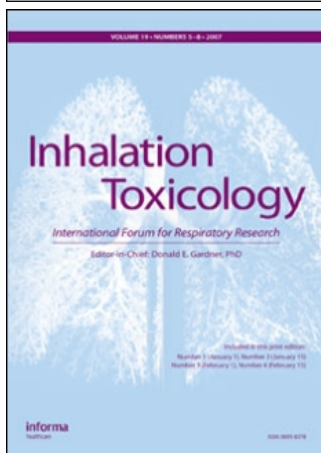


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Wolfgang Schober^a; Stefanie Lubitz^a; Benedetta Belloni^a; Gudrun Gebauer^a;
Jutta Lintelmann^b; Georg Matuschek^b; Ingrid Weichenmeier^a; Bernadette
Eberlein-König^{ac}; Jeroen Buters^a; Heidrun Behrendt^a

^a Division of Environmental Dermatology and Allergy GSF/TUM, ZAUM-Center for
Allergy and Environment, Technical University Munich, Munich, Germany

^b GSF-National Research Center for Environment and Health, Institute of Ecological
Chemistry, GSF-Focus: Aerosols and Health, Neuherberg, Germany

^c Department of Dermatology and Allergy, Biederstein, Technical University Munich,

Munich, Germany

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Wolfgang Schober, Stefanie Lubitz, Benedetta Belloni, and Gudrun Gebauer

Division of Environmental Dermatology and Allergy GSF/TUM, ZAUM–Center for Allergy and Environment, Technical University Munich, Munich, Germany

Jutta Lintelmann and Georg Matuschek

Institute of Ecological Chemistry, GSF-Focus: Aerosols and Health, GSF-National Research Center for Environment and Health, Neuherberg, Germany

Ingrid Weichenmeier

Division of Environmental Dermatology and Allergy GSF/TUM, ZAUM–Center for Allergy and Environment, Technical University Munich, Munich, Germany

Bernadette Eberlein-König

Division of Environmental Dermatology and Allergy GSF/TUM, ZAUM–Center for Allergy and Environment, Technical University Munich, Munich, and Department of Dermatology and Allergy, Biederstein, Technical University Munich, Munich, Germany

Jeroen Buters and Heidrun Behrendt

Division of Environmental Dermatology and Allergy GSF/TUM, ZAUM–Center for Allergy and Environment, Technical University Munich, Munich, Germany

Diesel exhaust particles (DEPs) have been implicated in the worldwide increased incidence of allergic airway diseases over the past century. There is growing evidence that DEP-associated polycyclic aromatic hydrocarbons (PAHs) participate in the development and maintenance of immunoglobulin (Ig) E-mediated allergic diseases. To address this issue we investigated the impact of U.S. Environmental Protection Agency (EPA) priority PAHs as well as of PAH-containing airborne extracts on antigen-induced CD63 upregulation and mediator release from human basophils. Whole blood samples from birch pollen allergic and control subjects were incubated in the presence of organic extracts of urban aerosol (AERex) or EPA-PAH standard with or without rBet v 1. Basophils were analyzed for CD63 expression as a measure of basophil activation by using multiparameter flow cytometry. In addition, purified basophils from birch pollen allergic donors were incubated for 2 h in the presence of 1 μ M benzo[*a*]pyrene (BaP) or phenanthrene (Phe) and then stimulated with rBet v 1 for 45 min. Supernatants were assayed for histamine, interleukin (IL)-4, and IL-8 by means of enzyme-linked immunosorbent assay (ELISA). Basophils exposed in vitro simultaneously to AERex or EPA-PAH standard and rBet v 1 expressed CD63 significantly more than with antigen alone. PAHs synergized with rBet v 1 dose dependently, but did not activate basophils from nonallergic donors. BaP and Phe significantly enhanced cytokine secretion (IL-4, IL-8) and histamine release from purified basophils without antigen added, and secretion was not further enhanced by rBet v 1 stimulation. In conclusion, PAHs from roadside emissions can directly activate sensitized basophils to cytokine secretion and drive proallergic processes through enhanced Fc ϵ RI-coupled mediator release from human basophils.

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Address correspondence to Wolfgang Schober, PhD, ZAUM–Center for Allergy and Environment, Technical University Munich, Biedersteiner Strasse 29, 80802 Munich, Germany. E-mail: schober@lrz.tum.de

Airborne particulates and gases related to human activities, or ambient air pollution, are important environmental issues that affect human health. In recent years, epidemiological studies have demonstrated an association between exposure to road-side emissions and increased symptoms of asthma and allergic rhinoconjunctivitis (Heinrich & Wichmann, 2004; Lemmen et al., 2004; Saxon & Diaz-Sanchez, 2005). Major components of inhaled particles less than 2.5 μm in size are diesel exhaust particles (DEPs) and their associated organic compounds, such as polycyclic aromatic hydrocarbons (PAHs). Experimental and clinical evidences indicate that DEPs can exacerbate allergic immune responses (Maejima et al., 1997; Nel et al., 1998) and increase total immunoglobulin (Ig) E in the human upper airway in vivo following intranasal challenge (Diaz-Sanchez et al., 1996; Takenaka et al., 1995).

Less attention has been attributed so far to the involvement of organic compounds of diesel emissions in IgE-mediated allergic inflammation at the level of mediator cells. Devouassoux and coworkers demonstrated spontaneous histamine release from and enhanced IL-4 production in basophils of allergic and non-allergic subjects exposed to DEP extracts (Devouassoux et al., 2002). Recent data provide evidence that DEP-associated PAHs may account for basophil activation and enhanced IL-4 production in response to crosslinking the high-affinity IgE receptor (Kepley et al., 2003).

To address this issue we investigated the impact of organic extracts of urban particulate matter (Schober et al., 2006) and single U.S. Environmental Protection Agency (EPA) priority PAHs on human basophils from birch pollen allergic donors as well as from nonatopic, healthy subjects and focused on possible synergistic effects of particle-associated organic compounds and Bet v 1, the major allergen of birch pollen grains, upon sensitized basophils.

MATERIALS AND METHODS

Sampling of Airborne Particulate Matter

Atmospheric fine dust was collected using an Andersen high-volume sampler (Andersen Instruments, Inc., USA) equipped with a 2.5- μm head as described in detail elsewhere (Schober et al., 2006). Particles were collected on quartz-fiber filters at an average flow rate of 52 m^3/h . A 1-day sample (AERex^{1d}) was collected on February 20–21, 2003 (23.93 h, total air volume 1270 m^3), and a 5-day sample (AERex^{5d}) on June 5–10, 2003 (119.89 h, total air volume 6230 m^3). The sampler was located near (<10 m) a highly frequented six-lane road (approximately 44,000 motor vehicles/day) in the north of Munich, Germany.

Preparation and Analysis of PM Extracts

AERex was prepared and characterized as described previously (Schober et al., 2006). Briefly, filters were heated for 2 h at 100°C and the aqueous condensate was online extracted by dropping through 2 ml *n*-hexane. After steam distillation *n*-hexane was replaced by dimethyl sulfoxide (DMSO), yielding

TABLE 1
HPLC analysis of particle-associated PAHs in AERex^{1d}
and AERex^{5d}

PAHs	AERex ^{1d} (ng/ml extract) ^a	AERex ^{5d} (ng/ml extract) ^b
Acenaphthene*	n.d.	n.d.
Anthanthrene	17.00	0.22
Anthracene*	51.15	5.34
Benzo[a]anthracene*	256.38	2.30
Benzo[b]fluoranthene*	513.02	0.35
Benzo[k]fluoranthene*	201.69	0.26
Benzo[g,h,i]perylene*	76.05	0.69
Benzo[a]pyrene*	164.70	0.20
Benzo[e]pyrene	808.01	0.98
Chrysene*	546.32	3.88
Coronene	n.d.	0.26
Dibenzo[a,h]anthracene*	12.35	0.19
Fluoranthene*	945.04	96.35
Fluorene*	n.d.	4.40
Indeno[1,2,3-c,d]pyrene*	92.46	n.d.
Naphthalene*	n.d.	9.21
Phenanthrene*	391.89	62.58
Pyrene*	730.62	66.39
Total PAH content [ng/m ³]	1.14	0.06

Note. From Schober et al. (2006). The samples were collected near (<10 m) a highly frequented six-lane road with 44,000 motor vehicles per day in the north of Munich, Germany. Asterisk indicates U.S. EPA priority pollutant. Solvent: DMSO; n.d., not detected.

^a4233.33 m^3 air/ml.

^b4152.73 m^3 air/ml.

two organic extracts with similar concentrations of air equivalents per milliliter. Variance of PAH composition was analyzed using a high-performance liquid chromatography (HPLC) system HP 1100 (Agilent Technologies, Germany) equipped with a diode array and a fluorescence detector. The HPLC method was externally calibrated for the quantification of 18 PAHs—15 of them are U.S. EPA priority pollutants—in the range from 2 $\text{pg}/\mu\text{l}$ up to 1000 $\text{pg}/\mu\text{l}$ using chromatographic peak areas versus concentration (Table 1).

Polycyclic Aromatic Hydrocarbons

The U.S. EPA-PAH standard was obtained from Dr. Ehrenstorfer GmbH, Germany. Benzo[a]pyrene and phenanthrene (Phe) were commercially available from Sigma-Aldrich, Germany.

Basophil Activation and Flow Cytometry

Antigen-induced basophil activation from birch pollen allergic and nonallergic individuals was analyzed using the BASOTEST (Orpegen Pharma, Germany) as previously reported in detail (Schober et al., 2006). Briefly, 100 μl heparinized whole

blood was first stimulated with 20 μ l phosphate-buffered saline (PBS) buffer containing interleukin (IL)-3 for 10 min at 37°C and then incubated with 100 μ l of rBet v 1 (Biomay, Austria) alone or in combination with AERex or EPA-PAH standard at different concentrations. In FACS analysis, basophils were gated by the presence of PE-conjugated anti-IgE, and expression of gp53 (CD63) was quantified on this gated cell population. Acquisition was performed on 1000 cells for each sample and results are given as the percentage of basophil expressing CD63.

Isolation of Peripheral Blood Basophils

Venous blood was taken from atopic individuals with known birch pollen allergy. Basophils from individual donors were purified by centrifugation over a Lymphoprep gradient (Axis-Shield, Norway) followed by negative selection with antibody-coated magnetic beads (Miltenyi Biotech, Germany) as described elsewhere (Gane et al., 1993). The final cell suspension consisted of 65% to 90% viable basophils as assessed by flow cytometry and Pappenheim staining.

Stimulation of Enriched Human Basophils

After purification, basophils were cultured in 96-well plates at a density of 50,000 cells/100 μ l and incubated with 1 μ M of BaP or Phe for 2 h prior to rBet v 1 stimulation.

Measurement of IL-4 and IL-8 Secretion and Histamine Release

Cell-free supernatants were assayed for IL-4, IL-8, and histamine by means of enzyme-linked immunosorbent assay (ELISA; R&D Systems and Beckman Coulter, USA).

Transmission Electron Microscopy

Enriched human basophils stimulated with extracts of airborne particles were fixed in 0.1 M cacodylate-buffered glu-

tardialdehyde (Serva, Germany), 2%, pH 7.2, 4°C, postfixed with 2% osmium tetroxide, dehydrated in an ascending series of ethanol, and embedded in Araldite Resin (Serva, Germany). Ultrathin sections were stained with uranyl acetate and lead citrate. Electron micrographs were taken in a Philips 400T electron microscope.

Statistical Analysis

Data are expressed as the arithmetic means $\pm$ SD. A paired *t*-test was performed to compare differences of CD63 expression and mediator release between mixtures or single PAHs and controls. A *p* value of $\leq .05$ was considered statistically significant.

RESULTS

Effects of AERex and EPA-PAH Standard on Basophil Activation

Both organic extracts have been found to synergize with rBet v 1 and significantly enhance basophil activation in all birch pollen allergic donors (Figure 1). However, stimulation with AERex^{1d} or AERex^{5d} in the absence of rBet v 1 exhibited no effect on CD63 expression in basophils from atopic subjects as well as from healthy controls, indicating no direct effect of AERex on human basophils in our study.

In 3 out of 6 patients basophil activation increased up to 90% after treatment with both EPA-PAH standard and rBet v 1. PAH-induced enhancement of CD63 upregulation of rBet v 1 in sensitized basophils was highly significant (Table 2) and occurred in a dose-dependent manner (Schober et al., 2006). In addition, transmission electron microscopy demonstrated basophil degranulation (Figure 2), and neutrophils—exhibiting leading lamellae as indicator of migratory activity—in the vicinity of the cells (not shown). None of the basophil samples from healthy controls

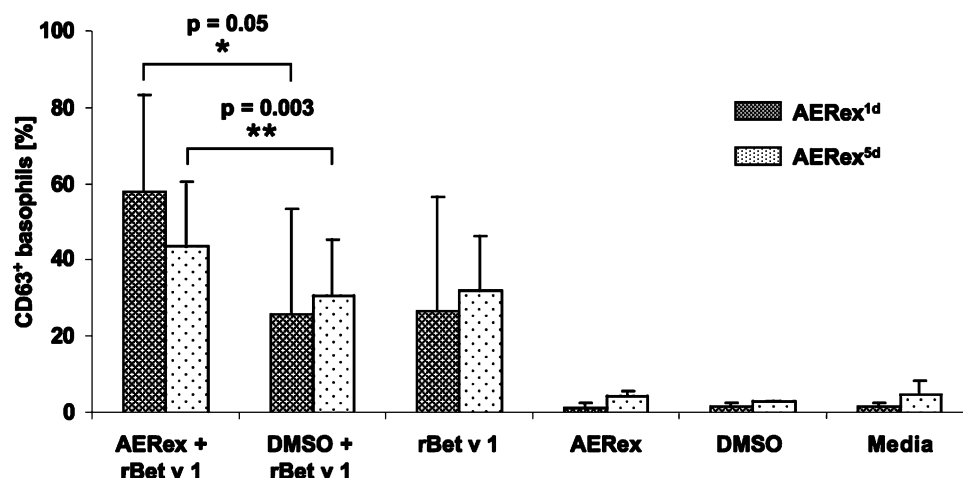


FIG. 1. AERex significantly enhances rBet v 1-induced basophil activation. Each column represents the mean $\pm$ SD of maximal dose-response relationships observed in three (AERex^{1d}) and five (AERex^{5d}) birch pollen allergic individuals after treatment with AERex and rBet v 1. From Schober et al. (2006).

TABLE 2

Effects of AERex, EPA-PAH standard, and single PAHs on mediator release from basophils of birch pollen allergic individuals

	Sensitized	Sensitized + rBet v 1
	Whole blood	
Mixtures		Upregulation:
AERex ^{1d}	No effect	CD63: +32 % ($p = .05$) ^a
AERex ^{5d}	No effect	CD63: +13 % ($p = .003$)
EPA-PAH ^{standard}	Not done	CD63: +66 % ($p = .007$)
PAHs	Effect	Effect
	Purified basophils	
BaP	IL-8: 133 % ($p = .006$) ^c IL-4: 135 % ($p = .05$) No release of histamine	IL-8: 138 % ($p = .001$) ^b IL-4 secretion unaffected No effect on histamine release
Phe	IL-8: 128 % ($p = .07$) IL-4: 137 % ($p = .03$) Histamine: 170 % ($p = .05$)	IL-8: 117 % ($p = .04$) IL-4 secretion unaffected No effect on histamine release

Note. Data are expressed as mean of 3 (AERex^{1d}, EPA-PAH^{standard}), 5 (AERex^{5d}), and 10 (PAHs) independent experiments.

^aChange of CD63 upregulation vs. rBet v 1 stimulation.

^{b,c}Change of mediator release vs. (b) rBet v 1 stimulation or (c) DMSO control (100%).

showed any upregulation of CD63 by EPA-PAH standard or rBet v 1.

Effects of BaP and Phe on Mediator Release From Purified Basophils

Benzo[a]pyrene (BaP) and phenanthrene (Phe) at concentrations of 0.1–5 μ M drove IL-8 secretion from sensitized basophils in a dose-dependent manner. The effect was more significant for BaP than for Phe, but could be observed with and without antigen added (Table 2). Moreover, both PAHs significantly enhanced

IL-4 secretion from purified basophils in the absence of antigen, suggesting an adjuvant role of DEP-PAHs in allergic sensitization. No relevant further increase in IL-4 secretion was seen in the presence of specific antigen. Furthermore, Phe but not BaP significantly enhanced histamine release from basophils of birch pollen allergic individuals in the absence of rBet v 1 (Table 2).

DISCUSSION

Increased industrialization in developed countries has been paralleled by increased prevalence of allergic asthma and rhinoconjunctivitis (Heinrich & Wichmann, 2004; Holgate, 1999) and has been grossly attributed to traffic related air pollution (Krämer et al., 2000; Saxon & Diaz-Sanchez, 2005). Controlled human studies clearly demonstrated that diesel exhaust particles (DEPs) participate in the development of allergic airway inflammation by enhancing allergen-specific IgE levels and Th2 cytokine production in response to common allergens (Diaz-Sanchez, 1997; Diaz-Sanchez et al., 1996). DEPs also act as adjuvants to coadministered antigen in nasal provocation challenges (Diaz-Sanchez et al., 1997) and can enhance the severity of clinical symptoms to allergen, presumably by mast-cell degranulation (Diaz-Sanchez et al., 2000).

Experimental data provide evidence that organic substances adsorbed to the particles (e.g., polycyclic aromatic hydrocarbons, PAHs) can interfere with ongoing allergic inflammatory processes independently from the particle nature (Bömmel et al., 2000, 2003; Devouassoux et al., 2002; Hitzfeld et al., 1992; Suzuki et al., 1993; Takenaka et al., 1995; Tsien et al., 1997). Due to their lipophilicity, such compounds are able to interact with a wide range of cells involved in the immune response, including basophilic leukocytes (Kepley et al., 2003).

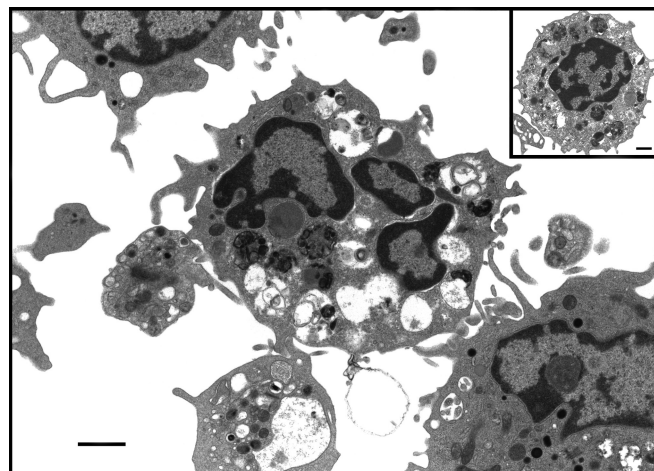


FIG. 2. Electron micrograph from enriched human basophils exposed to extracts of airborne particulate matter: basophil degranulation, attachment of platelets to the basophil and activated neutrophils in the vicinity of these cells. Inset: Unstimulated basophil from controls. Bar, 1 μ m.

In the present study we investigated whether DEP-associated PAHs directly affect human basophils or synergize with rBet v 1 in affecting basophil activation and mediator release. For these in vitro experiments, basophils of birch pollen allergic individuals were stimulated with organic extracts (AERex) of urban particulate matter (PM_{2.5}) and single U.S. EPA priority PAHs.

We recently published (Schober et al., 2006) that the mass concentration of the sum of all investigated PAHs in AERex^{1d} (1.14 ng/m³) was roughly 20 times higher than in AERex^{5d} (0.06 ng/m³). These values are similar to those values found in atmospheric particulate matter collected during previous studies in Munich (Lintelmann et al., 2005; Schauer et al., 2003; Schnelle-Kreis et al., 2001). Table 1 shows that depending on the specific PAH, 10- to 1500-fold higher concentrations could be measured in AERex^{1d} than in AERex^{5d}. In particular, the BaP content in AERex^{1d} was found to be 165 ng/ml, whereas only marginal amounts of this compound and other U.S. EPA priority PAH pollutants were recovered in AERex^{5d}, which was collected during summer.

Our data demonstrate that both AERex tested significantly enhance rBet v 1-induced basophil activation in all birch pollen-allergic subjects at ambient concentrations (Schober et al., 2006). Basophil activation is accompanied by extract-induced anaphylactic degranulation of the cells as seen by transmission electron microscopy (Figure 2). Strongest effects on basophil activation were monitored upon stimulation with the 1-day extract (AERex^{1d}), collected in winter 2003, which comprises a 20-fold higher total PAH content than the 5-day extract (AERex^{5d}), collected in summer 2003. This suggests that the biological activity of AERex is markedly correlated with the content of certain organic components in urban aerosols, in particular PAHs. Significant enhancement of antigen-induced basophil activation after treatment with single U.S. EPA priority PAHs supports this hypothesis and implicates a crucial role of DEP-PAHs on allergic inflammation by exacerbating the immediate-phase response.

Moreover, basophils are a major source of early IL-4 production in allergic patients and may be involved in the initiation of allergic inflammation. We found that BaP and Phe significantly enhance IL-4 secretion from sensitized basophils in the absence of rBet v 1 (Lubitz et al., 2006). Recently, an allergen-independent IL-4 production in DEPex (organic extract of diesel exhaust particles)-stimulated basophils from atopic donors has also been shown (Devouassoux et al., 2002). Furthermore, both PAHs drove IL-8 secretion from sensitized basophils with and without antigen added. IL-8 is an important activator and chemoattractant for neutrophils and has been implicated in a variety of both acute and chronic inflammatory processes. DEPs have been shown in human subjects to promote leukocyte infiltration and exacerbate local mucosa inflammation (Salvi et al., 2000). A key factor is the increase in IL-8 expression in resident cells of the airway tissue and lumen (Bömmel et al., 2003; Nightingale et al., 2000; Salvi et al., 2000). In addition to the impact of PAHs on cytokine secretion, our results demonstrate

that Phe strongly induced basophils from birch pollen allergic patients to degranulate in absence of rBet v 1. A similar effect of DEPex on histamine release from sensitized basophils has been previously described (Devouassoux et al., 2002). However, EPA-PAH standard synergized with rBet v 1 and significantly enhanced basophil CD63 expression, suggesting different mechanisms of cell activation by single PAHs and mixtures of organic air pollutants in atopic individuals.

Since basophils are mediator cells of allergy being involved in the initiation and propagation of allergic inflammation, including asthma, it is important to understand how traffic-related air pollutants affect their functional responses and signalling pathways. Our data suggest that diesel soot-associated PAHs not only can drive proallergic processes through enhanced FcεRI-coupled mediator release from human basophils, but also can initiate a proinflammatory Th2-dominated immune response via direct action of single PAHs on sensitized basophils.

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