

Diesel exhaust particles stimulate human airway epithelial cells to produce cytokines relevant to airway inflammation in vitro

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Background: Epidemiologic and experimental studies suggest that air pollution such as diesel exhaust particles (DEPs), one of the important air pollutants, may play a role in the increasing prevalence of allergic airway diseases.

Objective: We studied the effect of suspended particulate matter (SPM) and its main component, DEPs, on the production of IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) by human airway epithelial cells in vitro.

Methods: SPM obtained from high-volume air samplers and DEPs were added to cultured human nasal polyp-derived upper airway, normal bronchial, and transformed bronchial epithelial cells. Production of GM-CSF and IL-8 by airway epithelial cells was evaluated.

Results: Nontoxic doses of DEPs showed a significant stimulatory effect on IL-8 and GM-CSF production by these three kinds of epithelial cells in a dose- and time-dependent fashion. SPM had a stimulatory effect on GM-CSF, but not IL-8, production. These effects were abrogated by treatment with a protein synthesis inhibitor, cycloheximide, suggesting that the process required a de novo protein synthesis. On the double-chamber plates, airway epithelial cells responded to DEPs only when they were stimulated from the apical sides, which can be a model for in vivo environments. Neither charcoal nor graphite showed such stimulatory effects, indicating that the activity of DEPs did not derive from their particulate nature. Benzo(a)pyrene, one of the main aromatic hydrocarbons contained in DEPs, showed a stimulatory effect on the release of the cytokines, and this organic substance might have a causative effect on the potency of DEPs.

Conclusion: We conclude that SPM and DEPs, its main component, might be important air pollutants in the activation of airway epithelial cells for the release of cytokines relevant to allergic airway inflammation. (*J Allergy Clin Immunol* 1998; 101:778-85.)

Key words: Diesel exhaust particle, suspended particulate matter, cytokine, airway epithelial cell

Abbreviations used

ANOVA:	Analysis of variance
DEP:	Diesel exhaust particle
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
SPM:	Suspended particulate matter

Recent reports suggest that the prevalence rate of allergic disorders such as bronchial asthma and allergic rhinitis are clearly increasing in industrial areas and countries. It has been considered that air pollution influences human health, especially with regard to the respiratory system.¹⁻³ Among air pollutants, suspended particulate matter (SPM) and its main component, diesel exhaust particles (DEPs),⁴ have been demonstrated to have an adjuvant activity for IgE synthesis in mice.⁵⁻⁷ Repeated transtracheal inoculation of DEPs in animal models has been shown to induce bronchial hyperresponsiveness,⁸ which is an important characteristic of the pathophysiology of bronchial asthma. Diaz-Sanchez et al.⁹ demonstrated that transnasal challenges of DEP-derived extract in human beings enhanced local IgE production. They further showed that DEP induced local expression of various cytokines relevant to allergic inflammation.¹⁰ Recently, the same group¹¹ showed that direct exposure to the aromatic hydrocarbons derived from DEPs induced B cells to produce IgE in vitro.

A variety of exogenous agents, including DEPs, first contact airway epithelial cells. Today it is well established that human airway epithelial cells play a crucial role in the activation of inflammatory cells by releasing cytokines such as IL-6, IL-8, and GM-CSF.¹²⁻¹⁵

In this study we investigated the effect of SPM and DEPs on human airway epithelial cells in vitro in terms of GM-CSF and IL-8 production.

METHODS

Preparation of SPM and DEPs

SPM was collected by using a high-volume air sampler (Andersen type, Model AH-600; Sibata Scientific Technology Ltd., Tokyo, Japan). The air sampler was set up at a polluted district in the Tokyo metropolitan area and operated for 6 consecutive days.⁷ The materials collected during February and March 1987 were pooled and stored at 4°C. The principles of the classification of particles in this instrument were reported

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previously.¹⁶ We used SPM with sizes ranging from 7.0 μm to 10 μm .

The engine used for preparation of DEPs was the 4JB1 type, light-duty (2740cc), four-cylinder diesel engine (Isuzu Automobile Company, Tokyo, Japan). The engine was connected to an EDYC dynamometer (Meiden-Sya, Tokyo, Japan) and was operated by using a standard diesel fuel with the speeds of 2000 rpm under the load of 6 torque (kg/m). The exhaust was introduced into a stainless steel dilution tunnel (300 mm ϕ $\times$ 8400 mm). The DEPs were collected on a glass-fiber filter (203 mm $\times$ 254 mm) in a constant-volume sampler system attached to the end of the dilution tunnel. The temperature at the sampling point was below 50° C. The diameter of the particles was measured by an Anderson Air Sampler of the low-pressure type, and the mean diameter was 0.4 μm . Most of the shapes analyzed by a scanning electron microscope were globular. The details on the information of DEPs that we used was previously described.⁸ For some experiments, benzo(a)pyrene (Sigma Chemical Co., St. Louis, Mo.), one of the important components in DEP11, was used. Activated charcoal (HGS-082; Takeda Industry, Tokyo, Japan) and graphite (Japan Graphite Industry, Tokyo, Japan) were suspended in sterile media as described below and used.

Isolation of human airway epithelial cells

We isolated primary human nasal epithelial cells from nasal polyps as described previously.¹⁵ Briefly, nasal polyps obtained from consenting patients with chronic sinusitis were rinsed three times with Ham's F12 medium (GIBCO, Grand Island, N.Y.). They were incubated in 0.1% protease solution (Sigma) in Ham's F12 at 4° C for 16 hours. After incubation, fetal calf serum (heat-inactivated, GIBCO) was added to a final concentration of 10% to neutralize protease activity. The cells were detached from the tissue by gentle agitation, filtered through a 60 μm mesh, and centrifuged at 800 g for 10 minutes. The number of cells was counted by a standard hemocytometer, and the cell viability was assessed by the trypan blue dye exclusion technique.

For the preparation of human bronchial epithelial cells, a piece of macroscopically and microscopically normal human lobar or segmental bronchus (average width, 7 mm) was obtained either at the time of the resection of a lung tumor or at autopsy.¹² The bronchus was rinsed in sterile Hanks' balanced salt solution (GIBCO) without calcium and magnesium and incubated in Ham's F12 medium containing 0.1% protease (Sigma) at 4° C overnight. The bronchus was rinsed with Ham's F12 medium supplemented with 10% fetal calf serum, and the recovered cells were washed twice in Hanks' balanced salt solution. Cell number and viability were evaluated in a similar manner as described. The study was planned according to the ethical guidelines of the declaration of Helsinki and given institutional approval.

Culture of airway epithelial cells

The cells were plated onto collagen-coated, 24-well, flat-bottom tissue culture plates (Koken, Tokyo, Japan) at a density of 5×10^4 cells/well in hormonally defined Ham's F12 medium (HD-F12) as reported.^{12, 15, 17} HD-F12 contained 1% penicillin-streptomycin, 5 $\mu\text{g}/\text{ml}$ insulin (GIBCO), 5 $\mu\text{g}/\text{ml}$ transferrin (GIBCO), 25 ng/ml epidermal growth factor (Collaborative Research Corp., Lexington, Mass.), 15 $\mu\text{g}/\text{ml}$ endothelial cell growth supplement (Collaborative Research Corp.), 2×10^{-10} mol/L triiodothyronine (GIBCO), and 10^{-7} mol/L hydrocortisone (GIBCO). The cells were incubated in a humidified

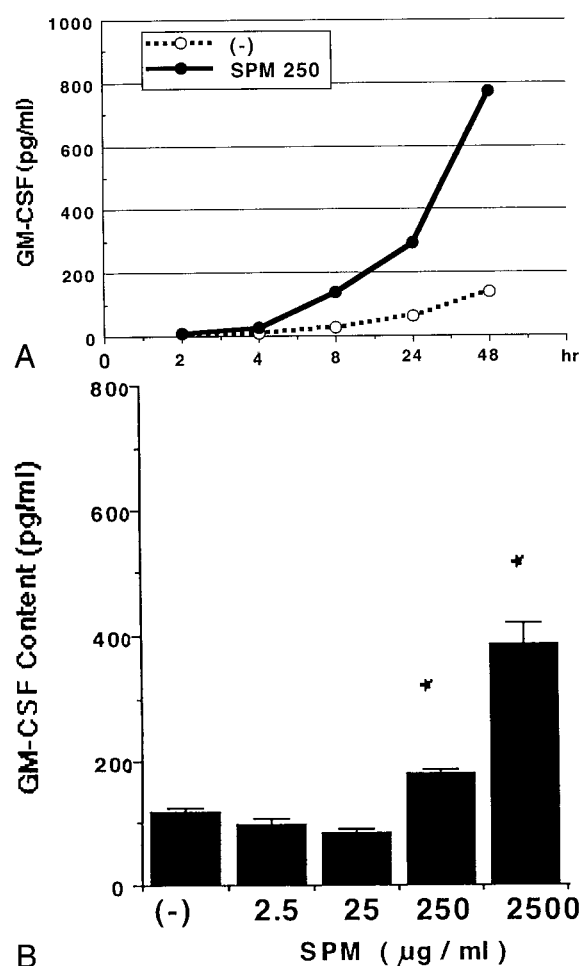


FIG. 1. Effect of SPM on release of GM-CSF by human primary nasal epithelial cells in vitro. Human primary nasal epithelial cells were isolated from resected nasal polyps and cultured in hormonally defined Ham' F12 medium until confluence. **A**, Cells were treated with and without 250 $\mu\text{g}/\text{ml}$ SPM. Supernatants were collected after different time periods, and concentration of immunoreactive GM-CSF was evaluated. There was a time-dependent increase in GM-CSF levels with SPM (250 $\mu\text{g}/\text{ml}$) compared with that of untreated cells. Results were representative among three different experiments. **B**, Different doses of SPM were added to cells, and concentration of GM-CSF was measured after 24 hours. SPM at doses of 250 and 2500 $\mu\text{g}/\text{ml}$ showed a significant stimulatory effect on GM-CSF production. * $p < 0.01$, $n = 3$ (ANOVA).

atmosphere at 37° C and 5% CO_2 . The medium was changed at day 1 and subsequently changed every 2 days. Confluent monolayers of epithelial cells were stained with anti-keratin (KL-1; Immunotech, Marseille, France) anti-vimentin (DAKO-Vimentin; DAKOPatts, Glostrup, Denmark), or control IgG1 monoclonal antibodies by using the avidin-biotin complex method.^{15, 17} In all preparations of primary nasal and bronchial epithelial cells, no less than 98% of the cells were positive to keratin but negative to vimentin, indicating that the cells were of epithelial cell origin as described.^{15, 17} Human bronchial epithelial cell line BEAS-2B¹⁸ (a kind gift from Drs. J. F. Lechner and C. C. Harris, National Cancer Institute, Bethesda, Mass.), was cultured in HD-F12 as reported.¹² Different con-

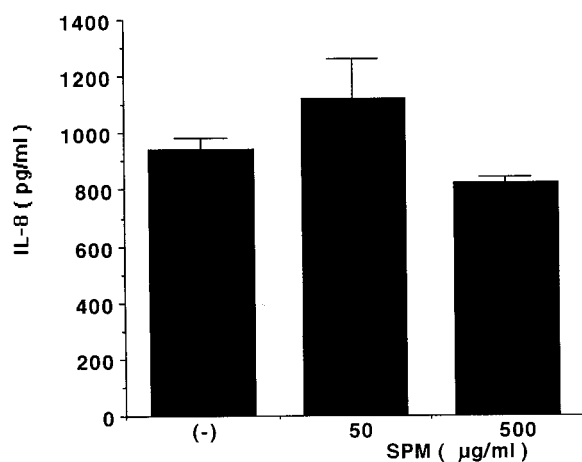


FIG. 2. Effect of SPM on IL-8 by human primary nasal epithelial cells in vitro. Cells were treated with different doses of SPM, and release of IL-8 into media was evaluated after 24 hours. SPM did not show statistically significant effect on IL-8 release.

centrations of SPM or DEPs suspended in the sterile medium were added to the cells, and the supernatants were harvested in different time periods.

In some experiments human bronchial epithelial cell line BEAS-2B was cultured on double-chamber plates to study the directional characteristics of IL-8 release on stimulus of DEPs. Briefly, BEAS-2B cells were plated at a density of 5×10^5 cells/well onto the upper chamber (average pore size, 3 µm in diameter), which was precoated with collagen type-I (Transwell, Corning). On confluence, different concentrations of DEPs or human recombinant IL-1β were added to either side of the plates, and the supernatant from each side was harvested. The samples were stored at -80°C until assay.

Cytokine assay

Specific immunoreactivity for IL-8 and GM-CSF in culture supernatants was measured by ELISA kits (R & D Systems, Inc., Minneapolis, Minn.).^{15,19} Each sample was assayed in duplicates as recommended by the manufacturer.

Statistical analysis

The results were analyzed by Student's *t* test for comparison between the two groups and by nonparametric equivalents of analysis of variance (ANOVA) for multiple comparison as reported.^{12,15,19}

RESULTS

SPM and DEPs stimulated airway epithelial cells to release GM-CSF, IL-8, or both

As shown in Fig. 1, *A*, SPM at 250 µg/ml had a significant stimulatory effect on GM-CSF release from human upper airway epithelial cells in a time-dependent fashion. After various concentrations of SPM were added to the cells, SPM showed a significant stimulatory effect on GM-CSF release at 250 and 2500 µg/ml (Fig. 1, *B*). SPM (250 µg/ml) also showed a significant stimulatory effect on GM-CSF release from human bronchial epithelial cells and their transformed BEAS-2B cells when measured after 24 hours ($152\% \pm 11.2\%$ and

$175\% \pm 23.6\%$, respectively; $n = 5$, $p < 0.01$ compared with baseline = 100%). This effect was not due to cytotoxicity because SPM at the concentrations used showed no effect on cell viability as assessed after 24 and 48 hours by trypan blue dye exclusion technique and LDH release assay (data not shown).²⁰ We measured lipopolysaccharide (LPS) levels in SPM by limulus assay,¹⁷ which showed a 100 ng/ml range, and this dose of LPS showed no significant effect on cytokine release (data not shown). In contrast, SPM had no significant effect on IL-8 production as shown in Fig. 2. Such was also the case in human bronchial epithelial cells and BEAS-2B cells (data not shown).

Evaluation by the trypan blue dye exclusion technique, LDH release assay, and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium (MTT) test demonstrated that DEPs at higher concentrations than 100 µg/ml were toxic to epithelial cells when evaluated after 24 and 48 hours. We studied the effect of DEPs in the range of 0 to 100 µg/ml because previous reports have shown that persons are exposed to a similar dose (0.2 mg) in a period of 1 day in Los Angeles.²¹ At nontoxic concentrations, DEPs showed a time- and dose-dependent stimulatory effect on GM-CSF release by human nasal epithelial cells (Fig. 3, *A* and *B*). DEPs also stimulated human primary bronchial epithelial cells (Fig. 3, *C*) and BEAS-2B cells at 50 µg/ml ($175\% \pm 34.5\%$ compared with baseline = 100%, $n = 5$, $p < 0.01$) to release GM-CSF when evaluated after 24 hours. DEPs also showed a significant stimulatory effect on IL-8 release from human nasal as well as bronchial epithelial cell line BEAS-2B (Fig. 4, *A* and *B*). IL-8 release by primary bronchial epithelial cells was also stimulated by DEPs at 50 µg/ml ($156\% \pm 22.7\%$ compared with control, $n = 3$). Addition of the protein synthesis inhibitor cycloheximide significantly blocked the increase in IL-8 and GM-CSF release by nasal and bronchial epithelial cells, suggesting a *de novo* synthesis of these cytokines by epithelial cells (Figs. 3 *B* and *C* and 4, *A* and *B*).

Nonhydrocarbon-containing particles showed no effect on cytokine release from airway epithelial cells

To determine whether the activity of DEPs shown was due to its particulate form in the preparation, we studied the activity of activated charcoal and graphite (average size, 0.7 µm) on IL-8 release from BEAS-2B cells. Neither charcoal nor graphite showed a statistically significant effect on IL-8 release as shown in Fig. 4, *C*.

Prototype aromatic hydrocarbon benzo(a)pyrene showed a stimulatory effect on GM-CSF release by BEAS-2B cells

We further evaluated the potency of benzo(a)pyrene, a prototype aromatic hydrocarbon contained in DEPs at noncytotoxic concentrations (assessed by MTT assay, 2.0 to 40 µg/ml). As shown in Fig. 5, benzo(a)pyrene (2.0 to 20 µg/ml) showed a dose-dependent stimulatory effect on GM-CSF release after 24 hours.

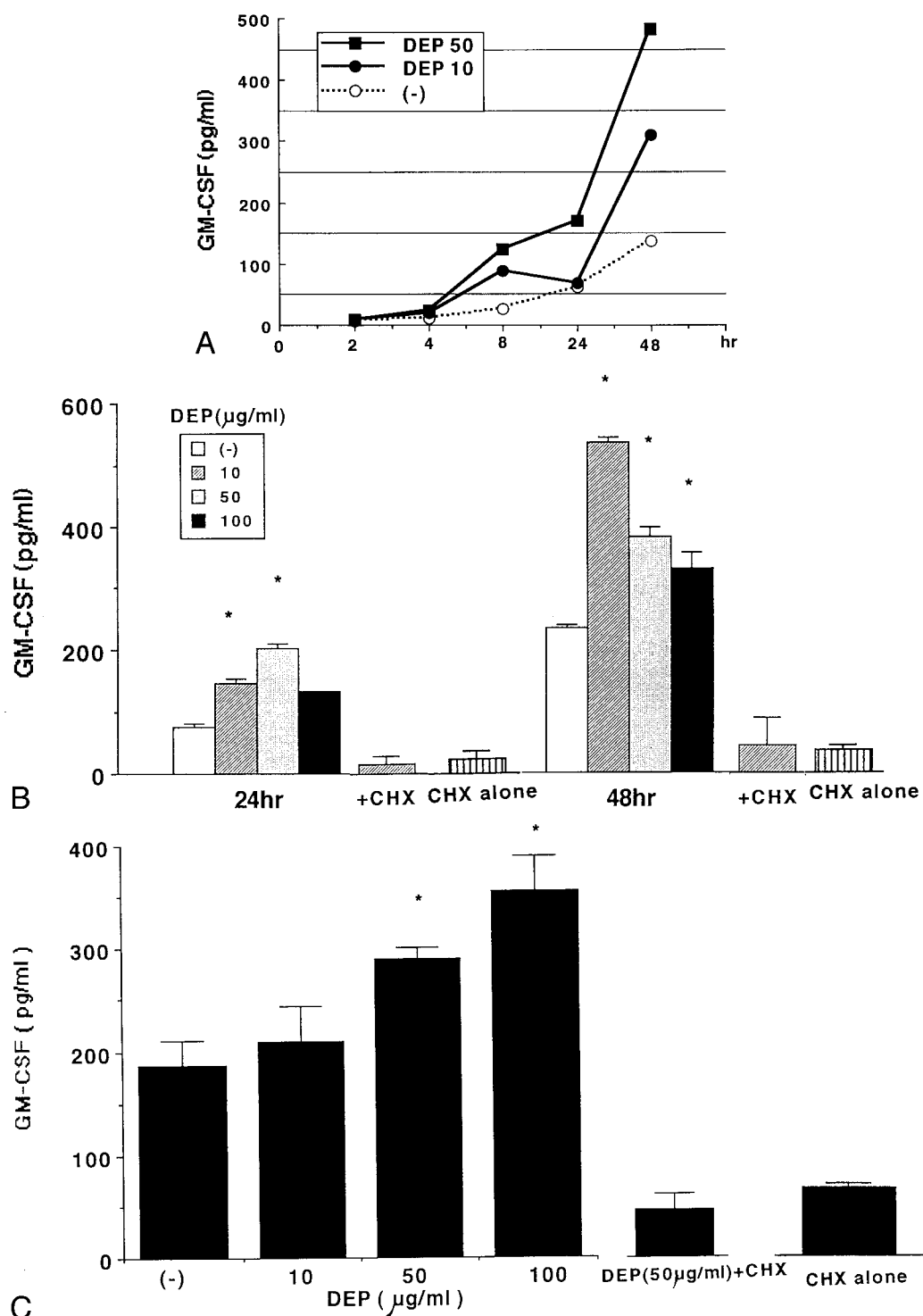


FIG. 3. Effect of DEPs on production of GM-CSF by human airway epithelial cells. **A**, On confluence, the primary nasal epithelial cells were treated with DEPs (10 and 50 $\mu\text{g/ml}$), and supernatants were harvested after different time periods. DEPs showed a time-dependent stimulatory effect of GM-CSF production. Results were representative among three different experiments. **B**, Dose-dependency of DEPs on GM-CSF release from nasal epithelial cells. DEPs (10 to 50 $\mu\text{g/ml}$) showed a significant stimulatory effect on GM-CSF release after 24 and 48 hours. $*p < 0.01$, $n = 3$ (ANOVA). **C**, DEPs showed a stimulatory effect on GM-CSF release from primary bronchial epithelial cells after 24 hours. $*p < 0.01$, $n = 3$ (ANOVA). Addition of protein synthesis inhibitor cycloheximide (CHX) (10 $\mu\text{g/ml}$) blocked increase in GM-CSF levels stimulated by DEPs, showing a de novo protein synthesis process as in **B** and **C**.

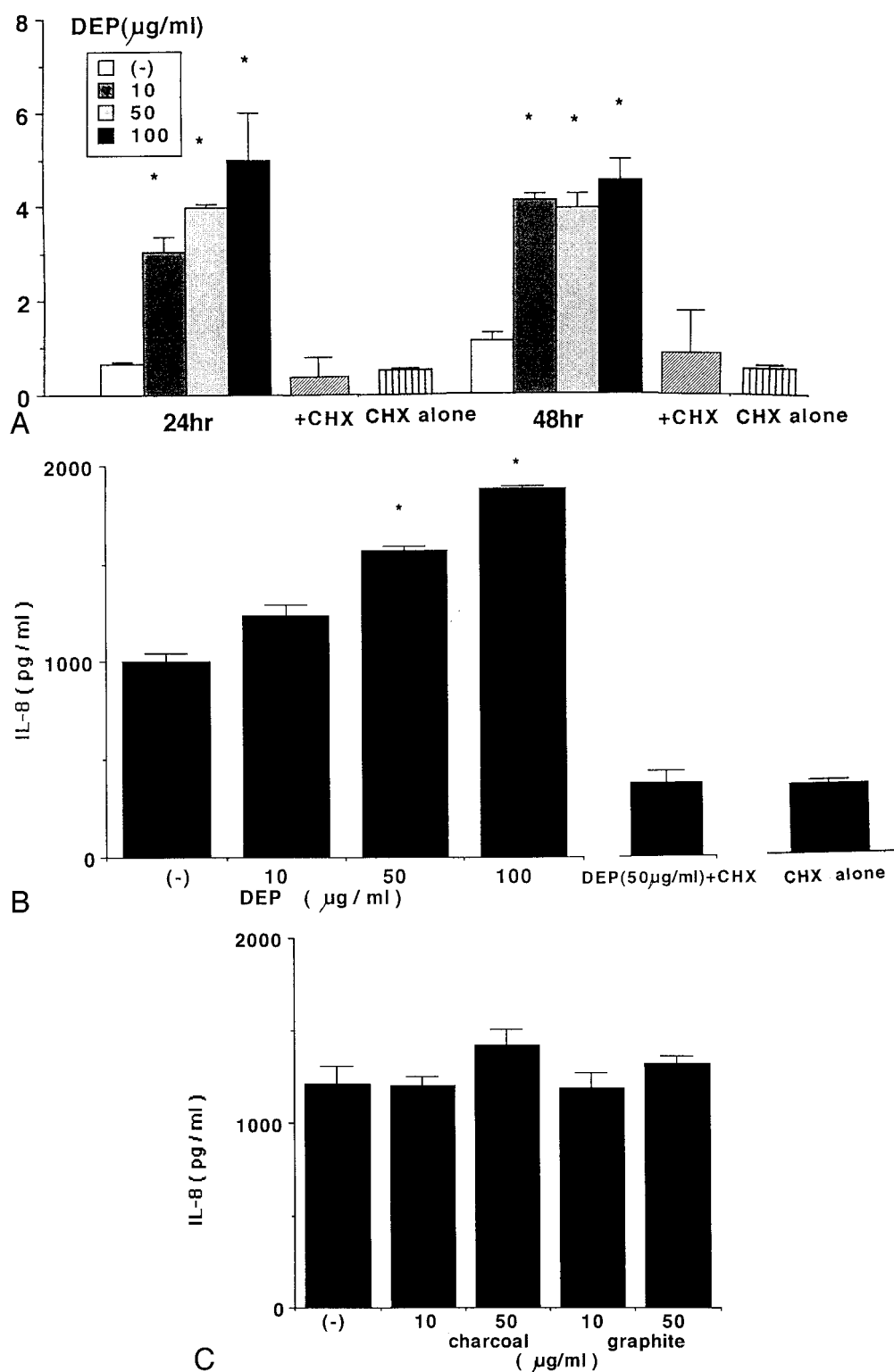


FIG. 4. Effect of DEPs on IL-8 release by human airway epithelial cells. **A**, DEPs showed stimulatory effect on IL-8 release by primary nasal epithelial cells in dose-dependent fashion after 24 and 48 hours. * $p < 0.01$, $n = 3$ (ANOVA). **B**, DEPs also showed stimulation on BEAS-2B cells after 24 hours. Addition of protein synthesis inhibitor cycloheximide (CHX) (10 $\mu\text{g/ml}$) blocked the increase in IL-8 levels stimulated by DEPs, showing de novo protein synthesis process. **C**, Effect of charcoal and graphite on the IL-8 release from BEAS-2B cells. Neither charcoal nor graphite showed significant effect on IL-8 release (24 hours) at noncytotoxic concentrations (10 and 50 $\mu\text{g/ml}$).

Directional characteristics of IL-8 release by airway epithelial cells on double-chamber plates

To evaluate the vectorial potential of the airway epithelial cells to release cytokines in response to DEPs, the BEAS-2B cells were plated onto the upper side of the double-chamber plates. On confluence, human recombinant IL-1 β (5 ng/ml) or DEPs (50 μ g/ml) were added to either side of the plates, and after 24 hours the supernatant was obtained from each side of the chamber. The amount of immunoreactive IL-8 was evaluated by ELISA and expressed as picograms per well. As shown in Fig. 6, A, IL-1 β significantly stimulated BEAS-2B cells to release IL-8 when this proinflammatory cytokine was added from either the apical or basal side of the plates.

In contrast, the epithelial cells responded to DEPs only when they were stimulated from the apical side of the chamber (Fig. 6, B). The IL-8 release by BEAS-2B cells was increased in both directions by apical treatment with DEPs. This was also the case when human bronchial epithelial cells in primary culture were used in this system (data not shown). We studied the effect of DEPs on plates with epithelial cells plated on the reverse side. In this case DEPs were added to the basolateral sides of the cells. The results showed that DEPs did not show any significant effect (percent of IL-8 release: 109% $\pm$ 21.3% and 111% $\pm$ 43.1% in the lower and upper chambers, respectively; $p > 0.05$, baseline release = 100%).

The BEAS-2B cells responded to benzo(a)pyrene (2 μ g/ml) only when it was added to the upper side of the plates (IL-8 release in lower side of the plates after 24 hours: 165% $\pm$ 43.7%, $p < 0.05$; when added to the upper side, 109% $\pm$ 54.8%; basal release when added to the lower side = 100%; $n = 3$).

DISCUSSION

In this report we found that DEPs, one of the important air pollutants, stimulated cytokine production by human airway epithelial cells (Table I). The activity was not due to cytotoxicity, and it required a de novo protein synthesis as suggested by the experiment with cycloheximide. The two kinds of other particulate matter, charcoal and graphite, showed no significant effect on the production of IL-8 by airway epithelial cells, suggesting that the activity of DEPs was not merely a result of their particulate nature. We further studied the potential of benzo(a)pyrene, one of the important aromatic hydrocarbons included in DEPs, and found it activated airway epithelial cells to release cytokines in vitro. These findings suggested that DEP-derived substance or substances, especially aromatic hydrocarbons, were responsible for its activity on airway epithelial cells.

Epidemiologic studies have suggested that there may be a link between the incidence of allergic disease and the presence of air pollutants, including hydrocarbons, nitrogen dioxide (NO₂), ozone (O₃), SPM, and DEPs.²² SPM consisted mostly of exhaust particles and soil dust in urban districts in Japan,²³ and it has been reported that more than 90% of the exhaust particles were

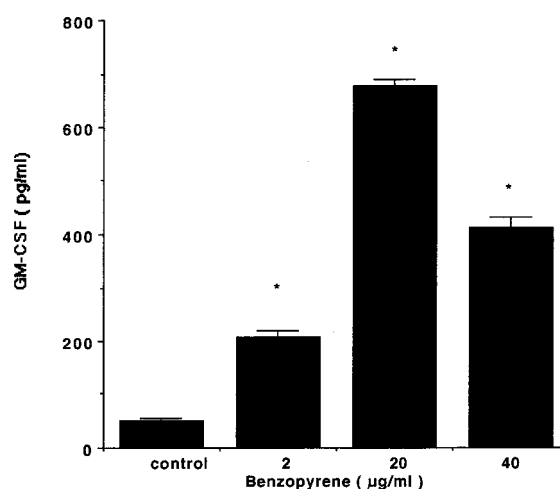


FIG. 5. Effect of benzo(a)pyrene on GM-CSF release from BEAS-2B cells. Prototype aromatic hydrocarbon benzo(a)pyrene had significant stimulatory effect on GM-CSF release from bronchial epithelial cells in dose-dependent fashion after 24 hours in vitro. * $p < 0.01$ compared with controls, $n = 3$ (ANOVA).

emitted from diesel vehicles in Japan.²⁴ DEPs have been demonstrated to have an adjuvant activity for IgE synthesis in vivo.^{5,6} Transnasal administration of DEPs or SPM in mice resulted in the increased IgE synthesis specific to the antigen.^{6,7} It has been speculated that DEPs might enhance IgE responses through their particulate nature. However, Takenaka et al.¹¹ recently demonstrated that aromatic hydrocarbons contained in DEPs have a direct biochemical effect on B cells to produce IgE in vitro. Diaz-Sanchez et al.⁹ indicated that transnasal challenges of DEP-derived extract in human beings enhanced local IgE production. They further showed that DEPs induced local expression of various cytokines relevant to allergic inflammation, but in vitro exposure of DEPs on lymphocytes failed to stimulate cytokine production.¹⁰ We therefore speculated on the possibility that SPM, DEPs, or both may have a direct effect on other types of cells that can contact air pollutants and increase the incidence of upper, as well as lower, respiratory diseases. Airway epithelial cells are the cells that first contact any noxious agents, including air pollutants, tobacco smoke, and bacterial and viral particles. These cells are not only the mechanical barriers, but also the potent modulators of immune and inflammatory responses in airway defense mechanisms. They are capable of expressing and releasing a variety of cytokines relevant to allergic airway inflammation such as IL-6,¹² IL-8,¹⁴ GM-CSF,¹⁵ and RANTES.²⁵ IL-8 is a potent chemotactic factor for eosinophils,²⁶ basophils,²⁷ and T lymphocytes,²⁸ as well as neutrophils.²⁹ Recent studies have shown that this C-X-C type chemokine plays an important role in eosinophil transmigration through the endothelium and epithelium.³⁰ Human recombinant IL-8 induced eosinophil infiltration in the airways and airway hyperresponsiveness in guinea pigs.³¹

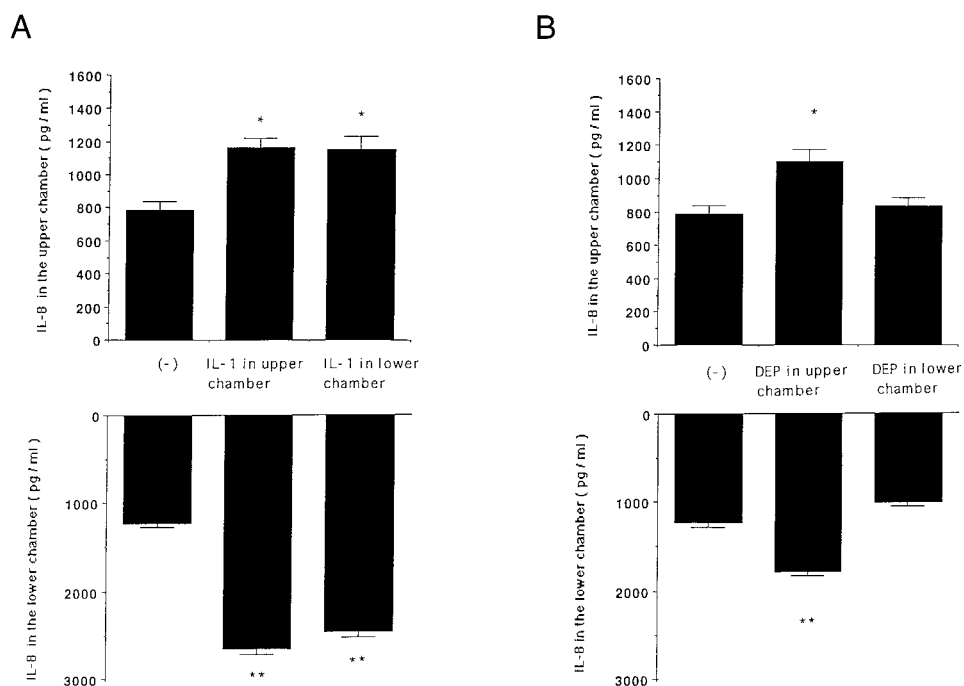


FIG. 6. Directional characteristics of IL-8 release by human bronchial epithelial cell line BEAS-2B on stimulus of IL-1 β and DEPs. Transformed human bronchial epithelial BEAS-2B cells were plated on upper side of double-chamber plate and cultured until confluence. **A**, Human recombinant IL-1 β (5 ng/ml), a potent stimulatory agent on cytokine production by epithelial cells, showed an upregulation of IL-8 production to both sides of chambers when it was added to upper and lower chambers. * $p < 0.01$ to control of upper-side release, ** $p < 0.01$ to control of lower-side release. **B**, BEAS-2B cells were cultured, and DEPs (10 μ g/ml) were added to either side of plate. DEPs showed significant stimulatory effect only when added to upper side of plate. * $p < 0.01$ to control of upper-side release, ** $p < 0.01$ to control of lower-side release.

TABLE I. Summary of the effect of SPM and DEPs on human airway epithelial cells

Pollutants	Epithelial cells	GM-CSF production	IL-8 production
SPM	Nasal	↑	→
	Bronchial	↑	→
	BEAS-2B	↑	→
DEPs	Nasal	↑	↑
	Bronchial	↑	↑
	BEAS-2B	↑	↑
Benzo(a)pyrene	BEAS-2B	↑	↑

GM-CSF is a crucial cytokine in the processes of maturing stages of granulocytes and macrophages. It also plays an important role in the maturation of eosinophils in the bone marrow. Recent studies further demonstrated that this cytokine induces prolongation of eosinophil survival in the local environments.³² Therefore DEPs might be involved in the activation of airway epithelial cells to release cytokines and thereby play an important role in the initiation and prolongation of allergic inflammation. Devalia et al. showed that potent air pollutants NO₂³³ and ozone³⁴ induced bronchial epithelial cells to pro-

duce inflammatory cytokines in vitro. Our findings added evidence that other important air pollutants (i.e., SPM and DEPs) had a potential to enhance production of cytokines important in allergic airway responses.

It remains to be elucidated how DEPs exert their effect on immune and inflammatory cells in vitro. Takenaka et al.¹¹ studied the activity of DEP-derived aromatic hydrocarbons, which were extracted in dichloromethane. We studied the effects of DEPs themselves directly because it was probable that these particulate matters, when inhaled, contact airway epithelial cells in vivo. We also compared the potency of charcoal and graphite to clarify whether the activity of DEPs was due to their particulate nature. The results showed that neither charcoal nor graphite had a significant effect on cytokine release from airway epithelial cells in nontoxic ranges. Benzo(a)pyrene is one of the prototypes of aromatic hydrocarbons contained in DEPs. Therefore we studied the activity of benzopyrene at nontoxic concentrations. It significantly activated airway epithelial cells to release GM-CSF. The enhancing effect of DEPs on cytokine release shown in this report therefore adds additional evidence that DEPs might interact with human cells.

Because airway epithelial cells have topographic characters, the vectorial characteristics of cytokine release is

important in the understanding of the mechanism of cell recruitment into the airways. In the double-chamber experiments, DEPs stimulated the epithelial cells only when they were added to the upper chamber (i.e., the apical side of the cell layer), whereas IL-1 stimulated the epithelial cells from either side of the plates. Preliminary studies with murine recombinant IL-1 showed that even after 24 hours, the murine cytokine was detected only in the added side of the plates when the epithelial cells reached confluence (unpublished data). Therefore it was unlikely that the directional characteristics were due to only its equilibration. Further studies would be necessary to clarify the mechanism of how DEPs affect the inflammatory responses in the airway mucosa. Investigation of the responsible substance or substances for the bioactivity of DEPs would be another issue of importance for the better environmental control of diesel-derived products.

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