

## Motorcycle Exhaust Particles Induce Airway Inflammation and Airway Hyperresponsiveness in BALB/C Mice

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Received February 11, 2004; accepted March 26, 2004

A number of large studies have reported that environmental pollutants from fossil fuel combustion can cause deleterious effects to the immune system, resulting in an allergic reaction leading to respiratory tract damage. In this study, we investigated the effect of motorcycle exhaust particles (MEP), a major pollutant in the Taiwan urban area, on airway inflammation and airway hyperresponsiveness in laboratory animals. BALB/c mice were instilled intratracheally (i.t.) with 1.2 mg/kg and 12 mg/kg of MEP, which was collected from two-stroke motorcycle engines. The mice were exposed 3 times i.t. with MEP, and various parameters for airway inflammation and hyperresponsiveness were sequentially analyzed. We found that MEP would induce airway and pulmonary inflammation characterized by infiltration of eosinophils, neutrophils, lymphocytes, and macrophages in bronchoalveolar lavage fluid (BALF) and inflammatory cell infiltration in lung. In addition, MEP treatment enhanced BALF interleukin-4 (IL-4), IL-5, and interferon-gamma (IFN- $\gamma$ ) cytokine levels and serum IgE production. Bronchial response measured by unrestrained plethysmography with methacholine challenge showed that MEP treatment induced airway hyperresponsiveness (AHR) in BALB/c mice. The chemical components in MEP were further fractionated with organic solvents, and we found that the benzene-extracted fraction exerts a similar biological effect as seen with MEP, including airway inflammation, increased BALF IL-4, serum IgE production, and induction of AHR. In conclusion, we present evidence showing that the filter-trapped particles emitted from the unleaded-gasoline-fueled two-stroke motorcycle engine may induce proinflammatory and proallergic response profiles in the absence of exposure to allergen.

**Key Words:** airway hyperresponsiveness; airway inflammation; asthma; IgE; intratracheal instillation; motorcycle exhaust particles.

There is growing evidence that environmental pollutants could have some impact on the immunologic function that is involved in the occurrence of asthma and chronic obstructive pulmonary disease (Diaz-Sanchez *et al.*, 1994, 1996; Li *et al.*,

1996; Nel *et al.*, 1998; Peterson and Saxon, 1996). This trend seems to be in concert with the increase in fossil fuel combustion and emission of particulate pollutants (Peterson and Saxon, 1996). For example, Japan has demonstrated that the discrepancy in incidence of rhino-conjunctivitis in the different regions seems to be a result of vehicle-exhaust pollution (Ishizaki *et al.* 1987). A study from Germany also showed that there was a significant correlation between an increase in the number of cars passing daily through a school district in Munich and a decrease in peak expiratory flow rate and the maximum expiratory flow of school children aged 9–11 years old (Wjst *et al.*, 1993). In addition, in a substantial number of cities, particularly in developing countries, WHO guidelines or national air quality standards are often exceeded for sulphur dioxide and suspended particulate matter consisting of particles with an aerodynamic diameter below 10  $\mu$ m (Schwela, 1996), which will cause serious damage to the respiratory system (Bascom, 1996). Several studies have shown that diesel exhaust particles (DEP) caused induction and propagation of allergic inflammation, including an increase of IgE and T-helper (cell)-2 (Th2) cytokine production, and mucosal inflammation in humans and animals challenged by allergens (Diaz-Sanchez *et al.*, 1994, 1996; Fujieda *et al.*, 1998; Takenaka *et al.*, 1995).

Asthma is a chronic inflammatory disease of the lung that has been increasing in prevalence, morbidity, and mortality over the last two decades, and is characterized by reversible bronchospasm, airway inflammation, and airway hyperresponsiveness (AHR) (NHLBI, 1995). According to clinical data and animal models of asthma, it is suggested that, in allergic asthma, the Th2 lymphocytes induce, via the production of cytokines, an inflammatory cascade comprised of eosinophil activation, IgE production, and mast cell activation, all of which in turn produce the necessary mediators causing airway hyperresponsiveness (AHR) (Chung and Barnes, 1999; Wills-Karp, 1999). Although numerous studies have shown that DEP can exert biological effects that lead to the development or exacerbation of chronic allergic airway disease, full identification of the active components and their mechanisms has not yet been achieved. Other environmental factors might also be involved in the proinflammatory and proallergic responses.

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In Taiwan, motorcycles are widely used, with more than 17 million registered in 2003, making the density as high as 0.85 motorcycles registered per person in the total population (MSTC, 2003). The use of motorcycles, especially the two-stroke engine, was estimated to introduce about 16,000 tons of total suspended particles (TSP) and 15,000 tons of particulate matter with a diameter of 10  $\mu\text{m}$  (PM10), per year in Taiwan (EPA, Taiwan, 1994). As compared with other countries, the mean concentration of PM10 per year is considerably higher in Taiwan (64  $\mu\text{g}/\text{m}^3$ ) compared to some other parts of the world (London, U.K., 14  $\mu\text{g}/\text{m}^3$ ; Paris, France, 14  $\mu\text{g}/\text{m}^3$ ) (EPA, Taiwan, 1996). Recent studies have highlighted the fact that PM10 or less can exacerbate asthma as well as chronic obstructive pulmonary disease (Diaz-Sanchez *et al.*, 1994; Nel *et al.* 1998; Peterson and Saxon, 1996). The impact of motorcycle exhaust particles (MEP) to the environment and its biological effects is relatively unclear. MEP contains a carbon black core, which absorb >110 different organic compounds, including C1–C20 chains of hydrocarbon compounds and polycyclic aromatic hydrocarbons (PAHs) (Ueng *et al.*, 2000). Studies have indicated that MEP is cytotoxic (Lee and Kang, 2002), mutagenic (Zhou and Ye, 1997), and genotoxic (Kuo *et al.* 1998) *in vitro*. It was also shown that MEP could impair the function of an isolated rat aorta (Cheng and Kang, 1999) and several metabolic enzymes in rat tissues (Ueng *et al.* 1998). However, no study has been carried out to examine the effects of MEP on the respiratory system, *in vivo*.

For the first time, the effect of MEP in inflammation-related lung parameters in laboratory animals was evaluated. We found that MEP, when applied intratracheally, would induce airway inflammation and AHR.

## MATERIALS AND METHODS

**Animals.** Female BALB/c mice were purchased from the Animal Center of the College of Medicine, National Taiwan University, Taiwan. All mice were 8–12 weeks old at the time of the experiments. The animals were allowed at least a 1-week acclimation period at the animal quarters with air conditioning and 12-h light/dark cycles. All animal treatments were approved by the IACUC of the College of Medicine, National Taiwan University, which followed the requirements of the animal welfare protection act of the Department of Agriculture, Executive Yuan, Taiwan.

**Collection and extraction of motorcycle exhaust particles.** The MEP were collected on 0.5- $\mu\text{m}$  quartz fiber filters (Advantec MFS, Inc., CA) from 50-cm<sup>3</sup> Yamaha Cabin or Suzuki two-stroke engines using 95% octane unleaded gasoline under the same conditions. The sampling apparatus consisted of, in sequence, a 30-cm long by 2.2-cm diameter stainless dilution tube, a filter holder, and a vacuum pump. Engines were running at 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 were extracted four times with methanol under sonication. Methanol was then removed by vacuum evaporator. The weight change of the residue was frequently measured, and the complete removal of solvent was achieved when the weight of the residue was constant. The final residue, the MEP, was collected and kept desiccated in darkness at  $-20^\circ\text{C}$ . Thirty-three  $\mu\text{g}$  of MEP could be extracted from one liter (l) of Yamaha motorcycle exhaust and 17  $\mu\text{g}$  MEP from 1 liter of Suzuki

motorcycle exhaust. In order to exclude the possible aging or any possible changes that might occur to the chemicals during storage, which might affect the biological activity of MEP. We have frequently treated the mice with MEP that has been stored for different periods of time after the preparation, and we have found that the MEP after storage for 2 months still gave the same effect as freshly prepared MEP.

For fractionation of the chemical components in MEP, the quartz filters with collected particles were extracted three times with 100 ml of hexane under sonication. The suspension was filtered and the residue was treated with the same volume of benzene, followed successively by chloroform, ethyl acetate, and methanol. The extracted fractions were filtered through a 0.22  $\mu\text{m}$  filter to remove the particles, and the solvents were then removed by vacuum evaporator. The extracted residues by different organic solvents were collected and kept desiccated at  $-20^\circ\text{C}$ . The following components are normally recovered from each gram of MEP: 0.81 g of *n*-hexane extract component, 0.09 g benzene extract component, 0.01 g chloroform extract component, 0.01 g ethyl acetate extract component, and 0.01 g methanol extract component.

**Study protocol.** On Days 0, 14, and 28, mice were first anesthetized with 0.75 mg of ketamine and 0.06 mg of combelen per mouse, by intramuscular injection, and then applied intratracheally instilled with 50  $\mu\text{l}$  of phosphate-buffered saline (PBS, pH 7.2) containing 24  $\mu\text{g}$  (1.2 mg/kg) or 240  $\mu\text{g}$  (12 mg/kg) MEP collected from the Yamaha motorcycle. On both Day one and Day 7 after the last intratracheal instillation, mice were studied for airway hyperresponsiveness, bronchoalveolar lavage, and cytokine levels in BALF, serum total antibodies, and lung histology. By using trypan blue as a marker, we found that even distribution of the particle instilled throughout the lung could be achieved by using the technique of Leong *et al.* (1998) (data not shown). For most of the experiment, the MEP collected from the Yamaha motorcycle was used unless otherwise specified. In one experiment, MEP collected from the Suzuki motorcycle was used for comparison and fractionation. In that experiment, we used both 500  $\mu\text{g}$  (25 mg/kg) of MEP or 500  $\mu\text{g}$  (25 mg/kg) of residues from organic solvent-extracted fractions. For the dosing regimen selected, 12 mg/kg of MEP, collected from the Yamaha motorcycle, was one-tenth of the minimum lethal dose from an acute toxicity study (unpublished result). From a comparison study, we have found that 25 mg/kg of MEP collected from the Suzuki engine was needed to induce a similar degree of airway inflammation as that of 12 mg/kg of MEP collected from the Yamaha engine.

**Lung histology.** The lungs were inflated with, and immersed in, 10% neutral phosphate-buffered formalin. Sections were prepared and stained with hematoxylin/eosin (H&E) to quantitate the number of infiltrating inflammatory cells under microscopy.

**Bronchoalveolar lavage.** After treatment, mice underwent bronchoalveolar lavage using 1.5 ml sterile Hank's balanced salt solution (HBSS), instilled bilaterally with a syringe. The lavage fluid was harvested by gentle aspiration (Miyabara *et al.*, 1998). This procedure was repeated three times. The fractions of lavage fluid were pooled, cooled to  $4^\circ\text{C}$ , and centrifuged at  $300 \times g$  for 5 min. Total cell counts were determined on fresh fluid specimens with the use of a hemocytometer. Differential cell counts were assessed on cytological preparations. The slides were prepared with the use of a Cytospin (Thermo Electron Corporation, Pittsburgh, PA) and stained with Liew staining. A total of 300 cells were counted under microscopy. Aliquots of supernatants were stored at  $-70^\circ\text{C}$  and analyzed for cytokines by ELISA.

**Determination of bronchoalveolar lavage cytokine level.** The lavage supernatants were utilized for determination of cytokine levels, IL-4, IL-5, TNF- $\alpha$ , and INF- $\gamma$ . Cytokine levels were assayed by the ELISA method, according to the procedure recommended by the manufacturer (R&D, Minneapolis, MN. for IL-4 [interleukin-4], TNF- $\alpha$ , and interferon- $\gamma$  (INF- $\gamma$ ) [Med-Systems Diagnostics GmbH, Vienna, Austria]; England. for IL-5.

**Measurement of airway responsiveness.** Airway responsiveness was measured in unrestrained animals by barometric whole-body plethysmography (from Buxco, Troy, NY). Briefly, mice were placed in the main chamber, and baseline readings were taken and averaged for 3 min. Aerosolized 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 are extrapolated to define the respiratory rate in breaths per min. Airway reactivity was expressed as an enhanced pause (Penh) and data were expressed as the ratio of  $Penh_{MCh}$  values to  $Penh_{PBS}$  from three independent experiments (Hamelmann *et al.*, 1997).

**Measurements of total serum IgE and IgG<sub>2a</sub>.** Total serum IgE (indicative of Th2 response) and IgG<sub>2a</sub> (of the Th1 response) were measured by sandwich ELISA as previously described (Wu *et al.*, 2000). Predetermined concentrations of anti-IgE and anti-IgG<sub>2a</sub> antibodies (Pharmingen, San Diego, CA) were coated onto ELISA plates and incubated at 4°C overnight. Plates were washed with PBS containing 0.05% Tween 20, and the diluted serum (50-fold dilution for IgE and 100-fold dilution for IgG<sub>2a</sub> determination) was added to the plates and incubated at room temperature for 2 h. Biotin-conjugated anti-IgE or anti-IgG<sub>2a</sub> antibodies and alkaline phosphatase-conjugated avidin (Sigma, St. Louis, MI) were added subsequently. Enzyme activity was evaluated using p-nitrophenyl phosphate (Sigma St. Louis, MI) 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. Standard curves were obtained from a series dilution of known concentrations of purified mouse IgE and IgG<sub>2a</sub> (Pharmingen, San Diego, CA).

**Statistical analysis.** All values refer to means  $\pm$  SEM (standard error of the mean) of at least three separate experiments. Statistically significant difference among groups was analyzed with one-way ANOVA followed by Scheffe for *post hoc* comparison using SPSS software. The minimal level of significance was a *p* value of  $<0.05$ .

## RESULTS

### MEP-induced Inflammatory Cell Infiltration in Bronchoalveolar Lavage Fluid (BALF) and Lung Tissue

The changes of cell numbers in the BALF of mice intratracheally instilled with MEP on Days 1 and 7 after the final treatment were investigated (Table 1). At the MEP concentration of 12 mg/kg, the total cell numbers in BALF were significantly

increased, and a slight or no change in cell numbers was seen with the lower dosage group (1.2 mg/kg) at Day 1. The numbers of neutrophils and eosinophils in BALF were changed dramatically in MEP-treated groups. Both cell types were increased in BALF about 5- to 8-fold after treatment with MEP 1.2 mg/kg and MEP 12 mg/kg, respectively, on Day 1 as compared with the saline group. The number of macrophages was relatively unchanged in the 1.2-mg/kg group but significantly increased in the 12-mg/kg group (a 57% increase in the number of macrophages, as compared to the saline group). The cell numbers at Day 7 were all decreased when compared to Day 1, but they were still significantly higher than the saline control group. Unlike other cells, the number of lymphocytes in MEP-treated groups was only slightly higher than the saline group at Day 1, but significantly increased at Day 7 after the final treatment (Table 1).

Histological examination of the lung sections from MEP-treated mice also showed the increase in infiltration of the inflammatory cells (Figs. 1A, 1B, and 1C). The intensity of inflammatory cells infiltrated into lung was significantly increased in the MEP 12-mg/kg group (Fig. 1D), as compared to the saline group.

### MEP Increased Cytokine Production In BALF

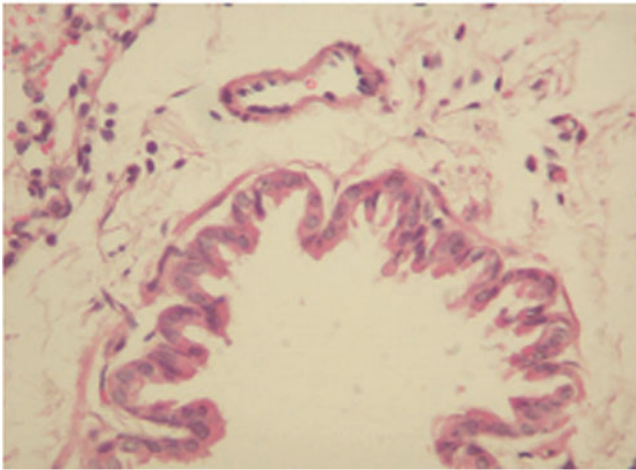
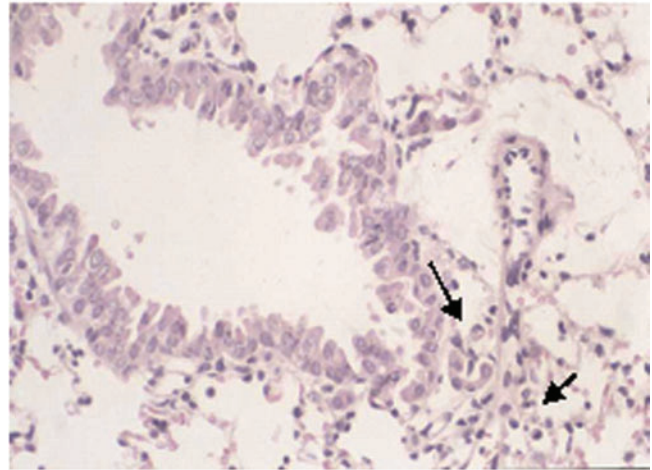
The levels of cytokines, including Th1 cytokine, IFN- $\gamma$ , and Th2 cytokines, IL-4, and IL-5 were analyzed in BALF of MEP-treated and control mice (saline group) at Day 1 and Day 7 after the last treatment. As shown in Table 2, IL-4 and IL-5 contents in BALF were significantly increased in both MEP 1.2-mg/kg- and 12-mg/kg-treated groups as compared with the saline group. The IFN- $\gamma$  levels were significantly higher in BALF only at MEP of 12 mg/kg. However, the pro-inflamma-

TABLE 1  
Cellular Profile of Bronchoalveolar Lavage Fluid after Treatment of MEP

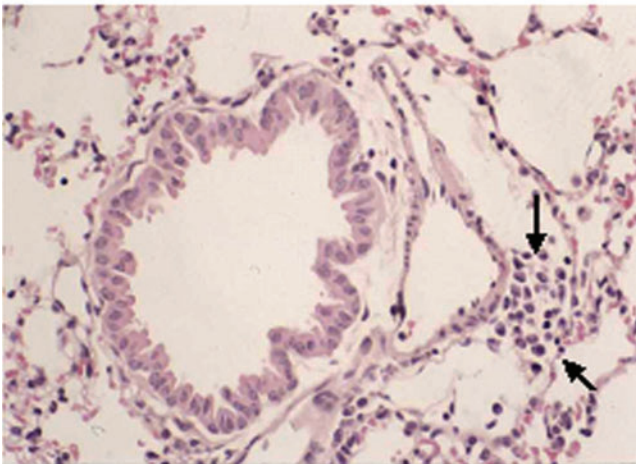
| Cell numbers ( $1 \times 10^3$ /total BAL supernatants) |                 |                 |                |                 |                  |
|---------------------------------------------------------|-----------------|-----------------|----------------|-----------------|------------------|
| Group                                                   | Total cell      | Macrophage      | Neutrophil     | Lymphocyte      | Eosinophil       |
| Day 1 after third intratracheal instillation            |                 |                 |                |                 |                  |
| Saline                                                  | 23.3 $\pm$ 2.0  | 23.0 $\pm$ 2.1  | 0.1 $\pm$ 0.1  | 0.1 $\pm$ 0.0   | 0.1 $\pm$ 0.1    |
| MEP 1.2 mg/kg                                           | 27.3 $\pm$ 2.3  | 25.5 $\pm$ 1.9  | 0.4 $\pm$ 0.1* | 0.2 $\pm$ 0.1   | 0.6 $\pm$ 0.1*   |
| MEP 12 mg/kg                                            | 41.4 $\pm$ 3.6* | 36.2 $\pm$ 4.5* | 0.5 $\pm$ 0.1* | 0.4 $\pm$ 0.1   | 0.6 $\pm$ 0.2*   |
| Day 7 after third intratracheal instillation            |                 |                 |                |                 |                  |
| Saline                                                  | 23.0 $\pm$ 2.7  | 21.9 $\pm$ 2.3  | 0.1 $\pm$ 0.0  | 0.2 $\pm$ 0.1   | 0.1 $\pm$ 0.0    |
| MEP 1.2 mg/kg                                           | 29 $\pm$ 4.3    | 27.4 $\pm$ 6.2  | 0.2 $\pm$ 0.1* | 0.6 $\pm$ 0.2*  | 0.2 $\pm$ 0.1**  |
| MEP 12 mg/kg                                            | 33.9 $\pm$ 3.9* | 31.2 $\pm$ 2.0* | 0.2 $\pm$ 0.1* | 0.7 $\pm$ 0.3** | 0.3 $\pm$ 0.1*** |

*Note.* Mice received intratracheal instillations of saline, MEP 1.2 mg/kg, and MEP 12 mg/kg, according to the study protocol in Materials and Methods, and BALF was derived on Day 1 and Day 7 after the final treatment. Total cell counts were determined with 500  $\mu$ l BALF, and different cell counts were determined by Liu staining, as described in Materials and Methods. Data were expressed as mean  $\pm$  SEM ( $n \geq 10$ ); \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , compared with saline group.

A saline (-)

B MEP 1.2mg/kg ( $\pm$ )

C MEP 12mg/kg (+)



D

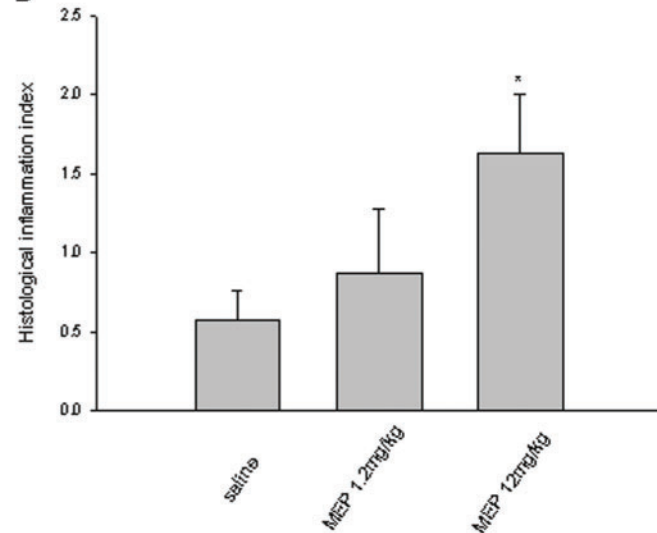


**FIG. 1.** Induction of pulmonary inflammation by MEP in BALB/c mice: Mice received intratracheal instillation of saline (A), 1.2 mg/kg MEP (B), and 12 mg/kg MEP (C). MEP collected from a Yamaha motorcycle according to the study protocol in Materials and Methods. Animals were sacrificed at 7 days after the final treatment. Lung sections were stained with H&E for measurement of inflammatory cells around the airways, as described in Materials and Methods. Arrows denote inflammatory cell infiltration. (D) Degree of lung inflammatory index was estimated. (-), none, score 0; ( $\pm$ ), very slight, score 1; (+), slight, score 2; (++) , moderate, score 3; (+++) , marked, score 4. Data were expressed as the mean  $\pm$  SEM ( $n \geq 10$ ). \* $p < 0.05$  compared with saline group.

tory cytokine, TNF- $\alpha$ , did not show any significant change in MEP-treated groups (data not shown).

#### *MEP Treatment Caused an Increase in Total Serum IgE Antibody Production*

The total serum IgE and IgG<sub>2a</sub> levels were measured and data summarized in Figure 2. Total serum IgE levels were significantly higher in mice intratracheally instilled with MEP (Fig. 2A). The serum IgE production increased from  $3.1 \pm 0.5$  nM (saline group) to  $5.2 \pm 0.5$  nM and  $5.8 \pm 0.8$  nM in mice treated with 1.2 mg/kg and 12 mg/kg MEP, respectively. No

significant changes of serum IgG<sub>2a</sub> were observed in the MEP treatment groups (Fig. 2B).

#### *Induction of Airway Hyperresponsiveness by MEP*

The effect of MEP treatment on AHR was investigated by using the noninvasive whole-body plethysmography method with a methylcholine (MCh) challenge (Fig. 3). The MCh (3.125 to 25 mg/ml) dose-dependent increase of the Penh ratio was significantly augmented in mice instilled intratracheally with both concentrations of MEP (1.2 mg/kg and 12 mg/kg).

*Effects of MEP and Extracted Components Collected from the Suzuki Motorcycle on Induction of Airway Inflammation and AHR*

For comparison, MEP collected from a different motorcycle was used to treat animals. We found that the MEP collected from the Suzuki motorcycle (MEPs) induced a similar proinflammatory and proallergic response profile as was seen with MEP collected from the Yamaha motorcycle (MEPy); however, with a weaker potency. Twenty-five mg of MEPs was needed to induce effects seen with 12 mg/kg of MEPy as judged by all parameters.

MEPs were sequentially extracted with different organic solvents, and the effect of each fraction was investigated. As seen previously, the cell numbers of macrophages, neutrophils, and eosinophils were increased in BALF at 25 mg/kg MEPs-treated mice (Table 3). In all the organic solvent extracts tested, only the benzene-extract fraction caused an increase in the numbers of neutrophil and eosinophil in BALF to a similar degree as that of the MEPs-treated group. Although the numbers of total cell and macrophage in BALF of the benzene extract-treated group were slightly increased, these were not significant when compared to the saline group.

The MEPs and benzene extract-treated mice showed significant increase in IL-4 (Fig. 4A), total serum IgE (Fig. 4B) levels and Penh ratio (Fig. 4C) when compared to the saline group.

## DISCUSSION

It is thought that the particulate and gaseous pollutants from the use of fossil fuels by factories and automobiles may be

**TABLE 2**  
Protein Levels of Cytokines in Bronchoalveolar Lavage Fluid

| Cytokine level (pg/total BAL supernatants)   |                  |                  |                 |
|----------------------------------------------|------------------|------------------|-----------------|
| Groups                                       | IL-4             | IFN- $\gamma$    | IL-5            |
| Day 1 after third intratracheal instillation |                  |                  |                 |
| Saline                                       | 3.03 $\pm$ 1.3   | 12.6 $\pm$ 1.5   |                 |
| MEP 1.2-mg/kg                                | 13.9 $\pm$ 3.9*  | 19.9 $\pm$ 1.5   |                 |
| MEP 12 mg/kg                                 | 16.5 $\pm$ 1.8** | 25.8 $\pm$ 3.8** |                 |
| Day 7 after third intratracheal instillation |                  |                  |                 |
| Saline                                       | 4.6 $\pm$ 1.1    | 17.9 $\pm$ 5.8   | 14.5 $\pm$ 10.7 |
| MEP 1.2 mg/kg                                | 10.1 $\pm$ 2.4*  | 29.9 $\pm$ 3.1*  | 18.2 $\pm$ 7.5  |
| MEP 12 mg/kg                                 | 12.8 $\pm$ 2.6** | 39.4 $\pm$ 8.3*  | 29.6 $\pm$ 8.3* |

*Note.* Mice received intratracheal instillations of saline, MEP 1.2 mg/kg, and MEP 12 mg/kg, according to the study protocol in Materials and Methods, and BALF was derived at Day 1 and Day 7 after the final treatment. Protein levels of each cytokine were analyzed by ELISA, as described in Materials and Methods. Data were expressed as mean  $\pm$  SEM ( $n \geq 10$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , compared with saline group.

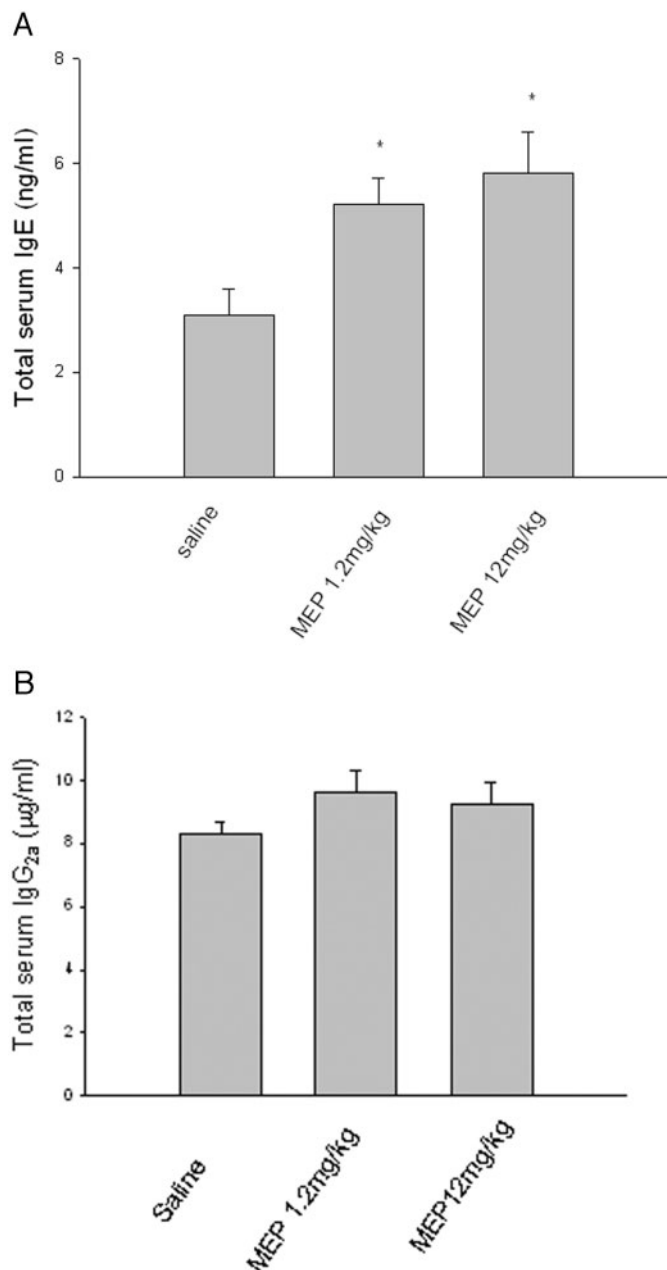


**FIG. 2.** Total serum antibody levels in BALB/c mice: Mice received intratracheal instillation of saline, MEP 1.2 mg/kg, and MEP 12 mg/kg, according to the study protocol in Materials and Methods. Blood samples were obtained at 7 days after the final treatment. Total serum IgE (A) and IgG<sub>2a</sub> (B) were analyzed by ELISA. Data were expressed as the mean  $\pm$  SEM ( $n \geq 10$ ). \* $p < 0.05$  compared with saline level.

important in the overall increase in the prevalence of allergic airway disease over the past century. In this study, we have presented evidence showing that the particulate fraction collected from motorcycle exhaust could induce pulmonary inflammation accompanied by an increase in alveolar cytokines, serum IgE production, and AHR induction in a mouse model.

Airway inflammation is characterized by an increase of

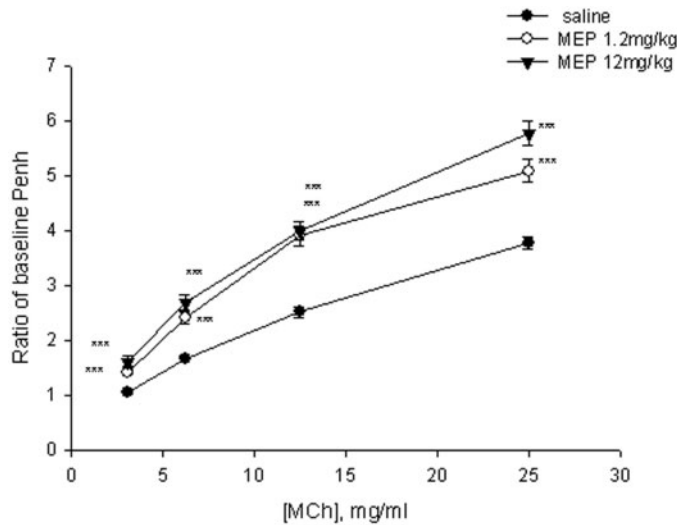


FIG. 3. Airway hyperresponsiveness induced by MEP in BALB/c mice: Airway hyperresponsiveness was measured by whole body plethysmography in mice receiving intratracheal instillation of saline, MEP 1.2 mg/kg, and MEP 12 mg/kg at 7 days after the final treatment. Data were expressed as the mean  $\pm$  SEM ( $n \geq 10$ ). \*\*\* $p < 0.001$  compared with saline group.

inflammatory-cell infiltration in that region. For example, airway inflammation in asthma is characterized by an eosinophilic inflammation (Bochner *et al.* 1994). We have found that the number of cells in BALF, including macrophage, neutrophil, lymphocyte, and eosinophil, were all increased after intratracheal instillation with MEP, especially at the 12-mg/kg concentrations. The infiltration and change of cell numbers of eosinophils and neutrophils was most obvious in the present study. The eosinophil is one of the major sources of cysteinyl leukotrienes that causes contraction of airway smooth muscle leading to AHR (Kay, 2001). Whether neutrophils have a direct role in the development of AHR remains controversial, although the presence of neutrophils has been documented in

allergic asthma and animal models of AHR (Devalia *et al.*, 1996; Kay, 2001; Takano *et al.*, 1997; Tomkinson *et al.*, 2001). In addition to eosinophils and neutrophils, infiltration of macrophages was also observed in the MEP group. The infiltration of macrophages could also release cysteinyl leukotrienes, which play an important role in allergic asthma and development of AHR (Kay, 2001). It has been shown that both eosinophils and neutrophils infiltrate BALF after repeated intratracheal instillation of DEP in mice (Sagai *et al.* 1996).

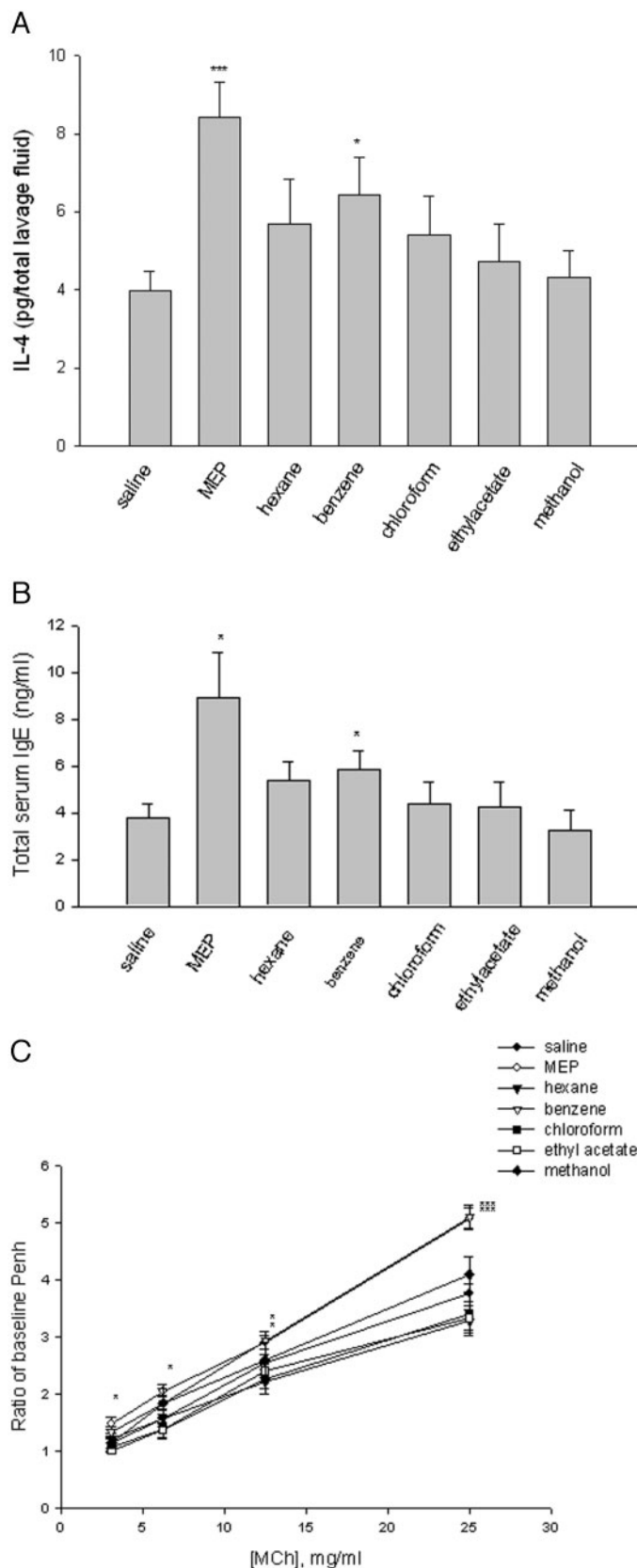
Particle overload in the lung has been shown to cause chronic pulmonary inflammation by an impairment of alveolar macrophage-mediated lung clearance, which eventually leads to accumulation of excessive lung burdens (Morrow, 1992; Oberdörster, 1995). One of the effective doses used in this study was 0.13 mg/g lung (1.2 mg/kg dosage), which was lower than that used in another particle overload experiment (1 mg dust/g lung tissue of F344 rat) (Morrow, 1986). It was also shown that mice are less sensitive to "particle overload" than rats (Muhle *et al.* 1990). In addition, the benzene-extracted fraction, which should be free of particles, induced similar effects as MEP. Based on these facts, we believe, although it needs to be proven, that the effects induced by MEP might be independent of particle overload.

In this study, we have found that MEP treatment could induce AHR, as judged by the increase of Penh, which represents a parameter of AHR by a noninvasive method. Different stimuli, direct and indirect, have been shown to induce AHR (Pauwel *et al.* 1988; Sterk *et al.* 1993). Direct stimuli cause AHR by direct action on the effector cells such as airway smooth muscle cells, bronchial vascular endothelial cells, and mucus-producing cells. Indirect stimuli cause AHR by an action on cells such as inflammatory and neuronal cells, which then interact with effector cells (Van Schoor *et al.* 2000). In a preliminary study, we have found that direct administration of MEP did not affect the contractility of the isolated trachea (unpublished result). This implies that induction of AHR by

TABLE 3  
Cell Profile of Bronchoalveolar Lavage Fluid after Treatment of MEP-Extracted Fractions

| Cell numbers ( $1 \times 10^3$ /total BAL supernatants) |                   |                   |                 |               |                  |
|---------------------------------------------------------|-------------------|-------------------|-----------------|---------------|------------------|
| Group                                                   | Total cell        | Macrophage        | Neutrophil      | Lymphocyte    | Eosinophil       |
| Saline                                                  | 20.8 $\pm$ 2.2    | 19.8 $\pm$ 2.0    | 0.1 $\pm$ 0.0   | 0.2 $\pm$ 0.1 | 0.1 $\pm$ 0.0    |
| MEP                                                     | 34.6 $\pm$ 2.2*** | 33.0 $\pm$ 2.3*** | 0.2 $\pm$ 0.0** | 0.4 $\pm$ 0.1 | 0.6 $\pm$ 0.1*** |
| Hexane                                                  | 20.8 $\pm$ 2.4    | 20.2 $\pm$ 2.4    | 0.1 $\pm$ 0.0   | 0.2 $\pm$ 0.1 | 0.2 $\pm$ 0.1    |
| Benzene                                                 | 26.8 $\pm$ 2.8    | 25.9 $\pm$ 2.7    | 0.2 $\pm$ 0.1*  | 0.4 $\pm$ 0.1 | 0.4 $\pm$ 0.1*** |
| Chloroform                                              | 21 $\pm$ 2.2      | 20.5 $\pm$ 2.2    | 0.1 $\pm$ 0.0   | 0.2 $\pm$ 0.1 | 0.1 $\pm$ 0.1    |
| Ethyl acetate                                           | 21.3 $\pm$ 1.9    | 21.5 $\pm$ 1.7    | 0.1 $\pm$ 0.0   | 0.3 $\pm$ 0.1 | 0.1 $\pm$ 0.0    |
| Methanol                                                | 23 $\pm$ 2.6      | 22.6 $\pm$ 2.5    | 0.1 $\pm$ 0.0   | 0.3 $\pm$ 0.1 | 0.1 $\pm$ 0.0    |

Note. Mice received intratracheal instillations of saline, MEP collected from Suzuki motorcycle 25 mg/kg, and organic solvent-extracted components, 25 mg/kg, according to the study protocol in Materials and Methods, and BALF was derived at Day 1 after the final treatment. Total cell counts were determined with 500  $\mu$ l of BALF, and different cell counts were assessed by Liu staining, as described in Materials and Methods. Data were expressed as mean  $\pm$  SEM ( $n \geq 10$ ); \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ , compared with the saline group.



MEP might be caused indirectly through the inflammatory cells. Inflammatory cells, especially eosinophils, could release a myriad of mediators that are potentially important in AHR, including the eosinophil-specific proteins, cytokines, and lipid mediators (Wills-Karp, 1999). In addition to the inflammatory cells, proinflammatory cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to play an important role in DEP-induced AHR in mice (Ohta *et al.*, 1999). The role of GM-CSF and other mediators in MEP-induced AHR is still awaiting further investigation.

IgE plays a major role in the pathogenesis of allergic inflammation in humans, both in the early and late phases of the mucosal response to allergens. In addition, IgE is shown to cause antigen-specific eosinophil degranulation (Kaneko *et al.*, 1995) and mast cell activation (Oettgen *et al.*, 1994). In the present study, we have seen that the serum IgE level was increased in MEP-treated mice. In contrast to IgE, the IgG<sub>2a</sub> level was not changed in MEP-treated mice, suggesting that the effect on IgE might be specific. Previous studies on DEP have shown that it could enhance the allergic inflammation by enhancing IgE production through different mechanisms, including cytokine production (Nel *et al.*, 1998) or direct activation of B cells (Takenaka *et al.*, 1995). In contrast to DEP, an *in vitro* study with isolated splenocytes showed that MEP did not cause cell proliferation by <sup>3</sup>H-thymidine incorporation assay or IgE production (data not shown). This implies that the cytokine production may play a role in the induction effect of IgE production seen with MEP.

In this study, we have also observed that the several cytokine levels were increased in the BALF of MEP-treated mice. IL-5 can influence the production, maturation, and activation of eosinophils and the local differentiation of tissue-infiltrating eosinophil precursors (Chung and Barnes, 1999). The Th2 type cytokine, IL-4, can evoke the switch of antibody isotypes from IgM to IgE, which further mediates the development of an allergic response (Hurst *et al.*, 2001). The increase of IL-4 expression in BALF of the MEP-treated mice seems to agree with the increase of IgE production. It is interesting to note, we also observed that the IFN- $\gamma$ , a Th1 type cytokine, increased production in BALF in the higher dose of MEP-treated mice, although it has shown that IFN- $\gamma$  can inhibit the synthesis of IgE and the differentiation of precursor cells to Th2 cells. However, some evidence from *in vivo* studies conflicts with this hypothesis. For example, the level of IFN- $\gamma$  is elevated in

**FIG. 4.** Airway inflammation and hyperresponsiveness induced by MEP collected from Suzuki motorcycle and organic solvent extracted components in BALB/c mice: Mice received intratracheal instillation of saline, MEP 25 mg/kg, or organic solvent extracted components, 25 mg/kg according to protocol in Materials and Methods. BALF and blood samples were collected on Day 1 after the final treatment. IL-4 levels in BALF (A) and total serum IgE (B) were analyzed by ELISA, and airway hyperresponsiveness (C) was measured by whole body plethysmography, as described in Materials and Methods on Day 1 after the final treatment. Data were expressed as the mean  $\pm$  SEM ( $n \geq 10$ ); \* $p < 0.05$ , \*\*\* $p < 0.001$  as compared with saline control.

the serum of severe asthma patients (Corrigan *et al.*, 1990) in supernatants from cultures of unstimulated and stimulated cells in BALF (Cembrzynska-Nowal *et al.*, 1993). In the murine model, Hansen and colleagues (1990) have found that introduction of Th1 cells worsened the underlying airway inflammation. Therefore, that the Th2 hypothesis is an answer in the MEP-inducing pathogenesis of asthma-like reaction seems an oversimplification; other possible mechanisms might be also involved (Salvi *et al.*, 2001).

A previous study by Kawasaki *et al.* (2001) has shown that the benzene fraction of DEP was important for the DEP-induced IL-8 gene expression in human epithelial cells. They also believed that BaP, a carcinogenic PAH, is an important active and major component of the benzene fraction, since BaP also induced IL-8 gene expression. We have fractionated the MEP by using organic solvents including hexane, benzene, chloroform, ethyl acetate, and methanol. We found that the benzene fraction caused similar effects as seen with MEP, such as an increase in eosinophil and neutrophil infiltration, IL-4 and IgE production, and AHR. This suggested that the chemicals responsible for inducing the effects observed might be enriched in the benzene fraction. Although both DEP and MEP could induce airway inflammation and AHR in mice, different chemical compositions and quantity might exist between MEP and DEP. The exhaust emitted from two-stroke motorcycle engines contained more hydrocarbons, both in quantity and species, than the diesel fuel (Chan and Nien, 1995; Jemma *et al.*, 1995). Studies also showed that although the chemical composition of MEP, in terms of PAH content, is similar to that of DEP, the percentages are different (Barfknecht *et al.*, 1982; Hiura *et al.*, 1999; Ueng *et al.*, 2000). The PAHs composition found in DEP was: phenanthrene 52%, fluorenes 15%, naphthalene 13%, pyrene 10%, and fluoranthene 10% (Barfknecht *et al.*, 1982), and 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 in the benzene-extracted fraction of DEP might be different from MEP. In addition, it has never been shown that BaP could induce airway inflammation by itself. Therefore, an important question to be answered is whether PAHs or other components are responsible for the effect seen with MEP. It is interesting to note that none of the MEP-extracted fractions induced an increase in the numbers of macrophages in BALF, as was observed in MEP. This implies that the carbon-black core, but not the chemicals absorbed in MEP, might be important in the induction of macrophage infiltration in the lung.

In conclusion, we have presented evidence showing that the filter-trapped particulate emissions from the unleaded-gasoline-fueled, two-stroke motorcycle engine may induce proinflammatory and proallergic response profiles in the absence of exposure to an allergen.

## ACKNOWLEDGMENTS

This work was supported by a research grant from the National Science Council, Taiwan.

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