

This article was downloaded by:[Advanced Institute of Sanita]
On: 19 June 2008
Access Details: [subscription number 790389265]
Publisher: Taylor & Francis
Informa Ltd Registered in England and Wales Registered Number: 1072954
Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Toxicology and Environmental Health, Part A Current Issues

Publication details, including instructions for authors and subscription information:
<http://www.informaworld.com/smpp/title~content=t713667303>

Motorcycle Exhaust Particles Augment Antigen-Induced Airway Inflammation in BALB/c Mice

Chen-Chen Lee ^a; Yu-Wen Cheng ^b; Jiunn-Wang Liao ^c; Bor-Luen Chiang ^d; Yih-Loong Lai ^e; Jaw-Jou Kang ^f

^a Department of Microbiology, School of Medicine, China Medical University, Taichung, Taiwan

^b School of Pharmacy, Taipei Medical University, Taipei, Taiwan

^c Graduate Institute of Veterinary Pathology, College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan

^d Graduate Institute of Clinical Medicine, Taipei, Taiwan, Republic of China

^e Department of Physiology, Taipei, Taiwan, Republic of China

^f Institute of Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China

Online Publication Date: 01 January 2008

To cite this Article: Lee, Chen-Chen, Cheng, Yu-Wen, Liao, Jiunn-Wang, Chiang, Bor-Luen, Lai, Yih-Loong and Kang, Jaw-Jou (2008) 'Motorcycle Exhaust Particles Augment Antigen-Induced Airway Inflammation in BALB/c Mice', Journal of Toxicology and Environmental Health, Part A, 71:6, 405 — 412

To link to this article: DOI: 10.1080/15287390701801687

URL: <http://dx.doi.org/10.1080/15287390701801687>

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: <http://www.informaworld.com/terms-and-conditions-of-access.pdf>

This article maybe used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

Motorcycle Exhaust Particles Augment Antigen-Induced Airway Inflammation in BALB/c Mice

Chen-Chen Lee¹, Yu-Wen Cheng², Jiunn-Wang Liao³, Bor-Luen Chiang⁴,
 Yih-Loong Lai⁵, and Jaw-Jou Kang⁶

¹Department of Microbiology, School of Medicine, China Medical University, Taichung, Taiwan,
²School of Pharmacy, Taipei Medical University, 250 Wu-Hsing St., Taipei, Taiwan, ³Graduate Institute
 of Veterinary Pathology, College of Veterinary Medicine, National Chung Hsing University, Taichung,
 Taiwan, ⁴Graduate Institute of Clinical Medicine, ⁵Department of Physiology, and ⁶Institute of
 Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China

Evidence indicates that environment pollutants from fossil fuel combustion compromise the immune system by enhancing allergic reactions and damaging the respiratory tract. This study was performed to investigate the effects of motorcycle exhaust particles (MEP), a major air pollutant especially in the urban areas of Taiwan, on allergen-induced airway inflammatory reactions in lab animals. BALB/c mice were intratracheally instilled with ovalbumin (OVA), MEP, or phosphate-buffered saline, 3 times every 2 wk. Airway hyperresponsiveness was measured in unrestrained mice by barometric plethysmography. Bronchoalveolar lavage fluid (BALF) and serum from treated animals were collected for cytokine and antibody determination by enzyme-linked immunosorbent assay (ELISA). Lung tissue stained with hematoxylin/eosin was examined. Data showed that MEP augmented OVA-induced airway inflammation; characterized by infiltration of eosinophils and neutrophils in BALF and lung tissue inflammation. The combination of OVA and MEP markedly increased interleukin-4 (IL-4), interleukin-5 (IL-5), and tumor necrosis factor- α (TNF- α) protein levels in BALF. In addition, MEP also augmented OVA-induced rise in OVA-specific immunoglobulin (Ig) G1 and IgE and airway hyperresponsiveness. Pretreated lavage cells with mitogen-activated protein kinase (MAPK) inhibitors showed that TNF- α release was significantly inhibited. This study found that MEP augmented antigen-induced allergic airway inflammation and airway hyperresponsiveness through a Th2-dominant pathway.

Allergic diseases in general and asthma in particular have become increasing problem for public health, especially in developed countries (Patino & Martinez, 2001). The observed

increases in the prevalence of asthma may be due to multiple factors. Several studies showed that exposure to certain outdoor pollutants such as exhaust from fossil fuel combustion and emission of particulate pollutants adversely impacted immunologic functions involved in the occurrence of asthma and chronic obstructive pulmonary disease (Diaz-Sanchez et al., 1994, 1996; Fernvik et al., 2002; Li et al., 1996a; Nel et al., 1998; Peterson & Saxon, 1996).

Asthma is a multifactorial disorder, involving numerous resident and recruited inflammatory cells (dendritic cells, T-helper 2 [Th2] lymphocytes, B lymphocytes, mast cells, basophils, eosinophils, CD8⁺ T lymphocytes, and bronchial fibroblasts) (Malerba & Pignatti, 2005) and inflammatory molecules, including antibodies (immunoglobulin [Ig] E and IgG1), Th2 cytokines (IL-4, IL-5, IL-9, and IL-13) (Kay et al., 2002; Renaud, 2001), proinflammatory cytokines (tumor necrosis factor [TNF]- α and transforming growth factor [TGF]- β) (Bandeira-Melo & Weller, 2003), and vasoactive mediators (prostaglandins and leukotrienes) (Wenzel, 2003). Cytokine expression results in the recruitment of eosinophils, leading to chronic airway inflammation, mucus hyperproduction, airflow obstruction, and airway hyperresponsiveness (AHR) (Malerba & Pignatti, 2005).

Motor vehicles emissions are a major source of airborne pollutants. In Taiwan, motorcycles are widely used. More than 1.276 million motorcycles were registered in 2004, making density as high as 564 motorcycles registered per thousand persons and ranking Taiwan as having the highest density in the world (Ministry of Transportation and Communication, Republic of China [ROC], 2004). Motorcycle use, especially with the two-stroke engine, was estimated to introduce per year about 16,000 tons of total suspended particles (TSP) and 15,000 tons with particulate matter (PM) with diameter <10 μ m (PM₁₀) in Taiwan (ROC Environment Protection Agency [EPA], 1994). The concentration of PM₁₀ is considerably

Received 1 August 2007; accepted 30 October 2007.

Funding research support: National Science Council, Taiwan.

Address correspondence to Jaw-Jou Kang, Institute of Toxicology, College of Medicine, National Taiwan University, No. 1 Jen-Ai Road, Section 1, Taipei, Taiwan. E-mail: jjkang@ntu.edu.tw

higher in Taiwan ($64 \mu\text{g}/\text{m}^3$) compared to other parts of the world (London, $14 \mu\text{g}/\text{m}^3$; Paris, $14 \mu\text{g}/\text{m}^3$) (ROC EPA, 1996). Increased ambient PM was linked to increases in adverse cardiovascular and respiratory health events (Dockery et al., 1993; Krewski et al., 2005; Schwartz, 1994).

Motorcycle exhaust particles (MEP) contain a carbon black core, which absorbs >110 different organic compounds, including C1–C20 chains of hydrocarbon compounds and polycyclic aromatic hydrocarbons (PAH) (Ueng et al., 2000). Studies indicate that MEP is cytotoxic (Lee & Kang, 2002), mutagenic (Zhou & Ye, 1997), and genotoxic (Cheng et al., 2004) *in vitro*. It was also shown that MEP impaired function of an isolated rat aorta (Cheng & Kang, 1999), induced proinflammatory and proallergic effects in BALB/c mice (Lee et al., 2004), and induced interleukin (IL)-8 production in airway epithelial cells (Lee et al., 2005). However, the environmental impact of MEP and its biological effects are relatively unclear, especially on the immune system. In this study the effects of MEP were investigated on the antigen-induced allergic immune responses in lab animals.

MATERIALS AND METHODS

Mice

Female BALB/c mice aged 6–8 wk (18–20 g) were obtained from the Animal Center of the College of Medicine. National Taiwan University Animal Care and Handling protocols were approved by the Animal Committee of National Taiwan University.

Collection and Extraction of Motorcycle Exhaust Particles

Motorcycle exhaust particles (MEP) were collected on $0.5\text{-}\mu\text{m}$ quartz fiber filters (Advantec MFS, Inc., Dublin, CA) from 50-cm^3 Yamaha Cabin engines using 95 octane unleaded gasoline. The sampling apparatus consisted of a 30-cm-long by 2.2-cm-diameter stainless-steel dilution tube in sequence with a filter holder and a vacuum pump. The engine was run at an idle speed of 150 rpm on an empty load, and the vacuum pump was set at a flow rate of 20 L/min to collect MEP for 1 h twice daily. The quartz filters with collected particles were kept from light and left to dry in a desiccator and repeatedly extracted four times with methanol under sonication. The methanol was then removed by a vacuum evaporator. The final residues, the motorcycle exhaust particles (MEP), were collected and kept desiccated at -20°C . Basically, $32.7 \mu\text{g}$ MEP was derived from 1L of Yamaha motorcycle exhaust.

Study Protocol

Mice were divided into three groups with 10 mice per group. On d 0, 14, or 28, mice were anesthetized with 0.75 mg ketamine and 0.06 mg combelen by intramuscular injection and instilled intratracheally (IT) with 50 μL

phosphate-buffered saline (PBS, pH 7.2) containing ovalbumin (OVA) at 2.5 mg/kg (Sigma, St. Louis, MO) or combined with MEP at 12 mg/kg. Both 1 d and 7 d after the last instillation, mice were evaluated for (1) airway hyper-responsiveness, (2) cell profile in bronchoalveolar lavage fluid (BALF), (3) cytokine levels in BALF, (4) serum antibodies, and (5) lung histology. The dosing regimen selected, 12 mg/kg MEP collected from motorcycles, was 1/10 of the minimum lethal dose based on a previous acute toxicity study (unpublished data).

Determination of Cytokine Expression

Interleukin (IL)-4, IL-5, interferon (INF)- γ , and TNF- α levels were assayed by the enzyme-linked immunosorbent assay (ELISA) method according to the procedure recommended by the manufacturer (R&D, Minneapolis, MN).

Bronchoalveolar Lavage and Culture of Bronchoalveolar Lavage Cells

Bronchoalveolar lavage (BAL) was completed using 1 mL Hanks buffered saline solution (HBSS), instilled bilaterally with a syringe. Lavage fluid was harvested by gentle aspiration three times and then centrifuged (Miyabara et al., 1998). Differential cell counts were assessed on cytologic preparations. Cell slides were prepared with use of a cytospin and stained with Liu staining. In total, 300 cells were counted under microscopy. Bronchoalveolar lavage (BAL) fluid supernatants were assayed by ELISA.

For evaluation of the role of the mitogen-activated protein kinase (MAPK) pathway in cytokine release, BAL cells from ovalbumin (OVA)-treated mice were isolated, 1×10^5 BAL cells cultured in 96-well plates in RPMI medium (Invitrogen, Carlsbad, CA) with 10% heat-inactivated fetal bovine serum and supplement with 4 mM L-glutamine, 25 mM HEPES (pH 7.2), 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin, and pretreated with SB203580 at 2 μM (p38 inhibitor), SP600125 at 20 μM (c-Jun NH₂-terminal kinase [JNK] inhibitor), or PD98059 at 20 μM (extracellular signal-related kinase [ERK] inhibitor) for 10 min and then incubated for 24 h with OVA at 10 $\mu\text{g}/\text{mL}$, MEP at 20 $\mu\text{g}/\text{mL}$, or OVA at 10 $\mu\text{g}/\text{mL}$ combined with MEP 20 $\mu\text{g}/\text{mL}$. Cell medium was collected and centrifuge at $300 \times g$ for 5 min at 4°C . Supernatants were isolated for determination of cytokine levels, IL-4, IL-5, and TNF- α by ELISA as described later.

Lung Histology

After bronchoalveolar lavage, the lungs were fixed with 10% neutral phosphate-buffered formalin. Sections were prepared and stained with hematoxylin/eosin (H&E) to quantitate the number of infiltrating inflammatory cells using microscopy.

Measurement of Airway Hyperresponsiveness

Airway responsiveness was measured in unrestrained animals by barometric whole-body plethysmography (Buxco, Troy, NY) (Hamelmann et al., 1997). Briefly, mice were placed in the main chamber, and baseline readings were taken and averaged for 3 min. Aerosolized phosphate-buffered saline (PBS) or methacholine (MCh) in increasing concentration (3.125 to 25 mg/ml) was nebulized through an inlet of the main chamber for 3 min, and readings were taken and averaged for 3 min after each nebulization. Recordings of every 10 breaths were extrapolated to define the respiratory in breaths per min. Airway reactivity was expressed as an enhanced pause (Penh), and data were expressed as the ratio of $Penh_{MCh}$ values compared to $Penh_{PBS}$ from three independent experiments.

Measurements of OVA-Specific IgE, IgG1, and IgG_{2a}

OVA-specific IgE, IgG1, and IgG_{2a} were measured by sandwich ELISA as previously described (Wu et al., 2000). OVA (10 µg/ml) was coated to ELISA plates and incubated at 4°C overnight. After washing with PBS containing 0.05% Tween 20, diluted serum was added to the plates and incubated at room temperature for 2 h. Biotin-conjugated anti-IgE, anti-IgG1, and anti-IgG_{2a} antibodies (Pharmingen, San Diego, CA) and alkaline phosphatase-conjugated streptavidin (Sigma, St. Louis, MO) were added subsequently. Enzyme activity was evaluated using *p*-nitrophenyl phosphate (Sigma St. Louis, MO) as the substrate and read using an enzyme-linked immunosorbent assay (ELISA) reader (MRX-TC; Dynex Technology, Chantilly, VA). Readings at 405 nm

were converted to ng/ml by using values obtained from standard curves. OVA-specific antibodies data were expressed as ELISA units (sample OD-blank OD/positive OD-blank OD). Positive OD was detected using serum that expresses OVA-specific antibodies (Ab).

Statistical Analysis

All values refer to mean ± SEM. (standard error of the mean) of at least three separate experiments. Statistical significant difference among groups was analyzed with one-way ANOVA followed by Newman-Keuls post hoc comparisons. The criterion significance was set at *p* values < .05.

RESULTS

The Addition Effect of Antigen-Induced Inflammatory Cells Infiltration by MEP

Changes in cell numbers in BALF of mice instilled IT with OVA alone or in combination with MEP (OVA+MEP) at 1 d and 7 d after final treatment were investigated (Table 1), and the protocol used is shown in Figure 1A. In the OVA group, total cell numbers in BALF were not significantly different from control. However, cell numbers rose markedly increased in OVA+MEP-treated mice, compared to saline. OVA challenge elevated the numbers of eosinophils harvested by BAL. The numbers of eosinophils increased markedly in the in the OVA+MEP group 1 d after final treatment. The combination of OVA plus MEP exposure elevated eosinophils by 18.6- and 3.5-fold, as compared to saline and OVA, respectively. Neutrophils in BALF were increased in the OVA+MEP group at 1 d and 7 d post-exposure,

TABLE 1
Cell Profile of Bronchoalveolar Lavage Fluid

Cell numbers (1×10 ³ /total BAL supernatants)					
Group	Total cells	Macrophages	Neutrophils	Lymphocytes	Eosinophils
Day 1 after final treatment					
Saline	23.25 ± 1.96	22.99 ± 2.08	0.06 ± 0.06	0.14 ± 0.02	0.11 ± 0.11
OVA	33.21 ± 5.17	26.83 ± 3.95	0.33 ± 0.1	0.16 ± 0.07	0.59 ± 0.08*
MEP	41.4 ± 3.6*	36.2 ± 4.5*	0.5 ± 0.1*	0.4 ± 0.1	0.6 ± 0.2*
OVA+MEP	43.62 ± 7.43*	42.63 ± 7.65*	0.72 ± 0.02*†	0.36 ± 0.18	2.05 ± 0.16*†
Day 7 after final treatment					
Saline	22.99 ± 2.68	21.92 ± 2.32	0.04 ± 0.01	0.21 ± 0.06	0.04 ± 0.01
OVA	29.34 ± 4.08	26.83 ± 3.95*	0.05 ± 0.02	1.49 ± 0.42*	0.20 ± 0.04*
MEP	33.9 ± 3.9*	31.2 ± 2.0*	0.2 ± 0.1*	0.7 ± 0.3*	0.3 ± 0.1*
OVA+MEP	39.67 ± 8.65*	38.31 ± 3.42*	0.21 ± 0.08*†	1.92 ± 0.76*	0.53 ± 0.23*†

Note. Mice received intratracheal instillation of saline, OVA, or OVA+MEP, according to the protocol outlined in Materials and Methods section, and BALF was obtained 1 d and 7 d after final treatment. Total cell counts were determined with 500 µl BALF and cell types were assessed by Liu staining, described in Methods section. Data are expressed as mean ± SEM (*n* ≥ 10). Significant differences indicated by asterisk for *p* < .05, compared with saline, and dagger for *p* < .05, compared with OVA.

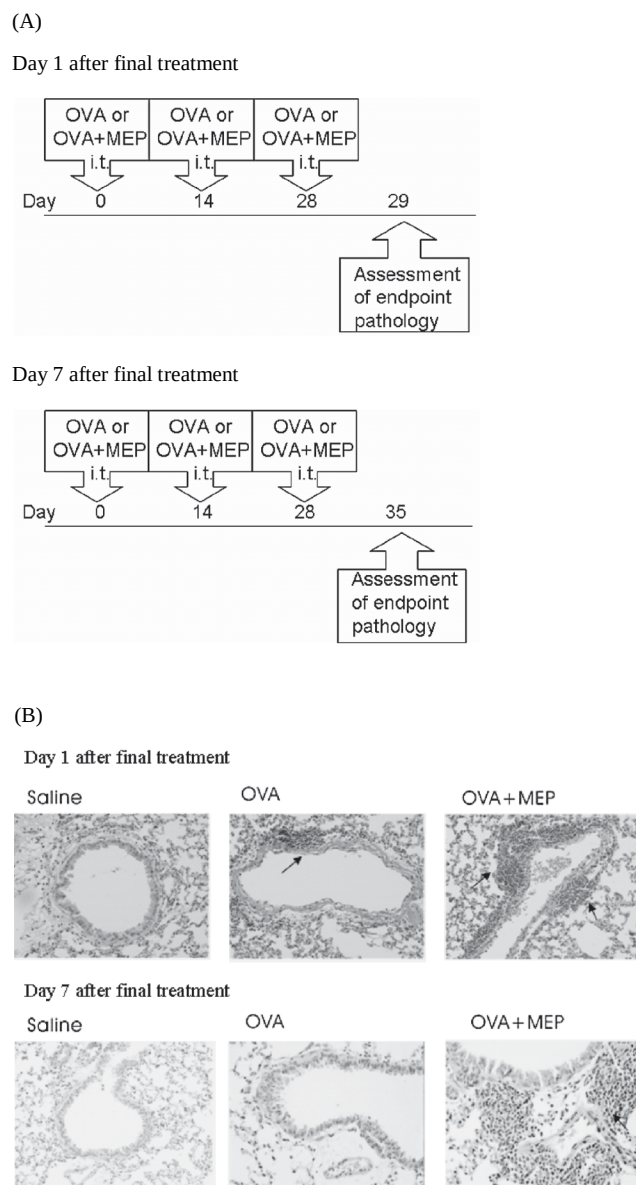


FIG. 1. OVA-induced airway inflammation by MEP on BALB/c mice. (A) Brief protocol of animal study. Mice received intratracheal (IT) instillation of saline, OVA (2.5 mg/kg), and OVA (2.5 mg/kg)+MEP (12 mg/kg). Animals were sacrificed 1 d and 7 d after final treatment. (B) Lung sections were stained with hematoxylin and eosin (H&E) for measurement of inflammatory cells (arrowhead with solid line) around the airways. Original modification, $\times 200$.

while lymphocytes rose significantly following either OVA alone or in combination with MEP at 7 d postexposure (Table 1). In addition, OVA alone exposure showed eosinophils infiltration that was sustained until d 7 after treatment, but neutrophils infiltration returned to control levels. The level of MEP-induced inflammation after treatment of mice with MEP alone was reported previously (Lee et al., 2004) to be similar to exposure to OVA alone but less than for OVA+MEP and 33.9 total cells,

31.2 macrophages, 0.2 neutrophils, 0.7 lymphocytes, and 0.3 eosinophils at d 7 post MEP exposure.

Pathologic examination of lung sections from OVA-treated mice revealed only a slight increase in infiltration of inflammatory cells above control (Figure 1B). After OVA+MEP treatment, the intensity of inflammatory cells infiltration in lung tissue was augmented compared to OVA (Figures 1B). The OVA-induced inflammatory cell infiltration in lung was decreased after d 7 postexposure, while the inflammation at d 7 after OVA+MEP treatment was sustained.

BALF Cytokine Expression and Involvement of MAPK Pathway in Cytokine Release by BAL Cells

Cytokine levels, including Th1 cytokine, IFN- γ , Th2 cytokines, IL-4 and IL-5, and the pro-inflammatory cytokine, TNF- α , were analyzed in BALF. As shown in Table 2, IL-4 and IL-5 levels in the OVA-treated group were not markedly altered. In contrast, OVA+MEP exposure resulted in significant increases in IL-4 and IL-5 of 6.3- and 3.8-fold, respectively, as compared to control. OVA+MEP also significantly enhanced production of IFN- γ and TNF- α , compared to the control. In addition, the OVA+MEP effect was greater than OVA alone for IL-4 and TNF- α release. The extent of MEP-induced rise in BALF cytokine was similar to OVA treatment.

To further investigate mechanisms for the induction of TNF- α production, BAL fluid (BALF) cells from mice 7 d after OVA exposure were isolated and treated with OVA, MEP, or OVA+MEP. Data showed that both MEP and OVA groups had increased TNF- α release compared to the control. OVA+MEP treatment led to enhanced TNF- α release in a near-additive manner as compared to OVA under no pretreatment with inhibitors (Table 3). Cells were pretreated with MAP kinase inhibitors and data showed that TNF- α release was inhibited by ERK, JNK, and p38 kinases inhibitors in cells stimulated in culture but not in control cells (Table 3). IL-4 and IL-5 levels from cultured BAL cells were also evaluated; however, the IL-4 and IL-5 levels were below ELISA kit detection levels (data not shown).

MEP-Treatment-Augmented Antigen-Induced Specific IgE and IgG1 Antibodies Production

Allergen-stimulated Th2 cells induce B cells to switch antibody isotypes from IgM to IgE or IgG1 in the murine model, which further elicits responses mediated in allergic development. The effect of MEP on OVA-induced allergic response was further investigated by measuring specific antibody levels in serum (Figure 2). OVA-specific IgE and IgG1 were induced by treatment with OVA. This effect was significantly augmented after OVA+MEP exposure. No changes in OVA-specific IgG2a level were observed in any group.

TABLE 2
Protein Levels of Cytokines in Bronchoalveolar Lavage Fluid

Cytokine level (pg/total BAL supernatants)				
Group	IL-4	IL-5	IFN- γ	TNF- α
Day 1 after final treatment				
Saline	3.03 $\pm$ 1.3	14.43 $\pm$ 4.14	13.27 $\pm$ 1.63	8.2 $\pm$ 1.24
OVA	16.1 $\pm$ 3.34	22.82 $\pm$ 3.4	19.09 $\pm$ 1.59	13.54 $\pm$ 3.1
MEP	16.5 $\pm$ 1.8**		25.8 $\pm$ 3.8**	
OVA+MEP	27.5 $\pm$ 6.7* [†]	31.6 $\pm$ 4.0*	32.1 $\pm$ 5.6*	38.89 $\pm$ 9.36* [†]
Day 7 after final treatment				
Saline	4.63 $\pm$ 1.1	17.94 $\pm$ 5.8	14.68 $\pm$ 10.65	16.56 $\pm$ 3.47
OVA	7.38 $\pm$ 1.89	32.4 $\pm$ 8.6	23.46 $\pm$ 5.69	15.46 $\pm$ 3.2
MEP	12.8 $\pm$ 2.6**	39.4 $\pm$ 8.3*	29.6 $\pm$ 8.3*	19.69 $\pm$ 5.13
OVA+MEP	29.11 $\pm$ 3.77* [†]	67.66 $\pm$ 17.57*	36.88 $\pm$ 6.89*	49.38 $\pm$ 20.3* [†]

Note. Mice received intratracheal instillation of saline, OVA, or OVA+MEP, according to the protocol outlined in Materials and Methods section, and BALF was derived 1 d and 7 d after the final treatment. Protein levels of each cytokine were analyzed by ELISA. Data are expressed as mean $\pm$ SEM ($n \geq 10$). Significant differences indicated by asterisk for $p < .05$, compared with saline, and dagger for $p < .05$, compared with OVA.

TABLE 3
Inhibition of MEP-Aggravated Antigen-Induced TNF- α Production by MAP Kinase Inhibitors

Medium TNF- α level (pg/ml)				
	Control	MEP	OVA	MEP+OVA
No pretreatment	464.9 $\pm$ 20.73	874.0 $\pm$ 48.25*	943.2 $\pm$ 35.37*	1667 $\pm$ 113.9* ^{‡§}
JNK inhibitor (SP600125)	210.4 $\pm$ 17.78	344.0 $\pm$ 81.52 [†]	688.6 $\pm$ 37.30	724.3 $\pm$ 84.00 [†]
P38 inhibitor (SB203580)	376.7 $\pm$ 38.48	458.2 $\pm$ 76.35 [†]	489.4 $\pm$ 114.7 [†]	1047 $\pm$ 133.8 [†]
ERK inhibitor (PD98059)	353.8 $\pm$ 54.84	504.9 $\pm$ 67.80 [†]	738.1 $\pm$ 142.5	1042 $\pm$ 54.60 [†]

Note. BAL cells were collected at 7 d after final treatment and 1×10^5 BAL cells cultured in 96-well plates, pretreated with MAP kinase inhibitors for 10 min and then treated with OVA at 10 μ g/ml or combined with MEP at 20 μ g/ml for a further 24 h. Medium was collected and TNF- α level was detected by ELISA. Data were expressed as mean $\pm$ SEM ($n \geq 3$). Significant difference indicated by asterisk for $p < .05$, compared with no pretreatment control group;

[†] $p < .05$, compared with respective no pretreatment group;

[‡] $p < .05$, compared with OVA no pretreatment group;

[§] $p < .05$, compared with MEP no pretreatment group.

Addition of OVA-Induced Airway Hyperresponsiveness by MEP

Effect of MEP treatment on airway responsiveness was investigated using noninvasive whole body plethysmography with a methacholine (MCh) challenge (Figure 3). MCh (3.125–25 mg/ml)-induced increases of Penh ratio were significantly higher in mice instilled it. with OVA for 5 wk compared to saline. It showed significantly addition effect in mice treated with OVA+MEP. The level of MEP-only-induced increase Penh ratio seen in our earlier study was similar to that of the OVA-only group here. (Data has been repeated in our previous study, Lee et al. 2004).

DISCUSSION

It is clear that inhalation of particulate and gaseous pollutants from use of fossil fuels by factories and automobiles contribute to the overall increase of allergic airway diseases (Fernvik et al., 2002; Steerenberg et al., 2003). Continued research is necessary to further detail mechanisms by which air pollutants produce adverse health effects. Particular attention needs to be given to means of intervention and, particularly, prevention of pollutant-induced health complications. In our previous study, data showed that particulate fractions collected from motorcycle exhaust (MEP) may induce proinflammatory and proallergic response profiles in the absence of exposure to an allergen (Lee et al.,

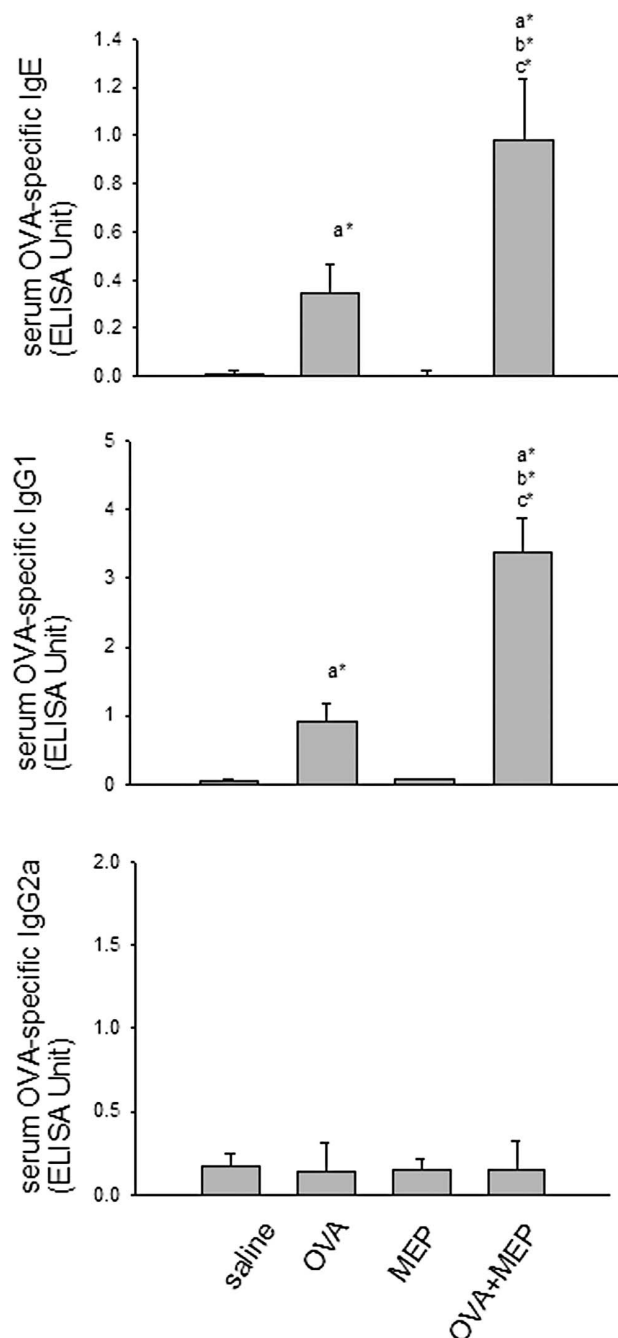


FIG. 2. OVA-specific antibodies levels in BALB/c mice. Blood samples were obtained 7 d after final treatment. OVA-specific IgE, OVA-specific IgG1, and OVA-specific IgG2a were analyzed by ELISA. Data are expressed as mean $\pm$ SEM ($n \geq 10$). Significance indicated by $a^* p < .05$, compared with saline group; $b^* p < .05$, compared with OVA; and $c^* p < .05$, compared with MEP.

2004) and directly induced IL-8 production in alveolar epithelial cells (Lee et al., 2005). In this study the effects of MEP were examined on allergen-induced allergic inflammation *in vivo*. Data showed that MEP co-exposure with antigen showed additional effects of airway allergic reactions accompanied with IL-4,

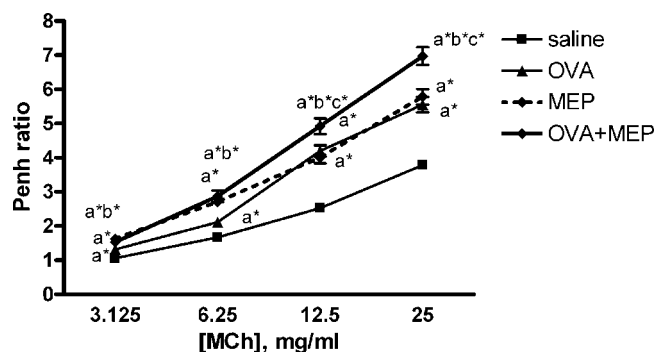


FIG. 3. OVA-induced airway hyperresponsiveness was augmented by coexposure to MEP in BALB/c mice. Airway resistance was measured by whole-body plethysmography 7 d after final treatment. Data expressed as mean $\pm$ SEM of the $Penh_{MCh}$ values in a ratio against $Penh_{PBS}$ values in three independent experiments ($n \geq 10$). Significance indicated by: $a^* p < .05$, compared with saline group; $b^* p < .05$ compared with OVA group.

IL-5, TNF- α cytokine release, inflammatory cells infiltration, and induction of airway hyperresponsiveness (AHR) and augmented antigen-induced IgE production.

Among the MEP co-exposure with OVA-induced cells infiltrating the airways, eosinophils were the predominant cell involved in the chronic inflammatory processes, characteristic of the late-phase response through release of an array of proinflammatory mediators, including cysteinyl leukotrienes, cationic protein, and cytokines (IL-3, IL-5, granulocyte-macrophage colony-stimulating factor [GM-CSF], IL-13), which correlate with development and progression of symptoms. In addition to eosinophils, neutrophils also play an important role in allergic asthma and development of AHR (Suzuki et al., 1996; Chung & Barnes, 1999). Addition of neutrophil infiltration was also observed in OVA cotreated with MEP. In addition, the validity of using Penh to measure airway reactivity has been questioned and it was shown that Penh measurement may be affected by factors other than airway resistance. (Lundblad et al. 2002; Alder et al. 2004). Our findings on increased AHR after OVA and MEP exposure will need to be validated by other methods in future studies.

The molecular mechanisms responsible for the MEP-induced addition of antigen-induced airway inflammation and AHR were assessed by measuring a Th1 cytokine (IFN- γ), Th2 cytokines (IL-4 and IL-5), and a proinflammatory cytokine (TNF- α) release in BALF. Data showed that the addition effects of MEP co-exposure with OVA-induced airway inflammation and hyperresponsiveness were through both Th1 cytokine and Th2 cytokines release. However, it is possible that the Th2-dependent pathway plays an important part in MEP-induced addition of OVA-induced airway inflammation because it was seen that there were concurrent treatment-increased OVA-specific IgE (IL-4-dependent) production and IgG1 (IL-5-dependent) production, but not OVA-specific IgG2a (IFN- γ -dependent) formation. The latter finding was

unexpected in light of the observed rise in IFN- γ release in BALF from OVA+MEP-treated mice. Increases in this Th1 cytokine were in agreement with the *in vivo* findings showing an elevation of IFN- γ in serum of severely asthmatic patients (Corrigan & Kay, 1990) and association between IFN- γ and a worsening of airway inflammation in a murine model (Salvi et al., 2001).

IL-4, IL-5, and TNF- α gene expression is controlled by transcriptional factors such as NF-AT, AP-1, and nuclear factor (NF)- κ B (Su et al., 1994; Li et al., 1996b), which are regulated by MAP kinase Schafer et al., 1999; Mori et al., 1999). To investigate mechanisms underlying MEP-augmented OVA-induced cytokine release, the effects from three MAPK pathway inhibitors were initially assessed. Levels of IL-4 and IL-5 were below ELISA kits' detection levels; therefore, it was only possible to document TNF- α expression in isolated cells from BALF. Data showed that all three inhibitors against JNK (SP600125), p38 (SB203580), and ERK (PD98059) inhibited MEP-augmented OVA-induced TNF- α release by the cells. The precise mechanism underlying MEP-augmented of OVA-induced cytokines release needs further investigation.

Previous studies demonstrated that individual polycyclic aromatic hydrocarbons (PAH) (Kanoh et al., 1996), including pyrene, anthracene, fluoranthene, benzo[a]pyrene, or DEP-associated PAH (Diaz-Sanchez, 1997), exert an adjuvant effect on allergen-induced IgE production. In addition, studies showed that the core carbon particles also exerted an adjuvant effect on allergen-induced IgE production (Lovik et al., 1997; Heo et al., 2001; Steerenberg et al., 2003). Which components augment OVA-induced airway inflammation and AHR remains to be determined. Lee et al. (2004) fractionated MEP using organic solvents including hexane, benzene, chloroform, ethyl acetate, and methanol and found that benzene fraction produced effects similar to those reported with MEP, including rise in eosinophil and neutrophil infiltration, IL-4 and IgE production, and AHR. This suggests that the chemicals responsible for inducing observed effects might be enriched in the benzene fraction and engine load (Madden et al., 2003); however, whether the benzene fraction produced augmentation of antigen-induced allergic inflammation and AHR as MEP needs further investigation.

Although both DEP and MEP induced antigen-induced airway inflammation and AHR in mice, it is likely that they differ in chemical composition, and quantitative differences might exist between MEP and DEP. Exhaust emitted from two-stroke motorcycle engines contains more hydrocarbons, both in quantity and in engine load, than diesel exhaust (Chan & Nien, 1999; Jemma et al., 1995; Madden et al., 2003). Studies showed that the chemical composition of MEP, in terms of PAH content, is similar qualitatively to DEP but different quantitatively (Ueng et al., 2000; Barfknecht et al., 1985; Hiura et al., 1999). PAH composition found in DEP was phenanthrene 52%, fluorenes 15%, naphthalene 13%, pyrene 10%,

and fluoranthene 10%, while the composition found in MEP was naphthalene 68%, anthracene 5%, acenaphthylene 4%, acenaphthene 4%, pyrene 4%, fluorine 3%, fluoranthene 2%, and others 10% (Ueng et al., 2000). Therefore, the constitution and quantity of chemicals might modify the antigen-induced airway inflammation and AHR in DEP compared to MEP. Therefore, whether PAH or other components are responsible for effects observed with MEP is an important question to be answered. Although we do not know the MEP exposing condition in the real world at this moment, we roughly estimated the exposure of MEP. As roughly 33 μ g of the MEP represent 1 L of exhaust from the motorcycle, then assuming that the MEP absorption percentage is 100%, the engine was run at an idle speed of 150 rpm on an empty load and the flow rate of 20 L/min, a 70 kg body weight rider waiting at the traffic light for 3 min and 10 times per day, the MEP exposure will be about 280 μ g/kg body weight.

In conclusion, in the present study, data demonstrated that MEP coexposure augmented OVA-induced airway inflammation and AHR. Allergic patients may present with increased symptom severity as a result of exposure to both motorcycle exhaust and allergens because of a synergistic effect of the two stimuli. This coexposure may lead to overexpression of inflammatory mediators potentially responsible for recruitment of a broad set of inflammatory cells. Data in this study identified at least one active pathway involved in the deleterious effects attributed to MEP on allergic inflammation.

REFERENCES

- Adler, A., Cieslewicz, G., and Irvin, C. G. 2004. Unrestrained plethysmography is an unreliable measure of airway responsiveness in BALB/c and C57BL/6 mice. *J. Appl. Physiol.* 97:286–292.
- Bandeira-Melo, C., and Weller, P. F. 2003. Eosinophils and cysteinyl leukotrienes. *Prostaglandins Leukocytes Essential Fatty Acids* 69:135–143.
- Barfknecht, T. R., Hites, R. A., Cavaliers, E. L., and Thilly, W. G. 1982. Human cell mutagenicity of polycyclic aromatic hydrocarbon components of diesel emissions. *Dev. Toxicol. Environ. Sci.* 10:277–294.
- Chan, C. C., and Nien, C. K. 1995. Comparison of tail-pipe emissions from motorcycles and passenger cars. *J. Air Waste Manage Assoc.* 45:116–124.
- Cheng, Y. W., and Kang, J. J. 1999. Inhibition of agonist-induced vasoconstriction and impairment of endothelium-dependent vasorelaxation by extract of motorcycle exhaust particles *in vitro*. *J. Toxicol. Environ. Health A* 56:75–87.
- Cheng, Y. W., Lee, W. W., Li, C. H., Lee, C. C., and Kang, J. J. 2004. Genotoxicity of motorcycle exhaust particles *in vivo* and *in vitro*. *Toxicol. Sci.* 81:103–111.
- Chung, K. F., and Barnes, P. J. 1999. Cytokines in asthma. *Thorax* 54:825–857.
- Corrigan, C. J., and Kay, A. B. 1990. CD4 T-lymphocyte activation in acute severe asthma: Relationship to disease severity and atopic status. *Am. Rev. Respir. Dis.* 141:970–977.
- Diaz-Sanchez, D. 1997. The role of diesel exhausts particles and their associated polyaromatic hydrocarbons in the induction of allergic airway disease. *Allergy* 52:52–56.
- Diaz-Sanchez, D., Dotson, A. R., Takenaka, H., and Saxon, A. 1994. Diesel exhaust particles induce local IgE production *in vivo* and alter the pattern of IgE messenger RNA isoforms. *J. Clin. Invest.* 94:1417–1425.
- Diaz-Sanchez, D., Tsien, A., Casillas, A., Dotson, A. R., and Saxon, A. 1996. Enhanced nasal cytokine production in human beings after *in vivo* challenge with diesel exhaust particles. *J. Allergy Clin. Immunol.* 98:114–123.

- Dockery, D., Pope, C. III, Xu, X., Spengler, J., Ware, J., Fay, M., Ferris, B., and Speizer, F. 1993. An association between air pollution and mortality in six US cities. *N. Engl. J. Med.* 329:1753–1759.
- Fernvik, E., Scharnweber, T., Knopp, D., Niessner, R., Vargaftig, B. B., and Peltre, G. 2002. Effects of fractions of traffic particulate matter on TH2-cytokines, IgE levels, and bronchial hypersponsiveness in mice. *J. Toxicol. Environ. Health A* 65:1025–1045.
- Hamelmann, E., Schwarze, J., Takeda, A., Oshiba, A., Larsen, G. L., Irvin, C. G., and Gelfand, E. W. 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care* 156:766–775.
- Heo, Y., Saxon, A., and Hankinson, O. 2001. Effect of diesel exhaust particles and their components on the allergen-specific IgE and IgG1 response in mice. *Toxicology* 159:143–158.
- Hiura, T. S., Kaszubowski, M. P., Li, N., and Nel, A. E. 1999. Chemicals in diesel exhaust particles generate reactive oxygen radicals and induce apoptosis in macrophages. *J. Immunol.* 163:5582–5591.
- Jemma, C. A., Shore, P. R., and Widdicombe, K. A. 1995. Analysis of C1–C16 hydrocarbons using dual-column capillary GC: Application to exhaust emissions from passenger car and motorcycle engines. *J. Chromatogr. Sci.* 33:34–48.
- Kanoh, T., Suzuki, T., Ishimori, M., Ikeda, S., Ohasawa, M., Ohkuni, H., and Tunetoshi, Y. 1996. Adjuvant activities of pyrene, anthracene, fluoranthene and benzo(a)pyrene in production of anti-IgE antibody to Japanese cedar pollen allergen in mice. *J. Clin. Lab. Immunol.* 48:133–147.
- Kay, A. B., Gelfand, E. W., Smith, L. J., and Tashkin, D. 2002. Natural history of asthma from childhood to adulthood. *Clin. Exp. Allergy Rev.* 2:117–127.
- Krewski, D., Burnett, R., Jerrett M., Pope, A., Rainham, D., Calle, E., Thurston, G., and Thun, M. 2005. Mortality and long-term exposure to ambient air pollution: Ongoing analyses based on the American cancer society cohort. *J. Toxicol. Environ. Health A* 68:1093–1109.
- Lee, C. C., and Kang, J. J. 2002. Extract of motorcycle exhaust particles induced macrophages apoptosis by calcium-dependent manner. *Chem. Res. Toxicol.* 15:1534–1542.
- Lee, C. C., Liao, J. W., and Kang, J. J. 2004. Motorcycle exhaust particles induce airway inflammation and airway hyperresponsiveness in BALB/c mice. *Toxicol. Sci.* 79:326–334.
- Lee, C. C., Cheng, Y. W., and Kang, J. J. 2005. Motorcycle exhaust particles induce IL-8 production through NF- κ B action in human airway epithelial cells. *J. Toxicol. Environ. Health A* 68:1537–1555.
- Li, X. Y., Gilmour, P. S., Donaldson, K., and MacNee, W. 1996a. Free radical activity and pro-inflammatory effects of particulate air pollution (PM 10) in vivo and in vitro. *Thorax* 51:1216–1222.
- Li, W., Whaley, C. D., Mondino, A., and Mueller, D. L. 1996b. Blocked signal transduction to the ERK and JNK protein kinases in anergic CD41 T cells. *Science* 271:1272–1276.
- Lovik, M., Hogseth, A. K., Gaarder, P. I., Hagemann, R., and Eide, I. 1997. Diesel exhaust particles and carbon black have adjuvant activity on the local lymph node response and systemic IgE production to ovalbumin. *Toxicology* 121:165–178.
- Lundblad, L. K. A., Irvin, C. G., Adler, A., and Bates, H. T. 2002. A reevaluation of the validity of unrestrained plethysmography in mice. *J. Appl. Physiol.* 93:1198–1207.
- Madden, M. C., Dailey, L. A., Stonehuerner, J. G., and Harris, D. B. 2003. Responses of cultured human airway epithelial cells treated with diesel exhaust extracts will vary with the engine load. *J. Toxicol. Environ. Health A* 66:2281–2297.
- Malerba, G., and Pignatti, P. F. 2005. A review of asthma genetics: Gene expression studies and recent candidates. *J. Appl. Genet.* 46:93–104.
- Ministry of Transportation and Communications. 2004. *Monthly statistics of transportation and communications in 2005*. Taipei: Ministry of Transportation and Communications, Republic of China.
- Miyabara, Y., Yanagisawa, R., Shimojo, N., Takano, H., Lim, H. B., Ichinose, T., and Sagai, M. 1998. Murine strain differences in airway inflammation caused by diesel exhaust particles. *Eur. Respir. J.* 11:291–298.
- Mori, A., Kaminuma, O., Miyazawa, K., Ogawa, K., Okudaira, H., and Akiyama, K. 1999. p38 mitogen-activated protein kinase regulates human T cell IL-5 synthesis. *J. Immunol.* 163:4763–4771.
- Nel, A. E., Diaz-Sanchez, D., Ng, D., Hiura, T., and Saxon, A. 1998. Enhancement of allergic inflammation by the interaction between diesel exhaust particles and the immune system. *J. Allergy Clin. Immunol.* 102:539–554.
- Patino, C. M., and Martinez, F. D. 2001. Interactions between genes and environment in the development of asthma. *Allergy* 56:279–286.
- Peterson, B., and Saxon, A. 1996. Global increases in allergic respiratory disease: the possible role of diesel exhaust particles. *Ann. Allergy Asthma Immunol.* 77:263–268.
- Republic of China Environment Protection Agency. 1994. *Environment data of Taiwan in 1994*, pp. 31–77. Taipei: Environmental Protection Agency, Executive Yuan, Republic of China.
- Republic of China Environment Protection Agency. 1996. *Environment data of Taiwan in 1996*. Taipei: Environmental Protection Agency, Executive Yuan, Republic of China.
- Renauld, J. C. 2001. New insights into the role of cytokines in asthma. *J. Clin. Pathol.* 54:577–589.
- Salvi, S. S., Suresh, B. K., and Holgate, S. T. 2001. Is asthma really due to a polarized T-cell response toward a helper T cell type-2 phenotype? *Am. J. Respir. Crit. Care Med.* 164:1343–1346.
- Schafer, P. H., Wadsworth, S. A., Wang, L., and Siekierka, J. 1999. p38 alpha mitogen-activated protein kinase is activated by CD28-mediated signaling and is required for IL-4 production by human CD4+CD45RO+ T cells and Th2 effector cells. *J. Immunol.* 162:7110–7119.
- Schwartz, J. 1994. Air pollution and daily mortality: A review and metaanalysis. *Environ. Res.* 64:36–52.
- Steenenbergh, P. A., Withagen, C. E. T., Dormans, J. A. W. A., van Dalen, W. J., van Loveren, H., and Casee, F. R. 2003. Adjuvant activity of various diesel exhaust and ambient particles in two allergic models. *J. Toxicol. Environ. Health A* 66:1421–1439.
- Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., and Ben-Neriah, Y. 1994. JNK is involved in signal integration during costimulation of T lymphocytes. *Cell* 77:727–736.
- Suzuki, T., Wang, W., Lin, J. T., Shirato, K., Mitsuhashi, H., and Inoue, H. 1996. Aerosolized human neutrophil elastase induces airway constriction and hyperresponsiveness with protection by intravenous pretreatment with half-length secretory leukoprotease inhibitor. *Am. J. Respir. Crit. Care Med.* 153:1405–1411.
- Ueng, T. H., Hu, S. H., Chen, R. M., Wang, H. W., and Kao, M. L. 2000. Induction of cytochrome P450 1A1 in human hepatoma HepG2 and lung carcinoma NCI-H322 cells by motorcycle exhaust particulate. *J. Toxicol. Environ. Health A* 60:101–119.
- Wenzel, S. E. 2003. The role of leukotrienes in asthma. *Prostaglandins Leukocytes Essential Fatty Acids* 69:145–155.
- Wu, W. M., Suen, B. F., Lin, B. F., and Chiang, B. L. 2000. Tamoxifen alleviates disease severity and decreases double negative T cells in autoimmune MRL-lpr/lpr mice. *Immunology* 100:110–118.
- Zhou, W., and Ye, S. H. 1997. Mutagenicity of scooter exhaust particulate matter. *J. Toxicol. Environ. Health* 52:34–44.