Organic Extracts of Urban Aerosol (<PM2.5) Enhance rBet v 1-Induced Upregulation of CD63 in Basophils from Birch Pollen–Allergic Individuals

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Epidemiological studies have linked the high prevalence rates of IgE-mediated allergic diseases to an increase in exposure to traffic-related air pollutants such as diesel exhaust particles (DEPs). There is growing experimental evidence that organic compounds of DEPs, predominantly polycyclic aromatic hydrocarbons (PAHs), participate in the development and maintenance of allergic airway diseases.

The aim of the present study is to characterize the impact of organic extracts of urban aerosol on human basophils from birch pollen–allergic and nonatopic, healthy subjects and to identify possible synergistic effects of particle-associated organic compounds and Bet v 1, the major allergen.

Key Words: basophils; CD63 expression; organic compounds of urban aerosol; PM2.5, IgE-mediated allergic diseases.
of birch pollen grains, upon basophil stimulation from sensitized individuals.

**MATERIALS AND METHODS**

**Sampling of airborne particulate matter (PM).** Atmospheric fine dust was collected using an Andersen High-Volume Sampler (Andersen Instruments Inc., U.S.A.) equipped with a 2.5-μm head. Particles were collected on a quartz-fibre filter (GF 20, 203 × 254 mm, Schleicher & Schuell, Germany) which was cleaned by heating at 500°C over 3 h before use. Air was drawn through the filter at an average flow rate of 52 m³/h. An organic extract of urban aerosol, 1-day sample (AERexId), was collected on February 20–21, 2003 (23.93 h, total air volume 1270 m³), and a 5-day sample (AERexId) between June 5 and June 10, 2003 (119.89 h, total air volume 6230 m³). The sampler was located near (<10 m) a highly frequented six-lane road (Ingolstädter Landstrasse: 44,000 motor vehicles/day; personal communication ''Planungsreferat München'') at the main entrance of the GSF research campus in the north of Munich, Germany. The campus has a wide lawn with some trees (no birch trees). The GSF boiler house is 800 m away from the sampling site. Sampling was performed 2 m above ground.

**Preparation of PM extracts.** One-third of the 1-day filter and two-thirds of the 5-day filter were extracted by steam distillation. Each filter was placed into a flask with 250 ml water. The flask was heated for 2 h at 100°C. At this temperature the majority of allergens are destroyed by denaturation, including Bet v 1. The steam was cooled down by passing through a Liebig cooler, and the aqueous condensate was online extracted by dropping through 2 ml n-hexane. After 2 h the process was stopped, and the n-hexane phase was removed. In order to compare both extracts, 0.1 ml dimethyl-sulfoxide (DMSO) for AERex 1d and 1 ml DMSO for AERex 5d were added. The n-hexane was vaporized under a gentle stream of nitrogen at 40°C, yielding two organic extracts with similar concentrations of air equivalents per ml.

**GC-MS analysis.** Identification of compounds was performed using a Hewlett-Packard gas chromatograph–mass spectrometer (GC-MS) system (GC 6890 series, with a 5973N mass selective detector). The MS was operated as a Hewlett-Packard gas chromatograph–mass spectrometer (GC-MS) system. Preparative (1a) was purchased from Biomay (Vienna, Austria). Purity of approximately 99% was determined by SDS-PAGE and staining with Coomassie Brilliant Blue R-250. The lyophilized protein was reconstituted with ultrapure water to a concentration of 1 mg/ml and aliquoted. Aliquots were stored at −80°C to final use. Skin prick test solution was commercially available from Allergopharma (Reinbek, Germany).

**Basophil activation and flow cytometry.** For quantitative determination of rBet v 1–induced activation of human basophils, a commercially available test system was used (BASOTEST®). Brieﬂy, 100 μl heparinized whole blood was first stimulated with 20 μl phosphate buffered saline (PBS) buffer containing IL-3 for 10 min at 37°C and then incubated for 20 min with 100 μl of rBet v 1 alone or in combination with AERex at concentrations from 0.007 to 0.755 m³ air equivalents. 1 μM of the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (f-MLP) was used as positive control, PBS solution served as negative background control. The degranulation process was stopped by storing the samples on ice for 5 min. 20 μl of a two-color antibody reagent consisting of PE-conjugated anti-IgE and Blue R-250. The lyophilized protein was reconstituted with ultrapure water to a concentration of 1 mg/ml and aliquoted. Aliquots were stored at −80°C to final use. Skin prick test solution was commercially available from Allergopharma (Reinbek, Germany).

### TABLE 1

**Characteristics of Atopic and Nonatopic Donors**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Gender</th>
<th>Age</th>
<th>SPT birch</th>
<th>RAST class birch/Bet v 1</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I f</td>
<td>26</td>
<td>++++</td>
<td>4/3</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>II m</td>
<td>24</td>
<td>++++</td>
<td>3/3</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>III f</td>
<td>21</td>
<td>++++</td>
<td>4/4</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>IV f</td>
<td>39</td>
<td>++++</td>
<td>4/4</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>V m</td>
<td>38</td>
<td>++++</td>
<td>3/3</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>VI f</td>
<td>23</td>
<td>++++</td>
<td>3/3</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I f</td>
<td>24</td>
<td>neg.</td>
<td>0/0</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>II m</td>
<td>32</td>
<td>neg.</td>
<td>0/0</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>III f</td>
<td>26</td>
<td>neg.</td>
<td>0/0</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>IV m</td>
<td>31</td>
<td>neg.</td>
<td>0/0</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>V f</td>
<td>23</td>
<td>neg.</td>
<td>0/0</td>
<td>no</td>
<td></td>
</tr>
</tbody>
</table>

**Note.** Age, gender, specific IgE concentration (CAP-RAST class 0–6), and skin prick test (SPT) birch (+++++: diameter of wheat > 6 mm, erythema > 20 mm) of individuals tested are shown; neg., negative.
Flow cytometric analysis was performed within 2 h using a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany) and CellQuest™ software. Basophilic granulocytes were gated by the presence of PE-conjugated anti-IgE, and expression of gp53 (CD63) was analyzed on this gated cell population. Acquisition was performed on 1000 cells for each sample, and results are given as the percentage of basophils expressing CD63. Results with more than 15% of activated basophils were regarded as positive, according to the manufacturer instructions (Orpegen Pharma, 1997). All basophil activation tests were performed outside the birch pollen season, which in Munich lasted from April 10 until May 1, 2003.

Statistical analysis. Data are expressed as the arithmetic means ± SD. A paired t-test was performed to compare differences of CD63 expression between AERex and controls. A p value of ≤0.05 was considered statistically significant.

RESULTS

Characterization of AERex

At least nine organic compound classes were identified by GC-MS analysis in AERex<sup>1d</sup> and AERex<sup>5d</sup> (Table 2). In particular, aromatic components such as PAHs and their derivates could be detected.

The mass concentration of the sum of all investigated PAHs in AERex<sup>1d</sup> (1.14 ng/m<sup>3</sup>) was roughly 20 times higher than in AERex<sup>5d</sup> (0.06 ng/m<sup>3</sup>). 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 3 shows that, depending on the specific PAH, 10- to 1500-fold higher concentrations could be measured in AERex<sup>1d</sup> than in AERex<sup>5d</sup>. In particular, the benzo[a]pyrene (B[a]P) content in AERex<sup>1d</sup> was found to be 165 ng/ml, whereas only marginal amounts of this compound and other EPA priority PAH pollutants were recovered in AERex<sup>5d</sup>, which was collected during summer. No LPS reactivity was found in both filter extracts tested at a 1:100 dilution. AERex concentrations used in basophil activation assay were therefore below the detection limit of LAL test (0.05 E.U./ml).

### TABLE 2

<table>
<thead>
<tr>
<th>Compound classes</th>
<th>AERex&lt;sup&gt;1d&lt;/sup&gt;</th>
<th>AERex&lt;sup&gt;5d&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>PAHs</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>PAH ketones and quinones</td>
<td>17.00</td>
<td>0.22</td>
</tr>
<tr>
<td>Anthracene</td>
<td>51.15</td>
<td>5.34</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>256.38</td>
<td>2.30</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>513.02</td>
<td>0.35</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>201.69</td>
<td>0.26</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>76.05</td>
<td>0.69</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>164.70</td>
<td>0.20</td>
</tr>
<tr>
<td>Chrysene</td>
<td>546.32</td>
<td>3.88</td>
</tr>
<tr>
<td>Coronene</td>
<td>n.d.</td>
<td>0.26</td>
</tr>
<tr>
<td>Dibenzo[a,h]anthracene</td>
<td>12.35</td>
<td>0.19</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>945.04</td>
<td>96.35</td>
</tr>
<tr>
<td>Fluorene</td>
<td>n.d.</td>
<td>4.40</td>
</tr>
<tr>
<td>Indeno[1,2,3-c,d]pyrene</td>
<td>92.46</td>
<td>n.d.</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>n.d.</td>
<td>9.21</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>391.89</td>
<td>62.58</td>
</tr>
<tr>
<td>Pyrene</td>
<td>730.62</td>
<td>66.39</td>
</tr>
<tr>
<td>Total PAH content [ng/m&lt;sup&gt;3&lt;/sup&gt;]</td>
<td>1.14</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Note. 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. Solvent: DMSO; n.d., not detected.

### CD63 Upregulation of Human Basophils Induced by rBet v 1

Antigen-induced regulation of CD63 expression on basophils of allergic donors was analyzed by incubation with serial dilutions of rBet v 1. Basophil activation was seen to be dose dependent in all patients, reaching a maximum upon stimulation with 4.5 ng/ml rBet v 1 (Fig. 1). For comparable studies on modulating effects of AERex, basic CD63 expression by rBet v 1 was adjusted individually for each donor to >10% and <50% basophils. As indicated by Figure 1, a dose range of 0.05–2.3 ng/ml rBet v 1 was found to be appropriate for basic activation in all atopic donors tested. None of basophil samples from healthy controls showed any upregulation of CD63 by rBet v 1.

### Effect of AERex on Basophil Activation

Basophil CD63 expression was analyzed either after stimulation with rBet v 1 or upon AERex exposure, or both, to investigate possible synergy between AERex and IgE-mediated basophil activation in birch pollen–allergic donors and healthy controls.
Activation with 0.05–2.3 ng/ml rBet v 1 induced 10% to 50% of basophils to upregulate CD63 (Fig. 2) except for donor I being below 10%. rBet v 1 upregulation of CD63 was not influenced by presence of DMSO (Fig. 3). After treatment with both AERex and allergen, basophil activation increased in all patients up to 90%, but was higher with AERex1d than with stimulation with AERex5d (Fig. 2). Compared to AERex5d, 5- to 50-fold lower concentrations of AERex1d were required for maximal effect on basophil activation, which is in accordance with the 20-fold higher total PAH content found in the 1-day sample (Table 3). AERex-induced enhancement of CD63 upregulation of rBet v 1 in sensitized basophils occurred in a dose-dependent manner as is shown exemplarily for one donor in Figure 4.

Both organic extracts synergized with rBet v 1 and significantly enhanced basophil activation in all birch pollen–allergic donors (Fig. 3). However, stimulation with AERex1d or AERex5d alone exhibited no effects on CD63 expression in basophils from atopic donors as well as from healthy controls, indicating no direct effect of AERex on human basophils in our study. In addition, both AERex extracts showed no enhancement of CD63 upregulation in f-MLP-stimulated basophils from birch pollen–allergic donors.

FACS analysis of propidium iodide–labeled cells revealed that both organic extracts were not toxic to basophils at dilutions of 1:1000 and higher (<0.1% DMSO). AERex-mediated enhancement of basophil activation was only seen at dilutions >1:5000.

**DISCUSSION**

Environmental particles (≤PM2.5: Particulate matter that is 2.5 μm and smaller in size) at ambient levels have been implicated in a great number of human health effects including atopic...
diseases. An epidemiological association between allergic airway diseases and exposure to atmospheric pollutants has been demonstrated and suggested to be one factor in the increasing prevalence of asthma and rhinoconjunctivitis (Heinrich and Wichmann, 2004; Krämer et al., 2000; Studnicka et al., 1997; van Vliet et al., 1997). In human subjects, studies clearly demonstrated that diesel exhaust particles (DEPs) participate in the development of allergic airway inflammation by enhancing specific IgE and Th2 cytokine production in response to common allergens (Diaz-Sanchez, 1997; Diaz-Sanchez et al., 1996, 1997). However, there is growing experimental evidence that organic substances adsorbed to the particles (polycyclic aromatic hydrocarbons, PAHs) are responsible for adjuvant effects of DEPs 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).

In the present study we investigated whether AERex directly affects human basophils or synergizes with rBet v 1 in stimulating basophil activation. For these in vitro experiments, we prepared and used two organic extracts of urban particulate matter (≤PM2.5) comprising quantitatively different PAH patterns.

Several studies have provided new insights into the possible involvement of basophils in initiation and amplification of the specific IgE-dependent allergic inflammatory response and fueled speculations that extend beyond their recognized role as effector cells (Devouassoux et al., 1999; Falcone et al., 2000). During the course of allergic reactions, basophils have been shown to migrate into tissues participating in inflammatory reactions (Durham, 1998). Upon crosslinking of high affinity IgE receptors (FceRI) or in response to other stimuli, basophils rapidly release mediators such as histamine and leukotrienes (Marone, 1988) as well as regulatory cytokines such as IL-4 (Arock et al., 1993; Brunner et al., 1993) and IL-13 (Gibbs et al., 1996; Li et al., 1996; Ochensberger et al., 1996). Organic extracts of DEPs have been shown to induce IL-4 expression and histamine release in highly enriched basophil populations, suggesting basophils to be a direct target for organic compounds present in diesel soot (Devouassoux et al., 2002).

We tested basophils from nonatopic as well as from allergic donors for synergy between AERex and allergen, because basophils from each donor group have differing activation requirements (Devouassoux et al., 1999). Our data demonstrate that both AERex extracts tested significantly enhance rBet v 1–induced basophil activation in all birch pollen–allergic subjects at ambient concentrations. This effect of AERex occurs in a dose-dependent manner. Synergy was only observed in basophils from atopic individuals, but not in healthy controls. This is in good accordance with studies reporting synergy of DEPs and antigen on Th2-type cytokine production after in vivo nasal or bronchial challenges in allergic subjects (Diaz-Sanchez et al., 1996, 1997). A similar effect of organic extract of diesel exhaust particles (DEPex) on allergen-induced degranulation of mast cells has been previously described (Diaz-Sanchez et al., 2000). Recent findings showed that DEPex is able to efficiently initiate IgE-independent basophil cytokine production and histamine release (Devouassoux et al., 2002). In contrast to this study, we used organic extracts of freshly sampled urban particulate matter (≤PM2.5) with different PAH content. Separation of urban PM10 into fine and coarse size particles revealed that the main proinflammatory response (TNF, IL-6, COX-2) in alveolar macrophages was driven by material present in the coarse PM, containing 90 to 95% of the stimulatory material in PM10 (Becker et al., 2005). Primary epithelial cells also responded to the coarse fraction with higher levels of IL-8 and COX-2 expression than that induced by fine or ultrafine PM. All size PM induced oxidant stress in epithelial cells. Compared to ambient PM, diesel PM induced only a minimal cytokine response in both alveolar macrophages and epithelial cells. Both coarse and

FIG. 3. AERex significantly enhances rBet v 1–induced basophil activation. Each column represents the mean ± SD of maximal dose-response relationships observed in three (AERex<sup>1d</sup>) and five (AERex<sup>5d</sup>) birch pollen–allergic individuals after treatment with AERex and rBet v 1.

FIG. 4. Dose-dependent enhancement of rBet v 1–induced basophil activation by AERex<sup>5d</sup>. Basophils of donor II were incubated with rBet v 1 and different concentrations of AERex<sup>5d</sup> (0.755–0.007 m<sup>3</sup>) to exemplify the dose-response relationship of AERex on rBet v 1–induced basophil activation. The mean ± SD of three independent experiments is shown, and results are expressed as the percentage of maximal CD63 expression (100%) upon stimulation with 0.3 ng/ml rBet v 1.
fine ambient air PM were also found to inhibit LPS-induced TNF release, while silica, volcanic ash, or carbon black had no inhibitory effect. Diesel particles did not affect cytokine mRNA induction nor protein accumulation but interfered with the release of cytokine from the cells. Ambient coarse and fine PM, on the other hand, inhibited both mRNA induction and protein production. Furthermore, Lin and coworkers (2005) found that nanoparticles contain more of traffic-related metals (Pb, Cd, Cu, Zn, Ba, and Ni) than particles of other sizes, although crustal metals accounted for over 90% of all the particulate metals. The Ag, Ba, Cd, Pb, Sb, V, and Zn contents in nanoparticles were strongly associated with diesel fuel. Divergent chemical composition and different distribution of fine and ultrafine particles in ambient PM and diesel PM might explain the absence of synergistic effects of DEPex and allergen on basophils in Devouassoux’s study (Devouassoux et al., 2002).

Strongest basophil activation was monitored upon stimulation with AERex, collected in winter 2003, which comprises a 20-fold higher total PAH content than AERex, collected in summer 2003. This suggests that the biological activity of AERex is markedly correlated to the content of certain organic components in urban aerosols, in particular PAHs. PAH concentrations fluctuate significantly over the year, with generally higher PAH levels in the winter months (Caricchia et al., 1999; Schauer et al., 2003). At low temperatures motor vehicles need a longer time to reach the optimum working temperature, which is characterized by an increased emission of PAHs (Lintelmann et al., 2005). In addition, significant changes of PAH levels at the end and beginning of the heating period indicate an influence of PAHs emitted by domestic heating. Inversion layers, which are typical for winters in the Munich area, inhibit exchange in the atmosphere, leading to accumulation of pollutants in the lowest layer (Lintelmann et al., 2005; Schauer et al., 2003). Furthermore, spring and summer in 2003 were extremely hot, leading to high UV radiation and high ozone concentrations. This caused photodegradation and oxidation of PAHs and easier atmospheric dispersion of pollutants (Lintelmann et al., 2005).

Overall, the composition of the organic phase of urban aerosols is dependent on seasonality of sampling, location, weather conditions, and other factors. This phase consists of over 20,000 different compounds, of which only one third could be identified to date. In our extracts, we can not exclude that other compounds than PAHs contribute to the observed effects. However, HPLC analysis of AERex and comparison with other studies investigating adjuvant effects of polyaromatic compounds (Bömmel et al., 2000; Kepley et al., 2003; Tsien et al., 1997) strongly indicate that, for the most part, PAHs contribute to the enhancement of rBet v 1–induced basophil activation in birch pollen–allergic patients. Since both organic extracts were sampled in different seasons and over different periods of time, this enhancement has to be considered a general effect of AERex on human basophils.

The bioavailability of PM-associated PAHs is known from cancer studies. To explore the basic relationship between a model PAH and a typical carrier particle, Gerde and coworkers (2001) investigated the rate and extent of release and metabolic fate of B[a]P adsorbed on the carbonaceous core of diesel soot. The native organic content of the soot had been denuded by toluene extraction. Exogenous B[a]P was adsorbed onto the denuded soot as a surface coating corresponding to 25% of a monomolecular layer. Dogs were exposed by inhalation to an aerosol bolus of the soot-adsorbed B[a]P. Following deposition in the alveolar region, a fraction of B[a]P was rapidly desorbed from the soot and quickly absorbed into the circulation. Release rates then decreased drastically. When coatings reached approximately 16% of a monolayer, the remaining B[a]P was not bioavailable and was retained on the particles after 5.6 months in the lung. However, the bioavailability of particles transported to the lymph nodes was markedly higher; after 5.6 months the surface coating of B[a]P was reduced to 10%. B[a]P that remained adsorbed on the soot surface after this period was approximately 30% parent compound. In contrast, the rapidly released pulse of B[a]P, which was quickly absorbed through the alveolar epithelium after inhalation, appeared mostly unmetabolized in the circulation, along with low concentrations of phase I and phase II B[a]P metabolites. The results indicate that absorption through the alveolar epithelium is an important route of entry to the circulation of organic compounds, in particular PAHs.

PAHs are bifunctional inducers that lead to the production of reactive oxygen species (ROS) through the induction of cytochromes P4501A (Prochaska and Talalay, 1988) and monofunctional inducers (quinones and derivatives) that can directly drive oxidative stress (Pinkus et al., 1996). ROS can activate a number of signaling pathways and cellular events. These effects are suspected to cause or to aggravate health impairments like chronic inflammatory processes or acute symptomatic responses in the respiratory tract (Dellinger et al., 2001; Li et al., 2002; Saxon and Diaz-Sanchez, 2005; Squadrito et al., 2001). The involvement of such metabolic pathways in human polymorphonuclear granulocytes has been previously reported (Devouassoux et al., 2002; Hitzfeld et al., 1997) and could also account for the modulating effects of AERex on human basophils (Kepley et al., 2003). Recent studies have shown that genetic variants of glutathione-S-transferases, which participate in ROS metabolism and detoxification of xenobiotics, affect susceptibility to DEP’s enhancement of allergic responses (Gilliland et al., 2004). This may also reflect on the cellular level of basophils we studied.

In summary, the capability of AERex to increase Bet v 1–induced basophil activation in birch pollen–allergic subjects at ambient concentrations suggests an important role of organic compounds, predominantly PAHs, in the maintenance and aggravation of IgE-mediated allergic diseases. Since basophils represent a major source of early IL-4 production in allergic
patients after specific antigen activation (Devouassoux et al., 1999), synergy between AERex and natural Bet v 1 exposure may also contribute to primary sensitization to other ubiquitous allergens.

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