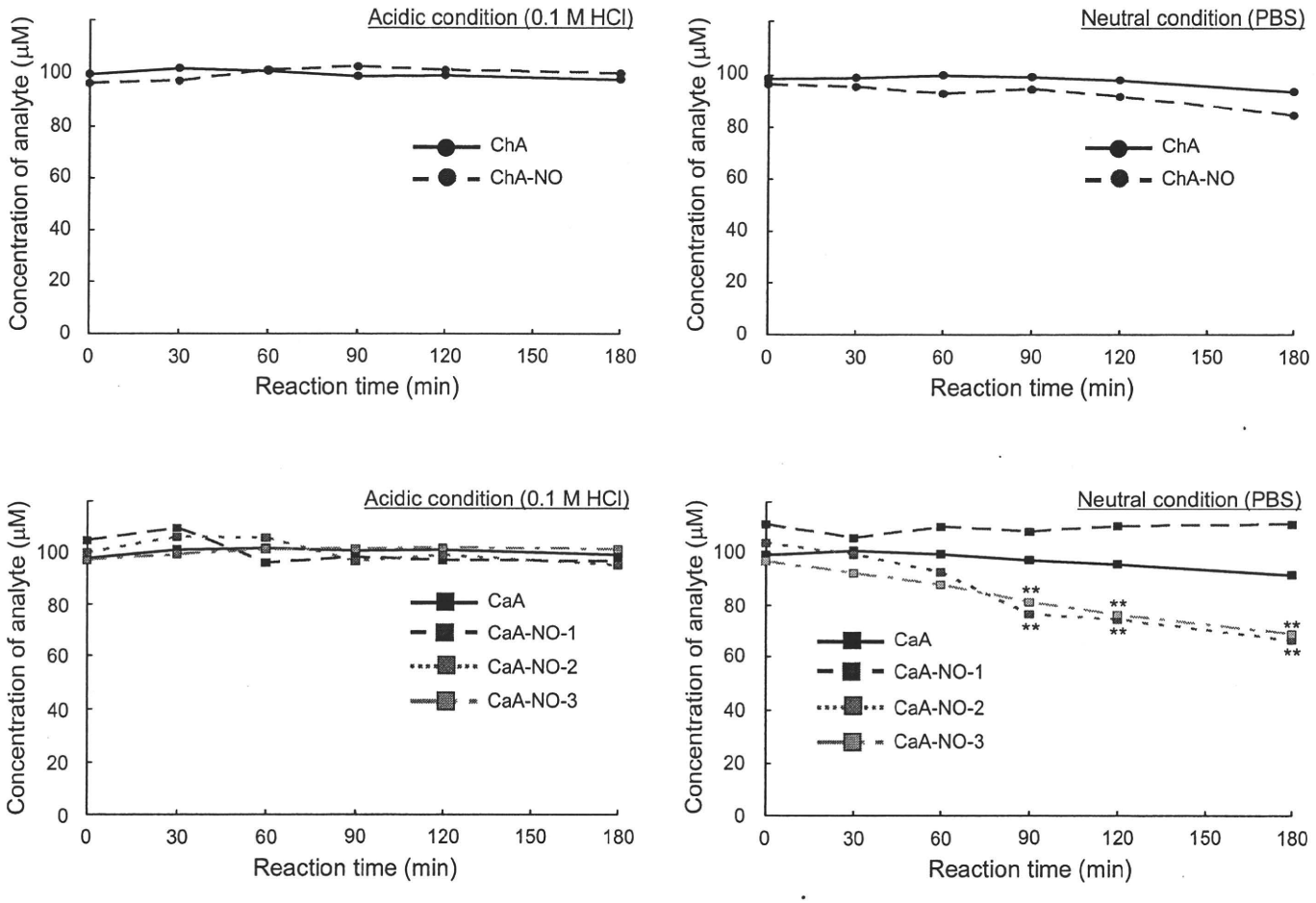


Figure 4 Sample stability of phenolic and nitrated phenolic compounds under acidic amd neutral condtions

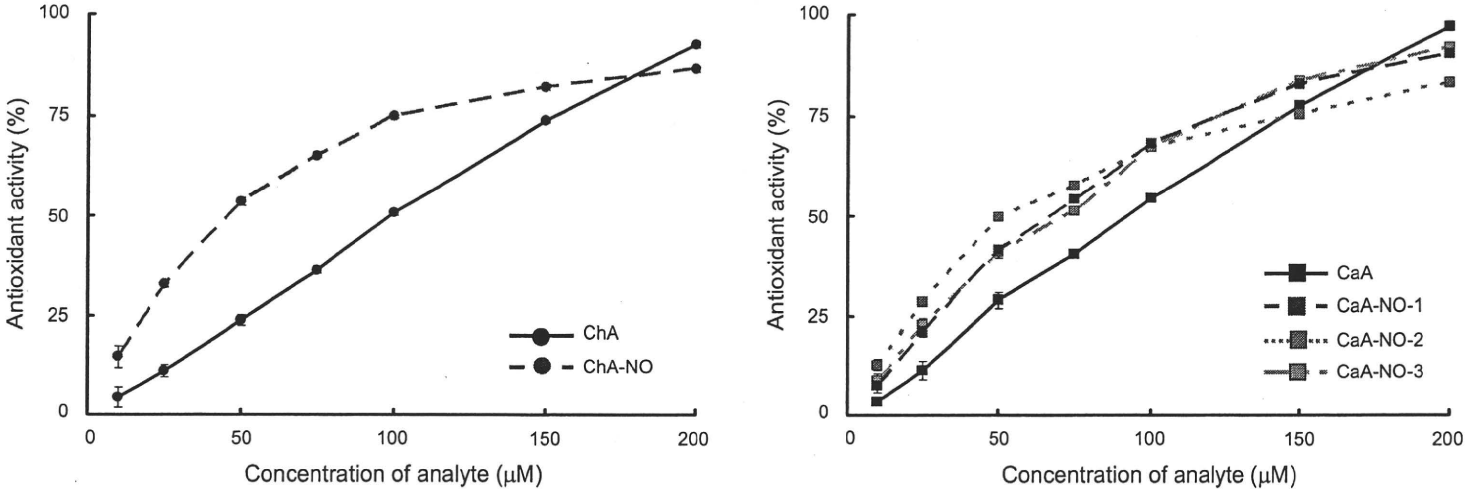


Figure 5 Antioxidant activities of phenolic compounds as assessed by DPPH assay

DPPH concentration was 1 mM. Data points represent means ± SD (n = 3).

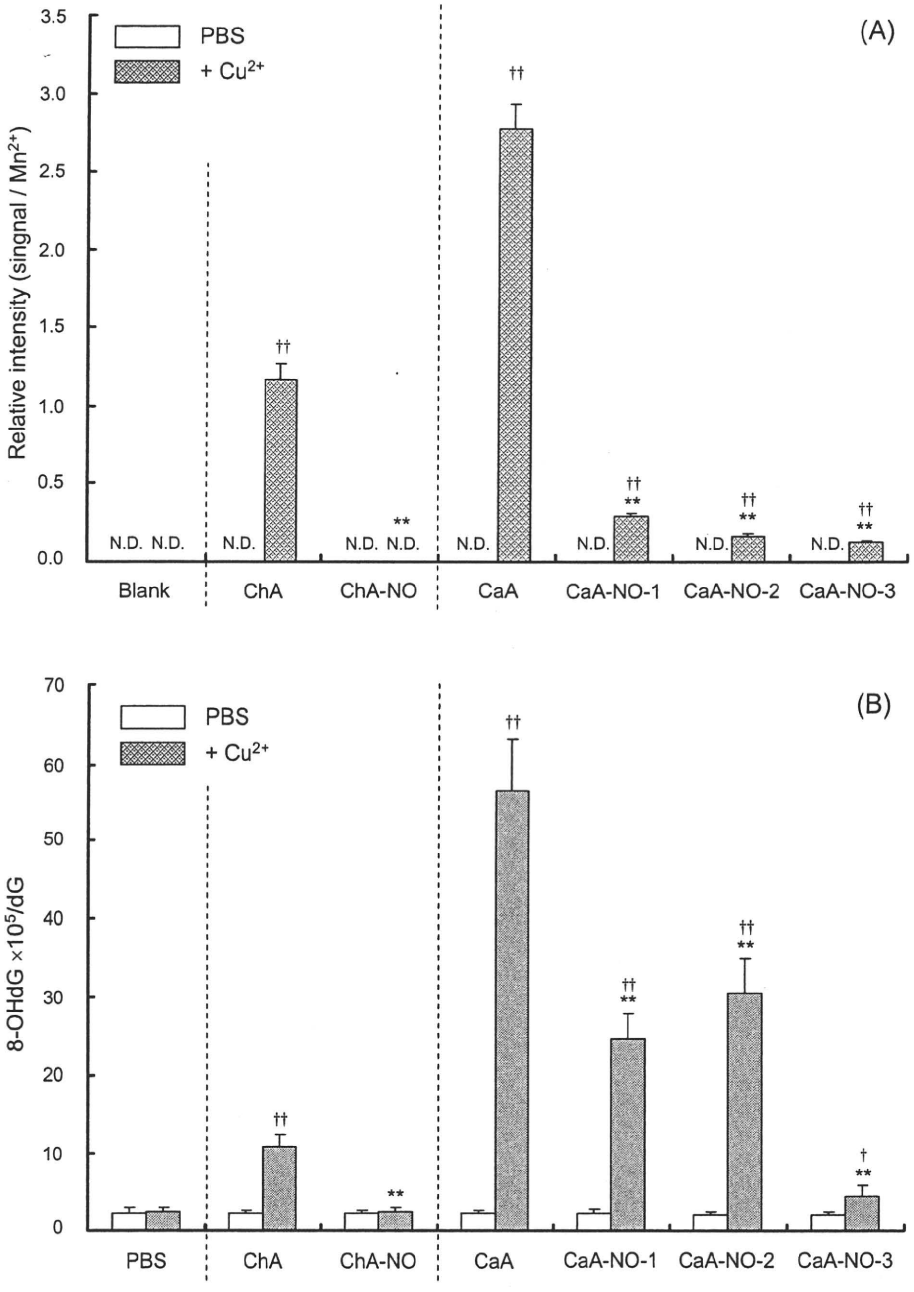


Figure 6 Pro–oxidant activities in the interaction between phenolic compounds and copper

(A) ESR (B) HPLC/UV/ECD

The data represents the mean ± S.D. (n = 6). * and ** : statistically significant ($P < 0.05$ and $P < 0.01$ vs. phenolic compound + copper). † and †† : statistically significant ($P < 0.05$ and $P < 0.01$ vs. phenolic compound alone).

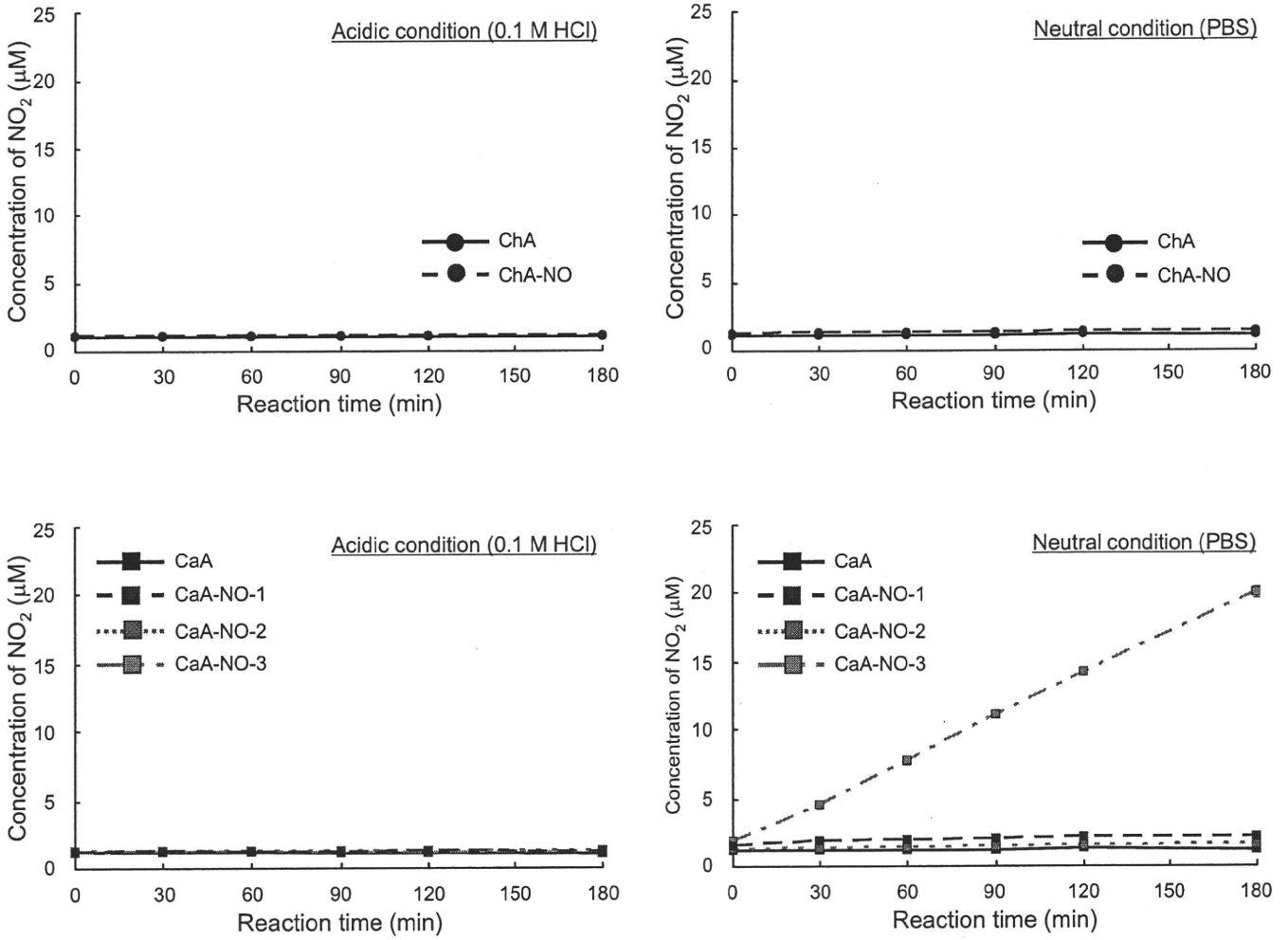


Figure 7 Concentration of NO_2 as assessed by Griess assay

Data points represent means ± SD (n = 6).

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	該当なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Fukuyama, T., Kosaka, T., Tajima, Y., Ueda, H., Hayashi, K., Shutoh, Y., and Harada, T.	Prior exposure to organophosphorus and organochlorine pesticides increases the allergic potential of environmental chemical allergens in a local lymph node assay.	Toxicol Lett.	199	347-56	2010
Fukuyama, T., Kosaka, T., Tajima, Y., Hayashi, K., Shutoh, Y., and Harada, T.	Detection of thymocytes apoptosis in mice induced by organochlorine pesticides methoxychlor.	Immunopharmacol Immunotoxicol.	33	193-200	2011

研究成果の刊行物・別刷

Toxicology Letters 199 (2010) 347–356

Contents lists available at ScienceDirect

Toxicology Letters

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

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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ARTICLE INFO

Article history:
Received 30 August 2010
Received in revised form
23 September 2010
Accepted 24 September 2010

Keywords:
Allergy
Immune disorder
Parathion
Methoxychlor
2,4-D-Butyl
Eugenol

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.

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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doi:10.1016/j.toxlet.2010.09.018

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.

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 ≥ 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.

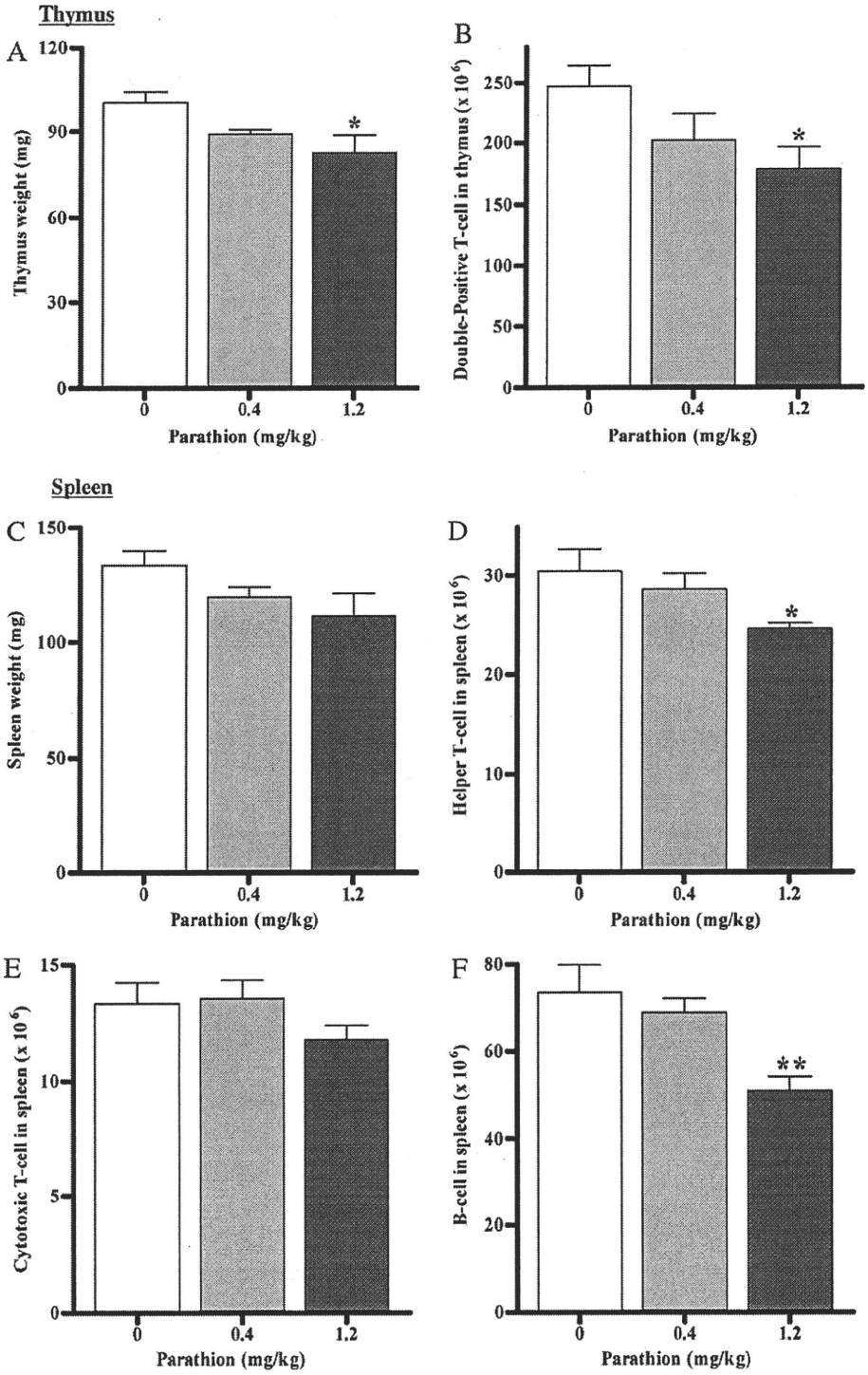

Fig. 1. Thymus weights (A), double-positive ($CD4^+CD8^+$) T-cell counts (B), spleen weights (C), helper ($CD4^+CD8^-$) T-cell counts (D), cytotoxic T-cell ($CD4^-CD8^+$) counts (E), and B-cell (CD45R/B220+) counts (F) of mice treated with parathion. Values are expressed as mean ± SD ($n = 6$ per group). 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

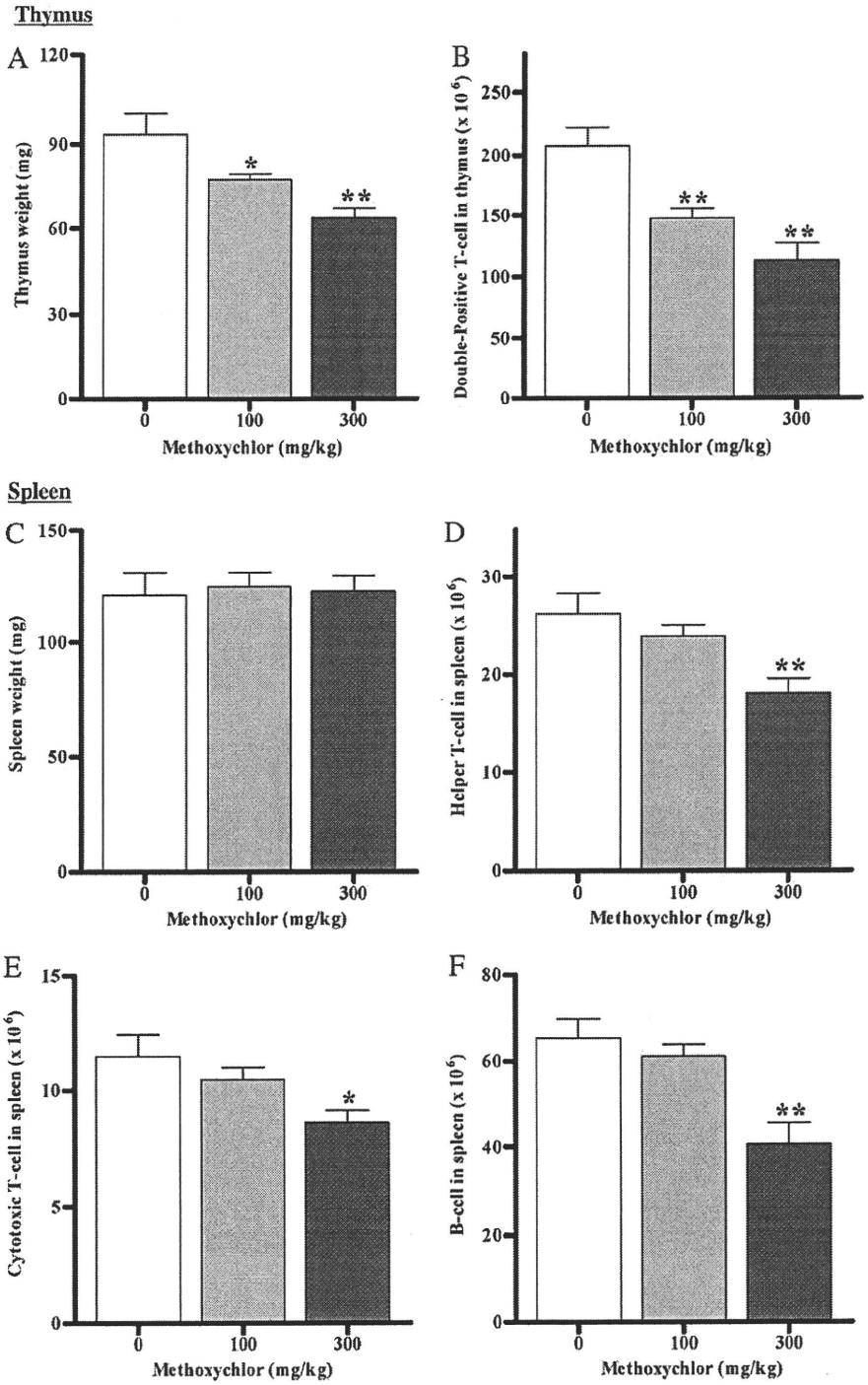

Fig. 2. Thymus weights (A), double-positive ($CD4^+CD8^+$) T-cell counts (B), spleen weights (C), helper ($CD4^+CD8^-$) T-cell counts (D), cytotoxic T-cell ($CD4^-CD8^+$) counts (E), and B-cell (CD45R/B220+) counts (F) of mice treated with methoxychlor. Values are expressed as mean ± SD ($n = 6$ per 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

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.

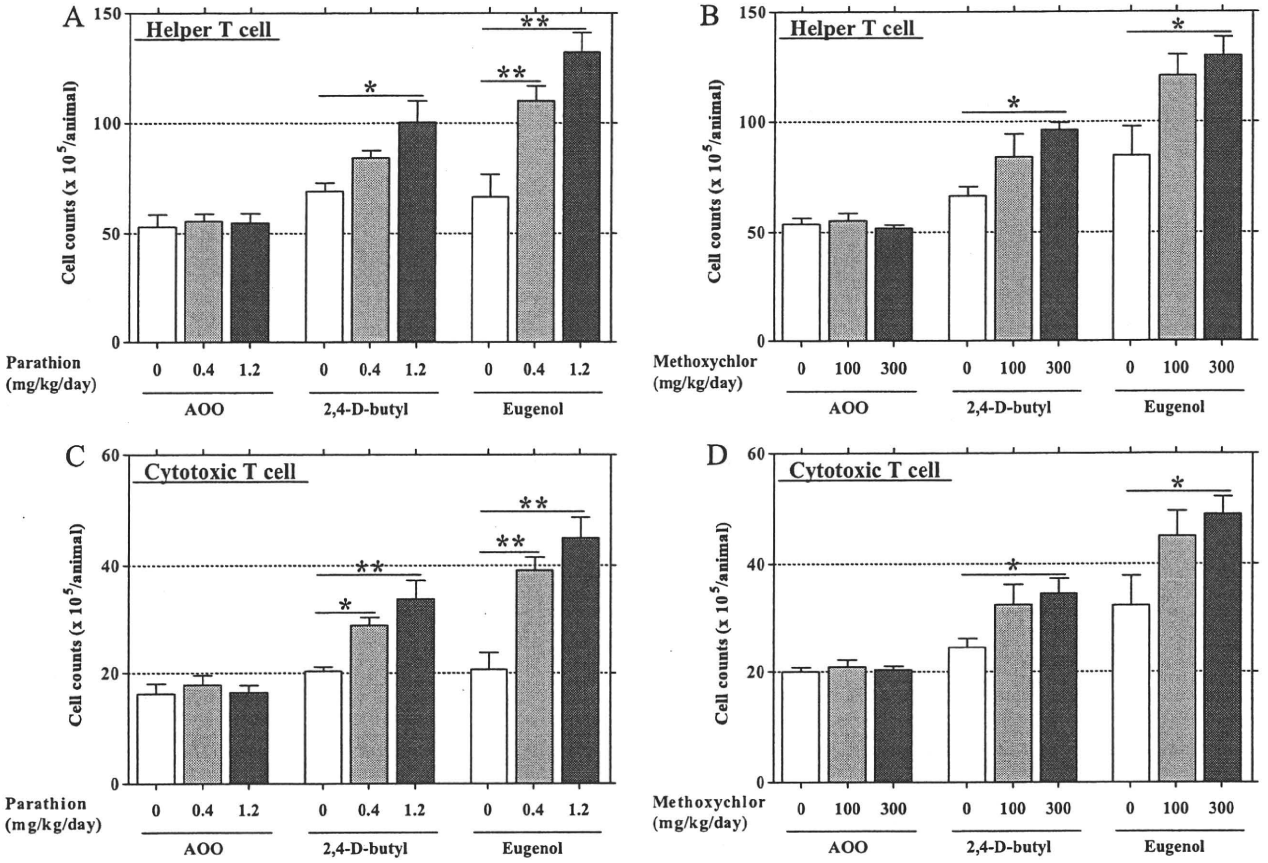

Fig. 3. Helper ($CD4^+CD8^-$) (A: Parathion; B: Methoxychlor) and cytotoxic ($CD4^-CD8^+$) (C: Parathion; D: Methoxychlor) T-cell counts in auricular lymph nodes of mice treated with test solution of mice. Values are expressed as mean ± SD ($n = 6$ per group). Statistical significance is marked by asterisks: $^*P < 0.05$, $^{**}P < 0.01$ (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).