RESEARCH REPORT

HEALTH EFFECTS INSTITUTE

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Health Effects of Acute Exposure to Air Pollution

Part I: Healthy and Asthmatic Subjects Exposed to Diesel Exhaust

Stephen T Holgate, Thomas Sandström, Anthony J Frew, Nikolai Stenfors, Charlotta Nördenhall, Sundeep Salvi, Anders Blomberg, Ragnberth Helleday, and Margaretha Södenberg

Part II: Healthy Subjects Exposed to Concentrated Ambient Particles

Stephen T Holgate, Robert B Devlin, Susan J Wilson, and Anthony J Frew

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The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI supports research on all major air pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate matter) and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 220 projects at institutions in North America and Europe and has published over 140 research reports.

Typically, HEI receives half its funds from the US Environmental Protection Agency and half from 28 manufacturers and marketers of motor vehicles and engines in the US. Occasionally, funds from other public and private organizations either support special projects or provide a portion of the resources for an HEI study. Regardless of funding sources, HEI exercises complete autonomy in setting its research priorities and in reaching its conclusions. An independent Board of Directors governs HEI. The Institute's Health Research and Health Review Committees serve complementary scientific purposes and draw distinguished scientists as members. The results of HEI-funded studies are made available as Research Reports, which contain both the Investigators' Report and the Review Committee's evaluation of the work's scientific quality and regulatory relevance.

HEI STATEMENT

Synopsis of Research Report 112

Effects of Particles on Lung Inflammation in Healthy and Asthmatic Volunteers

BACKGROUND

Epidemiologic studies have noted that short-term increases in ambient levels of particulate matter (PM) are associated with hospital admissions and deaths from cardiovascular and respiratory disorders. These studies have suggested that individuals with preexisting diseases, such as cardiovascular disease or asthma, are more susceptible to the effects of air pollution than healthy individuals. However, the biologic mechanisms that underlie this association and the role that the composition and size of PM may have in causing adverse health effects are not well understood.

One hypothesis about how PM may exert its effects is that when it deposits in the airways, it activates a cascade of inflammatory events, a part of the body's natural defense systems. Dr Holgate and an international team of collaborators had observed some of these inflammatory changes in lung fluids and blood from humans exposed to PM. They wanted to investigate whether such changes could also be observed in lung tissues. They proposed that such changes may be related to the chemical composition of PM (diesel exhaust vs concentrated ambient particles [CAPs]), and expected that changes would be more pronounced in people with asthma than in healthy people. The Swedish government and the US Environmental Protection Agency funded the human exposure studies (diesel exhaust in Sweden; CAPs in the US) that provided tissue samples. Recognizing an opportunity for one laboratory to analyze tissues obtained after different exposures, HEI funded Holgate's lung tissue studies in the United Kingdom. Part I of the Investigators' Report describes the effects of diesel exhaust on inflammatory markers in healthy and asthmatic participants. Part II describes the effects of CAPs from the eastern US on inflammatory markers in healthy participants only.

APPROACH

At the Swedish laboratory, 25 healthy and 15 asthmatic participants were exposed for 2 hours to diesel exhaust (100 $\mu g/m^3$ PM concentration) or to filtered air on separate days. At the US laboratory, 12 healthy participants were exposed for 2 hours to filtered air and 30 different healthy participants were exposed to a range of CAPs concentrations (25–311 $\mu g/m^3$; median 108 $\mu g/m^3$). In both studies, lung function was assessed before and immediately after exposure. To obtain lung tissues and fluids, all participants in Sweden underwent bronchoscopy 6 hours after exposure; in the US, lung tissues were obtained 18 hours after exposure from 11 control and 10 CAPs-exposed participants (range 38–311 $\mu g/m^3$; median 84 $\mu g/m^3$). Tissue samples were sent to Dr Holgate in the United Kingdom for analysis of inflammatory markers, including numbers of specific white blood cells, expression of activation markers, and levels of cytokines. Whereas Holgate's laboratory focused on analyzing lung tissues, the other laboratories also analyzed lung function, lung fluids, and blood. To appropriately interpret Holgate's results, it is important to also consider the results from the other laboratories.

RESULTS AND INTERPRETATION

Part I: Exposure of Healthy and Asthmatic Subjects to Diesel Exhaust

For both healthy and asthmatic participants, the investigators observed a small increase in airway resistance (meaning the airways were slightly more constricted) immediately after exposure to diesel exhaust.

For healthy participants, of the many biochemical markers of inflammation that were assessed, some were significantly changed 6 hours after exposure to diesel exhaust. For example, in lung fluids, the percentages of neutrophils and lymphocytes increased and the percentage of macrophages decreased (a complementary response). This group also showed increased levels of cytokines measured in lung fluids (interleukins [IL] 6 and 8) and in lung tissues (IL-8), and increased levels of cellular adhesion molecules in lung tissues (vascular cell adhesion molecule 1 and P-selectin). Protein and

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Dr Stephen T Holgate at the University of Southampton, Southampton, UK. The following Research Report contains two detailed Investigators' Reports and a Critique on the study prepared by the Institute's Health Review Committee.

messenger RNA levels of other inflammatory markers were unchanged. It is difficult to arrive at a simple unifying description of these complex inflammatory changes and their biological relevance.

For participants with asthma, the only inflammatory changes observed after exposure to diesel exhaust were a decrease in the percentage of eosinophils in lung tissues and fluids and an increase in levels of the cytokine IL-10 in lung tissues. (IL-10 has antiinflammatory effects and is currently being considered as a treatment for asthma.) These findings warrant further research.

The investigators expected that asthmatic participants would show more inflammatory changes than healthy participants because epidemiologic studies had found an association between increased asthma symptoms and high levels of air pollution on certain days. However, Holgate and colleagues did not find inflammatory changes in asthmatic participants after controlled exposure to diesel exhaust. It is possible that exposure at these relatively low levels would be less likely to cause inflammation. In addition, the small number of participants studied and the greater variability (compared with healthy participants) observed in their inflammatory parameters heighten uncertainty and lessen the likelihood of finding significant effects. In addition, it is unclear whether exposure affected ventilation differently in the two groups; a change in ventilation could affect the number and size of particles that reached the deeper lung.

Part II: Exposure of Healthy Subjects to Concentrated Ambient Particles

The investigators found no changes in lung function immediately after exposure. At 18 hours after exposure to CAPs but not to filtered air, they observed an increase in blood fibrinogen levels, and a higher percentage of neutrophils and a lower percentage of macrophages in lavage fluids (again, a complementary response). They found no differences in any of the inflammatory markers evaluated in bronchial tissues.

DISCUSSION OF BOTH STUDIES

Comparing the results of these two studies with results from other PM studies is difficult. In the current diesel exhaust study, many markers of inflammation were studied but few changed; of those that changed, the magnitude of the change was modest. Other studies have shown greater inflammatory effects after diesel exhaust exposure. Several reasons for the differing results among studies are plausible. The times at which endpoints were assessed differed among studies: Given that the spectrum of inflammatory events spans hours to days after exposure, the current diesel exhaust study focused on early inflammatory events by measuring markers at 6 hours after exposure. Other studies, including the current CAPs study, investigated inflammatory responses at 18 to 24 hours after exposure. Furthermore, because so few markers of inflammation changed in the current studies, it is possible that these changes occurred by chance.

One unresolved issue is that the exposure atmospheres included gaseous pollutants. In the CAPs study, gases were present at ambient levels and are unlikely to have influenced the results. In the diesel study, however, levels of nitrogen dioxide, for example, were relatively high. Because gases such as ozone and nitrogen dioxide are known to affect lung function and inflammation at fairly low concentrations, it is possible that small amounts of these gases influenced the results.

CONCLUSIONS

The current study is an early effort to investigate and compare effects of diesel exhaust and CAPs exposure on lung tissues obtained via bronchial biopsies from humans. Amidst a number of negative results, the study found that, after exposure to diesel exhaust, (1) lung function (airway resistance) changed modestly in both healthy participants and participants with asthma; and (2) healthy participants exhibited small changes in some markers of inflammation but participants with mild asthma did not. After exposure to CAPs, the study found no changes in bronchial tissues; however, the small number of participants and the variability in CAPs concentrations complicate interpreting and comparing these findings with results from the diesel exhaust exposures.

A consistent pattern of inflammation after exposure to a variety of PM mixtures in many studies has not emerged to date. In part, this may be due to different experimental approaches and to measuring different inflammatory markers at different times after exposure.

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HEALTH EFFECTS INSTITUTE

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Research Report 112

Health Effects of Acute Exposure to Air Pollution

HEI STATEMENT

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

INVESTIGATORS' REPORTS

When an HEI-funded study is completed, the investigators submit a final report. The Investigators' Report is first examined by three ouside 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.

Part I: Healthy and Asthmatic Subjects Exposed to Diesel Exhaust

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Part II: Healthy Subjects Exposed to Concentrated Ambient Particles

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Continued

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.

RELATED HEI PUBLICATIONS

Publishing History: In December 2002, Part I of this document was posted as a preprint on *www.healtheffects.org*; in December 2003, Part I was slightly revised and both Parts I and II were posted to the Web and finalized for print.

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When specifying Part I, Part II, or another section of this report, cite it as a chapter of the whole document.

INVESTIGATORS' REPORT

Health Effects of Acute Exposure to Air Pollution

Part I: Healthy and Asthmatic Subjects Exposed to Diesel Exhaust

Stephen T Holgate, Thomas Sandström, Anthony J Frew, Nikolai Stenfors, Charlotta Nördenhall, Sundeep Salvi, Anders Blomberg, Ragnberth Helleday, and Margaretha Söderberg

ABSTRACT

The purpose of this study was to assess the impact of short-term exposure to diluted diesel exhaust on inflammatory parameters in human airways. We previously exposed control subjects for 1 hour to a high ambient concentration of diesel exhaust (particle concentration 300 µg/m^3—a level comparable with that found in North Sea ferries, highway underpasses, etc). Although these exposures did not have any measurable effect on standard indices of lung function, there was a marked neutrophilic inflammatory response in the airways accompanied by increases in blood neutrophil and platelet counts. Endothelial adhesion molecules were upregulated, and the expression of interleukin 8 messenger RNA (IL-8 mRNA*) was increased in a pattern consistent with neutrophilia.

Individuals with asthma have inflamed airways and are clinically more sensitive to air pollutants than are control subjects. The present study was designed to assess whether this clinical sensitivity can be explained by acute neutrophilic inflammation or an increase in allergic airway inflammation resulting from diesel exhaust exposure. For this study, we used a lower concentration of diesel exhaust (100 µg/m^3 PM_{10}) for a 2-hour exposure.

At this concentration, both the control subjects and those with asthma demonstrated a modest but statistically significant increase in airway resistance following exposure to diesel exhaust. This increase in airway resistance was associated with an increased number of neutrophils in the bronchial wash (BW) fluid obtained from control subjects (median after diesel exhaust 22.0 vs median after air 17.2; $P = 0.015$), as well as an increase in lymphocytes obtained through bronchoalveolar lavage (BAL) (15.0% after diesel exhaust vs 12.3% after air; $P = 0.017$). Upregulation of the endothelial adhesion molecule P-selectin was noted in bronchial biopsy tissues from control subjects (65.4% of vessels after diesel exhaust vs 52.5% after air). There was also a significant increase in IL-8 protein concentrations in BAL fluid and IL-8 mRNA gene expression in the bronchial biopsy tissues obtained from control subjects after diesel exhaust exposure (median IL-8 expression 65.7% of *adenine phosphoribosyl transferase [APRT]* gene expression value after diesel exhaust vs 51.0% after air; $P = 0.007$). There were no significant changes in total protein, albumin, or other soluble inflammatory markers in the BW or BAL fluids. Red and white blood cell counts in peripheral blood were unaffected by diesel exhaust exposure.

Airway mucosal biopsy tissues from subjects with mild asthma (defined as forced expiratory volume in 1 second [FEV_1] greater than or equal to 70% of the predicted value) showed eosinophilic airway inflammation after air exposure compared with the airways of the corresponding control subjects. However, among the subjects with mild asthma, diesel exhaust did not induce any significant change in airway neutrophils, eosinophils, or other inflammatory cells; cytokines; or mediators of inflammation. The only clear effect of diesel exhaust on the airways of subjects with asthma was a significant increase in IL-10 staining in the biopsy tissues.

This study demonstrated that modest concentrations of diesel exhaust have clear-cut inflammatory effects on the airways of nonasthmatic (or control) subjects. The data suggest a direct effect of diesel exhaust on IL-8 production leading to upregulation of endothelial adhesion molecules and neutrophil recruitment. Despite clinical reports of increased susceptibility of patients with asthma to diesel exhaust and other forms of air pollution, it does not appear that this susceptibility is caused either directly by induction

* A list of abbreviations and other terms appears at the end of the Investigators' Report.

This Investigators' Report is Part I of Health Effects Institute Research Report 112, which also includes Part II, a Critique by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr Stephen T Holgate, University of Southampton, Southampton General Hospital, Mail Point 810, Level D, Centre Block, Tremona Road, Southampton, SO16 6YD, UK; *sth@soton.ac.uk*.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award R82811201 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

of neutrophilic inflammation or indirectly by worsening of preexisting asthmatic airway inflammation. The increased level of IL-10 after diesel exhaust exposure in airways of subjects with asthma suggests that this pollutant may induce subtle changes in airway immunobiology. This is an important topic for further investigation. Other possible explanations for the apparent lack of response to diesel exhaust among subjects with asthma include (1) the time course of the response to diesel may differ from the response to allergens, which peaks 6 to 8 hours after exposure; (2) a different type of inflammation may occur that was not detectable by the standard methods used in this study; and (3) the increased sensitivity of patients with asthma to particulate air pollution may reflect the underlying bronchial hyperresponsiveness found in asthma rather than any specific increase in inflammatory responses.

INTRODUCTION AND BACKGROUND

There is increasing concern about the possible adverse effects of diesel exhaust particles on human health. Over the past forty years, the atmospheric concentrations of conventional pollutants such as black smoke and sulfur dioxide have declined significantly as the result of clean air legislation (Guidotti 1996). However, the volume of road traffic has increased substantially over the same period and concerns have been raised about the possible effects on human health of both motor vehicle exhaust (Bascom et al 1996a,b) and particles released from diesel-fueled power plants (Quality of Urban Air Review Group 1996). This change in the pattern of pollution has been paralleled by a progressive increase in the proportion of children and adults affected by asthma and other allergic diseases (Britton 1992; Ninan and Russell 1992; Nystad et al 1997). Evidence from Japan suggests that individuals who live near major highways are more likely to develop allergies (Yokoyama et al 1985; Ishizaki et al 1987). In addition, an adjuvant effect of diesel particles on the development of immunoglobulin E (IgE) specifically directed against airborne allergens has been shown both in vitro (Diaz-Sanchez et al 1994; Takenaka et al 1995; Fujeida et al 1998) and in vivo (Suzuki et al 1996). Nevertheless, considerable controversy remains about the extent to which these changes are due to a more toxic environment or to a more susceptible population (Seaton et al 1994).

More recently, a clear association has been demonstrated between cardiovascular morbidity and airborne concentrations of particulate matter (Dockery et al 1993; Anderson et al 1996; Samet et al 2000). Some researchers have hypothesized that this association reflects changes in plasma viscosity, coagulability, or both that have been documented during air pollution episodes (Peters et al 1997). Associations have also been found between concentrations of fine particles and many other health outcomes (Kaiser 1997), particularly respiratory disorders including asthma episodes that require hospital admission (Schwartz et al 1993; Dockery and Pope 1994) and decrements in lung function in patients with asthma (Peters et al 1999). The mechanisms underlying these associations are not clear, especially in light of the presence of the association even at low particle concentrations (Schwartz et al 1993; Walters et al 1994; Wordley et al 1997).

Asthma is characterized by a complex and specific form of inflammation affecting the airway mucosa. Over the past 12 years, this type of inflammation has been carefully characterized and we now know a great deal about the pathological changes associated both with asthma (Djukanović et al 1990; Bradley et al 1991; Kraft et al 1999) and with exacerbations of asthma (Corrigan and Kay 1990; Faul et al 1997). In particular, clinical manifestations include damage to the epithelial layer, deposition of collagen below the basement membrane (Roche et al 1989), submucosal edema, and infiltration of the epithelium and submucosa with a variety of inflammatory cells, among which eosinophils and T cells are particularly evident (Djukanović et al 1990; Bradley et al 1991).

In our laboratory in Southampton, we have developed methods to accurately quantify the infiltrating cells (Synek et al 1996), analyze the activation of endothelial cells thought to be involved in cellular recruitment (Montefort et al 1994), and assess the presence and amount of various protein messengers involved in cellular activation (cytokines) and in cellular recruitment (chemokines) (Robinson et al 1992; Bradding et al 1994). Particular attention has been focused on the cytokines IL-4, IL-5, and interferon-γ (IFN-γ) because IL-4 and IFN-γ are critically involved in the regulation of IgE (Maggi et al 1992) and IL-5 is implicated in the growth, differentiation, and survival of eosinophils (Hamid et al 1991; Sanderson 1992). Unlike cytokines, which have fairly specific receptors and act on specific cell types, chemokines overlap much more in action and receptor specificity (Nickel et al 1999). Researchers studying asthma are particularly interested in eotaxin, RANTES (Regulated on Activation, Normal T cell Expressed, and presumably Secreted), macrophage inflammatory protein-1α (MIP-1α), IL-8, and growth-related oncogene-α (GRO-α). These molecules are associated with eosinophil and neutrophil recruitment (Nickel et al 1999)

and they are expressed in patients with mild asthma (Berkman et al 1996; Cruikshank et al 1995).

In an earlier collaboration between Dr Sandström and colleagues at University Hospital, Umeå, Sweden, and Holgate's laboratory in Southampton, a control group of human volunteers was exposed to diesel exhaust for 1 hour at a particle concentration of 300 μg/m^3; the material obtained after exposure was analyzed both in Umeå and in Southampton. That study found evidence of an acute inflammatory response in the control airways, with increased numbers of neutrophils in the bronchial epithelium, submucosa, and BW fluid; increased numbers of T cells in the bronchial epithelium and submucosa; increased numbers of mast cells in the submucosa; and increased numbers of B cells in BAL fluid. These cellular changes were associated with increased concentrations of histamine and fibronectin in the BAL fluid and upregulation of the endothelial adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1) (Salvi et al 1999a). Diesel exhaust exposure was associated with increased gene transcription for IL-8 and, to a lesser extent, for IL-5 (Salvi et al 2000). In addition, protein concentrations of IL-8 and GRO-α were elevated in the exposed airways.

In order to further explore the possible mechanisms of these health effects, programs of controlled exposure of human volunteers to diluted fresh diesel exhaust (Umeå, Sweden) or concentrated ambient particles (Chapel Hill, North Carolina) were begun. The Southampton group provided technical and analytical support to both exposure facilities. The effects of these exposures were analyzed in terms of lung function, blood parameters of inflammation and coagulation, the contents of the airway lumen, and the structure of the airway mucosa. Part I of this Research Report focuses exclusively on the diesel exhaust studies performed with control subjects and subjects with asthma. The studies with concentrated ambient particles are presented in Part II of this Research Report. Although the exposure protocols and the analytic work performed in Umeå and in Chapel Hill were not funded by HEI, a description of the protocols and the analytic results are included in this Research Report to provide a context for Holgate's analyses of bronchial biopsy tissues, which was the portion of work funded by HEI.

The hypothesis for these studies was that suspended particles, especially those smaller than 2.5 μm in aerodynamic diameter ($PM_{2.5}$) have the capacity to produce toxic effects by promoting an inflammatory cascade that starts in the epithelium and spreads to involve other cell types present in the airways.

SPECIFIC AIMS

The specific aim of this study was to assess the effect of short-term exposure to diesel exhaust on the airways of healthy control subjects and subjects with mild atopic asthma. The principal endpoints were measurements of lung function, cellular recruitment, and cytokine expression in the airways of the subject groups after exposure to diluted diesel exhaust or filtered air. The Health Effects Institute funded the analyses of biopsy tissues. All other aspects of the study were funded by the Swedish government.

METHODS

SUBJECTS

Subjects with asthma and control subjects were recruited by advertising for volunteers. The asthma group consisted of 15 subjects (5 female, 10 male) with mild atopic asthma (Table 1); the mean age was 30 years (range 23 to 52 years). They all had a diagnosis of asthma and had positive skin tests to one or more common airborne allergens. All 15 subjects met three criteria: They had hyperreactive airways based on methacholine challenge (the concentration that provoked a 20% fall in FEV_1 [PC_{20}] was between 0.5 and 4 mg/mL); their FEV_1 was greater than 70% of the predicted value; and their only medication consisted of inhaled β_2-agonists as required. The control group consisted of 25 subjects (9 female, 16 male); the mean age was 25 years (range 19 to 42 years) (Table 1). The control subjects had normal lung function and negative skin tests to common airborne allergens. None of the control subjects was taking any regular medication. None of the subjects in either group smoked; and all subjects had to be free from respiratory infection for at least 6 weeks before the study and had to remain so throughout the study. Subjects were not allowed to take any anti-inflammatory drugs or antioxidant vitamin supplements (vitamin C or vitamin E) during the study.

DIESEL EXHAUST EXPOSURE CHAMBER

The diesel exposure chamber (Figure 1) was designed and developed in the Department of Occupational and Environmental Medicine, University of Umeå, for carrying out controlled human exposures to diluted diesel exhaust (Rudell et al 1994, 1996, 1999). It was built with a metallic frame (3 × 3 × 2.3 m); the walls were made of a thin plastic polythene foil (0.15 mm thick) so that they could be

Table 1. Characteristics of Control and Asthmatic Subjects

	Gender	Age (years)	Height (cm)	Weight (kg)	FVC (L)	FEV_1 (L)	FEV_1/FVC (%)[a]
Subjects with Mild Asthma							
1	Male	28	176	76	6.31	4.24	67.0
2	Male	24	169	70	5.17	3.98	77.0
3	Female	26	172	64	5.28	3.96	75.0
4	Male	23	180	90	5.19	4.38	84.0
5	Male	30	175	71	5.35	3.85	72.0
6	Female	34	164	58	4.10	3.27	80.0
7	Female	28	164	69	3.89	3.32	85.0
8	Female	27	175	65	5.03	3.68	73.0
9	Male	25	180	87	5.52	4.54	83.0
10	Male	27	174	78	6.98	4.22	60.0
11	Male	27	200	91	6.79	4.72	70.0
12	Male	32	180	85	5.29	4.05	77.0
13	Male	52	175	85	4.11	3.24	79.0
14	Female	25	175	110	4.65	4.05	87.0
15	Male	47	192	99	6.65	4.10	62.0
Mean		**30.3**	**176.7**	**80**	**5.35**	**3.97**	**75.40**
SD		8.4	9.4	14	0.97	0.44	8.16
Control Subjects							
1	Male	25	178	86	5.76	4.71	82.0
2	Male	23	190	88	6.08	4.59	75.0
3	Male	32	181	74	5.09	4.20	83.0
4	Female	23	167	66	4.54	3.54	78.0
5	Male	24	181	80	6.47	5.39	83.0
6	Female	24	175	70	4.97	4.22	85.0
7	Male	32	184	78	6.41	5.34	83.0
8	Male	24	186	85	7.73	6.04	78.0
9	Male	19	186	92	6.63	4.38	66.0
10	Male	28	179	74	6.15	4.57	74.0
11	Female	22	161	52	3.75	3.11	83.0
12	Female	22	164	66	4.08	3.35	82.0
13	Female	24	162	60	4.17	3.50	84.0
14	Male	24	179	85	6.64	4.96	75.0
15	Female	26	160	52	3.68	3.20	87.0
16	Female	23	163	62	4.12	3.20	78.0
17	Female	22	167	67	3.91	3.38	86.0
18	Female	21	171	68	4.73	3.81	81.0
19	Male	42	177	83	6.16	4.56	74.0
20	Male	23	181	73	6.71	4.98	74.0
21	Male	24	180	70	6.17	4.86	79.0
22	Male	26	178	74	4.84	3.96	82.0
23	Male	24	191	86	7.72	5.37	70.0
24	Male	24	178	74	5.87	4.74	81.0
25	Male	23	177	64	4.79	3.75	78.0
Mean		**25.0**	**169.4**	**73**	**5.49**	**4.31**	**79.24**
SD		4.6	32.8	11	1.21	0.81	5.13

[a] FEV_1/FVC ratio as a percentage of the predicted value.

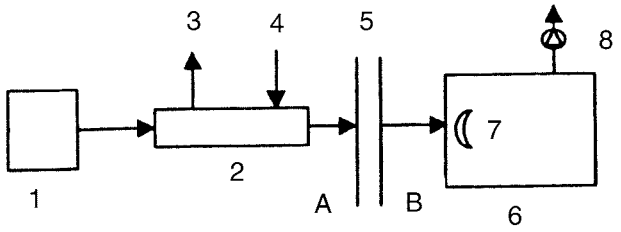

Figure 1. Schematic view of the engine room (A) and the exposure chamber (B). 1 = Engine; 2 = shunt dilutor; 3 = shunt tube; 4 = air dilutor; 5 = brick wall; 6 = exposure chamber; 7 = diesel exhaust inlet and cone-shaped flow distributor; and 8 = evacuation pipe and fan.

changed regularly. The inlet for diesel exhaust was in the middle of one wall 45 cm above the floor. The outlet with the waste tube fan was located above the roof 65 cm from the middle of the wall opposite the inlet. Air in the chamber was changed every 2.3 minutes. The diesel engine was situated in an adjacent soundproofed room. With the use of a shunt tube and adjustable valve, the exhaust from the diesel engine was diluted with ambient air to less than 10% of the total volume. (The ambient air was obtained from office space elsewhere in the building and contained less than 0.1 parts per million [ppm] of nitrogen oxides [NO_x].) The diluted exhaust was fed into the exposure chamber at a constant flow of 1.5 m^3/min with the use of a flexible tube, which was preheated and held at 200°C to avoid condensation. A predetermined concentration of diluted diesel exhaust in the chamber was achieved by varying the diameter of the diaphragm in the shunt-dilutor or in the valve opening of the shunt tube. The evacuation fan created a small negative pressure within the chamber to avoid exhaust leaking from the chamber into the surrounding room in which staff monitored the exposure. Thus, very small but constant amounts of air entered the chamber via minimal slots in the corners of the chamber. During the exposures the temperature and humidity inside the chamber were maintained at 20°C and 50%, respectively.

Diesel exhaust was generated from a Volvo diesel engine-TD1F Intercooler, a 6-cylinder, 4-stroke direct-injection turbo-charged diesel engine, model 1990. At the time of the exposures, the truck had been driven 4,500 km. The engine idled at 900 rpm during the exposures. The fuel used was a low-sulfate diesel (OK Promil 1, OK Petroleum, Stockholm, Sweden) with the following composition: cetane number 51, aromatics 25% volume, polycyclic aromatic hydrocarbons 0.5% volume, sulfur 0.06% weight, carbon 86.4% weight, hydrogen 13.5% weight, nitrogen < 0.02% weight, oxygen < 0.1% weight. The 10% volume boiling point was 200°C, 50% volume boiling point was 282°C, and 95% volume boiling point was 355°C.

During the initial evaluation studies (Rudell et al 1994), the size and shape of the particles in the diesel exhaust obtained at the opening of the tail pipe and in the exposure chamber did not differ. Using scanning electron microscopy, no visible changes were found in the size and shape or further agglomeration of the submicronic particles in the chamber (Rudell et al 1994). No gradients of nitrogen dioxide (NO_2) or particles were found between the breathing zone of the exposed volunteers and the inlet for the analyzing instruments in the exposure chamber. Predetermined concentrations of nitrogen oxides and particles could be achieved inside the chamber within 5 to 7 minutes and maintained at desired levels throughout the exposure period. The initial evaluation confirmed that a predetermined steady-state concentration of diesel exhaust was achieved, and exposures could be maintained at a standard dose for every subject (Rudell et al 1994).

Continuous analysis of carbon monoxide (CO) during the exposures was performed with a Miran 1-A instrument (Foxboro Co, East Bridgewater MA). Nitric oxide (NO) and NO_2 were continuously monitored with the help of a chemiluminescence instrument (CSI 1600 NO_x analyzer, Columbia, Scientific Industries Corp, Austin TX). Total hydrocarbons (HC) were analyzed continuously with a flame ionization detection instrument (model 3-300, JUM Engineering, Munich, Germany) that has a heated prefilter (180°C) and was calibrated with propane. The number and mass of exhaust particles were monitored using a condensation particle laser counter (model 3022, TSI, St Paul MN). Formaldehyde (HCHO) was collected on glass-fiber filters (diameter 13 mm) impregnated with 2,4-dinitrophenylhydrazine and analyzed by high-performance liquid chromatography (model WISP 712, Millipore, Milford CT). Airborne sulfate concentrations were not measured because the fuel used was a low-sulfate diesel fuel and the level of airborne sulfates was found to be negligible in previous studies.

The mean steady-state concentrations for PM_{10} and gases in the exposure chamber are given in Table 2. Differential mobility particle sizer measurements of diesel exhaust indicated a calculated geometric mean electrical mobility equivalent diameter for particle number of 0.073 µm, for particle surface of 0.12 µm, and for particle volume of 0.28 µm.

Particle mass distribution over space was examined in a separate experiment (Figure 2). The variability within the central space where the subjects were located was within ± 3% (points B, C, and E in Figure 2). For the very distal points on the diagonal, the variability was 9% (point A) and 6% (point D). Due to limitations in resources, particle size distribution over time and space was not evaluated during exposures.

The subjects were exposed for 2 hours once to diesel exhaust and once to filtered air in a randomized sequence and were blinded to the nature of each exposure. The exposure protocols were identical in all respects except that when the subject was exposed to filtered air, the diesel engine exhaust fumes were vented outside the building rather than being introduced into the exposure chamber. During the 2-hour period, the subjects exercised on a stationary bicycle for 15-minute periods alternating with 15-minute rest periods; this achieved a minute ventilation of approximately 15 to 20 L/m^2/min. Nasal lavage samples were obtained before, during, and after the exposure and by fiber-optic bronchoscopy 6 hours after the end of exposure. (Nasal lavage samples were obtained and stored for antioxidant measurements, which are not reported here.) The investigators analyzing samples were blinded to the randomized exposure order for all subjects.

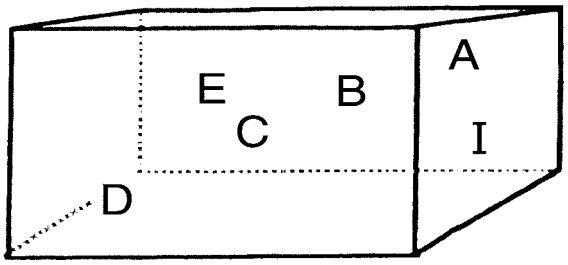

Figure 2. Variability of particle mass measured within the exposure chamber diagonal. I is the inlet, B–C is the area closest to the breathing zone of the subjects, E is the space more lateral to the wall, and A and D are the extremes along the diagonal.

BLOOD TESTS

Venous blood samples were drawn at four time points: immediately before exposure, after 1 hour of exposure, immediately after the end of exposure, and immediately before bronchoscopy (performed 6 hours after the end of exposure). Only data from before exposure and 6 hours after the end of exposure are reported here. Samples were sent to the routine laboratory in Umeå for cell counts, and plasma samples were stored for later analysis of antioxidant concentrations (not reported here).

BRONCHOSCOPIES

Fiberoptic bronchoscopies were performed at the Department of Respiratory Medicine and Allergy at the University Hospital of Northern Sweden, Umeå. Successful bronchoscopies were performed in 24 of 25 control subjects and 14 of 15 asthmatic subjects. Subjects were premedicated with subcutaneous atropine 30 minutes before the procedure and mildly sedated with intravenous propofol just before the procedure. Lidocaine was used for topical anesthesia. A flexible fiberoptic bronchoscope (Olympus BF IT 10, Tokyo, Japan) was inserted through a mouth piece with the patient in the supine position. During each bronchoscopy, five endobronchial mucosal biopsies were performed (three for immunohistochemistry and two for reverse transcriptase–polymerase chain reaction [RT-PCR] enzyme-linked immunosorbent assay [ELISA]) with a fenestrated forceps (Olympus FB-21C, Tokyo, Japan). Tissue samples were taken either from the anterior aspect of the main carina and the subcarinae of the third- to fourth-generation airways on the right side or from the posterior aspect of the main carina and the corresponding subcarinae on the left side. Biopsy tissues were obtained from one side, and lavage was carried out on the contralateral side. This procedure was reversed during the second bronchoscopy to avoid biopsy artifacts. BW with 20 mL (two times) and BAL with 60 mL (three times) sterile phosphate-buffered saline (PBS; pH 7.3, at 37°C) were performed after the tip of the bronchoscope was carefully wedged into the lingula or middle-lobe bronchus. The fluid was gently suctioned into a siliconized container placed in iced water. The material recovered from the first and second 20-mL BWs was analyzed separately (BW-1 and BW-2), whereas the fluid recovered from the three 60-mL BALs was pooled. The BW results reported here are from the first (BW-1) sample; the BW-2 samples were sent for antioxidant analysis, which is not reported here.

Table 2. Characteristics of Chamber Air During Diesel Exhaust Exposures

	CO (ppm)	NO (ppm)	NO_2 (ppm)	NO_x (ppm)	HC (ppm)	PM_{10} (μg/m^3)	HCHO (μg/m^3)
Minimum	0.6	0.5	0.1	0.8	1.3	94.0	33.0
Maximum	2.5	0.6	0.3	0.9	1.8	124.1	53.0
Mean	**1.7**	**0.6**	**0.2**	**0.8**	**1.4**	**108.3**	**43.5**
SD	0.4	0.02	0.02	0.02	0.11	11.6	5.9

ANALYSIS OF BW AND BAL FLUIDS

The BW and BAL samples recovered into the siliconized container were filtered through a nylon filter (pore diameter 100 μm, Syntab AB, Malmo, Sweden) and centrifuged at 400*g* for 15 minutes. The supernatants were separated from the cell pellets and immediately analyzed for albumin and total protein by an autoanalyzer using commercial kits from Boehringer Mannheim (Mannheim, Germany). Supernatants were then stored at –70°C for later cytokine and mediator analyses (transported by air to Southampton). Measurements of IL-8 and soluble ICAM-1 were performed in Southampton with commercial ELISA kits (R & D Systems, Minneapolis MN). Methylhistamine was measured with commercial kits from Pharmacia (Uppsala, Sweden) and Dako (High Wycombe, UK). The complement products C3a and C5a were quantified using radioimmunoassay kits (Amersham, UK).

Cell pellets were resuspended in PBS at 10^6 cells/mL and the total number of cells in the lavage and wash fluids was counted in a Burker chamber (VWR International). Cytocentrifuged specimens with 5×10^4 cells/slide were prepared using a cytospin 3 centrifuge (Shandon Southern Instruments, Sewikly PA) at 1000 rpm for 5 minutes; differential counts were measured after staining with May-Grunwald Giemsa, counting 400 cells/slide. Lymphocyte subsets were determined by flow cytometry (FACS Scan, Becton Dickinson).

TISSUE SAMPLE EMBEDDING, PROCESSING, AND CUTTING

The endobronchial biopsy tissues obtained at bronchoscopy were placed in ice-cooled acetone containing protease inhibitors (phenylmethyl-sulfonyl fluoride [2 nM] and iodoacetamide [2 nM]), cooled rapidly to −20°C, and left overnight. The following day the sample was put in acetone for 15 minutes and in methyl benzoyl for an additional 15 minutes, with both solvents at room temperature. The tissue was then immersed in glycol methacrylate JB4 solution A (Polysciences, Northampton, UK) at 4°C for 7 hours, during which the glycol methacrylate solution was changed three times. The tissue was finally embedded in glycol methacrylate resin (prepared by mixing glycol methacrylate monomer, *N,N*-dimethylaniline in polyethelene glycol 400, and benzoyl peroxide), which was left for polymerization overnight at 4°C. The blocks were then stored in airtight containers at −20°C until used for immunostaining with monoclonal antibodies (Table 3) and a standard peroxidase technique (Britten et al 1993). Embedded biopsy tissues were transported by air freight to Southampton for further analysis. Using a microtome, 2-μm sections were obtained, floated onto ammonia water (1:500), picked onto 0.01% poly-L-lysine–coated glass slides, and allowed to dry at room temperature for 1 hour. Slides were initially stained with toluidine blue to assess tissue structure and select the best sections for immunostaining. Sections on slides were then kept at −40°C in aluminum foil to preserve tissue antigenicity.

QUANTITATION OF INFLAMMATORY CELLS AND ADHESION MOLECULES

Stained inflammatory cells were counted separately in the epithelium and in the submucosa excluding mucosal

Table 3. Names and Sources of Monoclonal Antibodies Used for Immunohistochemistry

Antibody[a]	Marker	Cells	Source
ICAM-1	ICAM-1	Endothelial cells	Serotec, UK
VCAM-1	VCAM-1	Endothelial cells	Sanbio, UK
E-selectin	E-selectin	Endothelial cells	Serotec, UK
P-selectin	P-selectin	Endothelial cells	Immunotech, UK
EN4	Endothelium	Pan-endothelial marker	Sanbio, UK
AA1	Tryptase	Mast cells	Dr A Walls, Southampton, UK
NE	Neutrophil elastase	Neutrophils	DAKO, High Wycombe, UK
EG2	Cationic protein	Eosinophils	DAKO, High Wycombe, UK
CD14	CD14	Macrophages	DAKO, High Wycombe, UK
CD19	CD19	B cells	DAKO, High Wycombe, UK
CD3	CD3	T cells	DAKO, High Wycombe, UK
CD4	CD4	Helper T cells	Becton Dickinson, UK
CD8	CD8	Suppressor T cells	DAKO, High Wycombe, UK

[a] See the section Abbreviations and Other Terms for these definitions.

glands, blood vessels, muscle, and any region of torn or folded tissue. The length of the epithelium and the area of the submucosa in which cell counts were performed were determined using computer-assisted image analysis Color Vision Software (Improvision System, Birmingham, UK). The results were expressed as cells/mm of epithelium and cells/mm^2 of submucosa (Djukanović et al 1990; Bradding et al 1994; Synek et al 1996).

In the case of endothelial adhesion molecules, the number of blood vessels stained with a specific monoclonal antibody at a standard dilution was compared with the total complement of the microvessels identified by staining with the endothelial marker endothelium clone 4 (EN4) in subjacent sections 2 µm apart. The number of positively stained blood vessels was expressed as a percentage of the total vessel number (Montefort et al 1994).

The cytokines and chemokines IL-6, IL-8, IL-10, granulocyte-macrophage colony–stimulating factor (GM-CSF), GRO-α, tumor necrosis factor α (TNF-α), and nuclear factor κB (NF-κB) were stained with monoclonal antibodies as described above and their expression in the epithelium was expressed as a percentage of the area of epithelial cells.

RT-PCR ELISA

This method was developed in the Division of Respiratory Cell and Molecular Biology Research at the University of Southampton Medical School, to provide semiquantitative measurements of mRNA in small tissue samples (Salvi et al 1999b). Unless otherwise specified, all chemicals were obtained from Sigma (Poole, Dorset, UK).

RNA Extraction from Bronchial Biopsy Tissues

A second set of bronchial biopsy tissues were stored in liquid nitrogen at −70°C and transported by air freight to Southampton for further analysis. They were immediately suspended in a 0.1-mL, sterile, ground-glass minihomogenizer containing 2 µL glycogen (Roche Diagnostics) and 100 µL TRIZOL (Life Technologies). The samples were thoroughly homogenized until the tissue was completely lysed. The homogenate was transferred to a 500-µL sterile RNase-free eppendorf tube. An additional 100 µL TRIZOL was added to the glass minihomogenizer to wash the remaining RNA and was then pooled into the same eppendorf tube. A volume of 40 µL chloroform (20% volume of TRIZOL) was added to the eppendorf tube, which was then vortexed thoroughly for 30 seconds and incubated on ice for 15 minutes. The tubes were then centrifuged at 12,000*g* at 4°C for 15 minutes. This separated the homogenate into an upper aqueous phase, an intermediate phase, and a lower organic phase. RNA, which remains exclusively in the upper phase, was removed into a sterile RNase-free 500-µL eppendorf tube using a sterile fine-tip minipastette. An equal volume of isopropranol (approximately 80 µL) was added to the eppendorf tube, which was vortexed thoroughly for 30 seconds and kept at −40°C overnight to precipitate the total RNA. Following centrifugation at 12,000*g* at 4°C for 15 minutes the next day, RNA was visible as a tiny white opaque pellet at the bottom of the tube. The isopropranol was tipped off with a sterile fine-tip pastette, and the RNA pellet was washed twice with 500 µL cold 80% ethanol by centrifuging at 12,000*g* at 4°C for 5 minutes for each wash. The ethanol was tipped off, and the RNA pellet was allowed to air dry. Then 5 µL of ultra high-quality water treated with diethyl pyrocarbonate (DEPC) was added to the RNA pellet, which was dissolved by heating at 60°C for 10 minutes.

RNA Extraction from the BW Cell Pellet

A volume of 200 µL TRIZOL and 2 µL glycogen was added to the BW cell pellet in a 500-µL sterile RNase-free eppendorf tube, which was vortexed thoroughly and kept on ice for 30 minutes. The remaining part of the procedure was the same as that described above.

RNA Measurement

Total RNA was quantified from the RNA solution with the use of a Gene Quant instrument (Pharmacia, UK) to measure the optical density at 260 nm. The total RNA was expressed as micrograms per milliliter. Sample purity was assessed by measuring the ratio of the optical densities obtained at 260 nm and 280 nm and expressing it as the percentage of purity. During RT, equal amounts of RNA were reverse-transcribed between paired samples to ensure consistency and comparability between samples. During the entire procedure of RT-PCR ELISA, the investigator was blinded regarding type of exposure (air or diesel exhaust). The overall purity of the RNA was 85% to 90% for this study.

RT Protocol

RT was performed with a commercial kit (Promega, UK). A master mixture was prepared as shown in Table 4 (with amounts given for a 20-µL reaction). A volume of 5 µL total RNA (approximately 1 µg) was added to 15 µL of the master mixture in a 50-µL PCR tube and incubated at 42°C for 60 minutes with use of the PerkinElmer thermal cycler. The RT enzyme was inactivated by heating the tube at 94°C for

Table 4. Composition of Master Mixture Used for RT Protocol

Composition	Volume (µL)	Final Concentration
$MgCl_2$ (25 mM)	4	5 mM
RT buffer (10×)	2	
dNTP (10 mM)	2	1 mM for each dNTP
Oligo dT[p(dT)$_{15}$][a]	1	0.5 µg/µg RNA
RNase inhibitor	1	1 unit/µL
Avian myeloblastosis virus reverse transcriptase	1	15 units/µL
DEPC water	4	

[a] Oligo dT[p(dT)$_{15}$] = deoxythymidine oligomer, 15 dT in length.

3 minutes. The resultant 20-µL complementary DNA (cDNA) was removed from the cycler and diluted with 20 µL ultra high-quality water to obtain a total volume of 40 µL cDNA from each bronchial biopsy tissue or BW cell pellet sample.

PCR ELISA Digoxigenin Labeling

For the PCR amplification, primer pairs specific for the constitutively expressed gene *APRT* and the genes for the cytokines IL-1β, IL-5, IL-8, TNF-α, IFN-γ, and GM-CSF were used. We designed the primers according to published sequences (Table 5) (Salvi et al 1999b). For APRT and each cytokine, 2.5 µL cDNA was amplified using *Taq* DNA polymerase in the presence of 15 pmol of both primers, 2.5 µL digoxigenin labeling mix (Boehringer Mannheim), and magnesium-free thermophilic PCR buffer

Table 5. Nucleotide Primer Sequences for Capture Probes

Protein or Enzyme	Nucleotide Sequence of the Gene	Position from Transcription Site
Capture Probes for PCR ELISA		
APRT	Sense (5′) 5′-GCT GCG TGC TCA TCC GAA A G-3′	316–335
	Antisense (3′) 5′-CCT TAA GCG AGG TCA GCT CC-3′	542–561
IL-1β	Sense (5′) 5′-AAC AGG CTG CTC TGG GAT TC-3′	27–46
	Antisense (3′) 5′-TAA GCC TCG TTA TCC CAT GT-3′	405–424
IL-4	Sense (5′) 5′-CTG CAA ATC GAC ACC TAT TA-3′	46–65
	Antisense (3′) 5′-GAT CGT CTT TAG CCT TTC-3′	477–494
IL-5	Sense (5′) 5′-CTG AGG ATT CCT GTT CCT GT-3′	148–167
	Antisense (3′) 5′-CAA CTT TCT ATT ATC CAC TC-3′	385–404
IL-8	Sense (5′) 5′-GCA GCT CTG TGT GAA GGT GCA-3′	46–69
	Antisense (3′) 5′-CAG ACA GAG CTC TCT TCC AT-3′	215–234
TNF-α	Sense (5′) 5′-CGA GTG ACA AGC CTG TAG CC-3′	251–270
	Antisense (3′) 5′-CAT ACC AGG GCT TGG CCT CA-3′	555–574
IFN-γ	Sense (5′) 5′-GGT CAT TCA GAT GTA GCG GA-3′	121–140
	Antisense (3′) 5′-GCG TTG GAC ATT CAA GTC AG-3′	371–390
GM-CSF	Sense (5′) 5′-GCA TGT GAA TGC CAT CCA GG-3′	93–112
	Antisense (3′) 5′-GCT TGT AGT GGC TGG CCA TC-3′	288–307
Biotin-Labeled Internal Capture Probes		
APRT	5′-AGG GCG TCT TTC TGA ATC TC-3′	402–421
IL-1β	5′-TCT CCG ACC ACC ACT ACA GC-3′	241–259
IL-4	5′-CCG TAA CAG ACA TCT TTG CT-3′	220–239
IL-5	5′-GGG AAT AGG CAC ACT GGA GA-3′	207–226
IL-8	5′-TGA AGA GGA CCT GGG AGT AG-3′	173–192
TNF-α	5′-CAC TCT TTT GGA TGC TCT GG-3′	402–421
IFN-γ	5′-CAC TCT TTT GGA TGC TCT GG-3′	258–277
GM-CSF	5′-AGA CCC GCC TGG AGC TGT AC-3′	218–237

(Promega). Magnesium chloride ($MgCl_2$) was added in a concentration optimal for each primer pair. Target cDNA was amplified for 30 cycles using primer annealing temperatures optimized for each cytokine.

Digoxigenin Detection

Digoxigenin detection was performed with a commercial kit (Boehringer Mannheim). A volume of 10 µL PCR product was denatured with 40 µL PCR ELISA denaturation solution. The gene-specific biotinylated capture probe (50 ng/mL in 450 µL of the kit hybridization buffer) was then hybridized to each complementary digoxigenin-labeled PCR product at 37°C for 3 hours with constant shaking. During this time, the digoxigenin-labeled PCR product–biotinylated probe hybrids were immobilized onto streptavidin-coated microtiter plates. After thorough washing to remove free antibody, the bound PCR products were detected using peroxidase-conjugated antidigoxigenin antibody. The PCR products were then made visible with the peroxidase substrate 2,2′-azino-di-[3-ethyl benzthiazoline sulfonate] (ABTS). After 20 minutes of development, the wells contained a green color in proportion to the original amount of mRNA, and the signals were measured by reading absorbance at 405 nm.

STATISTICAL ANALYSIS

Blood, BW, BAL, and immunohistochemistry indices were analyzed using the Wilcoxon paired rank test. The Mann-Whitney *U* test was used to compare baseline values for control subjects with those for the subjects with asthma. The Mann-Whitney *U* test was also used to compare the cellular and soluble mediator responses between the two groups. To evaluate the effects of exposures, lung function results at the different time points were compared with preexposure levels and expressed as preexposure and postexposure ratios. Because these data were parametric and normally distributed, the paired *t* test was used to compare diesel exhaust exposure with air exposure. Similarly, the independent-sample *t* test was used to compare control subjects with subjects with asthma. Resultant *P* values < 0.05 were regarded as significant.

ETHICAL APPROVAL

All studies reported here were approved by the relevant ethics committees in Umeå or Southampton.

RESULTS

At the tested concentration of diesel exhaust (100 µg/m^3), both the control subjects and those with asthma demonstrated a modest but statistically significant increase in airway resistance at the end of the 2-hour exposure to diesel exhaust (Table 6). However, there were no significant changes in standard measures of dynamic lung function (Table 7). There were also no changes in blood hemoglobin, total white cell counts, or in neutrophil counts 6 hours after the end of diesel exhaust exposure (Table 8).

The increase in airway resistance after diesel exhaust exposure was associated with an increased number of neutrophils in the BW fluid from control subjects (median 22.0% after diesel exhaust vs 17.2% after air; $P = 0.015$) (Figure 3; Table 9). There were no significant increases in the numbers of other cell types. In the BAL fluid from control subjects, there was an increase in the proportion of lymphocytes after diesel exhaust (15.0% after diesel exhaust vs 12.3% after air; $P = 0.017$) and a smaller (but not statistically significant) change in neutrophil numbers (1.8% after diesel exhaust vs 1.3% after air) (Table 10). There were no significant changes in total protein or albumin in the BW or BAL fluids (Table 11). The granule products for eosinophils (eosinophilic cationic protein) and for neutrophils (myeloperoxidase) were measured in BW fluid to assess degranulation, but there were no differences in these values between the diesel exhaust and filtered air exposures (Table 12). Similarly, there was no change after exposure to diesel exhaust in soluble ICAM-1, which was measured as an index of epithelial activation. Extracellular superoxide dismutase was measured as a marker of response to oxidative stress; it did not change after exposure to diesel exhaust (Table 12). However, after diesel exhaust exposure there were increases in the proinflammatory cytokine IL-6 and the chemokine IL-8 in BW fluid, but this was observed only in the control subjects. These latter changes are consistent with epithelial activation, epithelial injury, or both, but the degree of injury fell short of that required to cause protein leakage.

Recruitment of neutrophils and T cells is usually dependent upon the upregulation of adhesion molecules on the endothelial cells that line the small blood vessels. These are upregulated in a coordinated and sequential fashion when endothelial injury or activation occurs: P-selectin is upregulated very rapidly, E-selectin appears at 4 to 8 hours, and ICAM-1 from 4 to 24 hours (Montefort et al 1993). VCAM-1 is associated with eosinophil and T cell recruitment, and is seen at 12 to 36 hours. In the control subjects, exposure to diesel exhaust induced upregulation of the adhesion molecule P-selectin (65.4% of vessels after diesel exhaust vs 52.5% after air). Expression of VCAM-1 also increased after exposure to diesel exhaust in the control subjects ($P < 0.05$) (Figures 4 and 5; Table 13).

Table 6. Airway Resistance[a] Before, During, and After Exposure to Diesel Exhaust or Filtered Air

	Filtered Air			Diesel Exhaust		
Subject	Before Exposure	After 1 Hour of Exposure	After 2 Hours of Exposure	Before Exposure	After 1 Hour of Exposure	After 2 Hours of Exposure
Subjects with Mild Asthma						
1	0.136	0.134	0.118	0.225	0.233	0.225
2	0.088	0.085	0.086	0.124	0.138	0.140
3	0.125	0.131	0.131	0.138	0.151	0.157
4	0.123	0.122	0.125	0.119	0.128	0.127
5	0.106	0.105	0.105	0.102	0.110	0.110
6	0.115	0.110	0.104	0.078	0.092	0.095
7	0.092	0.095	0.085	0.126	0.125	0.127
8	0.142	0.129	0.124	0.163	0.171	0.168
9	0.119	0.122	0.121	0.114	0.129	0.147
10	0.143	0.166	0.150	0.191	0.216	0.213
11	0.181	0.145	0.136	0.129	0.134	0.140
12	0.192	0.195	0.175	0.154	0.184	0.181
13	0.100	0.120	0.109	0.122	0.134	0.155
14	0.173	0.182	0.186	0.179	0.199	0.218
15	0.090	0.120	0.125	0.139	0.144	0.148
Mean	**0.128**	**0.131**	**0.125**	**0.140**	**0.153**	**0.157**[b]
SD	0.033	0.031	0.028	0.037	0.040	0.039
Control Subjects						
1	0.148	0.143	0.120	0.155	0.134	0.161
2	0.084	0.082	0.087	0.095	0.108	0.116
3	0.100	0.099	0.102	0.102	0.113	0.107
4	0.118	0.120	0.121	0.102	0.121	0.123
5	0.061	0.060	0.059	0.059	0.062	0.064
6	0.093	0.100	0.109	0.107	0.128	0.133
7	0.079	0.085	0.075	0.070	0.077	0.082
8	0.109	0.095	0.096	0.107	0.128	0.134
9	0.154	0.157	0.154	0.147	0.159	0.164
10	0.082	0.089	0.085	0.097	0.109	0.107
11	0.133	0.132	0.133	0.184	0.189	0.201
12	0.097	0.100	0.094	0.161	0.157	0.137
13	0.097	0.099	0.099	0.127	0.131	0.135
14	0.108	0.148	0.102	0.101	0.101	0.108
15	0.123	0.125	0.135	0.109	0.112	0.108
16	0.171	0.172	0.167	0.171	0.210	0.220
17	0.074	0.082	0.077	0.077	0.081	0.091
18	0.087	0.093	0.089	0.100	0.118	0.138
19	0.068	0.068	0.068	0.069	0.078	0.084
20	0.096	0.093	0.096	0.078	0.092	0.095
21	0.097	0.100	0.117	0.096	0.096	0.091
22	0.099	0.098	0.094	0.103	0.106	0.105
23	0.088	0.091	0.090	0.096	0.107	0.115
24	0.092	0.094	0.092	0.078	0.107	0.102
25	0.172	0.173	0.166	0.148	0.153	0.154
Mean	**0.105**	**0.108**	**0.105**	**0.110**	**0.119**[c]	**0.123**[d]
SD	0.030	0.030	0.028	0.034	0.034	0.036

[a] Airway resistance is measured in (kPa × sec)/L.

[b] Significant increase in airway resistance after 2 hours of diesel exhaust exposure ($P = 0.005$). The relative change in airway resistance from before exposure to the end of a 2-hour exposure was used to compare air exposures with diesel exhaust exposures by paired *t* tests.

[c] Significant increase in airway resistance after 1 hour of diesel exhaust exposure ($P = 0.026$ by paired *t* test).

[d] Significant increase in airway resistance after 2 hours of diesel exhaust exposure ($P < 0.001$ by paired *t* test).

Table 7. Dynamic Lung Function[a] Before and After Exposure to Diesel Exhaust or Filtered Air

	Filtered Air				Diesel Exhaust			
	FVC		FEV_1		FVC		FEV_1	
Subject	Before Exposure	After 2 Hours of Exposure	Before Exposure	After 2 Hours of Exposure	Before Exposure	After 2 Hours of Exposure	Before Exposure	After 2 Hours of Exposure
Subjects with Mild Asthma								
1	6.31	6.45	4.24	4.37	6.36	6.46	4.14	4.22
2	5.17	5.14	3.98	4.07	5.21	4.98	4.08	4.04
3	5.28	5.13	3.96	4.01	5.09	4.97	4.08	3.97
4	5.19	5.24	4.38	4.37	5.32	5.28	4.51	4.46
5	5.35	5.21	3.85	3.81	5.44	5.33	3.95	3.90
6	4.10	4.04	3.27	3.38	3.96	3.98	3.27	3.30
7	3.89	3.83	3.32	3.32	3.79	3.74	3.25	3.25
8	5.03	4.93	3.68	3.75	4.89	4.88	3.73	3.76
9	5.52	5.54	4.54	4.60	5.71	5.60	4.65	4.68
10	6.98	6.80	4.22	4.33	— [b]	6.62	— [b]	4.09
11	6.79	6.59	4.72	5.15	6.82	6.56	4.97	5.03
12	5.29	5.58	4.05	4.25	5.63	5.43	4.22	4.12
13	4.11	4.35	3.24	3.39	4.22	4.31	3.28	3.43
14	4.65	4.98	4.05	3.83	4.56	4.34	3.41	3.38
15	6.65	6.67	4.10	4.09	6.32	6.59	3.99	4.33
Mean	**5.35**	**5.37**	**3.97**	**4.05**	**5.24**	**5.27**	**3.97**	**4.00**
SD	0.97	0.93	0.44	0.50	0.91	0.96	0.54	0.52
Control Subjects								
1	5.76	5.78	4.71	4.70	5.79	5.82	4.75	4.79
2	6.08	6.30	4.59	4.81	6.17	6.21	4.85	4.77
3	5.09	5.31	4.20	4.44	5.30	5.37	4.46	4.38
4	4.54	4.54	3.54	3.56	4.58	4.70	3.69	3.77
5	6.47	6.39	5.39	5.35	6.46	6.36	5.49	5.37
6	4.97	4.76	4.22	4.10	5.04	4.75	4.30	4.13
7	6.41	6.31	5.34	5.35	6.35	6.39	5.56	5.61
8	7.73	7.71	6.04	5.99	8.00	7.65	6.38	6.07
9	6.63	6.78	4.38	4.30	6.40	6.63	4.27	4.27
10	6.15	6.09	4.57	4.44	6.21	6.45	4.62	4.72
11	3.75	3.70	3.11	3.14	3.79	3.78	3.19	3.20
12	4.08	4.00	3.35	3.32	4.14	4.10	3.53	3.43
13	4.17	4.08	3.50	3.54	4.05	3.96	3.47	3.47
14	6.64	6.69	4.96	5.12	6.68	6.73	5.21	5.01
15	3.68	3.54	3.20	3.13	3.75	3.74	3.27	3.21
16	4.12	4.27	3.20	3.11	4.32	4.39	3.23	3.24
17	3.91	3.91	3.38	3.45	4.19	3.97	3.65	3.55
18	4.73	4.50	3.81	3.71	4.22	4.19	3.32	3.27
19	6.16	6.48	4.56	4.81	6.55	6.44	4.89	4.86
20	6.71	6.76	4.98	5.09	6.67	6.77	4.79	4.92
21	6.17	6.00	4.86	4.76	5.70	5.76	4.60	4.58
22	4.84	4.84	3.96	3.90	4.97	4.84	4.08	4.09
23	7.72	7.67	5.37	5.23	7.84	7.84	5.31	5.41
24	5.87	6.03	4.74	4.88	6.01	6.11	4.92	5.00
25	4.79	4.96	3.75	3.83	5.19	5.20	4.10	4.17
Mean	**5.49**	**5.50**	**4.31**	**4.32**	**5.53**	**5.53**	**4.40**	**4.37**
SD	1.21	1.24	0.81	0.82	1.22	1.23	0.85	0.83

[a] All data are shown in L.

[b] Data for before diesel exhaust exposure are missing for subject 10.

Table 8. Numbers of Peripheral Blood Cells Before and 6 Hours After a 2-Hour Exposure to Diesel Exhaust or Filtered Air[a]

	Control Group (n = 24[c])			Mild Asthma Group (n = 14[c])		
Time of Measurement[b]	Median	25th Percentile	75th Percentile	Median	25th Percentile	75th Percentile
Hemoglobin (g/L)						
Before air	143	132	153	141	135	150
6 Hours after air	143	128	150	135	127	149
Before DE	142	129	149	142	134	151
6 Hours after DE	142	127	151	142	134	147
White Blood Cells						
Before air	5.6	4.9	6.6	5.4	4.7	5.7
6 Hours after air	6.8	5.6	8.0	6.3	5.7	7.6
Before DE	5.7	5.0	6.2	5.3	4.4	5.9
6 Hours after DE	6.5	6.1	7.6	6.8	5.7	7.8
Polymorphonuclear Neutrophils						
Before air	2.7	2.2	3.0	2.7	2.3	2.8
6 Hours after air	4.0	3.3	4.7	3.5	3.0	4.4
Before DE	3.0	2.2	3.6	2.5	2.1	3.1
6 Hours after DE	4.1	3.4	5.2	3.8	3.4	4.4
Lymphocytes						
Before air	2.3	1.9	2.6	1.9	1.7	2.3
6 Hours after air	2.1	1.6	2.4	2.1	1.5	2.3
Before DE	1.9	1.7	2.2	1.8	1.7	2.0
6 Hours after DE	2.2	1.6	2.3	2.0	1.8	2.4
Monocytes						
Before air	0.5	0.5	0.7	0.5	0.5	0.6
6 Hours after air	0.5	0.5	0.6	0.5	0.4	0.6
Before DE	0.5	0.4	0.6	0.4	0.3	0.5
6 Hours after DE	0.6	0.4	0.6	0.5	0.4	0.6

[a] Values are given as $\times 10^9$ cells/L unless otherwise noted.

[b] "6 Hours" refers to 6 hours after the end of a 2-hour exposure.

[c] One control subject underwent only diesel exhaust exposure and one asthmatic subject was unable to complete either bronchoscopy; therefore we omitted their data from all analyses.

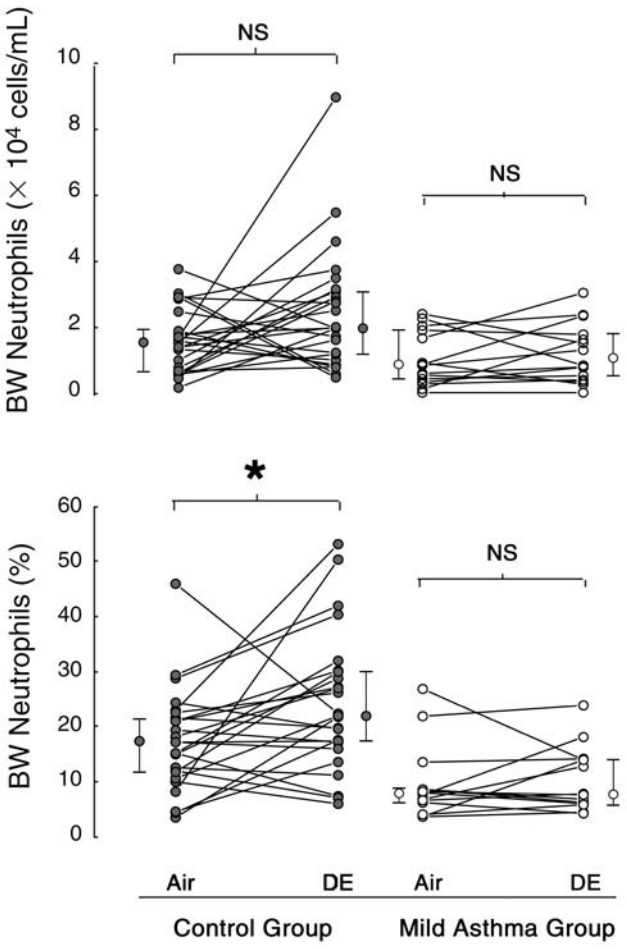

Figure 3. Neutrophils in BW fluid obtained 6 hours after control subjects (n = 24) and subjects with asthma (n = 14) were exposed to air or diluted diesel exhaust. Paired BW fluid samples were obtained from 14 of 15 asthmatic and 24 of 25 control subjects; one subject in each group did not complete the full bronchoscopy protocol. Data are illustrated as individual responses with group medians and interquartile ranges (expressed as error bars). NS = not significant; * = statistically significant at $P < 0.05$ by Wilcoxon signed rank test.

Neutrophil recruitment requires upregulation of endothelial adhesion molecules and a chemotactic gradient in order to induce cells to leave blood vessels and migrate toward extravascular sites of tissue injury. One of the chemokines that has been implicated in neutrophilic inflammation is IL-8 (Nickel et al 1999). In order to explore the pattern of cytokine expression in bronchial biopsy tissues, we developed a novel PCR ELISA method that allows semiquantitative analysis of mRNA in small tissue samples (Salvi et al 1999b). The principal advantages of the PCR ELISA technique are its high sensitivity, high specificity, and quantitative output. Six cytokines were selected for analysis: IL-1β, IL-5, IL-8, IL-13, IFN-γ, and TNF-α. After diesel exhaust exposure, there were significant increases in IL-8 in the BW fluid (Table 12; Figure 6) and in IL-8 mRNA in the bronchial biopsy tissue samples obtained from control subjects (median 65.7% of *APRT* value after diesel exhaust vs median 51.0% after air; $P = 0.007$) (Table 14; Figure 7). These results for control subjects were consistent with data obtained in our previous study using diesel exhaust at the higher concentration of 300 μg/m^3 (Salvi et al 1999a).

The subjects with mild asthma showed a modest degree of submucosal eosinophilic airway inflammation after exposure to filtered air compared with the airways of the control subjects (Table 9; Table 15, Figure 8). This observation is consistent with previously published work showing eosinophilic airway inflammation in the submucosa in people with all grades of asthma (Djukanović et al 1990). The time point selected (6 hours after exposure ended) was chosen to correspond with the time point at which peak cellular response had been observed in studies of allergen challenge (Montefort et al 1994). However, unlike the control subjects, the subjects with asthma did not show any significant change in submucosal or epithelial cellular parameters, cytokine gene transcription, or concentration of inflammatory mediators after exposure to diesel exhaust (100 μg/m^3 PM_{10} for 2 hours) (Tables 9 through 16; Figures 3 through 6). If anything, there was a reduction in the percentage of eosinophils among subjects with asthma after diesel exposure (Table 9, Figure 8).

Immunostaining for cytokine and chemokine protein in the respiratory epithelium showed a higher baseline expression of TNF-α in people with asthma compared with control subjects (median 0.18% in subjects with asthma vs 0.0% in control subjects; $P < 0.05$; see Table 17). GM-CSF expression was also increased in subjects with asthma; however, this did not achieve significance ($P = 0.069$). In contrast, IL-10 was expressed at lower density in the biopsy tissues from airways in the group with asthma (0.22% in subjects with asthma vs 1.29% in control subjects; $P = 0.001$). Following diesel exhaust exposure, IL-10 staining increased markedly in subjects with asthma (0.22% after filtered air exposure vs 0.92% after diesel exhaust exposure; $P = 0.002$), whereas biopsy tissues from control subjects showed a trend toward a significant reduction in IL-10 staining (1.29% after filtered air exposure vs 0.39% after diesel exhaust exposure; $P = 0.11$) (Figure 9; Table 17). There were no significant changes in epithelial IL-6, IL-8, GM-CSF, GRO-α, RANTES, TNF-α, and NF-κB staining in either group following exposure to diesel exhaust (Table 17).

Table 9. Relative Cell Counts in BW Fluid 6 Hours After a 2-Hour Exposure to Diesel Exhaust or Filtered Air[a]

	Control Group (n = 24[b])			Mild Asthma Group (n = 14[b])		
Exposure	Median	25th Percentile	75th Percentile	Median	25th Percentile	75th Percentile
Polymorphonuclear Neutrophils						
Air	17.2[c]	11.2	22.1	7.8	5.7	9.9
Diesel exhaust	22.0[d]	16.6	30.1	7.7	6.0	14.1
Lymphocytes						
Air	2.0	0.6	3.5	1.6	0.6	3.2
Diesel exhaust	1.6	0.7	3.5	2.5	1.1	5.2
Eosinophils						
Air	0.0	0.0	0.3	0.9[e]	0.2	1.5
Diesel exhaust	0.2[f]	0.0	0.4	0.4[g]	0.0	0.9
Mast Cells (per 10^3 total cells)						
Air	0.5	0.0	0.8	1.0[h]	0.6	1.4
Diesel exhaust	0.5	0.1	1.3	1.4	0.3	2.2
Macrophages						
Air	80.8	76.0	86.4	89.0[i]	85.6	90.5
Diesel exhaust	73.5[j]	68.3	81.1	86.6	79.8	90.6

[a] Data are expressed as percentages of total cell counts unless otherwise specified.

[b] Paired BW fluids were available for 14 of 15 asthmatic and 24 of 25 control subjects; one subject in each group did not complete the full bronchoscopy protocol.

[c] Control subjects had higher baseline levels of neutrophils than asthmatic subjects (P = 0.015 by Mann-Whitney U test).

[d] Increase in neutrophils after exposure to diesel exhaust in control subjects (P = 0.015 by Wilcoxon paired rank sum test).

[e] Asthmatic subjects had higher levels of eosinophils at baseline than control subjects (P < 0.001 by Mann-Whitney U test).

[f] When comparing absolute changes in cell numbers after air vs diesel exhaust exposure, the response in eosinophils differed between the two groups (P = 0.004 by Mann-Whitney U test).

[g] Decrease in eosinophils after exposure to diesel exhaust in asthmatic subjects (P = 0.044 by Wilcoxon paired rank sum test).

[h] Asthmatic subjects had higher levels of mast cells at baseline than control subjects (P = 0.021 by Mann-Whitney U test).

[i] Asthmatic subjects had higher levels of macrophages at baseline than control subjects (P = 0.013 by Mann-Whitney U test).

[j] Decrease in macrophages after exposure to diesel exhaust in control subjects (P = 0.014 by Wilcoxon paired rank sum test).

Table 10. Relative Cell Counts in BAL Fluid 6 Hours After a 2-Hour Exposure to Diesel Exhaust or Filtered Air[a]

	Control Group (n = 24[b])			Mild Asthma Group (n = 14[b])		
Exposure	Median	25th Percentile	75th Percentile	Median	25th Percentile	75th Percentile
Polymorphonuclear Neutrophils						
Air	1.3	1.0	3.0	1.0	0.8	1.4
Diesel exhaust	1.8	0.9	2.6	1.5	0.8	2.7
Lymphocytes						
Air	12.3	10.7	15.4	9.2	5.7	16.0
Diesel exhaust	15.0[c]	11.4	19.2	8.7	5.3	11.1
Eosinophils						
Air	0.1	0.0	0.4	0.3	0.2	0.7
Diesel exhaust	0.2	0.0	0.4	0.3	0.0	1.0
Mast Cells (per 10^3 total cells)						
Air	0.6	0.3	1.3	1.0	0.2	2.9
Diesel exhaust	0.8	0.4	1.6	0.6	0.3	1.8
Macrophages						
Air	83.8	82.5	87.4	87.6	83.1	92.3
Diesel exhaust	82.8[d]	79.7	87.2	88.8	85.8	92.5

[a] Data are expressed as percentages of total cell counts unless otherwise noted.

[b] Paired lavages were performed on 14 of 15 asthmatic and 24 of 25 control subjects; one subject in each group was unable to complete the full bronchoscopy protocol.

[c] Increase in lymphocytes after exposure to diesel exhaust in control subjects (P = 0.017 by Wilcoxon paired rank sum test).

[d] Decrease in macrophages after exposure to diesel exhaust in control subjects (P = 0.049 by Wilcoxon paired rank sum test).

Table 11. Total Protein and Albumin in BW and BAL Fluids 6 Hours After a 2-Hour Exposure to Diesel Exhaust or Filtered Air[a]

	Control Group (n = 24[b])			Mild Asthma Group (n = 14[b])		
Exposure	Median	25th Percentile	75th Percentile	Median	25th Percentile	75th Percentile
Protein in BW Fluid						
Air	54.5	46.0	70.0	38.0	21.0	66.8
Diesel exhaust	52.0	22.5	80.5	27.5	17.3	44.0
Protein in BAL Fluid						
Air	64.0	38.0	81.0	48.0	25.5	81.0
Diesel exhaust	54.5	41.0	96.8	38.2	24.6	56.5
Albumin in BW Fluid						
Air	29.0	20.8	38.5	35.5	16.0	66.0
Diesel exhaust	30.0	18.0	36.5	23.5	19.3	36.8
Albumin in BAL Fluid						
Air	44.5	29.0	54.0	42.0	23.0	65.0
Diesel exhaust	40.0	34.3	58.8	34.5	24.0	49.8

[a] All concentrations are given as µg/mL.

[b] Paired lavages were performed in 14 of 15 asthmatic and 24 of 25 control subjects; one subject in each goup was unable to complete the full bronchoscopy protocol.

Table 12. Concentrations of Soluble Mediators of Inflammation in BW Fluid 6 Hours After a 2-Hour Exposure to Diesel Exhaust or Filtered Air

	Control Group (n = 24[a])			Mild Asthma Group (n = 14[a])		
Exposure	Median	25th Percentile	75th Percentile	Median	25th Percentile	75th Percentile
Myeloperoxidase (µg/L)						
Air	6.4	4.5	10.6	7.2	4.2	9.3
Diesel exhaust	6.2	4.3	16.7	7.0	5.6	9.3
Eosinophilic Cationic Protein (µg/L)						
Air	0.9	0.9	1.3	1.7[b]	1.0	2.0
Diesel exhaust	1.0	0.9	1.3	1.2	0.9	1.9
Methylhistamine ([µg/L] $\times 10^{-2}$)						
Air	5.5	0.0	6.8	9.0[c]	7.0	10.0
Diesel exhaust	6.0	5.0	9.0	7.5	0.0	10.0
Soluble ICAM-1 (ng/mL)						
Air	33.0[d]	19.5	48.0	13.6	6.8	37.5
Diesel exhaust	34.0	21.0	49.0	27.5	12.6	37.0
IL-6 (pg/mL)						
Air	3.3	1.8	5.4	2.0	1.6	6.0
Diesel exhaust	5.1[e]	1.8	9.3	3.8	2.3	10.9
IL-8 (pg/mL)						
Air	42.5	28.5	57.8	35.5	25.5	53.5
Diesel exhaust	54.0[f]	30.0	74.8	44.0	27.3	51.3
GM-CSF (pg/mL)						
Air	0.0	0.0	0.6	0.0	0.0	0.7
Diesel exhaust	0.0	0.0	0.6	0.6	0.0	0.0
Extracellular Superoxide Dismutase (ng/mL)						
Air	1.8	1.1	2.5	1.8	1.6	2.9
Diesel exhaust	2.0	1.6	2.7	2.0	1.7	2.6

[a] Paired lavages were performed in 14 of 15 asthmatic and 24 of 25 control subjects; one subject in each group was unable to complete the full bronchoscopy protocol.

[b] Asthmatic subjects had higher levels of eosinophilic cationic protein at baseline than control subjects (P = 0.038 by Mann-Whitney U test).

[c] Asthmatic subjects had higher levels of methylhistamine at baseline than control subjects (P = 0.001 by Mann-Whitney U test).

[d] Control subjects had higher levels of soluble ICAM-1 at baseline than asthmatic subjects (P = 0.014 by Mann-Whitney U test).

[e] Increase in IL-6 after exposure to diesel exhaust in control subjects (P = 0.046 by Wilcoxon paired rank sum test).

[f] Increase in IL-8 after exposure to diesel exhaust in control subjects (P = 0.036 by Wilcoxon paired rank sum test).

Table 13. Expression of Cell Adhesion Molecules on the Vascular Endothelium in Bronchial Biopsy Tissue Samples Obtained 6 Hours After a 2-Hour Exposure to Diesel Exhaust or Filtered Air[a]

	Control Group (n = 24[b])			Mild Asthma Group (n = 14[b])		
Exposure	Median	25th Percentile	75th Percentile	Median	25th Percentile	75th Percentile
ICAM-1						
Air	63.7[c]	58.1	71.2	56.2	45.7	65.8
Diesel exhaust	66.4	59.3	79.4	52.7	41.2	59.8
VCAM-1						
Air	4.8	2.1	10.4	12.5	6.4	18.7
Diesel exhaust	8.8[d]	4.5	12.7	8.3	2.2	11.8
P-Selectin						
Air	52.5	45.6	65.3	50.7	36.7	58.6
Diesel exhaust	65.4[e]	58.2	75.8	54.5	44.2	63.9
E-Selectin						
Air	20.2	11.3	27.2	16.0	9.9	23.7
Diesel exhaust	20.3	15.1	30.4	16.7	11.4	23.5

[a] Molecules are quantified as a percentage of the total number of vessels stained with EN4.

[b] Paired bronchoscopies were performed on 14 of 15 asthmatic and 24 of 25 control subjects; one set of biopsy tissues from an asthmatic subject was inadequate for analysis.

[c] Control subjects had higher baseline levels of ICAM-1 than asthmatic subjects (P = 0.049 by Mann-Whitney U test).

[d] Increase in VCAM-1 after exposure to diesel exhaust in control subjects (P = 0.045 by Wilcoxon paired rank sum test). Asthmatic subjects had higher baseline levels of VCAM-1 than control subjects (P = 0.019 by Mann-Whitney U test). When comparing absolute (P = 0.016) and relative (P = 0.009) changes in VCAM-1 after air vs diesel exhaust exposure, the response differed between the groups (Mann-Whitney U test).

[e] Increase in P-selectin after exposure to diesel exhaust in control subjects (P = 0.002 by Wilcoxon paired rank sum test).

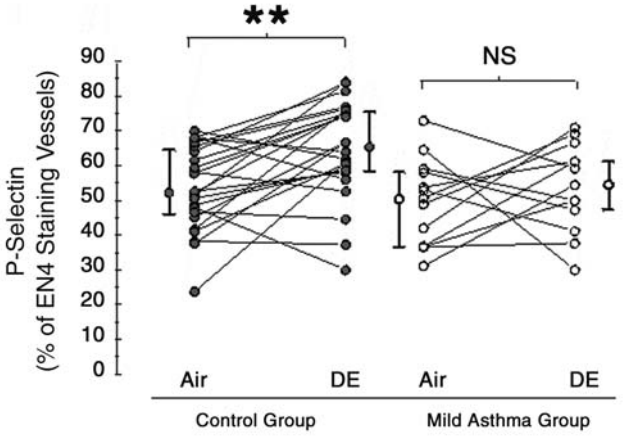

Figure 4. Expression of the adhesion molecule P-selectin on vascular endothelium in bronchial biopsy tissues obtained 6 hours after control subjects (n = 24) and subjects with asthma (n = 14) were exposed to air or diluted diesel exhaust. Paired biopsy tissue samples were available for 14 of 15 asthmatic and 24 of 25 control subjects; one subject in each group did not complete the full protocol. Data are illustrated as individual responses with group medians and interquartile ranges (expressed as error bars). NS = not significant; ** = statistically significant at $P < 0.01$ by Wilcoxon signed rank test.

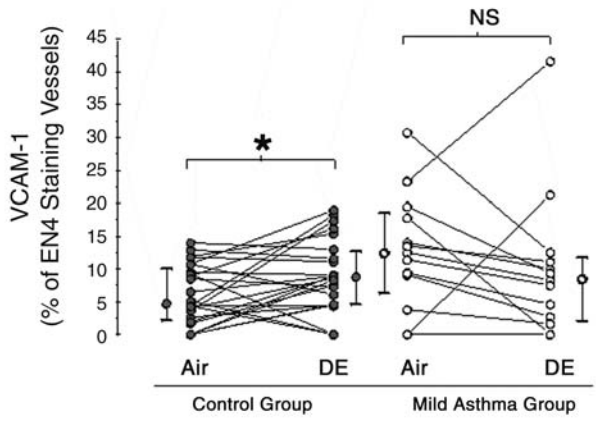

Figure 5. Expression of the adhesion molecule VCAM-1 on vascular endothelium in bronchial biopsy tissues obtained 6 hours after control subjects (n = 24) and subjects with asthma (n = 14) were exposed to air or diluted diesel exhaust. Paired biopsy tissue samples were available for 14 of 15 asthmatic and 24 of 25 control subjects; one subject in each group did not complete the full protocol. Data are illustrated as individual responses with group medians and interquartile ranges (expressed as error bars). NS = not significant; * = statistically significant at $P < 0.05$ by Wilcoxon signed rank test.

Table 14. Cytokine mRNA Expression in Bronchial Biopsy Tissue Samples Obtained 6 Hours After a 2-Hour Exposure to Diesel Exhaust or Filtered Air[a]

	Control Group (n = 21[b])			Mild Asthma Group (n = 12)		
Exposure	Median	25th Percentile	75th Percentile	Median	25th Percentile	75th Percentile
IL-1β						
Air	14.7	7.4	25.5	5.7	2.5	8.5
Diesel exhaust	14.3	8.4	29.6	4.6	2.5	7.1
IL-5						
Air	15.5	3.0	26.7	7.7	3.6	20.5
Diesel exhaust	17.8	7.9	26.0	4.5	1.7	13.0
IL-8						
Air	51.0	13.4	65.1	71.0	56.9	90.1
Diesel exhaust	65.7[c]	30.7	84.5	46.9	35.9	90.9
IL-13						
Air	1.0	0.0	4.8	ND[d]		
Diesel exhaust	0.8	0.0	4.0	ND		
TNF-α						
Air	8.9	3.2	33.2	21.8	9.5	39.8
Diesel exhaust	12.0	7.2	23.5	12.6	2.4	28.6
IFN-γ						
Air	8.1	2.6	19.5	2.8	1.3	25.7
Diesel exhaust	7.7	3.3	16.2	3.7	0.8	7.7

[a] Values are expressed as the percentage of *APRT* gene expression.

[b] Samples suitable for mRNA analysis were available from 12 of 15 asthmatic and 21 of 25 control subjects.

[c] Significant difference between diesel exhaust and air exposure (P = 0.007 by Wilcoxon nonparametric test).

[d] ND = not done.

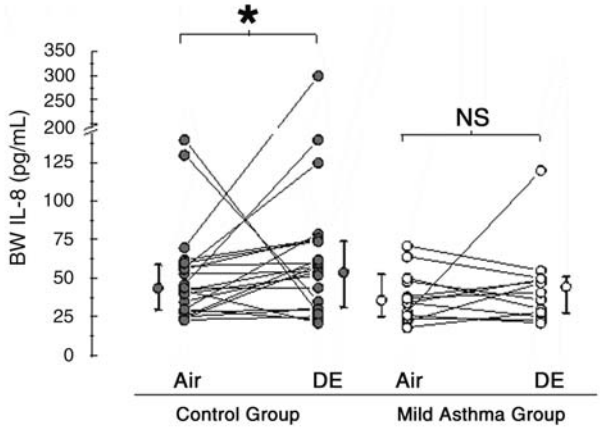

Figure 6. Concentrations of IL-8 protein in BW fluid obtained 6 hours after control subjects (n = 24) and subjects with asthma (n = 14) were exposed to air or diluted diesel exhaust. Paired BW fluid samples were obtained from 14 of 15 asthmatic and 24 of 25 control subjects; one subject in each group did not complete the full bronchoscopy protocol. Data are illustrated as individual responses with group medians and interquartile ranges (expressed as error bars). NS = not significant; * = statistically significant at $P < 0.05$ by Wilcoxon signed rank test.

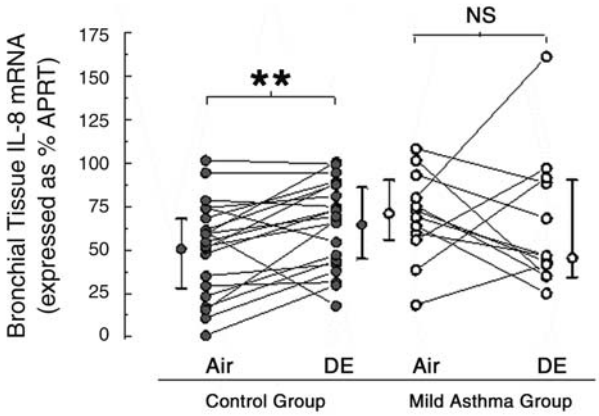

Figure 7. Expression of mRNA for IL-8 in bronchial biopsy tissues obtained 6 hours after control subjects (n = 21) and subjects with asthma (n = 12) were exposed to air or diluted diesel exhaust. Suitable paired biopsy tissue samples were available from 12 of 15 asthmatic and 21 of 25 control subjects. Data are illustrated as individual responses with group medians and interquartile ranges (expressed as error bars). NS = not significant; ** = statistically significant at $P < 0.01$ by Wilcoxon signed rank test.

Table 15. Markers of Inflammation Expressed in Bronchial Submucosa 6 Hours After a 2-Hour Exposure to Diesel Exhaust or Filtered Air[a]

	Control Group (n = 24)			Mild Asthma Group (n = 14[b])		
Exposure[c]	Median	25th Percentile	75th Percentile	Median	25th Percentile	75th Percentile
Neutrophil Elastase						
Air	43.4	15.6	94.6	30.7	15.6	58.1
Diesel exhaust	24.6	16.8	57.1	27.4	18.1	44.2
AA1						
Air	16.5	11.6	28.8	23.4	15.4	31.7
Diesel exhaust	16.9	12.3	27.2	21.7	11.7	30.8
EG2						
Air	0.0	0.0	1.8	4.9[d]	2.4	10.3
Diesel exhaust	0.9	0.0	1.7	1.8[d]	0.8	5.4
CD3						
Air	30.3	12.1	86.0	31.7	20.0	60.8
Diesel exhaust	30.5	18.5	74.6	43.2	7.5	64.7
CD4						
Air	15.9	9.9	47.0	15.8	7.8	38.5
Diesel exhaust	16.6	10.9	41.3	20.2	4.1	37.7
CD8						
Air	11.5	8.5	41.7	8.8	3.8	22.0
Diesel exhaust	9.8	7.5	29.1	10.1	1.4	14.5
CD14						
Air	0.0	0.0	1.3	0.4	0.0	1.5
Diesel exhaust	0.0	0.0	0.7	0.0	0.0	0.0

[a] Values are expressed as cells/mm^2.

[b] Paired biopsy tissues were available from 14 of 15 asthmatic and 24 of 25 control subjects; one subject from each group was unable to complete the full bronchoscopy protocol.

[c] See the section Abbreviations and Other Terms for these definitions.

[d] Subjects with asthma had higher baseline levels of EG2-staining cells than control subjects ($P < 0.001$ by Mann-Whitney U test). When comparing absolute changes in EG2 after air vs diesel exhaust exposure, the response differed between the two groups ($P = 0.023$ by Mann-Whitney U test).

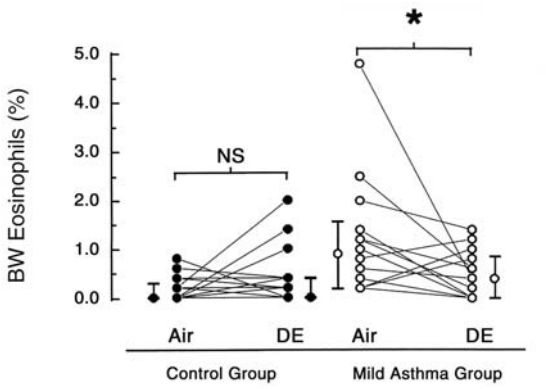

Figure 8. Eosinophils in BW fluid obtained 6 hours after control subjects (n = 24) and subjects with asthma (n = 14) were exposed to air or diluted diesel exhaust. Paired BW fluid samples were obtained from 14 of 15 asthmatic and 24 of 25 control subjects; one subject in each group did not complete the full bronchoscopy protocol. Data are illustrated as individual responses with group medians and interquartile ranges (expressed as error bars). NS = not significant; * = statistically significant at $P < 0.05$ by Wilcoxon signed rank test.

Table 16. Markers of Inflammation Expressed in Bronchial Epithelium 6 Hours After a 2-Hour Exposure to Diesel Exhaust or Filtered Air[a]

	Control Group (n = 24[b])			Mild Asthma Group (n = 14[b])		
Exposure[c]	Median	25th Percentile	75th Percentile	Median	25th Percentile	75th Percentile
Neutrophil Elastase						
Air	0.6	0.0	1.9	0.2	0.0	0.8
Diesel exhaust	0.5	0.3	0.9	0.3	0.0	1.7
AA1						
Air	0.0	0.0	1.3	0.8	0.0	1.4
Diesel exhaust	0.3	0.0	1.1	0.2	0.0	0.7
EG2						
Air	0.0	0.0	0.0	0.0	0.0	0.4
Diesel exhaust	0.0[d]	0.0	0.0	0.0	0.0	0.1
CD3						
Air	3.0[e]	1.5	6.1	1.0	0.5	3.3
Diesel exhaust	1.7[f]	0.4	3.1	1.2	0.2	4.1
CD4						
Air	0.4	0.0	1.1	0.2	0.0	1.0
Diesel exhaust	0.2	0.0	1.1	0.0	0.0	0.8
CD8						
Air	1.2	0.5	4.8	0.2	0.0	2.0
Diesel exhaust	1.7	0.5	2.8	0.3	0.0	1.2
CD14						
Air	0.0	0.0	0.0	0.0	0.0	0.0
Diesel exhaust	0.0	0.0	0.0	0.0	0.0	0.0

[a] Values are expressed as cells/mm^2.

[b] Paired biopsy tissues were available from 14 of 15 asthmatic and 24 of 25 control subjects; one subject from each group was unable to complete the full bronchoscopy protocol.

[c] See the section Abbreviations and Other Terms for these definitions.

[d] Although median values are identical, we noted a statistically significant decrease in EG2-staining cells in bronchial epithelium after exposure to diesel exhaust in control subjects (P = 0.043 by Wilcoxon paired rank sum test).

[e] Control subjects had higher baseline levels of CD3-staining cells than asthmatic subjects (P = 0.007 by Mann-Whitney U test).

[f] Decrease in CD3-staining cells in bronchial epithelium after exposure to diesel exhaust in control subjects (P = 0.011 by Wilcoxon paired rank sum test).

Table 17. Cytokines and Chemokines in Bronchial Epithelium 6 Hours After a 2-Hour Exposure to Diesel Exhaust or Filtered Air[a]

	Control Group (n = 24[b])			Mild Asthma Group (n = 14[b])		
Exposure	Median	25th Percentile	75th Percentile	Median	25th Percentile	75th Percentile
IL-6						
Air	0.33	0.17	0.77	0.31	0.10	1.00
Diesel exhaust	0.22	0.11	0.69	0.50	0.20	0.68
IL-8						
Air	2.93	1.55	6.43	1.53	0.56	4.20
Diesel exhaust	2.52	0.31	5.29	0.91	0.50	2.43
IL-10						
Air	1.29[c]	0.43	2.20	0.22	0.16	0.53
Diesel exhaust	0.39	0.11	1.58	0.92[d]	0.69	1.07
TNF-α						
Air	0.0	0.0	0.0	0.18[e]	0.0	0.68
Diesel exhaust	0.0	0.0	0.0	0.18	0.0	0.46
GM-CSF						
Air	0.0	0.0	0.0	0.0	0.0	0.27
Diesel exhaust	0.0	0.0	0.0	0.0	0.0	0.29
GRO-α						
Air	2.30	1.42	4.58	2.40	1.58	5.96
Diesel exhaust	1.92	1.14	2.81	2.54	1.49	4.29
NF-κB						
Air	1.15	0.48	1.53	1.05	0.74	3.17
Diesel exhaust	1.16	0.58	1.67	1.01	0.63	1.87
RANTES						
Air	0.19	0.12	0.49	0.26	0.09	0.73
Diesel exhaust	0.14	0.04	0.56	0.09	0.05	0.23

[a] Values are expressed as a percentage of the area of epithelial cells.

[b] Paired biopsy tissues were available from 14 of 15 asthmatic and 24 of 25 control subjects; one subject from each group was unable to complete the full bronchoscopy protocol.

[c] Control subjects had higher baseline levels of IL-10 than subjects with asthma (P = 0.001 by Wilcoxon paired rank sum test).

[d] Increase in IL-10 after diesel exhaust exposure in subjects with asthma (P = 0.002 by Wilcoxon paired rank sum test).

[e] Subjects with asthma had higher baseline levels of TNF-α than control subjects ($P < 0.05$ by Wilcoxon paired rank sum test).

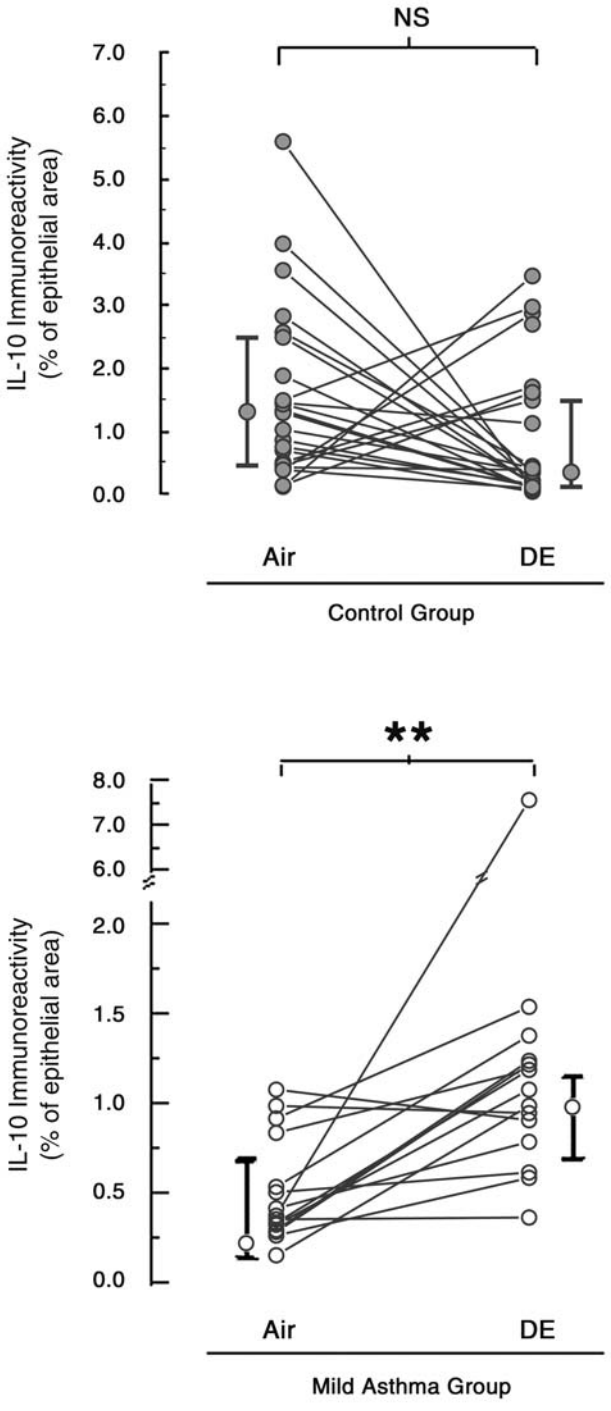

Figure 9. Area of bronchial epithelium staining for IL-10 in biopsy tissues obtained 6 hours after control subjects (n = 24) and subjects with asthma (n = 14) were exposed to air or diluted diesel exhaust. Paired BW fluid samples were obtained from 14 of 15 asthmatic and 24 of 25 control subjects; one subject in each group did not complete the full bronchoscopy protocol. Individual and group median values are shown. Data are illustrated as individual responses with group medians and interquartile ranges (expressed as error bars). NS = not significant; ** = statistically significant at $P < 0.01$ by Wilcoxon signed rank test.

DISCUSSION

This study has demonstrated that control subjects (people who do not have asthma) who are exposed to diluted diesel exhaust show an inflammatory response characterized by neutrophilic airway inflammation with transcription of IL-8 mRNA and upregulation of endothelial adhesion molecules. This inflammatory response is not accompanied by any significant change in standard parameters of lung function, although there are detectable changes in airway resistance. Subjects with mild asthma also showed an increase in airway resistance, but they did not show any change in airway neutrophils, IL-8, or endothelial adhesion molecules. The group with mild asthma had evidence of baseline allergic airway inflammation (marked by eosinophils and VCAM-1), but this did not increase after diesel exhaust exposure. The group with asthma did show a marked increase in staining for IL-10 after diesel exhaust exposure, although the degree of staining after diesel exhaust was comparable in both groups of subjects and the increase in the asthma group was due to the lower preexposure level for that group.

The particle concentration of diesel exhaust used in our initial study (300 $\mu g/m^3$) (Salvi et al 1999a) was comparable with levels observed in a number of occupational settings and in many cities of the developing world. Similar levels can also be encountered in traffic underpasses and special situations such as the loading docks of freight companies and car ferries. The lower concentration used in the present study (100 $\mu g/m^3$) is more widely encountered in the developed world. We elected to use fresh diesel exhaust on the grounds that the composition and size distribution of particles change over time, and we felt that it was more realistic to use fresh diesel exhaust, including the gaseous components and fresh particles, rather than rely on stale particle material. Diesel exhaust is a complex mixture of particles and gases derived from the complete and incomplete combustion of fuel and lubricating oil, as well as metallic components from the engine. The principal component of diesel exhaust particles is inorganic carbon, with some sulfate particles, metal traces, inorganic salts and oxides as well as a soluble organic fraction. It is known that the physical and chemical properties of diesel particles change over time, both in the atmosphere and in storage (Atkinson 1988; Scheepers and Bos 1992). In discussions with scientific colleagues since we started these studies, we have encountered a range of opinions regarding the appropriate concentration of particles to be used in such experiments. There is a legitimate question whether any observed inflammatory response should be attributed to the particulate or the gaseous content of the diesel exhaust. Our

parallel studies with NO_2 (Blomberg et al 1997) indicate that NO_x have only a minor effect on cellular inflammation. In those studies, the low degree of neutrophilia in BW samples after exposure to NO_2 (2 ppm for 2 hours) exceeded the neutrophilia encountered in the present study. We believe that the bulk of the inflammatory changes observed here are due to the particulate components of diesel exhaust, but we recognize that the effect of particle vs gaseous components has not been formally assessed.

Broadly speaking, the present study confirmed the main findings of our previous study (Salvi et al 1999a). Both studies showed proximal airway neutrophilia and distal airway lymphocytosis. Neither study found a statistically significant change in fluid-phase markers of injury. IL-8 mRNA expression was increased in both studies, but only in the present study was IL-8 protein increased in lavage fluid. Increases in peripheral blood neutrophils and platelets were seen in the first study but not in the present work. Adhesion molecule expression was increased in the control subjects in both studies: There was elevation in VCAM-1 in both studies, an elevation in ICAM-1 in the initial study, and an increase in E-selectin in the present study. With consideration that the total particle exposure in the present study was two thirds of that used in the initial study, the results are remarkably consistent. The absence of a measurable systemic effect in the present study probably reflects the lower intensity of exposure. Taken together, these studies confirm that airways of control subjects respond to diesel exhaust with airway neutrophilia, activation of IL-8, and upregulation of endothelial adhesion molecules. Some new markers such as IL-10 were only studied in the present study. Unfortunately, there was insufficient material remaining from the first study to go back and cross-reference these results.

The impact of air pollution on mechanisms of cellular recruitment is a subject of great interest. We focused on a number of cytokines and chemokines that seem relevant to allergic and nonallergic airway inflammation. The most clear-cut changes in control subjects were observed in IL-8 mRNA and protein expression. These changes are consistent with the neutrophilia and activation of endothelial adhesion molecules seen in the control subjects after diesel exhaust exposure. Conversely, the absence of any such changes in chemokines or adhesion molecules in the group with asthma is consistent with the absence of diesel exhaust–induced neutrophilia in this group. Previous in vitro work has shown that cultured human epithelial cells release IL-6, IL-8, and GM-CSF in a time- and dose-dependent manner when exposed to diesel exhaust particles, and this response is inhibited by protein synthesis inhibitors, suggesting de novo synthesis (Ohtoshi et al 1998; Steerenberg et al 1998).

Clinical and epidemiological experience suggests that people with asthma are much more susceptible to the effects of solvents, irritants, and particles in terms of lung function and disease exacerbation (Schwartz et al 1993; Dockery and Pope 1994). This could occur through a variety of mechanisms. The main aim of the present study was to explore which, if any, of the available inflammatory parameters were operational in asthmatic airways exposed acutely to diesel exhaust. However, diesel exhaust exposure did not induce a neutrophilic response in subjects with asthma such as was found in control subjects. Neither did diesel exhaust exposure induce any increase in the allergic airway inflammation characteristic of asthma.

This lack of cellular and mediator response in the group with asthma could occur for several different reasons. First, it may be a question of timing. The 6-hour time point was chosen to reflect the timing of the peak granulocyte response to allergen challenge. It is possible that the peak response to diesel exhaust might occur later. It might be instructive to look at a similar group of subjects 18 hours after exposure, a period when a lymphocyte response might be expected. Second, it may be difficult to detect small changes given the degree of background inflammation in the airways of people with asthma. We know that a modest degree of inflammation can have more pronounced physiologic effects in people with asthma compared with control subjects, so some caution is needed in interpreting the lack of clear cellular signals in the group with asthma. Third, it is possible that inflammatory markers other than those studied here may be more relevant to a diesel exhaust–induced response. Fourth, it is possible that counter-regulatory mechanisms are already active in people with asthma, and therefore the inflammatory effects of diesel exhaust are damped or masked. Fifth, it is possible that other counter-regulatory mechanisms are induced by diesel exhaust. The postexposure induction of IL-10 in the airways of people with asthma is one such mechanism.

IL-10 is generally regarded as an anti-inflammatory cytokine. It was originally described as cytokine synthesis inhibitor factor, a product of murine T_H2 T-cell clones that inhibited the production of cytokines by T_H1 T cells (de Waal Malefyt et al 1991; Fiorentino et al 1991). IL-10 is produced by T cells, macrophages, monocytes, and epithelial cells (Fiorentino et al 1989; de Waal Malefyt et al 1991; Bonfield et al 1995). It inhibits monocyte and macrophage function, suppressing the production of several monokines including TNF-α, IL-1β, IL-6, MIP-1α, and IL-8 (de Waal Malefyt et al 1991; Fiorentino et al 1991; Seitz et al 1995). IL-10 inhibits the release of the chemokines RANTES and

IL-8 from airway smooth-muscle cells (John et al 1997, 1998a) and inhibits the production of IFN-γ and IL-2 by T_H1 cells (Fiorentino et al 1989). This latter effect is thought to operate through inhibition of the antigen-presenting function of monocytes and macrophages. In contrast, IL-10 acts on B cells to enhance their viability, proliferation, and secretion of immunoglobulins, as well as favoring isotype switching (Jeannin et al 1998). IL-10 is a growth factor for mast cells (Thompson-Snipes et al 1991) and activates the transcription of mast cell protease genes. Most studies of human asthma have suggested that, as in the present study, baseline levels of IL-10 are low in people with asthma (Borish et al 1996; Koning et al 1997; John et al 1998b; Takanashi et al 1999). However, after allergen challenge the number of T cells that express IL-10 mRNA increases in the lung (Robinson et al 1996) and similar responses are seen in the nose (Klein et al 1999). Recent studies in animal models of allergic sensitization have suggested that IL-10 may provide a mechanism by which allergic inflammatory processes are downregulated in the lung (Stampfli et al 1999). However, other work in antigen-sensitized mice has reported that IL-10 augments airway reactivity, despite reducing T_H2 cytokine production and reducing airway eosinophilia (van Scott et al 2000).

These in vitro and in vivo properties of IL-10 suggest that the IL-10 response seen in the subjects with asthma after exposure to diesel exhaust is consistent with the lack of neutrophil, IL-6, or IL-8 response in this group. In addition, the IL-10 response could serve as a contributory factor toward a more long-term change in airways of people with asthma, leading to enhanced IgE production, increased numbers of mast cells in the airways, and increased airway reactivity. Further work will be required to explore and validate this conclusion.

Previous epidemiological and in vitro studies have suggested that diesel exhaust may act as an adjuvant for the production of IgE (Ishizaki et al 1987; Diaz-Sanchez et al 1994), although the precise mechanism of this effect remains unclear. If diesel exhaust does not directly cause airway inflammation, it might worsen asthma by altering the immunobiology of the airway so that the airway responds differently when exposed to allergens. In view of the respective roles of IL-5 and IL-4 in stimulating eosinophil growth and regulating IgE switching (Maggi et al 1992), these two cytokines were of particular interest for our studies. In the initial study we found a modest increase in IL-5 expression in control subjects (Salvi et al 2000), but we did not find any change in IL-5 expression in either group in the present study. The increase observed in IL-10 staining supports the hypothesis that airway immunobiology may be altered through a combination of stimulating mast cell growth and favoring IgE switching. This argument needs to take into account the relatively low level of IL-10 in the baseline biopsy tissues from the airways of subjects with asthma: To be biologically important, the relative expression of IL-10 would need to be more relevant than its absolute level.

CONCLUSIONS AND RECOMMENDATIONS

This study has demonstrated that exposure to concentrations of diesel exhaust encountered in occupational settings can induce airway and systemic inflammatory responses in control subjects that are not detectable by conventional lung function tests alone. The increased expression of endothelial adhesion molecules in control subjects after diesel exhaust exposure offers a possible clue to the increased cardiovascular morbidity that has been reported in epidemiological studies in association with fine particles. The present study also found consistent changes in cellular recruitment, endothelial activation, and cytokine activation in control subjects. These findings indicate that nonasthmatic airways can become inflamed and damaged when exposed to ambient concentrations of diesel exhaust.

Subjects with mild asthma showed a small but definite increase in airway resistance after diesel exhaust exposure. However, this was not associated with any worsening of the inflammatory changes that have been linked to the pathophysiology of asthma. Neither was there any sign of the neutrophilic reaction that we had observed in the control subjects. An increase in IL-10 immunostaining in the biopsy tissues taken from people with asthma raises the possibility that exposure to diesel exhaust may cause subtle changes in their airways that make them more likely to generate allergic-pattern responses. Further work is required to address later time points and susceptible subgroups. However, on the basis of the current data, the airways of people with mild asthma do not show marked inflammatory response to acute diesel exhaust as judged by standard parameters of airway inflammation 6 hours after exposure. Due to the higher degree of airway responsiveness, any small change in airway resistance is likely to have more clinical consequences in people with asthma than in control subjects. The reported clinical sensitivity of people with asthma to diesel exhaust pollution could reflect a nonspecific irritant response rather than a specific inflammatory response. However, the IL-10 data offer a tantalizing window that suggests that diesel exhaust may have subtle effects on airway immunobiology leading to alterations in immunological and physiological reactivity, alterations that may explain the heightened clinical sensitivity to diesel exhaust of people with asthma.

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ABBREVIATIONS AND OTHER TERMS

AA1	antibody against protease tryptase
ABTS	2,2′-azino-di-[3-ethyl benzthiazoline sulfonate]
AMV	avian myeloblastosis virus
APRT	adenine phosphoribosyl transferase
BAL	bronchoalveolar lavage
BW	bronchial wash
CAPs	concentrated ambient particles
CD terms	surface proteins on T and B cells
cDNA	complementary DNA
CO	carbon monoxide
DEPC	diethyl pyrocarbonate
dNTP	deoxyribonucleoside triphosphate
EG2	antibody against eosinophil cationic protein
ELISA	enzyme-linked immunosorbent assay
EN4	endothelium clone 4
FEV_1	forced expiratory volume in 1 second
FVC	forced vital capacity
GM-CSF	granulocyte-macrophage colony–stimulating factor
GRO-α	growth-related oncogene-α
HC	hydrocarbons

HCHO	formaldehyde
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
IFN-γ	interferon-γ
IgE	immunoglobulin E
$MgCl_2$	magnesium chloride
MIP-1α	macrophage inflammatory protein-1α
mRNA	messenger RNA
NE	neutrophil elastase
NF-κB	nuclear factor κB
NO	nitric oxide
NO_2	nitrogen dioxide
NO_x	oxides of nitrogen
PBS	phosphate-buffered saline
PC_{20}	concentration that provoked a 20% fall in FEV_1
PEF	peak expiratory flow
PM	particulate matter
$PM_{2.5}$	particles smaller than 2.5 μm in aerodynamic diameter
PM_{10}	particles smaller than 10 μm in aerodynamic diameter
ppm	parts per million
RANTES	Regulated on Activation, Normal T cell Expressed, and presumably Secreted
Raw	airway resistance
RIA	radioimmunoassay
RT-PCR	reverse transcription–polymerase chain reaction
TNF-α	tumor necrosis factor α
VCAM-1	vascular cellular adhesion molecule-1

INVESTIGATORS' REPORT

Health Effects of Acute Exposure to Air Pollution

Part II: Healthy Subjects Exposed to Concentrated Ambient Particles

Stephen T Holgate, Robert B Devlin, Susan J Wilson, and Anthony J Frew

ABSTRACT

The purpose of this study was to assess the impact of short-term exposure to concentrated ambient particles (CAPs*) on lung function and on inflammatory parameters in blood and airways of healthy human subjects. Particles were concentrated from the ambient air in Chapel Hill, North Carolina, using a Harvard/EPA (US Environmental Protection Agency) ambient fine particle concentrator (HAPC). Each of 38 subjects was exposed either to filtered air (n = 8) or to CAPs (n = 30) for two hours, during which all subjects intermittently exercised. Blood was obtained immediately before and 18 hours after exposure. Also at 18 hours after exposure, viable bronchial biopsy tissues and lavage samples were obtained from 10 CAPs-exposed and 7 control subjects by fiberoptic bronchoscopy. To balance these two groups, additional biopsy tissues were obtained from 4 control subjects participating in an identical protocol for another study.

For the CAPs-exposed group, the concentration of particulate matter measuring 2.5 µm or less in aerodynamic diameter ($PM_{2.5}$) in the exposure aerosols varied from 23.1 to 311.1 µg/m^3; for the filtered air group, mean particle concentration was 2.9 µg/m^3. For comparative analyses, the CAPs-exposed subjects were separated into three tertiles on the basis of the final concentration of particles to which they were exposed.

Lung function, assessed by spirometry and plethysmography before and immediately after exposure, was unaffected by CAPs. Of the inflammatory parameters studied in blood, subjects exposed to CAPs showed mean increases in fibrinogen of 40 to 48 mg/dL with no obvious differentiation by dose, whereas subjects exposed to filtered air showed no change; red and white blood cell counts were unaffected by CAPs exposure. In bronchoalveolar lavage fluid from CAPs-exposed subjects, neutrophils showed a dose-dependent increase both when analyzed as an absolute cell count and as a percentage of total lavaged cells. Bronchial biopsy tissues from 10 CAPs-exposed subjects and 11 control subjects did not show any consistent effect of CAPs exposure on cell counts or adhesion molecule expression.

We conclude that CAPs induced a modest degree of airway inflammation as judged by lavage, but this effect was not reflected in biopsy tissues from proximal airways. This discrepant finding may mean that the inflammatory effect of CAPs occurs in more distal airways or that the health effects of PM are driven by processes other than those investigated in this study.

INTRODUCTION AND BACKGROUND

Concern has been increasing about the possible adverse effects of particulate pollution on human health (Bascom et al 1996). Several studies have identified an association between cardiovascular morbidity and airborne PM concentrations (Dockery et al 1993; Anderson et al 1996). Peters and colleagues (1997) have suggested that cardiovascular morbidity may be associated with documented changes in plasma viscosity, coagulability, or both during air pollution episodes. Associations have also been found between concentrations of fine particles and a number of other health outcomes (Kaiser 1997), particularly respiratory distress measured, for example, by hospital admissions for asthma (Schwartz et al 1993; Dockery and Pope

* A list of abbreviations and other terms appears at the end of the Investigators' Report.

This Investigators' Report is one part of Health Effects Institute Research Report 112, which also includes Part I, a Critique by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning this Investigators' Report may be addressed to Dr Stephen T Holgate, University of Southampton, Southampton General Hospital, Mail Point 810, Level D, Centre Block, Tremona Road, Southampton SO16 6YD, UK; *sth@soton.ac.uk*.

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1994) and by decrements in lung function in patients with asthma (Peters et al 1999). The mechanisms underlying these associations are not clear, especially because they seem to operate even at low particle concentrations (Schwartz et al 1993; Walters et al 1994; Wordley et al 1997).

Although exposure to fresh diesel exhaust is a useful model for assessing the possible effects of diesel exhaust particles on human health (see Part I of this Research Report), the particles found in the ambient environment are more complex both in source and in composition. Moreover, good evidence suggests that particles change as they age, becoming aggregated into larger clusters and losing volatile material from their surfaces. To assess the health effects of ambient particles, it is necessary to concentrate them from the ambient environment. The HAPC allows ambient particles between 0.15 and 2.5 µm in aerodynamic diameter to be concentrated by approximately ten times (Sioutas et al 1997). Due to the mechanical properties of the concentrator, particles larger than 2.5 µm are to a great extent eliminated and ultrafine particles (< 0.15 µm) are not concentrated. The CAPs in a range of concentrations can then be used in human and animal exposure studies.

To explore the possible mechanisms of the health effects from particulate exposure, human subjects were exposed to filtered air or CAPs at Dr Devlin's laboratory in Chapel Hill, North Carolina. The effects of the CAPs exposures were analyzed by the Devlin group for changes in lung function, blood parameters of inflammation and coagulation, contents of the airway lumen, and structure of the airway mucosa. (The exposure protocol and analyses carried out by the Devlin group were funded by the EPA and have been reported elsewhere [Ghio et al 2000; Harder et al 2001; Huang et al 2003].) The Health Effects Institute funded Dr Holgate's laboratory in Southampton, United Kingdom, to analyze the bronchial biopsy tissues obtained from Devlin's subjects to assess whether the differences seen in lavage fluids (analyzed by the Devlin group) were also reflected in cellular recruitment and in the expression of cytokines and endothelial adhesion molecules in the tissues. The Devlin exposure protocol and results are summarized here along with the Holgate group's analyses of the biopsy tissues.

SPECIFIC AIMS

The specific aim of this study was to assess the effects of short-term exposure to CAPs on the airways of healthy subjects. The principal endpoints analyzed were measurements of lung function, blood coagulation factors, cellular recruitment, and cytokine expression in the airways of subjects after exposure to CAPs or filtered air. The Health Effects Institute funded the analyses of biopsy tissues by the Holgate group; all other aspects of the study were performed by the Devlin group and were funded by the US EPA.

METHODS

AMBIENT PARTICLE EXPOSURE SYSTEM

Particles with aerodynamic diameters from 0.15 to 2.5 µm were concentrated from Chapel Hill air using an HAPC. The concentrator has three-stages of virtual impactors (Figure 1). Working by inertial separation, only the particles are concentrated with no effect on the gaseous composition of the air (Sioutas et al 1997).

Outside air is first drawn through a conventional Anderson high-volume impactor with a 2.5-µm cutoff size at a flow rate of 5000 L/min. The exit flow from the Anderson impactor is drawn into the first stage of the HAPC, in which five virtual impactor slits are arranged in parallel (1000 L/min per slit). A virtual impactor consists of two parts: the upper part has a sharp-edged slit, which receives impinging particles; the lower part has a rectangular nozzle that directs air either to the outside atmosphere (major air flow) or through the receiving slit to the second stage of the concentrator (minor air flow). Each virtual impactor operates at a minor-to-total flow ratio of 0.2, so 80% of the airflow ejected from the rectangular nozzle is deflected to the outside atmosphere (ie, major flow at 4000 L/min) and 20% of the airflow proceeds straight down into the receiving slit (ie, minor flow at 1000 L/min). In this way, particles larger than 0.15 µm achieve sufficient momentum to cut across the deflecting major air flow stream and reach the receiving slit, whereas particles < 0.15 µm are not concentrated. Ideally, if all particles between 0.15 and 2.5 µm were condensed into the minor flow and concentrated, the overall particle concentration would increase by 5-fold. In the present system, particles between 0.15 and 2.5 µm are concentrated about 2.5-fold to 3-fold during the first stage.

The combined flow from the five receiving slits in the first stage is drawn into the second stage at a flow rate of 1000 L/min. The second stage consists of a single virtual impactor identical to those in the first stage. Here the ambient particles are again concentrated 2.5-fold to 3-fold and are drawn into the third stage at a flow rate of 200 L/min. The third stage is also a single virtual impactor; it operates at a minor-to-total flow ratio of 0.4 and concentrates particles about 2-fold at a flow rate of

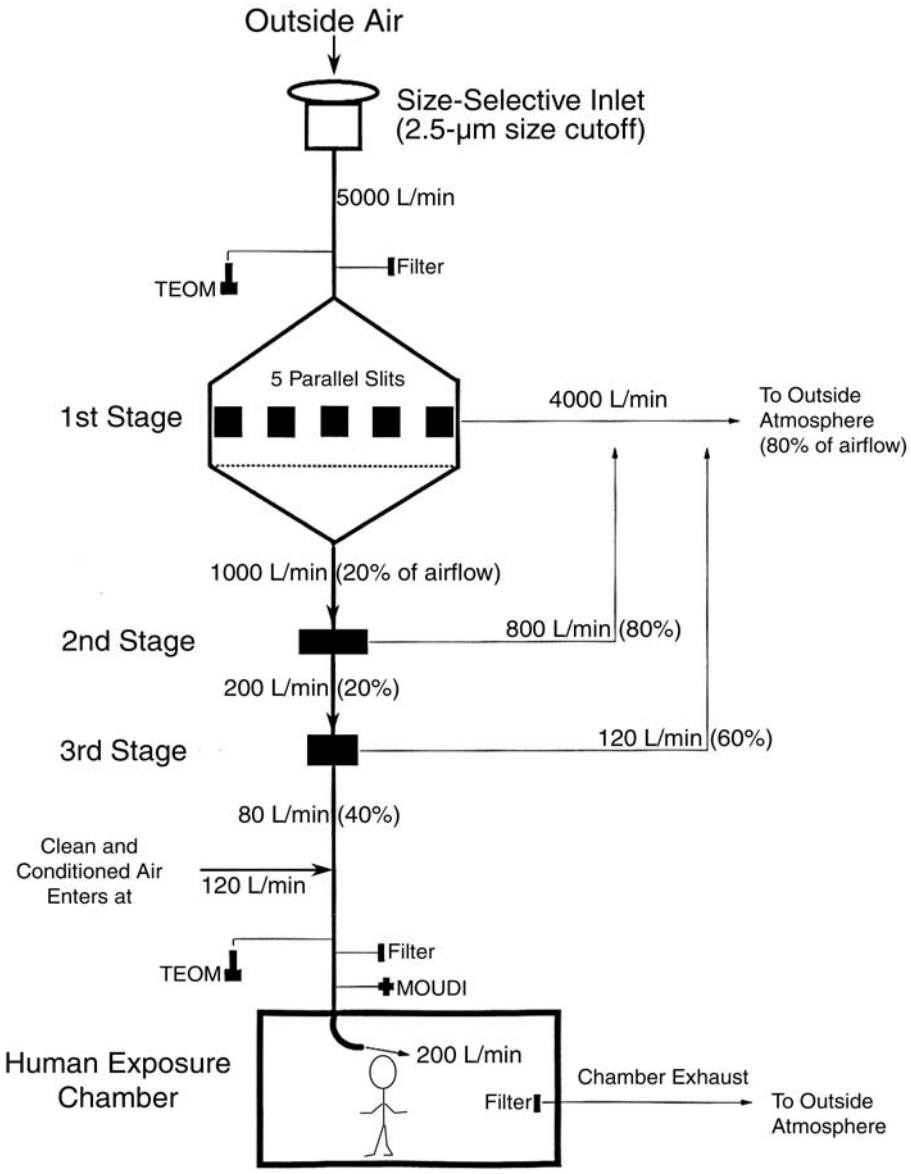

Figure 1. Schematic diagram of a Harvard Ambient Particle Concentrator. Outside air is drawn through a high-volume impactor with a size-selective inlet and a cutoff size of 2.5 µm in aerodynamic diameter. A small portion is diverted to a TEOM, which continuously monitors particle concentrations, and 10 L/min is diverted to a filter, from which total particle mass and composition are measured. The first stage of the HAPC has five virtual impactor slits arranged in parallel. Each impactor operates at a minor-to-total flow ratio of 0.2, so 80% of the airflow is deflected to the outside atmosphere and 20% of the airflow proceeds into the receiving slit. Thus, particles larger than 0.15 µm achieve sufficient momentum to cut across the deflecting major air flow stream and reach the receiving slit, whereas particles < 0.15 µm are not concentrated. The combined flow from the five receiving slits is drawn into the second and third stages, each of which consist of a single virtual impactor. The concentrated aerosol leaving the third stage is mixed with clean and conditioned air. Before the aerosol is drawn into the exposure chamber, small portions are again diverted to a TEOM and a filter; a third portion is diverted to a MOUDI, which assesses the particle size distribution. The resulting conditioned aerosol is delivered into the exposure chamber at a rate of 200 L/min. The net effect is particles concentrated by 6-fold to 10-fold (as measured at the inlet of the chamber). (Adapted from Ghio et al 2000.) (Devlin's Group)

80 L/min. The concentrated aerosol leaving the third stage is mixed with 120 L/min of clean and conditioned air (20°C and 50% relative humidity).

The resulting conditioned aerosol was delivered into the exposure chamber at a rate of 200 L/min. Adding the conditioned air diluted the aerosol concentration, but ensured consistent temperature and humidity. Overall, the net effect was particles concentrated 6-fold to 10-fold (as measured at the inlet of the chamber). The maximum concentration of aerosol delivered to the human exposure chamber varied depending on the concentration of particles in the ambient air (usually 5 to 30 $\mu g/m^3$) on the day of an exposure procedure and on the day-to-day functioning of the HAPC. Filtered air exposures were conducted using 200 L/min of conditioned air without any air from the HAPC.

The exposure chamber is constructed with aluminum panels and heavy-duty clear Plexiglas for doors and windows; the whole unit measures 4.0 × 6.7 × 7.5 feet. Because the air-pumping units are located downstream of the chamber and the HAPC, the chamber operates under a slightly negative pressure (10–12 inches H_2O). Concentrated aerosols or filtered air entered the chamber via a 6-inch-diameter curved duct positioned on the top and middle of the chamber and exited via an exhaust duct positioned in the middle of one of the vertical walls (Figure 1). A subject sat between the inlet and exit ducts with his or her head positioned within 18 inches of the inlet duct. A series of tests conducted during the present series of exposures showed that the particle concentration at the subject's head position was at least 90% of the particle concentration at the inlet duct.

PARTICLE CHARACTERIZATION

The particle air stream moving through the concentrator was characterized by several methods. First, air was sampled just before it entered the HAPC and again just before it entered the exposure chamber via the inlet duct (Figure 1); particles were collected on preweighed 47-mm Teflon filters (2-μm pores, Gelman Sciences, Ann Arbor MI) at a flow rate of 10 L/min for 2 hours during each exposure. These filters were analyzed in three ways: (1) They were weighed on an electrobalance (Mettler UMT2; Mettler-Toledo, Columbus OH) in a room with controlled temperature and humidity. The net filter weight at the end of each exposure protocol, the sampling time, and the flow rate were used to calculate the particle concentration in micrograms per cubic meter. (2) Filters with sequestered PM (both before and after passing through the concentrator) were analyzed for metals, elemental and organic carbon, sulfates, and nitrates using x-ray fluorescence and inductively coupled plasma–atomic emission spectrometry (see Appendix Tables A.1 and A.2 for metals and sulfate composition). We found no appreciable differences in chemical composition of particles before and after they were concentrated (data not shown). (3) The filters were used to determine the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) of the particles (Table 1).

Second, during the exposure, particle concentrations were continuously monitored using a tapered element oscillating microbalance (TEOM; series 1400a, Rupprecht & Patashnick, Albany NY) just before air entered the concentrator and just before it entered the exposure chamber.

Table 1. Physical Characteristics of Particles[a] (Devlin's Group)

	Filtered Air (n = 8)[b]	Low CAPs (Tertile 1) (n = 10)	Middle CAPs (Tertile 2) (n = 10)	High CAPs (Tertile 3) (n = 10)	Total CAPs[c] (n = 30)
$PM_{2.5}$ before concentrator ($\mu g/m^3$)[d]	—	9.9 ± 3.0	30.3 ± 3.8	37.6 ± 4.6	26.4 ± 3.0
$PM_{2.5}$ after concentrator ($\mu g/m^3$)[d,e]	2.9 ± 1.9	47.2 ± 5.3	107.4 ± 9.3	206.7 ± 19.2	120.4 ± 14.1
Concentration factor[f]	—	9.4 ± 2.3	4.1 ± 0.7	6.4 ± 1.0	6.5 ± 0.9
MMAD (μm)	—	0.54 ± 0.06	0.67 ± 0.05	0.72 ± 0.03	0.65 ± 0.03
GSD of MMAD	—	2.44 ± 0.16	2.39 ± 0.14	2.24 ± 0.08	2.35 ± 0.72

[a] Reprinted from Ghio et al 2000 with permission from the American Review of Respiratory and Critical Care Medicine, official journal of the American Thoracic Society. The data are presented as means ± SE.

[b] The Devlin study had 8 control subjects (see Ghio et al 2000); 4 more subjects from a different study with the same exposure protocol were added for the bronchoscopies only (performed at 18 hours after exposure).

[c] The value for Total CAPs is the mean ± SE of all 30 PM exposure values.

[d] The values for "before concentrator" and "after concentrator" were calculated from the gravimetric analysis of filters.

[e] The "after concentrator" data are the means ± SE of the 8 filtered air or 10 CAPs exposure values for each group.

[f] Calculated as [after concentrator] / [before concentrator] for each subject and then averaged for each exposure group.

Third, the particle-size distribution was assessed just before the air stream entered the exposure chamber using a microorifice uniform deposit impactor (MOUDI; MSP Corporation, Minneapolis MN); this is an eight-stage cascade impactor containing a series of microorifices that collect particles onto preweighed 37-mm Teflon filters (2-µm pores, Gelman Sciences). Aerosols were sampled from the inlet duct at a flow rate of 30 L/min for 2 hours.

Ozone levels also were measured inside the exposure chamber; they did not exceed 0.05 ppm.

STUDY POPULATION

Volunteers were recruited by newspaper advertisement. Those who responded were prescreened over the telephone to ensure that they met the entry criteria: age 18 to 40 years; nonsmoker for at least 5 years before the study; no history of allergies or respiratory diseases; and not presently taking any physician-prescribed medication other than oral contraceptives. All female subjects were given a urine pregnancy test; anyone with a positive result was excluded from further participation in the study. Subjects' characteristics and individual exposure concentrations are provided in Table 2.

Before participating in the study, subjects were informed of the protocol, procedures, and potential risks from their participation; each signed a statement of informed consent. The protocol and consent form were approved by the University of North Carolina School of Medicine Committee on the Protection of the Rights of Human Subjects. The screening procedures for each subject included a Minnesota Multiphasic Personality Inventory, medical history, physical examination, chest x-ray, and routine blood tests (hematology and biochemistry).

EXPOSURE TO CAPs OR FILTERED AIR

Each subject was exposed once for two hours to either filtered air or CAPs. The sequence of exposure atmospheres was randomized and no subject was exposed to both CAPs and to filtered air. Technicians and data analysts were blinded as to which subjects were exposed to which atmospheres. Subjects were continuously monitored by telemetry for cardiac rhythm, heart rate, and arterial oxygen saturation. During the two-hour exposure, the subject sat on a recumbent bicycle ergometer and exercised for a total of one hour in increments of 15 minutes of cycling and 15 minutes of rest repeated four times. Exercise intensity (ie, ergometer workload) was adjusted so that the subjects breathed at a ventilatory rate, normalized for body surface area, of 25 L/m^2 per minute. For most subjects, this amounted to about 50 L/min (ie, an oxygen consumption per unit of time [$\dot{V}O_2$] of approximately 1.0 L/min). This physiologic response required a cycle ergometer setting of 75 to 100 W.

BLOOD SAMPLING AND PULMONARY FUNCTION TESTING

Venous blood was taken from an antecubital site before and 18 hours after exposure; samples were analyzed for complete blood count, ferritin, blood viscosity, and fibrinogen (Labcorp, Burlington NC). Before and immediately after exposure, spirometry was used to assess lung function (forced vital capacity [FVC], forced expiratory volume in one second [FEV_1], and peak expiratory flow [PEF]) and plethysmography was used to measure airway resistance (Raw) with methods previously described (Devlin et al 1991).

BRONCHOSCOPY, BIOPSY, AND LAVAGE

Each fiberoptic bronchoscopy was performed as described by Ghio and colleagues (1998) by a qualified bronchoscopist at the Human Studies Facility in Chapel Hill. Bronchoscopy, lavage, and biopsy were performed 18 hours after exposure. Biopsy tissues suitable for analyses were obtained from 10 CAPs-exposed subjects and 7 control subjects (Table 2). To equalize the group numbers for analytic purposes, four additional biopsy tissues were obtained from control subjects exposed to filtered air followed by bronchoscopy. (These four subjects were enrolled in an ongoing protocol at Chapel Hill in which samples were obtained for methods development or as a source of alveolar macrophages for in vitro toxicology studies. As with all studies performed in the EPA Human Research Facility, this protocol was approved by the University of North Carolina Investigative Review Board as well as by the designated EPA Human Ethics Official.)

For lavage, four aliquots of sterile isotonic saline were instilled in a segmental bronchus of the lingula and immediately aspirated. The first aliquot (20 mL) was labeled as the bronchial wash (BW) sample; the remaining three aliquots (50 mL each) were pooled to form the bronchoalveolar lavage (BAL) sample. The procedure was repeated on the right middle lobe, again using 170 mL saline. The right and left lobe samples were pooled to form a single BW sample and a single BAL sample. All samples were put on ice immediately after aspiration and then centrifuged at 300*g* for 10 minutes at 4°C. Cells were washed once in RPMI medium and viability determined by trypan blue exclusion. Cell viability exceeded 85% in all cases and there were no differences in rates of viability between control subjects and CAPs-exposed subjects. Cell numbers were determined using a hemocytometer. Cell differentials were performed on cytocentrifuged cells stained with a modified Wright stain (Leukostat solution, Fisher Scientific, Pittsburgh PA). At least 200 cells were counted per slide.

Table 2. Characteristics of Subjects Exposed to CAPs or Filtered Air (Devlin's Group)[a]

Exposure Group	Subject ID	Exposure Date	$PM_{2.5}$[b] ($\mu g/m^3$)	Ethnicity[c]	Gender	Age	Height (cm)	Weight (kg)	Biopsy
Control	233	8-Sep-98	0.0	W	M	29	171	64	Yes
	235	9-Sep-98	0.0	W	F	34	158	61	Yes
	230	30-Sep-98	0.0	B	M	29	175	74	Yes
	242	6-Oct-98	0.0	H	M	31	178	96	Yes
	246	17-Feb-99	0.0	W	M	24	185	81	Yes
	247	24-Mar-99	0.0	W	M	24	175	71	Yes
	243	14-Dec-98	8.2[d]	W	F	24	167	79	Yes
	237	15-Dec-98	15.1[d]	W	M	22	175	71	No
	315[e]	11-Jan-00	0.0	H	F	27	153	51	Yes
	316[e]	3-Feb-00	0.0	W	F	33	169	80	Yes
	317[e]	15-Feb-00	0.0	W	M	29	183	96	Yes
	318[e]	17-Feb-00	0.0	A	M	29	164	69	Yes
Low CAPs (Tertile 1)	244	8-Dec-98	23.1	NA	NA	NA	NA	NA	No
	241	16-Feb-99	24.8	W	M	31	175	85	No
	238	23-Sep-98	37.6	W	M	25	182	77	Yes
	206	22-Apr-98	39.7	W	M	25	181	98	Yes
	205	9-Dec-97	39.9	W	M	29	177	76	No
	239	22-Sep-98	52.6	W	M	25	179	88	No
	219	15-Jul-98	57.0	W	M	24	179	88	No
	216	14-Jul-98	60.5	B	M	20	175	77	No
	201	2-Dec-97	66.3	W	M	29	177	80	Yes
	240	27-Oct-98	70.1	B	M	22	171	57	No
Middle CAPs (Tertile 2)	204	16-Dec-97	72.2	W	M	23	196	97	Yes
	220	30-Jun-98	73.5	W	M	25	191	106	Yes
	227	19-Aug-98	74.3	W	M	24	180	73	No
	208	5-May-98	94.7	W	M	26	188	85	Yes
	231	25-Aug-98	107.6	W	M	34	182	83	Yes
	209	13-May-98	109.1	B	M	27	175	97	No
	226	4-Aug-98	120.7	W	M	23	181	83	No
	229	11-Aug-98	135.9	W	M	25	182	73	No
	214	3-Jun-98	141.0	W	M	29	178	74	No
	234	16-Sep-98	148.6	W	M	26	193	93	No
High CAPs (Tertile 3)	202	18-Nov-97	152.2	B	M	26	179	75	No
	215	2-Jun-98	154.6	A	M	30	171	78	No
	212	19-May-98	161.7	W	M	22	171	79	No
	213	7-Jul-98	168.0	W	M	30	186	107	Yes
	221	8-Jul-98	183.3	W	M	22	177	76	No
	236	15-Sep-98	184.1	W	M	21	168	77	No
	207	6-May-98	199.6	W	M	37	181	72	Yes
	222	28-Jul-98	248.8	W	M	30	171	89	No
	218	23-Jun-98	303.6	W	M	32	183	96	No
	225	29-Jul-98	311.1	W	M	28	172	75	Yes

[a] NA = data not available.

[b] Actual concentration of particles to which subject was exposed. On the basis of these values, CAPs-exposed subjects were categorized into tertiles.

[c] A = Asian; B = black; H = Hispanic or Latino; W = white.

[d] Two control subjects were exposed to small amounts of PM, possibly from heavy winter clothing.

[e] The original exposure study had 8 control subjects (see Ghio et al 2000); 4 more control subjects from a different study with the same filtered air exposure protocol were added for the bronchoscopies only.

ANALYSIS OF LAVAGE FLUID

Recovery of BW or BAL fluid samples did not differ between control subjects and CAPs-exposed subjects, and all fluid recoveries were within 10% of each other. The supernatants were assayed for concentrations of total protein, interleukin-8 (IL-8), and IL-6 by enzyme-linked immunosorbent assay (ELISA; R & D Systems, Minneapolis MN). These soluble components were normalized per milliliter of fluid. Cell pellets were resuspended in phosphate-buffered saline at 10^6 cells/mL and the total number of cells in the BW and the BAL fluids were counted in a Burker chamber (VWR International). Cytocentrifuged specimens with 5×10^4 cells per slide were prepared using a Cytospin 3 centrifuge (Shandon Southern Instruments, Sewikly PA) at 1000 rpm for 5 minutes. Differential counts were made after staining with May-Grunwald Giemsa, counting 400 cells per slide. Lymphocyte subsets were determined by flow cytometry (FACS Scan, Becton Dickinson).

TISSUE SAMPLE EMBEDDING AND PROCESSING

The endobronchial tissue samples obtained at bronchoscopy were placed in ice-cooled acetone containing the protease inhibitors phenylmethyl-sulphonyl fluoride (2 nM) and iodoacetamide (2 nM), cooled rapidly to −20°C, and left overnight. The following day the samples were put in acetone at room temperature for 15 minutes and then in methylbenzoyl for 15 minutes. The tissue was then immersed in glycol methacrylate (GMA) JB4 solution A (Polysciences, Northampton, UK) at 4°C for 7 hours, during which time the GMA solution was changed three times. The tissue was finally embedded in GMA resin (prepared by mixing GMA monomer, *N,N*-dimethylaniline in polyethylene glycol 400, and benzoyl peroxide), and left to polymerize overnight at 4°C. The blocks were then stored in airtight containers at −20°C and transported by air freight from Chapel Hill NC, USA, to Southampton for further analysis.

METHODS FOR WORK PERFORMED BY THE HOLGATE GROUP

Tissue Preparation

Embedded biopsy tissues were maintained at −20°C until they were used for immunostaining with monoclonal antibodies (Table 3) and a standard peroxidase technique (Britten et al 1993).

Thin sections (2 μm) were cut using a microtome, floated onto ammonia water (1:500), picked onto 0.01% poly-L-lysine–coated glass slides, and allowed to dry at room temperature for 1 hour. Slides were initially stained

Table 3. Names and Sources of Monoclonal Antibodies Used for Immunohistochemistry (Holgate's Group)

Antibody[a]	Marker	Cells	Source
ICAM-1	ICAM-1	Endothelial cells	Serotec, UK
VCAM-1	VCAM-1	Endothelial cells	Sanbio, UK
E-selectin	E-selectin	Endothelial cells	Serotec, UK
P-selectin	P-selectin	Endothelial cells	Immunotech, UK
EN4	Endothelium	Pan-endothelial marker	Sanbio, UK
AA1	Tryptase	Mast cells	Dr A Walls, Southampton, UK
NE	Neutrophil elastase	Neutrophils	DAKO, High Wycombe, UK
EG2	Cationic protein	Eosinophils	DAKO, High Wycombe, UK
CD14	CD14	Macrophages	DAKO, High Wycombe, UK
CD19	CD19	B cells	DAKO, High Wycombe, UK
CD3	CD3	T cells	DAKO, High Wycombe, UK
CD4	CD4	Helper T cells	Becton Dickinson, UK
CD8	CD8	Suppressor T cells	DAKO, High Wycombe, UK
CD25	CD25	IL-2 receptor cells (activated T cells)	DakoCytomation, Ely, UK
LFA-1	CD11a	Lymphocyte function–associated antigen 1	Chemicon International, Harrow, UK
VLA-4	CD49d	Very late activation 4	Chemicon International, Harrow, UK

[a] See the section Abbreviations and Other Terms for these definitions and the Glossary in the Health Review Committee's Critique for more explanation of the terms.

with toluidine blue to assess tissue structure and to select the best sections for immunostaining. Sections on slides were then wrapped in aluminum foil and kept at −40°C to preserve tissue antigenicity.

Quantitation of Inflammatory Cells and Adhesion Molecules

Stained inflammatory cells were counted separately in the epithelium and in the submucosa, excluding areas of mucosal glands, blood vessels, muscle, and any region of torn or folded tissue. Inflammatory cells counted were mast cells, eosinophils, monocytes (CD14), neutrophils, CD3 (all T cells), CD4 (T helper and regulatory cells), CD8 (T cells with cytotoxic function), and CD25 (the α chain of the IL-2 receptor; a marker of T-cell activation). [Refer to the Glossary in the Health Review Committee's Critique for a more complete explanation of these and related terms.] The length of the epithelium and the area of the submucosa in which cell counts were performed were determined using computer-assisted image analysis Color Vision Software (Improvision System, Birmingham, UK). The results were expressed as cells per millimeter of epithelium or as cells per square millimeter of submucosa (Djukanovic et al 1990; Bradding et al 1994; Synek et al 1996).

For endothelial adhesion molecules (intercellular adhesion molecule 1 [ICAM-1], vascular cell adhesion molecule 1 [VCAM-1], E-selectin, and P-selectin), the number of blood vessels stained with a specific monoclonal antibody (eg, for ICAM-1 or E-selectin) at a standard dilution was compared with the total complement of microvessels stained with the endothelial marker endothelium clone 4 (EN4) in subjacent sections (2 µm apart). The number of positively stained blood vessels was then expressed as a percentage of the total number of vessels examined (Montefort et al 1994).

STATISTICAL ANALYSIS

Data are expressed as mean values ± standard errors. Differences between air-exposed and CAPs-exposed groups were tested using the *t* test of independent means. For those variables that were significantly altered, the population was divided post hoc into four groups: a control group that received filtered air and three tertiles of CAPs-exposed subjects, divided according to the particle concentration to which they were exposed. Differences between groups were then assessed by one-way analysis of variance (Colton 1974). The post hoc test employed was the Scheffé test. Relations between pairs of continuous variables (before and after exposure) were evaluated by linear regression analyses in which two-tailed tests of significance were used. Comparisons within and between groups for immunohistochemical staining were undertaken using Wilcoxon tests. For histological markers of inflammation, differences between air-exposed and CAPs-exposed groups were assessed by the Mann-Whitney test for nonparametric unpaired variables. P values < 0.05 were regarded as significant.

RESULTS

Thirty-eight exposures were conducted in the Chapel Hill study (see Ghio et al 2000 for a full discussion of the results). The mean steady-state $PM_{2.5}$ concentrations in the exposure chamber during CAPs exposures varied between 23.1 and 311.1 µg/m^3 (Table 2) with an overall average $PM_{2.5}$ concentration of 120.4 ± 14.1 µg/m^3 (Table 1). For analytical purposes, the CAPs-exposed subjects were divided post hoc into three tertiles on the basis of the concentration of CAPs to which they were exposed. The mean CAPs concentrations for these tertiles were 47.2 µg/m^3, 107.4 µg/m^3, and 206.7 µg/m^3, respectively (Table 1). The subjects exposed to CAPs included 30 males (mean age 25.7 years); the eight subjects exposed to filtered air included 6 males and 2 females (mean age 27.1 years).

CAPs exposure did not appear to affect lung function in that we observed no trends across the exposure groups in the four parameters we tested (FVC, FEV_1, PEF, and Raw) (Table 4). Likewise, we found no changes in blood hemoglobin, white cell counts (neutrophils, lymphocytes, and monocytes [CD14]), platelet counts, ferritin, or plasma viscosity after CAPs exposure (Table 5); but the blood fibrinogen concentration increased in all three CAPs-exposed groups when compared with the control group (Table 5; Figure 2).

In contrast, in the BAL fluid, we saw a dose-dependent increase in neutrophils after CAPs exposure, whether the increase was expressed as absolute cell counts (Figure 3) or as a proportion of the total lavaged cells (Table 6). Epithelial cells and lymphocytes in the BAL fluid did not vary across the groups, whereas macrophages showed a downward trend, which is consistent with the increase in the percentage of neutrophils (Table 6). Similar results were found with the BW fluid (data not shown; see Ghio et al 2000).

Biopsy tissues suitable for histologic examination were not available from all subjects who underwent bronchoscopy primarily because some tissues were too small for analysis. All tissues that were suitable (ie, with an intact epithelium and submucosa) were examined; data for the histologic studies are thus based on 10 CAPs-exposed subjects and 7 control subjects from the exposure groups selected for this study. To obtain sufficient control samples, we included biopsy tissues from 4 additional control subjects who were exposed to filtered air in an identical

Table 4. Changes in Lung Function After Exposure to CAPs or Filtered Air[a,b] (Devlin's Group)

Group	Filtered Air Control (n = 8)	Low CAPs Tertile 1 (n = 10)	Middle CAPs Tertile 2 (n = 10)	High CAPs Tertile 3 (n = 10)	All Subjects Exposed to CAPs (n = 30)
$PM_{2.5}$ ($\mu g/m^3$)[c]	2.9 ± 1.9	47.2 ± 5.3	107.4 ± 9.3	206.7 ± 19.2	120.4 ± 14.1
FEV_1 (L)	−0.05 ± 0.05	−0.06 ± 0.04	−0.04 ± 0.04	−0.04 ± 0.02	−0.05 ± 0.02
FVC (L)	−0.09 ± 0.07	0.05 ± 0.06	−0.04 ± 0.09	0.07 ± 0.09	−0.05 ± 0.02
Raw (cm H_2O/L/sec)	0.12 ± 0.08	0.05 ± 0.06	−0.04 ± 0.09	0.07 ± 0.09	0.03 ± 0.04
PEF (L/sec)	−0.15 ± 0.23	0.43 ± 0.45	−0.11 ± 0.21	0.16 ± 0.32	−0.16 ± 0.19

[a] Adapted from data provided in Ghio et al 2000.

[b] Spirometry was performed before and immediately after exposure. Data were calculated by subtracting the preexposure value from the postexposure value for each subject and are presented as the mean ± SE for the group. No significant differences were observed between any pairs of groups for any of the pulmonary endpoints measured.

[c] The group PM exposure level is the mean ± SE of the 8 control or 10 exposed subjects in each group. The value for All Subjects is the mean ± SE of all 30 PM exposure values.

Table 5. Changes in Blood Parameters After Exposure to CAPs or Filtered Air[a,b] (Devlin's Group)

Group	Filtered Air Control (n = 8)	Low CAPs Tertile 1 (n = 10)	Middle CAPs Tertile 2 (n = 10)	High CAPs Tertile 3 (n = 10)	All Subjects Exposed to CAPs (n = 30)
$PM_{2.5}$ ($\mu g/m^3$)[c]	2.9 ± 1.9	47.2 ± 5.3	107.4 ± 9.3	206.7 ± 19.2	120.4 ± 14.1
Red blood cells/mL	0.10 ± 0.09	−0.01 ± 0.11	0.1 ± 0.05	0.08 ± 0.07	0.02 ± 0.05
Hemoglobin (g/dL)	0.14 ± 0.24	0.05 ± 0.34	0.00 ± 0.12	0.28 ± 0.19	0.12 ± 0.13
Hematocrit (%)	1.10 ± 0.83	0.09 ± 0.90	0.31 ± 0.54	0.88 ± 0.54	0.44 ± 0.37
Neutrophils/mL	−0.31 ± 0.13	−0.26 ± 0.17	−0.29 ± 0.36	−0.14 ± 0.23	−0.22 ±0.14
Lymphocytes/mL	−0.05 ± 0.04	0.06 ± 0.10	0.11 ± 0.09	−0.02 ± 0.09	0.03 ± 0.05
Monocytes (CD14)/mL	0.00 ± 0.03	0.04 ± 0.03	0.01 ± 0.03	−0.02 ± 0.03	0.02 ± 0.02
Platelets/mL	6.29 ± 16.03	−7.00 ± 4.07	−7.22 ± 6.80	−5.70 ± 3.22	−7.47 ± 2.69
Ferritin (ng/mL)	3.00 ± 2.43	1.50 ± 3.57	4.33 ± 4.16	5.44 ± 3.88	1.90 ± 2.71
Viscosity[d]	0.13 ± 0.08	−0.04 ± 0.03	−0.02 ± 0.02	0.11 ± 0.03	0.01 ± 0.02
Fibrinogen (mg/dL)	− 5.3 ± 10.6	38.9 ± 17.8	43.3 ± 25.3	41.7 ± 28.8	41.2 ± 12.7[e]

[a] Adapted from data provided in Ghio et al 2000.

[b] Blood samples were obtained before and 18 hours after exposure. Data were calculated by subtracting the preexposure value from the postexposure value for each subject and are presented as the mean ± SE for the group.

[c] The group PM exposure level is the mean ± SE of the 8 control or 10 exposed subjects in each group. The value for All Subjects is the mean ± SE of all 30 PM exposure values.

[d] Blood viscosity data are expressed in relation to saline (ie, a value of 1.5 would mean a viscosity 1.5 times more dense than saline).

[e] $P < 0.05$ comparing all CAPs-exposed subjects with the control group (assessed by ANOVA and the Scheffé test).

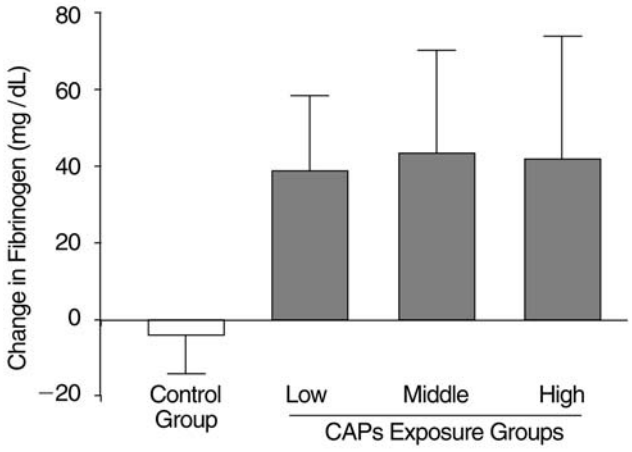

Figure 2. Changes in blood fibrinogen concentrations after exposure to filtered air (*n* = 8) or to CAPs (*n* = 30). CAPs-exposed subjects were grouped into tertiles according to the CAPs concentration to which they were exposed. Values shown are means with SE bars. (Adapted from data provided in Ghio et al 2000.) (Devlin's Group)

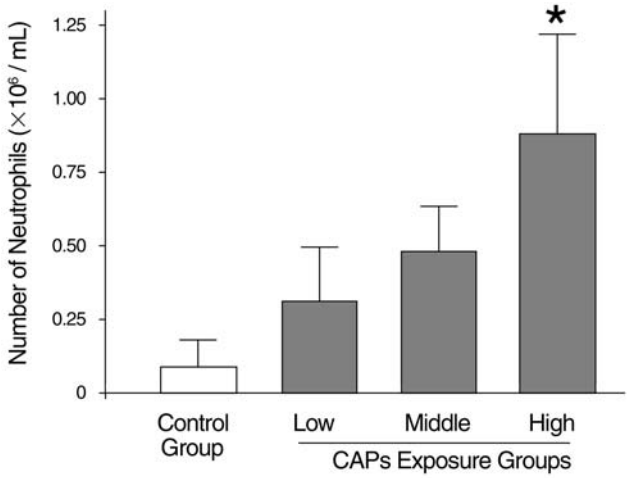

Figure 3. Number of neutrophils in alveolar lavage samples obtained 18 hours after exposure to filtered air (*n* = 8) or to CAPs (*n* = 30). CAPs-exposed subjects were grouped into tertiles according to the CAPs concentration to which they were exposed. Values shown are means with SE bars. The asterisk indicates a significant difference ($P = 0.05$) from the control group on the basis of one-way analysis of variance. (Adapted from Ghio et al 2000.) (Devlin's Group)

protocol for another study in the Chapel Hill laboratory. Adding these subjects resulted in suitable biopsy tissues from 11 control subjects.

Although we found increased numbers of neutrophils in the lavage fluids from CAPs-exposed subjects compared with the fluids from control subjects, immunohistochemical analysis of the bronchial biopsy tissues did not show any increase in mast cells, eosinophils, monocytes (CD14), or neutrophils (Figure 4, Table 7).

Recruitment of neutrophils is dependent upon the upregulation of adhesion molecules on endothelial cells that line small blood vessels. Adhesion molecules are upregulated in a coordinated and sequential fashion such that preformed P-selectin is rapidly expressed on the endothelial cell surface, E-selectin appears at 4 to 8 hours, and ICAM-1 appears at 4 to 24 hours (Montefort et al 1993). VCAM-1 is associated with eosinophil and T-cell recruitment and is seen at 12 to 36 hours. Compared with the tissues from control subjects, the tissues obtained after

Table 6. Cells in BAL Fluid After Exposure to CAPs or Filtered Air[a,b] (Devlin's Group)

Group	Filtered Air Control ($n = 8$)	Low CAPs Tertile 1 ($n = 10$)	Middle CAPs Tertile 2 ($n = 10$)	High CAPs Tertile 3 ($n = 10$)	All Subjects Exposed to CAPs ($n = 30$)
$PM_{2.5}$ ($\mu g/m^3$)[c]	2.9 ± 1.9	47.2 ± 5.3	107.4 ± 9.3	206.7 ± 19.2	120.4 ± 14.1
Total cell count ($\times 10^6$)	15.9 ± 1.9	20.3 ± 3.0	23.0 ± 2.1	20.8 ± 2.0	21.4 ± 1.3[d]
Macrophages (%)	80.6 ± 2.9	82.4 ± 2.2	82.9 ± 1.3	75.4 ± 3.7	80.2 ± 1.6
Neutrophils (%)	0.8 ± 0.3	1.4 ± 0.4	2.0 ± 0.4	4.2 ± 1.7	2.5 ± 0.6[d]
Lymphocytes (%)	16.8 ± 2.4	13.1 ± 1.7	13.1 ± 1.3	19.0 ± 3.1	15.1 ± 1.3
Monocytes (CD14) (%)	1.2 ± 0.3	0.9 ± 0.2	1.3 ± 0.3	2.2 ± 0.5	1.4 ± 0.2
Epithelial cells (%)	1.3 ± 0.3	0.8 ± 0.1	0.8 ± 0.1	1.4 ± 0.3	1.1 ± 0.1

[a] Reprinted from Ghio et al 2000 with permission from the American Review of Respiratory and Critical Care Medicine, official journal of the American Thoracic Society.

[b] Lavage was performed 18 hours after exposure. Data are presented as means ± SE.

[c] The group PM exposure level is the mean ± SE of the 8 control or 10 exposed subjects in each group. The value for All Subjects is the mean ± SE of all 30 PM exposure values.

[d] $P < 0.05$ comparing all CAPs-exposed subjects with the control group (assessed by ANOVA and the Scheffé test).

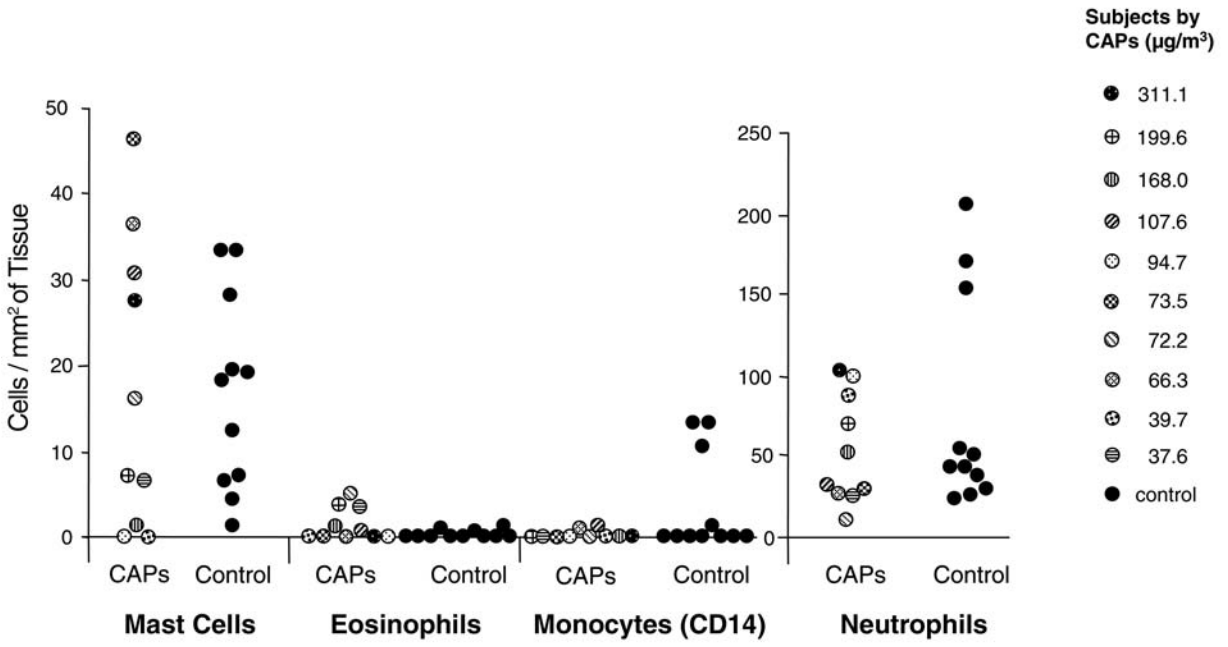

Figure 4. Numbers of inflammatory cells in the bronchial submucosa of biopsy tissues obtained 18 hours after exposure to filtered air (*n* = 11) or to CAPs (*n* = 10). Each data point is one subject; data are unpaired because each subject underwent bronchoscopy only after exposure and not before. Note the different scale of values for neutrophils. (Holgate's Group)

Table 7. Numbers of Inflammatory Cells in the Bronchial Submucosa of Biopsy Tissues Obtained 18 Hours After Exposure to Filtered Air or to CAPs[a] (Holgate's Group)

$PM_{2.5}$ (µg/m^3)		Mast Cells		Eosinophils		Monocytes (CD14)		Neutrophils	
CAPs	Filtered Air	CAPs	Filtered Air	CAPs	Filtered Air	CAPs	Filtered Air	CAPs	Filtered Air
37.6	0.0	6.6	4.4	3.3	0.0	0.0	0.0	25.4	50.7
39.7	0.0	0.0	19.4	0.0	0.0	0.0	0.0	86.5	44.7
66.3	0.0	36.5	6.5	0.0	0.6	1.0	1.2	28.0	26.0
72.2	0.0	16	1.2	4.9	0.0	0.0	0.0	11.1	40.5
73.5	0.0	46.4	18.2	0.0	0.0	0.0	0.0	29.3	25.0
94.7	0.0	0.0[b]	33.3	0.0[b]	0.0	0.0[b]	13.3	99.5[b]	170.0
107.6	0.0	30.7	12.5	0.6	0.0	1.1	0.0	32.1	153.1
168.0	0.0	1.4[b]	19.2	1.4[b]	1.2	0.0[b]	13.4	52.1[b]	29.1
199.6	0.0	7.1	33.3	3.6	0.8	0.0	10.5	69.8	205.6
311.1	0.0	27.5[b]	7.0	0.0[b]	0.0	0.0[b]	0.0	103.3[b]	46.5
	8.2		28.2[b]		0.0 [b]		0.0[b]		53.5[b]

[a] Biopsy tissues suitable for analysis were obtained from 10 subjects exposed to CAPs and 11 subjects exposed to filtered air (see Table 2). Data as cells/mm^2 are presented for each subject; they are unpaired data because each subject underwent bronchoscopy only after exposure and not before.

[b] Small biopsy tissue (area less than 0.46 mm^2).

CAPs exposure showed no consistent increase in any of these adhesion molecules (Figure 5, Table 8).

Immunocytochemical analysis of the bronchial tissues did not show any increase in CD25 (Figure 6 and Table 9) or in adhesion molecule ligands (LFA-1 and VLA-4; Figure 7) of the CAPs-exposed subjects compared with the control subjects. However, in the expression of CD3 T cells and the CD4 fraction, CAPs exposure produced a small but significant reduction in cell counts; a trend in the same direction was noted for the expression of the CD8 fraction (Figure 6).

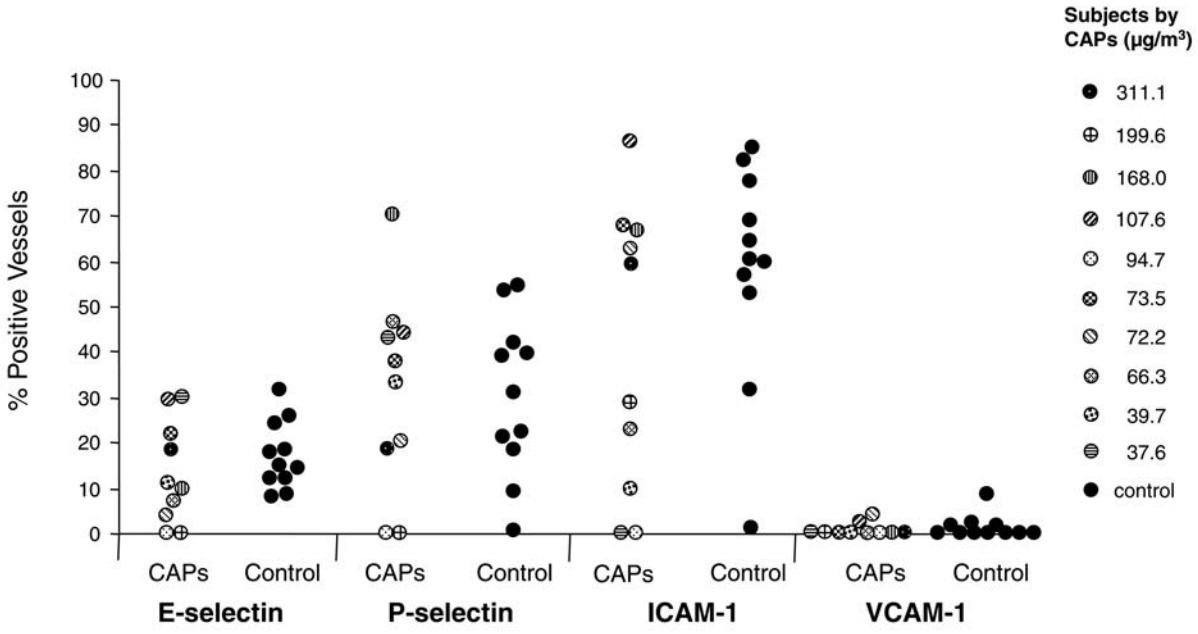

Figure 5. Expression of adhesion molecules on the vascular endothelium in bronchial biopsy tissues obtained 18 hours after exposure to filtered air (*n* = 11) or to CAPs (*n* = 10). Values are expressed as the percentage of blood vessels that showed positive expression of the adhesion molecule. Each data point is one subject; data are unpaired because each subject underwent bronchoscopy only after exposure and not before. (Holgate's Group)

Table 8. Expression of Cell Adhesion Molecules on the Vascular Endothelium in Bronchial Biopsy Tissues Obtained 18 Hours After Exposure to Filtered Air or to CAPs[a] (Holgate's Group)

$PM_{2.5}$ (µg/m³)		E-selectin		P-selectin		ICAM-1		VCAM-1	
CAPs	Filtered Air	CAPs	Filtered Air	CAPs	Filtered Air	CAPs	Filtered Air	CAPs	Filtered Air
37.6	0.0	30.0	12.1	43.3	39.8	0.0	85.2	0.0	8.6
39.7	0.0	11.1	17.7	33.3	0.3	10.0	60.3	0.0	0.0
66.3	0.0	6.7	24.2	46.7	39.0	23.1	1.2	0.0	0.0
72.2	0.0	4.0	31.8	20.0	54.6	62.5	57.1	4.2	0.0
73.5	0.0	21.6	7.9	37.8	18.4	68.0	60.0	0.0	0.0
94.7	0.0	0.0[b]	15.2	0.0[b]	9.1	0.0[b]	77.4	0.0[b]	0.0
107.6	0.0	29.5	14.3	44.5	21.4	86.0	69.2	2.2	0.0
168.0	0.0	10.0[b]	18.2	70.0[b]	31.1	66.7[b]	64.5	0.0[b]	1.6
199.6	0.0	0.0	25.7	0.0	41.8	28.6	52.9	0.0	2.1
311.1	0.0	18.2[b]	8.9	18.2[b]	22.2	59.3[b]	31.7	0.0[b]	0.0
	8.2		12.0[b]		53.7[b]		82.0[b]		1.8[b]

[a] Biopsy tissues suitable for analysis were obtained from 10 subjects exposed to CAPs and 11 subjects exposed to fitered air (see Table 2). Data as the percentage of positive vessels are presented for each subject; data are unpaired because each subject underwent bronchoscopy only after exposure and not before. Values are expressed as the percentage of blood vessels that showed positive expression of the adhesion molecule.

[b] Small biopsy tissue (area less than 0.46 mm^2).

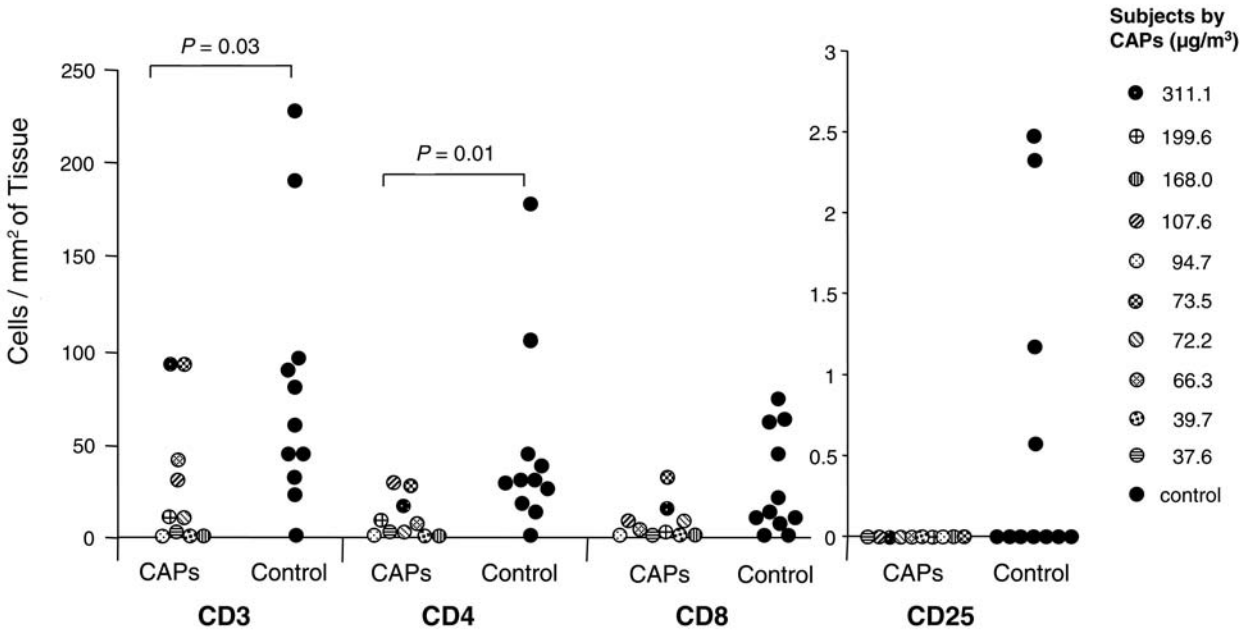

Figure 6. Numbers of total T cells and T-cell subsets expressing the indicated markers in the bronchial submucosa of biopsy tissues obtained 18 hours after exposure to filtered air (*n* = 11) or to CAPs (*n* = 10). Each data point is one subject; data are unpaired because each subject underwent bronchoscopy only after exposure and not before. The numbers of CD3 and CD4 cells after CAPs exposure were significantly lower than after filtered air exposure by the Mann-Whitney test. Note the different scale of values for the T-cell subset CD25. (Holgate's Group)

Table 9. Numbers of T Cells and T-Cell Subsets Expressed in the Bronchial Submucosa of Biopsy Tissues Obtained 18 Hours After Exposure to Filtered Air or to CAPs[a] (Holgate's Group)

$PM_{2.5}$ (µg/m³)		CD3[b]		CD4[b]		CD8		CD25	
CAPs	Filtered Air	CAPs	Filtered Air	CAPs	Filtered Air	CAPs	Filtered Air	CAPs	Filtered Air
37.6	0.0	0.8	58.7	0.8	37.7	0.0	9.4	0.0	0.0
39.7	0.0	0.0	44.1	0.0	17.1	0.0	9.4	0.0	1.2
66.3	0.0	40.5	43.1	6.2	25.4	3.1	5.9	0.0	0.0
72.2	0.0	8.6	0.0	1.2	0.0	7.4	0.0	0.0	0.0
73.5	0.0	91.5	79.5	26.9	29.5	31.7	20.5	0.0	0.0
94.7	0.0	0.0[c]	190.0	0.0[c]	103.3	0.0[c]	73.3	0.0[c]	0.0
107.6	0.0	29.6	21.9	28.0	12.5	7.1	0.0	0.0	0.0
168.0	0.0	0.0[c]	94.2	0.0[c]	43.6	0.0[c]	61.6	0.0[c]	0.6
199.6	0.0	9.5	227.2	7.1	177.2	1.2	60.5	0.0	2.5
311.1	0.0	91.8[c]	88.4	16.1[c]	29.1	13.8[c]	43.0	0.0[c]	2.3
	8.2		31.0[c]		28.2[c]		12.7[c]		0.0[c]

[a] Biopsy tissues suitable for analysis were obtained from 10 subjects exposed to CAPs and 11 subjects exposed to filtered air (see Table 2). Data as cells/mm² are presented for each subject; they are unpaired data because each subject underwent bronchoscopy only after exposure and not before.

[b] The numbers of CD3 and CD4 cells after CAPs exposure were significantly lower than after filtered air exposure by the Mann-Whitney test (CD3 $P = 0.03$; CD4 $P = 0.01$).

[c] Small biopsy tissue (area less than 0.46 mm²).

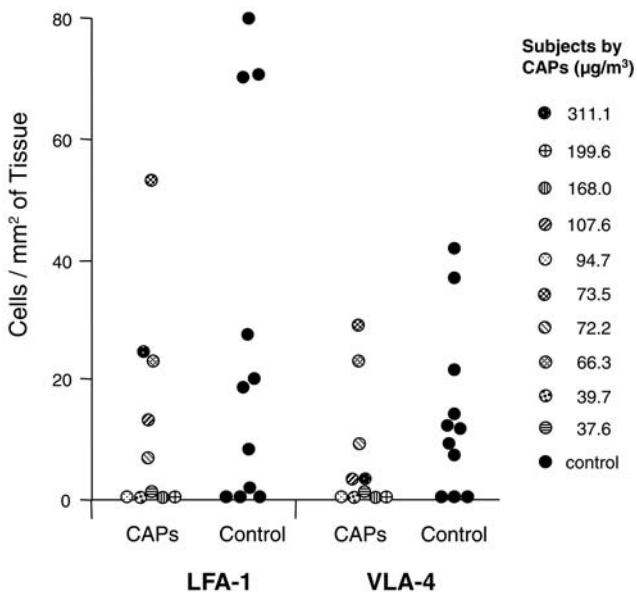

Figure 7. Numbers of cells that expressed adhesion molecule ligands in the bronchial submucosa of biopsy tissues obtained 18 hours after exposure to filtered air (*n* = 11) or CAPs (*n* = 10). Each data point is one subject; data are unpaired because each subject underwent bronchoscopy only after exposure and not before. (Holgate's Group)

DISCUSSION

The Devlin group's portion of this study (see Ghio et al 2000) has demonstrated that healthy subjects exposed to realistic levels of ambient air particles develop mild inflammation in both the airways and the peripheral lung. The influx of neutrophils into the lungs of CAPs-exposed individuals was dose-dependent; the subjects exposed to the highest CAPs concentrations had the most marked increases in neutrophils in BAL fluids. The number of neutrophils present in BW [data not shown] or BAL fluid was comparable to the levels found in healthy young subjects exposed to low levels (100 ppb) of ozone (Devlin et al 1991). The level of neutrophils found in the BW fluid after CAPs exposure [data not shown] was comparable to that seen in the BW fluid of humans exposed to diesel exhaust (300 µg/m^3 for 1 hour) (Salvi et al 1999). Although that study did not find an increased number of neutrophils in the BAL fluid, this difference may reflect differences between the two studies in the particle sources, the timing of bronchoscopies (6 hours after diesel exhaust exposure versus 18 hours after CAPs exposure), or in the total particle doses delivered to the lungs.

Another noteworthy outcome of the Devlin group's analyses is the finding that fibrinogen concentrations in the blood were increased after CAPs inhalation (Figure 2, Table 5; see also Ghio et al 2000). This appeared to be a specific elevation of an acute-phase reactant, because other proteins participating in the acute-phase response were not affected (eg, ferritin). High levels of fibrinogen may reflect a disruption in the normal equilibrium between coagulation and fibrinolysis. This disturbance was not sufficient to be reflected in the assays that measure the in vitro clotting time for either prothrombin or partial thromboplastin (data not shown). However, independent of other measures of coagulation, increases in blood fibrinogen concentrations can be associated with ischemic heart disease, including myocardial infarction and sudden death. This may provide an explanation for elevation in cardiovascular death rates without any significant lung injury.

CAPs exposure did not induce fibrinogen increases in a dose-dependent manner as it did with neutrophils; this suggests a threshold effect for the increase in fibrinogen and a graded response for airway inflammation. These observations may indicate that increases in neutrophils and blood fibrinogen are independently regulated and, furthermore, that the effect on blood coagulability may occur at particulate concentrations below that which triggers airway inflammation. This has important implications both for setting regulatory standards and for understanding the epidemiologic data that suggest increased risks of adverse cardiac and respiratory events associated with particulate air pollution.

In contrast to the Holgate group's studies of diesel exhaust exposure (see Part I of this Research Report), we found no associated upregulation of vascular adhesion molecules in the proximal airway tissues that we studied; nor any clear-cut change in mast cell, neutrophil, lymphocyte, or macrophage numbers in the tissue samples.

The main advantage of using CAPs for exposure studies is that the composition of the PM is environmentally relevant in that it contains the types of particles that everyone in the sampling area breathes on a regular basis. In contrast, our diesel exhaust exposure studies (see Part I) are artificial in the sense that "real-world" PM pollution does not come from a single source, even though levels of diesel exhaust particles comparable to those used in our diesel exhaust exposure studies are encountered in some occupational settings (Salvi et al 1999). Moreover, the diesel exhaust used in our studies was fresh, whereas ambient PM changes as it ages, both in size and in surface chemistry. However, the HAPC does not concentrate ultrafine particles (< 0.15 µm) so any observed effect of the CAPs is predominantly attributable to PM in the 0.15 to 2.5 µm size range. The main disadvantage of using CAPs is that the concentration and composition of the CAPs aerosol varies among exposures depending on the ambient environment on any given day and on the day-to-day variation in the

functioning of the concentrator. Moreover, the general type of PM will vary from site to site, with a quite different composition in North Carolina from what would be expected in California, New York, or Europe. The technology does not currently permit exposures to be standardized in terms of mass concentrations, although in theory it might be possible to measure PM concentrations in real time and appropriately dilute the aerosol to make a series of exposures at a standardized, relatively low CAPs concentration.

Immunohistochemical analysis of the bronchial biopsy tissues from subjects exposed to CAPs did not show any increase in mast cells, eosinophils, monocytes (CD14), or neutrophils (Figure 4), or in T cells (Figure 6) compared with tissues from the control subjects. In part this may reflect the wide range of CAPs concentrations that were used; even if there were an effect, it might have been lost at the lower end of the dose range for CAPs exposures. However, we found no evidence of any dose-dependent trend within the biopsy tissue data. Thus, it seems more likely that the lack of response is due either to a general lack of cellular response to CAPs, or to a response that may occur deeper in the lung than the regions that can be reached by bronchoscopic biopsy. Given that we saw no cellular response in these tissues, it is not surprising that we also saw no difference in adhesion molecule expression between the CAPs-exposed and control subjects.

Although it would be interesting to compare the effects of diesel exhaust and CAPs on human airways, two significant methodologic issues make this difficult: (1) achieving comparable dosimetry, and (2) knowing the appropriate timepoints at which to assess human airway responses to diesel exhaust or CAPs, none of which are well established. The two studies described in Parts I and II of this report were freestanding, independently planned studies with distinct hypotheses, different challenge strategies, different subject populations, and different sampling times. Because both exposure laboratories (Umeå, Sweden, where the diesel exhaust exposures were conducted, and Chapel Hill, North Carolina) acquired biopsy samples, we could capitalize on the availability of tissues and apply similar histologic techniques to analyze them; nevertheless, even those data cannot be directly compared between studies.

This work leaves several open questions. First, the epidemiologic studies have assessed morbidity and mortality risks in relation to PM mass concentration. This factor is independent of particle surface area or composition, both of which may be relevant to PM's toxicity. What type or source of PM should, therefore, be studied to try to understand the effects of ambient PM on human health? Second, we need to determine which cellular and molecular endpoints reflect relevant mechanisms that might lead to the outcomes of interest. The mortality risks from PM exposure appear to relate to cardiovascular endpoints; although these endpoints may be the end result of PM inhalation, we may not see any tangible airway changes that account for or lead to the cardiovascular risk. Perhaps the effect the Devlin group noted with fibrinogen is a clue as to the type of phenomenon that may drive the thrombotic and dysrhythmogenic risks likely to underpin the increased risk of cardiac events. Although the present data did not show a convincing dose-response effect on fibrinogen, this observation warrants further investigation. Third, we know that small changes in PM concentration can exacerbate airway disease in people with asthma or chronic obstructive pulmonary disease. Hitherto, studies have concentrated on the acute inflammatory response to PM, but the mechanism of clinical exacerbation may be more subtle. For example, PM could alter the immunologic biology of the airway. If PM exposure, for example, biases the way airway immune cells handle inhaled allergens and microorganisms, this could profoundly alter the outcome of exposure to viruses or allergens to which a person with compromised lungs is sensitized. PM exposure could also alter the function and growth patterns of airway structural cells. This might increase mucus cell numbers or drive increased mucus production, thereby altering both the physiology of the airway and its susceptibility to bacterial infection. Further work should address these and other hypotheses to clarify the nature and scale of the risk to human health from PM.

CONCLUSIONS AND RECOMMENDATIONS

This study has demonstrated that exposure to CAPs has an acute inflammatory effect in healthy subjects, as shown by increased numbers and percentages of neutrophils in BAL fluid and by increases in blood fibrinogen. The increased expression of endothelial adhesion molecules after diesel exhaust exposure (Part I) and the increases in blood fibrinogen after CAPs exposure (Part II) offer some possible clues to the increased cardiovascular morbidity that has been reported in epidemiologic studies associating PM with cardiorespiratory morbidity and mortality. Further work is needed to assess how these responses in healthy subjects relate to the populations who appear to be most at risk from particulate pollution. We also need to address other aspects of PM-induced airway damage if we wish to understand more about the mechanisms that underlie the epidemiologic associations of PM exposure with cardiac and respiratory morbidity and mortality.

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APPENDIX A. Elemental Composition of Particles

Table A.1. Metal Elemental Composition of Particles Determined by X-ray Fluorescence (Devlin's Group)

				Element (ng/cm^2)[a]					
Exposure Group	Subject ID	Date	$PM_{2.5}$ (μg/m^3)	V	Fe	Zn	Ni	Cu	As
Control	233	8-Sep-98	0.0	ND	55	ND	ND	ND	ND
	235	9-Sep-98	0.0	ND	ND	ND	ND	ND	ND
	230	30-Sep-98	0.0	ND	ND	21	ND	ND	ND
	242	6-Oct-98	0.0	ND	102	ND	ND	ND	ND
	246	17-Feb-99	0.0	ND	162	ND	ND	ND	ND
	247	24-Mar-99	0.0	ND	151	ND	ND	ND	ND
	243	14-Dec-98	8.2	ND	727	30	ND	55	ND
	237	15-Dec-98	15.1	ND	898	60	ND	ND	ND
Low CAPs (Tertile 1)	244	8-Dec-98	23.1	ND	108	ND	ND	ND	ND
	241	16-Feb-99	24.8	ND	453	ND	ND	34	ND
	238	23-Sep-98	37.6	ND	513	96	ND	ND	ND
	206	22-Apr-98	39.7	ND	ND	ND	ND	ND	ND
	205	9-Dec-97	39.9	ND	82	7	ND	5	ND
	239	22-Sep-98	52.6	ND	456	65	ND	ND	ND
	219	15-Jul-98	57.0	—	—	—	—	—	—
	216	14-Jul-98	60.5	ND	62	ND	ND	ND	ND
	201	2-Dec-97	66.3	ND	203	17	ND	32	ND
	240	27-Oct-98	70.1	—	—	—	—	—	—
Middle CAPs (Tertile 2)	204	16-Dec-97	72.2	ND	446	17	ND	14	ND
	220	30-Jun-98	73.5	ND	171	ND	ND	ND	ND
	227	19-Aug-98	74.3	ND	660	36	ND	ND	ND
	208	5-May-98	94.7	ND	98	ND	ND	ND	ND
	231	25-Aug-98	107.6	ND	424	71	ND	ND	ND
	209	13-May-98	109.1	ND	119	16	ND	ND	ND
	226	4-Aug-98	120.7	ND	154	15	ND	ND	ND
	229	11-Aug-98	135.9	ND	126	ND	ND	6	ND
	214	3-Jun-98	141.0	ND	123	ND	12	ND	ND
	234	16-Sep-98	148.6	ND	770	156	ND	ND	ND
High CAPs (Tertile 3)	202	18-Nov-97	152.2	—	—	—	—	—	—
	215	2-Jun-98	154.6	ND	97	ND	ND	ND	ND
	212	19-May-98	161.7	ND	288	13	ND	ND	ND
	213	7-Jul-98	168.0	ND	111	19	ND	ND	ND
	221	8-Jul-98	183.3	ND	140	11	ND	ND	ND
	236	15-Sep-98	184.1	ND	755	118	ND	ND	ND
	207	6-May-98	199.6	ND	121	12	ND	ND	ND
	222	28-Jul-98	248.8	ND	88	10	ND	ND	ND
	218	23-Jun-98	303.6	ND	1236	56	ND	ND	ND
	225	29-Jul-98	311.1	ND	61	7	ND	ND	ND

[a] ND = not detected; — = not measured.

Table A.2. Metal and Sulfate Composition of Particles as Determined by Inductively Coupled Plasma–Atomic Emission Spectrometry (Devlin's Group)

				Element (ng/filter)[a]						
Exposure Group	Subjects ID	Date	$PM_{2.5}$ ($\mu g/m^3$)	V	Fe	Zn	Ni	Cu	As	SO_4 (μg)
Control	233	8-Sep-98	0.0	ND	54.3	12.0	0.5	7.8	ND	ND
	235	9-Sep-98	0.0	ND	33.6	6.9	0.2	4.1	ND	0.8
	230	30-Sep-98	0.0	ND	42.6	4.6	0.4	1.1	ND	ND
	242	6-Oct-98	0.0	0.2	29.2	12.6	0.6	3.9	0.2	ND
	246	17-Feb-99	0.0	0.2	ND	9.3	0.4	0.6	ND	0.8
	247	24-Mar-99	0.0	ND	ND	2.9	ND	0.3	0.5	0.0
	243	14-Dec-98	8.2	0.8	19.0	41.3	2.1	9.3	ND	2.3
	237	15-Dec-98	15.1	2.1	14.6	44.1	1.3	6.3	0.5	2.3
Low CAPs (Tertile1)	244	8-Dec-98	23.1	7.6	14.2	15.3	1.6	2.4	ND	13.2
	241	16-Feb-99	24.8	0.9	35.9	68.9	0.8	13.1	ND	3.9
	238	23-Sep-98	37.6	0.7	25.1	81.0	1.2	4.5	0.5	14.7
	206	22-Apr-98	39.7	3.7	45.9	82.4	ND	12.6	2.7	17.6
	205	9-Dec-97	39.9	5.0	14.2	103.8	1.9	75.0	4.2	9.7
	239	22-Sep-98	52.6	1.0	33.3	72.4	1.0	5.5	1.7	21.7
	219	15-Jul-98	57.0	1.6	ND	38.6	1.3	6.6	1.6	13.2
	216	14-Jul-98	60.5	1.6	17.0	60.4	ND	6.6	2.2	5.9
	201	2-Dec-97	66.3	4.0	34.1	52.0	2.4	47.9	0.4	ND
	240	27-Oct-98	70.1	3.9	77.5	100.0	1.2	32.0	3.0	31.7
Middle CAPs (Tertile 2)	204	16-Dec-97	72.2	1.5	26.5	96.8	1.8	42.0	1.4	ND
	220	30-Jun-98	73.5	4.0	41.1	15.9	ND	0.5	0.7	14.3
	227	19-Aug-98	74.3	0.5	ND	59.3	629.7	11.3	2.8	33.3
	208	5-May-98	94.7	0.6	54.0	66.4	ND	20.9	1.4	53.2
	231	25-Aug-98	107.6	2.3	65.5	69.5	1.7	14.1	2.5	54.2
	209	13-May-98	109.1	9.1	225.4	154.4	2.8	19.6	1.8	42.2
	226	4-Aug-98	120.7	17.3	164.1	106.8	8.2	21.5	3.6	54.0
	229	11-Aug-98	135.9	1.8	397.1	48.0	1.3	13.5	1.9	104.4
	214	3-Jun-98	141.0	2.9	164.4	56.4	98.8	10.5	4.3	59.2
	234	16-Sep-98	148.6	3.8	162.5	152.0	1.6	18.5	2.8	87.3
High CAPs (Tertile 3)	202	18-Nov-97	152.2	—	—	—	—	—	—	—
	215	2-Jun-98	154.6	7.3	105.4	97.4	ND	17.1	4.5	62.3
	212	19-May-98	161.7	5.1	202.4	179.4	ND	21.7	5.4	69.7
	213	7-Jul-98	168.0	9.2	144.1	126.8	3.3	40.8	5.0	78.4
	221	8-Jul-98	183.3	11.3	137.1	55.8	2.1	13.7	4.0	103.4
	236	15-Sep-98	184.1	7.8	121.5	105.0	3.2	15.4	4.3	103.0
	207	6-May-98	199.6	2.1	397.4	152.4	0.4	34.3	4.4	175.7
	222	28-Jul-98	248.8	4.4	158.1	79.8	2.2	20.7	5.9	166.4
	218	23-Jun-98	303.6	6.4	409.5	125.0	10.0	24.0	5.3	210.7
	225	29-Jul-98	311.1	3.2	372.4	112.4	ND	22.4	6.6	196.7

[a] ND = not detected; — = not measured.

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ABBREVIATIONS AND OTHER TERMS**

AA1	antibody against protease tryptase
CAPs	concentrated ambient particles
CD terms	surface proteins on T lymphocytes
CD3	all T cells
CD4	T helper and regulatory cells
CD8	T cells with cytotoxic function
CD14	monocytes
CD25	a marker of T-cell activation
EG2	antibody against eosinophil cationic protein
ELISA	enzyme-linked immunosorbent assay
EN4	endothelium clone 4
EPA	Environmental Protection Agency (US)
FEV_1	forced expiratory volume in one second
FVC	forced vital capacity
GMA	glycol methacrylate
GSD	geometric standard deviation
HAPC	Harvard/EPA ambient fine particle concentrator
ICAM-1	intercellular adhesion molecule 1
IL	interleukin
LFA-1	leukocyte function-associated antigen
MMAD	mass median aerodynamic diameter
MOUDI	microorifice uniform-deposit impactor
NE	neutrophil elastase
PEF	peak expiratory flow
PM	particulate matter
$PM_{2.5}$	particulate matter less than 2.5 µm in aerodynamic diameter
Raw	airway resistance
TEOM	tapered element oscillating microbalance
VCAM-1	vascular cell adhesion molecule 1
VLA-4	very late antigen-4

** Refer to the Glossary in the Health Review Committee's Critique for more information about some of these terms.

CRITIQUE
Health Review Committee

INTRODUCTION

Epidemiologic studies have found an association between short-term increases in ambient levels of particulate matter (PM*) and increases in hospital admissions and deaths from cardiovascular and respiratory disorders (Schwartz et al 1993; Dockery and Pope 1994). These studies suggested that individuals with preexisting disease, including patients with asthma, might be more susceptible than healthy individuals to the effects of air pollution. Indeed, numerous studies have found that short-term exposure to particulate pollution exacerbates the symptoms of asthma (eg, Schwartz et al 1993; Burnett et al 1995; Anderson et al 1997; Choudhury et al 1997; Koren and Utell 1997; Lipsett et al 1997; Medina et al 1997) and can decrease lung function in people with asthma, including children (eg, Pope et al 1991; Romieu et al 1996; Gielen et al 1997; Pekkanen et al 1997; Peters et al 1997; Vedal et al 1998).

However, the biologic mechanisms underlying the association between increased levels of ambient PM and increased morbidity and mortality are not well understood. In 1996, HEI issued RFA 96-1, *Mechanisms of Particle Toxicology: Fate and Bioreactivity of Particle-Associated Compounds,* for studies that would improve our understanding of toxicologically relevant characteristics of ambient particles. In response, Stephen Holgate and colleagues at the University of Southampton, United Kingdom (UK), proposed to study and compare the effects of diesel exhaust and concentrated ambient particles (CAPs) on inflammatory markers in the lungs of healthy subjects and subjects with asthma. The proposed project was a collaboration between Dr Holgate, Dr Thomas Sandström at the University of Umeå, Sweden, and Dr Robert Devlin at the US Environmental Protection Agency in Research Triangle Park, North Carolina. Healthy and asthmatic subjects would be exposed to diesel exhaust by Sandström in Sweden in a study funded by the Swedish Government; healthy subjects would be exposed to CAPs by Devlin in North Carolina in a study at the US Environmental Protection Agency. Lung tissue samples from subjects in both studies would be analyzed for inflammatory markers by Holgate and colleagues in the UK. The proposal from Holgate requested HEI funding only for the lung tissue analyses performed in his laboratory.†

HEI funded the proposal because the Research Committee thought that assessing inflammatory endpoints in lung tissues after controlled human exposures might provide important information about a possible mechanism for PM's effects on the respiratory systems of both healthy and asthmatic people. Although the protocols for the two studies differed in many ways, the Committee hoped that comparative data on the effects of particles of different composition could be obtained. Part I of the Investigators' Report describes the effects of diesel exhaust on inflammatory markers in tissues from both healthy and asthmatic subjects. Part II describes the effects of CAPs on inflammatory markers in tissues from healthy subjects. Each Part of the Investigators' Report includes a description of the exposure protocols and the results of the analyses of lung function, lavage fluids, and blood parameters to provide a context for the Holgate group's analyses of bronchial tissues. This Critique is intended to aid HEI sponsors and the public by highlighting the strengths of the studies, pointing out alternative interpretations, and placing the findings into scientific perspective.

BACKGROUND

One of the working hypotheses for a mechanism of action by which particles cause adverse health effects is that particles, upon deposition in the airways, trigger a cascade of inflammatory events, in which several types of white blood cells (or leukocytes [see Glossary]) are activated and migrate from the blood into the airways. As a consequence of these inflammatory events, PM may exert its effects on the cardiovascular and respiratory systems via changes in blood coagulation (Seaton et al 1995).

Lung inflammation is the body's response to foreign agents (eg, bacteria, viruses, particles) that have entered lung tissue. Inflammation may occur with or without acute injury to the lung epithelium and is manifested by increases

*A list of abbreviations and other terms appears at the end of the Investigators' Report.

† Dr Holgate's eighteen-month study, *Inflammatory Mechanisms with Exposure to Air Pollution Particles*, began in January 1998. Total expenditures were $242,796. The draft Investigators' Report from Dr Holgate and colleagues was received for review in January 2000. Part I of a revised report, on the effects of diesel exhaust, was received in June 2000 and accepted for publication in February 2001. Part II of the revised report, on the effects of CAPs, was received in July 2002 and accepted for publication in September 2002. During the review process, the HEI Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in both the Investigators' Report and in the Review Committee's Critique.

GLOSSARY

Adhesion molecule: Molecule that mediates the binding of one cell to other cells, or to integrins or selectins. Adhesion molecules are important in the operation of the immune system. Examples: Intercellular adhesion molecules (ICAMs), vascular adhesion molecules (VCAMs).

Airway resistance (Raw): A measure defined as the driving pressure of air through the airways divided by the airflow rate. An increase in airway resistance is associated with bronchoconstriction (narrowing of the airways).

Albumin: A protein that makes up 35–50% of the total serum protein in humans and most animals. It serves several functions, including maintenance of the blood volume within blood vessels and balancing fluids and salts in the plasma.

B lymphocyte (B cell): A cell derived from bone marrow that expresses immunoglobulin on its surface as an antigen-specific receptor. It is the precursor of the plasma cell, which produces antibody.

Basophil: A polymorphonuclear leukocyte that contains granules that stain with basic dyes. Basophils are thought to have a function similar to mast cells. They are a rich source of vasoactive mediators, such as histamine and platelet activating factor. Additionally, they produce chemokines that activate neutrophils and eosinophils.

CD molecules: Nomenclature for proteins expressed on the surface of cells. The expression of one or more of these molecules can be used as a marker to identify a particular cell or set of cells (see below). Cells that express a particular surface molecule are designated *positive* (eg, $CD3^+$). The expression pattern of the CD molecules in the current study is:

- **CD3:** All T lymphocytes.
- **CD4:** The subset of T cells usually referred to as T helper cells, and includes T regulatory cells. Mature $CD4^+$ T cells are also $CD3^+$ but $CD8^-$.
- **CD8:** The subset of T cells with cytotoxic function. Mature $CD8^+$ cells are also $CD3^+$ but $CD4^-$.
- **CD14:** Monocytes.
- **CD19:** B lymphocytes.
- **CD25:** The α chain of the interleukin (IL)-2 receptor; a marker of T-cell activation.

Chemokines: Small cytokines of relatively low molecular weight, released by a variety of cells, and involved in the migration and activation of phagocytic cells and lymphocytes through chemotaxis (chemical attraction). Chemokines have a central role in inflammatory responses.

Complement: A series of serum proteins involved in the removal of infectious organisms. Activation occurs in a coupled sequence ("complement cascade") and generates fragments with multiple activities. Examples: C3a, C5a.

Cytokines: Soluble substances secreted by cells, which have a variety of effects on other cells. Examples: Interleukins (ILs), interferons, transforming growth factor-β, tumor necrosis factor (TNF) α, granulocyte-macrophage colony–stimulating factor (GM-CSF).

Endothelium: A thin layer of epithelial cells that lines blood and lymph vessels.

Enzyme-linked immunosorbent assay (ELISA): An assay in which the conversion of a colorless substrate to a colored product by an enzyme linked to an antibody is a measure of bound enzyme and, hence, the amount of bound antibody.

Eosinophil: White blood cell thought to be important in defense against parasitic infections. It plays a major role in allergic and asthmatic responses.

Eosinophilic cationic protein (ECP): A protein that is found in the granules of eosinophils.

Epithelium: Membranous tissue composed of one or more layers of cells covering most internal and external surfaces of the body.

Extracellular superoxide dismutase (EC-SOD): An antioxidant enzyme that scavenges superoxide, one of the reactive oxygen species that are associated with inflammation.

Forced expiratory volume in one second (FEV_1): The volume exhaled during the first second of a forced expiration following maximal inspiration.

Forced vital capacity (FVC): The total volume of air expelled from the lungs following maximal inspiration.

Granulocyte: A leukocyte that contains cytoplasmic granules. **See polymorphonuclear leukocyte**.

Granulocyte-macrophage colony–stimulating factor (GM-CSF): A cytokine that promotes growth of granulocytes and macrophages.

Growth related oncogene (GRO): A chemokine found predominantly in activated monocytes but also expressed in endothelial cells and several tumor cell lines. The expression of the GRO gene is induced by interleukins (ILs) and tumor necrosis factor (TNF).

Hemoglobin (Hb): The oxygen-carrying pigment of the red blood cells.

Inflammation: An acute or chronic response to tissue injury or infection involving accumulation of leukocytes, plasma proteins, and fluid.

Integrins: A family of cell-surface adhesion molecules found on leukocytes. They are important in leukocyte migration into tissues. Examples: Leukocyte function–associated antigen-1 (LFA-1), very late antigen-4 (VLA-4).

Intercellular adhesion molecules (ICAMs): Adhesion molecules on the surface of several cell types, including T cells that interact with integrins.

in the numbers of certain types of leukocytes in lung tissue or lung fluids (eg, macrophages, neutrophils, eosinophils, T lymphocytes, and B lymphocytes). Recruitment of cells into the lung is promoted by vascular adhesion molecules: Expression of these molecules by cells in the endothelium slows down cells in the blood, helps them attach to the endothelium, and allows them to pass through the endothelium into the lung tissue. Polymorphonuclear leukocytes are the first type of leukocyte to move into the lung and physically remove particles from the airways. During recruitment into the lung and subsequent stages of the inflammatory process, activated leukocytes release cytokines and chemokines, which facilitate communication among cells. One method for establishing whether inflammation has developed is to measure the numbers of leukocytes and the levels of adhesion molecules, cytokines, and chemokines in lung fluid and lung tissue.

Different sets of cytokines are secreted by different subsets of $CD4^+$ T lymphocytes. The T_H1 subset synthesizes cytokines (such as interferon-γ [IFN-γ] and tumor necrosis factor α [TNF-α]) that activate macrophages and cytotoxic T cells ($CD8^+$), some of the effector cells of cell-mediated immunity. The T_H2 subset synthesizes cytokines such as interleukin (IL)-4, which switches B cells to produce immunoglobulin E, and IL-5, which activates eosinophils. T_H2 cells and cytokines are involved in the response to allergens and are believed to play a role in asthma (Barnes 2001). Diesel exhaust has been shown to enhance T_H2-type responses under certain conditions (Diaz-Sanchez et al 1996; Bayram et al 1998a,b; Miyabara et al 1998; Nel et al 1998).

Evidence that exposure to PM triggers inflammatory events has been found in studies using lung cells in vitro, in animal studies, and in human controlled-exposure studies. For example, in vitro studies using human or rat

Interferons (IFNs): A group of proteins (IFN-α, -β, -γ) having antiviral activity and capable of enhancing and modifying the immune response. IFN-γ activates macrophages and inhibits the function of the T_H2 subset of $CD4^+$ T cells.

Interleukins (ILs): Cytokines secreted by a variety of leukocytes that have effects on other leukocytes. At least 18 different types have been characterized.

Leukocyte: White blood cells, comprising monocytes/macrophages, lymphocytes and polymorphonuclear cells.

Leukocyte function–associated antigen (LFA): An adhesion molecule on the T cell surface, which is a heterodimer of surface molecules CD11a and CD18.

Ligand: A molecule or part of a molecule that binds to a receptor.

Lymphocytes: Bone marrow-derived cells that express antigen-specific receptors. The major subsets are known as B and T cells. Small leukocytes with virtually no cytoplasm, found in blood, tissues, and lymphoid organs such as lymph nodes and the spleen.

Macrophage: Large leukocyte found in tissues, which is derived from a blood monocyte. Macrophages ingest (phagocytose) foreign particles.

Macrophage inflammatory protein (MIP): A chemokine that attracts monocytes, T cells, basophils and dentritic cells.

Mast cell: A bone marrow-derived granule-containing cell found in connective tissues. Activated mast cells release mediators such as histamine and cytokines and play a major role in allergic responses.

Methylhistamine: A major metabolite of histamine, which is released by mast cells during inflammation. Histamine opens endothelial cell junctions and upregulates adhesion molecules.

Monocyte: A phagocytic leukocyte found in the blood. Monocytes are precursors to tissue macrophages.

Monocyte chemotactic protein (MCP): A chemokine that attracts monocytes, T cells, basophils and dendritic cells. MCP activates macrophages and stimulates basophil histamine release.

Myeloperoxidase (MPO): A lysosomal enzyme that is critical to the oxygen-dependent killing of bacteria by phagocytic leukocytes.

Neutrophil: This is the most numerous granulocyte. The name stems from the relatively neutral color that its granules have upon staining. Neutrophils form in bone marrow and are fully mature when released into the circulation. They function in the first line of cellular defense by ingesting foreign objects.

Neutrophil elastase (NE): A protease produced by neutrophils during inflammation.

Nuclear factor-κB (NF-κB): A transcription factor that regulates the expression of several genes involved in the immune response.

Polymerase chain reaction (PCR): A technique that produces large amounts of DNA from a sequence by repeated cycles of synthesis.

Polymorphonuclear leukocyte (PMN): A subset of leukocytes (white blood cells) also known as granulocytes. PMNs, which include neutrophils, eosinophils, basophils, and mast cells, contain characteristic cytoplasmic granules and a multilobed nucleus.

Protease: An enzyme that breaks down proteins.

Radioimmunoassay (RIA): A widely used technique for measuring the level of a biological substance in a sample, by measuring the binding of antigen to radioactively labeled antibody (or vice versa).

RANTES (Regulated on Activation, Normal T cell Expressed, and presumably Secreted): A chemokine that attracts monocytes, T cells, basophils and eosinophils. RANTES degranulates basophils and activates T cells.

Receptor: Generally a transmembrane molecule, which binds to a ligand on the exterior surface of the cell, leading to biochemical changes inside the cell.

Selectins: A family of cell-surface adhesion molecules found on leukocytes and endothelial cells. Examples: E-selectin, P-selectin.

T helper cells (T_H): T cells required for B cells to make antibodies in response to most antigens.

- **T_H1:** A subset of $CD4^+$ T cells that synthesizes the cytokines IL-2, IL-3, IFN-γ, tumor necrosis factor β (TNF-β), and GM-CSF. These cytokines activate macrophages and $CD8^+$ T cells.
- **T_H2:** A subset of $CD4^+$ T cells that synthesizes the cytokines IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, and GM-CSF. These cytokines predominate in the response to allergens and parasites (eg, eosinophil activation).

T lymphocyte (T cell): The set of lymphocytes that requires the thymus for differentiation.

Transforming growth factor β (TGF-β): A cytokine that inhibits activation of monocyte and T-cell subsets.

Tryptase: A protease that is used as a marker for mast cells. Tryptase inactivates bronchodilating peptides and thereby may increase airway tone in allergic asthma. It also may prevent deposition of fibrin and act as an extravascular anticoagulant. In the current study, the antibody **AA1** was used in immunocytochemical assays to detect mast cell tryptase.

Tumor necrosis factor α (TNF-α): A cytokine with various actions including the selective killing of tumor cells.

Vascular cell adhesion molecules (VCAM): Adhesion molecules on the surface of blood vessels.

Western blotting: A technique to identify a specific protein in a mixture. Proteins separated by gel electrophoresis are blotted onto a nitrocellulose membrane; the protein of interest is detected by adding radiolabeled antibody specific for the protein.

macrophages grown in cell cultures have shown increased production of several cytokines after the cells were exposed to a wide variety of particles, such as suspended ambient urban PM (Becker et al 1996), coal mine dust (Gosset et al 1991), asbestos fibers and silica particles (Dubois et al 1989), titanium oxide and iron oxide particles (Driscoll and Maurer 1991; Warheit and Hartsky 1993; Geiser et al 1994; Oberdörster 1995), diesel particles (Yang et al 1997), or oil fly ash (Killingsworth et al 1997). Whereas these results indicate a possibility of inflammation occurring after exposure to particles, they need to be confirmed in vivo.

In fact, inflammation has been reported from studies with rodents after chronic intratracheal instillation of diesel or carbon black particles (Mauderly et al 1994; Nikula et al 1997), and after acute pulmonary instillation of oil fly ash (Dreher et al 1997; Killingsworth et al 1997; Kodavanti et al 1997), diesel particles (Sagai et al 1996; Ichinose et al 1997; Takano et al 1997) and urban particles collected from filters (Li et al 1997). However, the inflammation observed after instillation of high concentrations of particles may not be representative of effects of inhalation of much lower ambient levels of particles (Jakab et al 1990; Oberdörster 1995; Warheit et al 1997). Fewer studies have investigated the effects of lower doses of inhaled particles in laboratory animals; in one study, chronic inhalation of urban particles, diesel exhaust, or coal dust was shown to cause inflammation in rat lungs (Nikula et al 1997). In another study, ozone exposure followed by acute inhalation of urban particles was shown to enhance the inflammatory response in rat lungs (Vincent et al 1997).

Controlled human exposure studies to air pollutants that obtained bronchoalveolar or nasal lavage fluid have investigated single compounds or mixtures; for example, ozone

(Aris et al 1993b; Balmes et al 1996), nitric acid with or without ozone (Aris et al 1993a), magnesium oxide particles (Kuschner et al 1997), diesel exhaust (Rudell et al 1996), and diesel exhaust particles (Blomberg et al 1998). In these human controlled-exposure studies, endpoints measured included inflammatory markers (such as leukocyte numbers and levels of cytokines, chemokines, and adhesion molecules in lavage fluid or peripheral blood) as well as measurements of lung function. (Usually, lung function is monitored before and after exposure to assess whether exposure to air pollutants causes bronchoconstriction.) More recently, technical advances in generating particle exposures and reliably and safely performing lung biopsies in healthy volunteers or people with mild airway disease have enabled investigators to study inflammatory responses to PM in humans more directly.

At the time Holgate submitted his proposal to HEI, an instrument capable of concentrating ambient particles had recently been developed (Sioutas et al 1997). For the first time, the particle concentrator offered an opportunity to conduct human controlled-exposure studies with an aerosol that closely resembled ambient air but at higher concentrations. Devlin's group at the US Environmental Protection Agency initiated a series of studies in which volunteers were exposed to CAPs (Ghio et al 2000; Harder et al 2001). Holgate and colleagues proposed a collaboration in which tissue samples obtained via biopsy by Devlin would be analyzed for inflammatory markers in the UK. The results of this collaboration are described in Part II of the Investigators' Report.

Because air pollution has been associated with aggravation of symptoms in patients with pulmonary disease, several studies have investigated the effect of exposure to low concentrations of air pollutants in people with reduced lung function such as those who smoke (Frampton et al 1995) and those with asthma (Yang and Yang 1994; Scannell et al 1996; Torres et al 1997). Asthma is a chronic disease of the lower airways characterized by inflammation, decreased lung function, mucus hypersecretion, and airway hyperresponsiveness. Allergic asthma is associated with T_H2 cytokines (such as IL-4, IL-5, and IL-10), which trigger immunoglobulin E production by B cells and the recruitment of eosinophils into the airways (for a review see Chung and Barnes 1999). The epidemiologic association between ambient PM levels and asthma exacerbation (see, eg, Gielen et al 1997; Peters et al 1997; Timonen and Pekkanen 1997) suggests a possible role for inflammatory mechanisms and the need for studying mechanisms of action. Considerable effort has been devoted recently to studying the effects of air pollution on lung function and inflammation in subjects with asthma (Anderson et al 1992; Yang and Yang 1994; Diaz-Sanchez et al 1996; Devalia et al 1999), often in combination with allergen exposure (eg, Nel et al 1998; Higgins et al 2000; Svartengren et al 2000).

In an earlier collaborative study, Holgate, Sandström, and colleagues found differences in inflammatory endpoints between healthy volunteers who were exposed for 1 hour to diesel exhaust (300 $\mu g/m^3$ particle concentration) and those exposed for 1 hour to clean air (Salvi et al 1999a, 2000). The present study was aimed at using a lower concentration of diesel exhaust and extending the investigation to subjects with asthma. These results are described in Part I of the Investigators' Report.

STUDY GOALS AND APPROACH

Holgate and colleagues investigated whether ambient PM (especially particles smaller than 2.5 µm in aerodynamic diameter [$PM_{2.5}$]) has the capacity to produce toxic effects by promoting an inflammatory cascade that may start in the lung epithelium and spread to other cell types of the airways and blood vessels in the lung.

The specific objectives were:

1. To measure the effects of diesel exhaust (100 $\mu g/m^3$ particle concentration) on inflammatory markers in lung lavage fluid and bronchial tissues from healthy and asthmatic subjects (Part I).
2. To measure the effects of CAPs (concentrations ranged from 23 to 311 $\mu g/m^3$) on inflammatory markers in lung lavage fluid and bronchial tissues from healthy subjects (Part II).

Lung fluids (from bronchial wash [BW] and bronchoalveolar lavage [BAL]) and bronchial tissues (from lung biopsies) were obtained through fiberoptic bronchoscopy at 6 hours (diesel study) or 18 hours (CAPs study) after exposure. The technique of bronchoscopy and the differences between BW and BAL are briefly described in the sidebar. The tissue samples were treated, fixed, and subsequently sent to Holgate's laboratory for analysis.

The diesel and CAPs studies included assessment of lung function immediately before and after 2 hours of exposure to particles or to clean air. Lung function measures reported for both studies were forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), and airway resistance (Raw). The CAPs study also reported peak expiratory flow (PEF).

Holgate and colleagues assessed cell numbers and several inflammatory mediators (see Glossary) either as protein levels or messenger RNA (mRNA) expression. They can be grouped as follows:

1. Numbers of leukocytes recruited from the circulation into the lungs. Specific endpoints included numbers of monocytes ($CD14^+$ cells), neutrophils, eosinophils, mast cells, and B ($CD19^+$) and T ($CD3^+$) lymphocytes and T lymphocyte subsets ($CD4^+$, $CD8^+$, and $CD25^+$ cells).
2. Levels of vascular adhesion molecules as markers of endothelial activation. The investigators measured P-selectin, E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which are sequentially expressed.
3. Levels of cytokines and chemokines. The investigators measured the cytokines IL-1β, IL-5, IL-6, IL-10, IL-13, TNF-α, GM-CSF, and IFN-γ and the chemokines IL-8 and RANTES.
4. Other endpoints such as levels of the transcription factor NF-κB, myeloperoxidase, eosinophilic cationic protein (EG2), methylhistamine, extracellular superoxide dismutase, tryptase (AA1), neutrophil elastase, growth-related oncogene-α (GRO-α), total protein and albumin were also assessed.

QUANTIFICATION OF INFLAMMATORY ENDPOINTS

Cell numbers and levels of inflammatory markers were assessed in BW and BAL fluids by the Sandström and Devlin groups. A small amount of fluid was used for a total cell count; the remainder was centrifuged to separate cells from fluid. Portions of resuspended cell pellet were used for a differential cell count, in which numbers of alveolar macrophages, lymphocytes, neutrophils, eosinophils, and other leukocytes were determined. Levels of soluble components in the supernatant were quantified by radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA).

Fixed biopsy tissues from both the Sandström and Devlin groups were embedded in plastic and shipped to Holgate's laboratory, where they were sliced and immunocytochemically stained with antibodies to proteins or analyzed for messenger RNA. Cells were stained and counted per length of epithelium or area of submucosa, using computer-assisted image analysis. Adhesion molecules were stained and the number of positively stained blood vessels was quantified as the percentage of total vessels. Cytokines were stained with monoclonal antibodies and stained cells were expressed as a percentage of the area of epithelial tissue. In addition, mRNA levels in bronchial tissue were quantified using a reverse transcription–polymerase chain reaction (RT-PCR)-ELISA method developed in Holgate's laboratory to provide semiquantitative measurements of mRNA in small tissue samples (Salvi et al 1999b).

OBTAINING CELL AND TISSUE SAMPLES BY FIBEROPTIC BRONCHOSCOPY

Fiberoptic bronchoscopy is a technique used in clinical investigations to obtain samples of lung fluid and lung tissue from healthy volunteers or patients with mild airway disease (American Thoracic Society 1990; Liu and Calhoun 1998). Under local anesthesia, a bronchoscope is lowered into the airways via the mouth and wedged into the third or fourth generation of branching bronchi. *Bronchoalveolar lavage* (BAL) is performed by instilling and withdrawing multiple small volumes (50–60 mL) of saline through the bronchoscope. In total, 100 to 250 mL of fluid may be recovered. BAL allows the recovery of both cellular and noncellular components from the epithelial surface of the lower respiratory tract. It has been estimated that approximately 1.5 to 3.0% of the lung and approximately one million alveoli are sampled. *Bronchial wash* (BW) is a similar sampling technique that collects cells and fluid from the large airways. Both BAL and BW may be performed during one bronchoscopy.

In airway *biopsies*, a small section of tissue (1–2 mm in diameter) is removed from the bronchial lining using forceps. The sampling locations can vary among the more easily accessible upper airways and the less accessible lower airways. Biopsies are performed after the lavage has been completed to avoid excess blood in the lavage fluid.

The exact location of cells or markers in epithelium or interstitium may be determined using biopsy tissues. Whereas BAL and BW collect cells and soluble components limited to the airway side of the epithelial barrier, the biopsy collects a tissue sample that includes both airway and vascular sides. Both techniques provide important and complementary information about cell numbers and levels of inflammatory markers in the airways.

ADDITIONAL MEASUREMENTS

Several additional endpoints were assessed in lavage fluid and peripheral blood at the laboratories in Sweden (diesel exposure) and the United States (CAPs exposure). The diesel study measured hemoglobin, leukocytes, neutrophils, lymphocytes and monocytes in blood. The CAPs study measured coagulation-related endpoints (such as red blood cells, hemoglobin, hematocrit, platelets, and fibrinogen) as well as ferritin. The results on lung function and blood endpoints in the CAPs study were previously published (Ghio et al 2000) and have been included in Part II of the Investigators' Report.

TECHNICAL EVALUATION OF PART I: DIESEL EXHAUST

BRIEF OVERVIEW OF THE DIESEL STUDY

At the laboratory of Sandström and colleagues, 25 healthy subjects and 15 asthmatic subjects were exposed for 2 hours to diesel exhaust (100 $\mu g/m^3$ particle concentration) and to filtered air on separate days. The design of this study was identical to their previous study in which subjects were exposed to diesel exhaust (300 $\mu g/m^3$ particle concentration) for 1 hour (see Salvi et al 1999a, 2000). Subjects were nonsmokers between 19 and 42 years old. Asthmatic subjects had mild atopic asthma with a positive skin test for one or more common allergens. For the exposure, exhaust from an idling Volvo diesel engine (TD1F Intercooler, 6-cylinder 4-stroke direct-injection turbo-charged, model 1990) using low-sulfur fuel was diluted and routed to an exposure chamber in which one subject at a time was exposed. Conditions in the current study were identical, except for a secondary dilution of the diesel exhaust with ambient air to achieve the lower concentration and longer exposure duration. During exposure, subjects exercised on a stationary bicycle for 15 minutes alternating with 15-minute rest periods. Subjects were exposed twice, once to diesel exhaust and once to filtered air, in random sequence. On control days the diesel engine was running but fumes were vented outside instead of into the exposure chamber and filtered air was delivered to the chamber.

Lung function was assessed before and immediately after exposure; bronchoscopy to obtain lung fluid and bronchial tissue was performed 6 hours after the end of exposure. Six hours was chosen because it correlates with changes in inflammatory markers in asthmatic subjects challenged with allergens in other studies. Blood sampling and nasal lavage were conducted before, during, immediately after, and 6 hours after exposure.

SUMMARY OF KEY RESULTS

Holgate, Sandström, and colleagues report that immediately after a 2-hour exposure to diesel exhaust an increase in airway resistance, an indication of bronchoconstriction, was observed in both healthy and asthmatic subjects compared with filtered air exposure. No other changes in lung function were observed after exposure. However, asthmatic subjects were reported to have an increased baseline airway resistance. Of the many biochemical measures assessed 6 hours after exposure in blood, BW fluid, BAL fluid, and lung tissue, some measures were significantly changed in healthy subjects but not in asthmatic subjects. However, asthmatic subjects did have inflammation representative of asthma before exposures started. An overview of statistically significant changes after exposure to diesel exhaust is presented in the Critique Table and summarized here.

In *healthy subjects*, the percentage of neutrophils was increased in BW fluid and the percentage of lymphocytes was increased in BAL fluid at 6 hours after exposure to diesel exhaust. The percentage of macrophages was decreased in both BW and BAL fluids. In lung tissue, levels of the cell adhesion molecules VCAM-1 and P-selectin were increased. In addition, IL-6 and IL-8 levels were increased in BW fluid. mRNA for IL-8 indicating IL-8 synthesis was also increased in tissue biopsies. Levels of other molecules or their mRNA were unchanged (eg, IL-5, IL-10, TNF-α, NF-κB, and $CD4^+$ T lymphocytes). These results indicate some inflammatory changes in lungs of healthy subjects after exposure to diesel exhaust, notably in IL-6, IL-8, and cellular adhesion molecules.

In *asthmatic subjects*, none of the changes in levels of inflammatory mediators found in healthy subjects after exposure to diesel exhaust were observed. However, other changes occurred, such as a decrease in the percentage of eosinophils in BW fluid and submucosal lung tissue, and an increase in IL-10 levels in lung epithelium. Whereas asthmatic subjects did not show increased inflammation after exposure to diesel exhaust, they did show baseline inflammation after exposure to filtered air, compared with healthy subjects. For asthmatic subjects, the authors reported lower baseline levels of neutrophils and soluble ICAM-1 in BW fluid; lower baseline levels of ICAM-1, IL-10 protein, and $CD3^+$ cells in bronchial tissue; higher baseline levels of mast cells, eosinophils, macrophages, eosinophilic cationic protein, and methylhistamine in BW fluid; and higher baseline levels of VCAM-1, TNF-α, and eosinophils in bronchial tissue compared with healthy subjects. Presumably, these differences reflect the increased inflammatory status of the airways in people with asthma.□

DISCUSSION

This study was the result of an international collaboration in which human subjects were exposed to diesel exhaust in Sweden and lung tissues and fluids were provided for detailed analysis to investigators in the United Kingdom. The Swedish investigators have conducted several human controlled-exposure studies using gaseous pollutants (such as nitric oxide) and particle-plus-gas atmospheres (specifically, diluted diesel exhaust). The investigators in the United Kingdom have conducted extensive work on lung inflammation and asthma. This study was among the first to analyze lung tissues obtained via bronchial biopsies in human subjects exposed to diesel exhaust.

Critique Table. Key Results: Comparison of Current and Previous Studies[a]

	Earlier Collaboration Between Holgate and Sandström Groups[b]	Current Study Part I: Holgate and Sandström Groups		Current Study Part II: Holgate and Devlin[c] Groups
Exposure atmosphere	Diesel exhaust[d]	Diesel exhaust[d]		CAPs[e]
Particle mass	300 µg/m^3	100 µg/m^3		23–311 µg/m^3
Exposure duration	1 hour	2 hours		2 hours
Subjects	Healthy	Healthy	With Asthma	Healthy
Lung Function[f]				
Airway resistance	NM	↑	↑	=
Markers of Lung Inflammation [g,h]				
Cell Numbers				
Neutrophils in BW[i]	↑	↑	=	↑
Eosinophils in BW	NM	=	↓	NM
Eosinophils in biopsy tissues[j]	=	=	↓	=
Macrophages in BW	↑(trend)	↓	=	↓
Macrophages in BAL[i]	NM	↓	=	=
Lymphocytes in BAL	↑	↑	=	=
T lymphocytes (CD3$^+$) in biopsy tissues[j]	↑	↓	=	↓
Cytokines				
IL-6 in BW	NM	↑	=	=
IL-8 in BW	NM in BW; = in BAL	↑	=	=
IL-8 mRNA in biopsy tissues[j]	↑	↑	=	NM
IL-10 in biopsy tissues[j]	NM	=	↑	NM
Adhesion molecules				
VCAM-1 in biopsy tissues[j]	↑	↑	=	=
P-selectin in biopsy tissues[j]	=	↑	=	=

[a] An arrow indicates a significant change in the positive (↑) or negative (↓) direction; an = means no change compared with exposure to filtered air; NM means not measured.

[b] As reported in Salvi et al 1999a, 2000.

[c] As reported in Ghio et al 2000.

[d] In the two diesel exhaust exposure studies, all subjects were exposed twice: once to filtered air and once to diesel exhaust. The exposure conditions were identical except for the exhaust concentrations and the exposure durations.

[e] In the CAPs exposure study, subjects were exposed only once to either CAPs or to filtered air.

[f] Lung function was measured immediately after exposure in all studies.

[g] All biochemical parameters were assessed in fluids or tissues acquired either 6 hours after exposure (diesel exhaust studies) or 18 hours after exposure (CAPs study).

[h] In addition to the results presented in the table, the following endpoints were significantly increased in the earlier diesel exhaust study (Salvi et al 1999a, 2000) but not in the current diesel exhaust study: In BAL fluid—methylhistamine and fibronectin; in biopsy tissues—neutrophils, mast cells, CD4$^+$ cells, CD8$^+$ cells, IL-5 mRNA (upward trend), IL-8 mRNA, endothelial ICAM-1, LFA-1$^+$, and VLA-4$^+$ (upward trend); in bronchial epithelium—GRO-α. In the CAPs study, blood fibrinogen was significantly increased.

[i] For a description of BW and BAL, see sidebar (Obtaining Cell and Tissue Samples by Fiberoptic Bronchoscopy).

[j] Holgate studies funded by HEI.

Inflammatory Markers and Lung Function in Healthy Subjects

The investigators assessed a large number of inflammatory markers that are relevant to airway inflammation associated with lung disease or exposure to irritants, pollutants, or allergens. They noted some changes in inflammatory endpoints that were similar to those found in their previous study, which used a higher concentration of diesel exhaust (300 μg/m^3 particle concentration for 1 hour; Salvi et al 1999a, 2000). The most consistent results were found with the cytokine IL-8, for which increased protein and mRNA were observed. IL-8 plays a role in chemotaxis and activation of neutrophils. In addition, increased percentages of neutrophils in BW fluid and lymphocytes in BAL fluid were observed in both studies, as well as increased VCAM-1 expression in tissue (Critique Table).

Some results also differed between the two studies. For instance, in the previous study the investigators reported increases in neutrophils, mast cells, $CD4^+$ and $CD8^+$, T lymphocyte subsets, ICAM-1, and leukocyte function–associated antigen-1 (LFA-1) in bronchial tissues (Salvi et al 1999a), and increased GRO-α protein expression in bronchial epithelium (Salvi et al 2000). Exposure-related changes in these endpoints were not found in the current study.

These findings are of potential interest, but some caution in interpretating the results is warranted. The magnitude and consistency of changes in inflammatory markers shown in the current study compared with other studies is modest; because many markers were studied and few changed, these changes may have occurred by chance. It is possible that the more modest response in the current study is due in part to the lower concentration of diesel exhaust, which (taking into account the longer exposure duration) provided two-thirds of the effective diesel dose of the first study.

The change in airway resistance but not in FEV_1 or FVC in the current study confirms findings from two earlier studies. Salvi and colleagues (1999a) observed no changes in FEV_1 or FVC, whereas Rudell and colleagues (1996) observed a change in airway resistance and specific airway resistance (ie, corrected for lung volume). Information on specific airway resistance was not provided in the current study.

Since the start of this project, several studies have investigated the health effects of diesel exhaust in cell cultures, animals, and human subjects (reviewed by Sydbom et al 2001). For example, in vitro studies using human bronchial epithelial cells or lung slices have reported increases in the expression of ICAM-1 (Bayram et al 1998a; Takizawa et al 2000), the cytokines IL-1β, IL-6, IL-8, granulocyte-macrophage colony–stimulating factor, and TNF-α (Bayram et al 1998b; Ohtoshi et al 1998; Steerenberg et al 1998; Boland et al 1999; Abe et al 2000; Le Prieur et al 2000), and the transcription factor NF-κB (Baeza-Squiban et al 1999; Takizawa et al 1999). Using intranasal challenge with diesel exhaust particles and allergens, Diaz-Sanchez and colleagues (1996) found increases in gene transcription for IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, and IFN-γ in nasal lavage fluid from healthy subjects.

Two recent studies assessed inflammatory markers in induced sputum in healthy human subjects exposed to diesel exhaust (Nordenhäll et al 2000) or diesel exhaust particles (Nightingale et al 2000). Nordenhäll and colleagues exposed healthy subjects for 1 hour to filtered air or diesel exhaust (300 μg/m^3 particle concentration) in a crossover design and reported increases in the percentage of sputum neutrophils, IL-6, and methylhistamine. No changes were observed in eosinophilic cationic protein, GRO-α, IL-8, myeloperoxidase, or TNF-α. Nightingale and colleagues exposed healthy subjects for 2 hours to filtered air or 200 μg/m3 diesel exhaust particles in a crossover design and reported an increase in sputum neutrophils and myeloperoxidase, as well as in levels of exhaled carbon monoxide; no changes were observed in cardiovascular endpoints, lung function, or inflammatory markers in blood.

In summary, many studies have found that diesel exhaust or diesel exhaust particles induce changes in inflammatory markers in nasal and lung lavage fluids in humans and other species and in cell cultures. A consistent pattern has not emerged, however, possibly due to differences in experimental approaches and study design, methods of exposure, and measurement of different sets of inflammatory markers among studies. There are many markers of inflammation, which may show changes at different time intervals after exposure. The current study assessed early changes, at 6 hours after the end of exposure. Other studies, including the study described in Part II of the Investigators' Report, have investigated inflammatory responses that occur later, for example, 24 hours after exposure (eg, Dye et al 1999).

Inflammatory Markers and Lung Function in Asthmatic Subjects

The lack of changes in inflammatory endpoints observed in asthmatic subjects exposed to diluted diesel exhaust compared with healthy subjects in the current study is surprising. It is difficult to reconcile the lack of response in asthmatic subjects with the observation that short-term exposure to particulate air pollution causes aggravation of asthma, that is, increased number of attacks and hospitalizations on high-pollution days. A few changes were observed in the asthmatic subjects: They had decreases in lung function and in eosinophils in lung fluids and tissues, and an increase in IL-10 levels in lung tissues. IL-10 is a potent

inhibitor of macrophage function, suppressing the production of many proinflammatory cytokines. Lower production of IL-10 has been reported in alveolar macrophages obtained from people with atopic asthma (Chung 2001). IL-10 was not measured in any of the previous human exposure studies, but has increasingly been seen as a potential target for asthma treatment options (Barnes 2001). These findings warrant further research.

The investigators reported several differences in baseline levels of inflammatory markers between asthmatic and healthy subjects, presumably reflecting changes in the lungs of asthma patients due to chronic mild inflammation (Holgate et al 2000). These subjects had mild asthma symptoms, characterized by a slight decrease in lung function (FEV_1 more than 70% of predicted). The comparisons of asthmatic with healthy subjects were complicated, however, by differences between the subject groups. For example, the mean age for the healthy and asthmatic subjects was 30.3 and 25.0 years, respectively, and no data on past smoking have been reported. In addition, there were only 15 subjects with asthma compared with 25 healthy subjects, which means that measurements of endpoints averaged over subjects were more variable for asthmatic than healthy subjects. Furthermore, it is unclear if exposure may have affected minute ventilation (air exchange in liters per minute) between groups, thereby changing the dose of particles reaching the lower lung or changing the location in the airway where the particles are deposited.

Many epidemiologic studies have investigated health effects of air pollution on asthma symptoms (see Strachan 2000; Gavett and Koren 2001; Pandya et al 2002). Most information is available on changes in lung function; because of difficulties in obtaining airway samples, fewer studies have measured inflammatory markers. One study reported increased blood levels of soluble ICAM-1, soluble VCAM-1, and RANTES in asthmatic children compared with healthy children in relation to their exposure to air pollution (Ando et al 2001). These studies indicated worsening of symptoms and the occurrence of inflammatory responses due to particulate air pollution exposure. Some of these health effects may be ascribed to ozone exposure, however, which has been shown to aggravate asthma symptoms and increase levels of inflammatory markers in nasal and BAL fluids (Scannell et al 1996; Hiltermann et al 1997). A controlled-exposure study by Nordenhäll and colleagues of the effects of exposure to diesel exhaust (300 $\mu g/m^3$ particle concentration) on subjects with moderate asthma found increased airway hyperresponsiveness and resistance, as well as increased levels of IL-6 in sputum (Nordenhäll et al 2001). No changes were reported in sputum levels of methylhistamine, eosinophilic cationic protein, myeloperoxidase, or IL-8.

Finally, some uncertainty remains regarding the exact nature of the exposure that both the healthy and asthmatic subjects encountered in the current study in terms of particle size and composition. For details on the diesel exposure setup, the investigators refer to previous studies, which used higher concentrations of diesel exhaust (Rudell et al 1994, 1996; Salvi et al 1999a, 2000). One important difference between the current study and the Sandström group's previous studies was an extra dilution step, which may have altered the particle size distribution in the exposure chamber for the current study; however, particle size distribution for the further diluted diesel exhaust was not provided. Information on sulfur concentrations or numbers of ultrafine particles was also not available, nor is it clear whether subjects may have encountered low levels of gaseous components during filtered air exposure.

TECHNICAL EVALUATION OF PART II: CAPs

BRIEF OVERVIEW OF THE CAPs STUDY

At the laboratory of Dr Devlin and colleagues, 30 healthy subjects were exposed once for 2 hours to CAPs (23–311 $\mu g/m^3$; median 108 $\mu g/m^3$) and 8 different subjects were exposed for 2 hours to filtered air. Four additional subjects, exposed to filtered air in a separate study, were included only to provide more biopsy tissue samples. Subjects were between 19 and 42 years old and had not smoked for at least 5 years; they had no allergies or respiratory infections. (Unlike Part I, no subjects with asthma were assessed in this study.) Ambient air was concentrated using a Harvard/EPA concentrator (Sioutas et al 1997). This instrument concentrates particles between 0.15 and 2.5 µm in aerodynamic diameter approximately 6-fold to 10-fold. Particles larger than 2.5 µm are excluded; particles smaller than 0.15 µm are not concentrated, but are not excluded. Throughout the exposure, subjects exercised on a stationary bicycle for 15 minutes alternated with 15-minute rest periods.

Lung function was assessed before and immediately after exposure; bronchoscopy to obtain lung fluids and bronchial tissues was performed 18 hours after exposure. This time frame was chosen because inflammatory responses in the airways of humans and other species are frequently observed at that time after exposure to airborne stimuli (eg, Oberdörster et al 2000). Blood was sampled before and 18 hours after exposure. Lung function, lung fluids, and blood were all analyzed in Dr Devlin's laboratory; biopsy tissue samples were analyzed in Dr Holgate's laboratory.

SUMMARY OF KEY RESULTS

Results of Work Performed by the Devlin Group

The investigators (Ghio et al 2000; Harder et al 2001) reported no changes in the lung function of healthy subjects immediately after a 2-hour exposure to CAPs (Critique Table). An increase in blood fibrinogen levels was observed in individuals exposed to CAPs but not in those exposed to filtered air; no other changes were observed in blood parameters. In lavage fluids, the percentage of neutrophils was higher and the percentage of macrophages was lower after CAPs exposure than after filtered air exposure.

Results of Biopsy Tissue Analysis by the Holgate Group

Bronchial tissues were successfully obtained from 10 CAPs-exposed and 11 filtered air–exposed subjects. In the bronchial tissues, no differences were observed between subjects exposed to CAPs or filtered air in the numbers of neutrophils, mast cells, eosinophils, monocytes, and the two T-cell subsets $CD8^+$ and $CD25^+$, nor in the levels of adhesion molecules (see Glossary). The only significant result was that the numbers of all T cells ($CD3^+$) and of the T-cell subset $CD4^+$ were somewhat lower after CAPs exposure than after filtered air exposure.

Because the particle exposure concentrations varied greatly (38–311 $\mu g/m^3$; median 84 $\mu g/m^3$) among the CAPs-exposed subjects who provided bronchial tissues, the investigators also evaluated the data from each individual to detect whether subjects exposed to a higher level of CAPs showed a larger change in a particular inflammatory endpoint than subjects exposed to lower levels. These analyses, however, did not reveal any trends for changes related to the exposure concentrations among subjects exposed to CAPs.

DISCUSSION

This study was among the first to analyze lung tissues obtained via bronchoscopy in human subjects exposed to CAPs. The study was the result of an international collaboration in which human subjects were exposed to CAPs in the United States and lung tissues were provided for detailed analysis in the United Kingdom. The US investigators have extensive experience conducting controlled exposure studies with human subjects using gaseous pollutants (such as nitrogen dioxide and ozone) and, more recently, CAPs. The UK investigators have conducted extensive work on lung inflammation and asthma.

Inflammatory Response (Healthy Subjects)

The overall evidence of lung inflammation observed in analyses by the Devlin group was modest. At 18 hours after exposure to CAPs but not to filtered air, they found an increase in blood levels of fibrinogen, which is a marker of inflammation as well as coagulation, and a higher percentage of neutrophils and a lower percentage of macrophages in lung lavage fluids. No airway inflammation was observed in bronchial tissues obtained from a subset of individuals exposed to CAPs compared with individuals exposed to filtered air; that is, no differences were observed in cell numbers, interleukins, or adhesion molecules.

Several factors may have contributed to the lack of inflammation in bronchial tissues after exposure to CAPs. First, viable biopsy tissues were obtained from only ten subjects who had been exposed to a wide range of particle concentrations (38–311 $\mu g/m^3$). To complicate this, subjects exposed to CAPs were not also exposed to filtered air, which removed the possibility of using each subject as his or her own control. As a result of these two factors, the power to detect small changes may have been low due to greater variability in the data set. Second, inflammation occurs over a period of time ranging from a few hours to days after exposure. In the CAPs study, the Devlin group collected biopsy tissues 18 hours after exposure. It is possible that inflammatory changes occurred at earlier time points, which would be similar to the changes observed in the subjects exposed to diesel exhaust at 6 hours after exposure (Investigators' Report, Part I). Third, as the investigators point out in their report, it is possible that effects may have occurred in areas deeper in the lung than the position from which they obtained tissues.

CAPs Exposures

One of the difficulties encountered in this type of study is variation in the concentration and composition of CAPs to which subjects are exposed; this is due to the daily variation in ambient PM concentration and composition and to differences in the day-to-day performance of the particle concentrator. Some of this day-to-day variation in operating the concentrator may be due to variation in the negative operating pressure of the instrument, high ambient particle concentrations, or high humidity (Godleski et al 2000). As discussed above, the small number of subjects and large variation in CAPs concentrations may have reduced the likelihood of finding significant changes.

The Harvard/EPA particle concentrator has been used in several animal and human exposure studies and its advantages and limitations have been discussed in detail elsewhere (eg, Godleski et al 2000). Its main advantage is that it provides particle concentrations at higher-than-ambient levels and therefore can be used on days when ambient pollutant levels are low. One disadvantage is that it does not remove most ambient gaseous pollutants, which also

vary from day to day. (Ozone is likely removed by reacting with the concentrator surface.) Subjects are therefore exposed to unknown levels of gaseous pollutants, which may have increased the variability in the endpoints measured (see General Discussion of Both Studies below).

Particle Composition

Evaluating the effects that daily variations in particle composition may have on health endpoints in a small number of subjects is a difficult task. In Part II of the Investigators Report, Holgate and Devlin provided data on PM composition measured via two methods, x-ray fluorescence (XRF) and inductively coupled plasma–atomic emission spectrometry (ICP-AES) (Appendix Tables A.1 and A.2). The data produced by the two methods are quite different. For example, although the XRF method produced much higher iron content values than the ICP-AES method, it appeared to be much less sensitive in general because it assessed many elements as being below the limit of detection. Therefore, the ICP-AES data seemed to be more useful in trying to assess how elemental composition may be associated with changes in inflammatory endpoints. The investigators did not attempt to make such associations in this study. However, a follow-up data analysis by Devlin and colleagues (Huang et al 2003) used factor analysis to explore whether certain particle components (as assessed by the ICP-AES method) would show a stronger association with those health endpoints that were changed after exposure to CAPs. They found that a sulfate/iron/selenium factor was associated with a higher percentage of neutrophils in bronchoalveolar lavage fluids, and that a copper/zinc/vanadium factor was associated with increased blood fibrinogen levels (Huang et al 2003). These results suggest that particle composition may influence the biologic response and provide a possible means of relating specific sources of particles to health effects. However, further research is needed to understand what factors may be related to specific health effects.

GENERAL DISCUSSION OF BOTH STUDIES

CONTROLLED EXPOSURE TO POLLUTANT MIXTURES

The studies described here are among the few thus far to expose human subjects to real-world PM mixtures in the forms of diesel exhaust or CAPs. These studies have shown that exposure to relatively low levels of diesel exhaust caused small changes in lung function and that both diesel exhaust and CAPs caused small changes in markers of lung inflammation in healthy subjects (Critique Table).

To date, few other studies have been completed in which human subjects were exposed to diesel exhaust or CAPs. One study in which healthy and asthmatic subjects were exposed for 2 hours to CAPs (99–224 $\mu g/m^3$) in Los Angeles showed few significant effects on lung function, lung or systemic inflammatory responses, or cardiovascular endpoints (Gong et al 2003a,b). Two other studies, neither of which evaluated lung function or inflammation, found changes in cardiovascular parameters after elderly humans were exposed to CAPs (Devlin et al 2003) and after healthy adults were exposed to CAPs and ozone (Brook et al 2002).

One unresolved issue is that the exposure mixtures in the diesel and CAPs studies described here included gaseous pollutants as well as particles. For example, the diesel study subjects were exposed to carbon monoxide, nitrogen oxides, formaldehyde, and other volatile components. Although the levels of carbon monoxide and formaldehyde were low, the level of nitrogen dioxide (NO_2) was noteworthy at 0.2 ppm. Because gases such as ozone and NO_2 have been shown to affect lung function and inflammation at fairly low concentrations (eg, 0.2 ppm ozone [Aris et al 1993a]; 2.0 ppm NO_2 [Devlin et al 1999]; 0.27 ppm NO_2 [Barck et al 2002]), it is possible that small amounts of these gases in the exposure atmospheres may have influenced the results. In the CAPs study, particles were concentrated but gases were not scrubbed from the mixture and may therefore have been present at ambient levels (except for ozone, which reacts with the concentrator surface and is removed). In addition, most health studies of particulate mixtures have not assessed ambient concentrations of air toxics, such as aldehydes, which may irritate the upper respiratory tract (reviewed by Leikauf 1992), or volatile organic compounds, which have been shown to cause airway irritation and neutrophil influx in nasal lavage fluid (Koren et al 1992). A more precise assessment of the gaseous components and the concentrations of air toxics in the diesel exhaust and CAPs mixtures would be useful in future studies.

COMPARISON OF DIESEL EXHAUST AND CAPs STUDIES

The goal of this project was to take advantage of two separate, existing human studies to compare changes in health endpoints in response to different pollutant mixtures. Unfortunately, the results are not easily compared. In the diesel exhaust study, for healthy subjects, Sandström and Holgate reported increased IL-8 messenger RNA and VCAM-1 in bronchial tissues; these changes were accompanied by increased levels of lymphocytes and neutrophils and decreased levels of macrophages in lavage fluids. For the CAPs study, which examined only healthy subjects, the Holgate group found no inflammatory changes in bronchial

tissues; the Devlin group, on the other hand, reported increased levels of fibrinogen in blood, and a higher percentage of neutrophils and a lower percentage of macrophage levels in lung lavage fluids after exposure to CAPs but not filtered air (similar to those observed in healthy subjects in the diesel study). (The inflammatory changes found in the current diesel study were similar to those found in a previous study by the Sandström and Holgate groups using a higher concentration of diesel exhaust [Salvi et al 1999a, 2000].)

Several methodologic issues, all of which pertain to the exposure and bronchoscopy protocols and not to the biopsy tissue analyses performed by the Holgate group, may elucidate why it is difficult to compare these results. Some uncertainties are associated with the subjects selected for the two studies. First, neither the Sandström group nor the Devlin group collected information on the lifetime smoking histories of the subjects, although the Devlin group did select subjects who had not smoked in the past 5 years. Second, the subject groups differed in age distribution and in distribution of females. Third, use of asthma medications may have influenced the inflammatory markers in the asthma group. What influence these factors may have had on health endpoints remains unclear.

The most obvious difference between the current diesel and CAPs studies is the time points at which lavage fluids and bronchial tissues were obtained: the diesel study at 6 hours after exposure, which is early in the inflammatory process, and the CAPs study at 18 hours after exposure. To investigate how influential this difference may have been, the Sandström and Holgate groups jointly conducted another study to see if inflammatory changes observed at 6 hours after diesel exposure may have still been present at 18 hours. They again exposed healthy subjects and subjects with allergic asthma or rhinitis to 100 $\mu g/m^3$ diesel exhaust for 2 hours (Brown et al 2002). They reported persistent neutrophilic inflammation in the bronchial submucosa of biopsy tissues from healthy subjects 18 hours after exposure, but the inflammatory responses they had observed at 6 hours after exposure in the current diesel study had resolved by 18 hours. Thus, in the current CAPs study, it remains possible that CAPs exposure could have caused inflammatory changes at 6 hours that had resolved by 18 hours when they acquired bronchial tissues.

Other possible explanations for the different outcomes between the studies may have been due to small differences in the techniques used to perform the bronchoscopies (American Thoracic Society 1990). Health endpoints measured in lavage fluid may vary due to the precise location where the bronchoscope is lodged, the amount of fluid used to wash the airways, and the length of time the fluid is left in place before it is extracted. In addition, application of local anesthesia may alter the outcomes. Similar to lavage fluids, tissues obtained at one position are different from those obtained elsewhere in the airways. Especially when the goal is to evaluate the effects of inhaled particles, it is important to obtain tissues where the particles are most likely to deposit; because particle deposition varies among the points where airways divide (the subcarinae) and other locations. Furthermore, prompt processing of tissue samples is essential. In these studies, the bronchoscopies and subsequent lavage fluid and tissue preservation were conducted in Sweden and the United States; the tissues were then transported to the United Kingdom for analysis. Thus, the sampling, handling, storage, and shipping conditions may have introduced small variations in the health endpoints assessed. To what extent these factors may have influenced the results remains unclear.

CONCLUSIONS

The current studies found an assortment of results. For healthy subjects exposed to either diesel exhaust or CAPs, the studies showed (1) small changes in inflammatory endpoints in lavage fluids obtained via bronchoscopy, and (2) small changes in inflammatory endpoints in bronchial tissues after diesel exhaust exposure but not after CAPs exposure. As discussed above, several factors may have contributed to this lack of effects in bronchial tissues after CAPs exposure, including the small number of subjects in the exposure group and the variability in exposure levels.

For asthmatic subjects exposed to diesel exhaust, the study showed (1) baseline inflammation of the airways (before exposure), (2) a small change in lung function after exposure, (3) few changes in inflammatory endpoints in lavage fluids after exposure, and (4) an increase in IL-10 levels but no other changes in bronchial tissues. The increase in IL-10 is a novel observation that needs to be corroborated. The lack of changes in inflammatory endpoints after subjects with asthma were exposed to diluted diesel exhaust is not consistent with the epidemiologic reports that asthma symptoms increase in association with particulate air pollution. Most epidemiologic studies assess possible associations between air pollution and health in large numbers of individuals and thus can detect events even if they occur in vulnerable people who represent a small percentage of the population. In comparison, controlled exposure studies conducted in a small number of individuals have less power to detect effects and may not include the most vulnerable people. The subjects with asthma in this study had mild symptoms and may not be

as sensitive as individuals with more severe asthma or other conditions. Furthermore, it is not entirely clear how the kinds of measurements made in controlled exposure studies relate to health outcomes observed in some of the epidemiologic studies.

One conclusion from these studies is that the controlled exposures as performed here appear to be relatively safe for healthy subjects or subjects with mild lung disease. Future studies could therefore expose subjects to higher concentrations of particles or include subjects with moderate lung or cardiovascular disease, who may be more susceptible to the effects of air pollutant exposure. Caution would obviously be needed because such subjects may be at increased risk of severe symptoms and possibly death. Furthermore, subjects with asthma are often on extensive daily medication regimens, which would likely interfere with experimental evaluations and a study design would need to accommodate this possible source of confounding. In a controlled experimental setting, therefore, it remains difficult to determine whether people with severe asthma or other lung diseases are more susceptible to the adverse effects of air pollutants.

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