Asthma is an inflammatory disease of the airways, and exacerbations of this disease have been associated with high levels of air pollution. The objective of this study was to examine whether ambient air pollution and/or allergen exposure induces inflammatory changes in the upper airways of asthmatics. Sixty patients with intermittent to severe persistent asthma visited the Hospital's Out Patient Clinic every 2 wk for a period of 3 mo, and on each visit a nasal lavage was obtained. Associations between nasal inflammatory parameters and seasonal allergens and/or air pollution exposures were analyzed using linear regression analysis. The study ran from July 3 to October 6, 1995, during which period ozone (8-h mean: 80 μg/m³) and PM₁₀ (24-h mean: 40 μg/m³) were the major air pollutants; the major aerallergen was mugwort pollen (24-h mean: 27 pollen grains/m³). Effects on both cellular and soluble markers in nasal lavage were demonstrated for both ozone and mugwort pollen, but not for PM₁₀. Ambient ozone exposure was associated with an increase in neutrophils (112% per 100 μg/m³ increase in 8-h average ozone concentration), eosinophils (176%), epithelial cells (55%), IL-8 (22%), and eosinophil cationic protein (ECP) (19%). Increases in environmental mugwort pollen counts were associated with an increase in nasal eosinophils (107% per 100 pollen/m³) and ECP (23%), but not with neutrophils, epithelial cells, or IL-8. This study demonstrated that both ambient ozone and allergen exposure are associated with inflammatory responses in the upper airways of subjects with asthma, although the type of inflammation is qualitatively different. Hiltermann TJN, de Bruijne CR, Stolk J, Zwinderman AH, Spieksma FThM, Roemer W, Steerenberg PA, Fischer PH, van Bree L, Hiemstra PS. Effects of photochemical air pollution and allergen exposure on upper respiratory tract inflammation in asthmatics. AM J RESPIR CRIT CARE MED 1997;156:1765-1772.
inflammatory parameters in the nose and exposure to ambient air pollution and allergen.

METHODS

Subjects

Two hundred seventy asthmatic patients from the Pulmonary Out Patient Clinic of the Leiden University Hospital were asked to complete a questionnaire form in the late spring of 1995. From the responding subjects (150), 62 patients with intermittent to severe persistent asthma (GINA criteria) were recruited (16). We selected our subjects based on a clinical history of asthma and airway hyperresponsivity (PC_{20} to methacholine < 9.6 mg/ml). Two subjects were excluded from the analysis because they dropped out of the study for personal reasons within the first 14 d. All II subjects completed the study (for subject characteristics, see Table 1). The study was approved by the Medical Ethical Board of Leiden University Medical Centre, and informed consent was obtained from each subject.

Study Design

A II nonsmoking subjects willing to participate were screened for their atopic status and hypersensitivity to methacholine. Subjects were instructed on how to use a peak flow meter and how to fill in a diary card. Subjects were monitored for 3 mo (July 3 to October 6, 1995). During this period they were asked to record in the diary: morning and evening peak flow, respiratory symptoms, exposure to environmental tobacco smoke, and medication use. Every 2 wk subjects visited the Out Patient Clinic, on which occasion a nasal lavage was performed.

Atopic Status

The atopic status of all participants was determined by measuring specific IgE levels to a panel of common inhalant allergens (Pharmacia CAP System; Pharmacia AB, Uppsala, Sweden) in the patient’s serum. If this measurement indicated that a subject was atopic, the specific IgE-response to relevant individual inhalant allergens was determined (Pharmacia CAP System).

Pulmonary Function Measurements and Bronchoprovocation Testing

Spirometry was performed on a dry rolling-seal spirometer (Morgan Spiroflow, Rainham, UK), according to recent standardization recommendations (17). Inhalation challenge tests were performed by using doubling doses of methacholine bromide (Janssen Pharmaceutica, Beerse, Belgium) inhaled by the standardized 2-min tidal breathing method (18). The response was measured by FEV_{1}. The tests were discontinued if FEV_{1} decreased by more than 20% from baseline or when a concentration of 9.6 mg/ml (≈ 40 mM) had been administered. A fter the last methacholine dose, 400 μg salbutamol were inhaled per metered-dose inhaler.

Subjective Symptoms

For the present study, a diary was used to record symptoms, exposure to environmental tobacco smoke, and medication use that was based on a diary used in previous epidemiologic studies in The Netherlands (19). Medication use was subdivided into: inhaled (lung) steroid use, nasal steroid use, short-acting bronchodilators, and other medication use.

Nasal Lavage

A II nasal lavages were performed in the morning, between 8:30 and 12:00 a.m. Subjects always attended the Out Patient Clinic at the same time of day. Nasal lavage was performed as described previously (20). Each nostril was lavaged with 10 ml phosphate-buffered saline (PBS). The lavement was expelled over a sieve, collected in a Falcon tube (Tube no. 1), and kept on ice. The nasal mucus that stayed behind in the sieve was treated with 6 ml Sputolysin (Calbiochem Corp., La Jolla, CA) diluted to 0.05% w/vol dithirotol, and collected in a separate Falcon tube (Tube no. 2). Tube no. 2 was subsequently placed in a shake water bath at 37 °C for 15 min. Immediately thereafter both Tubes no. 1 and no. 2 were centrifugated at 250 × g for 10 min. The supernatant of Tube no. 1 was aspirated and stored at −70 °C pending analysis of proteins. The supernatant of Tube no. 2 was discarded. Both pellets were resuspended and pooled together, and total cell counts were determined by an automated cell counter (Coulter Counter model ZM; Coulter Electronics, Bedfordshire, UK) to obtain an estimate of the total number of cells. Subsequently, the sample was diluted with PBS to a final concentration of 0.4 × 10^6 cells/ml. Fifty microliters per bucket of this suspension was used to prepare cytospin preparations (Shandon Southern Instruments, Sewickley, PA). Differential cell counts of eosinophils, neutrophils, lymphocytes, macrophages, and epithelial cells were performed on cytocpins stained with Diff-Quik (Dade Int. Inc., Deerfield, IL). In each sample, at least 400 nucleated cells were counted and the percentage of each cell type was calculated.

Total protein was determined using the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as the standard. The IL-8 levels were determined by enzyme-linked immunosorbet assay (ELISA; range, 0.24 to 3.75 pg/ml; CLB, Amsterdam, The Netherlands), and eosinophil cationic protein (ECP) levels were determined by fluoroimmunosay (FEIA) using the Pharmacia CAP System (range, 2 to 200 ng/ml; Pharmacia) according to the manufacturer’s directions.

Air Pollution and Allergen Data

A ir pollution and meteorologic data were taken from the Dutch National Air Quality Monitoring Network operated by the National Institute of Public Health and the Environment in Bithoven, The Netherlands, as described previously (19). Data from three stations were obtained (De Zilk, Zegveld, and The Hague Center). All II patients lived within the area covered by these three stations. Because the correlation of the level of the air pollutants between the stations was high (r > 0.88), the pollutant levels of Zegveld only were used in the analysis. M easured air pollutants were: ozone, black smoke, SO_2, PM_{10}, and NO_2. For ozone the maximal 8-h moving average, and for all other air pollutants the 24-h mean was used in the analysis (1). During the study period relevant allergens were grass pollen (Poaceae) and mugwort pollen (A rtemisia vulgaris) (21). The pollen data, obtained with a Burkard pollen sampler placed at rooftop level in Leiden, were used in the analysis as 24-h mean airborne pollen concentrations. For mugwort pollen the data were adjusted for ground-level concentrations (22), by multiplication with a factor 4.

Analysis

D ata were analyzed using SA S (Statistical Analysis System version 6.08; SA S Institute Inc., Cary, NC). Because all inflammatory parameters demonstrated a skewed distribution, these data were first natural log (ln) transformed. The association between air pollutants and inflammatory parameters was analyzed using air pollution data of the first day and the mean levels of the first two and three days (Lag1,
Mean2, and Mean3), prior to the day that the nasal lavage was performed, and the average air pollutant concentration of the previous week. We analyzed the association between air pollutants and inflammatory parameters using a linear regression model, accounting for repeated measurements (Proc Mixed) (23). The hereby estimated population mean of the regression slope parameter was transformed into an "estimated effect." Because the data on inflammatory parameters were log-transformed in the regression model, the inverse log of the regression slope parameter was used and expressed as estimated effect in percent change of the population mean value. The estimated effect was calculated over the range of the analyzed pollutants and allergens. Therefore, the estimated effect was expressed as percent change per 100 μg/m³ for ozone, 50 μg/m³ for PM₁₀, and 100 grains/m³ for mugwort pollen for Lag1. When calculated over periods of a week the ranges of air pollutants and allergen concentrations were about half those based on daily values. In order to be able to compare the effect of Lag1 with those of 1 wk, the estimated effect for the average weekly air pollutant concentration was expressed per 50 μg/m³ for ozone, 25 μg/m³ for PM₁₀, and 50 grains/m³ for mugwort pollen. In addition, the estimated effect for the levels of Mean2 and Mean3 were expressed per 90 μg/m³ and 75 μg/m³ for ozone, per 35 μg/m³ and 30 μg/m³ for PM₁₀, and per 95 grains/m³ and 90 grains/m³ for mugwort pollen, respectively.

Confounders considered were time trends in inflammatory parameters, exposure to aeroallergens (grass or mugwort pollen), exposure to environmental tobacco smoke, and nasal steroid use. Differences at p values < 0.05 were considered statistically significant.

**RESULTS**

**Nasal Lavage**

For each subject an average of seven nasal lavages (range, six to nine) were performed, with a total of 421 nasal lavages during the study period. The study ran for a total of 96 d, of which on 70 d nasal lavages were performed. The mean volume of recovery was 12.2 ml (range, 4.6 to 17.6). IL-8 (geometric mean, 365 pg/ml; range, 16 to 6,140) and total protein (geometric mean, 187 μg/ml; range, 63 to 1,388) were measurable in all samples. ECP (geometric mean, 4.4 ng/ml; range, < 2.0 to 82.2) could be determined in 277 samples; in 144 samples the level was below the detection limit of the assay. A value of 2.0 ng/ml was used in the analysis for these samples. The main cell types in the lavage fluid were neutrophils (geometric mean, 2.1 × 10⁶/ml; range, 0 to 4.8 × 10⁶), epithelial cells (geometric mean, 1.7 × 10⁵/ml; range, 0 to 8.8 × 10⁵), and eosinophils (geometric mean, 405/ml; range, 0 to 1.4 × 10⁵) (Table 2). The numbers of lymphocytes and monocytes were too low to count (demonstrable in less than 22 and 2% of the nasal lavages, respectively), and were therefore left out of the analysis. There was a marked correlation between the log mean individual number of neutrophils and the log mean individual IL-8 level (r = 0.69; p < 0.01) (Figure 1).

**Air Pollution and Allergen Data**

The maximal 8-h moving ozone average, the 24-h average concentration levels of PM₁₀, and mugwort-pollen counts during the study period are shown in Figure 2. Ozone was the most prominent air pollutant present during the study, with 16 d that exceeded the European WHO G guideline for the maximal 8-h moving average of 120 μg/m³. PM₁₀ was moderately elevated (mean, 40 μg/m³; 95% CI, 21 to 76). The daily average concentrations of NO₂, black smoke and SO₂ were all low (maximal detected levels < 43, < 23, and < 17 μg/m³, respectively). Therefore, these data were not included in the analysis. There was a relatively strong correlation between the levels of ozone and PM₁₀ (r = 0.64). Both ozone and PM₁₀ levels were highly correlated with ambient maximal temperature (r ≈ 0.70). Ozone levels showed a stronger correlation with mugwort pollen (r = 0.57) than PM₁₀ (r = 0.23). During the study period the grass-pollen season was in its ending stage and counts were generally low (mean, 29 grains/m³; 95% CI, 0 to 145). One grass-pollen peak was observed at the start of the study period (July 5 to 11; maximum, 253 grains/m³). The distribution of mugwort pollen during the study demonstrated at least three discrete peaks (mean, 27 grains/m³; 95% CI, 0 to 155) (Figure 2).

**Nasal Lavage and Ambient Air Pollution and/or Allergen Exposure**

The inflammatory parameters in the nasal lavage tended to increase during the study period (as illustrated for neutrophils...
and eosinophils in Figure 3); therefore, a linear correction for a
time trend (day number) was made in the linear regression
model. The estimated effect, of an increase of 100 μg/m³ (for
Ozone Lag1), 90 μg/m³ (for Ozone Mean2), 75 μg/m³ (for
Ozone Mean3), or 50 μg/m³ (for Ozone Week) in the 8-h aver-
age ozone concentration is depicted in Table 3. Ozone was asso-
ciated with both a neutrophilic and eosinophilic inflammation,
with the strongest effect on cellular parameters with ozone
levels of the previous day (112 to 176% increase in neutrophils
and eosinophils, respectively) (Figure 4), and strongest effect
on soluble mediators with mean ozone levels of 3 d before (22
and 19% increase in IL-8 and ECP, respectively). Ozone lev-
els were also associated with increased amounts of epithelial
cells (largest effect 104% increase with mean ozone levels of
2 d prior to the nasal lavage). The mean ozone concentration
of the week was significantly associated with neutrophils, ep-
ithelial cells, and IL-8, but the effects were generally smaller.
Mugwort pollen counts were primarily associated with an
eosinophilic inflammation (Table 4). The strongest effects of
mugwort pollen on eosinophils were demonstrable with Lag1
(107% increase for an increase of 100 grains/m³). The most
consistent effects of mugwort pollen were demonstrable on
ECP levels, with the maximal effect observed with mugwort
Mean2 prior to the nasal lavage (23% increase) (Figure 5).
PM₁₀ was not associated with a change in inflammatory pa-
rameters, except for an association of epithelial cell numbers
with the mean weekly PM₁₀ levels (44% increase) (Table 5).

Nasal steroid use and allergen exposure are possible con-
founders that may influence the observed association of in-
flammatory parameters with ozone. After adjustment for these
possible confounders, the estimated effects of ozone on the
eosinophilic inflammatory reaction were no longer significant,

Figure 2. Daily ambient ozone (closed circles), PM₁₀ (open triangles),
and mugwort pollen (open circles) during the study period. Data
are presented as mean 24-h values (12 midnight to 12 midnight)
for PM₁₀ and mugwort pollen. For ozone, data are presented as 8-h
moving average.

Figure 3. Daily mean neutrophil and eosinophil number during the study period; representative figure for
all inflammatory parameters.
although the magnitude of the effect was still considerable (91% increase for eosinophils) (Table 6). In contrast, the effect of ozone on IL-8 levels and neutrophil and epithelial cell numbers was largely unaffected by correction for these confounders. We also considered the possibility that ozone exposure and nasal steroid use had affected the observed associations with mugwort pollen. After adjustment for these confounders, the association of mugwort pollen with nasal ECP levels was not affected, but the effects on eosinophil numbers were no longer significant (data not shown).

**DISCUSSION**

The results from the present study demonstrate an association of inflammatory parameters in nasal lavage of patients with intermittent to severe persistent asthma with ambient ozone and allergen exposure, but not with exposure to PM$_{10}$. The inflammatory profile observed in association with ozone was markedly different from that associated with allergen exposure. Whereas ozone exposure was associated with both an increase in neutrophils, eosinophils, and epithelial cells, allergen exposure was mainly associated with increased eosinophil numbers and ECP levels. There was no marked difference between the magnitude of the effects of ozone or allergen on nasal lavage eosinophil numbers or ECP levels.

To our knowledge, this is the first epidemiologic study in patients with intermittent to severe persistent asthma to demonstrate a difference between the inflammatory reaction of the upper airways associated with ozone and that associated with allergen exposure. In line with our observations, increased neut-
Eosinophil numbers and epithelial shedding have been shown to be associated with ambient ozone exposure in healthy children (11) and adults (10). However, the effects of air pollution on the eosinophil inflammatory response have only been demonstrated in controlled experimental settings so far (8, 24), except for a report by Frischer and colleagues (11) demonstrating increased nasal lavage ECP levels in children on days with high versus those with low ozone levels. If we stratify our data on mugwort sensitization (25% sensitized, see Table 1), no major difference in the nasal inflammatory response was observed for ozone and mugwort pollen exposure between the two subgroups (data not shown). Yet, the mugwort-sensitized subjects had a significant two-fold higher eosinophil number during the study period than did the asthmatics who were not sensitized to mugwort pollen (data not shown). Our observation that the effects of either ozone or mugwort pollen on levels of the neutrophil chemoattractant chemokine IL-8 in nasal lavage are limited is in line with previous observations after laboratory exposure to allergen and/or ozone (8). Despite these limited effects, we did observe a strong correlation between neutrophil numbers and levels of IL-8 in nasal lavage. This is in line with the in vivo neutrophil chemoattractant activity of IL-8 that was demonstrated by an increase in neutrophil numbers in both nasal lavage and mucosa after intranasal instillation of IL-8 (25). Finally, a similar association as found in the present study between nasal lavage IL-8 levels and nasal leukocyte counts has been demonstrated after a controlled experimental exposure to ozone in asthmatics (9). Whereas such associations can be explained by the neutrophil chemoattractant activity of IL-8, they might also be explained by the fact that neutrophils may be a source of IL-8 themselves (26). We did not observe an association between ozone and nasal lavage total protein levels. Others have reported an increase in total protein in upper or lower airway lavage after

**TABLE 5**

<table>
<thead>
<tr>
<th></th>
<th>PM10</th>
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<tbody>
<tr>
<td>Lag1</td>
<td>Mean2</td>
</tr>
<tr>
<td>Total protein</td>
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</tr>
<tr>
<td>IL-8</td>
<td>-12</td>
</tr>
<tr>
<td>ECP</td>
<td>9</td>
</tr>
<tr>
<td>Neutrophils</td>
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</tr>
<tr>
<td>Eosinophils</td>
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<tr>
<td>Epithelial cells</td>
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<tr>
<td>Total cells</td>
<td>14</td>
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</tbody>
</table>

**TABLE 6**

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<td>Lag1</td>
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</tr>
<tr>
<td>IL-8</td>
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</tr>
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<td>ECP</td>
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</tr>
<tr>
<td>Neutrophils</td>
<td>110†</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>91</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>63‡</td>
</tr>
<tr>
<td>Total cells</td>
<td>34</td>
</tr>
</tbody>
</table>

For definition of abbreviations, see Tables 2 and 3.

‡ p < 0.05.

† p < 0.01.

‡ p < 0.05.

For eight-hour maximal ozone concentration: mean, 80 µg/m³; range, 12 to 185 µg/m³. Estimated effect = % change per 100 µg/m³ (for Lag1), 90 µg/m³ (for Mean2), 75 µg/m³ (for Mean3), or 50 µg/m³ (for Week) increase in O3 concentration.
exposure to ozone levels above the ambient level (5, 8), but these changes were not observed at ambient levels (27, 28). A t these levels, IL-8 (28) and neutrophil numbers (27) appeared to be more sensitive markers for ozone exposure, which is in accordance with our observation. Therefore, this study extends previous data demonstrating that ambient air pollution may induce damage to the nasal epithelium and increases both neutrophil and eosinophil numbers and IL-8 and ECP levels in the upper airways of patients with asthma.

During this study there was a trend toward an increase in inflammatory parameters in the nasal lavage (Figure 3). This may have been due to increases in other relevant environmental exposures such as viruses and house dust mite during the course of the study. However, this study was not designed to study the effects of these exposures. We addressed the potential bias introduced by this trend by taking the study day into account in our linear regression model. A nother confounder may have been nasal steroid use during the study period. Steroids are likely to dampen the inflammatory process (29, 30) and thereby the associations found in this study. Therefore, if anything, the observed association may be an understimation of the inflammatory response. In the final analysis (Table 6) we corrected for both allergen exposure and steroid use, although steroid use did not appear to affect the observed associations dramatically (data not shown). Despite the large within-subject variability in inflammatory parameters observed in this study (Table 2), we detected distinct signals from ozone, PM$_{10}$, and mugwort pollen exposure. Therefore, we do not think it likely for chance alone to explain the observed associations.

Whereas we could not demonstrate effects of PM$_{10}$ on nasal inflammatory parameters, animal experiments suggest that ambient PM$_{10}$ samples collected from various sites are able to induce oxidative and inflammatory reactions both in vivo and in vitro (31, 32). In the present study our volunteers may not have been exposed intensively to those ambient PM$_{10}$ fractions responsible for inflammatory reactions, e.g., to PM$_{10}$ fractions from urban areas with high (diesel) traffic emissions. A nother alternative may be that PM$_{10}$ is not sufficiently deposited in the nasal mucosa but passes through the nose and precipitates into the lung.

This study is the first epidemiologic study demonstrating an association of ambient ozone exposure with both a neutrophil and an eosinophil influx in asthmatics. It has to be noted that after adjustment for mugwort exposure an effect of ambient ozone exposure on eosinophil influx was still demonstrable; however, this effect was no longer significant. Epidemiologic studies have demonstrated an inflammatory reaction mainly dominated by neutrophils in both healthy children (11, 33) and adults (10). In addition, most controlled experimental studies demonstrating an ozone-induced neutrophilic inflammatory reaction in the lungs have been carried out in healthy subjects (1, 6, 7). However, recent studies have demonstrated that subjects with asthma have a greater (5, 9) and different ozone-induced inflammatory reaction (8, 34, 35) as compared with healthy subjects. In contrast to healthy volunteers, in asthmatics both a neutrophil and an eosinophil influx have been demonstrated in BAL (34, 35) and nasal lavage (8) after ozone exposure. In vitro, exposure to ozone of human epithelial cell lines results in an increase in the production of IL-8 and other proinflammatory mediators (1). Recent in vitro studies demonstrate that supernatants obtained from ozone-exposed bronchial epithelial cell cultures induce an increased chemotaxis and adherence of eosinophils to endothelial cells (24). This effect can be attenuated significantly by the addition of anti-GM-CSF, anti-IL-8, and anti- RANTES neutralizing monoclonal antibodies, suggesting that these cytokines may be produced upon ozone exposure (24). In addition, Peden and colleagues (8) have elegantly demonstrated in vivo that the influx of eosinophils can be attributed to a direct effect of ozone. Moreover, they demonstrated that there may be a synergistic effect of allergen and ozone exposure on both eosinophil influx and activation (8). In the present study we did not attempt to distinguish between these two phenomena, although both events will have occurred. On the basis of our data, we speculate that ozone and allergen differentially regulate eosinophil influx and activation. This is suggested by the more consistent effect of mugwort pollen exposure on ECP levels than ozone exposure, whereas the eosinophil influx demonstrated a similar trend for both mugwort pollen and ozone exposure. An interesting finding is that exposure to ozone prior to an allergen challenge enhances bronchial responsiveness to inhaled allergen in both subjects with asthma and those with rhinitis, but not in healthy control subjects (12). Short-term increases in airway inflammation can be triggered by many different irritants, including indoor and outdoor allergens, viruses, cigarette smoke, and air pollutants such as ozone (16). The higher levels of inflammatory mediators at the end of the study period, when ozone and mugwort pollen levels were low, suggest that other factors such as viruses and house dust mite exposure may be more clinically relevant. The contribution of air pollution relative to other stimuli that may induce an inflammatory reaction in the airways of patients with asthma is difficult to assess.

In the present study we were able to demonstrate that ambient ozone and allergen exposures result in distinct inflammatory changes within the nose. Nasal eosinophils and ECP levels may be useful markers for allergen exposure, but also ambient ozone exposure, to direct anti-inflammatory therapy for asthma in the future. We propose that inflammatory changes in nasal lavage may yield information relevant to the events occurring in the lower airways. Hence, we speculate that during episodes with both increased allergen levels and high ambient photochemical air pollution, asthma exacerbations are more likely to develop than during periods with either increased allergens or ambient photochemical air pollution alone.

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