を含む成分を体重減少目的で 6 カプセル（カテキン 600mg 相当）/day を半年間摂取した 42 歳の女性が劇症肝炎になり、肝移植を受けた事例を発表した。その後、文献上でもカテキンによる肝機能障害が 13 例報告されている。また、培養肝細胞を用いた実験では、エピガロカテキンガレート(EGCG)はミトコンドリア膜を直接破壊し、酸化ストレスを誘導することが明らかとなった。これらの報告を受け、2007 年 6 月に米国薬局方の DSI-EC (Dietary Supplements Information Expert Committee) は、緑茶成分を含むサプリメントには肝障害の危険性を示した警告文をつけるように指導している。日本では緑茶抽出物を利用した製品は多く販売されているが、現時点まで類似の報告はない。フェノール性抗酸化物質を高濃度に含む製品の消費量が年々増加していることから、今後、我が国でも同様の健康被害が発生する事が懸念される。

本研究では、フェノール性抗酸化物質の毒性発現機構について化学的な解析を行う。そして、毒性発現に関わる生物的、化学的因子を明らかにすることで、フェノール性抗酸化物質の薬物代謝酵素、生体環境、化学物質等との複合影響を予測し、毒性発現を事前に予測・予防するための情報提供を行う。平成 25 年度は、フェノール性抗酸化物質の毒性発現における複合影響を明らかにすることを目的として、薬物代謝酵素による酸化反応を経由するカテキンの毒性発現機構について化学的解析を行った。その結果、カテキンは塩基性条件下では酸化の過程で酸素をスーパーオキシドアニオンに還元することができた。金属イオンを伴うフェノール性抗酸化物質の毒性は in vitro 試験で既に報告されているが、塩基性条件下で活性酸素の発生を直接証明できたことで、複合影響として金属イオンを伴う毒性がヒトでも発症する可能性が強く支持された。一方、カテキンは抱合反応を受けて排泄されるが、薬物代謝酵素によって酸化反応が進行するとカテコール部分がキノン体へと酸化されることが予想される。しかし、キノン構造は化学的には非常に不安定で単離精製することができない為、キノン酸化体を経由する毒性経路は実証されていない。そこで我々は、生体高分子のモデル化合物の存在下、カテキンの酸化反応を行ったところ、キノン酸化体と生体高分子との付加体を得る事ができた。この結果は、カテキンはキノンへと酸化体されると毒性を発現することを示唆している。従って、キノン体への酸化を引き起こす薬物代謝酵素を促進する因子は、フェノール性抗酸化物質の複合影響として毒性を増強させる可能性を明らかにすることができた。

キノン構造を有する化合物は求核性の生体高分子と付加体を形成するとともに、NADH 等の補酵素やアスコルビン酸等の生体内還元物質が存在すると酸素分子を還元活性化して活性酸素を発生する。活性酸素は DNA を酸化して突然変異を誘発したり、脂質やタンパク質を酸化して神経細胞に損傷を与えたり、動脈硬化を促進する。そこで平成 26 年度は、フェノール性抗酸化物質の複合影響に資する、キノンへの酸化代謝と活性酸素の生成を伴う毒性の評価系を確立と，カテキン等の代謝活性化を伴う活性酸素毒性について評価を行う。

B．研究方法

1．pBR322 を用いた DNA 切断反応

エッペンドルフチューブにフェノール性抗酸化物質のアセトン溶液と 2,2-diphenyl-1-picrylhydrazyl (DPPH) の溶液それぞれ 2.5μl を混合後、3 分間 37℃でインキュベートした。その後、氷浴上で 50μM bp pBR322 の 50mM pH7.2 カコジル酸溶液を 40μl を添加、さらに 10mMNADH の 50mM pH7.2 のカコジル酸溶液を 40μl 添加した、37℃で 2 時間インキュベートした後、0.1% bromophenol blue in 30% glycerol を 5μL 添加し、泳動用サンプルとした。Ethydium bromide を添加した 1%アガロースゲルを作成し、各サンプル 10μl について電気泳動を行った（100V、5 時間）後、ゲルを洗浄後、UV トランスイルミネーター上で撮影した。

フェノール性抗酸化物質に対する DPPH の量は，異なる比率のカテキンと DPPH(2, 4, 8, 16 倍量)の d6-アセトン溶液について NMR を測定し、スペクトルにカテキンのピークが消失し、キノン酸化体のみが生成する条件を検討して決定した。

（倫理面への配慮）

本研究は全て化学系の実験であり、倫理面への問題はない。

C．研究結果

1．カテキンの酸化を伴う DNA 切断反応

酸化酵素によるカテキンからキノンへの酸化反応を一電子酸化剤の DPPH によって再現できるか NMR で確認した。その結果、カテキンに対して 4 倍量の DPPH を用いることで、ほぼ全てのカテキンがキノン体へ酸化されることがわかった。そこで、エッペンドルフチューブ中、この条件でキノンへの酸化を行い、さらにこの溶液に pBR322DNA と NADH を添加してキノン酸化体による DNA 切断反応を行った。

その結果（図１）、カテキンは DPPH による酸化の後、NADH を添加することで DNA の切断反応が進行することが確認された。この切断反応はカテキンの濃度に依存して増強した（図２）。また、この切断反応は DPPH と NADH の両方を添加することで進行することがわかった。この結果より、カテキンは DPPH による酸化によって生成したキノン体が NADH 存在下、酸素を還元活性化して活性酸素を発生し、DNA を切断することがわかった。

そこで次に緑茶抽出物の主なポリフェノールで健康被害が最も多いエピガロカテキンについて、同様の条件で DNA 切断実験を行った（図３）。その結果、エピガロカテキンも DPPH と NADH 存在下で DNA 切断反応が進行した。反応はエピガロカテキンの濃度に依存して増強すること、また、DPPH によってキノンへ酸化させても NADH が存在しないと反応が進行しないことがわかった。次に、エピガロカテキンの切断活性の強さをカテキンと比べてみた（図４）。pBR322DNA はスーパーコイルの Form I が切断が入るとオープンサーキュラーの Form II になり、さらに切断されるとリニアーの Form III に転換される。カテキンは濃度依存的に Form II の生成量が増えてくるが、2mM でも Form I が残っている。一方、エピガロカテキンは高濃度では Form II への転換とともに Form III の生成が確認され、Form I は完全に消失した。この結果より、エピガロカテキンのキノン体への酸化を伴う DNA 切断反

応はカテキンと比べて非常に強力であることが明らかとなった。

2. DNA 切断反応の活性種の解析

キノン酸化体の生成と NADH が関与する DNA 切断反応がどのような活性酸素種によって進行しているか明らかにするため、カテキンおよびエピガロカテキンによる DNA 切断反応に対する活性酸素消去剤の影響を検討した（図５）。活性酸素の消去剤としてスーパーオキシドを過酸化水素に還元する SOD、過酸化水素を水に還元するカタラーゼ、ヒドロキシルラジカルを水に還元する DMSO を DNA 切断反応の系に添加したところ、全ての消去剤は DNA 切断を完全に抑制した。この結果は、これらの活性酸素の中で最も酸化力の強いヒドロキシルラジカルがスーパーオキシドと過酸化水素を経由して生成し、それが DNA に対し強力な切断活性を示していることを示している。

D．考察

フェノール性抗酸化物質は比較的安全性が高い化合物として知られているが、海外では緑茶抽出物を摂取することで肝炎を発症した事例が報告されている。多くのフェノール性抗酸化物質は第 II 相薬物代謝酵素によって抱合体となって排泄される。しかし、健康食品等によって第 I 相薬物代謝酵素が誘導されると、フェノール性化合物はキノン酸化体へと代謝されることが予想される。キノン代謝物は化学的には不安定で核酸や蛋白質と結合したり、活性酸素を発生して遺伝毒性を示すことが懸念される。近年、健康食品に含まれている第 I 相酵素を誘導する成分が、医薬品の作用に影響を与えることが問題となった。軽度の鬱状態の改善作用によって健康食品としての利用が高まっているセントジョーンズワートは第 I 相薬物代謝酵素を誘導することによって、インジナビル（抗 HIV 薬）、ジゴキシン（狭心症薬）、シクロスポリン（免疫抑制薬）の効果を減少させることから、平成 12 年５月、厚生労働省は医療関係社に注意喚起を行った。複合影響の観点から健康食品による第 I 相薬物代謝酵素の誘導は、フェノール性抗酸化物質においてはてキノン酸化体の生成を伴う毒性の発現が懸念される。そこで、フェノール性抗酸化物質の毒性を明らかにすることを目的として、化学的な反応モデルによるカテキンからのキノンへの酸化と活性酸素毒性の構築を試みた。

第 I 相薬物代謝酵素によるキノンへの酸化は化学的にはガルビノキシルラジカルや DPPH などの一電子酸化剤を用いることによって再現することができた。生成するキノン酸化体は不安定で単離生成することが困難であるので、NMR のサンプルチューブの中で反応を行い、そのままスペクトルを測定することで、キノン酸化体の生成を確認した．その結果、カテキンに対して 4 倍量の DPPH を用いるとほぼ定量的にキノン酸化体が生成することがわかった。そこで、この反応系をエッペンドルフチューブで行い、そこに pBR322DNA と NADH を添加してインキュベートし、アガロースゲル電気泳動によって解析したところ、pBR322DNA の切断反応が進行していることが確認できた。また、発生している活性酸素種を検討したところ、ヒドロキシルラジカルによる DNA 鎖の切断が進行していることがわかった。以上の結果より、フェノール性抗酸化物質

がキノン体へと酸化されると、NADHによる一電子還元反応によって生成するセミキノンラジカルアニオンが酸素を還元してスーパーオキシドアニオンラジカルが発生することがわかった（図６）。また、スーパーオキシドアニオンラジカルは過酸化水素を経由してヒドロキシルラジカルを生成することでDNA鎖への切断反応が進行していることが明らかとなった。

以上、フェノール性抗酸化物質のキノンへの酸化と活性酸素毒性の発現を化学的な系で構築することができた。肝炎の発症が報告されているエピガロカテキンについてこの反応系でDNA切断活性を解析したところ、カテキンと比べて非常に強力であることがわかった。この結果は、フェノール性抗酸化物質のヒトでの毒性には第Ⅰ相酵素によるキノン酸化体の生成を伴う活性酸素毒性が関与している可能性を示唆している。この反応系はフェノール性抗酸化物質の生体における毒性発現のモデルとして有用であり、DNA切断活性の強さを指標とした毒性評価系として利用できることがわかった。今後は他のフェノール性抗酸化物質についてもこの反応系を利用した評価を行い、複合影響の観点からヒトへの健康影響を明らかにする。

Ｅ．結論

複合影響の観点からフェノール性抗酸化物質のヒトへの影響を評価するためには、機能性食品等によって誘導された第Ⅰ相薬物代謝酵素の影響を検討する必要がある。フェノール性抗酸化物質は第Ⅰ相薬物代謝酵素によってキノン体へと酸化されると、生体分子との付加や酸化ストレスを引き起こす可能性がある。しかしながら、非常に不安定なキノン酸化体について毒性を評価することは難しい。そこで、化学的な系でキノンへの酸化を伴う毒性の再現を試みた。一電子酸化剤によってキノン酸化体を生成させた後、この反応系にNADHとpBR322DNAを添加すると、活性酸素の生成によるDNA鎖の切断反応が進行することがわかった。この系を利用してカテキンとエピガロカテキンについて毒性を再現したところ、肝炎が報告されているエピガロカテキンはカテキンよりも非常に強力にDNA切断反応が進行した．この結果より、フェノール性抗酸化物質はヒトではキノン酸化体を経由する発現機構で毒性を発現している可能性が示唆された。この反応系は健康食品やサプリメントなどに含まれる薬物代謝酵素を誘導する化合物と、フェノール性抗酸化物質との複合影響を評価する試験系としての活用が期待される。

Ｆ．研究発表

1.　論文発表

1) Takizawa, Y., Nakata, R., Fukuhara, K., Yamashita, H., Kubodera, H., Inoue, H., The 4'-hydroxyl group of resveratrol is functionally important for direct activation of PPARα, *PLoS One*, 10, e0120865 (2015)

2.　学会発表

なし

Ｇ．知的財産権の出願・登録状況

1）特許出願 2014-174387 号、発明の名称：「新規スチルベン誘導体」、福原　潔、熊本浩樹、樋口明弘、坪田一男

2）特許出願 2014-174332 号、発明の名称：

「スチルベン誘導体を有効成分とする角結膜疾患又は老視の予防及び／又は治療剤」	樋口明弘、坪田一男、福原　潔

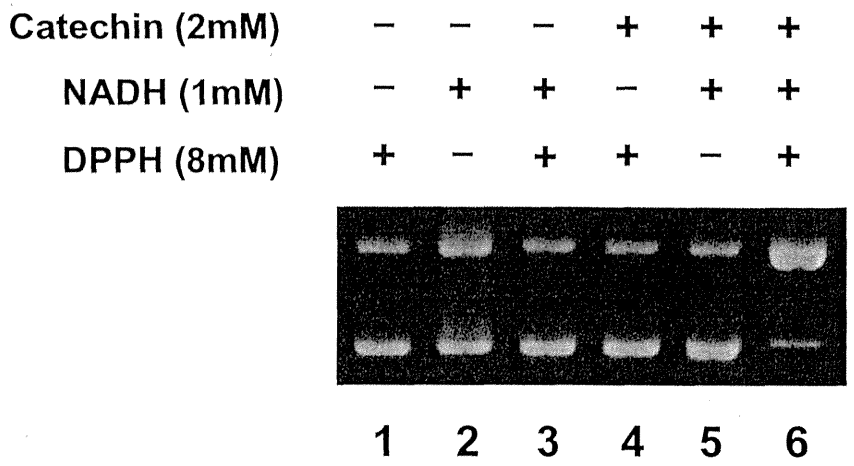

図1. (+)-カテキンのキノンへの酸化を伴うDNA切断反応：一電子酸化剤(DPPH)と還元剤(NADH)の影響

Assays were performed in 50 mM sodium cacodylate buffer, pH7.2, containing 45μbp of pBR322DNA, for 2h at 37°C. Lane 1: 1mM NADH, lane 2: 8mM DPPH, lane 3: 1mM NADH and 8mM DPPH, lane 4: 2mM catechin and 1mM NADH, Lane 5: 2mM catechin and 8mM DPPH, lane 6: 2mM catechin, 1mM NADH and 8mM DPPH.

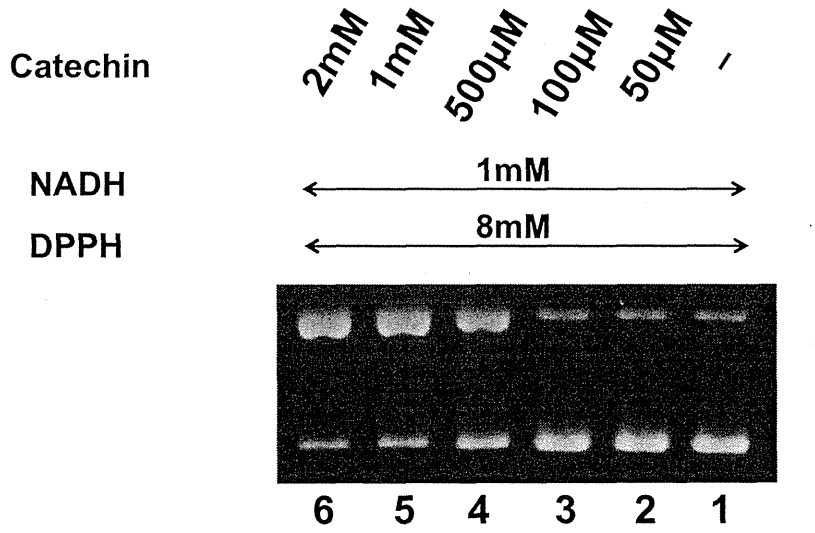

図2. (+)-カテキンのキノンへの酸化を伴うDNA切断反応：濃度依存性

Assays were performed in 50 mM sodium cacodylate buffer, pH 7.2, containing 45 μbp of pBR322DNA, 1 mM NADH, 8 mM DPPH for 2 h at 37 °C under aerobic condition. Lane 1 – 6: 0, 50 μM, 100 μM, 500 μM, 1 mM and 2 mM.

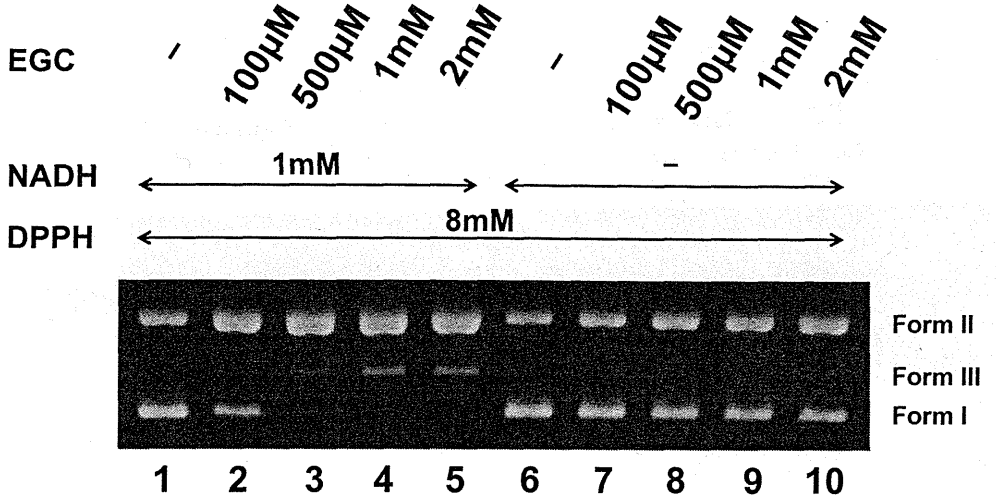

図3. エピガロカテキン(EGC)のキノンへの酸化を伴うDNA切断反応

Assays were performed in 50 mM sodium cacodylate buffer, pH7.2, containing 45μbp of pBR322DNA, 8mM DPPH, for 2 h at 37 °C under anaerobic condition. Lane 1 – 5: 0, 100 μM, 500μM, 1 mM and 2 mM EGC in the presence of 1mM NADH. Lane 6 – 10: 0, 100 μM, 500μM, 1 mM and 2 mM EGC in the absence of NADH.

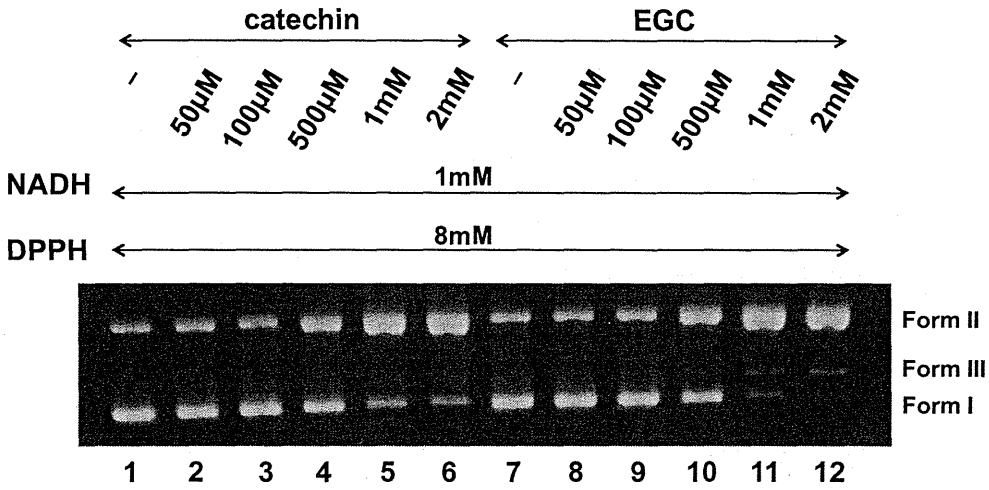

図4. (+)-カテキンとエピガロカテキンの酸化を伴うDNA切断反応

Assays were performed in 50 mM sodium cacodylate buffer, pH 7.2, containing 45 μbp of pBR322DNA, 1 mM NADH and 8mM DPPH for 2h at 37 °C under aerobic condition. Lane 1 – 5: 0, 50 μM, 100 μM, 500μM, 1 mM and 2 mM of catechin. Lane 6 – 10: 0, 50 μM, 100 μM, 500μM, 1 mM and 2 mM of EGC.

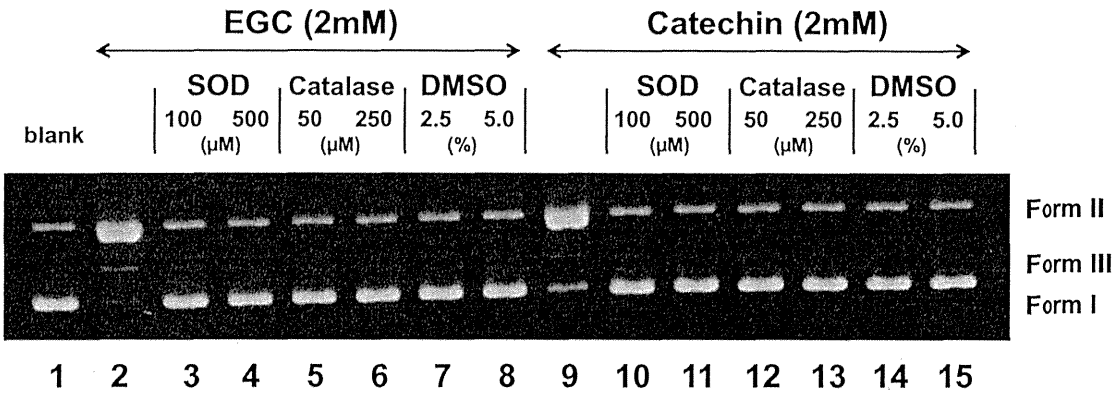

図5. (+)-カテキンとエピガロカテキンの酸化を伴うDNA切断反応：活性酸素阻害剤の影響

Assays were performed in 50 mM sodium cacodylate buffer, pH7.2, containing 45μbp of pBR322DNA, 1mM NADH, 8mM DPPH, 2mM EGC (lane 2 - 8) and 2mM catechin (lane 9 - 15), for 2 h at 37 ℃ under anaero bic condition. Lane 3 and 10: 100 μM SOD, lane 4 and 11: 500 μM SOD, lane 5 and 12: 50 μM catalase, lane 6 and 13: 250 μM catalase, lane 7 and 14: 2.5 % DMSO, lane 8 and 15: 5.0 % DMSO.

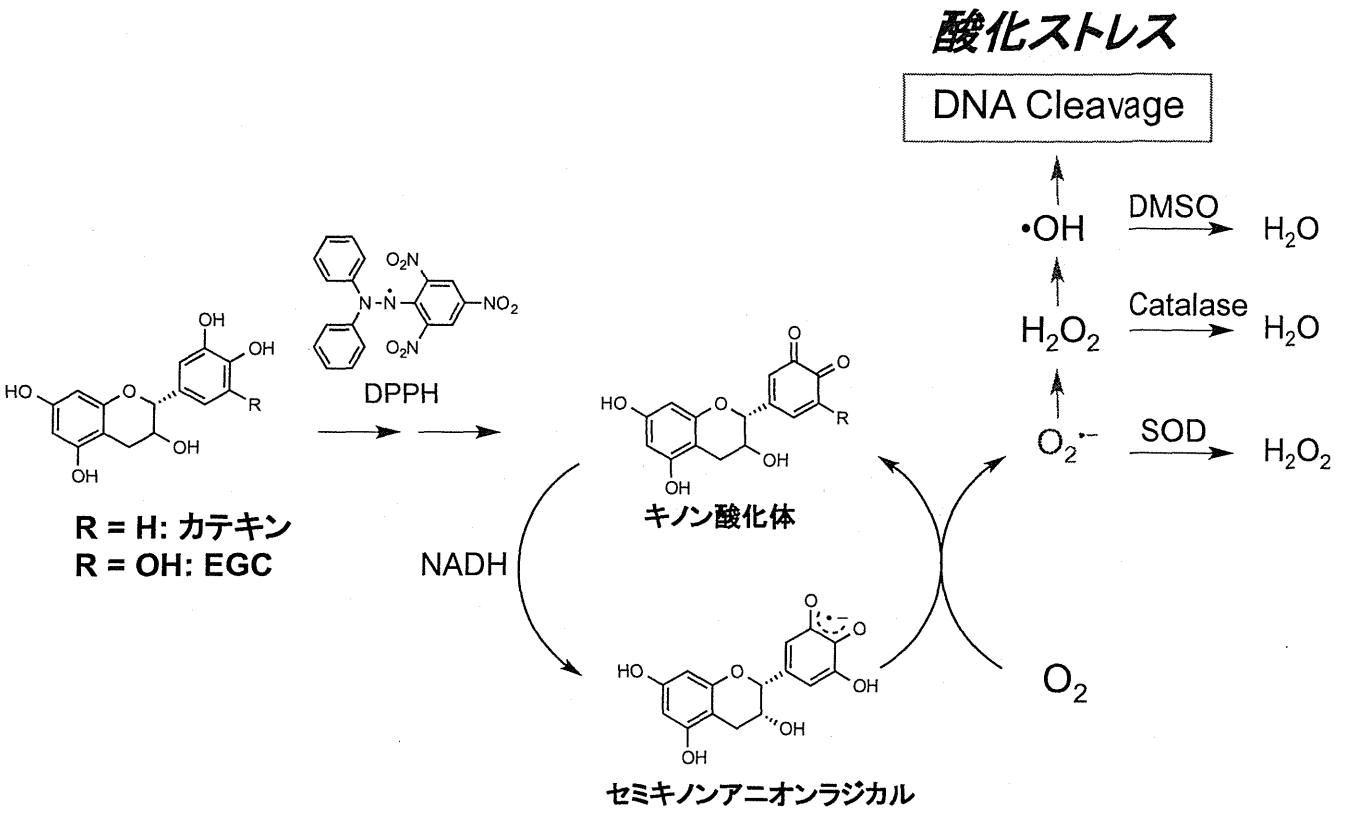

図6. (+)-カテキンの酸化代謝を伴うDNA切断反応機構

研究成果の刊行に関する一覧表レイアウト（参考）

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., Nishino, R., Kosaka, T., Watanabe, Y., Kurosawa, Y., Ueda, H., and Harada, T.	Effects of prior oral exposure to combinations of environmental immunosuppressive agents on ovalbumin allergen–induced allergic airway inflammation in Balb/c mice.	Immunopharmacology and immunotoxicology	36(4)	261-270	2014
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Takizawa, Y., Nakata, R., Fukuhara, K., Yamashita, H., Kubodera, H., Inoue, H.	4'-hydroxyl group of resveratrol is functionally important for direct activation of PPARα	*Plos One.*	10	E0120865	2015

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RESEARCH ARTICLE

Effects of prior oral exposure to combinations of environmental immunosuppressive agents on ovalbumin allergen-induced allergic airway inflammation in Balb/c mice

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Abstract

Humans are exposed daily to multiple environmental chemicals in the atmosphere, in food, and in commercial products. Therefore, hazard identification and risk management must account for exposure to chemical mixtures. The objective of the study reported here was to investigate the effects of combinations of three well-known environmental immunotoxic chemicals – methoxychlor (MXC), an organochlorine compound; parathion (PARA), an organophosphate compound; and piperonyl butoxide (PBO), an agricultural insecticide synergist – by using a mouse model of ovalbumin (OVA)-induced allergic airway inflammation. Four-week-old Balb/c mice were exposed orally to either one or two of the environmental immunotoxic chemicals for five consecutive days, prior to intraperitoneal sensitization with OVA and an inhalation challenge. We assessed IgE levels in serum, B-cell counts, and cytokine production in hilar lymph nodes, and differential cell counts and levels of related chemokines in bronchoalveolar lavage fluid (BALF). Mice treated with MXC + PARA or PBO + MXC showed marked increases in serum IgE, IgE-positive B-cells and cytokines in lymph nodes, and differential cell counts and related chemokines in BALF compared with mice that received the vehicle control or the corresponding individual test substances. These results suggest that simultaneous exposure to multiple environmental chemicals aggravates allergic airway inflammation more than exposure to individual chemicals. It is expected that the results of this study will help others in their evaluation of immunotoxic combinational effects when conducting assessments of the safety of environmental/occupational chemicals.

Keywords

Allergic airway inflammation, environmental chemicals, methoxychlor, parathion, piperonyl butoxide

History

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Introduction

Every day, humans are exposed concurrently or sequentially to a huge number of products that contain environmental agents (e.g. cosmetics, pesticides, drugs, and biotechnology-derived products) and to multiple environmental chemicals in the atmosphere and in food[1–5]. Because of this constant exposure, safety assessments must take into consideration the effects of combined exposure. However, most current toxicity assessments are based on exposure to individual substances and, as such, the effects of combined exposure to environmental chemicals remain unclear. The objective of the studies reported here was to investigate the combined toxicologic effects of multiple chemicals, focusing on their influence on the immune system.

In our previous studies, we demonstrated the combined effects of three well-known environmental immunotoxic chemicals – methoxychlor (MXC), an organochlorine compound; parathion (PARA), an organophosphate compound; and piperonyl butoxide (PBO), an agricultural insecticide synergist – by using a short-term oral exposure method that we developed[6]. Briefly, Balb/c mice exposed to MXC + PARA or PBO + MXC had lower immune responses to subsequent immunization with sheep red blood cells (SRBCs), including lower SRBC-specific IgM production and fewer SRBC-specific T and B cells, than mice exposed to the corresponding individual chemicals. This suggests that environmental chemicals can have additive immunotoxic effects when encountered simultaneously. In the current study, to develop the advanced mechanisms of the combined immunotoxicologic effects of multiple chemicals, we used a mouse model of ovalbumin (OVA)-induced allergic airway inflammation and focused on the mechanisms by which the allergic potential is modulated[7].

Recent reports suggest that prior exposure to environmental immunosuppressive chemicals induces allergic diseases in immature rodents[8,9]. Our laboratory previously demonstrated that prior exposure to immunosuppressive environmental chemicals aggravates T cell-mediated allergic reactions[10],

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mite- and chemical allergen-induced atopic dermatitis-like immunoreactions[11,12]. These data indicate that immunosuppression by environmental chemicals is closely related to the aggravation of allergic reactions.

In the current study, we demonstrate the immunomodulatory effects of exposure to MXC + PARA and PBO + MXC compared to exposure to the individual agents in a mouse model of OVA-sensitization and challenge, one of the most popular allergic airway inflammation models[7]. In this study, to explore the mechanisms of airway inflammation development, we analyzed leukocyte numbers, chemokine production, and immunoglobulin (Ig) E levels in serum and B-cells and cytokine production in hilar lymph node (LN) cells.

Materials and methods

Chemicals

Standard MXC ($C_{16}H_{15}Cl_3O_2$, >97% pure), standard PARA ($C_{10}H_{14}NO_5PS$, 99.5% pure), and standard PBO ($C_{19}H_{30}O_5$, >98% pure) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil was purchased from Hayashi Chemicals (Tokyo, Japan). Albumin from chicken egg white (ovalbumin, OVA) was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan) and Sigma-Aldrich Japan K.K. (Tokyo, Japan). Aluminum hydroxide hydrate gel suspension (ALUM) was purchased from Cosmo Bio Co., Ltd. For the intraperitoneal (i.p.) sensitization, OVA (10 μg) was dissolved in PBS (0.2 ml/animal) containing ALUM (2 mg). For the inhalation challenge, OVA was dissolved in PBS to 0.1% (w/v). MXC, PARA, or PBO diluted in corn oil to a fixed final volume was orally administered to mice. Based on the EPA Immunotoxicity Guidelines (1998), which states that doses should "not produce significant stress, malnutrition, or fatalities", doses used in this study were <1/5 the median lethal dose (LD_{50}; dose at which ≥50% of animals would be expected to die) and were administered to avoid induction of clear general or immune toxicity (i.e., changes in appearance, posture, behaviour, respiration, consciousness, neurologic status, temperature, excretion, etc.[12]; The single-chemical dosages used in this study were: MXC, 100 mg/kg day; PARA, 1.0 mg/kg day; and PBO, 100 mg/kg day. Combination dosages (MXC + PARA and PBO + MXC) were prepared by mixing each chemical so that the final concentration of each chemical was half that of the single dosage. As the objective in this study was to investigate the relationship between combined exposure to immunosuppressive chemicals and allergic responses in a mouse model, we selected relatively high doses compared with actual human exposures. The examination of the relationship of allergic responses to doses comparable to human exposures is now underway.

Animals

Balb/cAnN mice (female, 3-wk-old) were purchased from Charles River Laboratories (Atsugi, Kanagawa, Japan) and housed individually in cages under controlled lighting (lights on, 07:00–19:00), temperature (22 ± 3 °C), humidity (55% ± 15%), and ventilation (at least 10 complete fresh-air changes/h). Standard rodent chow (Certified Pellet Diet MF; Oriental Yeast Co., Tokyo, Japan) and filtered water were available *ad libitum*. All aspects of the current study were conducted in accordance with the Animal Care and Use Program of the Institute of Environmental Toxicology, Japan (IET IACUC Approval No. 12103).

Experimental protocol

The experimental protocol used in this study is depicted in Figure 1. Following a 6-d acclimatization period, healthy Balb/c mice (now 4-wk-old) were subjected to grouping. All animals were weighed individually on the day of initiation of treatment and those with extremely high or low body weights were eliminated. The remaining animals were allocated to each dose group ($n = 8$ mice per group) for dosing, vehicle control, or no treatment (intact group), through the stratified random sampling method on their body weights. After allocation, it was confirmed that all individual body weights were within a range of 80–120% of the mean value. According to our preliminary study, animals receiving only OVA sensitization or challenge showed almost the same patterns of allergic airway inflammation as the intact group. Therefore, data of only OVA sensitization or challenge are not shown in this article. On Days 1–5, mice were given an oral dose (by gavage, without any anaesthesia) of a single or combination test solution (MXC, PARA, PBO, MXC + PARA, PBO + MXC), or vehicle only. For sensitization, OVA/ALUM was injected intraperitoneally into each mouse on days 29, 36, and 42. For the challenge, OVA/PBS was administered by inhalation (see section "Method of inhalation exposure") on days 43, 45, 47, 50, 52. One day after the last challenge (day 53), all animals were anesthetized and sacrificed by pentobarbital sodium injection (75 mg/kg, i.p.). Blood samples were taken from the inferior vena cava, and serum samples were assayed for IgE levels. Bronchoalveolar lavage fluid (BALF) was collected by cannulating the trachea and lavaging the lungs 3 times with 1 mL PBS supplemented with 1% heat-inactivated foetal calf serum (FCS; Life Technologies Co., Ltd., Tokyo, Japan). The first BALF fraction from each animal was centrifuged at 350 × *g* for 5 min, and chemokine levels were measured. The cell pellets of all three fractions were resuspended, pooled, and centrifuged at 350 × *g* for 5 min. The supernatants were removed, and the cell pellets were used for differential cell counts. Hilar LN removed from each mouse were stored in RPMI 1640 medium (Life Technologies Co., Ltd., Carlsbad, CA). Single-cell suspensions were prepared from LNs by passage through a sterile 70-μm nylon cell strainer in 1 mL RPMI 1640 supplemented with 5% FCS. Single-cell suspensions were used to analyze the IgE-positive B-cells and cytokine production.

Method of inhalation exposure

For the inhalation challenge, animals were exposed to 0.1% OVA/PBS mist continuously for 30 min/d. The appearance of the inhalation exposure system is shown in Figure 1. The animals were individually held in animal holders (Tokiwa Kagakukikai Co., Ltd., Tokyo, Japan) attached to a nose-only exposure chamber (total volume 31.2 L, Tokiwa Kagakukikai Co., Ltd.) so that only their noses were exposed to the chemical mist. The mist was generated by an atomizer

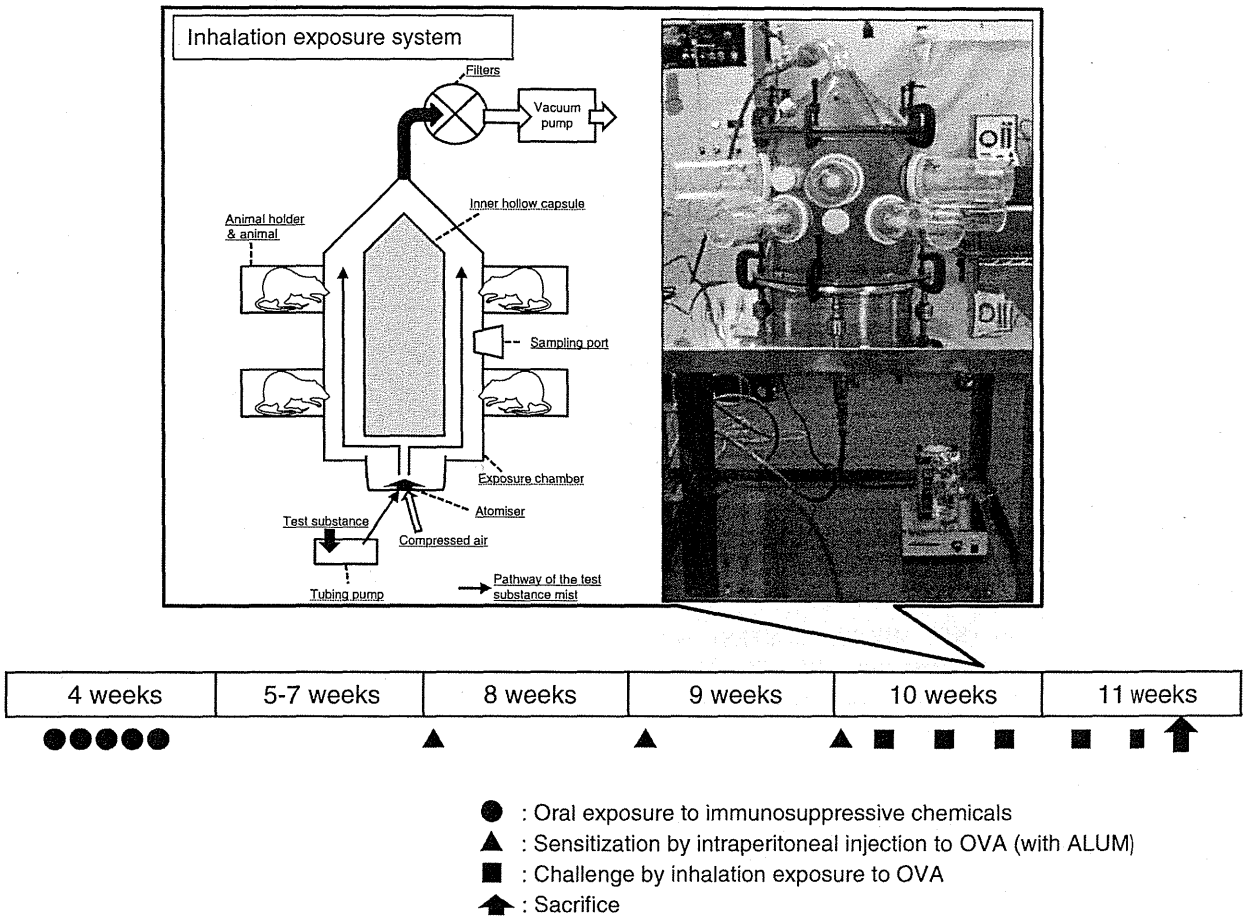

Figure 1. Inhalation exposure system and experimental protocol. See the "Materials and Methods" section for a detailed description. OVA, ovalbumin; ALUM, Aluminum hydroxide hydrate gel suspension.

(Ikeuchi Co., Ltd., Tokyo, Japan) with compressed air (ES4AD-5, Kobelco, Tokyo, Japan) and supplied to the exposure chamber through an air filter (F3000-10-Y, CKD Corporation, Aichi, Japan). Airflow to the chamber was controlled by an area flowmeter (NSPO-4, Nippon Flow Cell, Tokyo, Japan) at a rate of 20 L/min. The chamber air was exhausted through an air filter system consisting of a glass wool filter, a mist trap, and an activated charcoal filter (Tokiwa Kagakukikai Co., Ltd.) and was emitted to the atmosphere by means of a blower (TFO-K4P, Hitachi, Ltd., Tokyo, Japan). The actual concentration, mass median aerodynamic diameter (MMAD), and geometric standard deviation (GSD) were monitored by gravimetric analysis by using an air sampler (MF-200, Oct Science Co., Ltd., Osaka, Japan) and an Andersen-type personal sampler (Model 1312S, Kanomax Japan, Inc., Osaka, Japan). The mean values of the actual concentration of active ingredient, MMAD, and GSD were kept at approximately 5 mg/m^3, 4.0 μm, and 2.0, respectively, throughout the inhalation exposure.

Assay for total and OVA-specific IgE levels in serum

Total (BD OptEIA Mouse IgE ELISA Set, BD Pharmingen, Tokyo, Japan) and OVA-specific (Mouse OVA-IgE ELISA KIT, AKRIE-030, Shibayagi, Gunma, Japan) IgE levels in serum were measured using enzyme-linked immunosorbent assays (ELISAs) in accordance with the manufacturer's protocols.

Flow cytometric analysis of BALF and hilar LN cells

The following antibodies used for flow cytometric analysis were purchased from BD Pharmingen: fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgE (clone R35-72), FITC-conjugated anti-mouse Gr-1 (RB6-8C5), phycoerythrin (PE)-conjugated hamster anti-mouse CD11c (HL3), and allophycocyanin-cyanin-7-conjugated anti-mouse CD45R/B220 (RA3-6B2). To avoid nonspecific binding, 1×10^6 cells were incubated with 1 μg Mouse BD Fc BlockTM (BD Pharmingen) for 5 min at 4 °C, followed by incubation with the monoclonal antibodies for 30 min at 4 °C in the dark. Cells were washed twice with 5% FCS in PBS, resuspended at 1×10^6 cells per tube in 500 μL PBS, and analyzed on a FACSVerse flow cytometer (BD Pharmingen) using FACSuite (BD Pharmingen). For each sample, 20 000 events were collected and analyzed for expression of antigens.

For differential cell counts, half of the cells were used for Cytospins (Shandon Inc., Pittsburgh, PA) and the remaining cells were used for flow cytometric analysis. The Cytospin preparations were stained with Giemsa stain (Wako Pure

Chemical Industries), and 200 cells in each sample were examined to differentiate the numbers of macrophages, eosinophils, and neutrophils present (according to standard leukocyte typing). According to the results, the differential cell counts from the cytospin preparations showed almost similar patterns as those from the flow cytometric analysis, therefore data are not shown.

Cytokine production in LN cells

To stimulate T-cell receptor signalling, we cultured single-cell suspensions recovered from LNs (1×10^6 cells/well) for 24 or 96 h with Dynabeads Mouse T-Activator CD3/CD28 (25 μg/well) antibodies (Life Technologies Co., Ltd., Tokyo, Japan) in 24-well plates at 37 °C in a 5% CO_2 atmosphere. The levels of cytokines (interleukin [IL]-4, -5, and -13 in supernatants [cell culture medium]) were measured using a cytometric bead array (BD CBA Mouse Flex Set, BD Pharmingen) in accordance with the manufacturer's protocol.

Chemokine levels in BALF

The levels of chemokines (KC, MIP-1β, RANTES) were measured using a cytometric bead array (BD CBA Mouse Flex Set, BD Pharmingen) in accordance with the manufacturer's protocol.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to evaluate the results. For significant results, differences between vehicle control and treatment groups were then assessed by using Dunnett's multiple comparison test. Statistical significance of differences between single-chemical and combination-treatment groups was determined by using Tukey's multiple comparison test. Statistical significance was defined as $p < 0.05$.

Results

No abnormal clinical signs (e.g. decreased activity) or changes in body weight were observed in any of the treatment groups (data not shown).

Total and OVA-specific IgE levels in serum

The MXC + PARA mice displayed significantly higher total IgE levels in serum compared with the values for the vehicle control (2754 versus 1392 pg/ml, $p < 0.01$), MXC-treated mice (1694 pg/ml, $p < 0.01$), and PARA-treated mice (1671 pg/ml, $p < 0.01$) (Figure 2). The PBO + MXC mice had average total IgE levels in mice treated with vehicle control, MXC, and PBO, respectively (2254 versus 1392, 1694 and 1562 pg/ml), but this increase was not significant.

The MXC + PARA mice had significantly higher OVA-specific IgE levels than mice treated with vehicle control (19.5 versus 10.5 U/ml, $p < 0.05$) and PARA (10.8 U/ml, $p < 0.05$). Mice treated with MXC + PARA also had 152% the levels of mice treated with MXC alone (12.8 U/ml); however, this increase was not significant. The PBO + MXC mice had significantly higher OVA-specific IgE values than mice treated with the vehicle control (19.1 versus 10.5 U/ml, $p < 0.05$) or PBO (10.9 U/ml, $p < 0.05$). Mice treated with PBO + MXC also had 149% the levels in mice treated with MXC alone; however, this increase was not significant.

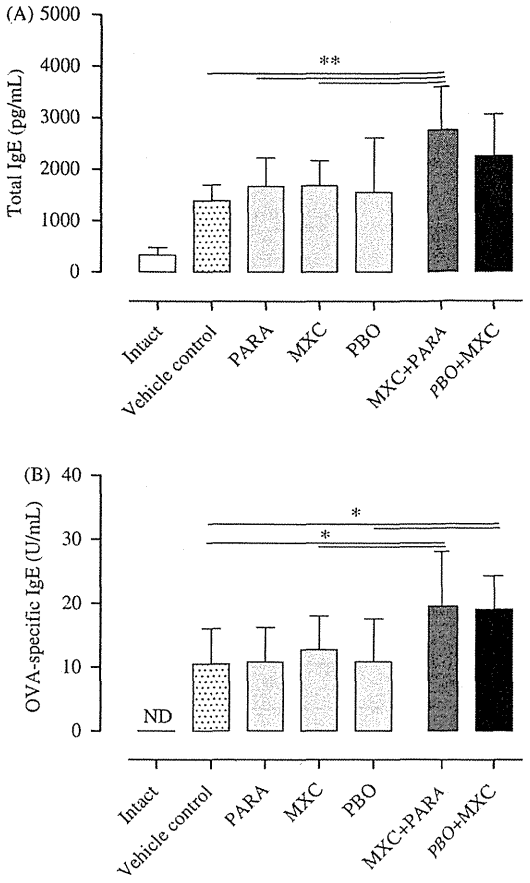

Figure 2. Total and OVA-specific IgE levels in serum. Mice were treated with nothing (intact), vehicle, methoxychlor (MXC), parathion (PARA), piperonyl butoxide (PBO), or the indicated combinations of the agents, and total (A) or OVA-specific (B) IgE levels in serum were measured. IgE levels are expressed as mean ± SD (titre; $n = 8$ per group). $^*p < 0.05$ and $^{**}p < 0.01$ (Dunnett's multiple comparison test) versus vehicle control group; $^*p < 0.05$ and $^{**}p < 0.01$ (Tukey's multiple comparison test) versus MXC-, PARA- or PBO-treated group.

BALF analysis

To assess the allergic airway inflammation in the lung, we counted eosinophils, lymphocytes, macrophages, and neutrophils (Figure 3) and measured the levels of related chemokines (KC, MIP-1β, and RANTES; Figure 4) in BALF. In the MXC, PARA, and PBO treatment groups, all responses in BALF were comparable with those of the vehicle control group (Figures 3 and 4).

The MXC + PARA and PBO + MXC mice had significantly higher numbers of eosinophils, lymphocytes, and neutrophils than mice treated with the vehicle control, MXC, or PARA. The change in eosinophil counts was particularly large: the MXC + PARA mice had 447, 305, and 307% the numbers in mice treated with vehicle control, MXC, and PARA (7881 versus 1764, 2566 and 2584 pg/ml), respectively, and the PBO + MXC had eosinophil counts 415, 284, and 310% the counts in mice treated with vehicle

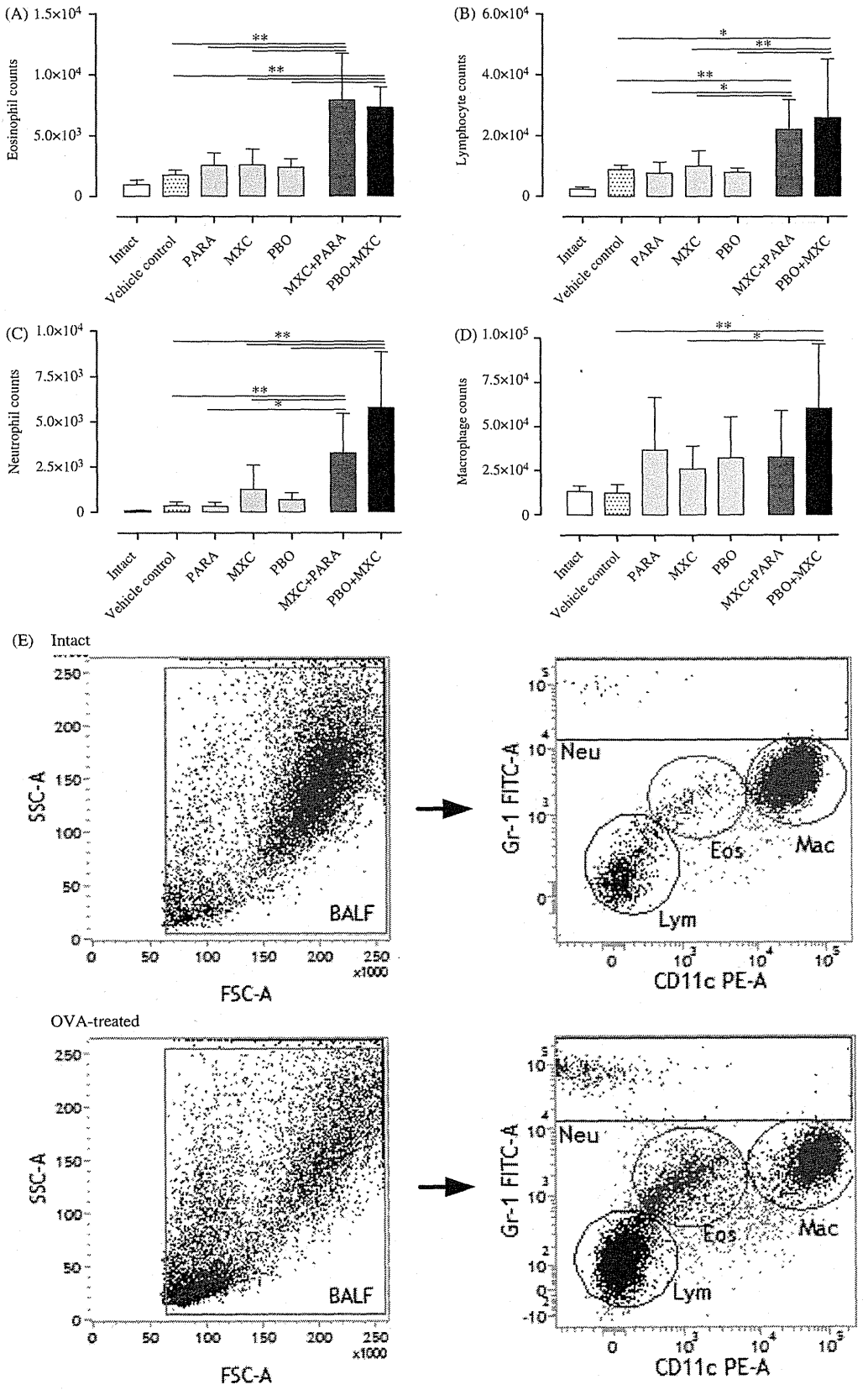

Figure 3. Immune cells counts in BALF. Designations of treatments are as in Figure 2. (A) Eosinophils, (B) lymphocytes, (C) neutrophils, (D) macrophages, (E) representative dot plots from an intact (untreated) mouse and a mouse exposed to OVA only. Cell counts are expressed as mean ± SD ($n = 8$ per group). *$p < 0.05$ and **$p < 0.01$ (Dunnett's multiple comparison test) versus vehicle control group; *$p < 0.05$ and **$p < 0.01$ (Tukey's multiple comparison test) versus MXC-, PARA- or PBO-treated group.

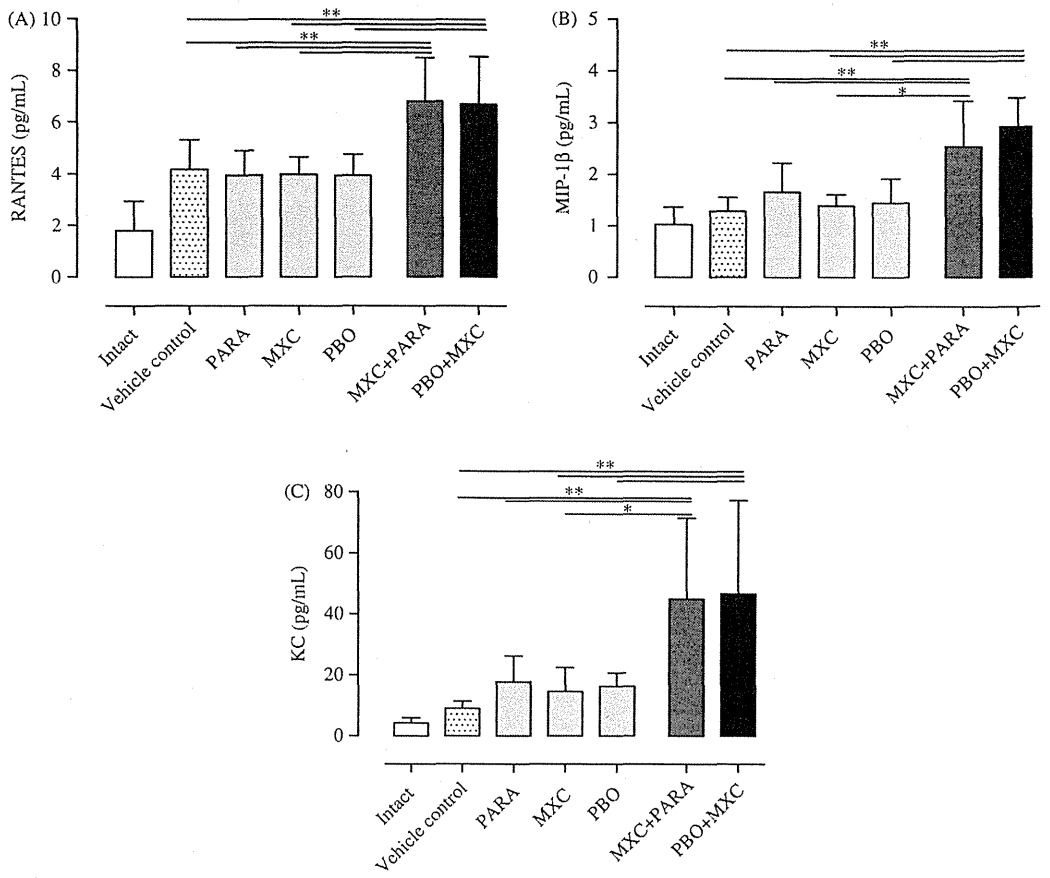

Figure 4. Chemokine levels in BALF. The levels of (A) RANTES, (B) MIP-1β, and (C) KC are expressed as mean ± SD (pg/mL; $n = 8$ per group). Designations of treatments are as in Figure 2. $^{**}p < 0.01$ (Dunnett's multiple comparison test) versus vehicle control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ (Tukey's multiple comparison test) versus MXC-, PARA- or PBO-treated group.

control, MXC, and PBO (7328 versus 1764, 2584 and 2365 pg/ml), respectively. The PBO + MXC mice also had significantly higher numbers of macrophages than mice treated with the vehicle control ($p < 0.01$) or MXC ($p < 0.05$), and also had 188% the number of macrophages in PBO-treated mice, although this increase was not significant. The MXC + PARA treatment group had numbers of macrophages comparable with those of the vehicle control, MXC-only, and PARA-only treatment groups.

The MXC + PARA mice displayed significantly higher levels of RANTES, MIP-1β, and KC than the levels in mice treated with the vehicle control, MXC, or PARA. The MXC + PARA mice had RANTES levels 163, 171, and 172% the levels in mice treated with vehicle control, MXC, and PARA (6.81 versus 4.18, 3.99 and 3.95 pg/ml), respectively. The PBO + MXC mice also had significantly higher levels of these chemokines than mice treated with the vehicle control, MXC, or PBO. The PBO + MXC mice had RANTES levels 161, 168, and 170% the levels in mice treated with vehicle control, MXC, and PBO (6.71 versus 4.18, 3.99 and 3.95 pg/ml), respectively.

Analysis of hilar LN cells

To evaluate the proliferation of IgE-positive B-cells following the single and combination treatments, isolated lymphocytes were stained with anti-B220 and anti-IgE antibodies. In the MXC, PARA, and PBO treatment groups, IgE-positive B-cell counts were comparable with those of the vehicle controls (Figure 5). The MXC + PARA-treated mice had significantly higher counts than mice treated with the vehicle control (1.11 versus 0.29×10^6 cells, $p < 0.01$), MXC (0.43×10^6 cells, $p < 0.01$), or PARA (0.59×10^6 cells, $p < 0.05$). The PBO + MXC-treated mice had significantly higher values than mice treated with the vehicle control (1.35 versus 0.29×10^6 cells, $p < 0.01$), MXC ($p < 0.01$), or PBO (0.52×10^6 cells, $p < 0.05$).

We also examined the production of related cytokines (IL-4, -5, and -13) from T-cells. In the MXC, PARA, and PBO treatment groups, production of all cytokines was comparable with that in the vehicle controls (Figure 6). The MXC + PARA mice had significantly higher levels of IL-4 than mice treated with the vehicle control (39.8 versus 20.4 pg/ml, $p < 0.01$), MXC (22.9 pg/ml, $p < 0.01$), and PARA (19.0 pg/ml, $p < 0.01$). The PBO + MXC mice had, on average the levels of IL-4 of mice treated with the vehicle control, MXC, or PBO (39.8 versus 20.4, 22.9 and 23.5 pg/ml), but this increase was not significant.

For the levels of IL-5, the MXC + PARA mice had, on average the levels in mice treated with the vehicle control, MXC, or PARA (55.7 versus 21.7, 27.9 and 29.5 pg/ml), but this increase was not significant. In contrast, the PBO + MXC

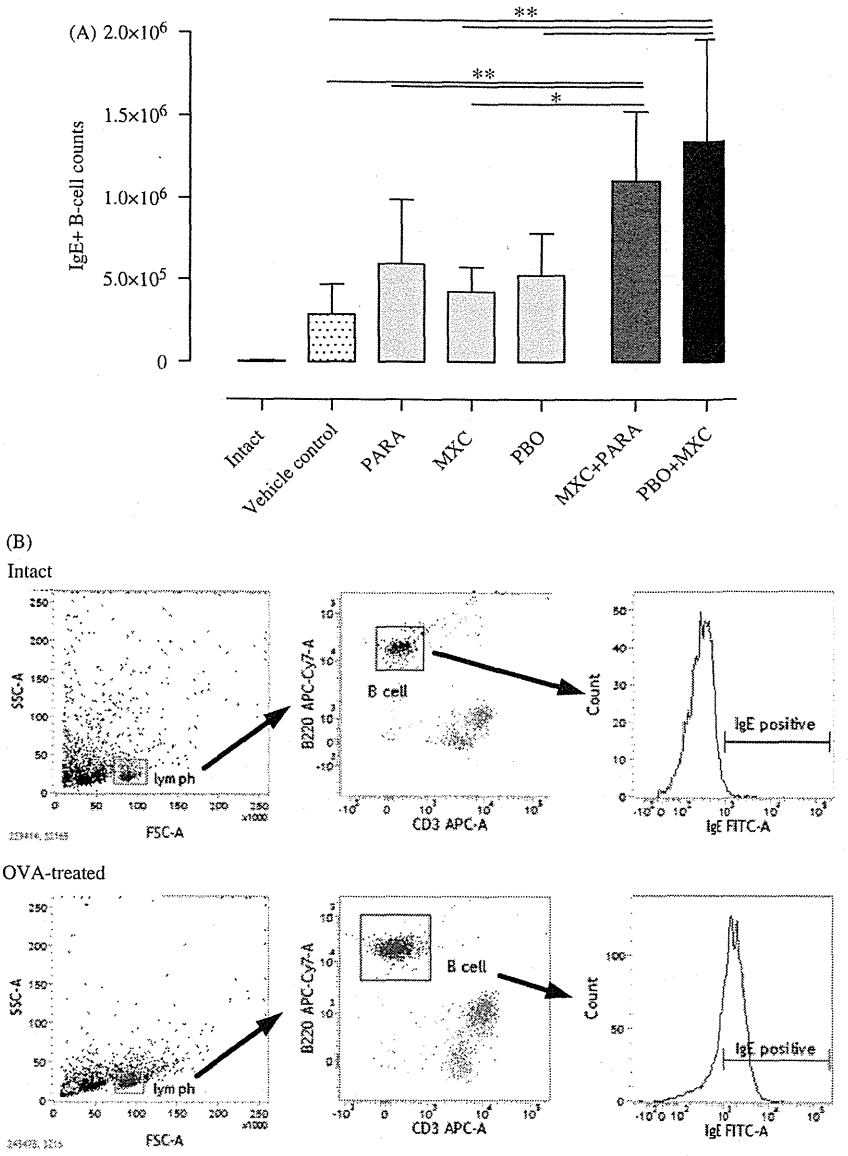

Figure 5. Responses in hilar LN cells. (A) IgE-positive B-cell counts; (B) representative histograms from an intact mouse and a mouse treated only with OVA. Designations of treatments are as in Figure 2. Cell counts and total IgE levels (ng/mL) are expressed as mean ± SD ($n = 8$ per group). $^{**}p < 0.01$ (Dunnett's multiple comparison test) versus vehicle control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ (Tukey's multiple comparison test) versus MXC-, PARA- or PBO-treated group.

mice had significantly higher levels of IL-5 than mice treated with the vehicle control (86.3 versus 21.7 pg/ml, $p < 0.01$), MXC ($p < 0.01$), or PBO (27.4 pg/ml, $p < 0.01$).

For the levels of IL-13, the MXC + PARA mice had significantly higher values than the mice treated with the vehicle control (129.7 versus 46.9 pg/ml, $p < 0.01$), MXC (73.9 pg/ml, $p < 0.01$), or PARA (77.0 pg/ml, $p < 0.05$). The PBO + MXC mice had significantly higher levels of IL-13 than mice treated with the vehicle control (144.7 versus 46.9 pg/ml, $p < 0.01$), MXC ($p < 0.01$), or PBO (78.1 pg/ml, $p < 0.01$).

Discussion

In the current study, we sought to better understand the immunological mechanisms by which combined exposures to three well-known environmental chemicals – MXC, PARA, and PBO – aggravate allergic airway inflammation. To that end, we examined the allergic effects of these chemicals in Balb/cAnN mice using an OVA-induced allergic airway inflammation model. Changes in the host immune status were assessed by measures of effects on total and OVA-specific IgE levels in serum, differential cell counts and related chemokine levels in BALF, and IgE positive B-cell counts and cytokine production from T-cells in the hilar lymph nodes.

MXC, PARA, and PBO were selected on the basis of previous studies: MXC exposure causes atrophy of $CD4^+CD8^+$ T-cells in the thymus[13]; PARA markedly inhibits antigen-specific IgM production[14]; and PBO depletes T-cells in the spleen and thymus, induces bone marrow hypoplasia, and inhibits T-cell proliferation in lymphoid tissues[15–17]. We also previously showed that exposure to MXC, PARA, or PBO results in increased thymocyte apoptosis, markedly inhibits and SRBC-specific IgM production[18,19]. We selected the doses of MXC (100 mg/kg day), PARA (1.0 mg/kg day) and PBO (100 mg/kg day) based on the results from acute oral LD50 doses (2900 mg/kg, 5 mg/kg and 2600 mg/kg,

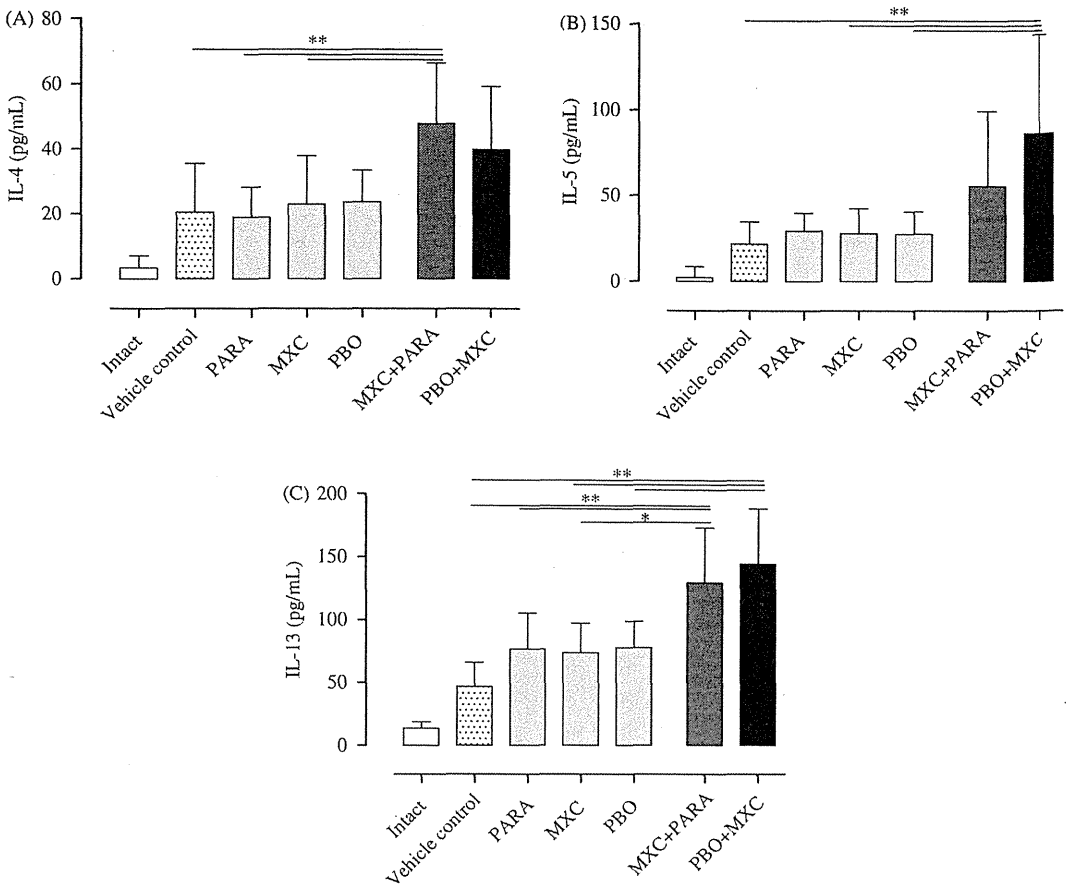

Figure 6. Cytokine production in hilar LN cells. The levels of (A) IL-4, (B) IL-5, and (C) IL-13 are expressed as mean ± SD (pg/mL; $n = 8$ per group). Designations of treatments are as in Figure 2. $^{**}p < 0.01$ (Dunnett's multiple comparison test) versus vehicle control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ (Tukey's multiple comparison test) versus MXC-, PARA- or PBO-treated group.

respectively) and subchronic general or immune toxicity. The objective in this study was to investigate the relationship between combined immunosuppressive chemicals and allergic responses using confirmed environmental immunosuppressive chemicals using a mice model focused on workers safety assessment. Therefore, we selected relatively-high doses (approximately 1/5 of LD50) and short-term exposure compared with estimator of human exposures in actual consumers. The examination focused on the safety assessment for consumers which exposure levels were equivalent to acceptable daily intake (ADI) is now underway.

OVA is a common antigen used to evaluate allergic airway inflammation. After sensitization and challenge with OVA, total and OVA-specific IgE responses in serum can be assessed with ELISAs. IgE antibodies play an important role in the development of allergic asthma, and can be used as a marker of OVA-induced allergic asthma[20]. In addition, in the development of the allergic response, activated B-cells can act as antigen-presenting cells for helper T-cells, resulting in hyperproduction of IgE[21,22]. According to the results of our current study, compared with the vehicle control mice, MXC + PARA and PBO + MXC mice had significant increases in serum IgE responses, whereas mice treated with MXC, PARA, or PBO alone did not. In addition, the total IgE responses of the MXC + PARA mice were significantly higher than those of mice treated with MXC or PARA alone, and the OVA-specific IgE responses with the MXC + PARA and PBO + MXC treatment led to significant increases relative to those seen in PARA and PBO mice. Along with serum IgE responses, the numbers of IgE-positive B-cell, which take a central role in the production of IgE, were significantly higher in the MXC + PARA and PBO + MXC mice than in mice treated with the vehicle control or single chemicals. These results suggested to us that MXC + PARA or PBO + MXC exposures induced more severe allergic airway inflammatory responses than exposure to any of the three chemicals individually.

Allergic airway inflammation, such as asthma, is recognized by the airway infiltration of eosinophils and other inflammatory cells, bronchial hyper-responsiveness, and airway obstruction[23]. In addition, degranulation and release of inflammatory mediators such as KC, RANTES, and MIP-1β by activated neutrophils and eosinophils may cause extensive tissue damage and propagate airway obstruction[22,24,25]. In the present study, the administration of MXC + PARA and PBO + MXC significantly exacerbated airway eosinophilic inflammation and increased the total inflammatory cell number and the production of KC, RANTES, and MIP-1β in the BALF, as compared to the administration of vehicle control or individual chemicals.

The observed changes in pulmonary inflammation possibly resulted from concomitant changes in the levels of KC, RANTES, and MIP-1β in the BALF[26]. These results suggested to us that MXC + PARA or PBO + MXC exposures induced a more severe induction in allergic airway inflammatory responses, including IgE reactions, than exposure to vehicle control or any of the three individual chemicals.

Allergic airway inflammation is orchestrated by Th2 cells, leading to IgE production and eosinophil activation[27]. Th2 cells produce and secrete a large quantity of Th2 cytokines, such as IL-4, IL-5, and IL-13, and these cytokines promote IgE production and eosinophil migration[28]. In the present study, the administration of MXC + PARA and PBO + MXC significantly increased the production of IL-4, -5, and -13 in the hilar LN cells as compared to the vehicle control and individual chemicals. These observations indicate that Th2 cytokines are important in the development of airway inflammation. Therefore, increased Th2 cytokine production in the hilar LN cells may subsequently result in aggravated airway inflammation, which was observed in groups treated with combined chemicals.

The results reported here demonstrate that prior exposure to combinations of immunosuppressive environmental chemicals such as MXC, PARA, and PBO can modulate immune functions and increase the severity of allergic airway inflammation compared to exposure to the vehicle control or any of the individual chemicals. This may occur through prevention of the central tolerance of autoreactive T or B cells, because mice exposed to immunosuppressive environmental chemicals showed significant decreases in Treg cell populations[12]. After the immunosuppressive compounds are withdrawn, recovery from immunosuppression occurs and an immune disorder develops in the mice, including increased production of antibodies and autoreactive T- or B-cells. Then, if antigens are encountered, abnormal hypersensitivity reactions are induced.

Overall, our data show that combined exposure to certain environmental chemicals can aggravate allergic inflammation beyond that caused by exposure to the individual chemicals in the mixtures, as shown by effects on IgE responses, eosinophil counts, and levels of related chemokines and cytokines. These syndromes may result in the activation of mast cell degranulation and aggravation of asthma-like symptoms such as labored respiration, increase airway resistance and wheezing[29]. In addition, the aggravation of allergic airway inflammation is in line with our preliminary study, which found that combined exposure to certain environmental chemicals can suppress acquired immune responses[12]. The combined toxicity may be affected by the age of the animal, chemical structure, receptor binding, and immune pathways involved; further studies are currently in progress. It is expected that the results of this study will help others in their evaluation of combinatorial immunotoxic effects when conducting assessments of the safety of environmental or occupational chemicals.

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Declaration of interest

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