

Effects of diesel organic extracts on chemokine production by peripheral blood mononuclear cells

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Background: Polyaromatic hydrocarbons (PAHs) associated with diesel exhaust particles (DEPs) are found in the atmospheric urban pollution. Such compounds have been shown to favor IgE production, bronchial hyperresponsiveness, and airway inflammation. Chemokines are a group of chemotactic cytokines involved in the recruitment of inflammatory cells.

Objective: We investigated the effect of DEP-PAHs on the release and mRNA expression of IL-8, MCP-1, and RANTES by PBMCs obtained from healthy subjects.

Methods: Protein production in supernatants was assessed by ELISA, and mRNA expression was evaluated by semiquantitative RT-PCR.

Results: Secretion of IL-8 and RANTES increased in a dose-dependent manner with increasing concentrations of DEP-PAHs (range, 0.5 ng to 50 ng/mL). On the contrary, the release of MCP-1 was significantly inhibited, also in a dose-dependent manner. Messenger RNA production coding for IL-8, RANTES, and MCP-1 showed parallel variations to the production of the correspondent proteins. Effects of DEP-PAHs became significant at 7 hours and up to 48 hours time culture for MCP-1, and up to 24 hours time culture for IL-8 and RANTES. Moreover, supernatants from DEP-PAH-activated cells, compared with those of controls, exhibited a significantly enhanced chemotactic activity for neutrophils and eosinophils, which was significantly inhibited by pretreatment with anti-IL-8 and anti-RANTES neutralizing antibodies, respectively.

Conclusion: These findings suggest that the chemokine pathways are modulated by DEP-PAHs at the transcriptional level, reinforcing the idea that the development of inflammatory reactions might be affected by diesel exhaust emission. (*J Allergy Clin Immunol* 1999;103:1115-24.)

Key words: Diesel, chemokines, PBMC, inflammation, air pollution

A dramatic increase in the prevalence of allergic airway diseases such as asthma and allergic rhinitis has been observed during the past century, at least in the industrialized countries for which data are available. As stated in many epidemiologic studies, one commonly

Abbreviations used

AhR:	Aryl hydrocarbon receptor
DEP-PAH:	Diesel exhaust particles–polyaromatic hydrocarbons
GAPDH:	Human glyceraldehyde phosphate dehydrogenase
HPF:	High-power field
RPMI:	Roswell Park Memorial Institute medium

proposed reason for this increase is the constant degradation of air quality, resulting in part from human industrial activity.¹ A large part of airborne particulate pollution encountered in urban air originates from diesel-powered engines. This statement is even more accurate in countries such as France and Japan, where the number of diesel cars in use has increased rapidly during the past 3 decades.² Although a direct relationship between diesel exhaust and exacerbation of pre-existing asthma has been studied extensively, the results are still extensively debated in epidemiologic studies.³ Asthma is characterized by a chronic inflammation of the bronchi. The two hallmarks are a production of IgE antibodies specific for the allergen that activate effector cells, such as mast cells, and the expression of a preferential type-2 cytokine profile (namely IL-4 and IL-5)⁴ that is in part responsible for an enhancement of the IgE production and for the recruitment and activation of eosinophils involved in the respiratory epithelium damage.^{5,6}

Recent studies have shown in vitro and in vivo evidences of the involvement of diesel exhaust particles–polyaromatic hydrocarbons (DEP-PAHs) in the modulation of the inflammatory reaction. In vitro studies on PBMCs have shown that DEP-PAHs can increase the amount of total IgE produced by already commuted B cells.^{7,8} In vivo, nasal challenges with DEPs in mice and humans have demonstrated an adjuvant activity on specific and total IgE production, respectively.^{9,10} DEPs are also able to potentiate cytokine mRNA expression in the human upper respiratory mucosa, enhancing the inflammation process by means of IgE production.¹¹ Beside regulatory cells, DEP-PAHs have been also shown to act directly on many effector cells such as neutrophils, mast cells, and eosinophils¹²⁻¹⁴ by indirectly recruiting them and by triggering the release of pro-inflammatory cytokines and other specific mediators. Finally, not only leukocytes but also epithelial cells have been shown to be affected by DEPs, again through the release of pro-inflammatory mediators.¹⁵ These

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Supported by a grant from ADEME and Institut Pasteur de Lille and by PRIMEQUAL-PREDIT grant No. 97034 from Ministère de l'Environnement.

Received for publication Dec 31, 1998; revised Feb 15, 1999; accepted for publication Feb 16, 1999.

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0091-6749/99 \$8.00 + 0 1/1/98189

results highlight the fact that DEPs and associated DEP-PAHs can trigger or affect many cells involved in the inflammatory reaction.

In the process of the inflammatory reaction, one important step is represented by the local attraction of the cells, in particular through the release of gradients of chemokines. Chemokines are a group of chemotactic cytokines of small molecular weight (8 to 9 kd) mainly devoted to the regulation of adhesion, transendothelial migration, and chemotactic movements of inflammatory cells toward an inflammatory site. Chemokines are classified into subfamilies according to their amino acid residues in a conserved N-terminal sequence. The two best described subfamilies are the CXC-type and the CC-type chemokines.¹⁶ IL-8, a CXC-type chemokine, is mainly active on neutrophils but is also active on lymphocytes, basophils, and eosinophils. Besides having a strong chemotactic effect, IL-8 has been shown to enhance neutrophil adhesion to endothelial cells and allow the release of lysosomal enzymes. MCP-1 and RANTES are two CC-type chemokines. MCP-1 is the strongest chemotactic and activating factor for monocytes and is also active on lymphocytes. Produced by stimulated mononuclear cells and monocytes, MCP-1 has a potent chemotactic and histamine-releasing activity on basophils.¹⁷ RANTES has a chemotactic activity on memory T lymphocytes and monocytes, as well as on eosinophils, and can induce degranulation of the latter.¹⁸ In addition, RANTES can enhance IgE production by human B cells.¹⁹ It appears that besides their well-known chemotactic activity for various cellular types, IL-8, RANTES, and MCP-1 are also directly involved in the activation of the actors of the allergic inflammatory reaction.

The aim of this study was to investigate whether DEP-PAHs could affect the early step of the inflammatory reaction by interfering with chemokine synthesis. To achieve this goal we studied the kinetics of production of the chemokines IL-8, RANTES, and MCP-1 by normal PBMCs under various concentrations of DEP-PAHs.

METHODS

Generation of diesel exhaust

DEPs were obtained from a light-duty diesel-powered passenger car (Renault, pollution department, Boulogne Billancourt) used under the standard European Community cycle, representing the typical conditions encountered while driving in an urban setting and during short trips in a nonurban setting. DEPs were impacted on a Pallflex filter in a cyclone equipped with a dilution tunnel. After 3 cycles, the filter was removed from the tunnel and immediately used for organic extraction of the DEP-PAHs.

Extraction of DEP-PAHs

DEP extract is an organic extract of PAH. To prepare DEP-PAHs, a high-capacity filter was extracted in dichloromethane (CH_2Cl_2) using a Soxhlet apparatus (Foss Tecator, Höganäs, Sweden). The resulting extract was then analyzed by HPLC to determine the concentration of its main organic constituents and stored at +4°C in the dark as a stock solution of 25 μg DEP-PAH/mL CH_2Cl_2 . The DEP-PAH solution was diluted freshly each time in

complete Roswell Park Memorial Institute (RPMI) medium for use.

Donors

Venous blood was obtained from healthy nonatopic subjects. None of the subjects had a history of allergic diseases. Results of skin prick tests and radioallergosorbent tests to common aeroallergens were negative for all subjects. Total IgE concentrations were below 150 IU/mL.

Cell culture

Venous blood from nonallergic donors was collected on heparin. Platelet-rich plasma was obtained by centrifugation (120g, 10 minutes) and discarded. The blood was diluted with an equal volume of RPMI 1640 (Sigma, St Louis, Mo) and layered over a Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden). After centrifugation (400g, 30 minutes), PBMCs were harvested, washed twice in RPMI 1640, and finally resuspended in complete RPMI (RPMI 1640 supplemented with 2 mmol/L glutamine, 100 IU/mL penicillin, 100 μg /mL streptomycin, and 10% FCS) and incubated at 37°C in 5% CO_2 . PBMCs (2×10^6 cells/well) were incubated in 12-well flat-bottomed microculture plates (Nunc-Roskilde, Denmark). PBMC contamination by granulocytes represented less than 1.5%. Preliminary experiments were performed at 2, 4, 7, 18, 24, and 48 hours with DEP-PAH concentrations of 0.5, 5, 25, and 50 ng/mL ($n = 3$). The following experiments were performed at 7, 18, 24, and 48 hours in the presence of DEP-PAH concentrations of 5 ng/mL and 50 ng/mL, CH_2Cl_2 , or medium alone.

Culture supernatants were collected, filtered through a 0.2 μm -pore-size filter (Sartorius AG, Göttingen, Germany), aliquoted, and stored at -20°C for further chemokine quantifications.

Cell viability

A trypan blue exclusion test was used to determine the effect of DEP-PAHs on PBMC viability. After removal of the culture supernatants, the cells were resuspended in a small volume of RPMI 1640, stained with trypan blue, and counted. Viability was calculated as follows: (alive cells/(alive cells + dead cells))•100(%).

Quantitation of chemokine levels in cell culture supernatants

Commercially available ELISA kits were used to determine the concentrations of the chemokines MCP-1, RANTES (R&D Systems, Abingdon, United Kingdom), and IL-8 (CLB, Amsterdam, Netherlands), according to the manufacturer's recommendations. The limits of detection were 15.6 pg/mL, 15.6 pg/mL, and 1.95 pg/mL, respectively.

Semiquantitative RT-PCR reaction

After removal of the supernatants, PBMCs were resuspended in Trizol reagent (Gibco BRL Life Technologies, Gaithersburg, Md), and total cellular RNA was extracted according to the manufacturer's procedure. RNA concentrations were measured with use of a spectrophotometer. RNA integrity was determined by visualizing the 18S and 28S ribosomal RNA bands with ethidium bromide after electrophoresis on a 2% formaldehyde gel. To synthesize cDNA, 1 μg of RNA was resuspended in a volume of 25 μL with 0.2 μg oligo(dT)₁₂₋₁₈ and 8 IU Rnasin. After annealing for 5 minutes at 70°C, the solution was cooled to 4°C. First Strand buffer (50 mmol/L Tris-HCl, 75 mmol/L KCl, 3 mmol/L MgCl_2 pH 8.3), 2 mmol/L of each dNTP (Pharmacia Biotech, Uppsala, Sweden), 8 IU Rnasin ribonuclease inhibitor (Promega, France), 40 μg /mL carrier tRNA, 4 mmol/L DTT (Gibco BRL), and 400 IU MMLV reverse transcriptase (Gibco BRL) were then added in a final volume of 50 μL . The reaction mixture was heated 1 hour at 42°C to synthesize

the complementary DNA strand, and the reaction was stopped by denaturing the enzyme at 95°C for 5 minutes.

The PCR reactions were carried out in a volume of 25 μ L and covered with oil. Ten μ L of each cDNA that was diluted 20 times was supplemented with 100 μ mol/L of dATP, dCTP, dGTP, 95 μ mol/L of dTTP, and 10 μ mol/L of digoxigenin-11-dUTP (Boehringer, Mannheim, Germany), 0.5 μ mol/L of each primer, 0.6U Platinum Taq DNA polymerase (Gibco BRL), and 1 mmol/L $MgCl_2$. PCR primers for human glyceraldehyde phosphate dehydrogenase (GAPDH), MCP-1, RANTES, and IL-8 were purchased from Eurogentec, Belgium. The sequences of the primers were as follows: GAPDH sense: 5'-GTCTTCACCACTGAG-3'; anti-sense: 5'-CCAAAGTTGTCA TGGATGACC-3'. MCP-1 sense: 5'-TCCAGCATGAAAGTCTCTGC-3'; anti-sense: 5'-TGG AATCCTGAACCCACTTC-3'. RANTES sense: 5'-TCCCATATTCCTCGGAC-3'; anti-sense: 5'-GATCTACTCCGAAGCCA-3'. IL-8 sense: 5'-TTGGCAGCCTTCCTCTGATT-3'; anti-sense: 5'-AACTTCTCCACAACCCTCTG-3'.

The number of amplification cycles for each chemokine was determined to define optimal conditions for linearity and to permit semiquantitative analysis of signal strength. Twenty-one cycles were performed for amplification of GAPDH gene, 22 for MCP-1 gene, and 25 for RANTES and IL-8 genes. Amplification was initiated by a 1-minute denaturation step at 94°C and was then followed by 21 to 25 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, followed by 7-minute extension at 72°C using a DNA thermal cycler (Mastercycler 5330, Eppendorf). Amplified PCR products were separated by gel electrophoresis in 1.5% agarose after ethidium bromide staining. DNA molecular weight marker VI DIG-labeled (0.15 to 2.1 kb) was purchased from Boehringer. The gel was then blotted overnight on a positively charged nylon membrane (Boehringer) by capillary action, and amplified products were detected using a digoxigenin nucleic acid detection kit (Boehringer). The intensity of each spot was calculated by densitometry analysis, and results were expressed as a percentage of each corresponding GAPDH housekeeping gene's optical density (Gel Analyst, Clara Vision). Specificity of the amplified products was ascertained using specific oligonucleotides probes as previously described.²⁰

Chemotaxis assay

Human neutrophils were obtained from nonallergic donors, purified using Boyüm's technique,²¹ and resuspended in complete RPMI medium at a concentration of 1.10^6 cells/mL. The purity of the preparation was more than 98% for all experiments. Supernatants from PBMCs cultured with CH_2Cl_2 or various DEP-PAH concentrations were assayed after a two times dilution. The assay was performed in a 48-well micro chemotaxis chamber (Neuro Probe, Cabin John, Md) using 3 μ m pore polycarbonate filters (Nucleopore Corp, Pleasanton, Calif) during 30 minutes at 37°C in 5% CO_2 . Neutrophils that had migrated through the filter were counted under a microscope with a magnification of 500-fold.

Human eosinophils obtained by Boyüm's method from peripheral blood of hypereosinophilic patients were purified using magnetic beads coated with anti-CD16 (MACS, Miltenyi Biotech, Bergisch Gladbach, Germany) as previously described.²² The negative immunomagnetic fraction of highly purified eosinophils (95.4 $\pm$ 1.6% as determined by microscopic examination of Giemsa-stained cytospin preparations; contaminating cells corresponding to 2.4 $\pm$ 1.4% lymphocytes and 2.2 $\pm$ 1.8% monocytes) was harvested and resuspended in complete RPMI at a concentration of 1.10^6 cells/mL. The chemotaxis protocol was the same as that previously described, except that the incubation of the chamber lasted 1 hour and 5 μ m pore polycarbonate filters were used.

Each condition was performed in triplicate, and at least 4 fields were counted for each well. The results were expressed as the sub-

traction of the mean number of migrating neutrophils or eosinophils with complete RPMI medium alone (negative control) from the mean number of migrating neutrophils or eosinophils with the samples or the positive controls: 25 ng/mL of recombinant human IL-8 (Endogen, Boston, Massachusetts) and a 10^{-7} mmol/L solution of recombinant human RANTES (PeproTech, Rocky Hill, NJ), respectively.

For neutralization assays, samples and controls were incubated 1 hour at 37°C with rabbit anti-IL-8 and anti-RANTES neutralizing antibodies (Endogen and R&D Systems, respectively) at a final concentration of 50 μ g/mL before the chemotaxis assay.

To distinguish chemotaxis from chemokinesis, neutrophil movements were compared under the following experimental conditions (solution in top chamber/solution in bottom chamber): control/control, control/test, test/control, and test/test. Test solutions used were those that induced maximal neutrophil migration in the standard chemotaxis assay (ie, supernatants from PBMC incubated with 50 ng DEP-PAHs).

Statistical analysis

Nonparametric statistical analyses of IL-8, MCP-1, and RANTES in culture supernatants were performed with Wilcoxon's paired rank test. Values of $P < .05$ were regarded as statistically significant. If not specified otherwise, all results were expressed as median and interquartile (Q1 and Q3) values. Statistical analysis was performed using software (Statview 4.11, Abacus Concepts, Berkeley, Calif) on a Macintosh computer.

RESULTS

PBMC viability

The viability of PBMCs cultured in complete RPMI medium and incubated with various concentrations of DEP-PAHs, CH_2Cl_2 , or medium alone during 7 to 48 hours time culture was first examined. The volume of CH_2Cl_2 or DEP-PAHs solubilized in dichloromethane added to 1 mL medium was limited to 2 μ L. The toxic effect on PBMCs was found to be nonexistent over 24-hour culture (viability >97% for all conditions) and remained very low even after 48 hours (viability for CH_2Cl_2 : 95.3 $\pm$ 1.7%; 5 ng DEP-PAHs: 94.7 $\pm$ 2%; 50 ng DEP-PAHs: 93.4 $\pm$ 1.4%). On the contrary, increasing volumes of the CH_2Cl_2 solvent was found to induce cell mortality. Indeed, after 24-hour culture, CH_2Cl_2 volumes of 2, 5, and 10 μ L resulted in a cell viability of 98%, 96%, and 75%, respectively (data not shown).

Chemokine concentrations in supernatants

At all time points, a control with medium only was performed that showed no significant difference with the negative CH_2Cl_2 control. Preliminary kinetics and dose-response experiments were performed to determine the optimal time points of action and doses of DEP-PAHs on the release of IL-8, RANTES, and MCP-1 by PBMCs. When a panel of DEP-PAH concentrations ranging from 0.5 to 50 ng/mL was used in the cell cultures, a dose-dependent response clearly could be observed for all 3 chemokines, the strongest effect being observed with the 50 ng DEP-PAHs dose. This dose-response was obtained at all time points from a 2-hour to 24-hour incubation for IL-8 and RANTES, and from a 7-hour to 48-hour culture for MCP-1 (Fig 1). For time points before 7 hours (name-

ly 2-hour and 4-hour incubation times), no significant difference between cultures exposed to DEP-PAHs and controls was observed. Baseline levels of RANTES decreased progressively with time, whereas IL-8 and MCP-1 levels tended to increase with incubation time. Following the kinetics results, the two concentrations of 5 ng/mL and 50 ng/mL DEP-PAHs were chosen, and experiments were conducted on PBMCs with incubation times of 7 hours, 18 hours, 24 hours, and 48 hours.

DEP-PAHs enhanced the release of IL-8 and RANTES. ELISA analysis of the culture supernatants demonstrated an increase in IL-8 and RANTES production when PBMCs were exposed to DEP-PAHs compared with the CH₂Cl₂ solvent control (Table 1). For both chemokines, this increase was statistically significant at the DEP-PAH concentration of 50 ng/mL and already after 7 hours' incubation ($P < .05$). It remained significant up to 24 hours' incubation ($P < .01$). At 48 hours, the average level of IL-8 in cell cultures exposed to DEP-PAH still appeared higher than did the concentrations found in controls, but the difference was not significant. The lower dose of 5 ng/mL DEP-PAHs had very little or no effect on the production of both IL-8 and RANTES. The effect of the 50-ng dose on RANTES (+58.6% maximum increase at 7 hours compared with the control) was comparatively less than that on IL-8 (+137.6% maximum increase at 24 hours compared with the control). Individual data for the 24-hour time point are shown in Fig 2.

DEP-PAHs decreased the release of MCP-1. Analysis of MCP-1 in cell supernatants showed that DEP-PAHs induced a decrease in the release of MCP-1 compared with the solvent control. A decrease in MCP-1 was already found to be statistically significant after a 7-hour incubation and remained so throughout the 48-hour time culture (Table 1). Contrary to what was observed for IL-8 and RANTES, both the maximal DEP-PAHs dose of 50 ng/mL and the intermediate dose of 5 ng/mL caused significant inhibition of MCP-1 production. This inhibitory effect on MCP-1 release was very strong compared with the enhancing effect shown for IL-8 and RANTES. Indeed, the lower 5-ng/mL DEP-PAHs dose induced a decrease ranging from 51.3% to 81% depending on the incubation time considered, with the higher dose of 50 ng inducing a decrease ranging from 76.5% to 94.6%. Individual data for the 24-hour time point are shown in Fig 2.

Chemokine mRNA expression

We examined the variations of chemokine mRNA production in PBMCs incubated with 5 ng/mL and 50 ng/mL DEP-PAHs compared with PBMCs incubated with dichloromethane solvent.

As shown in a representative experiment in Fig 3, after a 24-hour incubation, IL-8 and RANTES mRNA levels augmented with increasing concentrations of DEP-PAHs compared with controls (+105% and +44%, respectively, mean of 3 experiments). On the contrary, MCP-1 mRNA levels decreased with increasing concentrations of DEP-PAHs compared with cells incubated with solvent only (-82%, mean of 3 experiments). This effect on IL-8,

RANTES, and MCP-1 mRNA synthesis was also observed after a 7-hour and 18-hour incubation (data not shown).

Chemotaxis assay

The chemotactic activity of the supernatants of PBMCs incubated with DEP-PAHs was evaluated on neutrophils from normal donors and eosinophils from hypereosinophilic patients. As shown in Fig 4, supernatants incubated with the highest dose (50 ng) of DEP-PAHs exhibited a significantly enhanced capacity to attract neutrophils compared with control (medians and interquartiles (Q1:Q3): 13 (10.6:19.5) neutrophils/high-power field (HPF) and 3.5 (2.7:6.7) neutrophils/HPF, respectively, $n = 5$, $P < .05$). Chemotactic activity of supernatants from 50 ng DEP-PAHs-stimulated cells was reduced by $76 \pm 18\%$ ($n = 5$) when neutralizing anti-IL-8 antibodies were added to the supernatants before the chemotaxis assay.

In a similar fashion, the supernatants of the 50 ng DEP-PAHs-incubated cells exhibited a significantly enhanced capacity to attract eosinophils compared with controls (Fig 5) (14.1 [12:16.4] eosinophils/HPF and 2.8 [2:4.6] eosinophils/HPF, respectively, $n = 5$, $P < .05$). When using neutralizing anti-RANTES antibodies, chemotactic activity of supernatants from 50 ng DEP-PAHs-stimulated cells was reduced by $51.7 \pm 19\%$ ($n = 5$).

The lowest dose of 5 ng DEP-PAHs did not induce a statistically significant increase in the number of migrating neutrophils or eosinophils (5.8 [3.6:8.6] neutrophils/HPF and 7.8 [6.6:9.7] eosinophils/HPF, respectively, $n = 5$).

Controls using anti-IL-3 antibodies showed no inhibitory effect on the ability of DEP-PAHs supernatants to induce chemotactic movements of neutrophils or eosinophils. In addition, the enhanced migration of neutrophils and eosinophils was not the result of a chemokinetic effect of the PBMC supernatants. Indeed, the greatest neutrophil and eosinophil migrations detected were obtained when bottom wells contained test solutions and when upper wells contained control medium. All other combinations led to migration levels similar to those obtained with negative control (ie, complete RPMI medium in both chambers).

DISCUSSION

DEPs and associated DEP-PAHs have been shown in several studies to have a broad spectrum of action in many aspects of the inflammatory reaction. In this context, we sought to investigate whether DEP-PAHs could also affect the production of chemokines.

This study demonstrates that the organic extract of diesel exhaust particles can modulate the release of chemokines produced by mononuclear cells of normal donors. This effect was shown to be dose-dependent and to take place early, already a couple of hours after the beginning of the exposure. After a 7-hour incubation

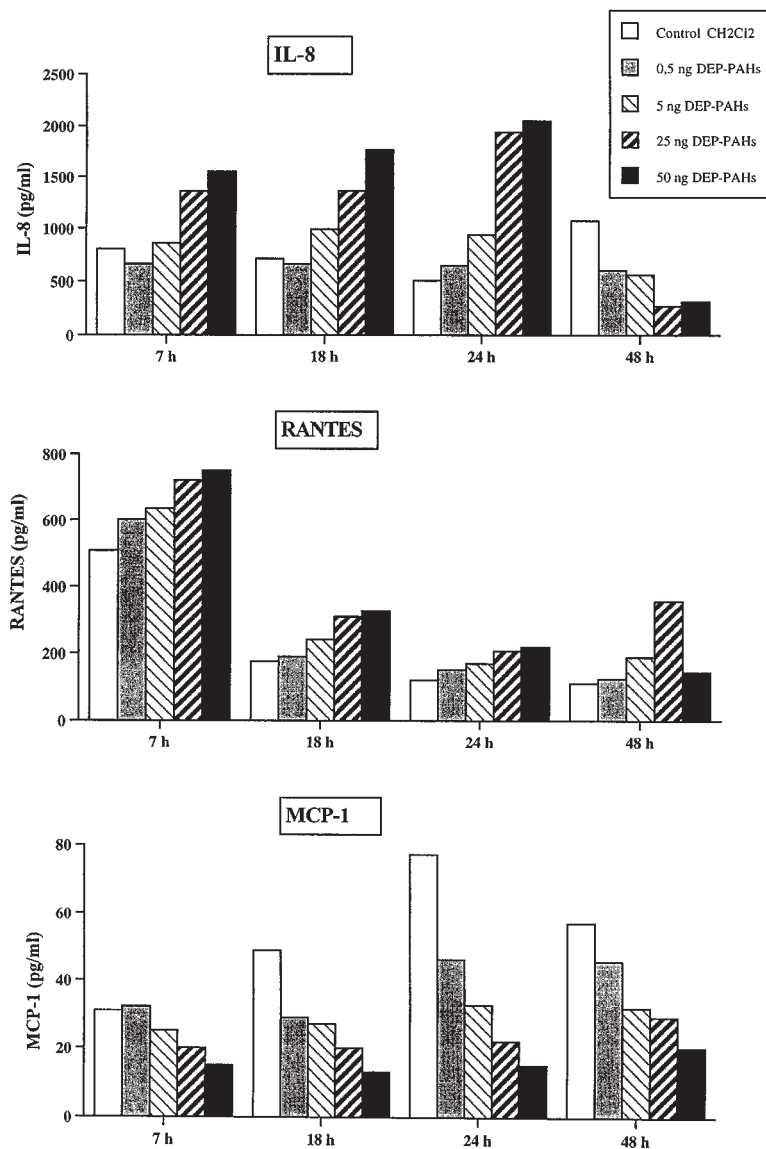


FIG 1. Kinetics of production and dose-response of chemokines RANTES, IL-8, and MCP-1 under various concentrations of DEP-PAHs. Each graph corresponds to 1 representative experiment out of 3.

with DEP-PAHs, IL-8 release was significantly enhanced, as was RANTES release. In contrast, the release of MCP-1 appeared to be nearly totally blocked by treatment with DEP-PAHs. These variations persisted up to a 24-hour culture for IL-8 and RANTES and up to a 48-hour culture for MCP-1. Messenger RNA expression showed similar variations to those observed at the protein level, indicating that DEP-PAHs were able to affect IL-8, RANTES, and MCP-1 at the transcriptional level.

A baseline production of chemokines was present in the supernatants of cells cultured with medium only, with some variations depending on the time and the donor. This finding is in agreement with previous studies, which showed that mononuclear cells produce baseline levels of chemokines because of plastic adherence.²³ In the case of RANTES, the regular decrease over time of average

concentrations was in contrast with the other chemokines, which showed average protein levels that tended to accumulate or remain constant within the different time points. RANTES has been shown to be produced in high quantities by platelets.²⁴ Although we took care to eliminate most platelets from mononuclear cell preparations, a 5% contamination occurred. The previous observation might be explained by the nonspecific release of preformed RANTES at early time points by the contaminating platelets.²⁵ Nevertheless, both solvent control and cells exposed to DEP-PAHs were subject to this nonspecific release of RANTES, and our results showed a significant increase in protein and mRNA production after DEP-PAHs treatment compared with solvent control.

Several studies demonstrated that DEPs can modulate

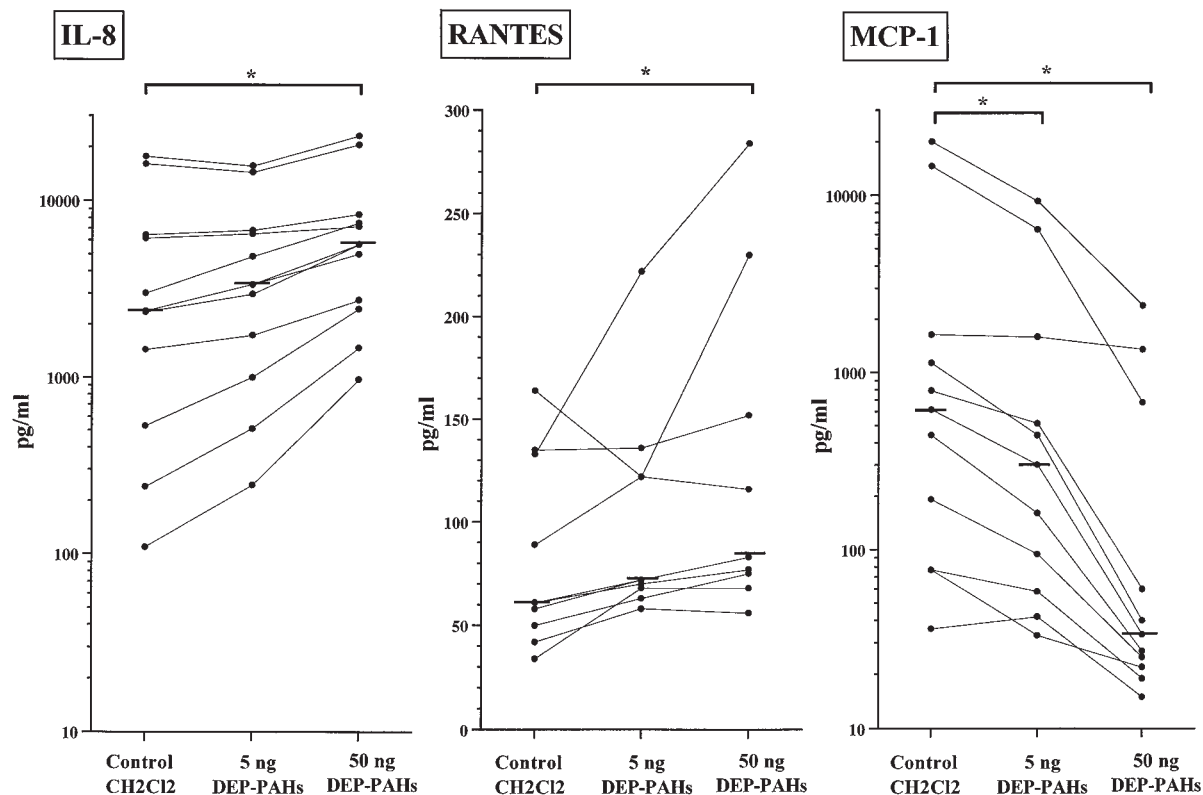


FIG 2. Effect of DEP-PAHs on production by PBMCs of chemokines IL-8 (n = 11), RANTES (n = 9), and MCP-1 (n = 11) after 24-hour incubation. Lines correspond to the medians. *P < .01.

TABLE I. IL-8, RANTES, and MCP-1 release by normal PBMCs

Supernatant	IL-8 (pg/mL)			RANTES (pg/mL)			MCP-1 (pg/mL)		
	Control	DEP-PAHs		Control	1DEP-PAHs		Control	DEP-PAHs	
		5 ng	50 ng		5 ng	50 ng		5 ng	50 ng
7 h	2955 (1068:5074)	2405 (1044:5755)	4708* (2580:7024)	312 (256:736)	296 (182:778)	495* (312:992)	788 (56:894)	150* (38:489)	66* (20:68)
18 h	4013 (739:6479)	4277 (789:9088)	6190† (2057:11268)	146 (93:174)	128 (93:195)	166* (122:249)	833 (94:1777)	333* (67:1718)	52† (29:343)
24 h	2380 (756:6331)	3343 (1179:6697)	5656† (2512:8133)	61 (48:133)	72 (67:125)	83† (73:171)	617 (77:1639)	300† (58:1587)	33† (22:677)
48 h	2887 (1150:6812)	3385 (748:7692)	4486 (1654:7885)	92 (52:113)	89 (42:110)	88 (50:119)	357 (98:4638)	119* (102:1605)	84* (41:1085)

Production of IL-8, RANTES, and MCP-1 by PBMCs incubated with CH₂Cl₂ control, 5 ng, and 50 ng DEP-PAHs.

Results are expressed as median and interquartiles (Q1:Q3). n ≥ 6 for all conditions or time points.

*P < .05.

†P < .01.

the functions of many cell types, consequently modifying various parameters of the inflammatory reaction. Indeed, cytokine production (IL-4 and IL-6) by mast cells is increased by treatment with DEP-PAHs.¹³ This effect, taken together with results showing the production of a type-2 cytokine profile after intranasal challenges,¹¹ suggests that DEP-PAHs can alter the balance of Th1/Th2 cytokines toward a Th2 pro-inflammatory type. Eosinophils have also been shown to respond to DEP-

PAHs by increasing their adhesiveness to nasal epithelial cells and to degranulate after exposure.¹⁴ Neutrophils are also indirect targets of DEP extracts through the activation of the complement alternative pathway. In particular, this leads to an overproduction of the C3a complement protein, a powerful chemotactic agent for neutrophils.¹² In this context, chemokines, as pluripotent molecules having several cellular targets and chemotactic and activating properties, might play an early role in the recruitment and

activation of these various cell types. We suggest that chemokine production alterations could be one possible mechanism by which diesel exhausts have a direct effect on inflammatory cells. DEP-PAHs, through an increase in IL-8 and RANTES, might induce and upregulate an inflammatory response. However, DEP-PAHs in our study also induced a decrease in MCP-1 protein levels. Given the major cellular targets of IL-8, RANTES, and MCP-1, respectively, neutrophils, eosinophils and memory T cells, and monocytes/macrophages, it could be hypothesized that diesel exposure may likely lead to the preferential recruitment of neutrophils, eosinophils, and memory T cells rather than monocytes/macrophages in an *in vivo* situation.

To assess the overall effect of DEP-PAH-treated supernatants on a functional point of view, the chemotactic activity of these supernatants was evaluated on neutrophils and eosinophils. Supernatants from cells incubated with DEP-PAHs at high concentrations exhibited a strong chemotactic activity toward neutrophils. Because IL-8 is the major chemotactic factor for neutrophils¹⁶ and because elevated levels of IL-8 were found in this study, we evaluated the effect of neutralizing anti-IL-8 antibodies on the neutrophil chemotactic effect. This treatment resulted in a strong inhibition of the chemotactic activity on neutrophils, suggesting that IL-8 may be the major factor involved in neutrophil recruitment after incubation of PBMCs with DEP-PAHs. However, one cannot exclude the possibility that other neutrophil chemotactic factors might be involved. Eosinophils also migrated preferentially in the presence of supernatants from PBMCs incubated with the highest dose of DEP-PAHs compared with controls. This effect is likely to be the result of the increased concentrations of RANTES in these cell supernatants, because RANTES is described as a potent chemotactic agent for human eosinophils,²⁵ and because our data demonstrated a significant inhibition of the chemotactic activity following incubation with a neutralizing anti-RANTES antibody. Eotaxin, an eosinophil-specific chemotactic molecule,²⁶ was also assessed but not detected in our supernatants except for some samples, just above the sensitivity level of the kit.

At the transcriptional level, mRNA coding for IL-8 and RANTES and for MCP-1 were, respectively, up-regulated and down-regulated in PBMCs incubated with DEP-PAHs. One of the best characterized receptors able to bind PAHs is the aryl hydrocarbon receptor (AhR). It has been shown that diesel engines can release many compounds that bind to the AhR, particularly the nitropolycyclic aromatic family members.²⁷ The AhR is a common cytosolic receptor-type transcription factor that is involved in the activation of many genes coding for drug-metabolizing enzymes such as cytochrome P4501A1 and glutathione-S transferase, which can in turn detoxify PAHs.²⁸ Once bound to its ligand, the AhR is translocated to the nucleus, where it forms a heterodimer with the AhR nuclear translocator.²⁹ The heterodimer complex binds a DNA response element localized upstream of the target gene.

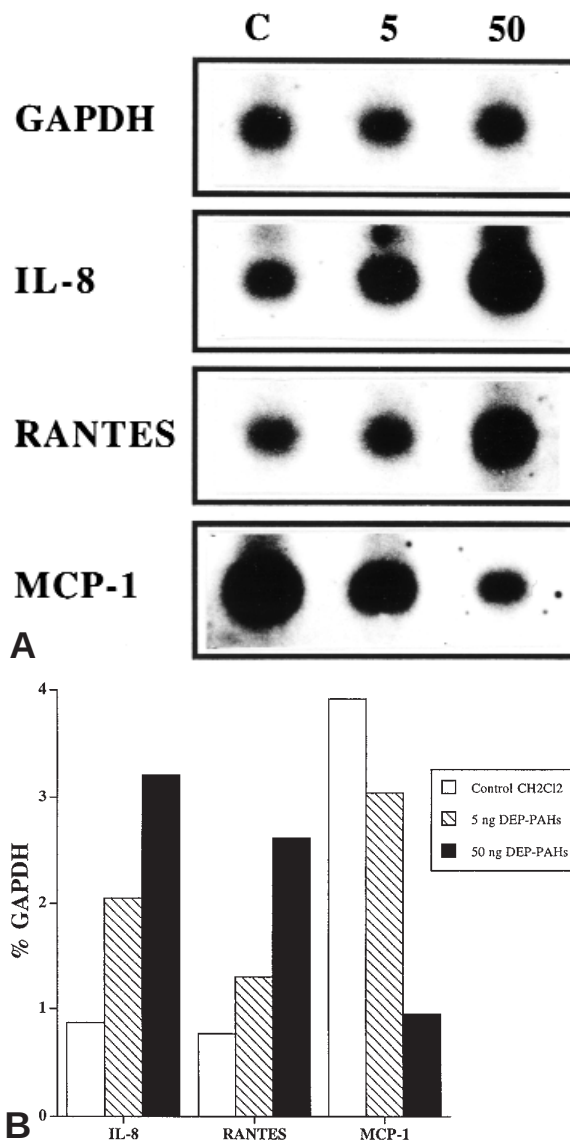


FIG 3. A, Analysis of IL-8, RANTES, and MCP-1 mRNA expression by PBMCs incubated 24 hours with CH₂Cl₂ solvent (lane C), 5 ng (lane 5), or 50 ng (lane 50) DEP-PAHs of 1 representative RT-PCR experiment out of 3. **B,** Densitometric analysis. Values of optical density for the chemokine signals were normalized against corresponding GAPDH.

This heterodimer ligand/AhR-AhR nuclear translocator has been reported to activate various genes, including non-metabolism-related genes such as those coding for plasminogen activator inhibitor and IL1 β .³⁰ Interestingly, the AhR can also act as an inhibitor of the expression of other genes by an alteration of the transcriptional complex.³¹ Such an antinomic activity of ligand-AhR complex at the transcriptional level might be of interest given the contrasting results obtained in the present study on IL-8 and RANTES for one part and on MCP-1 for the other part. Further investigations of the involved regulatory sequences in both genes, as well as experiments using specific molecular tools (PAH-inducible firefly luciferase

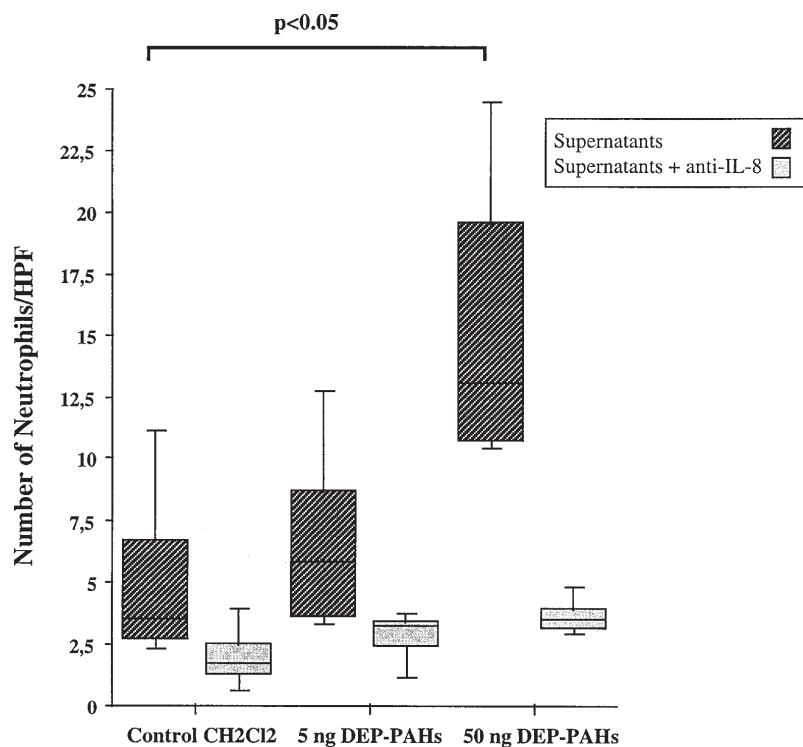


FIG 4. Neutrophil chemotaxis assay. All supernatants were harvested at the time point of 18 hours. Results represent the mean number of neutrophils per HPF after 30-minute incubation with various untreated supernatants (*striped bars*) or supernatants incubated with anti-IL-8 antibodies during 1 hour (*shaded bars*) (n = 5).

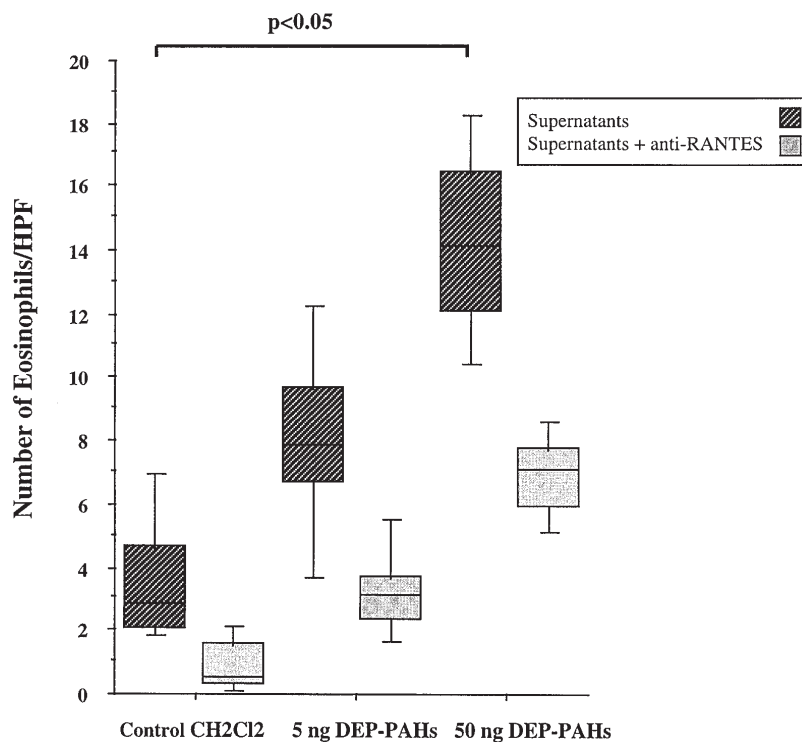


FIG 5. Eosinophil chemotaxis assay. All supernatants were harvested at the time point of 18 hours. Results represent the mean number of eosinophils per HPF after 1-hour incubation with various supernatants (*striped bars*) or supernatants incubated with anti-RANTES antibodies during 1 hour (*shaded bars*) (n = 5).

reporter gene construct, recombinant AhR molecules, and gel retardation analysis³²⁻³⁴), could help to understand the mechanisms involved in the opposite effects of DEP-PAHs on IL-8, RANTES, and MCP-1 gene expression. However, it is of note that DEP-PAHs form a very broad family of unsaturated small hydrocarbons (3 to 5 benzene rings) with a majority of derivatives from fluoranthene, pyrene, anthracene, and chrysene (HPLC results from Renault Lardy, data not shown). Thus many of its compounds have no known biologic receptor and can act via other pathways such as Ca⁺⁺-dependent mechanisms (protein kinase C, phospholipase A₂).²⁸

Recent studies indicate a possible direct relationship between allergic diseases such as asthma and DEPs. Chemokines are of particular interest when one considers the inflammatory reaction associated with allergic diseases.¹⁶ In a mouse model of allergic asthma, Takano et al³⁵ demonstrated an infiltration of eosinophils and lymphocytes in the bronchial epithelium after nasal instillation of DEPs. Such a recruitment, associated with a possible activation of eosinophils by RANTES, would likely increase the damage caused to the epithelium at the inflammation site after eosinophil and neutrophil degranulation.³⁶ Finally, it is of interest that RANTES has also been shown to directly enhance spontaneous IgE production but only in already switched surface IgE⁺ B cells.¹⁹ DEP-PAHs are known to increase IgE production,¹¹ and the dissection of the molecular mechanisms of action has shown that DEP-PAHs increase the level of productive but not germ line epsilon mRNA transcripts, therefore acting on postswitch IgE-producing cells.¹⁰ Thus the observed increased RANTES production by DEP-PAHs might be one mechanism involved in the augmentation of IgE synthesis. The effects of DEP-PAHs combined or not with the specific allergen on the chemokine production in allergic persons might be even more striking than in healthy subjects and are currently under investigation.

In conclusion, we show that DEP-PAHs can directly modulate chemokine production by PBMCs from healthy subjects, leading to chemoattraction of inflammatory cells. These findings support the hypothesis that the development of inflammatory reactions might be affected by diesel exhaust emissions.

We are grateful to Philippe Gosset, Philippe Lassalle, and Michel Joseph for critical review of this work. We thank Pascal Dorlhène, Gilles Jouvenot, and Michèle Chevrier from the staff of Renault Lardy for providing the DEP-PAHs and for the HPLC analysis.

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