Black-Pigmented Material in Airway Macrophages from Healthy Children: Association with Lung Function and Modeled PM$_{10}$

Jonathan Grigg, Neeta Kulkarni, Nevil Pierse, Lesley Rushton, Christopher O’Callaghan, and Andrew Rutman
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Black Material in Airway Cells from Healthy Children: Association with Lung Function and Modeled Levels of Particulate Matter

BACKGROUND

In the 1990s, several epidemiologic and controlled exposure studies suggested an association between exposure to air pollution from traffic-derived particulate matter (PM) — in particular the fraction of PM that is inhalable (i.e., has an aerodynamic diameter ≤ 10 µm [PM_{10}]) — and increases in symptoms of airway diseases, including exacerbations of asthma. Some studies had also suggested that exhaust from diesel engines — which are used in a large fraction of vehicles worldwide and particularly in Europe — contributed to these effects.

To address more fully the possible association between exposure to PM and the exacerbation of asthma and airway allergic conditions, HEI issued a request for applications, RFA 00-2, Effects of Diesel Exhaust and Other Particles on the Exacerbation of Asthma and Other Allergic Diseases. In response, Professor Jonathan Grigg, University of Leicester, United Kingdom, submitted an application titled “The relationship between pollutant particles in alveolar macrophages from normal children and proxy markers of PM_{10} exposure.” Professor Grigg proposed to evaluate whether the quantification of particles in airway macrophages — the principal cell type that ingests (or phagocytoses) agents that enter the airways — could be used as a marker of children’s exposure to PM_{10}. Professor Grigg also proposed to collect the airway macrophages by a noninvasive technique, the induction of sputum. HEI’s Research Committee recommended Professor Grigg’s proposal for funding; although the proposal was not directly responsive to the RFA, committee members thought the possible development of a noninvasive biomarker of exposure to PM would be useful for future studies of air pollutant effects.

AIMS

Grigg and colleagues’ primary hypothesis was that the level of particles detectable in the airway macrophages of healthy children correlates with modeled estimates of local, traffic-derived PM_{10} at the children’s home addresses. They were specifically interested in carbonaceous particles because carbon is a major component of particles derived from combustion sources such as traffic. A secondary objective was to determine whether the level of carbonaceous particles detected inside airway macrophages could be correlated with markers of airway inflammation. Although it was not part of their original aims, Grigg and colleagues also evaluated whether the area of carbonaceous particles inside the children’s macrophages correlated with several pulmonary function parameters they had measured. Because they did not definitively determine that the particulate material inside the macrophages was carbonaceous, the investigators refer to it as “black-pigmented material” in the Investigators’ Report.

Thus the report focuses on two associations: the association between the amount of black-pigmented material detected in airway macrophages and modeled estimates of locally derived PM_{10} at the child’s home address; and the association between the amount of black-pigmented material detected in airway macrophages and pulmonary function.

APPROACH

Grigg and colleagues recruited 116 healthy children aged 8 to 15 years in and around the city of Leicester, United Kingdom. To increase the possibility of detecting a correlation between modeled exposure and particles detected in macrophages, the investigators selected children with widely different...
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mobile-source-derived PM$_{10}$ exposures: the highest-exposure group (> 3.82 µg/m$^3$) and the lowest-exposure group (≤ 2.3 µg/m$^3$). The investigators measured baseline lung function in each participant: forced expiratory volume in 1 second (FEV$_1$), forced vital capacity (FVC), the ratio of FEV$_1$ to FVC (FEV$_1$/FVC), and forced expiratory flow between 25% and 75% of the forced vital capacity (FEF$_{25%-75%}$). They calculated the percentage of predicted values for each child, taking into account the child’s age, sex, ethnic origin, height, and weight. Based on the answers to a questionnaire they gave to the participants about their physical activity over the previous two weeks, the investigators calculated an activity score for each child.

Using nebulized hypertonic saline, Grigg and colleagues induced sputum in the participants and obtained 66 adequate samples for the study of airway macrophages. They excluded data from two children living at the same address because these children had much greater areas of black material in their macrophages than other children in the study. Of the remaining 64 participants, 40 identified themselves as white (i.e., they had parents of European extraction), 22 as Asian (i.e., their parents were from the Indian subcontinent), and 2 as “other.”

The investigators prepared slides of the sputum-derived cells and performed a differential cell count for leukocyte subsets. Leukocytes, and macrophages in particular, were further characterized on slides by light microscopy. Grigg and colleagues captured and analyzed two-dimensional images of 100 macrophages, deleting the nucleus from each image because the image-analysis software identified it as a large particle. They then calculated the total area of particles inside each cell in micrometers squared. They also measured levels of the cytokine interleukin-8 (IL-8) — a neutrophil chemoattractant — in the supernatant of the sputum samples.

For each child at his or her home address, Grigg and colleagues estimated an annual exposure to PM$_{10}$ derived from mobile sources. Estimates were obtained using the Airviro dispersion model, version 2.21, a geographic-information-system-based software that integrates meteorologic data and data on emissions of pollutants from different types of sources — specifically, point (such as industrial or commercial facilities), line (roads), and area (such as residential or large industrial estates) sources. For this study, only concentrations of PM$_{10}$ from roads were estimated.

Grigg and colleagues used linear regression to investigate the relationships between the variables of interest. The response variable for most regressions was either modeled exposure or a measure of lung function, such as FEV$_1$, and the predictor variable of interest was the median area of black material. They also assessed results using a nonparametric test — the Spearman rank correlation test.

RESULTS AND INTERPRETATIONS

The investigators’ main findings regarding their hypotheses were as follows:

- A weak correlation was found between particles detected in sputum-derived macrophages and annual PM$_{10}$ exposure at the child’s home; and
- No correlation was found between particles detected in sputum-derived macrophages and any marker of airway inflammation (the level of IL-8 and the percentage of neutrophils and eosinophils measured in sputum).

An additional and potentially important finding of the study was a negative correlation between the area of particles in children’s sputum-derived macrophages and both FEV$_1$ and FEF$_{25%-75%}$. A 1-µm$^2$ increase in the area of black material inside macrophages was associated with a decrease of 17% in predicted FEV$_1$ and 35% in FEF$_{25%-75%}$.

DISCUSSION AND CONCLUSIONS

In this study, Grigg and colleagues attempted to establish whether carbon particles found in the airway macrophages of healthy children in Leicester, England — a city with little or no heavy industrial sources of pollution — could be used as a biomarker of exposure to traffic-related PM$_{10}$.

Using light microscopy to identify black areas, presumably particles, in airway macrophages obtained by sputum induction, Grigg and colleagues found a weak correlation between the area of particles and estimates of annual PM$_{10}$ exposure at the child’s home address. At face value, these findings suggest that particles detected inside airway macrophages have the potential to be a useful marker of exposure to PM. However, several issues of study design and interpretation of data
suggest that the study’s main findings should be interpreted cautiously: one important uncertainty is the accuracy of the estimates of individual PM$_{10}$ exposures obtained by using the Airviro dispersion model without validating this approach. A second important issue is that the findings may be confounded by ethnic origin, that is, that children of Asian origin — one of the two major subgroups of study participants, who as a group may have had different lung function — may have had higher modeled exposure to PM and levels of particles in the macrophages than white children, the other major subgroup of participants. In addition, although obtaining sputum-derived macrophages is noninvasive and these macrophages may be more easily obtained than cells deeper in the lungs, they may not be the cells that most accurately reflect an individual’s exposure to PM$_{10}$. Particles detected in macrophages obtained from lower in the airways, particularly in the alveolar region, may be a more appropriate reflection of particle load. Furthermore, the investigators did not establish that the particles found inside the macrophages were carbonaceous or derived from traffic or, indeed, any other outdoor combustion source.

The investigators’ attempt to determine whether particles detected in airway macrophages correlated with markers of airway inflammation was worthy. The fact that the investigators did not find associations between the area of particles in airway macrophages and any marker of airway inflammation, however, was perhaps not surprising, because one-time measures of these markers are likely to be more variable within individuals than measures of pulmonary function, such as FEV. In addition to the possibility that there is no correlation with the markers assessed, other possible explanations include that the sampling methods were too insensitive or inadequate to detect changes in the levels of markers or that the level of exposure to pollution of the children in this study was too low for any inflammatory effects of particles to be observed in the airways.

The investigators’ finding of a negative correlation between the area of particles in the children’s macrophages and the pulmonary function parameters FEV$_1$ and FEF$_{25\%–75\%}$ is potentially important. At face value, it suggests that greater exposure to PM may lead to impairment of children’s lung function. Although the investigators did not specifically address the issue, particles identified in airway macrophages are likely to reflect long-term exposure to air pollution. Thus, Grigg and colleagues’ finding is consistent with other data suggesting that long-term exposure to air pollutants affects the development of children’s lung function. However, the magnitude of the changes in pulmonary function associated with increased particle area that Grigg and colleagues reported appears surprisingly large, casting doubt on the results of their regression modeling.

The main question explored by Grigg and colleagues — whether particles inside airway macrophages may be used as a biomarker of exposure to PM$_{10}$ (and, in particular, traffic-derived PM$_{10}$) — remains interesting and important. However, given the caveats regarding the study design and the interpretation of the results discussed here, this study has not answered the question. Nevertheless, the potential importance of the study’s main finding — that there are associations between particles detected in airway macrophages and a reduction in key lung function parameters — suggests that further studies are needed to investigate the reported associations.
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Jonathan Grigg, Neeta Kulkarni, Nevil Pierse, Lesley Rushton, Christopher O’Callaghan, and Andrew Rutman

Institute of Cell and Molecular Science, Barts and the London School of Medicine and Dentistry, London, United Kingdom; Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, United Kingdom; Department of Public Health, Wellington School of Medicine, University of Otago, New Zealand; Department of Epidemiology and Public Health, Imperial College London, London, United Kingdom

HEI STATEMENT

This Statement is a nontechnical summary of the Investigators' Report and the Health Review Committee's Critique.

INVESTIGATORS’ REPORT

When an HEI-funded study is completed, the investigators submit a final report. The Investigators’ Report is first examined by three outside technical reviewers and a biostatistician. The report and the reviewers’ comments are then evaluated by members of the HEI Health Review Committee, who had no role in selecting or managing the project. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, if necessary, revise the report.

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CRITIQUE  Health Review Committee

The Critique about the Investigators’ Reports is prepared by the HEI Health Review Committee and staff. Its purpose is to place the studies into a broader scientific context, to point out strengths and limitations, and to discuss remaining uncertainties and implications of the findings for public health.

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Black-Pigmented Material in Airway Macrophages from Healthy Children: Association with Lung Function and Modeled PM\textsubscript{10}

Jonathan Grigg, Neeta Kulkarni, Nevil Pierse, Lesley Rushton, Christopher O’Callaghan, and Andrew Rutman

ABSTRACT

Epidemiologic studies in children suggest that chronic inhalation of carbonaceous particulate matter \(\leq 10 \mu m\) in aerodynamic diameter (PM\textsubscript{10}*) attenuates the normal growth of lung function. However, the relation between markers of PM\textsubscript{10} exposure and the quantity of particles entering the pediatric airway is unclear. Experimental studies have shown that particles entering the lower airway remain visible in the cytoplasm of airway macrophages (AMs) for several months. We hypothesized that particle loading of AMs, detected as black-pigmented material, reflects individual exposure of healthy children to PM\textsubscript{10}. In this study, we aimed to establish the relation between the median area of black material in AMs (measured as the two-dimensional area of black material [“black area”] per AM per child) and (1) lung function, and (2) level of primary PM\textsubscript{10} at the child’s home address as estimated by dispersion modeling (referred to as “modeled primary PM\textsubscript{10}”). We also performed a series of exploratory analyses assessing the association between the median black area in AMs and (1) variables that could modify individual exposure, and (2) airway inflammation. To achieve these aims, AMs were sampled using induced sputum from children in Leicestershire, United Kingdom, and lung function was determined by spirometry. Data from 64 of 116 children who provided adequate induced sputum samples were analyzed. The area of the black material in AMs was determined by an analysis of digitized light-microscopic images of 100 randomly chosen AMs per child. There was a significant inverse association between size of black area in AMs and lung function: each 1.0-\(\mu m^2\) increase in the area of the black material in AMs was associated with a 17.0% (95% confidence interval [CI], 5.6 to 28.4) reduction in forced expiratory volume in one second (FEV\textsubscript{1}), a 12.9% (95% CI, 0.9 to 24.8) reduction in forced vital capacity (FVC), and a 34.7% (95% CI, 11.3 to 58.1) reduction in forced expiratory flow between 25% and 75% of forced vital capacity (FEF\textsubscript{25-75}). These associations were not affected by bronchodilator treatment. There was also an association between modeled exposure to primary PM\textsubscript{10} and area of black material in AMs: each 1.0-\(\mu g/m^3\) increase in primary PM\textsubscript{10} was associated with an increase of 0.10 \(\mu m^2\) (95% CI, 0.01 to 0.18) in black area in AMs. There was no significant association between the median black area in AMs and age, height, weight, sex, activity level, and levels of neutrophilic airway inflammation in the induced sputum. We conclude that the median area of black material in AMs in children is a promising marker of individual exposure to carbonaceous PM\textsubscript{10} and that our data strengthen the epidemiologic data suggesting that PM\textsubscript{10} impairs the growth of lung function in children.

INTRODUCTION

Epidemiologic studies have shown a consistent adverse effect of particulate air pollution on the cardiovascular health of adults (as reviewed by the U.S. Environmental Protection Agency in 2004). In children a major concern is that particulate pollution may impair lung development (Schwartz 2004). Two recent studies have provided insights into the effects of particulate matter with an aerodynamic diameter of 10 \(\mu m\) or less or 2.5 \(\mu m\) or less (PM\textsubscript{10} and PM\textsubscript{2.5}) on pediatric lung function. Ward and Ayres (2004) showed, in a systematic review of 22 panel studies in children 6 to 11 years of age, that short-term variations in levels of ambient PM\textsubscript{10} or smaller are associated with
changes in peak expiratory flow rate. Looking at long-term effects, Gauderman and colleagues (2004) reported that children living in communities with high mean levels of PM$_{2.5}$ have decreased growth of FEV$_1$ as a percentage of the predicted value, FVC, and FEF$_{25-75}$. The mechanism for these long-term effects is unclear, but they may reflect a reduction in the number of alveoli or the chronic inflammation of airways (Gauderman et al. 2004).

PARTICLES IN AIRWAY MACROPHAGES

The capacity of AMs to phagocytose and store particles over time has been used to detect occupational exposure to hazardous dusts (Rainey et al. 1994; Fireman et al. 1999), and there is evidence from several studies in animals and humans to suggest that the number of particles in AMs increases with increases in the inhaled dose. For example, Finch and colleagues (2002) exposed rats to four dilutions of biodiesel emissions (control, 17 µg/m$^3$; 40 µg/m$^3$; 200 µg/m$^3$; and 500 µg/m$^3$) by inhalation for 13 weeks. A semiquantitative method was used to assess the severity of the particle loading of AMs. In their study, in order to be classified as showing moderate loading, a macrophage had to have many particles in the cytoplasm with particles covering the nucleus. In the highest-exposure group, 34 of 40 rats had moderate particle loading, as compared with 7 of 40 in the intermediate- and none in the lowest-exposure groups. After a 28-day period of recovery after exposure, 27% of the particle-exposed rats had moderate amounts of black particulate material in AMs, compared with 85% immediately after exposure. In a similar study, when rats were exposed for 30 or 90 days to wood smoke, the number of AMs containing 10 particles or more was higher in the group exposed to 10 mg/m$^3$ of PM than in those exposed to 1 mg/m$^3$ (Tesfaigzi et al. 2002). There are no data showing a similar dose–response relation in humans. Airway obstruction induced by hypertonic saline can be prevented by pretreatment with a $\beta_2$-adrenergic–receptor agonist (e.g., albuterol [also known as “salbutamol”]) (Gibson et al. 2002) — a strategy that also improves the success rate (Jones et al. 2001). Recently, we used induced sputum in a pilot study to measure the amount of black material in AMs from a population of children in Ethiopia exposed to very high (biomass smoke) and much lower (fossil-fuel-derived emissions) concentrations of inhaled carbonaceous particles (Kulkarni et al. 2005). We found a significantly larger two-dimensional area of black material in AMs from children in the high-exposure population. These data are compatible with our hypothesis that the amount of black material in AMs obtained by sputum induction reflects exposure to carbonaceous PM$_{10}$.

AIRWAY INFLAMMATION

Because the induction of sputum also samples inflammatory cells and mediators, this method has the capability of detecting PM$_{10}$-induced airway inflammation. Although the cellular mechanism for PM$_{10}$-induced impairment of lung-function growth is unknown, airway inflammation may be a key step in PM$_{10}$-induced cardiovascular mortality in adults (van Eeden et al. 2001; Pope et al. 2004). Indeed, increased levels of the neutrophil chemotactant interleukin-8 (IL-8) and airway neutrophilia have been reported in healthy volunteers exposed to diesel exhaust (Salvi et al. 2000).

PM$_{10}$

The major constituents of PM are sulfate, nitrate, ammonium, chloride, elemental and organic carbon, crustal material, and biologic materials (Harrison and Jones 1995; Harrison and Yin 2000). Elemental carbon is derived mainly from combustion processes (mostly associated with road traffic in the developed world) and forms the core of the particle. The core often has a surface coating of semivolatile organic compounds. The toxicity of airborne PM depends on particle size and chemical composition, which may include varying levels of trace metals, strong acid, and sulfate (Harrison and Yin 2000).

A wide range of sources contribute to PM, which can be divided roughly into three categories. Primary particles originate from incomplete combustion in motor vehicle...
engines or stationary combustion plants, and most of these particles are of local origin (in Leicester, U.K., they are mostly traffic derived) (Leicester Air Quality Report Review and Assessment 2000). Secondary particles, formed in the atmosphere, consist of ammonium sulfates, ammonium nitrate, and secondary organic aerosols. Because secondary particles are formed relatively slowly, their contribution to PM$_{10}$ is more spatially uniform. The third category, coarse particles, includes natural and biologic matter (e.g., spores).

Despite convincing epidemiologic data showing the adverse effects of the total level of PM$_{10}$ on children’s health, uncertainties remain. First, it is unclear to what extent the level of PM$_{10}$ measured at a point distant from the home misclassifies individual exposure. For example, a monitoring station that uses a fixed tapered-element oscillating microbalance (TEOM) monitor cannot capture exposure variations resulting from time spent outside or in proximity to main roads. Personal PM$_{10}$ level monitoring provides data on short-term individual exposure, but it is expensive, and the equipment is bulky. In contrast, a combination of geographic information system (GIS) technology and dispersion modeling of pollutants is able to provide estimates of PM$_{10}$ exposure at any geographic location for large numbers of children (Moschandreas et al. 2002).

In the present study, we combined modeling of the dispersion of locally derived PM$_{10}$ (primary particles) with the induced sputum method of obtaining AMs from children within Leicestershire (United Kingdom). The major city in Leicestershire is Leicester, which stands in a broad, shallow valley, with major radial and tangential roads, but no heavy industry. The city runs the Airviro dispersion model (Leicester Air Quality Report Review and Assessment 2000), described later in the “Estimates of Level of Locally Derived Primary PM$_{10}$” section under “Methods and Study Design.”

**SPECIFIC AIMS**

The following primary aims were set for healthy children:

- to quantitate the amount of black-pigmented material in AMs;
- to assess the association between the median area of black material in AMs and lung function; and
- to determine the association between the median area of black material in AMs and mean level of annually locally derived (primary) PM$_{10}$ modeled at the home address.

The following secondary aims were set:

- to determine the association between the median area of black material in AMs and the percentage of neutrophils and level of IL-8 in sputum from each child; and
- to determine the association between the median two-dimensional area of black material per AM per child and the distance from the home address to the nearest main road.

To achieve these aims, we recruited healthy children from nonsmoking families in Leicestershire and sampled AMs using the induced sputum method. The median two-dimensional area of black pigment in 100 AMs per child was measured by image analysis. The level of locally derived PM$_{10}$ at the residence was modeled. Lung function was determined by spirometry before and after the application of bronchodilators. The associations between the size of the black area in AMs and both lung function and levels of locally derived PM$_{10}$ were determined.

**METHODS AND STUDY DESIGN**

**RECRUITMENT AND SCREENING OF SUBJECTS**

The study protocol and the informed consent forms were approved by the local Institutional Review Board (Leicestershire Research Ethics Committee). All parents gave written, informed consent and were financially compensated for expenses associated with their children’s participation. All children gave assent after the procedure was explained to them by the research fellow.

Healthy children from schools, healthy siblings of children attending an asthma clinic and of children listed in an asthma database, and healthy children and their siblings from a Leicester birth cohort were recruited (Figure 1). The availability of PM$_{10}$ data for screening the children from the Leicester birth cohort and their siblings enabled us to selectively recruit children in the lowest-exposure group. To be
Association of Black-Pigmented Material in Children's AMs with Lung Function and Modeled PM$_{10}$

To be included in the study, children had to (1) be between 8 and 15 years of age; (2) reside in Leicestershire; (3) have resided in the same house during the previous year; and (4) have normal reported levels of physical activity. In order to achieve the broadest range of exposures, locally derived levels of primary PM$_{10}$ (based on 1997 data) for each child's home address had to be in either the lowest- or highest-exposure group for use in screening. Thus children in the middle group of exposure were excluded. The lowest-exposure group was initially defined as those exposed to a mean level of locally derived PM$_{10}$ of 1.92 µg/m$^3$, and the highest-exposure group as >3.82 µg/m$^3$, but due to difficulties in recruiting children, the upper limit for the lowest-exposure group was increased to 2.3 µg/m$^3$. The exclusion criteria were (1) any chronic respiratory illness (e.g., asthma); (2) a history of an acute respiratory tract infection in the past 3 months; (3) exposure to tobacco smoke at home or any personal smoking; (4) use of open coal-fired heating in the home; (5) a holiday outside Leicestershire for >5 days during the previous 3 months; and (6) residence in an apartment above the first floor (U.S. definition: second floor).

Permission was obtained from the local education authority to approach the schools and subsequently from head teachers to distribute invitation letters to children between 8 and 15 years of age. Parents and children who expressed a wish for more information were contacted and were asked questions related to the inclusion and exclusion criteria. Addresses of children fulfilling the inclusion criteria were converted to grid coordinates to obtain the modeled PM$_{10}$ level.

Children fulfilling the inclusion criteria (a total of 116) were invited to come to the study clinic for sputum induction, which was performed from November 2002 through December 2003 at the Leicester Royal Infirmary. Children and parents were asked to provide information on the child's age, sex, number of siblings, and the birth order, as well as the parent's assessment of whether the family lived near a main or quiet road and details of other potential sources of pollution near the home apart from traffic.

To exclude significant exposure to cigarette smoke, salivary samples from 114 children were tested for cotinine (2 were insufficient for analysis). Samples were collected in a 1-mL Eppendorf container using a straw to drip in the sample. Samples were immediately frozen at −70°C, then transported in batches to ABS Laboratories (London, U.K.) on frozen gel pads. Salivary cotinine was expressed in ng/mL. The salivary cotinine analysis was done by gas–liquid chromatography with a detection limit of 0.1 ng/mL (Feyerabend and Russell 1990). In the cotinine assay, a level ≤ 14.3 ng/mL excludes active smoking.
(McNeill et al. 1987), and a level of 1.2 ng/mL represents the 90th percentile, indicating children with no adult smokers in the household (Jarvis et al. 1992). Table 1 summarizes the characteristics and exposure data of the 64 children from whom an adequate induced sputum sample was obtained (2 subjects with outlier values were excluded; see the section “Statistical Methods and Data Analysis” and Figure 2).

Table 1. Individual Data of the Children Who Produced an Adequate Induced Sputum Sample

<table>
<thead>
<tr>
<th>ID Number</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>Ethnic Origin</th>
<th>Screened PM$_{10}$ (µg/m$^3$)</th>
<th>Salivary Cotinine (ng/mL)</th>
<th>Baseline FEV$_1$ (% Predicted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPC 02</td>
<td>Male</td>
<td>13</td>
<td>32.35</td>
<td>154.1</td>
<td>Asian</td>
<td>7</td>
<td>0.3</td>
<td>76.4</td>
</tr>
<tr>
<td>TPC 04</td>
<td>Male</td>
<td>13</td>
<td>40.00</td>
<td>152.5</td>
<td>Asian</td>
<td>5.26</td>
<td>0.2</td>
<td>90.8</td>
</tr>
<tr>
<td>TPC 05</td>
<td>Male</td>
<td>12</td>
<td>34.10</td>
<td>142.0</td>
<td>Asian</td>
<td>5.94</td>
<td>1.6</td>
<td>85.1</td>
</tr>
<tr>
<td>TPC 06</td>
<td>Female</td>
<td>12</td>
<td>55.00</td>
<td>159.3</td>
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Table 1 (continued). Individual Data of the Children Who Produced an Adequate Induced Sputum Sample

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ACTIVITY SCORES

Children were also asked to fill in a questionnaire, called Kriska's Modifiable Activity Questionnaire (Kriska and Caspersen 1997), that measures leisure-time physical activity. The questionnaire results were used for calculation of metabolic (MET) activity and vigorous (VIG) activity, both in hours per week (Aaron et al. 1993). The number of days children did hard and light physical exercise over the previous 2 weeks and the time spent watching television were also recorded.

For calculation of the MET and VIG activity scores, all the activities that the child did more than 10 times in a year were given a MET value, which was obtained from existing charts (Kriska and Caspersen 1997). The calculation of MET using the questionnaire was done with the following formula:

\[
\text{MET}(\text{hr/wk}) = \frac{(\text{no. of mo}/\text{yr}) \times (4.3 \text{ wk}/\text{mo}) \times (\text{no. of dy/wk})}{60 \text{ (min/hr)} \times 52 \text{ (wk/yr)}}
\]

The total MET score was calculated by adding the MET scores for all the activities. VIG was calculated by averaging hours per week over the past year for activities with a MET score > 6.

LUNG FUNCTION AND SPUTUM INDUCTION

A Vitalograph 2120 spirometer with Vitalograph 2120 Spirotrac IV software (Vitalograph, Buckingham, U.K.) was used to record baseline spirometric measurements. The Vitalograph 2120 has a Fleisch-type pneumotachograph to detect flow. Spirotrac IV is a fully integrated spirometry data-management system compliant with the American Thoracic Society 1994 updates and the European Respiratory Society 1993 guidelines (http://www.vitalograph.ie/spirometry_normal_values_guidelines.html#spirometry). The software calculated the percentage of predicted values for each measure of pulmonary function after the child’s age, sex, ethnic origin, height, and weight were entered. The acceptability and reproducibility criteria were based on the American Thoracic Society recommendations (1995), modified for children. The FEV₁, FVC, FEV₁/FVC, and FEF₂₅%–₇₅% were calculated. Each flow volume loop was visually examined, and if the final expiratory phase was interrupted, but the first portion of the loop was acceptable, only the FEV₁ was calculated. The software used the Lam-Polgar regression equations for predicted values for Asian children (Lam et al. 1982; Polgar and Promadhat 1971; Polgar and Weng 1979). In addition, z (standard deviation [SD]) scores were calculated for all baseline values, as previously described by Rosenthal and colleagues (1993), using equations derived from a normal, healthy cohort of white British children. After baseline lung function measurements were obtained, children were treated with 200-µg albuterol, administered via a metered-dose inhaler and Volumatic spacer device (Allen & Hanbury’s, Middlesex, U.K.). Postbronchodilator lung function measurements were obtained at 15 minutes. Children with a postbronchodilator FEV₁ < 80% were excluded.

For children with FEV₁ ≥ 80%, the coughing technique to obtain induced sputum was demonstrated. Children were asked to rinse their mouths and blow their noses prior to sputum induction. Nebulized 4.5% saline was administered using an ultrasonic nebulizer with an output of approximately 1 mL/min (Sonix nebulizer, Clement Clarke International, Harlow, Essex, U.K.) in sequential 5-minute inhalations (Cataldo et al. 2001). Every 5 minutes, the FEV₁ was measured to detect saline-induced bronchoconstriction. If the FEV₁ fell more than 10% below baseline, an additional dose of the bronchodilator albuterol was given. Nebulized saline was discontinued if the fall in FEV₁ was > 20%, and 2.5% nebulized albuterol was administered. The FEV₁ had to be within 5% of the baseline value before a child could be discharged from the laboratory. The sputum sample obtained was immediately stored on ice and processed within 2 hours. No children had an adverse drop in FEV₁ or symptoms that needed “rescue” albuterol. Some children reported mild throat discomfort after sputum induction.

INDUCED SPUTUM AND LEUKOCYTE DIFFERENTIAL

Induced sputum samples were processed within 2 hours of collection at 4°C as described previously by Brightling and colleagues (2000) with some minor modifications. Sputum was placed on a petri dish, and plugs were weighed. Freshly prepared 0.1% dithiothreitol (Sigma, Poole, Dorset, U.K.) at 4 times the weight of the sputum was added, and the sample was homogenized by rocking on a Denley Spiramix 5 roller mixer (Denley Instruments, Billinghamurst, U.K.) for 15 minutes. An equal volume of Dulbecco’s phosphate-buffered saline (PBS) (Sigma, Poole, Dorset, U.K.) was then added, and the sample was mixed by vortexing for 15 seconds. The sample was filtered using premoistened 48-µm gauze (Sefar, Bury, U.K.), and the volume was recorded. The total cell count, cell viability, and level of squamous cell contamination were assessed using a Neubauer hemocytometer and the trypan blue dye exclusion method. A cell pellet was obtained by centrifuging the sample at 2000 rpm.
for 10 minutes. The supernatant was gently aspirated and stored at −70°C in aliquots of 1 mL for cytokine analysis. The cells were resuspended at 0.5 × 10^6/mL in PBS for cytocentrifugation. Two spins per subject were prepared using 75 µL of cell suspension at 450 rpm (18.1 g) for 6 minutes with a Shandon CytoSpin III cytocentrifuge (Shandon, Sewickley, Pa.). The air-dried slides were stained with Diff-Quik (Dade Behring, Düdingen, Switzerland). When the slides were dry, they were covered with a drop of XAM mounting medium (BDH, Poole, Dorset, U.K.) and a coverslip. An operator blinded to the black-area status of the AMs performed a differential count on 300 nonsquamous cells at a magnification of ×40.

BLACK-PIGMENTED MATERIAL IN AIRWAY MACROPHAGES

Digital color images of 100 AMs from each child were captured using a JVC digital camera and an Olympus BX50 microscope (Olympus Optical, Southall, U.K.) at ×1000 magnification under oil immersion. Before obtaining each image, the microscope was adjusted for Köhler illumination, and the slide and lenses were cleaned to remove any contaminating dust particles. All the AMs in randomly selected fields were identified by their characteristic features and imaged to include the whole cell, with its borders clearly visible. Images were obtained with the AM nucleus in focus and ensuring that there were no artifacts in the image. Scion Image software (Scion Corporation, Frederick, Md.) was used to acquire the images. An image of a stage micrometer graticule (S-12S stage micrometer, 0.1 mm/50 division; Pyser–SGI, Kent, U.K.) was also obtained at the same magnification. Scion software scaling was calibrated using the image of the stage micrometer graticule (246 pixels = 20 µm).

Each AM image was initially processed using Jasc Paint Shop Pro software (Paint Shop Pro 7, Jasc Software, Eden Prairie, Minn.) and PC USB Graphics Tablet (Medion Electronics, Swindon, U.K.). First, each AM image was “cut and pasted,” and then the nucleus was removed — because the image analysis software identified the darkly stained nucleus as a “large particle.” The Scion Image software was used to measure black areas in micrometers squared, employing an in-house macro to reduce the number of manual steps. The Density Slice command was adjusted to obtain the best fit of the black areas visible on the color image (Figure 3). Examination of the color image identified bacteria and dense cell wall areas that had been erroneously selected as “black” by the software (they appeared blue in the image). Bacteria were identified by morphology and color, and dense cell wall areas by position and color. These were manually removed using the Erase feature or excluded by selecting only the true black areas. The total black area for each AM and the summary variable were calculated for 100 randomly selected AMs. The maximum diameter of each black area was also measured in AMs from 36 of the individual children. For all analyses, the median area of black-pigmented material per AM per child was chosen a priori as the primary particle-loading variable.

INTERLEUKIN-8

The level of IL-8, found in the thawed induced sputum supernatants, was analyzed according to an established
enzyme-linked immunosorbent assay (ELISA), described previously by Brightling and colleagues (2000), by an operator blinded to the median area of black material in the AMs, using a BD OptEIA kit for human IL-8 (BD Biosciences Pharmingen, San Diego, CA). The level of IL-8 was expressed as ng/mL, after correction for dilution of the supernatant. The sensitivity level of the assay was 0.8 × 10⁻⁹ ng/mL.

ESTIMATES OF LOCALLY DERIVED PRIMARY PM₁₀ LEVEL

To assess the levels of locally generated PM₁₀ at the children’s home addresses, the Airviro, version 2.21, dispersion model (Swedish Meteorological and Hydrological Institute, Norrköping, Sweden) was used. First, the Airviro model incorporated information from an emissions database (Leicester Air Quality Report Review and Assessment 2000), traffic data, and meteorologic data to calculate the spatial distribution of primary PM₁₀ across the city. Then secondary and coarse components of PM₁₀ were added to the model results to obtain a “total” PM₁₀ value. A constant value of 5 µg/m³ was used to represent the coarse component in this study. Therefore,

\[
\text{total PM}_{10} = \text{primary PM (modeled)} + \text{secondary PM (sulfate monitoring)} + \text{coarse (constant)}
\]

The home addresses were converted to grid coordinates (Ordnance Survey, Southampton, U.K.), and the model output was searched using these coordinates. The Leicester City Council Pollution Control Group supplied the database (the Leicester Air Quality Report Review and Assessment 2000) with modeled hourly primary (i.e., locally generated) PM₁₀ levels for home coordinates for the year before the date on which the induced sputum sample was obtained (Leicester City Council, Pollution Control Group, 2000). The mean level of annual locally derived PM₁₀ at the home addresses was used as the primary variable.

Airviro Inputs

The Airviro dispersion model operates on a Unix workstation and includes modules for data collection, dispersion calculations (grid, Gaussian, or canyon), and an emissions database. Dispersion calculations are performed in the dispersion module using meteorologic data and local emissions data from the emissions database. A meteorologic mast was installed to provide meteorologic data for the Airviro model. Emission sources for modeling with Airviro are defined as point (e.g., industrial and commercial buildings), line (roads), or area (e.g., residential areas or large industrial estates) sources. The Airviro model can be run on either a city or county map, zooming in where greater detail is required. Emissions from all sources in the part of the map initially selected are used for dispersion calculations, even in cases where the zoom function has subsequently been used to select a smaller area for display. For this study, the model was run at a resolution of 50 m × 50 m.

Emission factors were obtained from the Strategy Directorate of the Greater London Authority. For this study, modeled concentrations of primary PM₁₀ for 2002 and 2003 were obtained using an updated emission inventory package, EMIT 2.0 (Emission Inventory Toolkit, CERC, Cambridge, U.K.) (Leicester City Council Air Quality Review and Assessment 2003). Up-to-date National Atmospheric Emission Inventory emission factors (Leicester City Council Air Quality Review and Assessment 2003) were loaded into EMIT 2.0. In the model, emissions were defined as emanating from line, point, or area sources. For line sources (i.e., roads), Airviro first calculated hourly emissions for each road link using hourly traffic flows multiplied by an emission factor for each vehicle. Traffic flow data were obtained from the Leicester City Council’s Greater Leicester Traffic Model. This model, based on the Transport Improvement Planning System (TRIP), is designed to calculate traffic flow from land use factors such as population, housing, and industrial activity. It also takes into account the physical characteristics of roads such as carriage way widths and speed limits. Six vehicle types for which emission factors were available from the London Research Centre were used in the output calculation. The traffic allocated to each link was described according to peak flows, road type, and traffic type. The speed allocated to each link was the statutory speed limit for the road, but was amended by inspecting individual links and available observations. A point source was defined as any source that had well-defined position and an emission of small restricted volume. Area sources were regarded as producing diffuse emissions from a defined area, and Airviro modeled the dispersion of emissions from area sources at a height of 2 meters. To reduce the time needed for full data entry, the time variation of emissions for each source was limited to the “standard” (as set by the Leicester City Council) of emission variations according to the daily, weekly, and seasonal pattern of emissions.

To assess the contribution of traffic-derived PM₁₀ in this study, only line source data were used. To calculate the dispersion of modeled primary PM₁₀, the grid reference point for each child’s home and the desired year of meteorologic data (the year before the study date) were selected. Each yearly model calculated hourly PM₁₀ level.
Distance from Home to Main Road

Address data were supplied in Excel format and imported into a Microsoft Access database to allow easy comparison with the Address-Point (Ordnance Survey, Southampton, U.K.) database for Leicestershire. The Address-Point database provides full address and National Grid coordinate data for all postal addresses in the United Kingdom. Address data from the study were matched with corresponding data in the Address-Point database, and their grid coordinates to a 1-meter resolution were extracted. The grid coordinates were imported into the ArcView (v3.2) GIS software (ESRI, Aylesbury, Buckinghamshire, U.K.) for analysis. The distance from each child’s home address to the nearest main road was calculated using a digitized meridian map of all the main roads in Leicestershire and ArcView Avenue scripts.

STATISTICAL METHODS AND DATA ANALYSIS

The data are summarized as means and standard deviations (SD) and as percentiles. The median area of black material was chosen a priori as our measure of particle loading in the AMs. Screened children were identified as living in the highest- and lowest-exposure areas using the model run based on emissions inventory information available in 1997; however, modeled, locally derived PM$_{10}$ levels at home addresses in the 12 months before sputum induction did not separate into two distinct categories probably because an updated emissions inventory was introduced between 1997 and 2001 (Figure 4). We therefore used normal linear models. Potential confounders (age, sex, ethnic origin, weight, height, MET, VIG, number of days active outside per week, days of light and hard exercise per week, time spent in decreased activity per day, and salivary cotinine level) were added to the normal linear model if they had a $P$ value of $< 0.05$, or, in the models where the main effect was significant, if they changed the slope of the main effect by $> 10\%$. The results were confirmed using a nonparametric test (Spearman rank correlation test). S-plus, version 6.2 (Insightful, Seattle, Wash.) and SPSS 12.0.1 for Windows (SPSS, Chicago, Ill.) were used to carry out all statistical analyses. GraphPad Prism (GraphPad Software, San Diego, Calif.) was used for graphic representation. Two Asian children living at the same address had abnormally large median areas of black material in AMs ($4.62 \, \mu m^2$ and $2.87 \, \mu m^2$). Questioning of the household adults revealed the regular use of incense sticks and an oil lamp; therefore, data from these 2 children were excluded.

RESULTS

QUALITATIVE ASPECTS

The demographic data, exercise scores, lung function values, and induced sputum results of 64 children are summarized in Table 2. The baseline lung function of children producing adequate samples for analysis was not significantly different from the lung function of those who could not produce a sample ($P = 0.34$). AMs showing a representative range of sizes of black-pigmented area are shown in Figure 5. The black areas were not uniformly distributed within the AMs of individual children. The distribution of the maximum diameter of black-pigmented areas from 36 of 64 children is shown in Figure 6. The unexpected spike at 0.1 µm is probably explained by the very small “dots” identified as “separate” by the image analysis program, but which are attached to larger areas. The morphology of black pigment in AMs when viewed by light microscopy suggests that the large areas result from aggregates of smaller particles (Figures 7A and 7B). Black pigment was not seen in other airway cells (neutrophils, columnar cells, and eosinophils), consistent with the major role of AMs in removing inhaled particles. Free black areas, which possibly resulted from freshly inhaled matter or ruptured cells, were very infrequently observed (Figure 7C).

CHARACTERISTICS AND PHYSICAL ACTIVITY

The median area of black material in AMs showed no significant association with age, height, weight, and number of siblings (Table 3). A larger median area of black...
Table 2. Summary of Demographic, Exercise, Lung Function, and Sputum Induction Data from Children Who Produced an Adequate Sample of Induced Sputum

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SEM</th>
<th>SD</th>
<th>25th</th>
<th>50th</th>
<th>75th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>11.47</td>
<td>0.29</td>
<td>2.34</td>
<td>9.00</td>
<td>12.00</td>
<td>13.75</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>45.54</td>
<td>1.68</td>
<td>13.44</td>
<td>35.10</td>
<td>44.60</td>
<td>54.95</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>149.77</td>
<td>1.64</td>
<td>13.15</td>
<td>138.68</td>
<td>151.35</td>
<td>158.43</td>
</tr>
<tr>
<td>MET (hr/wk)</td>
<td>39.93</td>
<td>5.00</td>
<td>39.99</td>
<td>15.38</td>
<td>29.09</td>
<td>54.39</td>
</tr>
<tr>
<td>VIG (hr/wk)</td>
<td>3.37</td>
<td>0.59</td>
<td>4.72</td>
<td>0.29</td>
<td>1.63</td>
<td>4.63</td>
</tr>
<tr>
<td>FEV₁ (% predicted)</td>
<td>100.12</td>
<td>1.38</td>
<td>11.06</td>
<td>91.25</td>
<td>99.15</td>
<td>106.58</td>
</tr>
<tr>
<td>FVC (% predicted) (n = 61)</td>
<td>103.02</td>
<td>1.44</td>
<td>11.21</td>
<td>95.15</td>
<td>103.00</td>
<td>110.85</td>
</tr>
<tr>
<td>Leukocyte viability (%) (n = 54)</td>
<td>69.92</td>
<td>2.21</td>
<td>16.23</td>
<td>60.97</td>
<td>71.40</td>
<td>81.76</td>
</tr>
<tr>
<td>Squamous cells (%) (n = 54)</td>
<td>5.45</td>
<td>0.99</td>
<td>7.31</td>
<td>0.00</td>
<td>2.60</td>
<td>8.48</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>31.85</td>
<td>3.65</td>
<td>29.20</td>
<td>5.67</td>
<td>20.63</td>
<td>56.00</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>1.63</td>
<td>0.63</td>
<td>5.04</td>
<td>0.00</td>
<td>0.25</td>
<td>0.75</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>65.95</td>
<td>3.72</td>
<td>29.79</td>
<td>41.38</td>
<td>78.63</td>
<td>93.25</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>0.15</td>
<td>0.03</td>
<td>0.21</td>
<td>0.00</td>
<td>0.00</td>
<td>0.25</td>
</tr>
<tr>
<td>Epithelial cells (%)</td>
<td>0.41</td>
<td>0.10</td>
<td>0.79</td>
<td>0.00</td>
<td>0.25</td>
<td>0.50</td>
</tr>
<tr>
<td>IL-8 (ng/mL) (n = 63)</td>
<td>14.87</td>
<td>3.44</td>
<td>27.33</td>
<td>0.96</td>
<td>6.26</td>
<td>12.06</td>
</tr>
</tbody>
</table>

*a n = 64 except where noted.

Figure 5. Representative images of AMs showing different sizes of black areas (each from a different child). Stained with Diff-Quik and imaged under oil immersion by light microscopy. In the last image (bottom center), a neutrophil is adjacent to the AM.
Figure 6. Distribution of the maximum two-dimensional diameter of black areas in the airway macrophages of 36 children (n = 3600 AMs). There are few particles >2 µm.

Figure 7. The morphology of black pigment in AMs when viewed by light microscopy. Stained with Diff-Quik and imaged under oil immersion (×1000). A and B: Single black area (black arrows) with a morphology consistent with aggregates of smaller particles in AM cytoplasm; C: Black area (black arrow) outside AM.

Table 3. Associations of Variables with Median Area of Black Material in AMs

<table>
<thead>
<tr>
<th>Variable</th>
<th>$r^2$</th>
<th>SE (Estimate)</th>
<th>Coefficient</th>
<th>SE (Coefficient)</th>
<th>P</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>0.003</td>
<td>0.232</td>
<td>0.005</td>
<td>0.012</td>
<td>0.690</td>
<td>−0.020 to 0.030</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>0.000</td>
<td>0.232</td>
<td>0.000</td>
<td>0.002</td>
<td>0.898</td>
<td>−0.004 to 0.005</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>0.002</td>
<td>0.232</td>
<td>−0.001</td>
<td>0.002</td>
<td>0.732</td>
<td>−0.005 to 0.004</td>
</tr>
<tr>
<td>Number of siblings</td>
<td>0.013</td>
<td>0.231</td>
<td>0.026</td>
<td>0.029</td>
<td>0.379</td>
<td>−0.032 to 0.084</td>
</tr>
<tr>
<td><strong>Exercise</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MET (hr/wk)</td>
<td>0.011</td>
<td>0.231</td>
<td>0.001</td>
<td>0.001</td>
<td>0.410</td>
<td>−0.001 to 0.002</td>
</tr>
<tr>
<td>VIG (hr/wk)</td>
<td>0.019</td>
<td>0.230</td>
<td>0.007</td>
<td>0.006</td>
<td>0.283</td>
<td>−0.006 to 0.019</td>
</tr>
<tr>
<td>Outdoor activity in past 7 days</td>
<td>0.002</td>
<td>0.227</td>
<td>−0.005</td>
<td>0.013</td>
<td>0.708</td>
<td>−0.032 to 0.022</td>
</tr>
<tr>
<td>Outdoor activity in typical week</td>
<td>0.006</td>
<td>0.226</td>
<td>−0.010</td>
<td>0.017</td>
<td>0.543</td>
<td>−0.043 to 0.023</td>
</tr>
<tr>
<td>Hard exercise (days)</td>
<td>0.038</td>
<td>0.230</td>
<td>−0.043</td>
<td>0.027</td>
<td>0.127</td>
<td>−0.098 to 0.012</td>
</tr>
<tr>
<td>Light exercise (days)</td>
<td>0.038</td>
<td>0.228</td>
<td>−0.037</td>
<td>0.024</td>
<td>0.124</td>
<td>−0.085 to 0.010</td>
</tr>
<tr>
<td>Television/video games (hr)</td>
<td>0.001</td>
<td>0.232</td>
<td>0.008</td>
<td>0.036</td>
<td>0.832</td>
<td>−0.063 to 0.079</td>
</tr>
</tbody>
</table>
material in AMs was seen in Asians than in whites, possibly because a greater number of Asian children lived in areas of higher modeled exposure ($P = 0.0001$). The median area of black material in AMs was not significantly associated with measures of increased activity (MET score, VIG score, number of days active outside in a week, and days of light and hard exercise) or decreased activity (time spent watching television and playing video games) (Table 3 and Figure 8).

**LUNG FUNCTION**

There was a significant exposure-dependent inverse correlation between median area of black-pigmented material in AMs and baseline (prebronchodilator) FEV₁ and FEF₂₅%–₇₅% as a percentage of predicted value ($P < 0.005$) and as a $z$ score (Figure 9, Table 4). Each 1.0-$\mu$m² increase in black area in AMs was associated with a 17.0% (95% CI, 5.6 to 28.4) reduction in FEV₁, a 12.9% (95% CI, 0.9 to...
24.8) reduction in FVC, and a 34.7% (95% CI, 11.3 to 58.1) reduction in FEF\textsubscript{25%–75%}. These associations were not affected by bronchodilator treatment, which indicates that the decrement in lung function is not the result of reversible bronchoconstriction. In turn, this would explain why there was no correlation between median area of black material in AMs and FEV\textsubscript{1}/FVC since the latter is the indicator for reversible obstruction. The association between percentage of predicted FVC and median area of black material in AMs was less consistent. There was, however, a significant inverse association ($P < 0.001$) between median area of black material in AMs and FVC when expressed as a $z$ score. The association between mean level of annual modeled primary PM\textsubscript{10} at the home address and lung function was weak ($P = 0.04$) for FEV\textsubscript{1} and nonsignificant for the other lung function variables (Table 5).
AIRWAY INFLAMMATION

The IL-8 level was above the assay detection limit in the induced sputum supernatant of all 63 samples measured. There was no significant correlation between the median area of black material in AMs and percentage of airway neutrophils, percentage of airway eosinophils, or IL-8 concentration (Table 6 and Figure 10). However, there was a significant association between the neutrophil differential count and the IL-8 concentration ($r = 0.67, P < 0.0001$; data not shown).

Table 5. Associations Between Locally Derived Annual PM$_{10}$ Level at Home and Lung Function

<table>
<thead>
<tr>
<th>Lung Function (% Predicted)</th>
<th>Linear Regression</th>
<th>Spearman Rank Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r^2$</td>
<td>SE (Estimate)</td>
</tr>
<tr>
<td>FEV$_1$</td>
<td>0.066</td>
<td>10.774</td>
</tr>
<tr>
<td>FVC</td>
<td>0.005</td>
<td>11.281</td>
</tr>
<tr>
<td>FEV$_1$/FVC</td>
<td>0.017</td>
<td>6.809</td>
</tr>
<tr>
<td>FEF$_{25%–75%}$</td>
<td>0.062</td>
<td>22.151</td>
</tr>
</tbody>
</table>

Table 6. Associations Between Inflammatory Variables and Median Area of Black Material in AMs

<table>
<thead>
<tr>
<th>Variable</th>
<th>Linear Regression</th>
<th>Spearman Rank Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r^2$</td>
<td>SE (Estimate)</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>0.027</td>
<td>29.028</td>
</tr>
<tr>
<td>Eosinophil (%)</td>
<td>0.000</td>
<td>5.079</td>
</tr>
<tr>
<td>IL-8 (ng/mL)</td>
<td>0.010</td>
<td>27.416</td>
</tr>
</tbody>
</table>

Figure 10. Association between the median area of black material in AMs and airway inflammation: A: sputum neutrophil percentage; B: sputum IL-8 concentration. The P values were not significant for all associations.
LOCALLY DERIVED PM$_{10}$ LEVEL AND DISTANCE FROM THE ROAD

There was a positive correlation between the median area of black material in AMs and mean level of annual locally derived PM$_{10}$ modeled at the home address (Figure 11). Thus, for each unit increase in level of modeled, locally derived PM$_{10}$ at the home address, we found a 0.10-$\mu$m$^2$ (95% CI, 0.01 to 0.18) increase in the median area of black material in AMs. The distance from the home to the nearest main road was not associated with the median area of black material (Figure 12), and there was no significant difference in the median area of black material in AMs in children living less than 150 m from a major road from that of children living further away ($P = NS$).

DISCUSSION

In this study, we recruited a group of healthy children living in a single geographic region of the United Kingdom and assessed the relation between the particle loading of black material in AMs and the level of modeled, locally generated PM$_{10}$ at the home address. The children at their home addresses were exposed to nearly uniform levels of PM$_{10}$ blown in from distant areas and countries. Thus, in children undergoing sputum induction, we assumed that variations in home exposure were the result of variations in the level of locally generated “primary” PM$_{10}$. Using a predefined measure of both the size of the black-pigmented area in AMs and the level of modeled, locally derived PM$_{10}$, we found an association between the median area of black material in AMs and modeled exposure over the previous 12 months. More relevant to potential health effects, we found an inverse association between the median area of black material in AMs and measures of lung function.

BLACK MATERIAL IN AIRWAY MACROPHAGES AND LUNG FUNCTION

Gauderman (2004) reported correlations of PM$_{2.5}$, NO$_2$, and elemental carbon with both reduced lung function growth in children and reduced final attained lung function. Even at relatively low ambient levels of traffic-derived air pollution, an Austrian study has shown a reduction in lung function growth with increased levels of air pollution in children over a 3-year period (Horak Jr. et al. 2002). Our finding that increased median area of black material in AMs is associated with a reduction in FEV$_1$ and FEF$_{25\%-75\%}$ in healthy children is compatible with these epidemiologic data. The size of this effect is surprising, since all children were exposed to relatively low, albeit variable, levels of PM$_{10}$. Although we adjusted for variables known to influence lung function, such as age, sex, ethnic origin, height, and weight, it remains possible that the association between the median area of black material in AMs and compromised lung function could be due to an unexpected confounding factor. An alternative, physiologic explanation is that lower levels of lung function increase the deposition of particles in the lower airways. The association between the median area of black material in AMs and a decrement in lung function is not, however, due to reversible bronchoconstriction, thus ruling out an association between median black area in AMs and “subclinical” asthma in our study group.
LEVEL OF LOCALLY GENERATED PM$_{10}$

The level (median of 1.21 µg/m$^3$) of locally generated PM$_{10}$, modeled in our study using Airviro, is consistent with the levels found in similar or busier cities in the United Kingdom (www.defra.gov.uk/environment/airquality/publications/particulate-matter/pdf/ch8.pdf). For example, the estimated contribution of traffic to PM$_{10}$ was 0.6 µg/m$^3$ in Birmingham and 1.2 µg/m$^3$ in Manchester.

One reason why we did not find a stronger association between the level of modeled, locally derived PM$_{10}$ and the median area of black material in AMs is because both measurements have limitations. Outdoor levels of PM$_{10}$ at the home address can, at best, describe only a portion of total individual exposure. However, living in a home with a high level of outdoor PM$_{10}$ will be associated with high exposure indoors and increased exposure when playing or traveling in the immediate vicinity.

A further limitation of our use of the Airviro model is that there was an insufficient number of TEOM sites for the validation of Airviro for modeling PM$_{10}$ in Leicester. That would require simultaneous measurement of PM$_{10}$ at multiple sites. Alternatively, future validation studies could use passive sampling of carbon monoxide, as a marker for primary PM$_{10}$ (Ebelt et al. 2001). Indeed, Airviro performs well when predicting the spatial distribution of traffic-associated carbon monoxide (Mukherjee and Viswanathan 2001).

Despite these limitations, Airviro probably represents an improvement over the method employing distance from home to main road, because Airviro integrates the contributions from all roads and takes into account traffic flows on these roads. Indeed the crudeness of the method using the distance from home to main road may explain why we found no association between the median area of black material in AMs and the distance variable. However, our group recently reported (Pierce et al. 2006) an association (adjusted odds ratio = 1.56) between the level of locally generated PM$_{10}$ and cough without cold and wheeze in a cohort of 4400 children using the distance between home and main road. In addition, an increased prevalence of respiratory symptoms with proximity to a main road has been reported (van Vliet et al. 1997; Venn et al. 2001) for both Dutch and British children in studies using that method. Any attempt to demonstrate an association between the median area of black material in AMs and distance from home to main road may therefore need to selectively recruit children living within 150 m of a main road, the zone where the effects of proximity are most pronounced (Venn et al. 2001).

LIMITATIONS OF STUDYING BLACK MATERIAL IN AIRWAY MACROPHAGES

Our hypothesis was that variations in the median area of black material in AMs reflect variations in the amount of PM being deposited in the lower airway. It is very likely that black areas (probably composed of carbon) in AMs from Leicester children are derived from fossil fuel combustion, since we excluded children exposed to environmental tobacco smoke and indoor coal fires. There is also evidence that carbonaceous matter is the major component of fine and ultrafine PM in the United Kingdom (Harrison and Jones 1995). Preliminary elemental analysis of AMs by electron energy loss spectroscopy (EELS) did not show spectra of silica, sulfur, titanium, and iron (M. Geiser, personal communication, August 11, 2005). The size of the black area in AMs is the result of an interaction between interrelated variables including mucociliary clearance rate, tidal volume to lung surface area, AM phagocytic capacity, residence time of AMs before moving out onto the mucociliary escalator (the cilia and mucus that lines much of the airways and that captures foreign material and moves it up to the throat), and “recycling” of PM by phagocytosis of apoptotic particle-containing AMs. Although the association between the median area of black material in AMs in humans and exposure to PM in experimental exposure studies has not been examined, the following observations have been reported: (1) an exposure-dependent increase in particles in AMs has been observed in rats exposed for 13 weeks to varying doses of biodiesel emissions (Finch et al. 2002); (2) light-microscopic analysis of AMs from stray dogs has demonstrated that animals living in areas of high ambient PM$_{10}$ levels have an increased proportion of AMs containing carbon (Calderón-Garcidueñas et al. 2001); and (3) children chronically exposed to high levels of PM$_{10}$ have a larger median carbon area in AMs than children exposed to lower concentrations of carbonaceous PM$_{10}$ (Kulkarni et al. 2005). It remains unclear whether the median area of black material in AMs reflects integrated exposure over several months, or short-term peaks of exposure, or some combination of both. A long-term exposure relationship is supported by reports that titanium dioxide persists within AMs for more than 3 years after exposure (Maatta and Arstila 1975) and that PM in AMs from smokers is present for more than 2 years after a lung from a smoker is transplanted into a nonsmoker (Marques et al. 1997).

Although sputum induction removes macrophages solely from the larger airways (bronchi), the current view is that the bronchial macrophages are alveolar macrophages that have moved out of the lung on the mucociliary escalator. Particle loading of macrophages in the larger airways,
AMs are composed of carbon, although preliminary analysis of occupationally exposed adults, Fireman and colleagues (1999) reported that “results showed that approximately 70% of particles internalized by macrophages that are present in induced sputum as well as in bronchoalveolar lavage samples were smaller than 2.5 µm (< PM$_{2.5}$).” They concluded that “comparison of BAL and IS [induced sputum] specimens in the evaluation of the study population yielded similar quantitative and qualitative results.”

Our success rate in inducing sputum (56%) is comparable to that from other reports in healthy children. Gibson and colleagues (2003), in a study of asthmatic children, included 37 healthy children and obtained adequate samples from 22 — a success rate of 59% (P. G. Gibson, personal communication). Similarly, Wilson and colleagues (2000) found that 17 of 27 healthy children (63%) produced an “adequate” induced sputum sample.

We used light microscopy and image analysis to measure the median area of black-pigmented material in AMs, but there are other ways of measuring the amount of PM in AMs. Flow cytometry analysis of AMs provides semiquantitative data on particle loading (Palecanda and Kobzik 2000), but the small numbers of AMs recovered from children preclude its use in this age group. We measured black-pigmented material in only one focal plane (but maximized the capture of intracellular black-pigmented material by obtaining images with a nucleus in focus). Three-dimensional particle loading can be measured using confocal microscopy, but inhaled particles have to be fluorescently labeled — something that is not possible for natural exposures.

We found no association between the median area of black-pigmented material in AMs and the amount of exercise, even though this could, by increasing respiratory rate, increase exposure to PM$_{10}$. There are some limitations to our assessment of exercise. First, we did not differentiate between indoor and outdoor activities. Second, we did not define the place and time of exercise. Third, values were dependent on recall by the parent or child and could have been open to bias.

**BLACK MATERIAL: CARBON OR NOT?**

We have not proven that the particles we found in the AMs are composed of carbon, although preliminary analysis by EELS of AMs from children, as described in the previous section, did not show presence of iron, titanium, silica, or sulfur. Since carbon is a major constituent of PM in the United Kingdom (Harrison and Jones 1995), it is most likely that the black particles visualized are carbonaceous. The majority of individual black areas were < 2.5 µm — a finding that is compatible with the hypothesis that PM$_{2.5}$ may be the component of PM$_{10}$ reaching the distal airways. Our data on particle size distribution collected through light microscopy are similar to the data of Dumortier (1994), who measured particle size as the “area-equivalent diameter.” To estimate the aerodynamic diameter from the actual diameter (assuming there is no aggregation or disaggregation in phagolysosomes), information on particle density (determined by composition) and shape would be required. The aerodynamic diameter of inhaled particles cannot therefore be reliably estimated from our data on the distribution of the size of the black areas.

**AIRWAY INFLAMMATION**

In animal models, the induction of neutrophilic airway inflammation has been implicated in the pathogenesis of lung injury induced by carbonaceous particulates (Ichinose et al. 1995), but data from human studies to support this are inconsistent. Airway neutrophilia and increased IL-8 level have been reported in BAL fluid in healthy humans exposed to diesel exhaust (Salvi et al. 2000; Stenfors et al. 2004). In contrast, other researchers have found no increase in IL-8 level or airway neutrophils in induced sputum after exposure to PM$_{10}$ or ambient particulates (Nordenhall et al. 2000; Gong, Jr. et al. 2003). Consistent with the latter studies, we found no association between IL-8 level or the neutrophil differential count in induced sputum and either the median area of black material in AMs or the level of modeled, locally derived PM$_{10}$.

**FUTURE DIRECTIONS FOR ANALYSIS OF BLACK MATERIAL IN AIRWAY MACROPHAGES**

Our data provide a point of reference for the analysis of the median area of black-pigmented material in AMs in other cities and in animal exposure experiments. Future work should include replication of this study in a separate cohort of children, ideally combined with the longitudinal assessment of lung function growth. In conclusion, we assessed the association between the level of modeled, locally derived PM$_{10}$ at the home address and the size of the black-pigmented area in AMs in a group of 64 healthy children. A weak but statistically significant positive association was found between the two variables. However, a stronger statistically significant inverse association was found between the median area of black material in AMs and lung function.
ACKNOWLEDGMENTS

We would like to acknowledge Catherine Mallon from the Pollution Control Group, Leicester City Council, for providing the monitored and the modeled PM data. Bob Harris of the Institute of Environment and Health, University of Leicester, provided data on the distance from roads and the grid reference points for all children. Lucy Woodman of the Glenfield General Hospital, Institute for Lung Health, Leicester, analyzed supernatant for IL-8. EELS was done in collaboration with Marianne Geiser and Nadine Kapp at the Institute of Anatomy, University of Bern, Switzerland. We would also like to thank the local education authorities, head teachers, parents, and children who participated in this study.

REFERENCES


APPENDIX A. HEI QUALITY ASSURANCE AUDIT STATEMENT

The conduct of this study was subjected to independent audits by Mr. David Bush of T&B Systems, Inc. Mr. Bush is an expert in quality assurance for air quality monitoring studies and data management. The audits included on-site reviews of study activities for conformance to the study protocol and operating procedures. The dates of the audits are listed in the table below with the phase of the study examined.

<table>
<thead>
<tr>
<th>Date</th>
<th>Phase of Study</th>
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<tr>
<td>January 12, 2004</td>
<td>The auditor conducted an on-site audit at the University of Leicester, Leicester, United Kingdom. Dr. Henry Gong and Ms. Kimberly Hudson from the Los Amigos Research and Education Institute also participated in this audit, providing expertise for the review of the particle imaging and counting technique and the clinical portions of the study. Several recommendations were presented for strengthening documentation for both the macrophage particle analysis and clinical data efforts.</td>
</tr>
<tr>
<td>February 14, 2008</td>
<td>The auditor reviewed the study final report and final database. Several data points for each parameter were traced through the entire data processing sequence to verify the integrity of the database. Some minor issues were noted. All were addressed by the authors, and none affected the study findings.</td>
</tr>
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Written reports of each inspection were provided to the HEI project manager, who transmitted the findings to the Principal Investigator. These quality assurance audits demonstrated that the study was conducted by an experienced team with a high concern for data quality. The report appears to be an accurate representation of the study.

David H. Bush, Quality Assurance Officer
ABOUT THE AUTHORS

Jonathan Grigg is a professor of child health at Barts and the London School of Medicine and Dentistry, London, United Kingdom, and was a senior lecturer in child health at the University of Leicester during the study. He is a fellow of the Royal College of Paediatrics and Child Health, with interests in the health effects of air pollution, nanotoxicology, and pediatric environmental health.

Neeta Kulkarni is locum consultant at the Leicester Primary Care Trust, United Kingdom, and was the clinical research fellow on this project. She received her M.D. from the University of Leicester and M.R.C.P. in the United Kingdom. Her work focuses on pediatric respiratory medicine, and she has a developing interest in the health effects of PM pollution from traffic and biomass burning.

Nevil Pierse was the statistician on the project. He has a master’s degree in statistics from the University College Cork, Ireland, where he was awarded the Donal McCarthy Postgraduate Research Scholarship in Statistics, as well as receiving an earlier degree in mathematics and statistics. He has worked on childhood asthma and air pollution for the University of Leicester and the M.R.C. Institute for Environment and Health at the university. He is currently working on indoor air pollution with the University of Otago (Wellington, New Zealand).

Lesley Rushton is a medical statistician and epidemiologist and was head of epidemiology at the M.R.C. Institute for Environment and Health, University of Leicester, United Kingdom. Her main research area is the epide- miologic aspects of occupational and environmental health, including environmental causes of childhood respiratory disease and the reduction of exposure of children to environmental tobacco smoke in the home.

Christopher O’Callaghan is a professor of pediatrics at the University of Leicester, United Kingdom. He wrote his M.D. thesis on aerosol delivery to infants and runs an aerosol research laboratory. His Ph.D. thesis was on ependymal cilia, and he runs a laboratory to investigate the effect of pathogens and toxins on the ciliated respiratory and ependymal epithelia.

Andrew Rutman is an electron microscopist in the Division of Child Health, University of Leicester, United Kingdom, with interests in imaging lung cilia and lower airway inflammatory cells.

OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH

ARTICLES


ABSTRACTS


# ABBREVIATIONS AND OTHER TERMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AM(s)</td>
<td>airway macrophage(s)</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>EELS</td>
<td>electron energy loss spectroscopy</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FEF&lt;sub&gt;25%–75%&lt;/sub&gt;</td>
<td>forced expiratory flow between 25% and 75% of forced vital capacity</td>
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<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>forced expiratory volume in 1 second</td>
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<tr>
<td>FVC</td>
<td>forced vital capacity</td>
</tr>
<tr>
<td>GIS</td>
<td>geographic information system</td>
</tr>
<tr>
<td>MET</td>
<td>metabolic activity score (hr/week)</td>
</tr>
<tr>
<td>IL-8</td>
<td>interleukin-8</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PM</td>
<td>particulate matter</td>
</tr>
<tr>
<td>PM&lt;sub&gt;10&lt;/sub&gt;</td>
<td>PM with an aerodynamic diameter ≤ 10 µm</td>
</tr>
<tr>
<td>PM&lt;sub&gt;2.5&lt;/sub&gt;</td>
<td>PM with an aerodynamic diameter ≤ 2.5 µm</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>TEOM</td>
<td>tapered-element oscillating microbalance (monitor)</td>
</tr>
<tr>
<td>VIG</td>
<td>vigorous activity score (hr/wk)</td>
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INTRODUCTION

In the 1990s, several epidemiologic and controlled exposure studies suggested an association between exposure to air pollution from traffic-derived particulate matter (PM*) — in particular the fraction of PM that is inhalable (i.e., has an aerodynamic diameter ≤ 10 µm [PM₁₀]) — and increases in symptoms of airway diseases, including exacerbations of asthma (Wjst et al. 1993; Edwards et al. 1994; Weiland et al. 1994; van Vliet et al. 1997). Some studies also suggested that exhaust from diesel engines — which are used in a large fraction of vehicles worldwide and particularly in Europe — contributed to these effects (Brunekreef et al. 1997; van Vliet et al. 1997).

To address more fully the possible association between exposure to PM and the exacerbation of asthma and airway allergic conditions, HEI issued a request for applications: RFA 00-2, Effects of Diesel Exhaust and Other Particles on the Exacerbation of Asthma and Other Allergic Diseases. Professor Jonathan Grigg of the University of Leicester, United Kingdom, responded by submitting an application titled, “The relationship between pollutant particles in alveolar macrophages from normal children and proxy markers of PM₁₀ exposure.” He proposed to evaluate whether the quantification of particles in airway macrophages — the major cell type that ingests (or phagocytoses) agents that enter the airways — could be used as a marker of children’s exposure to PM₁₀. He chose to measure carbonaceous particles, because carbon is a major component of particles derived from combustion sources such as traffic. In combination with these measurements, Grigg and colleagues also proposed to use models to estimate individual children’s exposures to PM₁₀. The HEI Health Research Committee recommended Professor Grigg’s proposal for funding; although the proposal was not directly responsive to the RFA, the committee members thought the development of a possible noninvasive biomarker of exposure to PM would be useful for future studies of air pollutant effects.

SCIENTIFIC BACKGROUND AND RATIONALE FOR THE STUDY

Before the start of the current study, several studies had reported that proximity to traffic was associated with adverse affects on lung function in children, especially in those with airway conditions such as asthma and allergic rhinitis (Wjst et al. 1993; Edwards et al. 1994; Weiland et al. 1994; van Vliet et al. 1997). Exposures in these studies were generally estimated by classifying exposure to traffic using either questionnaires or objective traffic counts. However, the extent to which these estimates of exposure captured the exposures of individual children living in the area was unclear. Because most PM₁₀ in U.K. cities that lack heavy industry is generated from road traffic (Holman 1999), Grigg and colleagues reasoned that a child’s major exposure to PM₁₀ in such a city would be derived from local — particularly, traffic-related — sources. By modeling exposures at locations where children spent most of their time — at home and school — Grigg and colleagues proposed to make more accurate estimates of their exposures to PM₁₀, as a surrogate marker of their exposures to traffic-related pollution.

As described in the report’s Introduction, Grigg and colleagues also hypothesized that the detection of carbon particles within airway macrophages would serve as a biomarker of PM₁₀ exposures. Many of the investigators’ assumptions about the use of particles detectable in airway macrophages as a marker of exposure appeared reasonable. Macrophages are long-lived cells found throughout the airways; they are the principal cells that phagocytose agents such as particles and potential airborne pathogens (e.g., bacteria, viruses, and allergens) that enter the airways. Most particles that enter the lungs are cleared rapidly (in a few hours) by transport up the mucociliary escalator, the cilia and mucus that line much of the airways and that...
capture foreign material and move it up to the throat. Nonetheless, a fraction of these particles is cleared much more slowly and can be retained for months (Lay et al. 1998). Furthermore, particles from occupational exposures such as mining can be detected in lung macrophages months or years after the exposure has ended (Rainey et al. 1994; Fireman et al. 1999). A transplantation study also supports the idea that lung macrophages maintain the characteristics of an exposure even years after the exposure has ended; in this study, the cytoplasm of lung macrophages contained inclusion bodies characteristic of smokers as long as 2 years after the transplantation of a lung lobe from a heavy smoker into a nonsmoker (Marques et al. 1997). Although this finding suggests that the lifetime of lung macrophages is long, it does not rule out the possibility that the cells may have a shorter life and pass on their contents via apoptotic or necrotic pathways to newly generated macrophages. Taken together, these results suggest that particles detectable in lung macrophages could reflect long-term or time-distant exposures to particles.

Before the current study, Grigg and colleagues also presented preliminary data suggesting that macrophages they had collected by bronchoalveolar lavage (BAL) from the lungs of 23 children in Leicester contained phagocytosed particles (Bunn et al. 2000). The investigators believed that these particles were carbonaceous. Because collection by BAL is invasive and hence of limited use in children, Grigg and colleagues also provided preliminary data suggesting that airway macrophages could be obtained from an adult by inducing sputum and that these macrophages contained carbonaceous particles.

### TECHNICAL EVALUATION

#### AIMS

Grigg and colleagues proposed to determine whether the amount of carbonaceous particles in sputum macrophages collected from healthy children in Leicester, United Kingdom, could be correlated with estimates of their exposures to locally derived PM$_{10}$. To estimate children’s exposures to PM$_{10}$, the investigators proposed to use a model of individual traffic-related exposures to PM that was based on the emissions inventory information of mobile sources. A secondary objective of the study was to determine whether the amount of carbonaceous particles inside airway macrophages could be correlated with markers of airway inflammation.

To assess the amount of carbonaceous particles inside a cell, Grigg and colleagues proposed to use light-microscopic techniques to measure the area occupied by particles in the cross-sectional area of the cell. To model exposures to PM$_{10}$, the investigators originally proposed to include estimates of exposures of different lengths (previous week, month, or year) at the child’s home and school addresses and to examine factors such as the level of activity, age, and time spent outdoors. During the study, the investigators focused on developing annual estimates of PM$_{10}$ at the child’s home address and on estimating the impact of the proximity of the home address to the nearest main road.

Although not part of their original aims, Grigg and colleagues also evaluated whether the size of the area of carbonaceous particles inside the children’s macrophages correlated with several pulmonary function parameters that they had measured when the children provided sputum samples. Because they did not definitively determine that the particulate material inside the macrophages was carbonaceous, the investigators refer to it as “black-pigmented material” in the Investigators’ Report.

Thus the report focuses on two associations: the association between the amount of black-pigmented material detected in airway macrophages and modeled estimates of locally derived PM$_{10}$ at the child’s home address; and the association between the amount of black-pigmented material detected in airway macrophages and pulmonary function.

#### STUDY DESIGN AND METHODS

##### Study Population

In 2002 and 2003, Grigg and colleagues recruited healthy children aged 8 to 15 years in and around the city of Leicester, United Kingdom, who met their criteria for inclusion in the study. In addition to age, the inclusion criteria were residence in Leicestershire and in the same dwelling during the previous year, and having normal reported levels of activity. Grigg and colleagues excluded children who had any chronic respiratory illness, such as asthma; a history of acute respiratory infection in the previous 3 months; exposure to tobacco smoke (at home or because the child smoked); use of open coal-fired heating in the home; a vacation of more than 5 days outside Leicestershire in the previous 3 months; or residence in an apartment above the first floor (U.S. definition: second floor).

To increase the possibility of detecting a correlation between modeled exposure and particles detected in macrophages, the investigators selected children who had widely different PM$_{10}$ exposures: the highest-exposure group (> 3.82 µg/m$^3$) and the lowest-exposure group (≤ 2.3 µg/m$^3$). To estimate individual children’s PM$_{10}$ exposures, Grigg and colleagues used information for 1997 from the Airviro model (discussed in detail later, in the section “Modeling of PM$_{10}$ Exposure”).
Pulmonary Function Measurements and Collection of Sputum

The investigators invited children who met the criteria for inclusion in the study to their research laboratory, where they measured baseline lung function: forced expiratory volume in 1 second (FEV₁), forced vital capacity (FVC), the ratio of FEV₁ to FVC (FEV₁/FVC), and forced expiratory flow between 25% and 75% of the forced vital capacity (FEF₂₅₋₇₅%). One hundred and sixteen children participated. The investigators calculated the percentage of predicted values for each child, taking into account the child’s age, sex, ethnic origin, height, and weight. For each lung function measurement, the investigators calculated a z score — a derived response variable that is, in effect, a standardization relative to a reference group; however, these results are difficult to interpret.

After baseline pulmonary function testing, the researchers gave the children an inhaled dose of the bronchodilator albuterol (also referred to as “salbutamol” outside of the United States) and measured lung function 15 minutes later. One child with a post-bronchodilator FEV₁ of less than 80% was excluded, and another did not want to continue to participate. Using nebulized hypertonic saline, Grigg and colleagues induced sputum in the remaining 114 children and obtained 66 adequate samples for study of airway macrophages.

Of the 66 participants who provided adequate samples, 40 identified themselves as white (i.e., parents of European extraction), 24 as Asian (i.e., parents from the Indian subcontinent, mainly India), and 2 as “other.”

Grigg and colleagues excluded from their analyses data from two Asian children living at the same address because these children had much greater areas of material in their macrophages than the other children in the study; the investigators subsequently ascertained from the children’s parents that incense and oil lamps were burned in their home. Given the exclusion of data from these children, however, it is noteworthy that the report contains no discussion rates for vehicles derived from emissions inventories; traffic flow and traffic patterns from major roads; the distance from each child’s home to the nearest main road; and meteorologic data.

Sputum Assays

The investigators homogenized sputum samples and centrifuged them to separate them into cellular and supernatant fractions. They prepared slides of the cells and performed a differential cell count for leukocyte subsets, evaluating squamous cell (i.e., nonleukocyte) contamination (approximately 5%). The predominant leukocytes were macrophages (66%) and neutrophils (32%), with small numbers of lymphocytes and eosinophils. Leukocytes, and macrophages in particular, were further characterized by light microscopy, as described in the following section. Using an enzyme-linked immunosorbent assay (ELISA), Grigg and colleagues measured the cytokine interleukin-8 (IL-8) — a neutrophil chemoattractant — in the supernatant. Levels of IL-8 in the supernatant and the percentage of neutrophils and eosinophils in the cellular fraction were used as markers of inflammation.

Characterization of Macrophages by Light Microscopy

Using a light microscope, the investigators identified macrophages on air-dried slides. They captured and analyzed two-dimensional images of 100 macrophages per child, deleting the nucleus from each image because the image analysis software identified it as a large particle (see Figure 3 of the Investigators’ Report; unless otherwise specified, subsequent figure numbers refer to the Investigators’ Report). They also removed images of bacteria, although the validity of the method used was not referenced. They then calculated the total area in micrometers squared of particles inside each cell.

Modeling of PM₁₀ Exposure

To estimate locally derived PM₁₀ exposures for each child at his or her home address, Grigg and colleagues used the Airviro, version 2.21, dispersion model developed by the Swedish Meteorological and Hydrological Institute (www.environmental-expert.com/software/smhi/smhi.htm). Airviro is known as a “dispersion model” because it models the dispersion of pollutants generated by local sources at various distances from the sources. It is geographic-information-system-based software that integrates meteorologic data and data on emissions of pollutants from different types of sources, specifically, point (such as industrial or commercial facilities), line (roads), and area (such as residential or large industrial estates) sources. For this study, only concentrations of PM₁₀ from roads were estimated. Thus, the model included emissions rates for vehicles derived from emissions inventories; traffic flow and traffic patterns from major roads; the distance from each child’s home to the nearest main road; and meteorologic data.

Measures of Children’s Activities

The children filled out a questionnaire detailing their leisure-time physical activity, and the investigators also recorded the number of days the children did hard and
light physical exercise over the previous 2 weeks and the time they spent watching television. The investigators then calculated activity scores, which represented the metabolic equivalents of energy expenditure (MET) and vigorous activity (VIG), both in hours per week.

**Statistical Analysis**

Grigg and colleagues used linear regression to investigate the relationships between the variables of interest. The response variable for most regressions was either modeled exposure or a measure of lung function, such as FEV₁, and the predictor variable of interest was the median area of black material. Potentially confounding and effect modifying variables — such as subject characteristics and measures of physical activity — shown to be highly correlated with the outcome were included in the regression model. The investigators also assessed results using a non-parametric test — the Spearman rank correlation test.

**RESULTS**

**Low Modeled Exposure in All Study Participants**

When the investigators recruited children for the study, they used 1997 emissions inventory data in the Airviro model to estimate each child's exposure to PM₁₀. For their analyses at the end of the study, however, the investigators used updated emissions inventory information for the year 2001. With this updated emissions information, they estimated exposures to PM₁₀ at each child's home in the 12 months prior to sputum induction. Notably, the investigators found that the range of primary PM calculated from the model with the new emissions inventory information was three times lower than the range calculated for 1997, which was used to select study participants. Thus, based on these new estimates, all the children in the study fell into a low-exposure category, from 0 to 2.5 µg/m³ (see Figure 4). It should be noted again that these are not total PM₁₀ concentrations but only the contribution estimated from mobile sources. The investigators did not discuss reasons for this difference in exposure estimates, and they did not report the correlation between the estimates for the different years.

**Identification of Black-Pigmented Material Within Macrophages**

Grigg and colleagues found black-pigmented areas in macrophages (Figure 5) and reported that these black areas could not be detected in other airway cells, including neutrophils, columnar cells, and eosinophils (data not shown). They also reported that higher median areas of black-pigmented material were observed in children of Asian origin than in white children. They attributed this finding to a greater proportion of children of Asian origin living in areas of higher modeled exposure than white children (data not shown).

Nearly all of the black areas within macrophages were less than 2 µm in maximum diameter, and most were 0.5 µm or smaller (Figure 6). The investigators interpreted the morphologic observations (Figures 7A and 7B) to suggest that large black areas within a cell result from the aggregation of smaller particles.

Preliminary elemental analyses of the macrophages by electron energy loss spectroscopy (EELS) did not detect silica, iron, titanium, or sulfur (data not shown). These data support, but do not establish, that the particles were carbonaceous.

**Weak Correlation Between Size of Black Areas Detected in Airway Macrophages and Annual PM Exposure at the Child's Home**

Grigg and colleagues found a weak, but significantly positive, correlation ($r^2 = 0.081; P = 0.022$) between the median area of black material detected in airway macrophages and annual modeled exposure to primary PM₁₀ at the children's home addresses (Figure 11). An increase of 1 µg/m³ in modeled primary PM₁₀ was associated with an increase of 0.10 µm² (95% CI, 0.01 to 0.18) in black area. Distance from the home to the nearest main road was not associated with the size of the black area in macrophages (Figure 12). However, few children lived very close to busy roads (< 150 m).

**Negative Correlation Between Size of Black Areas Detected in Airway Macrophages and Pulmonary Function Parameters**

The researchers reported a significant negative correlation between the median area of black material in airway macrophages and the baseline (i.e., prebronchodilator) FEV₁ and FEF₂₅₋₇₅%, expressed either as a percentage of the predicted value or as a z score (Figure 9 and Table 4). The investigators also noted that the associations were not affected by bronchodilator treatment, indicating that the decrease in lung function was not the result of reversible bronchoconstriction. The median black area was also significantly associated with the FVC expressed as a z score but not as the percentage of the predicted value. These results are summarized in Critique Table 1, which also shows the calculated decrease in the percentage of predicted lung function associated with a 1-µm² increase in the black area inside the macrophages.
No Correlation Between Size of Black Areas Detected in Airway Macrophages and Other Parameters, Including Markers of Airway Inflammation

The investigators found no significant association between the particles detected in the airway macrophages and the child’s age, height, weight, sex, and activity level; or markers of airway inflammation (the percentage of neutrophils and eosinophils and the level of IL-8 in induced sputum).

DISCUSSION

In their proposal for this study, Grigg and colleagues described their intention to examine whether carbonaceous particles detected in the sputum-derived macrophages of healthy children could be used as a noninvasive marker of PM10 exposure, and in particular exposure to traffic-derived particles. An additional objective of the study as stated was to correlate PM exposure and particles detected in airway macrophages with airway inflammation. Given the mounting evidence linking air pollution with adverse effects on children’s lung growth and pulmonary function and the interest in assessing traffic-related health effects, the research questions were timely and of great interest.

The investigators’ main findings regarding these hypotheses were as follows:

- A weak correlation between the median black area detected in airway macrophages and annual PM10 exposure at the child’s home; and
- No correlation between the median black area detected in airway macrophages and any marker of airway inflammation.

An additional and potentially important finding of the study was the following:

- A negative correlation between the median area of particles in children’s airway macrophages and both FEV1 and FEF25%–75%.

WEAK CORRELATION BETWEEN SIZE OF BLACK AREAS DETECTED IN AIRWAY MACROPHAGES AND ANNUAL PM EXPOSURE AT THE CHILD’S HOME

Grigg and colleagues’ finding of a weak correlation between the median area of black-pigmented material inside the airway macrophages of healthy children and estimates of annual PM10 exposure at their home addresses suggests, at face value, that the amount of particles detected inside airway macrophages has the potential to be a useful marker for PM exposures. Although the investigators did not specifically address the issue of whether the particles detected reflected recent or longer-term exposures to PM, their findings are consistent with previous data that particles identified in airway macrophages are retained for long periods and hence are likely to reflect long-term exposure to air pollution. Nonetheless, several issues of study design and interpretation of the data suggest that the study’s main findings should be interpreted cautiously, for the reasons described in the next few sections.

Estimates of Individual Exposures

One major uncertainty is the accuracy of the estimates of individual exposures. The Airviro dispersion model that Grigg and colleagues used was developed and verified to estimate ambient levels of gaseous pollutants such as NO2, but it is not clear that it can provide suitably accurate estimates for ambient levels of PM10. However, given the similarity of the main local source of NO2 and PM (namely, traffic), there is no compelling reason to presume that the model is not valid for locally generated PM. Nevertheless, there are many uncertainties in the parameters used to derive the final exposure estimates. These include estimates of traffic patterns and vehicle profiles and the emission rates for diesel and gasoline vehicles. In addition, the investigators did not present any validation of their modeled estimates of PM10. Such validation might have been provided by a comparison of the Airviro-derived estimates

<table>
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<tr>
<th>Lung Function Parameter</th>
<th>Association with Median Black Area</th>
<th>Decrease in % Predicted Lung Function Value Associated with 1-µm² Increase in Black Area Inside Macrophages</th>
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<tbody>
<tr>
<td>FEV1</td>
<td>$r^2 = 0.126, P = 0.004$</td>
<td>17.0 (CI = 5.6–28.4)</td>
</tr>
<tr>
<td>FEF&lt;sub&gt;25%–75%&lt;/sub&gt;</td>
<td>$r^2 = 0.130, P = 0.004$</td>
<td>34.7 (CI = 11.3–58.2)</td>
</tr>
<tr>
<td>FVC</td>
<td>$r^2 = 0.074, P = 0.034$</td>
<td>12.9 (CI = 1.0–3.4)</td>
</tr>
</tbody>
</table>

Critique Table 1. Association of Different Lung Function Parameters with Median Black Area in Airway Macrophages

Both the investigators and their colleagues found no significant association between the particles detected in the airway macrophages and other parameters, including markers of airway inflammation. The investigators did not specifically address the issue of whether the particles detected reflected recent or longer-term exposures to PM, their findings are consistent with previous data that particles identified in airway macrophages are retained for long periods and hence are likely to reflect long-term exposure to air pollution. Nonetheless, several issues of study design and interpretation of the data suggest that the study’s main findings should be interpreted cautiously, for the reasons described in the next few sections.

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of PM\textsubscript{10} levels with the levels of ambient PM\textsubscript{10} measured at the five monitoring sites in the City of Leicester’s network.

In addition, the lack of association between the distance from the children’s homes to the nearest main road — another and more direct measure of traffic-derived pollution — and the size of the area of black-pigmented material detected in macrophages also supports the interpretation that PM\textsubscript{10} derived from mobile sources does not have a major role in the reported observations.

A further concern about the use of Airviro is that it models only outdoor concentrations of pollutants and thus does not reflect concentrations indoors, where children spend most of their time (Janssen et al. 1997; Koutrakis et al. 2005). Indoor levels of PM\textsubscript{10} are determined by indoor sources and the penetration factor of PM\textsubscript{10} from outside to inside the home. This penetration factor can vary greatly depending on the season (e.g., owing to open versus closed windows) and factors such as temperature and wind speed. Thus, the children’s true exposure to PM\textsubscript{10} is not well represented by outdoor PM\textsubscript{10} values.

The fact that the investigators found only a weak association between particles in the children’s airway macrophages and modeled PM\textsubscript{10} exposure at the children’s home — exposure that the investigators hypothesized reflected primarily traffic as the emissions source — also suggests that traffic is not a major source of the particles detected.

**Confounding by Ethnic Origin**

A second major issue is that Grigg and colleagues’ findings may be confounded by ethnic origin, specifically, by the fact that children of Asian origin — one of the two major subgroups of study participants — may have had both higher modeled PM exposures and larger areas of black-pigmented material in their macrophages than white children. Thus, the parameters measured may simply reflect or substitute for the ethnic origin of the study participants.

Confounding by ethnic origin is difficult to rule out in the current study because the investigators did not show the analyses of responses in Asian and white children separately. They did mention that higher median areas of black-pigmented material were observed in children of Asian origin than in children of white origin, and that this finding might possibly be because a greater portion of children of Asian origin live in areas of higher modeled exposure than do white children. Because relationships between height, weight, and age, on the one hand, and lung function, on the other hand, are different between subjects in different ethnic groups (Aggarwal et al. 2005, 2007), it is quite possible that the effect of PM\textsubscript{10} on lung function is different as well.

The investigators did show an analysis by ethnic subgroup in their recent paper (Kulkarni et al. 2006) which uses data from the current study. Figure 3 of that paper shows nonsignificant correlations between the median area of particles in the macrophages of healthy children and modeled PM\textsubscript{10} at or near their home address in each subgroup (“whites” and “nonwhites”) analyzed separately. In the analysis of all children, however, this correlation was significant. The fact that the association was not significant in either subgroup individually but was in the overall population makes the interpretation of the results difficult. The most likely explanation is that the finding of significance in the overall population is driven by the values in Asian children being much higher on both measures than those in white children.

**Generalizability of the Findings**

Data from the investigators’ recent paper (Kulkarni et al. 2006) also suggest that it may be difficult to use particles detected in airway macrophages as a biomarker of PM exposure in children across the spectrum of health and disease. Using the same data from healthy children reported in the current study, the investigators compared the size of the particle area in the macrophages of healthy children to that in asthmatic children who also lived in Leicester (Kulkarni et al. 2006). They found much lower particle content in the macrophages of the asthmatic children, even though their modeled levels of PM\textsubscript{10} were somewhat higher than those of healthy children. Thus, these data suggest that the correlation between the size of the area of particles detected in airway macrophages and the levels of modeled PM\textsubscript{10} in children is influenced by the presence or absence of asthma and possibly other conditions that affect the airways, including inherent differences in airway caliber. Thus the relationship between PM detected in airway macrophages and PM exposures is not likely to be straightforward.

**Sputum-Derived Macrophages**

A key goal of the study was to determine whether particles detected in sputum-derived macrophages could be used as a noninvasive marker of exposure to PM\textsubscript{10}. However, although sputum-derived macrophages may be more easily obtained than cells deeper in the lungs, they may not be the cells in lung parenchyma that most accurately reflect an individual’s exposure to PM\textsubscript{10}. These macrophages are recovered from regions of the respiratory tract where there is an effective method to transport the sputum, in particular from the mucus-covered large airways. Particles detected in macrophages obtained from lower in the airways — particularly in the alveolar region — may be a more appropriate reflection of particle load. Preliminary results of a study...
using macrophages obtained from adults by BAL (i.e., from deeper in the lungs than sputum-induced macrophages) suggest associations between particles detected in these cells and modeled annual exposure to PM$_{10}$ (estimated using a model distinct from Airviro) and living close to a main road (Celis et al. 2007).

**Nature of Black Areas Detected and Their Emissions Sources**

The investigators did not unequivocally establish that the black areas found inside the macrophages were carbonaceous particles. Titanium dioxide particles, for example, are usually opaque and are indistinguishable from carbon or other opaque particles through conventional light microscopy. However, elemental analyses by EELS did rule out some elements, and so it is probable that the particles identified were carbonaceous.

**NO CORRELATION BETWEEN SIZE OF BLACK AREAS DETECTED IN AIRWAY MACROPHAGES AND MARKERS OF AIRWAY INFLAMMATION**

The investigators’ finding that there were no associations between the size of the black areas in airway macrophages and any marker of airway inflammation may have been due to the following: besides the possibility that there is no correlation with the markers assessed, it might be that the sampling methods were too insensitive or inadequate to detect changes in the levels of markers. Another possibility, in view of the fact that lung inflammation has been detected in children and dogs from areas of Mexico with high levels of ozone and PM (annual average PM$_{10}$ of 78 µg/m$^3$ in metropolitan Mexico City) (Calderón-Garcidueñas et al. 2001, 2007), is that the level of exposure to pollution of the children in this study was too low for inflammatory effects of particles in the lungs to be observed.

**NEGATIVE CORRELATION BETWEEN SIZE OF BLACK AREAS IN CHILDREN’S AIRWAY MACROPHAGES AND FEV$_1$ AND FEF$_{25}$–75%**

The investigators’ finding of a negative correlation between median area of particles in children’s airway macrophages and FEV$_1$ and FEF$_{25}$–75% has been reported elsewhere (Kulkarni et al. 2006). The finding suggests at face value that higher and possibly longer-term exposure to PM leads to the impairment of lung function in children. This is a potentially important finding that fits with other data, most notably from California (Gauderman et al. 2004) and Austria (Horak et al. 2002), which have suggested that long-term exposure to air pollutants affects the development of children’s lung function.

However, the reported magnitude of changes in pulmonary function associated with particle area in the current report is noteworthy: as Critique Table 1 shows, a 1-µm$^2$ increase in black area inside macrophages was associated with a decrease of 17% in predicted FEV$_1$ and 35% in FEF$_{25}$–75%. The magnitude of these effects casts doubts on the results of the regression modeling. In a cell the size of a macrophage with an approximate diameter of 20 µm and a cross-sectional area of 300 µm$^2$, this change in area represents a tiny fraction of the cell’s total cross-sectional area. In an earlier study of Ethiopian children exposed to biomass burning, Grigg and colleagues found that particles inside the children’s macrophages occupied 3.3 µm$^2$, approximately 6 to 10 times the area of particles detected in cells in the current study, and presumably reflecting much higher exposures to particulate pollution (Kulkarni et al. 2005).

**SUMMARY AND CONCLUSIONS**

In this study, Grigg and colleagues attempted to establish whether carbon particles found in the airway macrophages of healthy children in Leicester, England — a city with little or no heavy industrial sources of pollution — could be used as a biomarker of exposure to traffic-related PM$_{10}$. The researchers also examined the correlation between particles present in these macrophages and markers of airway inflammation and between the presence of macrophage particles and pulmonary function parameters.

Using light microscopy to identify black areas, presumably particles, in airway macrophages derived from sputum induction, Grigg and colleagues found a weak correlation between the area of particles and estimates of annual PM$_{10}$ exposure at the child’s home address. At face value, these findings suggest that particles detected inside airway macrophages have the potential to be a useful marker for exposures to PM. However, several issues of study design and interpretation of data imply that the study’s main findings should be interpreted cautiously: one major uncertainty is the accuracy of the estimates of individual PM$_{10}$ exposures obtained by using the Airviro dispersion model without validation of this approach. A second major issue is that the findings may be confounded by ethnic origin; that is, that children of Asian origin — one of the two major subgroups of study participants — may have had higher modeled exposures to PM and higher levels of particles in their macrophages than white children, the other major subgroup of participants. In addition, although obtaining sputum-derived macrophages is noninvasive and these macrophages may be more easily obtained than cells deeper in the lungs, they may not be the cells that most accurately reflect an individual’s exposure to...
PM$_{10}$. Particles detected in macrophages obtained from lower in the airways, particularly in the alveolar region, may be a more appropriate reflection of the particle load. Furthermore, the investigators did not establish that the particles found inside the macrophages were carbonaceous or derived from traffic or indeed any other outdoor combustion source.

The investigators’ attempt to determine whether particles detected in airway macrophages correlated with markers of airway inflammation was worthy. The fact that the investigators did not find associations between the area of particles in airway macrophages and any marker of airway inflammation was perhaps not surprising, because one-time measures of these markers are likely to be more variable within individuals than measures of pulmonary function, such as FEV. In addition to the possibility that there is no correlation with the markers assessed, other possible explanations include that the sampling methods were too insensitive or inadequate to detect changes or that the level of exposure to pollution of the children in this study was too low for any inflammatory effects of particles in the airways to be observed.

The investigators’ finding of a negative correlation between the area of particles in the children’s macrophages and the pulmonary function parameters FEV$_1$ and FEF$_{25\%–75\%}$ is potentially important. At face value, it suggests that higher exposure to PM may lead to impairment of children’s lung function. Although the investigators did not specifically address the issue, particles identified in airway macrophages are likely to reflect long-term exposure to air pollution. Thus, Grigg and colleagues’ finding is consistent with other data suggesting that long-term exposure to air pollutants affects the development of children’s lung function. However, the magnitude of the changes in pulmonary function associated with increased particle area that Grigg and colleagues reported appears surprisingly large, thus casting doubt on the results of their regression modeling.

In summary, the main question explored by Grigg and colleagues — whether particles inside airway macrophages may be used as a biomarker of exposure to PM$_{10}$ (and, in particular, traffic-derived PM$_{10}$) — remains interesting and important. However, given the caveats regarding the study design and the interpretation of the results discussed in this Critique, this study has not answered the question. Nevertheless, the potential importance of the study’s major finding — that there are associations between particles detected in airway macrophages and a reduction in key lung function parameters — suggests that further studies are needed to investigate the reported associations.

ACKNOWLEDGMENTS

The Health Review Committee thanks the ad hoc reviewers for their help in evaluating the scientific merit of the Investigators’ Report. The Committee is also grateful to Maria Costantini for her oversight of the study, to Gerardo Sanchez and Geoffrey Sunshine for their assistance in preparing its Critique, to Hilary Selby Polk for science editing and managing the publication process, and to Ruth Shaw for preparing this report for publication.

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Black-Pigmented Material in Airway Macrophages from Healthy Children: Association with Lung Function and Modeled PM$_{10}$

Jonathan Grigg, Neeta Kulkarni, Nevil Pierse, Lesley Rushton, Christopher O'Callaghan, and Andrew Rutman