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Effects of Exposure to Ultrafine Carbon Particles in Healthy Subjects and Subjects with Asthma

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Includes a Commentary by the Institute's Health Review Committee



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STATEMENT

Synopsis of Research Report 126

Effects of Controlled Exposure to Ultrafine Carbon Particles in Healthy and Asthmatic People

Ambient particles are a complex mixture whose size, chemical composition, and other physical and biological properties vary with location and time. Regardless of the characteristics at a single site, however, epidemiologic studies have reported that short-term increases in low levels of particles are associated with short-term increases in illness and death. Remaining questions about the health effects of particles include the specific characteristics of particles—especially size and chemical composition—and the biological mechanisms that may explain the reported associations.

To address some of these critical issues, Health Effects Institute issued Request for Applications (RFA) 98-1, "Characterization of Exposure to and Health Effects of Particulate Matter" in 1998. A component of the RFA was to promote evaluation of health effects of controlled exposure to particles in animals and healthy people as well as in those who might be more susceptible to particle exposure. People with asthma are one of the groups considered particularly susceptible to the effects of airborne particles.

HEI funded two studies to address this issue in human volunteers who were either healthy or had mild asthma. One was the current study, led by Dr Mark Frampton, University of Rochester School of Medicine and Dentistry, Rochester, New York, to evaluate the effects of inhalation exposure to ultrafine (<0.1 μm diameter) carbon particles, which some scientists believe are more effective than larger particles in causing health effects (the ultrafine hypothesis of particle toxicity). The second study, led by Dr Henry Gong of Los Amigos Research and Education Institute, Downey, California, investigated the effects of exposure to fine (0.1–2.5 μm in diameter) concentrated ambient particles (CAPs) derived from the Los Angeles area.

As one of the consequences of an HEI-sponsored workshop held before the studies began, Frampton

and Gong developed exposure and study protocols that were similar to those being used in CAPs studies underway at the US Environmental Protection Agency research facility in Chapel Hill, North Carolina. In this way, results of the studies could more easily be compared with each other. HEI published the results of the Gong study in 2003.

APPROACH

Healthy and mildly asthmatic men and women were exposed via a mouthpiece over 2 hours to laboratory-generated ultrafine carbon particles (average diameter approximately 0.025 μm) and on a different occasion to filtered air as follows:

- 12 healthy participants were exposed at rest to 10 $\mu\text{g}/\text{m}^3$ particles or air;
- 12 healthy participants were exposed to particles (10 and 25 $\mu\text{g}/\text{m}^3$) or air during intermittent exercise on a stationary bicycle (15-minute cycles of rest and exercise over 2 hours); and
- 16 asthmatic volunteers were exposed to either 10 $\mu\text{g}/\text{m}^3$ particles or air using the same intermittent exercise protocol used for the healthy exercising volunteers.

Frampton and colleagues hypothesized that ultrafine particle exposure would activate leukocytes (white blood cells) and endothelial cells (cells lining blood vessels to form part of the interface between blood and tissue cells) and lead to an inflammatory response in the airways and in the blood. The investigators further hypothesized that effects would be greater in people with asthma than in healthy people. Their proposed study also anticipated that particle exposure might affect respiration and cardiac electrophysiologic function. The investigators therefore measured multiple indices of pulmonary and cardiac function as well as blood parameters at different times before, during, and up to 21 hours (45 hours for asthmatics) after exposure to particles or filtered air. To

measure inflammatory responses in the airways, Frampton and colleagues collected sputum at screening and 22 hours after exposure. In addition, they calculated the number and mass of inhaled particles that deposited in the lungs of asthmatic and healthy participants.

RESULTS AND INTERPRETATION

Healthy resting people had no detectable changes in airway, systemic, or cardiac electrophysiologic endpoints at any time measured during or after inhalation exposure to 10 $\mu\text{g}/\text{m}^3$ particles. In exercising healthy and asthmatic participants, Frampton and colleagues did not detect changes in any airway inflammatory endpoint during or after exposure to 10 or 25 $\mu\text{g}/\text{m}^3$ particles. These findings are noteworthy in the light of the investigators' novel finding that, at the same inhaled concentration of particles, about 50% more particles deposited in lungs of asthmatic people than in healthy people. In addition, these researchers calculated that over 4 times as many particles were deposited in the lungs of exercising participants as in the lungs of resting participants. Thus, even though the dose of particles delivered to the lungs of exercising asthmatic people may have been approximately 6 times the dose delivered to the lungs of resting healthy people, ultrafine particles had no effects on the airways. Further, the concentration of particles of the size used in this study was 10 to 100 times higher than average concentrations of ultrafine particles reported in urban air.

Some systemic and cardiovascular changes were associated with particle exposure in healthy and asthmatic exercising volunteers. The pattern and magnitude of these changes were similar in the two groups, differing from the investigators' prediction of greater responses in the asthmatic participants.

Exposure to ultrafine particles was associated with changes in numbers of certain leukocytes in the blood of healthy and asthmatic exercising volunteers. The numbers of some types of leukocytes decreased but the total number of leukocytes did not change. Expression of some adhesion molecules on the surface of leukocytes, a characteristic of cell activation, was also changed. The significance of these observations is not clear. The investigators speculate that exposure to particles mildly constricts pulmonary blood vessels, activating vascular endothelial cells

and preventing outflow of activated leukocytes from the lungs into the circulation. An alternative possibility to explain the findings is that the particles are selectively toxic to activated cells. In addition, as the investigators appropriately note, the changes detected may also have been chance findings that can occur at random and are more likely to be seen when evaluating multiple endpoints.

The investigators also found small changes in cardiac repolarization, the time taken between the electrical stimuli governing contraction and relaxation of the heart. Again, the biological or clinical significance of these small changes in healthy and asthmatic individuals is unclear.

CONCLUSIONS

This innovative and technically complex study used state-of-the-art measurements to assess responses to inhaled ultrafine particles in healthy and asthmatic volunteers. The particles were generated in a laboratory and did not contain toxicologically important components such as metals and organic compounds, but they were relevant to real world exposures because carbon is a major component of airborne particles from urban settings. The concentration of ultrafine particles used, however, was 10 to 100 times higher than the average concentrations reported in urban air.

Frampton and colleagues found few airway, systemic, or cardiac electrophysiologic changes associated with ultrafine particle exposure. The clinical significance of any of the changes is not clear. Thus, in this limited set of healthy and mildly asthmatic participants, the effects of exposure to ultrafine carbon particles did not support the hypothesis that ultrafine particles are more toxic than larger components of the particle mix. This paucity of effects is consistent with the results of studies conducted in North America with human exposures to concentrated fine particles.

Future controlled exposures should include particles of different sizes and composition, different susceptible populations (such as those with cardiovascular disease), a larger number of participants, longer exposure durations and higher concentrations; and different endpoints to increase the statistical and scientific strength and thus provide a stronger test of the ultrafine hypothesis.



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HEI STATEMENT

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

INVESTIGATORS' REPORT

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

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COMMENTARY Health Review Committee

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

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Effects of Exposure to Ultrafine Carbon Particles in Healthy Subjects and Subjects with Asthma

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ABSTRACT

Increased levels of particulate air pollution are associated with increased respiratory and cardiovascular mortality and morbidity as well as worsening of asthma. Ultrafine particles (UFP*; less than 0.1 μm in aerodynamic diameter) may contribute to the health effects of particulate matter (PM) for a number of reasons. Compared with larger particles on a mass basis, UFP have a higher predicted pulmonary deposition, greater potential to induce pulmonary inflammation, larger surface area, and enhanced oxidant capacity. UFP also have the potential to cross the epithelium and enter the systemic circulation. We hypothesized that exposure to UFP causes airway inflammation in susceptible humans with activation of circulating leukocytes and vascular endothelium, a systemic acute phase response, and transient hypercoagulability. We further hypothesized that in people with asthma, UFP deposition would be increased and underlying airway inflammation enhanced. Our objectives were:

- to develop a system for controlled exposures of humans to UFP;
- to measure the pulmonary fractional deposition of UFP;
- to assess the effects of UFP exposure on blood leukocyte and endothelial adhesion molecule expression and activation, on airway nitric oxide (NO) production, on the

systemic acute phase response, on blood coagulability, and on cardiac electrical activity and repolarization; and

- to evaluate these responses in both healthy subjects and people with asthma.

We developed and validated a mouthpiece exposure system for human studies of carbon UFP and then conducted three clinical exposure studies: healthy subjects breathing filtered air and UFP (10 $\mu\text{g}/\text{m}^3$) at rest (UPREST); healthy subjects breathing air and UFP (10 and 25 $\mu\text{g}/\text{m}^3$) with intermittent exercise (UPDOSE); and subjects with mild asthma breathing air and UFP (10 $\mu\text{g}/\text{m}^3$) with intermittent exercise (UPASTHMA). All exposures were for 2 hours on the mouthpiece system. Exposures were separated by at least 2 (UPREST and UPDOSE) or 3 (UPASTHMA) weeks. Prior to and at intervals after each exposure, we assessed symptoms, pulmonary function, blood markers of inflammation and coagulation, and airway NO production. Sputum inflammatory cells were assessed 21 hours after exposure. Continuous 12-lead electrocardiography (ECG) recordings were analyzed for changes in heart rate variability, repolarization, and arrhythmias.

For healthy subjects, the fractional deposition of UFP at rest was 0.66 ± 0.11 (mean \pm SD) by particle number, confirming the high deposition for UFP predicted by models. Deposition further increased during exercise (0.83 ± 0.04). Asthmatic subjects showed higher UFP deposition than did healthy subjects when breathing at rest (0.76 ± 0.05).

During the UPREST protocol, there were no convincing effects for any outcome measures. Breathing 25 $\mu\text{g}/\text{m}^3$ UFP with exercise (UPDOSE) was associated with reductions in blood monocytes and activation of T lymphocytes in healthy females. In asthmatic subjects (UPASTHMA), breathing 10 $\mu\text{g}/\text{m}^3$ UFP was associated with reduced numbers of blood eosinophils and CD4⁺ T lymphocytes. In the UPDOSE group, monocyte expression of intercellular adhesion molecule-1 (ICAM-1) was reduced in a concentration-related manner ($P = 0.001$). In the UPASTHMA group, CD11b expression was reduced on monocytes and

*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 126, which also includes a Commentary by the Health Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr Mark W Frampton, University of Rochester Medical Center, 601 Elmwood Ave, Box 692, Rochester NY 14642-8692, mark_frampton@urmc.rochester.edu.

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.

eosinophils, and ICAM-1 expression was reduced on polymorphonuclear leukocytes (PMNs). ECG analyses of UPDOSE subjects showed transient reductions in parasympathetic influence on heart rate variability and a reduced repolarization (QT) interval. In UPASTHMA subjects, ECG analyses showed decreased QT variability, but no effect on the QT interval. There were no significant effects in any of the studies on symptoms, pulmonary function, or markers of airway inflammation. We found no increases in soluble markers of systemic inflammation or coagulation.

Our hypothesis that inhalation of carbon UFP causes pulmonary inflammation and an acute phase response was not confirmed. However, the observed subtle changes in leukocyte subsets and adhesion molecule expression are consistent with effects on vascular endothelial function. We also found effects on heart rate variability and on cardiac repolarization in healthy subjects. If confirmed, the finding that very low mass concentrations of particles have cardiovascular effects would have important implications for future PM regulatory strategies.

INTRODUCTION

A strong and consistent association has been observed between ambient particle concentrations and adjusted mortality rates. An increase of $50 \mu\text{g}/\text{m}^3$ in the concentration of PM less than $10 \mu\text{m}$ in aerodynamic diameter (PM_{10}) is associated with a 3% to 8% increase in the relative risk of death (US Environmental Protection Agency [EPA] 1996). The strongest associations are seen for respiratory and cardiac deaths, particularly among the elderly. Associations with increased mortality have been observed at mean PM_{10} concentrations well below the US National Ambient Air Quality Standard (NAAQS) of $150 \mu\text{g}/\text{m}^3$ as a 24-hour average (Dockery and Pope 1996; EPA 1996). The Health Effects Institute (HEI)-funded National Morbidity, Mortality, and Air Pollution Study confirmed these associations (Samet et al 2000a,b). After a methodologic flaw in the original analysis was discovered and original estimates were revised, the study reported an approximately 0.21% increase in total mortality (HEI 2003).

Increased levels of particulate air pollution are also associated with asthma exacerbations, increased respiratory symptoms, decreased lung function, increased medication use, and increased hospital admissions (EPA 1996). There is also strong evidence for long-term health consequences of exposure to PM. Using data collected on 1.2 million adults in the US from 1982 to 1998, Pope and colleagues (2004) found that a $10 \mu\text{g}/\text{m}^3$ increase in fine PM was associated with an 8% to 18% increase in cardiovascular causes of

death. PM-related mortality was chiefly due to ischemic heart disease, dysrhythmias, heart failure, and cardiac arrest.

Because of concerns about the potential public health implications of these findings, the EPA promulgated a new standard for fine particles in 1997. Nevertheless, the mechanisms by which particulate pollution at such low concentrations induce health effects remain unclear. Determining the biological mechanisms involved was identified in 1998 as a high priority research need by the EPA and the US National Academy of Sciences (National Research Council). Data from human clinical studies are also needed to determine the link between exposure and response in both healthy and susceptible populations (Utell and Drew 1998).

Hypotheses proposed to explain the characteristics of particles responsible for the adverse effects of PM have focused on particle acidity (Chen et al 1992), particle content of transition metals (Pritchard et al 1996), bioaerosols (Rylander and Snella 1983), and UFP (Oberdörster et al 1995; Seaton et al 1995). We suggest that ambient UFP are relevant for several reasons.

- UFP induce pulmonary inflammation at lower mass concentrations than larger particles (Oberdörster et al 1995; Li et al 1999).
- Ambient UFP have a much higher number concentration and surface area than larger particles at the same mass concentration. For example, in order to achieve an airborne concentration of $10 \mu\text{g}/\text{m}^3$, 2.4×10^6 20-nm particles/ cm^3 are needed; in contrast, only one 2.5- μm particle/ cm^3 is needed (Oberdörster et al 1995).
- Inhaled UFP have a high deposition efficiency predicted for the pulmonary region. For example, 20nm particles have up to 50% deposition efficiency (International Committee for Radiological Protection [ICRP] 1994).
- UFP have enhanced oxidant capacity when compared on a mass basis with larger particles (Brown et al 2001; Li et al 2003).
- UFP can easily cross the epithelium and reach interstitial sites (Stearns et al 1994). In humans, they may even enter the systemic circulation (Nemmar et al 2002).

SETTING AIR QUALITY STANDARDS

Although few studies have measured UFP in ambient air, one panel study of asthmatic subjects (Peters et al 1997b) found that peak flow varied more closely with the 5-day mean of UFP number than with fine particle mass concentration. This finding suggests that the UFP component of fine particle pollution contributes to airway effects in people with asthma. A recent HEI-funded study in

Erfurt, Germany, also found associations between ambient UFP and mortality (Wichmann et al 2000).

Determining the health effects of UFP exposure has considerable importance in setting appropriate air quality standards. The Committee on Research Priorities for Airborne PM of the US National Research Council has stated that "Present knowledge...strongly suggests that mass dose is not a sufficient metric for understanding health effects" (National Research Council 1998). Further, more stringent regulation of fine particle mass may, paradoxically, increase the number of ambient UFP, as was found in Erfurt, Germany (Ebelt et al 2001). This paradoxical increase occurs because reducing the number of particles in the accumulation mode reduces the agglomeration of UFP, thereby increasing ambient UFP number, despite an overall reduction in PM_{2.5} mass. Human studies of the health effects of exposure to UFP are needed to help identify the appropriate dose measure (particle number, surface area, or mass) for regulatory purposes.

EFFECTS ON CARDIOVASCULAR SYSTEM

Plausible mechanisms explaining the relation between particle exposure and changes in cardiovascular mortality have not been clearly defined (Utell et al 2002). Some studies have provided evidence that PM exposure has cardiovascular effects and have offered clues as to possible mechanisms. For example, PM exposure is associated with changes in heart rate and heart rate variability (Peters et al 1999; Pope et al 1999a,b; Gold et al 2000; Creason et al 2001); with increased systolic blood pressure (Ibald-Mulli et al 2001); with increased plasma viscosity (Peters et al 1997a), C-reactive protein (Peters et al 2001b), and fibrinogen (Pekkanen et al 2000); with reductions in hemoglobin concentration and packed red blood cell (RBC) volume (Seaton et al 1999); with increases in cardiac arrhythmia (Peters et al 2000); and with triggering of acute myocardial infarction (Peters et al 2001a). Seaton et al (1995) hypothesized that airway inflammation induced by UFP triggers cardiovascular events. Inflammation is accompanied by a systemic acute phase response, with increased plasma viscosity and increased propensity for blood coagulation, which could induce coronary artery thrombosis in people with atherosclerosis.

A few studies have specifically assessed the cardiovascular effects of UFP. In a recent study of patients with stable coronary artery disease (Pekkanen et al 2002), investigators performed repeated exercise tests concurrent with monitoring of ambient particle mass and number. Significant independent effects were found for both fine and UFP on the degree of ST segment depression during exercise.

For example, exposure to UFP (0.01 to 0.1 μm) was associated with an increased risk for greater than 0.1 mV of ST depression (odds ratio, 3.14; confidence interval, 1.56–6.32).

We proposed the following sequence of events in which PM exposure induces airway inflammation and cardiovascular effects:

1. Initial PM injury to epithelial cells is caused by the generation of reactive oxygen species. This event is accompanied by activation of nuclear regulatory factors, which lead to elaboration of proinflammatory cytokines (including interleukin [IL]-8 and IL-6) and increased expression of NO synthase (NOS) and result in increased NO in exhaled air.
2. These processes activate vascular endothelial and circulating PMNs, eosinophils, lymphocytes, and monocytes. Emigration of inflammatory cells from blood to tissue involves an increase in adhesion molecules on vascular endothelium (E-selectin, P-selectin, ICAM-1, and vascular cell adhesion molecules [VCAM]-1) and on circulating leukocytes (L-selectin, leukocyte function antigen-1 [LFA-1], Mac-1 [CD11b/CD18], very late activation antigen [VLA]-4 and ICAM-1 [Lukacs et al 1995]). The events in the process of leukocyte-endothelium binding include increased expression followed by shedding of adhesion molecules, as cells tether and roll. This is followed by cell activation, stable adhesion, and transmigration through the epithelium (Salmi and Jalkanen 1997). Endothelial activation may further contribute to the increase in exhaled NO concentrations associated with airway inflammation.
3. Increased release of IL-6 and tissue factor activates blood mononuclear cells. IL-6 initiates hepatic synthesis of acute phase proteins, including C-reactive protein serum amyloid A, and fibrinogen. Monocyte tissue factor and endothelial cell activation initiate the coagulation cascade.

The combined effects of these processes on endothelial function, vascular inflammation, cardiac rate control and repolarization, and blood coagulation may precipitate an acute coronary event or arrhythmia in susceptible individuals with critical coronary artery disease.

CONTRIBUTING EFFECT OF ASTHMA

People with asthma are at risk for health effects from airborne PM, which has been associated with increased emergency room visits for asthma in children and adults (Lipsett et al 1997; Tolbert et al 2000; Atkinson et al 2001). Further, PM exposure increases dyspnea and bronchodilator use in people with asthma (Hiltermann et al 1998).

Asthma is a disease characterized by airway inflammation. There is evidence for activation of lung leukocytes and pulmonary vascular endothelium, particularly during exacerbations (Ohkawara et al 1995). Soluble ICAM-1 and soluble E-selectin are increased in sera from asthmatic subjects during exacerbations, compared with stable asthmatics and healthy subjects (Montefort et al 1994); soluble ICAM-1 is increased in the serum of asthmatics after allergen challenge (Lee et al 1997). Activation of T lymphocytes with production of type 2 (IL-4, -5, -13) inflammatory cytokines drives the recruitment and retention of eosinophils in the airway (Corrigan and Kay 1990; Wilson et al 1992). Treatment with inhaled corticosteroids reduces expression of activation markers CD25 and HLA-DR in lymphocytes from bronchoalveolar lavage fluid and also reduces HLA-DR expression in blood lymphocytes (Wilson et al 1994). In asthma, blood CD4⁺ T cells express increased mRNA for IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF). IL-5 mRNA expression correlates with asthma severity and eosinophilia (Corrigan et al 1995). Allergen challenge in asthmatics causes a reduction in blood CD4⁺ T cells (Walker et al 1992) and an increase in airway CD4⁺ cells (Virchow et al 1995). Lymphocytes may play a role in regulating inducible NOS activity through release of interferon (IFN)- γ , which stimulates monocytes and macrophages to release cytokines such as tumor necrosis factor and IL-1, which in turn stimulate increased inducible NOS mRNA transcription in airway epithelium (Robbins et al 1994). Airway inflammation in asthma is associated with increased NO in the exhaled air (Barnes and Kharitonov 1996). Exhaled NO concentrations increase with asthma exacerbations and decrease with inhalation of corticosteroids (Kharitonov et al 1996) or inhibitors of NOS (Yates et al 1995).

UFP exposure may worsen asthma by further shifting lymphocyte responses to the type 2 phenotype, by further activating resident lymphocytes, by increasing the likelihood that lymphocytes will encounter antigen, and/or by increasing penetration of allergen through an injured epithelium. Anderson and colleagues (1990) demonstrated increased deposition of ultrafine sebacate particles in the airways of asthmatics compared with healthy subjects.

SPECIFIC AIMS

We hypothesized that exposure to UFP causes airway inflammation in susceptible humans. The mechanisms involve activation of circulating leukocytes and vascular endothelium, and the consequences include a systemic

acute phase response with transient hypercoagulability. We further hypothesized that, in people with asthma, UFP deposition and underlying airway inflammation would be increased.

These hypotheses were tested in a series of studies with specific aims:

1. Develop a system for controlled exposure of humans to UFP.
2. Measure the pulmonary fractional deposition of UFP in both healthy and asthmatic humans.
3. Assess the effect of inhalation of carbon UFP on leukocyte and endothelial adhesion molecule expression and activation.
4. Evaluate airway NO production as a marker for airway inflammation after carbon UFP exposure.
5. Measure the effects of carbon UFP exposure on markers of the systemic acute phase response and blood coagulability.
6. Document any effects of carbon UFP exposure on cardiac electrical activity and repolarization.
7. Assess subjective responses to UFP inhalation in both healthy and asthmatic subjects.

METHODS AND STUDY DESIGN

SUBJECT CRITERIA

The study was approved by the Institutional Review Board for Research Subjects of the University of Rochester Medical Center. Forty subjects were to be chosen to participate and paid a stipend. All were to be lifetime non-smokers, aged 18 to 40 years. Additional exclusion criteria were: regular marijuana use within the past five years, pregnancy, ischemic heart disease, active psychiatric disorder, occupation involving chronic exposure to heavy dust or PM, inability to complete the required exercise, inability to produce sputum with sputum induction, or current drug or alcohol abuse. Subjects were required to avoid the following medications for the indicated interval before and during the study: systemic steroids (1 month) and nonsteroidal antiinflammatory drugs (1 week, including aspirin, vitamins C and E, and antihistamines). Subjects with atopy or allergic rhinitis were not excluded as long as they did not require regular treatment with antihistamines or systemic steroids. Subjects were not studied within 6 weeks of a respiratory infection.

Healthy subjects were required to have normal spirometry (forced expiratory volume in 1 second [FEV₁] and forced

vital capacity [FVC] \geq 80% predicted, forced expiratory flow rate from 25% to 75% of FVC [FEF_{25–75}] \geq 60% predicted), a normal 12-lead ECG, and no history of chronic respiratory disease.

Subjects were considered to have asthma if they had: (1) a history of repetitive symptoms characteristic of intermittent bronchoconstriction (wheezing, shortness of breath), and (2) *either* improvement in FEV₁ of 12% or more with the administration of inhaled albuterol (if abnormally low values were obtained compared to predicted for airway conductance, FEV₁, or FEV₁/FVC) (Morris et al 1971) *or* airway hyperresponsiveness with methacholine challenge. For methacholine challenge, increasing concentrations of methacholine (0.00, 0.08, 0.16, 0.31, 0.63, 1.25, 2.50, 5.00, 10.00 mg/mL) in normal saline were administered at 4-minute intervals using a nebulizer (model 646, DeVilbiss Company, Somerset PA) with a dosimeter (Rosenthal-French model D-2A, Laboratory for Applied Immunology, Fairfax VA) calibrated to deliver 0.01 mL/breath. Subjects were instructed to take 5 breaths (each lasting 6 seconds), and FEV₁ was measured 30 seconds after the last breath. The concentration of methacholine that produced a 20% decrease in FEV₁ (PD₂₀) was determined by interpolation using the regression line of the methacholine dose response. Subjects with a PD₂₀ greater than 10 mg/mL were excluded from the study.

The severity of asthma in the study subjects recruited was to be consistent with mild intermittent, mild persistent, or moderate persistent asthma, according to US National Institutes of Health guidelines (National Institutes of Health 1997). Subjects with FEV₁ < 70% of predicted at baseline screening or with > 20% reduction in FEV₁ after the screening exercise were excluded. Subjects were considered atopic if they gave a history of environmental allergies or seasonal rhinitis or if the serum immunoglobulin E (IgE) level, obtained at the time of screening, was elevated.

STUDY DESIGN

Exposure Groups

This project involved three clinical studies of exposure to carbon UFP. All studies used a crossover design in which each subject was exposed to filtered air and to UFP. Exposure orders were randomized, and the randomization was blocked by order of presentation and gender. Exposures were blinded to both subjects and investigators.

The first study, UPREST, involved 12 subjects (6 female) who were exposed to filtered air and UFP (10 $\mu\text{g}/\text{m}^3$) at rest for 2 hours. Each subject had both exposures, which

were separated by at least 2 weeks. Followup extended for 21 hours after exposure.

The second study, UPDOSE, involved 12 subjects (6 female) with 3 exposures for each subject. Exposures were separated by at least 2 weeks: filtered air and UFP (10 and 25 $\mu\text{g}/\text{m}^3$). For safety reasons, the random order of exposure was restricted so that each subject received the 10 $\mu\text{g}/\text{m}^3$ UFP exposure before the 25 $\mu\text{g}/\text{m}^3$ UFP exposure. To simulate outdoor activities, subjects exercised on a bicycle ergometer for 15 minutes of each 30-minute period at an intensity adjusted to increase the minute ventilation (\dot{V}_E) to approximately 20 L/min/m² body surface area. Thus there was a total of 1 hour of rest and 1 hour of exercise per exposure. Followup extended for 21 hours after exposure.

The third study, UPASTHMA, involved 16 (8 female) subjects with asthma exposed to filtered air or 10 $\mu\text{g}/\text{m}^3$ UFP for 2 hours with intermittent exercise (as described for UPDOSE subjects) for a total of 2 exposures. Each exposure was separated by at least 3 weeks. Followup extended for 48 hours after exposure.

The experimental protocols for the three studies are summarized in Figure 1. The studies required 5 to 7 visits for each subject.

Screening Visit

At the screening visit, informed consent was obtained, and subjects completed a standardized screening questionnaire for assessment of respiratory symptoms, medical history, and smoking history. A physical examination was followed by routine pulmonary function tests: spirometry, diffusing capacity, and measurement of lung volumes. Subjects exercised on the bicycle ergometer for 15 minutes to determine the intensity necessary to achieve a minute ventilation of 20 L/min/m². For females, pregnancy testing was performed. Finally, subjects underwent sputum induction by inhaling nebulized saline.

Exposure Visits

On Day 1, at least 1 week after the screening visit, subjects arrived at 7:15 AM for the following procedures: measurement of blood pressure and heart rate; pulse oximetry; symptom questionnaire; attachment of a 12-lead Holter heart monitor (Mortara Instruments, Milwaukee WI) with a resting recording for 10 minutes; phlebotomy; measurement of exhaled NO; and spirometry. These procedures took about 2 hours.

For the symptom questionnaire, subjects ranked the severity of each symptom on a scale from 0 (“not present”)

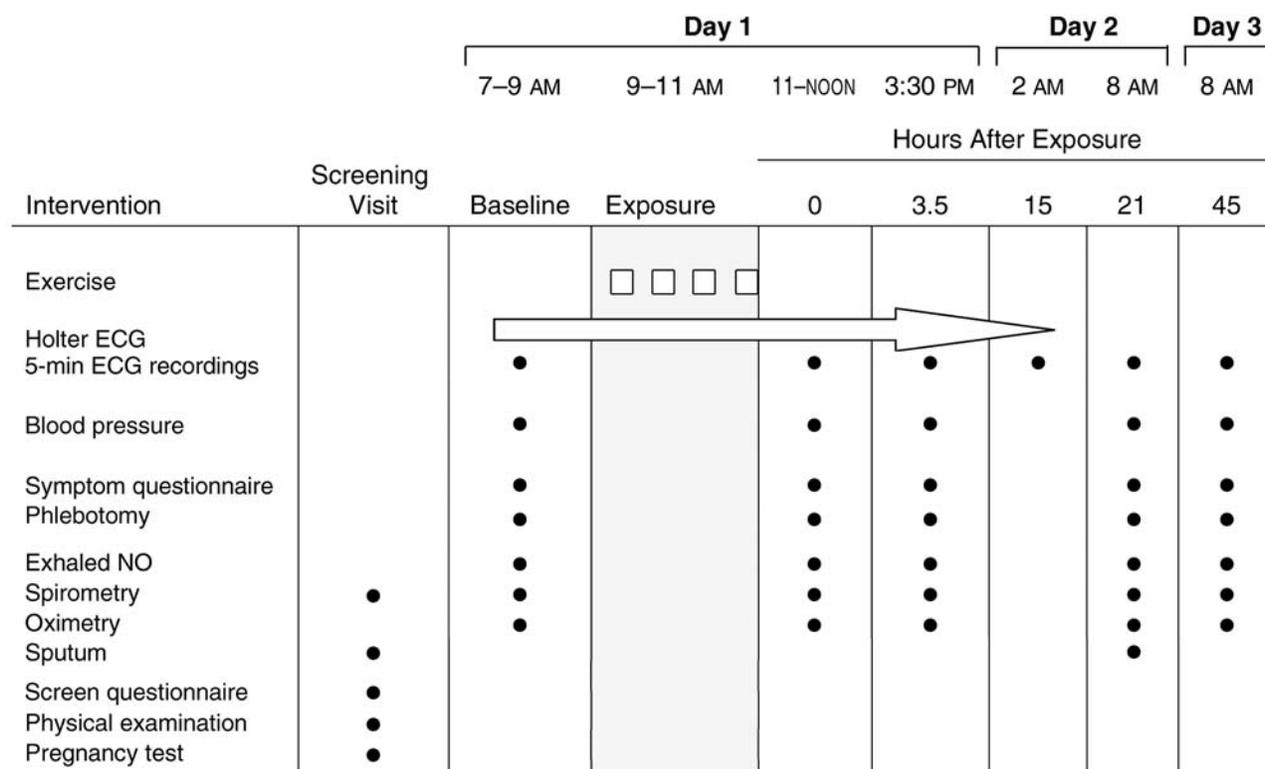


Figure 1. Protocols for screening visit and for Days 1 through 3 per exposure. Exercise lasted for 15 minutes of each half hour of the 2-hour exposure, yielding 1 hour total of exercise and 1 hour of rest. All exposures were 2 hours in duration. Only UPASTHMA subjects returned for Day 3.

to 5 (“incapacitating”). Subjects breathed room air on the mouthpiece system for 5 minutes before the exposure was started. They were then exposed by mouthpiece for 2 hours to either filtered air or UFP. After 1 hour of exposure, subjects were given a 10-minute break off the mouthpiece.

Immediately after the exposure, the measurements taken before exposure were repeated. The subjects were given lunch and remained in the Clinical Research Center. Measurements were taken again 3.5 hours after exposure. The subjects were then sent home with activity diaries.

For Day 2, subjects returned the next morning at 8:00 AM (21 hours after exposure). The same series of measurements was performed and sputum was induced. The Holter heart monitor was removed. For UPREST and UPDOSE subjects, this was the end of one exposure.

On Day 3 (48 hours after exposure), UPASTHMA subjects returned for an additional set of measurements, including a 10-minute digital ECG recording.

All subjects returned for subsequent exposures at least 2 weeks (UPREST and UPDOSE) or 3 weeks (UPASTHMA) after exposure, using an identical exposure protocol.

EXPOSURE SYSTEM

The exposure system was designed to meet several requirements.

- UFP had to be generated in real time during exposure to minimize agglomeration and diffusion losses.
- Simultaneous measurements of particle number, mass, and size distribution were necessary to characterize inhaled UFP.
- Determinations of particle characteristics were required at inspiration and expiration to determine deposition.
- Sufficient UFP aerosol was needed to meet the range of inspiratory flow demands during rest and exercise.

While typical minute ventilation at rest is 6 to 8 L/min, this measure increases several fold with exercise. Instantaneous or peak flow rates can reach 100 L/min. In order to meet peak demands of the subject, the flow rate into the mixing chamber on the inspiration side of the system was 120 L/min.

The exposures took place in an environmental chamber in the General Clinical Research Center at the University of

Rochester Medical Center. We chose a mouthpiece exposure system to facilitate accurate measurement of respiratory deposition. Details of particle generation and the mouthpiece exposure system have been described elsewhere (Chalupa et al 2002). The design is a one-pass, dynamic flow exposure system. Carbon UFP were generated from pure graphite electrodes by spark discharge in anhydrous argon, using a commercial generator (Palas Co, Karlsruhe, Germany). The use of argon served to exclude oxygen, water vapor, and other gases to minimize the formation of organic compounds and oxidation products. The particle size distribution was controlled by varying both the gap between the electrodes and the spark frequency. The generator continually adjusted the electrode position to keep the gap, and therefore the particle size, constant during particle generation. A constant flow (6 L/min) of argon through the spark chamber during generation minimized particle agglomeration. The generator settings were adjusted to provide a nominal particle count median diameter of 26 nm with a lognormal volume median diameter of 54 nm and geometric standard deviation of 1.6 nm. The mass and number concentrations of UFP emitted from the Palas generator were found to be stable over time (Table 4).

Particles were continuously generated, and the concentration was monitored and regulated during the exposure. Particles were passed through a charge neutralizer after generation to achieve the Boltzman equilibrium. All tubing was electrically conductive with lengths minimized to avoid particle loss. The ionized particles then entered a 28.4-L mixing reservoir. Particles in the reservoir entered the circuitry to the mouthpiece according to the demands of the subject. An overflow line removed the excess aerosol. Nonrebreathing valves (Hans Rudolph, Kansas City MO) at the mouthpiece insured one-way passage of the particles and allowed aerosol concentrations to be analyzed in real time on both the inspiratory and expiratory sides of the subject. The subject inhaled from a mouthpiece and wore a nose clip.

A resilient reservoir was placed on the expiratory side of the subject, loosely coupled to a dedicated filter and exhaust system. The system was designed to keep both sides of the nonrebreathing valves at atmospheric pressure, unaffected by the subject's respiration. Tubing on the expiratory side was heated to approximately $\sim 37^{\circ}\text{C}$ to avoid condensation. The intake supply flow rate was monitored with a differential pressure gauge (Magnehelic, Dwyer Instruments, Michigan City IN) calibrated using a dry test meter (Singer American Meter Company Division, Wellesley MA).

Particle characterization was accomplished by determination of particle mass concentration, number concentration, and size distribution. Condensation particle counters (TSI, St Paul MN) and an electrical detection mobility analyzer (TSI) determined particulate number, surface area, and volume concentrations of the inspired and expired aerosols. The target mass concentrations for exposures were 10 and 25 $\mu\text{g}/\text{m}^3$. The mass concentrations were measured with a tapered element oscillating microbalance (TEOM, Rupprecht and Patachnick, Albany NY). The TEOM provided mass concentrations in micrograms per cubic meter at averaging times of 1 minute to 24 hours, with lower limits of mass determination on the order of 5 $\mu\text{g}/\text{m}^3$. The TEOM mass balance is sensitive to pressure changes within the system; these were controlled by the system design. At the low mass concentrations in these studies of UFP (10 to 25 $\mu\text{g}/\text{m}^3$), relatively long averaging times of several hours were required to provide accurate mass determinations. For this reason, we determined a standard curve of particle mass versus number concentration to validate TEOM mass measurements with estimates based on particle number. The mass concentration was monitored continuously on the inspired side of the system, but we relied on real-time monitoring of particle number to assure constant levels of particle generation during exposures.

Electronic integration (HPChem Integrating Software, Hewlett-Packard, Gaithersburg MD) of a pneumotachographic airflow transducer (E for M Co, White Plains NY) on the expiratory limb provided continuous measurements of tidal volume (V_T), respiratory rate, and minute ventilation.

To determine particle losses, a reciprocal pump was used to simulate respiration. A resting minute ventilation of 10 L/min was simulated using a volume of 800 mL at 12.5 cycles/min. Mild exercise (22 L/min) was simulated using a volume of 1200 mL at 18.3 cycles/min. Continuous upstream and downstream measurements of particle number and volume were determined for the whole system and for a respiratory valve alone. Mass losses were calculated using particle volume determined by the electrostatic classifier. During exercise simulation, losses were 0% for particles of 23.7-nm midpoint diameter and larger; maximum losses were 3.9% for 7.5-nm particles. At resting conditions, maximum losses were 13.2% for 7.5-nm particles.

Dilution air and air used in control exposures was filtered through charcoal and high efficiency particle air filters. Particle mass in the intake diluting air was undetectable, and particle numbers ranged from 0 to 10 particles/ cm^3 . The environmental chamber in which the exposures took place was maintained at a temperature of 21.8 ± 1.0 (SD) $^{\circ}\text{C}$ and a

relative humidity of $28.7\% \pm 10.5\%$. During both UFP and control air exposures, the inspired air temperature and humidity were $\sim 22^\circ\text{C}$ and $\sim 12\%$, respectively.

PARTICLE DEPOSITION

The total respiratory deposition fraction (DF) was calculated for both particle number and mass concentrations (Daigle et al 2003). Inspiratory and expiratory UFP number concentrations were measured continuously and recorded every 5 seconds during the exposure. Particle number concentration was then averaged for the periods at rest and exercise. Particle size distribution from the inspiratory circuit was determined before and just after each exposure. Particle size distribution from the expiratory circuit was measured during one rest period and one exercise period each hour. For computational simplicity, data on particle size distribution from the electrical detection mobility analyzer were grouped into 8 particle size bins with mid-point count median diameter from 8.7 to 64.9 nm (particle count median diameter ranging from 7.5 to 75.0 nm), which included more than 98% of the particles. The mean size-specific inspiratory particle concentration was determined by multiplying the average inspiratory number concentration by the percentage of particles in each size bin in the inspiratory circuit. The mean size-specific expiratory particle concentration was determined by multiplying the average expiratory number concentration by the percentage of particles in each size bin in the expiratory circuit. The correction factors for system losses were subtracted from the measured inspired concentrations and added to the measured expired concentrations. The particle number DF was then calculated by subtracting the corrected expiratory number concentration from the corrected inspiratory number concentration and dividing the difference by the corrected inspiratory number concentration.

The particle mass DF was calculated as follows (Chalupa et al 2002): inspired and expired particle volume (mass) concentrations were determined for each size bin from the scanning mobility particle sizer data. The percentage of inspired and expired particles by volume per bin was determined by dividing each bin volume concentration by the total volume concentration (sum of individual bins). The mean expired mass concentration was calculated by multiplying the ratio of the total expired volume concentration to the total inspired volume concentration by the measured (TEOM) inspired mass concentration. The inspired mass concentration for each bin was calculated as the product of the inspired volume percentage of particles in each bin and the mean inspired mass concentration from the TEOM. The expired mass concentration for each bin was calculated as the product of the expired volume

percentage for each bin and the calculated overall expired mass concentration. These mass data were corrected for system losses by multiplying each bin by the loss correction factor for that bin, then subtracting that product from the inspired data and adding to the expired data. Finally, a loss-corrected DF was calculated as the loss-corrected inspired mass concentration minus the loss-corrected expired mass concentration, divided by the loss-corrected inspired mass concentration.

Theoretical total respiratory DFs were calculated using three models: (1) ICRP (1994), (2) US National Council on Radiation Protection and Measurements (NCRP; 1997), and (3) the Multiple Path Particle Deposition Model (MPPDep, Version 1.11, July 1999, Chemical Industry Institute of Toxicology) (Cassee et al 2002). For the MPPDep model, predictions were calculated for each subject using measured functional residual capacity, respiratory frequency, and V_T at rest and during exercise. The following values were used for all subjects: upper respiratory tract volume, 50 mL; inspiratory:expiratory ratio, 1:2; and nominal particle density, 1.5 g/cm^3 . Mouth breathing was assumed for all test subjects.

PULMONARY FUNCTION

Spirometric measurements of FVC and FEV_1 were performed with a pneumotachograph interfaced with a micro-computer (model CPF-S, Medical Graphics, St Paul MN). Lung volumes (by plethysmography) and diffusing capacity for carbon monoxide (DL_{CO}) were measured in the clinical pulmonary function laboratory, using equipment from Morgan Scientific (Haverhill MA).

AIRWAY NITRIC OXIDE

Measurement of airway NO production provides a noninvasive method for assessing airway inflammation (American Thoracic Society 1999). We have developed methods for separately measuring NO production in the conducting (or upper) airways ($\dot{V}_{\text{U,NO}}$), and in the alveolar (or lower) airways ($\dot{V}_{\text{L,NO}}$) (Hyde et al 1997; Pietropaoli et al 1999). The technique involves determination of the single-breath diffusing capacity for NO (DL_{NO}) (Perillo et al 2001) and measurement of the partial pressure of exhaled NO (PE) at differing constant expiratory flow rates. During all measurements, exhalation against positive pressure closed the nasopharyngeal velum and thus prevented contamination of the expired airway gases with NO from the nasopharynx.

DL_{NO} was measured by inhaling 10 ppm NO from a bag-in-box apparatus. After holding breath for 2 seconds, the subject exhaled at a constant flow rate of 0.5 L/sec into the recording spirometer. Expiratory flow was kept constant

by applying fixed expiratory resistance and asking the subject to maintain a constant expiratory pressure by watching a manometer. The rate of change of NO concentration at increments of exhaled volume provided the data to calculate DL_{NO} (Perillo et al 2001).

For determination of $\dot{V}_{U_{NO}}$ and $\dot{V}_{L_{NO}}$, subjects inhaled NO-free air to total lung capacity from the bag-in-box apparatus, held breath for 10 seconds, and then exhaled at each of six different constant expiratory flow rates from 6 to 1300 mL/sec. These flow rates were achieved by changing the level of expiratory resistance before individual exhalations. During each exhalation, the subject maintained the same constant expiratory pressure of 10 cm H₂O. Constant expiratory pressure applied against different, fixed expiratory resistances during individual exhalations resulted in a variety of constant expiratory flow rates. Exhalations at each flow rate were performed in duplicate and averaged. The partial pressure of NO in the alveoli P_A and $\dot{V}_{U_{NO}}$ were determined by plotting the inverse of the more rapid flow rates on the x-axis versus the corresponding exhaled NO measurements on the y-axis. P_A was determined as the y-intercept (infinite flow rate) of this plot, and $\dot{V}_{U_{NO}}$ was calculated from its slope. $\dot{V}_{L_{NO}}$ was determined as follows (Pietropaoli et al 1999):

$$\dot{V}_{L_{NO}} = DL_{NO} (P_A).$$

NO concentrations in the exhaled breath were measured with a rapidly responding chemiluminescence NO analyzer (model 270B, Sievers, Boulder CO) operating at a sampling rate of 250 mL/min. The analyzer was calibrated daily using serial dilutions of a gas containing 229 ppb NO. Reference gas samples of NO-free air were obtained by passing compressed air from a cylinder containing less than 2 ppb of NO (Scott Specialty Gases, Plumsteadville PA) through a filter packed with potassium permanganate (Purafil, Thermoenvironmental Instruments, Franklin MA). To correct for instrument drift, all measurements were corrected by subtracting the average of NO-free air readings taken immediately before and after each NO determination.

BLOOD MARKERS OF COAGULATION AND INFLAMMATION

The soluble markers of inflammation, coagulation, and leukocytes measured in this study are shown in Table 1. Venous blood was collected in sodium citrate. Plasma was separated, divided into aliquots, and stored at -80°C prior to analysis. Fibrinogen, factor VII, and von Willebrand factor (vWf) were analyzed in the laboratory of the Vascular Medicine Program (Dr Victor Marder, Orthopedic Hospital, Los Angeles CA). The principle for the fibrinogen assay is

the relation of thrombin clotting time to fibrinogen concentration. This assay is based on a modification of the technique of Ellis and Stransky (1961). An STA Compact Analyzer (Diagnostica Stago, Paris, France) or AMAX CS190 Analyzer (Sigma, St Louis MO) was used to perform the assay. The principle for the factor VII activity assay is the degree of correction of a prothrombin time-based assay of an unknown sample for factor VII-deficient plasma. This assay is based on a modification of the technique of Owen and Aas (1951) and performed on the equipment used for the fibrinogen assay. The principle for the vWf antigen assay is the degree of agglutination of anti-vWf-coated latex particles by plasma containing vWf (Furlan, Perret et al 1985). The vWf antigen assay was performed on the same equipment used for the fibrinogen and factor VII assays.

IL-6, serum amyloid A, soluble ICAM-1, soluble L-selectin, P-selectin, and E-selectin were determined using commercial enzyme-linked immunosorbent assays (ELISAs) that were validated using dilution and add-back experiments. For these assays, venous blood was collected in heparin anticoagulant. Aliquots of plasma were stored at -80°C prior to analysis.

IMMUNOFLUORESCENCE ANALYSIS

Flow cytometry provided a sensitive method for evaluating changes in cell differential counts and for assessing changes in phenotype and expression of activation markers and adhesion molecules on blood leukocytes. The cell surface molecules studied were chosen to delineate changes in lymphocyte subsets, cell activation, and expression of adhesion molecules, all of which may reflect responses to inflammation and endothelial activation (Table 1).

Fresh heparinized whole blood was stained with fluorochrome-labeled monoclonal antibodies (Becton Dickinson, Mountain View CA). Leukocytes were stained with the appropriate monoclonal antibody conjugated to fluorescein isothiocyanate (FITC), and simultaneously stained with both CD14-phycoerythrin (PE) and CD45-peridinin chlorophyll protein (PerCp, a Becton Dickinson fluorochrome with minimal wavelength overlap with FITC or PE). This staining procedure permitted determination of the relative expression of adhesion molecules and other markers separately on PMNs, eosinophils, lymphocytes, and monocytes.

Lymphocyte subsets were characterized using combination gating and selective markers: $CD3^{+}4^{+}$ (T-helper), $CD3^{+}CD8^{+}$ (T-cytotoxic-suppressor), $CD3^{+}\gamma\delta TCR^{+}$ (T-null), $CD3^{-}CD19^{+}$ (B cells), and $CD3^{-}CD16^{+}/56^{+}$ (natural killer cells).

Table 1. Markers of Inflammation and Coagulation

Abbreviation	Name	Description
Soluble Markers		
IL-6	Interleukin 6	Inflammatory cytokine
SAA	Serum amyloid A	Acute phase protein
sICAM-1	Soluble intercellular adhesion molecule-1	Shed adhesion molecule
sCD40L	Soluble CD40 ligand	Marker of inflammation and cardiovascular risk
sVCAM-1	Soluble vascular cell adhesion molecule-1	Shed adhesion molecule
sE-Selectin	Soluble E-selectin	Shed adhesion molecule
sL-Selectin	Soluble L-selectin	Shed adhesion molecule
sP-Selectin	Soluble P-selectin	Shed adhesion molecule
	Factor VII	Coagulation factor
	Fibrinogen	Coagulation factor and acute phase protein
vWf	von Willebrand factor	Coagulation factor and marker of endothelial activation
Leukocyte Markers		
CD3		T lymphocytes
CD4		T-helper lymphocytes
CD8		T-cytotoxic lymphocytes
CD11a	Part of lymphocyte function-associated antigen-1 (LFA-1)	Part of adhesion molecule
CD11b	Part of Mac-1	Part of adhesion molecule (subunit of complement receptor 3)
CD16	Fc γ RIII	Low-affinity receptor for IgG
CD18		Part of adhesion molecule, complexed with CD11a or CD11b
CD19		B lymphocytes
CD23	Fc ϵ RII	Low-affinity IgE receptor
CD25	Tac	α -chain of IL-2 receptor, activation marker on lymphocytes
CD32	Fc γ RII	Low-affinity receptor for IgG
CD45RO		Memory T lymphocytes
CD49d	Part of VLA-4	Part of adhesion molecule
CD54	Intercellular adhesion molecule-1 (ICAM-1)	Adhesion molecule
CD62L	L-selectin	Adhesion molecule
CD64	Fc γ RI	High-affinity receptor for IgG
CD154	CD40 ligand	Marker of activation and inflammation
Cellular Expression		

RBCs were lysed and remaining cells were analyzed on a flow cytometer (FACScan, Becton Dickinson) equipped with a 15-mW argon ion laser at 488 nm. Ten thousand events were collected from each sample in list mode using Cell Quest software (Becton Dickinson). Forward scatter, 90° side scatter, and 3-color fluorescence (FITC, 530/30 nm band pass; PE, 585/42 nm band pass; and PerCP, 650 nm long pass filter) were measured. The appropriate isotype control antibodies were run with each experiment to determine appropriate gate settings. Each leukocyte subset was determined as a percentage of gated cells; then that percentage was multiplied by the concentration of leukocytes from the complete blood count to express each subset as a concentration of cells. Standardized fluorescent microbeads (Quantium 24P and 25P, Bangs Laboratories, Fishers IN) were run with each experiment. These data were fitted with an exponential curve: $f(x) = Ae^{Bx}$ where x was the

channel number and A and B were constants determined from the regression fit. This standard curve was then used to convert mean channel numbers for the various markers to molecules of equivalent soluble fluorochrome (MESF) (Gavras et al 1994). This provided a correction for minor day-to-day instrument variations in fluorescence detection.

LYMPHOCYTE CYTOKINE PROFILE

Blood samples were collected from asthmatic subjects (UPASTHMA group) before exposure and at 21 and 45 hours after exposure. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Hypaque gradient centrifugation (Cellgro, Herndon VA) and were frozen in liquid nitrogen. The cells were assayed for expression of IFN- γ and IL-4 using the enzyme-linked immunospot assay (ELISPOT) (Karulin et al 2000) in 96-well plates (MAIP

S4510, Millipore, Bedford MA); the plates were coated for 2 hours at room temperature with 50 μ L of antibodies specific for either IFN- γ (10 μ g/mL, 1-D1K, Mabtech, Nacka, Sweden) or IL-4 (5 μ g/mL, 8D4, BD Pharmingen, San Diego CA). After 2 hours at room temperature, the plates were washed with RPMI 1640 medium with 8% fetal bovine serum. Dilutions of previously frozen human PBMCs (thawed using a rapid-sequence method yielding 70–80% recovery of cells) were added to each well and stimulated with 20 μ g/mL of phytohemagglutinin A (PHA-p; Sigma).

After 36 to 40 hours at 37°C and 5% carbon dioxide, the plates were washed with phosphate-buffered saline (PBS) with 0.1% Tween 20 (PBST) (EM Science, Gibbstown NJ). The biotinylated second antibodies (IFN- γ , biotinylated 7-B6-1 [Mabtech] at 1 μ g/mL; for IL-4, biotinylated 25D2 [BD Pharmingen] at 2 μ g/mL) were added in PBST with 1% bovine serum albumin (PBSTB), and left for 1 to 2 hours at room temperature. The plates were washed in PBST, and alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove PA) in PBSTB was added at a 1:1000 dilution. After washing with PBST, the plates were developed using an alkaline phosphatase substrate (kit III SK-5300, Vector Laboratories, Burlingame CA). Plates were dried and counted using the autcount blue setting on the automated Immunospot Reader (Cellular Technology, Cleveland OH). ELISPOTs were corrected for background and control standards. Only wells with 30 to 250 ELISPOTs were included in the analysis.

SPUTUM INDUCTION

The cells obtained in induced sputum are representative of those cells found in the lower airways and can be used to measure airway inflammation noninvasively. Sputum was induced as part of the baseline determinations on the Screening Day. Subjects unable to produce an adequate sample ($> 0.7 \times 10^6$ cells with $\geq 70\%$ nonepithelial cells) were excluded from the study. Sputum was also induced 22 hours after each exposure. Only one sputum induction was performed after each exposure because sputum induction itself induces a transient airway inflammatory response that influences repeated measurements (Holz et al 1998; Nightingale et al 1998).

Inhalations of increasing concentrations (3%, 4%, and 5%) of nebulized hypertonic saline (Devilbiss Ultra-Neb 99 Ultrasonic Nebulizer, Somerset PA) induced sputum production, using a method modified from Pizzichini and colleagues (1996). Spirometry was used before and during the procedure to detect any bronchoconstriction.

The sputum was processed on ice using the plug selection technique (Pizzichini et al 1996). This technique allows separation of lower airway cells and associated fluids from

contaminating saliva and squamous epithelial cells from the upper airway. A portion of the sputum was incubated with dithiothreitol to disperse the cells and filtered through a 48- μ m nylon mesh. The cells were then counted in a hemocytometer in the presence of trypan blue. Cytospin slides were prepared for microscopic differential counts (~500 cells counted). The cell-free supernatant was divided into aliquots and stored at -80°C for subsequent analysis of IL-6 and IL-8 using ELISA.

CARDIAC MONITORING

Each subject had a continuous digital 12-lead ECG recording that was started prior to exposure and ended the following morning. During the continuous ECG recordings, 5-minute supine resting ECG recording sessions were performed to evaluate ECG parameters in a controlled setting unaffected by physical activity or body position. These 5-minute sessions occurred before exposure, immediately after exposure, and 3.5 hours and 21 hours after exposure. In the UPASTHMA group, subjects returned 45 hours after exposure for an additional ECG session. Each 5-minute segment was preceded by a 2-minute resting period.

In addition, a 5-minute ECG segment was identified during the final 15 minutes of exposure as well as during the night (systematically at 2 AM). For UPDOSE and UPASTHMA subjects, the monitoring segment was chosen from the last 15 minutes of exposure during the last exercise period and a 5-minute ECG session was identified during the night (2 AM) after exposure.

ECG ANALYSES

The 24-hour ECG recordings were analyzed using the H-Scribe Mortara System (Mortara Instruments, Milwaukee WI). After automatic beat annotation, verified by a technician, the ECG analysis was completed using MISHA, a research version of Mortara's program, yielding quantitative measures of several ECG parameters, including beat-to-beat (RR) intervals, lead-specific and beat-to-beat ST segment levels, T wave amplitude, and T wave complexity. The QT interval was measured as the longest interval for each beat. Then an 8-beat-segment average was computed for QTc, corrected for heart rate with the Bazett formula (Bazett 1920). Subsequently, the means of 8-beat averages from a 5-minute period were calculated. The measurements of QTc interval durations were also performed manually in lead II. For measurements of T wave amplitude, original ECG leads I, II, and V1 to V6 were used, and the median value from these 8 original ECG leads was taken for each beat and averaged over 5 minutes. T wave complexity, describing the morphology of the T wave, was measured in each beat by principal component analysis based on all

12 leads and averaged over the 5-minute period (Priori et al 1997). Variability of T wave complexity was measured as a standard deviation over the 5-minute period and did not require correction for heart rate. ST segment analysis was focused on leads II, V2, and V5, and the median ST segment level over the 5-minute period was used.

The time-domain parameters of heart rate variability, SDNN (standard deviation of normal-to-normal (sinus beat intervals) and rMSSD (root mean square of successive differences in NN intervals), were calculated for each 5-minute segment of interest and for a 16-hour period starting 3.5 hours after exposure. The following frequency-domain parameters of heart rate variability were computed for each 5-minute segment using fast Fourier technique: high frequency power (0.15–0.40 Hz), low frequency power (0.04–0.15 Hz)—both expressed in normalized units—and the ratio of the two (Malik and Camm 1995).

This combination of analyses yielded information regarding autonomic nervous system effects (which might occur via direct reflexes from airways and/or inflammatory responses), myocardial vulnerability to arrhythmia, and the underlying health of the myocardial substrate (Zareba et al 2001). In addition, arrhythmias were quantitated as the total of atrial premature contractions and ventricular premature contractions for the entire recording period in each protocol.

DATA HANDLING AND STATISTICAL METHODS

UPREST and UPASTHMA

These studies utilized a standard, two-period crossover design in which each subject was exposed to both particles and filtered air. Equal numbers of males and females were included because some effects of particle exposure might depend on gender. The order of presentation was randomized separately for each gender, with half of each group of subjects receiving each of the two possible orders first (ie, UFP or air). The time between the two exposures (the washout period) was sufficiently long to expect carryover effects from the previous exposure to be minimal or nonexistent.

The standard analysis for continuous endpoints is a repeated-measures analysis of variance (ANOVA). In this analysis, order of presentation and gender are between-subject factors, while exposure substance, period (eg, first visit, second visit), and time (when there are repeated measurements after each exposure) are within-subject factors. The analyses addressed study period and carryover effects although the latter were expected to be minimal (because of the nature of the exposures and the length of the washout period). In subjects whose carryover effects were

highly significant, first exposure data were examined separately (Jones and Kenward 1989).

For some endpoints, repeated measurements were made at uniform intervals after each exposure. In these cases, the ANOVA included tests for the effect of time as well as interactions with other effects in the model. The ANOVA model and its interpretation have been discussed by Wallenstein and Fisher (1977). Each ANOVA included an examination of residuals as a check on the required assumptions of normally distributed errors with constant variance. If these assumptions were not satisfied, data transformations (for example, square-root transformation for cell counts) were considered. In the special case that only one measurement was made in each exposure period, the ANOVA simplifies to three different *t* tests, for exposure substance, period, and carryover effects (Jones and Kenward 1989). In this case nonparametric tests, such as the Wilcoxon rank sum test, can be used if the observations are not normally distributed.

UPDOSE

The study utilized a three-period crossover design in which each subject had exposures to low ($10 \mu\text{g}/\text{m}^3$) and high ($25 \mu\text{g}/\text{m}^3$) concentrations of particles, as well as air. For safety considerations, each subject received the $10 \mu\text{g}/\text{m}^3$ UFP exposure before the $25 \mu\text{g}/\text{m}^3$ UFP exposure. There were then three possible exposure sequences depending on the place of air exposure in the sequence. Equal numbers of subjects were randomly assigned to each exposure sequence. The statistical analysis was based on the usual ANOVA model for crossover designs (Jones and Kenward 1989). That is, the ANOVA included effects for exposure substance (3 levels) and period (3 levels). Because of the nature of the design, carryover effects were limited to an effect for having been previously (in the period just before the present one) exposed to $10 \mu\text{g}/\text{m}^3$ UFP (all subjects) and to $25 \mu\text{g}/\text{m}^3$ UFP (4 subjects). A random subject effect was included in the model. Interactions between subjects and both substance and period were included as random effects. Because the data were not balanced for this model, parameters for the model were estimated by the method of maximum likelihood (Searle et al 1992). Residuals were examined as a check on the assumptions, and when necessary, data transformations were considered. In some cases a logarithmic or square root transformation was necessary to stabilize the variance.

A *P* value of <0.05 was required for statistical significance. Because of the multifaceted nature of these studies, a fairly large number of significance tests were performed. Our strategy for interpretation was to rely on the pattern of

the significance tests, and specifically on concordant effects among biologically related variables, rather than individual *P* values. Data are shown as mean \pm SE unless otherwise indicated.

In terms of the statistical formalities of the ANOVA, a carryover effect is the same as (is confounded with) a treatment by period interaction, which allows the treatment effect to differ between the two periods; it is the interpretation that differs. Specifically, a carryover effect represents a residual effect of the treatment in the first period that is still present in the second period of observation. Carryover effects are the bane of crossover designs because their presence makes it impossible for treatment effects to be assessed using within-subject comparisons.

In designing a crossover study, it is important to choose a washout period of sufficient length that residual treatment effects from the first period will not be present in the second; this requires the use of treatments whose effects will indeed disappear with time. These considerations are essential in planning the study because the ability of the crossover design to detect such residual treatment effects can be limited. In the present studies the a priori hypothesis was that any effects of exposure to UFP would be transient and that the washout period was long enough that any residual effects of particle exposure would disappear well before subjects returned for the second visit.

An ANOVA indicates that such carryover effects are either present or not on a group basis. The interpretation of such effects depends primarily on the particular endpoint and well as the presence of such effects for related endpoints. In the present studies the pattern of carryover effects did not indicate any systematic persistence in the second period.

RESULTS

PARTICLE COMPOSITION

To confirm that the UFP were composed predominantly of elemental carbon, UFP were collected on Teflon filters and sent to the laboratory of Dr Glen Cass for analysis. The particles collected in this manner showed a surprisingly high content of organic carbon—approximately 22%. Others have also found organic compounds in particles produced by the Palas generator (McDonald et al 2001). For possible sources of the organic carbon, we considered the Palas generator itself. To eliminate any sources of organic material within the generator, all plastic parts, including the internal combustion chamber, the collars holding the

graphite electrodes, and the tubing were replaced with either Teflon, ceramic, or stainless steel materials. UFP from this rebuilt generator were again submitted to Dr Cass's laboratory for analysis, but the organic content was still high—approximately 24%.

We then considered whether the organic content was an artifact of collecting UFP on the filters. UFP have a great deal of surface area and thus can adsorb materials from ambient air. Possibly the handling and transport of filters facilitated adsorption of organic materials from ambient air.

To test this possibility, we utilized a new technique for single particle analysis of composition developed by Dr Kim Prather and colleagues (Su et al 2004), using aerosol time of flight mass spectrometry (ATOFMS). Dr Prather found that UFP analyzed immediately after emission from the rebuilt generator were predominantly elemental carbon. The single-particle mass spectra of the UFP were nearly identical to elemental carbon particles from gasoline- and diesel-powered emission sources measured previously: 82% of the particles showed short-chain fragmentation patterns of C1, C2, and C3; 14% showed longer chain fragmented peaks. Thus, 96% of the particles consisted of elemental carbon (Dr K Prather, personal communication). We therefore concluded that our subjects inhaled particles consisting of elemental carbon and that these were similar in composition to ambient elemental carbon particles.

SUBJECT CHARACTERISTICS

A total of 40 subjects were studied, 20 male and 20 female. Subjects were 18 to 40 years of age except for two subjects in the UPREST group, aged 42 and 52 years. Three subjects were Asian, one African American, and the rest white. Sixteen subjects met the criteria for mild asthma. All subjects provided written, informed consent. Table 2 shows the subject characteristics and baseline pulmonary function (at screening) for each of the three clinical studies. Table 3 provides further characterization of the asthmatic subjects. Most of the asthmatic subjects were atopic (15 of 16), and most (11 of 16) did not use inhaled steroids, long-acting bronchodilators, or leukotriene inhibitors.

PARTICLE DEPOSITION

Table 4 shows the actual average particle number and mass concentration for each 2-hour exposure. Figure 2 shows the particle size distribution for inspired and expired aerosols from one subject from each exposure protocol.

Table 5 shows the deposition data for all three study groups. In the UPREST group, the individual total DF by particle number ranged from 0.46 to 0.79. The highest DF

Table 2. Subject Characteristics and Baseline Pulmonary Function

	UPREST	UPDOSE	UPASTHMA
Age (yr) ^a	30.1 ± 8.9	26.9 ± 5.8	23.0 ± 2.7
M/F	6/6	6/6	8/8
Oral contraceptive use (females only)	6/6	4/6	5/8
Height (cm) ^a	167 ± 8	173 ± 10	170 ± 8
Weight (kg) ^a	70 ± 13	79 ± 11	80 ± 15
FEV ₁ (% predicted) ^a	103.8 ± 8.0	106.3 ± 16.6	97.6 ± 20.2
FVC (% predicted) ^a	99.9 ± 8.3	103.5 ± 15.6	106.2 ± 14.5
FEV ₁ /FVC (% ^a)	87.9 ± 4.2	86.8 ± 5.2	77.8 ± 6.9
FEF ₂₅₋₇₅ (% predicted) ^a	113.9 ± 24.2	106.3 ± 22.0	77.6 ± 29.7
DL _{CO} (% predicted) ^a	90.9 ± 11.5	89.8 ± 18.2	99.7 ± 12.5

^a Data are mean ± SD.

Table 3. Subjects with Asthma

Characteristic	Number of Subjects (<i>n</i> = 16)
Environmental allergies	12
Increased IgE	11
Atopic (allergies or increased IgE)	15
Medications	
Albuterol only	12
Inhaled steroid	4
Long-acting β-agonist	2
Leukotriene inhibitor	2
FEV ₁ PD ₂₀ (mg/mL) (<i>n</i> = 11) ^a	4.3 ± 5.5
Bronchodilator response (% increase in FEV ₁) (<i>n</i> = 5) ^a	19 ± 4

^a Data are mean ± SD. PD₂₀ = 20% decrease in FEV₁.

was seen with the smallest particles. The DF decreased for particle sizes up to 48.7 nm and then leveled off (Table 5). The expired aerosol showed a very slight increase in count mean diameter, most likely as a result of the more efficient deposition of the smaller particles.

In the initial exposures in the UPDOSE group, usable data were available for 7 of the 12 subjects. We found in the first few subjects that measurements of expiratory particle concentrations were inaccurate because of pressure changes associated with the subject's breathing during exercise. Repositioning the expiratory sampling port resolved this problem. No significant differences in DF were measured at 10 μg/m³ UFP or 25 μg/m³ UFP; therefore, these data were averaged. The individual total DF by particle number ranged from 0.55 to 0.66 at rest and 0.76 to 0.88 during exercise. The at-rest results were similar to those found in the UPREST study, and DF increased with exercise in all size bins. Figure 3 shows the DF for the 12 subjects in the UPREST group and the 7 subjects from the UPDOSE group with usable deposition data while breathing at rest. The DF during exercise also reached a plateau in the larger size bins (Table 5 and Figure 3).

Table 4. Particle Characterization^a

	UPREST	UPDOSE (Low)	UPDOSE (High)	UPASTHMA
Target mass (μg/m ³)	10	10	25	10
Measured mass (μg/m ³)	10.0 ± 2.1	14.0 ± 4.0	28.5 ± 5.1	11.1 ± 3.1
Number (× 10 ⁶ /cm ³)	1.88 ± 0.08	2.04 ± 0.06	6.96 ± 0.10	2.20 ± 0.10
Count median diameter (nm)	27.3 ± 2.5	25.2 ± 1.7	26.5 ± 1.5	23.1 ± 1.6
Geometric standard deviation	1.6 ± 0.0	1.6 ± 0.0	1.6 ± 0.0	1.6 ± 0.0

^a Data are mean ± SD.

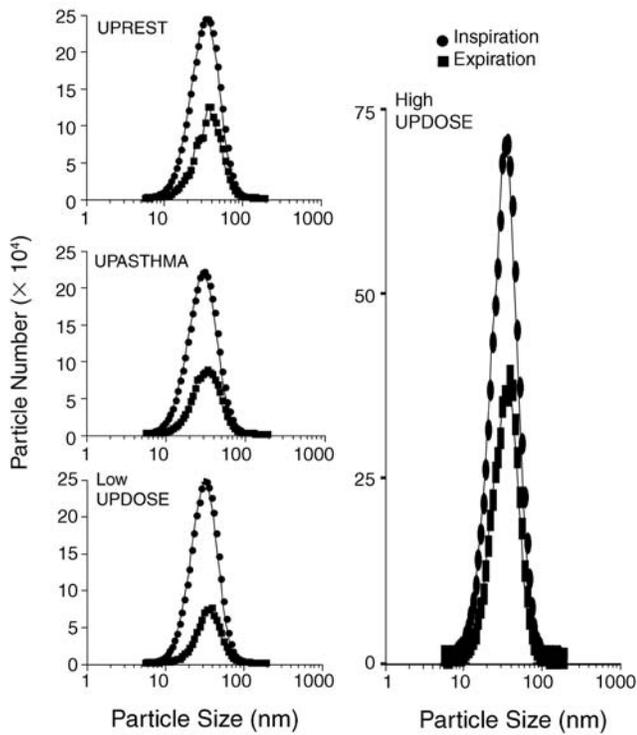


Figure 2. Inspiratory and expiratory particle size distributions for one subject each in the low UFP exposures (UPREST, UPDOSE, and UPASTHMA) and high UFP exposure (UPDOSE).

No significant gender differences were found. In UPREST, the particle number DF was 0.68 ± 0.13 (mean \pm SD) for men ($n = 6$) and 0.65 ± 0.12 for women ($n = 6$) ($P = 0.70$). The corresponding mass DFs were 0.60 ± 0.13 and 0.59 ± 0.14 , respectively ($P = 0.89$). There were not enough subjects with usable data in the UPDOSE group to make gender comparisons. Analysis of all 19 exposures at rest from both UPREST and UPDOSE groups also showed no significant gender difference.

The predicted overall DF was determined using three models. For the ICRP and NCRP models, input data were chosen to match our exposure conditions as closely as model parameters would allow. For the MPPDep model, respiratory and lung function data (including functional residual capacity, tidal volume, and respiratory frequency) were entered for each of the subjects in these studies. Figure 3 compares the experimental DFs with predictions using the MPPDep model. Overall, the models predicted very little increase in DF with exercise, and the experimental data significantly exceeded model predictions during exercise (Figure 3). For example, using the MPPDep model for 26 nm particles, breathing at 18 breaths/min with a V_T of 722 mL, the predicted total DF is 0.624. With 25 breaths/min and V_T of 1680 mL, the predicted DF increases slightly to 0.650. The tracheobronchial DF decreases from 0.232 to 0.170, and the alveolar DF increases from 0.333 to 0.428.

Table 6 shows the rest and exercise values for the number DF and total particle number deposition over a 1 hour. The combined effect of increased minute ventilation and an

Table 5. Mean Particle DF by Particle Size in Three Protocols^a

Midpoint Diameter (range, nm)	UPREST (12 subjects)	UPDOSE ^b (7 subjects)		UPASTHMA (16 subjects)	
	Rest	Rest	Exercise	Rest	Exercise
8.7 (7.5–10.0)	0.80 ± 0.09	0.74 ± 0.07	0.94 ± 0.02	0.84 ± 0.03	0.93 ± 0.02
11.6 (10.0–13.3)	0.78 ± 0.08	0.74 ± 0.06	0.91 ± 0.02	0.83 ± 0.04	0.91 ± 0.03
15.4 (13.3–17.8)	0.74 ± 0.09	0.68 ± 0.05	0.89 ± 0.03	0.80 ± 0.04	0.89 ± 0.03
20.5 (17.8–23.7)	0.70 ± 0.12	0.63 ± 0.05	0.84 ± 0.04	0.77 ± 0.06	0.86 ± 0.04
27.4 (23.7–31.6)	0.66 ± 0.13	0.60 ± 0.05	0.79 ± 0.05	0.72 ± 0.07	0.82 ± 0.05
36.5 (31.6–42.2)	0.59 ± 0.13	0.58 ± 0.05	0.75 ± 0.05	0.68 ± 0.08	0.77 ± 0.06
48.7 (42.2–56.2)	0.55 ± 0.14	0.58 ± 0.05	0.72 ± 0.07	0.66 ± 0.08	0.75 ± 0.06
64.9 (56.2–75.0)	0.55 ± 0.13	0.67 ± 0.07	0.72 ± 0.09	0.65 ± 0.09	0.73 ± 0.07
Total DF by particle number	0.66 ± 0.12	0.63 ± 0.03	0.84 ± 0.04	0.76 ± 0.05	0.86 ± 0.04
Total DF by particle mass	0.59 ± 0.13	0.60 ± 0.04	0.76 ± 0.06	0.69 ± 0.07	0.79 ± 0.06

^a Data are mean \pm SD.

^b DFs were usable for only 7 of the 12 subjects. Because they were similar, the DFs for 10 and 25 $\mu\text{g}/\text{m}^3$ were averaged together.

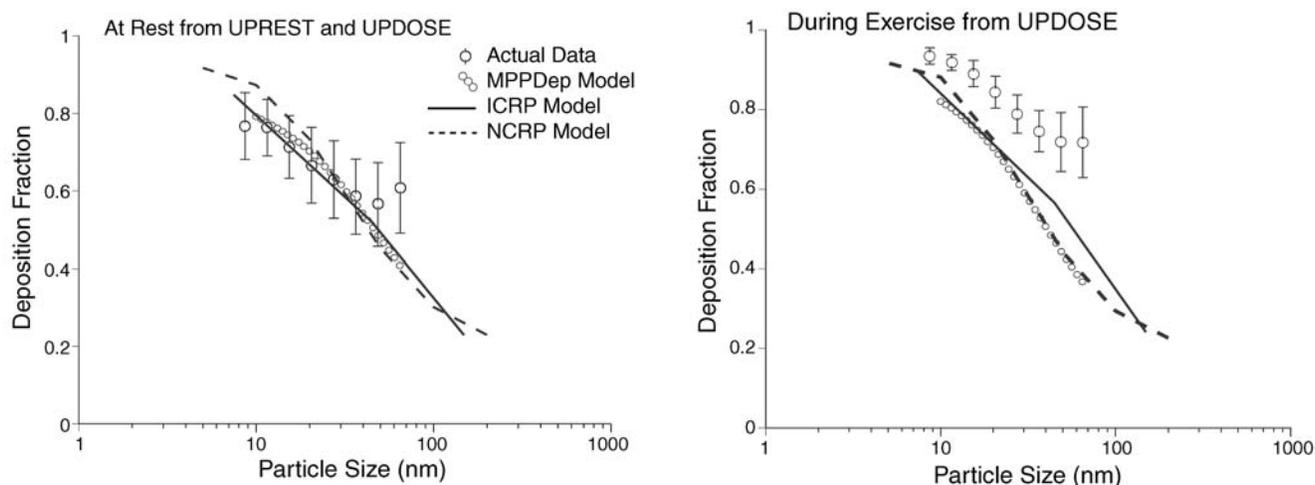


Figure 3. Observed and predicted total respiratory deposition of UFP ($10 \mu\text{g}/\text{m}^3$) in 19 healthy subjects at rest (UPREST and UPDOSE) and in 7 healthy subjects during moderate exercise (UPDOSE). Particle deposition was calculated separately for 8 particle size bins. **At rest.** Actual experimental data shown are the mean \pm SD. The predicted deposition for these subjects was calculated from the MPPDep (Chemical Industry Institute of Toxicology 1999). Total ICRP models (1994) were based on habitual mouth-breathing reference workers breathing at $1.2 \text{ m}^3/\text{hr}$ (Table F.1, page 416); NCRP models (1997) were based on the sum of naso-oro-pharyngo-laryngeal, tracheobronchial, and pulmonary deposition, (V_T 770 cm^3 , breathing frequency 13; Table 5.2, page 68). **At exercise.** ICRP predictions (1994) were based on habitual mouth-breathing reference workers breathing at $1.7 \text{ m}^3/\text{hr}$ (Table F.1, page 417); NCRP predictions (1997) based on a V_T of 2400 cm^3 and breathing frequency of 25.

Table 6. Comparison of Number DF and Total Particles Deposited in Healthy (UPDOSE) and Asthmatic (UPASTHMA) Subjects Exposed to $10 \mu\text{g}/\text{m}^3$ of UFP for 2 Hours^a

	UPDOSE		UPASTHMA	
	Rest	Exercise	Rest	Exercise
Number DF	0.63 ± 0.03	0.84 ± 0.04	0.76 ± 0.05	0.86 ± 0.04
Mass DF	0.59 ± 0.03	0.77 ± 0.05	0.69 ± 0.07	0.79 ± 0.06
Total number deposited ($\times 10^{12}$)	0.79 ± 0.24	3.35 ± 0.90	1.22 ± 0.23	4.79 ± 1.19
Total mass deposited (μg)	3.83 ± 0.39	15.31 ± 0.84	5.83 ± 2.37	22.56 ± 8.96

^a Mean \pm SD.

increase in DF with exercise increased particle number deposition more than fourfold for the healthy subjects over those with asthma.

For asthmatic subjects at rest, DF was significantly greater than for healthy subjects at rest, and DF increased further with exercise (Table 5). The number DF during breathing at rest was 0.76 ± 0.05 for subjects with asthma and 0.63 ± 0.03 for healthy subjects ($P < 0.001$). With exercise, DF was not significantly different between healthy and asthmatic subjects.

RESPIRATORY SYMPTOMS AND PULMONARY FUNCTION

There was no evidence for significant or meaningful effects on pulmonary function in these studies, even in

subjects with asthma. Figure 4 shows the changes from baseline for FEV_1 in UPDOSE and UPASTHMA subjects. ANOVA showed marginal significance ($P = 0.04$) for an interaction between exposure and gender for FEV_1 in the UPDOSE study; however, the changes were small and no concentration response was evident.

Exposure to UFP appeared to cause a small but statistically significant reduction in minute ventilation under certain conditions (Table 7). In UPDOSE subjects, the minute ventilation decreased with UFP exposure only during exercise, with a greater effect in males (UFP main effect, $P = 0.030$; exposure \times gender interaction, $P = 0.025$). In UPASTHMA subjects, minute ventilation decreased with UFP during breathing at rest (main effect, $P = 0.007$) with no gender difference. A small mean decrease was seen during

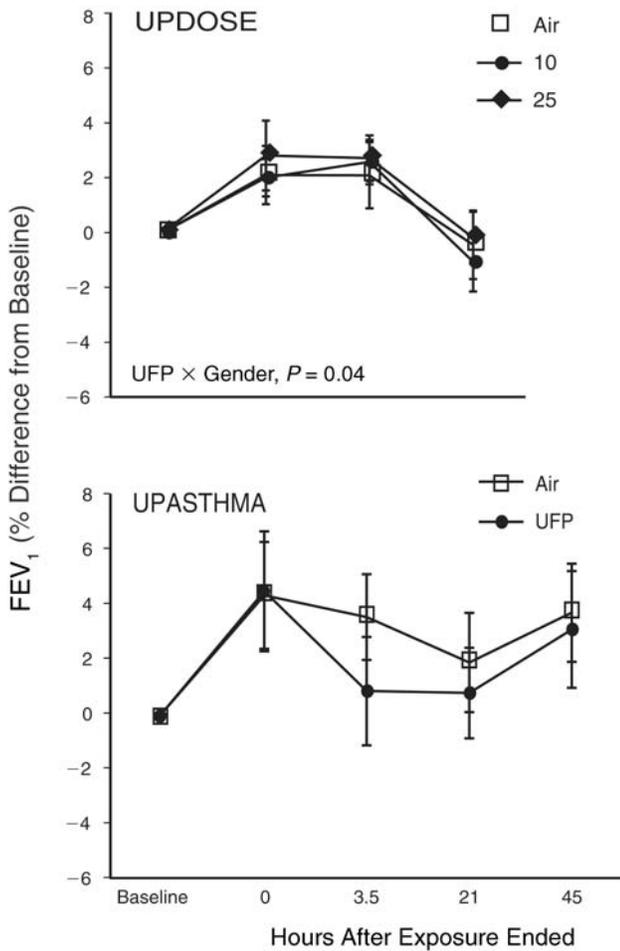


Figure 4. Change in FEV₁ between baseline and end of monitoring period (UPDOSE and UPASTHMA). Data represent means ± SE.

exercise as well, but was not statistically significant. For subjects with asthma, this reduction in minute ventilation was accounted for by a small but significant reduction in tidal volume ($P = 0.01$) with no change in respiratory rate. These findings suggest the possibility of very mild irritant effects of the UFP exposures.

UFP exposure had no measurable effect on respiratory symptoms. Total symptom scores and individual sums of scores for respiratory symptoms were similar with air and UFP exposures. Subjects were not able to identify the exposure atmosphere more often than expected by chance.

VITAL SIGNS AND OXYGEN SATURATION

No significant effects on systolic or diastolic blood pressure or heart rate were found in any of the studies. The UPREST data showed a marginally significant interaction of time, exposure, and gender on oxygen saturation ($P = 0.047$), with saturation slightly increased after UFP exposure relative to air in males. In the UPDOSE group, a significant exposure-gender interaction was apparent for an effect on oxygen saturation ($P = 0.02$) with oxygen saturation decreasing slightly in females at 21 hours after exposure (only with the higher concentration of UFP) (Figure 5). Oxygen saturation had no significant effect in the UPASTHMA group. We concluded that no convincing effects on oxygen saturation occurred with exposure to UFP at these concentrations.

MARKERS OF LOCAL AIRWAY INFLAMMATION

Airway inflammation was assessed using markers found in induced sputum and measurements of airway production of NO. Tables 8 and 9 compare markers of airway inflammation and airway NO production in healthy subjects and asthmatic subjects prior to exposure (baseline). As expected, sputum from subjects with asthma contained

Table 7. Respiratory Measurements During Exposure^a

	UPDOSE						UPASTHMA			
	Air		10 µg/m ³ UFP		25 µg/m ³ UFP		Air		10 µg/m ³ UFP	
	Rest	Exercise	Rest	Exercise	Rest	Exercise	Rest	Exercise	Rest	Exercise
\dot{V}_E (L/min)	11.9±3.3	39.1±5.1	11.3±2.0	34.2±8.5	10.9±2.1	34.5±10.0	13.8±2.3	43.4±9.5	12.8±1.8	41.9±9.0
V_T (L)	0.71±0.25	1.54±0.63	0.71±0.25	1.42±0.50	0.63±0.14	1.34±0.38	0.76±0.13	1.74±0.44	0.74±0.12	1.71±0.46
Respiration ^b	17.3±3.9	26.6±6.3	17.0±3.7	25.4±5.7	17.7±3.9	26.7±6.8	18.1±1.9	25.3±3.3	17.4±2.3	25.1±3.7

^a Data are mean ± SD.

^b Breaths per minute.

a higher percentage of eosinophils. In addition, $\dot{V}_{U_{NO}}$ was more than fivefold higher in asthmatic subjects compared with healthy subjects ($P = 0.014$). In contrast, P_A was more than 50% lower in subjects with asthma ($P = 0.004$), and $D_{L_{NO}}$ was also reduced ($P = 0.031$).

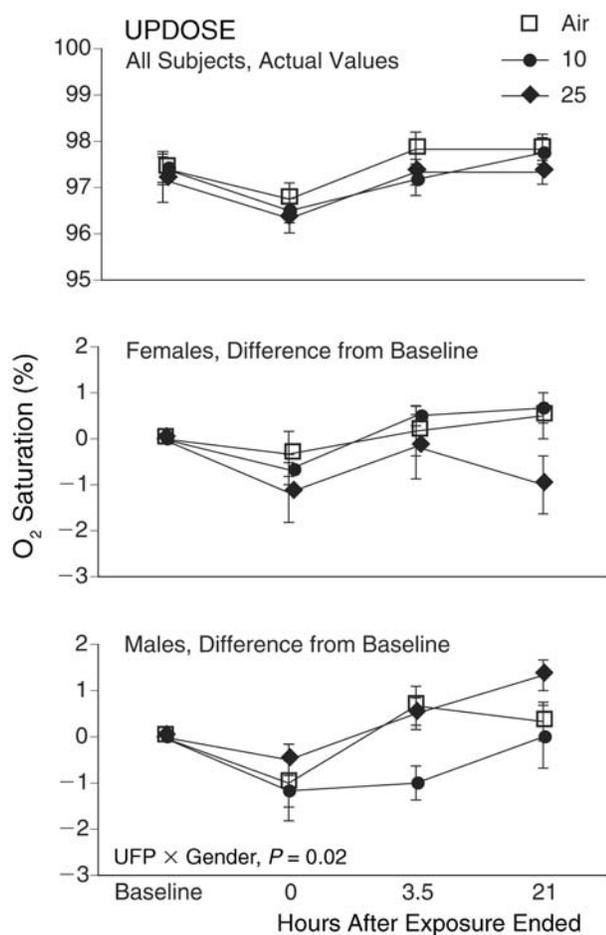


Figure 5. Blood oxygen saturation, UPDOSE. Actual values for all subjects and differences from baseline for females and males. Baseline values: air, $97.42\% \pm 0.36$; $10 \mu\text{g}/\text{m}^3$, $97.42\% \pm 0.31$; $25 \mu\text{g}/\text{m}^3$, $97.17\% \pm 0.49$. Data represent mean \pm SE.

Table 8. Cell Differential Counts from Sputum of Healthy Subjects (UPDOSE) and Asthmatic Subjects (UPASTHMA) at Screening^a

	UPDOSE	UPASTHMA	P Value
Alveolar macrophages	49.3 ± 6.3	48.9 ± 6.3	0.73
Lymphocytes	1.7 ± 0.5	1.4 ± 0.4	0.406
PMNs	40.5 ± 8.0	19.0 ± 4.8	0.019
Eosinophils	0.4 ± 0.3	10.2 ± 4.6	0.047
Epithelial cells	8.1 ± 3.7	20.5 ± 4.5	0.019

^a Data are percent of total cells (mean \pm SE).

No convincing evidence was found to suggest a significant airway inflammatory response to carbon UFP exposure in any of the three groups. UFP exposure had no effect on total sputum cell recovery (Figure 6). UFP exposure was associated with a small, but statistically significant,

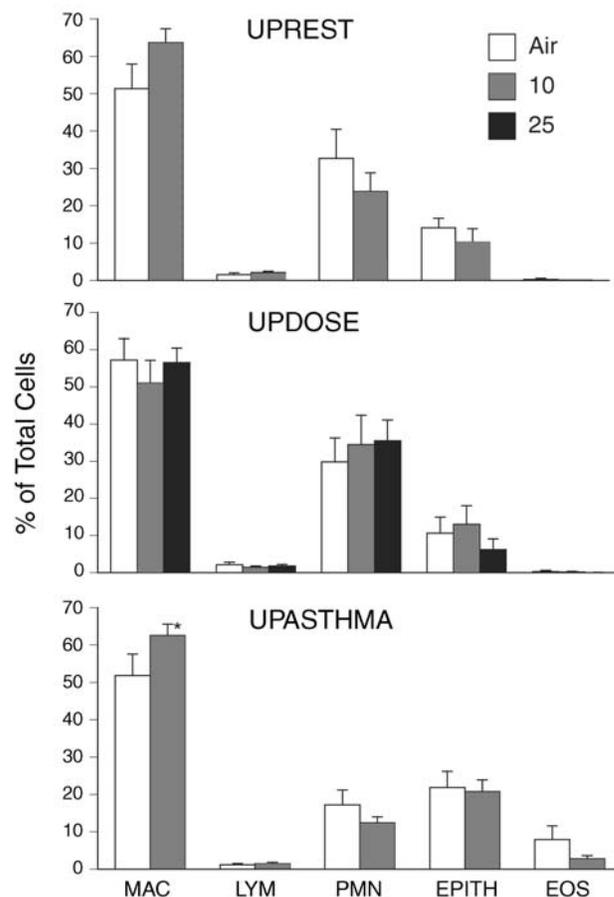


Figure 6. Sputum cell differential counts (21 hours after exposure ended) expressed as percentage of total cells. Data represent means \pm SE. * UFP exposure was associated with a significant increase in percentage of alveolar macrophages in subjects with asthma ($P = 0.019$). MAC = macrophages, LYM = lymphocytes, PMN = polymorphonuclear leukocytes, EPITH = epithelial cells, and EOS = eosinophils.

Table 9. Airway NO Production Before Exposure in Healthy Subjects (UPDOSE) and Asthmatic Subjects (UPASTHMA)^a

	UPDOSE	UPASTHMA	P Value
P_A (mm Hg $\times 10^{-6}$)	1.6 ± 0.2	0.74 ± 0.24	0.004
$\dot{V}_{U_{NO}}$ ($\mu\text{L}/\text{min}$)	0.06 ± 0.01	0.32 ± 0.09	0.014
$D_{L_{NO}}$ ($\text{mL}/\text{min} \times \text{mmHg}$)	166.3 ± 9.5	191.6 ± 19.0	0.246
$\dot{V}_{L_{NO}}$ ($\mu\text{L}/\text{min}$)	0.26 ± 0.02	0.14 ± 0.05	0.031

^a Data are mean \pm SE.

increase in the percentage of alveolar macrophages in subjects with asthma ($P = 0.019$). UFP exposure was not associated with significant changes in other cell types. In all 3 groups, concentrations of inflammatory cytokines IL-6 and IL-8 in the sputum sol were unaffected by UFP exposure. There were no convincing UFP effects on alveolar or conducting airway NO production in any group. In the UPDOSE group, the ANOVA suggested a significant interaction between UFP exposure and gender for \dot{V}_{LNO} ($P = 0.009$) with a slight reduction in males 3.5 hours after exposure to 25 $\mu\text{g}/\text{m}^3$ UFP compared with air and 10 $\mu\text{g}/\text{m}^3$ UFP. DL_{NO} increased slightly with exposure to 25 $\mu\text{g}/\text{m}^3$ UFP, compared with exposure to air ($P = 0.022$). There were no significant differences in NO production in UPASTHMA subjects. Overall, there was no evidence for increased airway inflammation in response to UFP exposure in either healthy subjects or subjects with asthma.

MARKERS OF SYSTEMIC INFLAMMATION

Plasma concentrations of the inflammatory cytokine IL-6, CD40 ligand, plasma nitrite/nitrate (NO_2/NO_3) levels, and the soluble adhesion molecules E-, P-, and L-selectin, VCAM-1, and ICAM-1 were measured as indicators of systemic or vascular inflammation. Serum amyloid A concentration assessed the presence of an acute phase response. At baseline, subjects with asthma showed higher concentrations of soluble VCAM-1 and L-selectin only, compared with healthy subjects (Table 10).

Table 10. Markers of Systemic and Vascular Inflammation in Healthy Subjects (UPDOSE) and Asthmatic Subjects (UPASTHMA) Before Exposure^a

	UPDOSE	UPASTHMA	<i>P</i> Value
IL-6 (pg/mL)	1.3 ± 0.2	1.6 ± 0.2	0.263
sICAM-1 (ng/mL) ^b	201.2 ± 19.6	158.8 ± 6.6	0.06
sCD40L (ng/mL) ^b	0.89 ± 0.3	3.9 ± 1.6	0.086
sVCAM-1 (ng/mL) ^b	297.2 ± 24.0	367.6 ± 14.9	0.022
sE-Selectin (ng/mL) ^b	43.8 ± 4.4	44.9 ± 4.5	0.868
sL-Selectin (ng/mL) ^b	830.3 ± 60.0	1123.3 ± 46.5	0.001
sP-Selectin (ng/mL) ^b	51.2 ± 5.1	49.1 ± 3.5	0.74
NO_2/NO_3 (μM)	29.8 ± 3.6	21.1 ± 2.6	0.065
SAA (ng/mL × 10 ³)	23.9 ± 13.7	8.1 ± 3.7	0.287
Factor VII (% of normal)	104.1 ± 13.4	97.3 ± 3.9	0.637
Fibrinogen (mg/dL)	256.2 ± 25.0	250.1 ± 9.0	0.824
vWf (% of normal)	95.8 ± 13.5	117.4 ± 16.7	0.324

^a Data are mean ± SE. SAA = serum amyloid A.

^b Soluble molecules.

In UPDOSE subjects, IL-6, soluble ICAM-1, and soluble VCAM-1 showed significant changes over time, possibly related to the effects of exercise during exposure and/or diurnal factors. IL-6 showed a marginally significant exposure-gender interaction ($P = 0.036$, Figure 7), similar to that seen with fibrinogen and vWf. IL-6 increased after UFP exposure in both males and females, likely in response to exercise. This IL-6 increase was blunted in association with UFP exposure (in males only) in a concentration-response fashion. A statistically significant main effect of UFP on soluble VCAM-1 concentrations was indicated by the ANOVA ($P = 0.032$); however, the change was small, without a concentration-related response (Figure 8). Soluble CD40 ligand also showed significant changes related to UFP exposure (Figure 9), with a UFP concentration-related decrease UFP 21 hours after exposure. However,

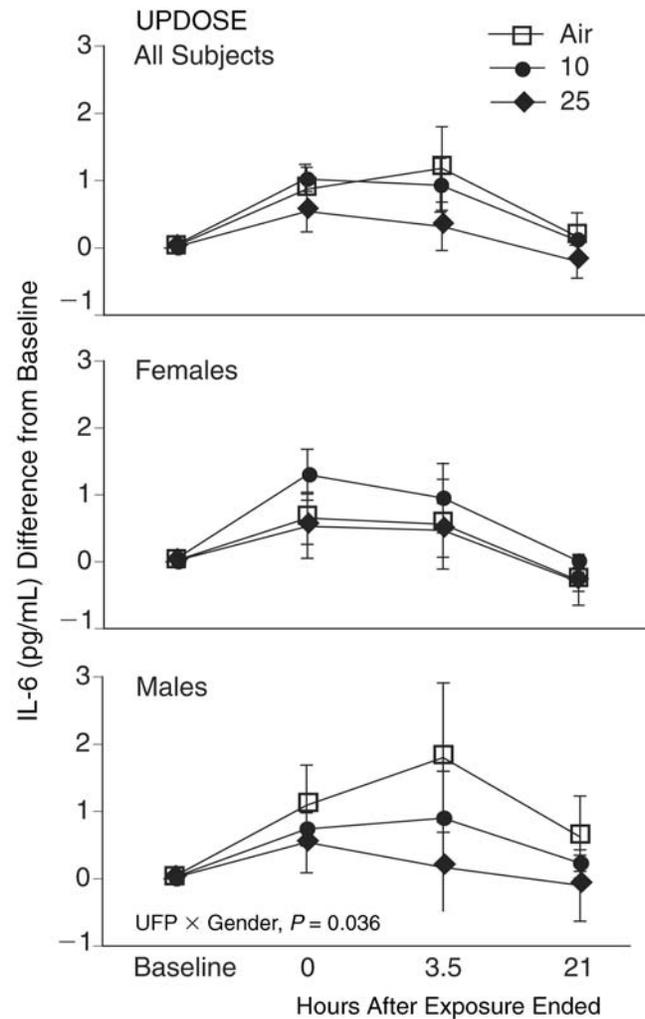


Figure 7. Plasma concentrations of IL-6, UPDOSE. Differences from baseline for all subjects, females, and males. Baseline values: air, 1.52 ± 0.44 pg/mL; 10 $\mu\text{g}/\text{m}^3$, 1.13 ± 0.19 pg/mL; 25 $\mu\text{g}/\text{m}^3$, 1.33 ± 0.21 pg/mL. Data represent means ± SE.

small differences among exposures were also seen at baseline, raising the possibility that the statistical findings were a chance occurrence. There were no significant changes in plasma NO₂/NO₃ concentrations.

In the UPASTHMA group, there were no significant UFP-related changes in IL-6 or serum amyloid A. Soluble E-selectin decreased ($P = 0.027$) in association with UFP exposure relative to air exposure (Figure 10). Overall, no evidence was found that UFP exposure increased blood cytokines or soluble adhesion molecules. However, the difference between air and UFP exposure was significant for plasma NO₂/NO₃ concentrations in the UPASTHMA group, with concentrations decreasing after air exposure with exercise and slightly increasing after UFP exposure in males (Figure 11).

BLOOD LEUKOCYTES

Cell distribution, phenotype, and adhesion molecule expression in blood were measured as indirect indicators of change in endothelial function and systemic inflammation. The complete blood count and differential leukocyte counts were measured using standard automated methods in the clinical laboratory of Strong Memorial Hospital. Lymphocyte differential counts were also measured using immunofluorescence labeling and flow cytometry (see Methods). In UPDOSE and UPASTHMA groups, there were significant time-related changes in white blood cells (WBCs), RBCs, hematocrit, and hemoglobin, likely related to exercise, diurnal effects, and repeated phlebotomy. Hemoglobin, hematocrit, percentage of eosinophils, and RBC counts were higher in subjects with asthma than in healthy subjects (Table 11).

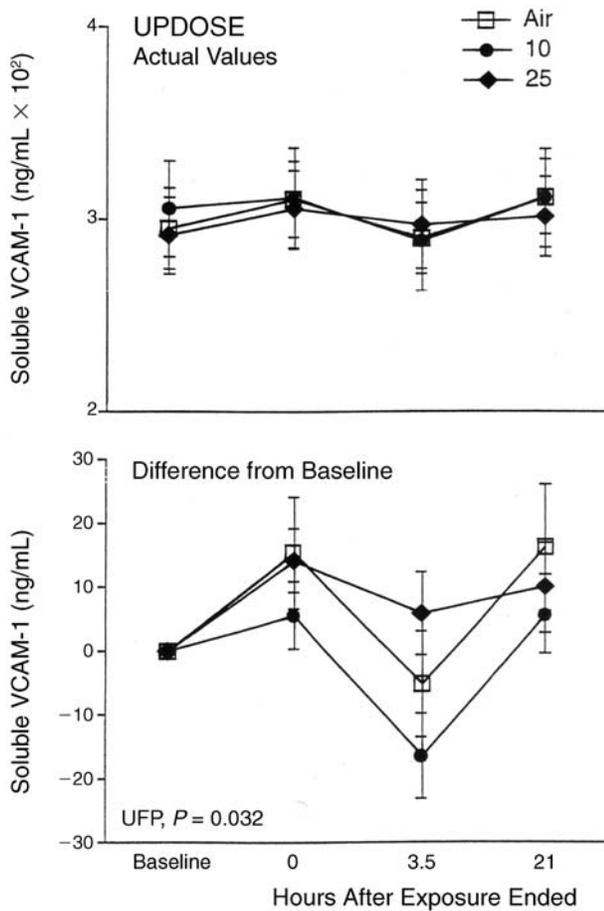


Figure 8. Plasma concentrations of soluble VCAM-1, UPDOSE. Actual values and difference from baseline. Data represent means ± SE.

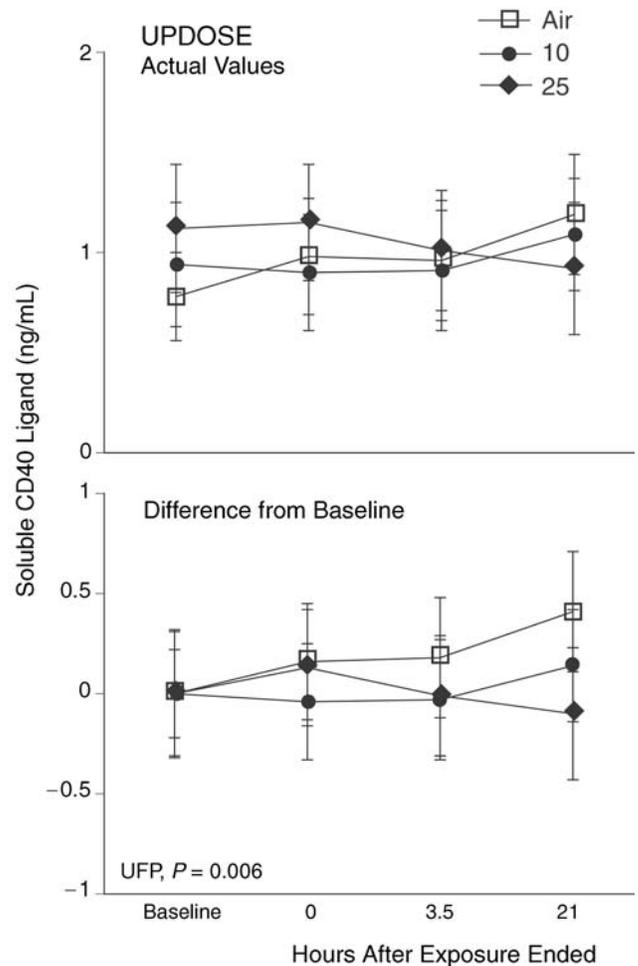


Figure 9. Plasma concentrations of soluble CD40 ligand, UPDOSE. Actual value and difference from baseline. Data represent means ± SE.

In healthy subjects at rest, UFP exposure did not alter total WBCs, differential counts, hemoglobin, or hematocrit. A significant interaction between time and UFP exposure was seen for hemoglobin, but there was no clear concentration response and no significant changes in RBCs, hematocrit, or RBC indices.

In the UPDOSE group, WBCs increased significantly after all exposures (time effect, $P < 0.001$) with a slightly smaller increase after exposure to $25 \mu\text{g}/\text{m}^3$ UFP that was not statistically significant (UFP effect, $P = 0.08$). PMN percentage increased after all exposures, whereas other leukocytes decreased. There was evidence for a concentration-related effect of UFP exposure on the percentage of blood monocytes, with the response differing by gender (Figure 12). With all exposures, monocyte percentage decreased after exposure, and then increased the following morning. In females, after exposure to $25 \mu\text{g}/\text{m}^3$ UFP, monocytes showed a greater reduction and did not return

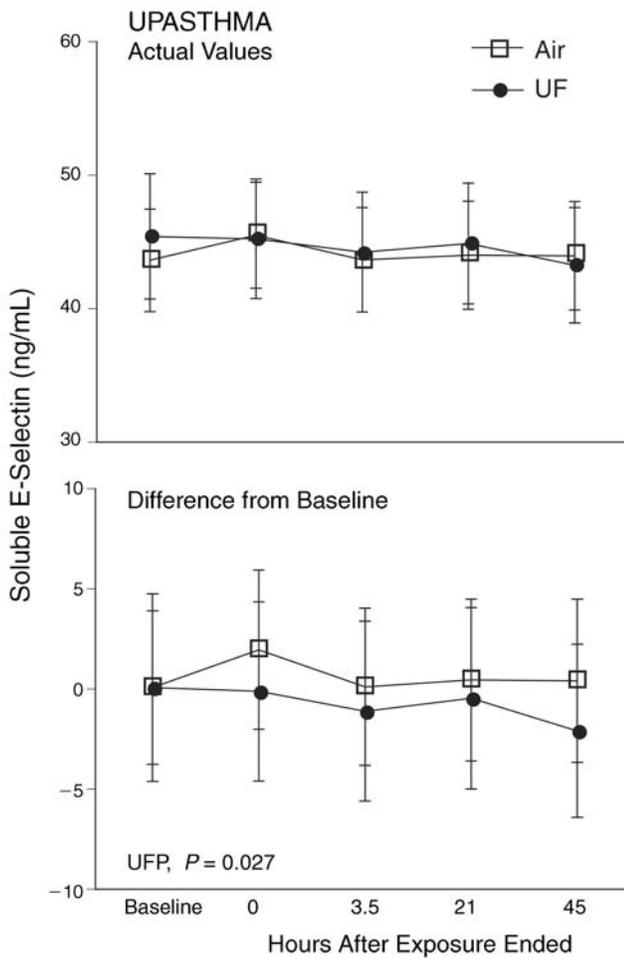


Figure 10. Plasma concentrations of soluble E-selectin, UPASTHMA. Actual values and difference from baseline. Data represent means \pm SE.

Table 11. Complete Blood Cell Counts Before Exposure in Healthy Subjects (UPDOSE) and Asthmatic Subjects (UPASTHMA)^a

	UPDOSE	UPASTHMA	P Value
WBC ($\times 10^3/\mu\text{L}$)	5.75 ± 0.4	6.0 ± 0.3	0.57
RBC ($\times 10^6/\mu\text{L}$)	4.4 ± 0.1	4.7 ± 0.1	0.028
Hemoglobin (g/dL)	13.1 ± 0.3	14.1 ± 0.3	0.026
Hematocrit (%)	38.1 ± 0.9	41.9 ± 1.0	0.009
Platelet count ($\times 10^3/\mu\text{L}$)	223.3 ± 9.2	210.1 ± 9.5	0.329
Neutrophils (%)	49.3 ± 2.6	51.5 ± 1.8	0.487
Lymphocytes (%)	38.1 ± 2.5	33.5 ± 1.8	0.144
Monocytes (%)	8.3 ± 0.5	8.3 ± 0.5	1.00
Eosinophils (%)	3.7 ± 0.5	5.9 ± 0.7	0.013
Basophils (%)	0.9 ± 0.1	0.9 ± 0.2	0.831

^a Data are mean \pm SE.

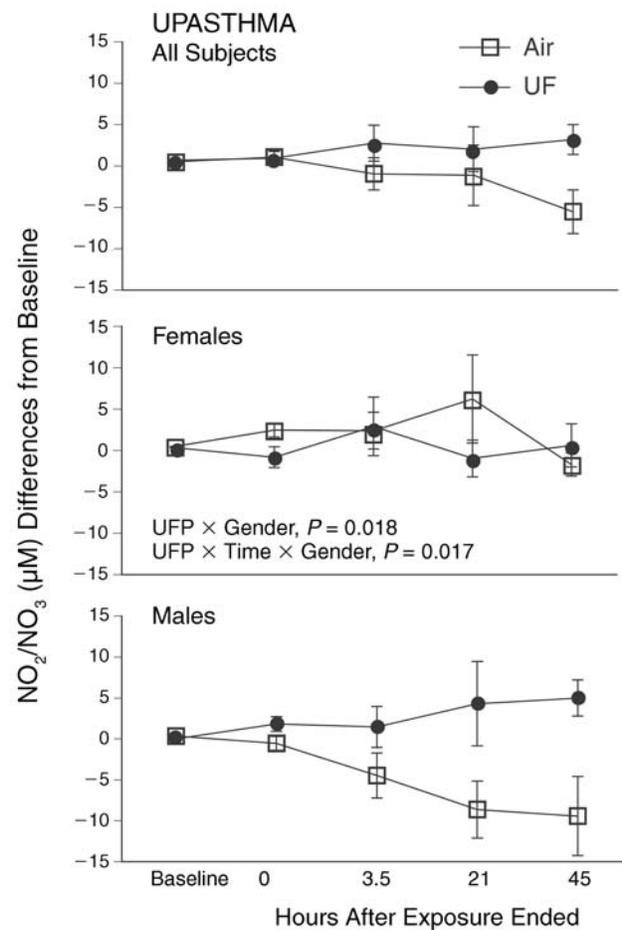


Figure 11. Plasma concentrations of NO_2/NO_3 , UPASTHMA, Differences from baseline. All subjects, females, and males. Baseline values: air, $23.59 \pm 3.28 \mu\text{M}$; UFP, $20.05 \pm 2.12 \mu\text{M}$. Data represent means \pm SE.

to baseline values 21 hours after exposure (Figure 12). The ANOVA indicated a significant exposure-gender interaction ($P = 0.002$). A similar reduction was seen when monocyte numbers were analyzed by flow cytometry (main effect, $P = 0.035$; exposure-gender interaction, $P = 0.002$). In females only, a significant decrease in blood basophils was seen with both UFP concentrations (exposure-gender interaction $P = 0.015$, Figure 13). These changes were accompanied by small, nonsignificant enhanced increases in the percentage of PMNs.

In the UPASTHMA group, UFP exposure had no significant effect on WBCs, RBCs, hemoglobin, or hematocrit. Time-related changes were seen as expected. A significant interaction of exposure and time was seen for basophils ($P = 0.02$; Figure 14), which decreased at 0 and 3.5 hours after UFP exposure compared with air exposure without a significant gender difference. The percentage of blood eosinophils was sufficient for enumeration both in the clinical

laboratory and by flow cytometry. Both methods showed reductions in eosinophil percentage at 0 and 3.5 hours after exposure, with greater reductions after UFP exposure than after air exposure. However, the differences were significant by ANOVA only for the flow cytometry measurements ($P = 0.049$, Figure 15). These data suggest that exposure to UFP causes reductions in blood monocytes in healthy females and reductions in basophils in both healthy and asthmatic subjects.

LYMPHOCYTE SUBSETS AND ACTIVATION

The percentage of T lymphocytes expressing the activation marker CD25 was higher in asthmatic subjects than in healthy subjects prior to exposure (UPDOSE: $27.0 \pm 2.5\%$ vs UPASTHMA: $33.0 \pm 3.3\%$, $P = 0.04$). For UPREST and UPDOSE subjects, there were no main UFP effects on lymphocyte subsets, expression of the activation marker CD25,

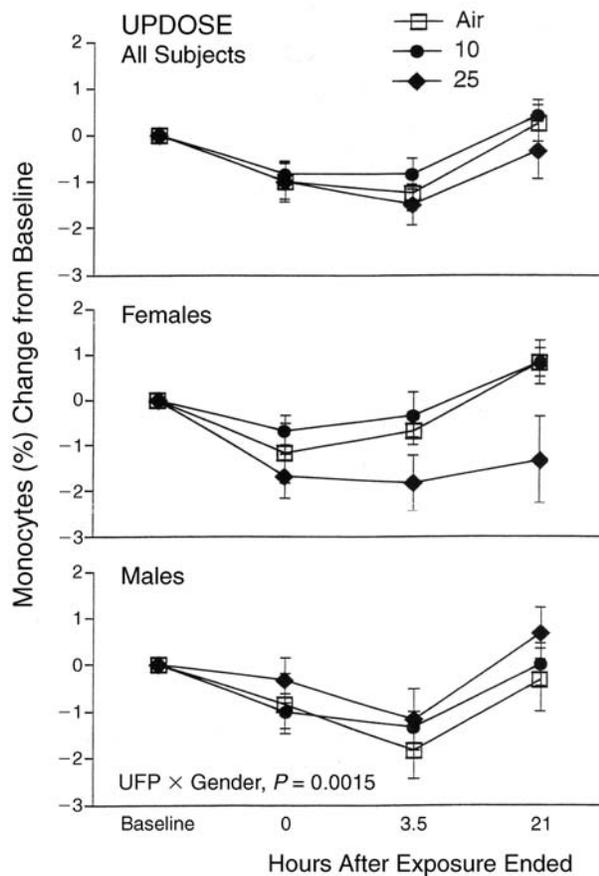


Figure 12. Percentage of monocytes, UPDOSE. Differences from baseline. All subjects, females, and males. Baseline values: air, $8.42 \pm 0.48\%$; $10 \mu\text{g}/\text{m}^3$, $8.33 \pm 0.40\%$; $25 \mu\text{g}/\text{m}^3$, $8.50 \pm 0.40\%$. Data represent means \pm SE.

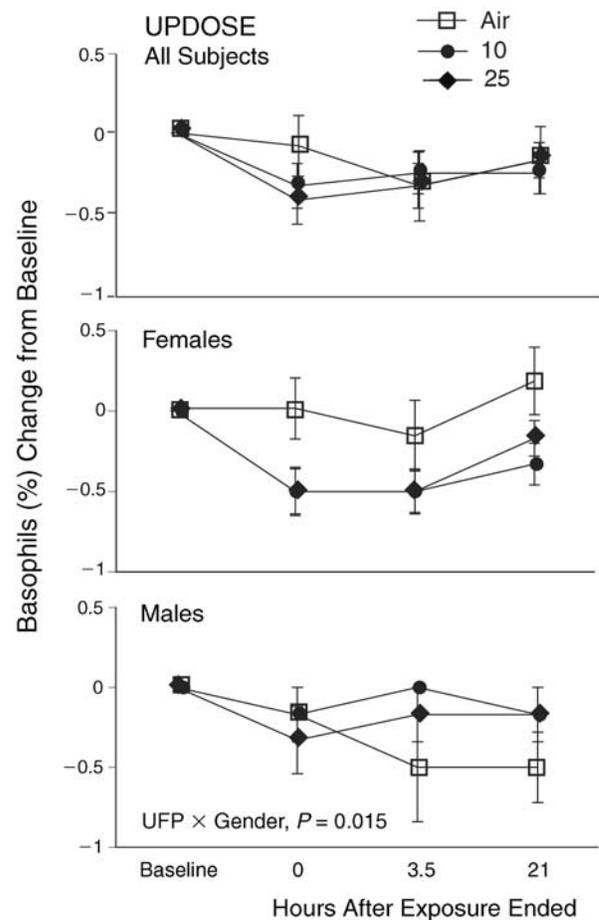


Figure 13. Percentage of basophils, UPDOSE. Differences from baseline. All subjects, females, and males. Baseline values: air, $0.92 \pm 0.15\%$; $10 \mu\text{g}/\text{m}^3$, $1.00 \pm 0.00\%$; $25 \mu\text{g}/\text{m}^3$, $1.08 \pm 0.08\%$. Data represent means \pm SE.

or memory T cells (CD45RO⁺). UPREST subjects showed a statistically significant time-exposure interaction for the percentage of T lymphocytes expressing neither CD4 nor CD8 (null T cells), but no other T lymphocyte subset changes were found. No effect on null T cells was seen in UPDOSE subjects. Also in the UPDOSE group, significant interactions were evident between UFP exposure and gender for CD25 expression on CD3⁺ T cells ($P = 0.002$, Figure 16) and for memory T cells ($P = 0.018$, data not shown). Expression of CD25 increased early after exposure to 25 $\mu\text{g}/\text{m}^3$ UFP in females only. Changes in memory T cells lacked clear relation to concentration in both males and females.

In the UPASTHMA group, the percentage of CD4⁺ T cells decreased immediately after exposure to UFP, compared with air (exposure-time interaction, $P = 0.021$; Figure 17). No significant effects were evident on other lymphocyte subsets or on CD25 expression. Overall, the data suggest that UFP exposure induced T-lymphocyte activation in healthy females and sequestration in asthmatic subjects.

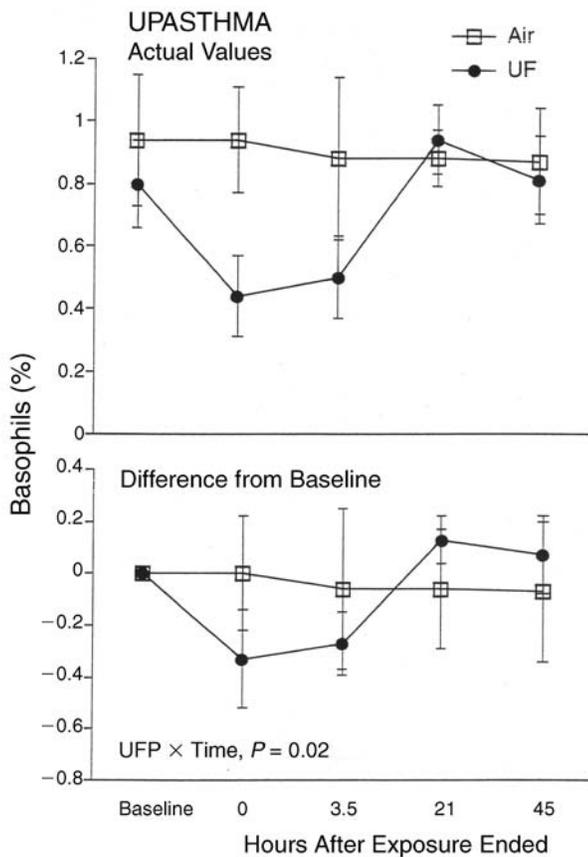


Figure 14. Percentage of basophils, UPASTHMA. Actual values and difference from baseline. Data represent means \pm SE.

LYMPHOCYTE CYTOKINE PROFILE

As shown in Table 12, approximately 0.1% of cells were positive for IFN- γ , and approximately 0.015% of cells were positive for IL-4 with no significant differences between air and UFP exposures.

BLOOD LEUKOCYTE EXPRESSION OF ADHESION MOLECULES AND Fc RECEPTORS

Cell surface expression of adhesion molecules on blood leukocytes reflects influences that are both direct and indirect. As noted in our original hypothesis, direct leukocyte activation by proinflammatory cytokines or other mediators, generated as part of an airway inflammatory response to UFP exposure, was expected to increase expression. Alternatively, activation of vascular endothelium may alter the apparent expression of adhesion molecules in venous blood leukocytes because cells with a higher density or affinity of surface adhesion molecules are more likely to be slowed or retained within the activated vascular bed.

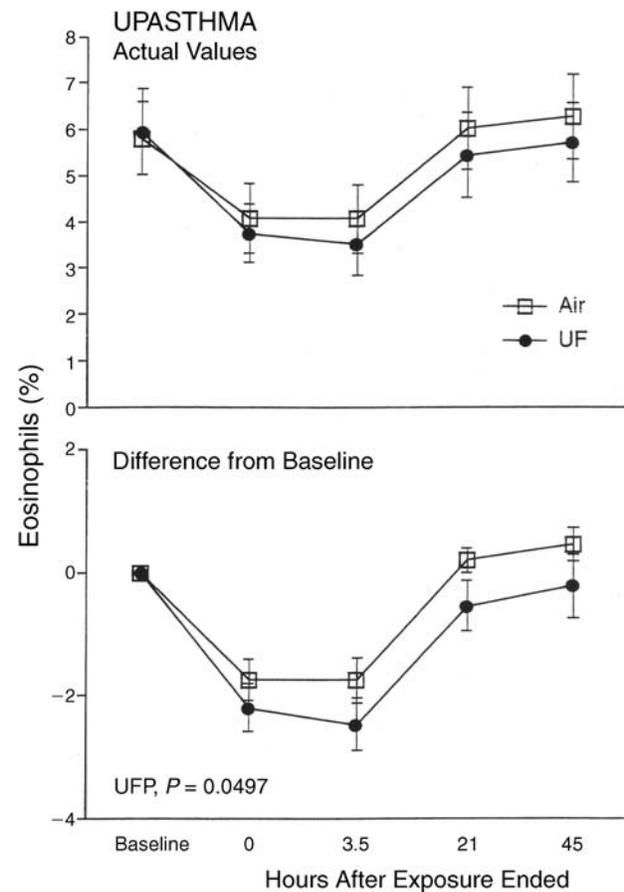


Figure 15. Percentage of eosinophils, UPASTHMA. Actual values and difference from baseline. Data represent means \pm SE.

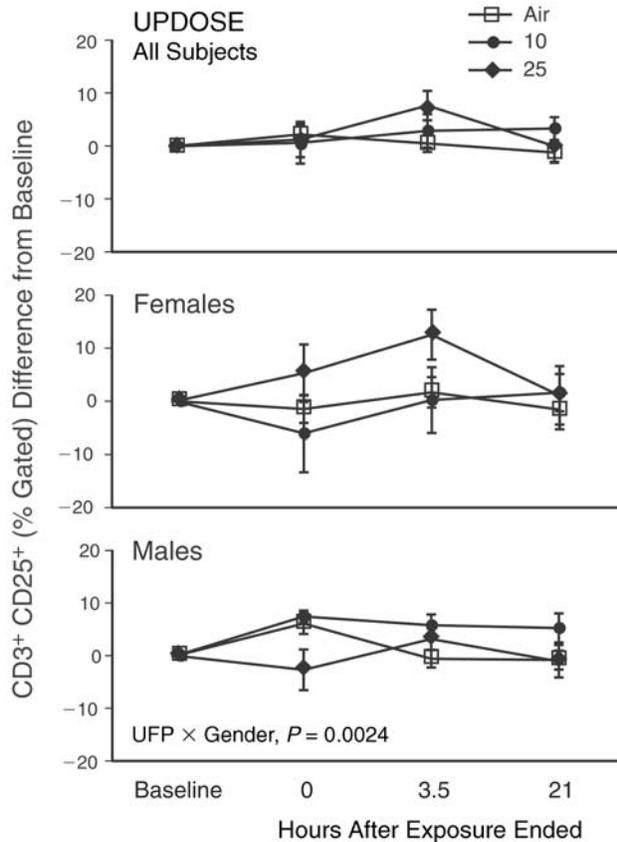


Figure 16. Percentage of blood T lymphocytes expressing CD3⁺CD25⁺, UPDOSE. Differences from baseline. All subjects, females, and males. Baseline values: air, 27.9 ± 2.7%; 10 µg/m³, 26.6 ± 3.1%; 25 µg/m³, 26.2 ± 2.6%. Data represent means ± SE.

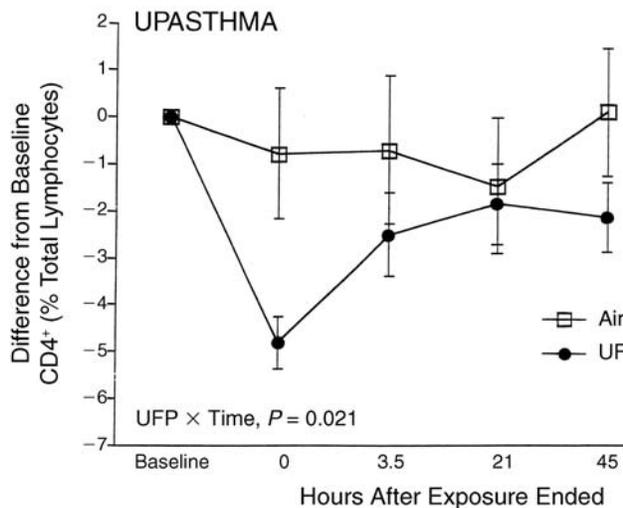


Figure 17. CD4⁺ as a percentage of total T lymphocytes, UPASTHMA. Differences from baseline. Baseline values: air, 46.3 ± 2.2%; UFP, 46.2 ± 2.8%. Data represent means ± SE.

Table 12. Lymphocyte Cytokine Expression^a

Exposure	IFN-γ		IL-4	
	Air	UFP	Air	UFP
Before	973 ± 117	967 ± 120	176 ± 27	153 ± 26
After				
21 hours	942 ± 151	911 ± 154	175 ± 31	139 ± 15
45 hours	1186 ± 230	916 ± 119	233 ± 44	187 ± 36

^a Positive cells/10⁶ mononuclear cells (mean ± SE).

Table 13. Blood Leukocyte Markers Before Exposure in Both Healthy and Asthmatic Subjects

	UPDOSE	UPASTHMA	P Value
Lymphocytes			
CD11a	41,710 ± 1844	14,575 ± 4161	< 0.001
CD11b	1460 ± 67	1784 ± 107	0.017
CD16	1538 ± 115	1740 ± 123	0.242
CD23	1580 ± 62	2850 ± 615	0.057
CD32	1594 ± 74	1986 ± 122	0.011
CD49d	8168 ± 335	10,486 ± 324	< 0.001
CD54	2381 ± 69	2964 ± 155	0.003
CD62L	21,468 ± 1246	25,003 ± 1497	0.081
CD64	1419 ± 42	1780 ± 78	< 0.001
Monocytes			
CD11a	64,155 ± 4041	26,220 ± 5260	< .001
CD11b	17,944 ± 915	25,047 ± 2751	0.025
CD16	3238 ± 162	4237 ± 222	0.001
CD23	3095 ± 117	4525 ± 556	0.023
CD32	55,199 ± 5314	87,051 ± 2675	< 0.001
CD49d	13,556 ± 915	17,089 ± 642	0.005
CD54	12,314 ± 401	17,942 ± 1065	< 0.001
CD62L	41,358 ± 3326	50,244 ± 5307	0.169
CD64	61,391 ± 2481	62,735 ± 4043	0.779
PMNs			
CD11a	28,358 ± 904	12,753 ± 2276	< 0.001
CD11b	16,868 ± 1055	24,178 ± 2705	0.021
CD16	330,263 ± 35,151	466,724 ± 31,525	0.008
CD23	4212 ± 313	5291 ± 437	0.056
CD32	65,813 ± 8320	111,986 ± 3953	< 0.001
CD49d	7189 ± 545	5292 ± 282	0.007
CD54	5045 ± 296	5661 ± 283	0.146
CD62L	63,591 ± 4614	80,656 ± 5954	0.032
CD64	8902 ± 593	8668 ± 522	0.769

As expected, we found differences between healthy and asthmatic subjects in leukocyte expression of surface markers at baseline (Table 13). For example, in subjects with asthma, monocyte CD54 (ICAM-1) expression was higher ($P = 0.003$) and CD11a expression lower ($P < 0.001$) than in healthy subjects.

In the UPREST group, there were few main changes after UFP exposure. Blood monocytes showed a small increase in expression of CD54 in females ($P = 0.028$; exposure \times gender, $P = 0.032$). Lymphocytes showed a small increase in expression of CD49d ($P = 0.013$) and CD11b in females (exposure \times gender, $P = 0.002$). Overall, significance levels for the surface marker endpoints were relatively modest. The data did not suggest a consistent biological response or a convincing gender difference in the overall response.

For the UPDOSE group, the ANOVA showed several gender-related differences in leukocyte surface marker expression. These differences suggest that the exercise in the UPDOSE protocol may have affected leukocytes differently

in men and women. For example, Figure 18 compares males and females in the UPDOSE group for the change from baseline in expression of CD11a and CD62L on PMNs, and CD49d and CD54 on monocytes. In general, females showed a reduction in expression of these markers after air exposure with exercise, compared with males.

UFP exposure led to a significant effect on PMN expression of Fc receptors for IgE (CD23, $P = 0.01$) and immunoglobulin G (IgG) (CD32, $P = 0.017$; CD64, $P = 0.001$) (Figure 19). Expression of CD32 and CD49d on PMNs showed significant exposure-gender interactions. CD32 expression showed a slight reduction after UFP exposure compared with air exposure, and CD64 expression also showed a slight reduction after exposure to $25 \mu\text{g}/\text{m}^3$ UFP. Lymphocyte CD23 expression showed significant differences among the 3 exposures ($P = 0.007$), but there was no clear concentration response. Monocyte expression of CD54 (ICAM-1) decreased after exposure in a concentration-response pattern ($P = 0.001$) with the greatest effect occurring at 0 and 3.5

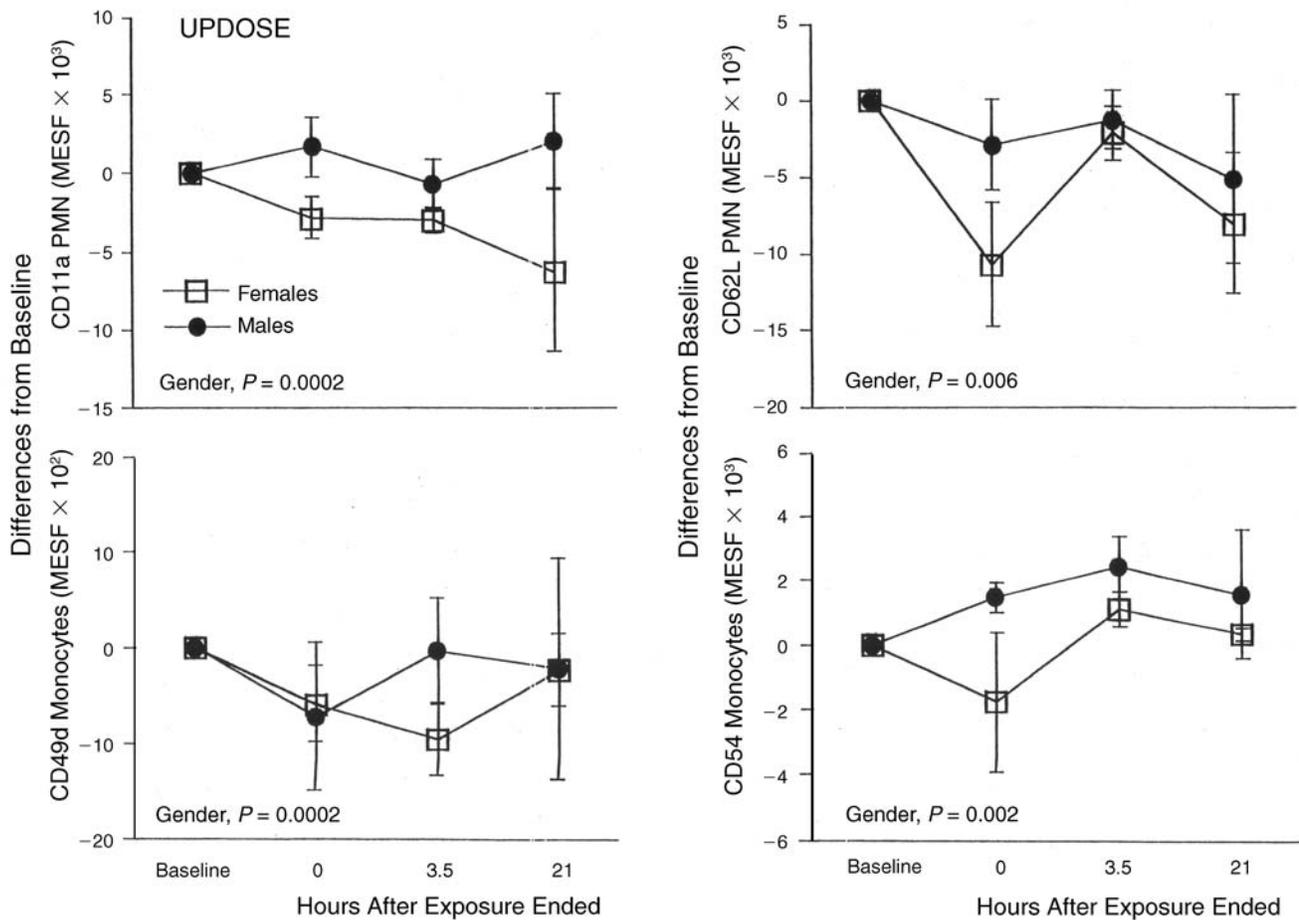


Figure 18. Expression of adhesion molecules after air exposure, UPDOSE. Data represent means \pm SE after air exposure only.

hours after exposure and the differences resolved by 21 hours after exposure (Figure 20). CD32 expression decreased slightly in a concentration-related manner ($P = 0.048$). Expression of CD62L (L-selectin) showed a significant exposure–gender interaction ($P = 0.006$, data not shown) with expression increasing in females but decreasing in males relative to air exposure. However, these findings lacked a clear concentration response.

Overall, the findings in the UPDOSE group appeared to provide evidence for modest effects of UFP exposure, with exercise, on blood monocyte number and leukocyte expression of surface markers. In general, surface marker expression decreased in association with UFP exposure; this change was consistent with retention of higher expressing cells within the capillary bed.

In the UPASTHMA group, blood monocytes showed a significant reduction in CD11b expression after exposure ($P = 0.029$, Figure 21). Expression of CD54 on PMNs decreased in a time-related fashion with the greatest difference between air and UFP exposures at 45 hours after

exposure; the related time–exposure interaction was significant ($P = 0.031$, Figure 22). Expression of CD62L on PMNs showed a significant exposure–gender interaction with an increase in expression of CD62L in males only (Figure 23). No convincing evidence was found for significant effects on blood lymphocyte adhesion molecule expression. Lymphocyte CD16 expression showed a significant main effect of UFP ($P = 0.017$, data not shown), but the data suggest a small difference at baseline with regression toward the mean.

The most significant effect on leukocyte surface molecule expression in asthmatic subjects appeared to be on eosinophils. As noted previously, a small exposure-related reduction was found in eosinophil percentage from the blood

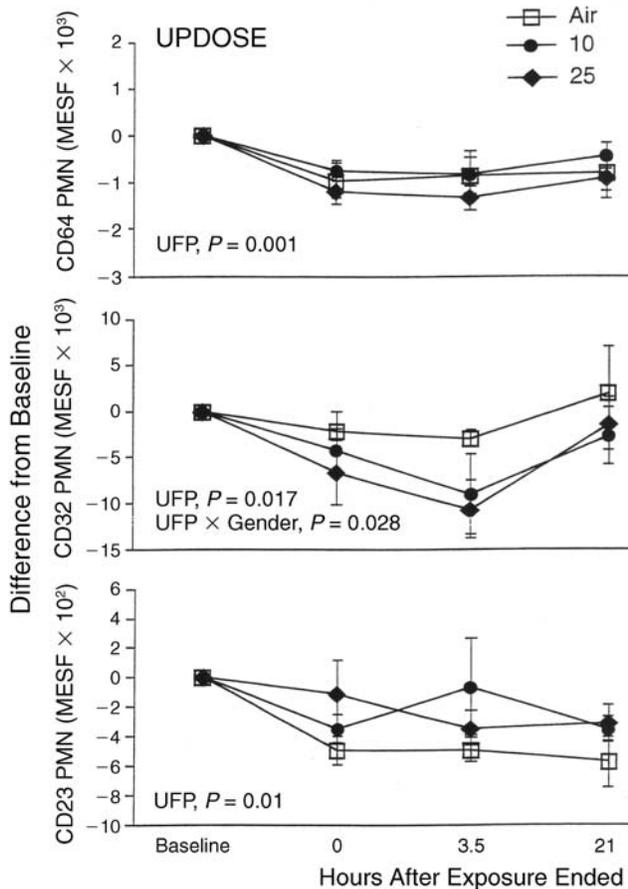


Figure 19. PMN expression of Fc receptors for IgG (CD64 and CD32) and IgE (CD23), UPDOSE. Difference from baseline. Data represent means \pm SE.

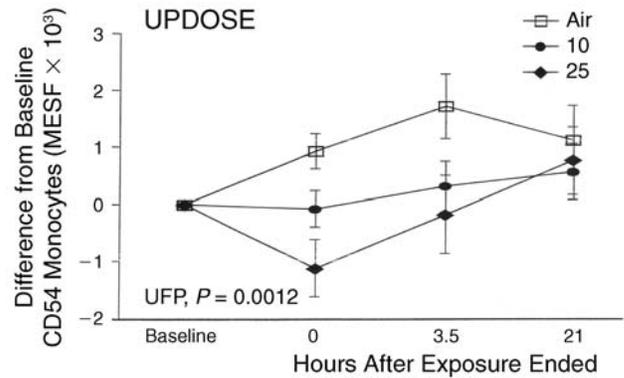


Figure 20. Monocyte expression of CD54 (ICAM-1), UPDOSE. Differences from baseline. Baseline values: air, $12.2 \pm 0.3 \times 10^3$ MESF; $10 \mu\text{g}/\text{m}^3$, $12.5 \pm 0.5 \times 10^3$ MESF; $25 \mu\text{g}/\text{m}^3$, $13.7 \pm 0.7 \times 10^3$ MESF. Data represent means \pm SE.

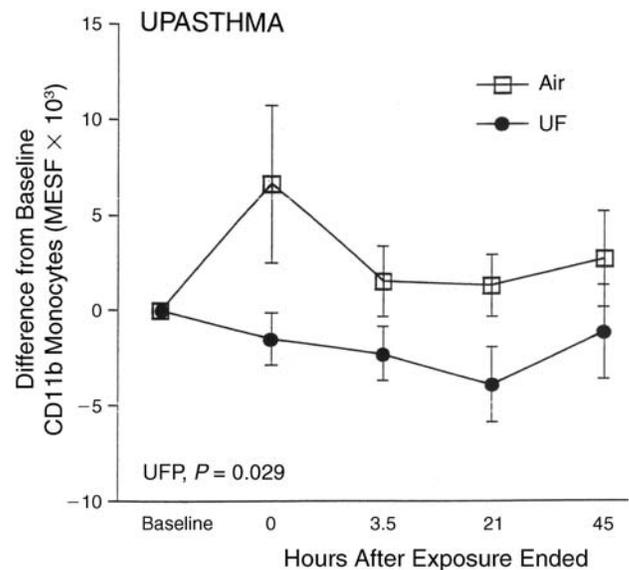


Figure 21. Monocyte expression of CD11b, UPASTHMA. Differences from baseline. Baseline values: air, $21.8 \pm 2.4 \times 10^3$ MESF; UFP, $23.9 \pm 3.1 \times 10^3$ MESF. Data represent means \pm SE.

leukocyte differential count. In addition to this early reduction in eosinophil number, eosinophil expression of CD32 (time-exposure interaction, $P = 0.015$) and CD11b (main effect, $P = 0.015$, Figure 24) showed a delayed reduction.

In summary, data on leukocyte expression of adhesion molecules in UPASTHMA subjects were similar to the findings for healthy subjects and suggested modest effects of UFP exposure with exercise. In general, the effect appears to be a reduction in expression, or a blunting of the postexposure increase seen after air exposure.

MARKERS OF BLOOD COAGULATION

UFP exposure had no main effects in any of the studies. In the UPDOSE group, the ANOVA for both fibrinogen and vWf yielded significant interactions between UFP exposure and gender ($P = 0.001$ and $P = 0.005$, respectively). As shown in Figure 25, both fibrinogen and vWf decreased, in males only, after exposure to $25 \mu\text{g}/\text{m}^3$ UFP, compared with air and $10 \mu\text{g}/\text{m}^3$ UFP. In the UPASTHMA group, there were no significant effects on coagulation factors. These data do not provide evidence for activation of the coagulation cascade by UFP exposure.

CARDIAC MONITORING

UPREST

Few significant changes in ECG parameters were observed after UFP exposure in young healthy subjects under resting conditions. Heart rate variability and repolarization parameters are shown in Tables 14 and 15. SDNN and

rMSSD showed somewhat increased values during, immediately after, and 3 hours after UFP exposure than after air, but the differences for SDNN were not significant (Figure 26). PNN50 and rMSSD showed significant exposure-time interactions ($P = 0.039$ and $P = 0.032$, respectively), but the differences were small. For rMSSD, a

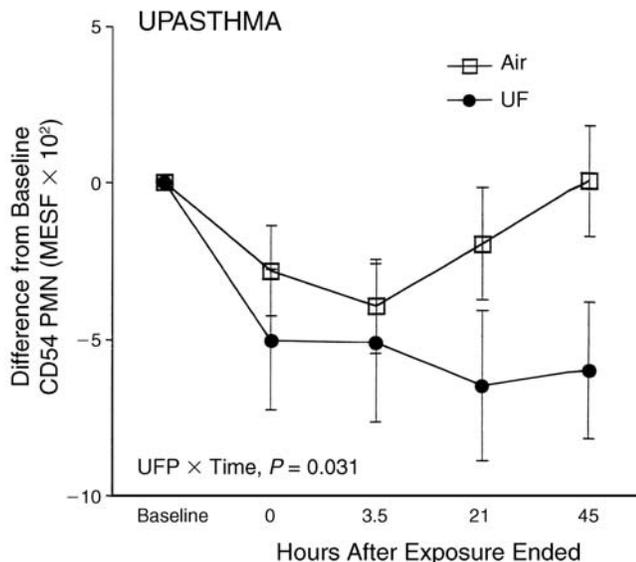


Figure 22. PMN expression of CD54 (ICAM-1), UPASTHMA. Differences from baseline. Baseline values: air, $56.8 \pm 2.3 \times 10^2$ MESF; UFP, $63.2 \pm 4.5 \times 10^2$ MESF. Data represent means \pm SE.

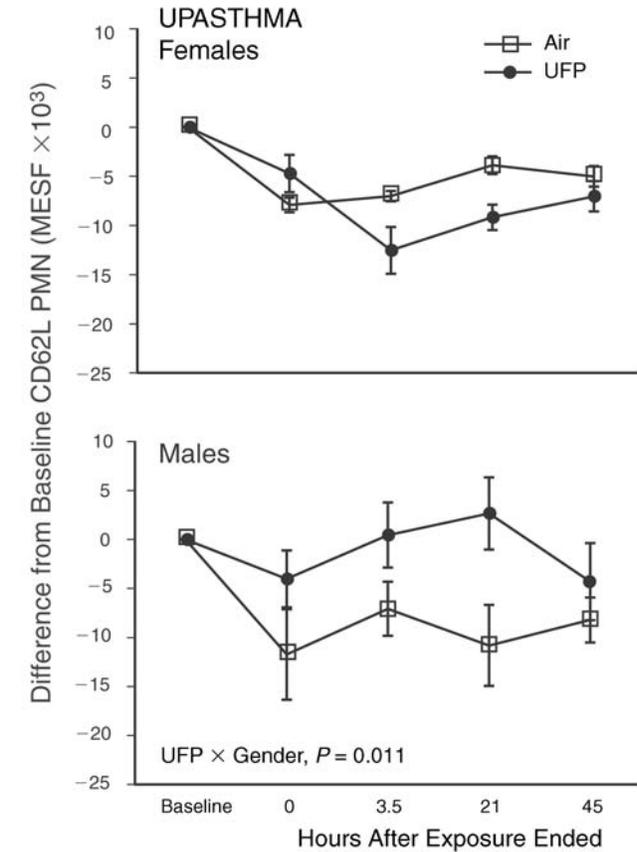


Figure 23. PMN expression of CD62L (L-selectin), UPASTHMA. Differences from baseline. Baseline values: air, $78.3 \pm 3.7 \times 10^3$ MESF; UFP, $78.1 \pm 6.3 \times 10^3$ MESF. Data represent means \pm SE.

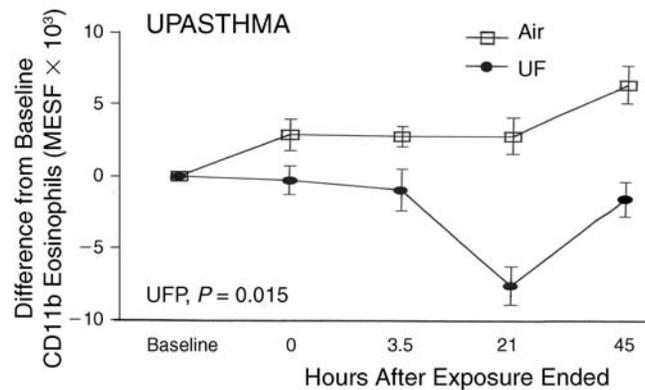


Figure 24. Eosinophil expression of CD11b, UPASTHMA. Differences from baseline. Baseline values: air, $59.8 \pm 3.5 \times 10^3$ MESF; UFP, $57.8 \pm 2.8 \times 10^3$ MESF. Data represent means \pm SE.

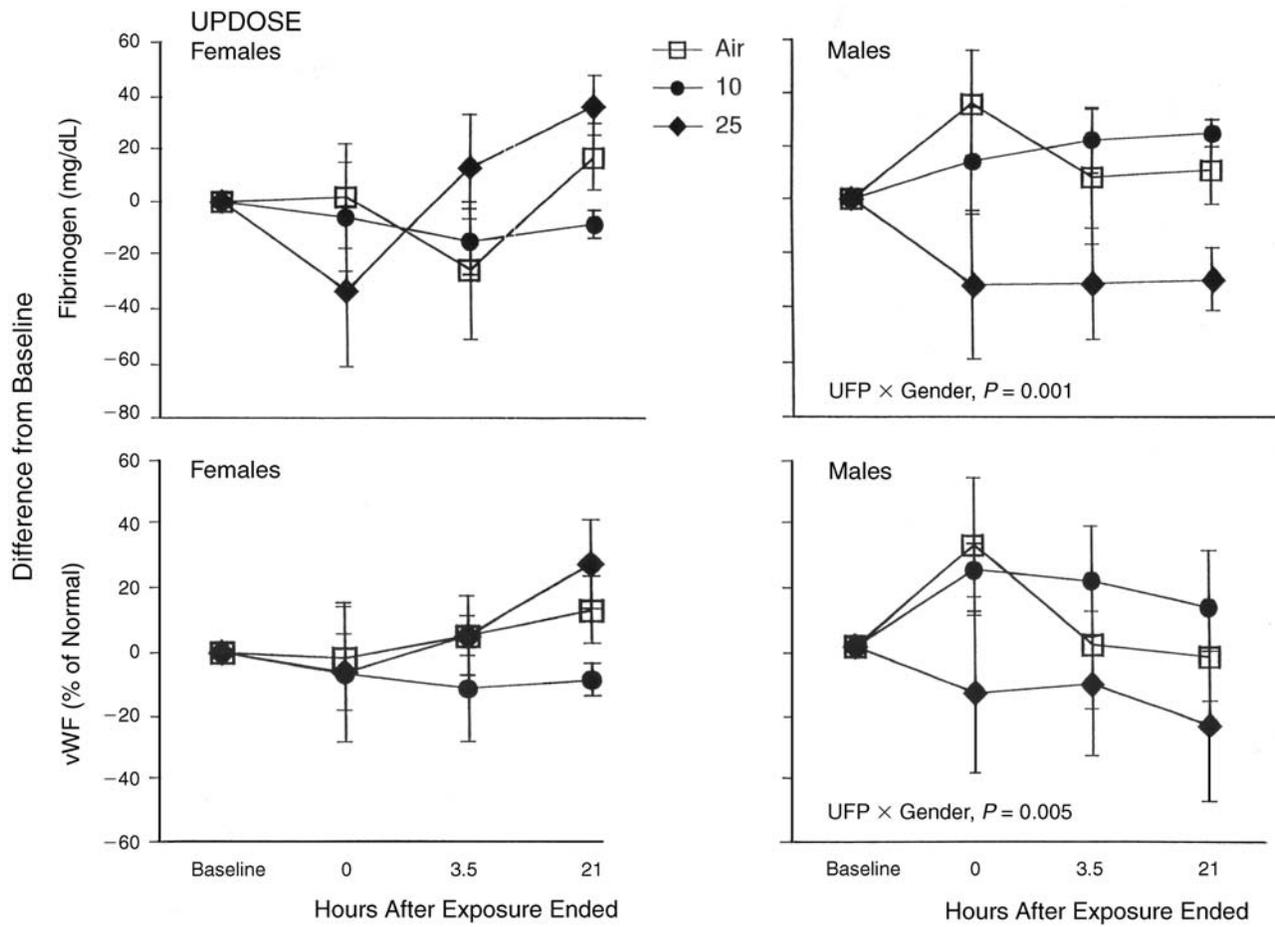


Figure 25. Plasma concentrations of fibrinogen and vWf, UPDOSE. Differences from baseline. Data represent means \pm SE.

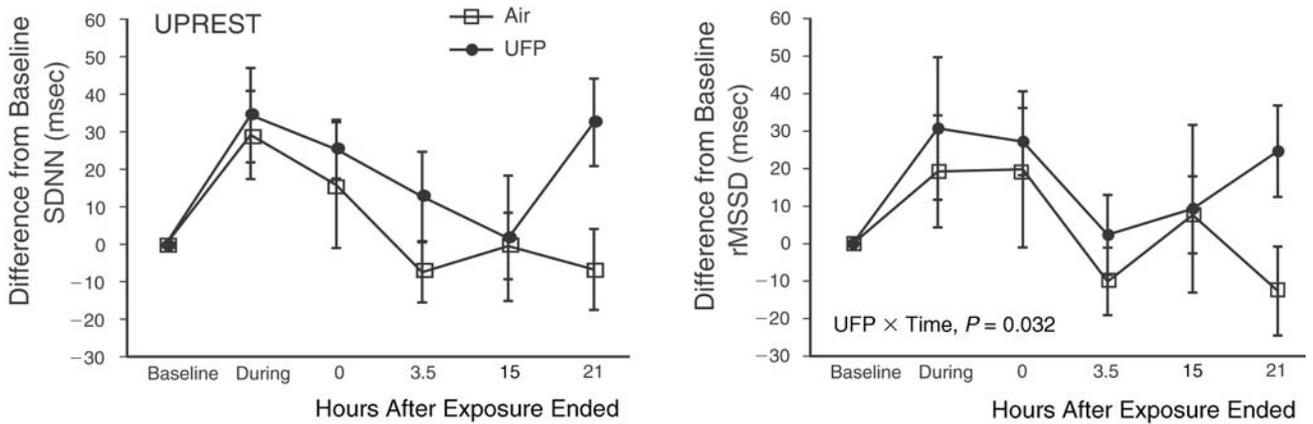


Figure 26. Time domain HRV, UPREST. Differences from baseline. SDNN and rMSSD. Data represent mean \pm SE.

Table 14. Heart Rate Variability in UPREST, Differences from Baseline^a

ECG Parameter/Exposure	During Exposure	Hours After Exposure Ended			
		0	3.5	15	21
RR (msec)					
Air	20 ± 42	169 ± 35	-4 ± 25	80 ± 48	-34 ± 39
UFP	16 ± 40	140 ± 31	-37 ± 45	71 ± 28	0.6 ± 21
SDNN (msec)					
Air	29 ± 12	16 ± 17	-7 ± 8	0 ± 9	-6 ± 11
UFP	35 ± 13	26 ± 8	13 ± 12	2 ± 17	33 ± 12
rMSSD (msec)					
Air	19 ± 15	20 ± 21	-10 ± 9	8 ± 10	-13 ± 12
UFP	31 ± 19	27 ± 9	2 ± 11	9 ± 22	25 ± 12
Low frequency (Hz)					
Air	1411 ± 707	1213 ± 1435	-402 ± 511	367 ± 529	-228 ± 791
UFP	1692 ± 449	693 ± 336	508 ± 321	486 ± 635	833 ± 482
High frequency (Hz)					
Air	2022 ± 1706	672 ± 1665	-917 ± 680	686 ± 537	-1188 ± 925
UFP	2566 ± 1459	1315 ± 696	44 ± 779	1022 ± 1655	2407 ± 1261
Low frequency/high frequency					
Air	0.68 ± 0.45	-0.46 ± 0.29	0.15 ± 0.25	0.12 ± 0.59	0.42 ± 0.42
UFP	0.30 ± 0.23	-0.28 ± 0.24	0.31 ± 0.58	0.10 ± 0.31	-0.03 ± 0.17

^a Data are mean ± SE.

Table 15. Cardiac Repolarization Parameters in UPREST Group, Differences from Baseline

ECG Parameter/Exposure	During Exposure	Hours After Exposure Ended			
		0	3.5	15	21
QTc (msec)					
Air	-8 ± 5	-11 ± 5	1 ± 3	-7 ± 12	3 ± 4
UFP	-3 ± 4	-4 ± 4	-0.4 ± 7	-6 ± 11	0.8 ± 5
T wave amplitude (µV)					
Air	26 ± 19	43 ± 21	-44 ± 24	-44 ± 21	2 ± 18
UFP	31 ± 17	23 ± 16	-50 ± 18	-37 ± 16	24 ± 12
PCA					
Air	-3 ± 1	-0.7 ± 0.9	-0.06 ± 1.9	1.5 ± 1	-1 ± 1
UFP	-3 ± 0.7	-0.4 ± 0.7	0.9 ± 0.5	0.5 ± 1	-0.8 ± 0.9
PCA variability					
Air	0.21 ± 0.14	0.02 ± 0.16	0.07 ± 0.13	0.15 ± 0.10	0.04 ± 0.08
UFP	0.37 ± 0.13	0.13 ± 0.08	0.61 ± 0.42	0.08 ± 0.11	0.37 ± 0.10
ST in L2 (µV)					
Air	8 ± 5	-1 ± 3	-12 ± 4	3 ± 4	-1 ± 5
UFP	10 ± 5	2 ± 3	-12 ± 2	10 ± 4	6 ± 4
ST in V2 (µV)					
Air	-4 ± 2	-3 ± 3	-6 ± 4	-10 ± 5	-9 ± 4
UFP	-2 ± 2	0 ± 2	-3 ± 2	-10 ± 4	-7 ± 4
ST in V5 (µV)					
Air	-2 ± 4	-4 ± 3	-16 ± 6	-7 ± 6	-12 ± 7
UFP	7 ± 2	4 ± 2	-7 ± 3	4 ± 3	0.3 ± 3

Table 16. Heart Rate Variability, Differences from Baseline, UPDOSE Protocol

ECG Parameter/ Exposure	During Exposure	Hours After Exposure Ended			
		0	3.5	15	21
RR (msec)					
Air	-300 ± 40	-110 ± 35	-117 ± 37	63 ± 53	-22.8 ± 30
10 µg/m ³ UFP	-275 ± 26	-84 ± 26	-91 ± 28	77 ± 35	27 ± 19
25 µg/m ³ UFP	-333 ± 50	-70 ± 44	-101 ± 30	37 ± 54	-72 ± 29
SDNN (msec)					
Air	-12 ± 12	3 ± 6	-7 ± 12	-9 ± 13	7 ± 7
10 µg/m ³ UFP	15 ± 10	5 ± 4	7 ± 8	3 ± 5	12 ± 7
25 µg/m ³ UFP	-17 ± 15	4 ± 17	-18 ± 13	-11 ± 15	-6 ± 7
rMSSD (msec)					
Air	-33 ± 13	11 ± 15	-20 ± 17	-5 ± 18	5 ± 7
10 µg/m ³ UFP	-8 ± 7	-3 ± 4	-2 ± 10	12 ± 7	14 ± 10
25 µg/m ³ UFP	-33 ± 19	-3 ± 23	-30 ± 18	-9 ± 20	-12 ± 6
Low frequency (Hz)					
Air	-622 ± 249	-583 ± 438	-354 ± 499	-272 ± 531	-41 ± 303
10 µg/m ³ UFP	380 ± 388	480 ± 329	534 ± 416	345 ± 287	389 ± 219
25 µg/m ³ UFP	-1734 ± 1030	147 ± 1657	-1264 ± 1134	-961 ± 1153	-436 ± 695
High frequency (Hz)					
Air	-1835 ± 1223	-997 ± 1132	-1610 ± 1273	-1025 ± 1375	518 ± 506
10 µg/m ³ UFP	-383 ± 151	175 ± 197	-32 ± 221	470 ± 323	1418 ± 1056
25 µg/m ³ UFP	-2295 ± 1644	100 ± 2071	-1864 ± 1662	-1314 ± 1665	-111 ± 252
Low frequency/high frequency					
Air	3 ± 1	-0.4 ± 0.6	0.1 ± 1.0	-0.5 ± 0.9	-1.3 ± 0.9
10 µg/m ³ UFP	3 ± 0.6	0.5 ± 0.3	1 ± 0.4	0 ± 0.3	-0.3 ± 0.2
25 µg/m ³ UFP	2 ± 1	-0.5 ± 1	-0.1 ± 1.0	-0.9 ± 0.8	-0.2 ± 0.4

difference in the preexposure values may have influenced the analysis results. Repolarization duration, measured by the QTc corrected using the Bazett formula, did not change. T-wave amplitude and complexity and ST segment position did not change. We concluded from these data that there were no significant effects of the resting UFP exposures on heart rate, heart rate variability, repolarization, or the ST segment in young, healthy subjects.

UPDOSE

Similar analyses were performed for the ECG recordings obtained in young, healthy subjects during the UPDOSE protocol with exercise. The results are shown in Tables 16 and 17 for parameters of heart rate variability and repolarization.

Exercise had profound effects on the ECG parameters recorded during exposure to air or either UFP concentration. The ANOVA showed highly significant time-related effects for most ECG parameters.

Similar to observations for the UPREST group, SDNN and rMSSD showed higher values with exposure to

10 µg/m³ UFP than with air exposure. Frequency domain parameters of heart rate variability showed a similar pattern, but this effect was not observed during exposure to 25 µg/m³ UFP. When analyzing these parameters using normalized units, we observed that the response of the parasympathetic system (measured by normalized units of HF components) was blunted during recovery from exercise immediately after UFP exposure in comparison to air exposure (Figure 27). This diminished vagal response was not evident 3.5 hours later.

Analysis of the QT interval duration also showed a blunted response after UFP exposure in comparison to air exposure. Figure 28 shows that the QT and QTc shortened during exercise but shortened more substantially during UFP exposure than during air exposure. Further, the QT and QTc intervals remained shortened for several hours after UFP exposure compared with air exposure. Simultaneously, T wave amplitude was slightly higher after exercise with UFP exposure than after exercise with air exposure ($P = 0.026$, data not shown).

Table 17. Cardiac Repolarization Parameters in UPDOSE Group, Differences from Baseline

ECG Parameter/ Exposure	During Exposure	Hours After Exposure Ended			
		0	3.5	15	21
QTc (msec)					
Air	37 ± 10	19 ± 9	21 ± 7	10 ± 5	23 ± 9
10 µg/m ³ UFP	23 ± 7	6 ± 5	5 ± 5	-3 ± 10	1 ± 5
25 µg/m ³ UFP	27 ± 11	0.1 ± 4	5 ± 3	-5 ± 9	7 ± 4
T wave amplitude (µV)					
Air	-75 ± 42	74 ± 26	-41 ± 36	-42 ± 22	6 ± 22
10 µg/m ³ UFP	-41 ± 38	106 ± 24	3 ± 25	-13 ± 22	38 ± 18
25 µg/m ³ UFP	-66 ± 40	86 ± 34	-9 ± 21	-18 ± 26	28 ± 18
PCA					
Air	-1 ± 2	-2 ± 2	1 ± 2	-1 ± 0.8	-1 ± 1
10 µg/m ³ UFP	-2 ± 1	-3 ± 1	-0.1 ± 0.8	1 ± 2	-0.3 ± 0.8
25 µg/m ³ UFP	0 ± 2	-2 ± 0.6	0.3 ± 1	-0.4 ± 0.9	-1 ± 1
PCA variability					
Air	3 ± 1	-0.01 ± 0.48	0.7 ± 0.6	-0.2 ± 0.4	-0.1 ± 0.5
10 µg/m ³ UFP	4 ± 1	-0.6 ± 0.4	-0.4 ± 0.5	0.2 ± 0.9	-0.03 ± 0.72
25 µg/m ³ UFP	4 ± 0.7	0.1 ± 0.4	0.5 ± 0.7	-0.1 ± 0.3	0.4 ± 0.5
ST in L2 (µV)					
Air	-20 ± 8	-0.3 ± 4	0.7 ± 6	0.8 ± 3	0 ± 5
10 µg/m ³ UFP	-18 ± 12	0.7 ± 5	2 ± 4	-1 ± 3	-3 ± 5
25 µg/m ³ UFP	-17 ± 7	2 ± 5	1 ± 4	-0.7 ± 2	0.5 ± 2
ST in V2 (µV)					
Air	-19 ± 7	-7 ± 4	-5 ± 4	-12 ± 5	-11 ± 5
10 µg/m ³ UFP	-19 ± 5	-4 ± 4	-2 ± 2	-10 ± 5	-4 ± 5
25 µg/m ³ UFP	-25 ± 6	-6 ± 4	-3 ± 3	-8 ± 5	-6 ± 3
ST in V5 (µV)					
Air	-10 ± 5	-3 ± 3	-2 ± 4	7 ± 5	-0.6 ± 4
10 µg/m ³ UFP	-14 ± 7	-3 ± 4	-2 ± 3	11 ± 4	2 ± 4
25 µg/m ³ UFP	-16 ± 5	-4 ± 4	-5 ± 3	7 ± 4	2 ± 2

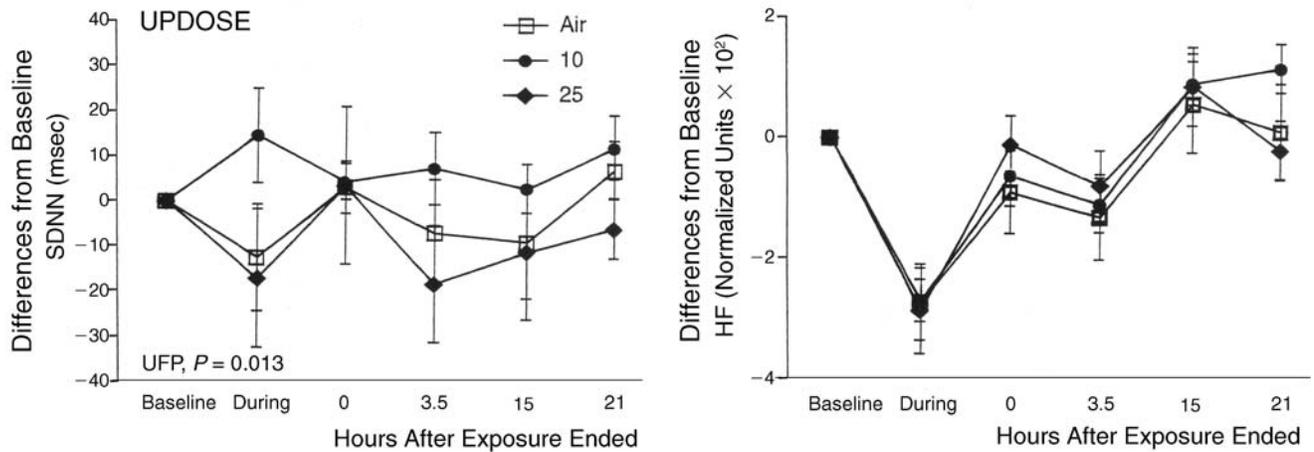


Figure 27. HRV, UPDOSE. Differences from baseline. SDNN and high-frequency HRV in normalized units. Data represent mean ± SE.

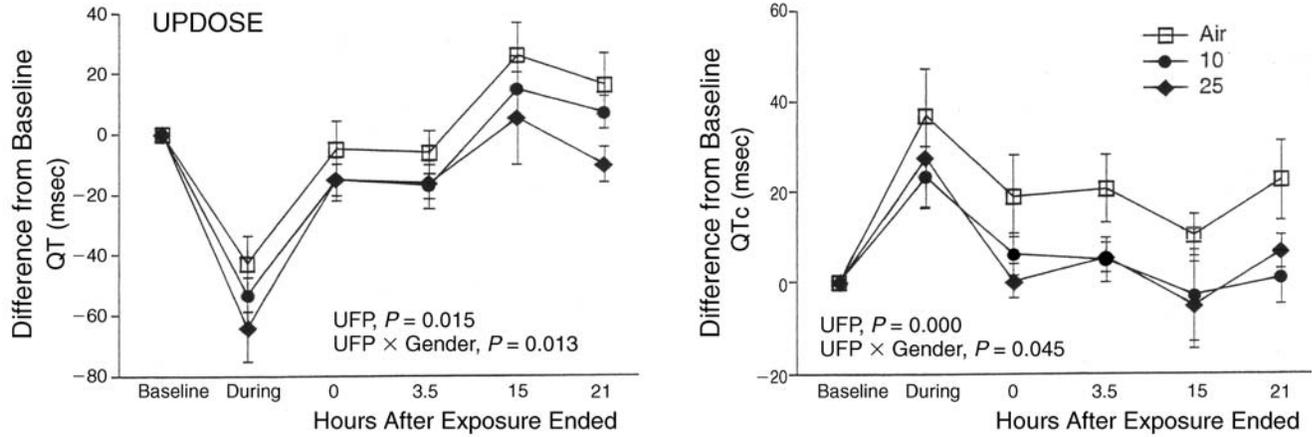


Figure 28. Cardiac repolarization, UPDOSE. Differences from baseline. QT interval and QT corrected with the Bazett formula. Data represent mean \pm SE.

Table 18. HRV in UPASTHMA Group, Differences from Baseline

ECG Parameter/ Exposure	During Exposure	Hours After Exposure Ended				
		0	3.5	15	21	45
RR (msec)						
Air	-309 \pm 45	-2 \pm 25	-86 \pm 20	74 \pm 23	-26 \pm 24	-24 \pm 26
UFP	-261 \pm 40	-7 \pm 21	-92 \pm 15	86 \pm 33	7 \pm 21	-13 \pm 18
SDNN (msec)						
Air	-33 \pm 11	6 \pm 7	-13 \pm 5	13 \pm 9	1 \pm 6	6 \pm 7
UFP	-24 \pm 10	-3 \pm 8	-19 \pm 7	-1 \pm 7	-14 \pm 6	-3 \pm 7
rMSSD (msec)						
AIR	-32 \pm 9	7 \pm 6	-18 \pm 6	14 \pm 7	-4 \pm 6	-11 \pm 8
UFP	-27 \pm 7	2 \pm 7	-22 \pm 4	11 \pm 8	-5 \pm 5	-8 \pm 4
Low frequency (Hz)						
AIR	-510 \pm 256	494 \pm 416	-120 \pm 313	804 \pm 537	315 \pm 221	17 \pm 191
UFP	-469 \pm 273	78 \pm 263	-390 \pm 233	1412 \pm 627	-166 \pm 133	33 \pm 251
High frequency (Hz)						
AIR	-1556 \pm 491	257 \pm 412	-960 \pm 315	105 \pm 418	-394 \pm 424	-904 \pm 485
UFP	-1006 \pm 342	124 \pm 357	-934 \pm 265	169 \pm 416	-260 \pm 346	-565 \pm 276
Low frequency/high frequency						
AIR	3 \pm 0.6	0.4 \pm 0.4	1 \pm 0.6	0 \pm 0.3	0 \pm 0.3	0.4 \pm 0.5
UFP	3 \pm 0.6	-0.4 \pm 0.5	0.5 \pm 0.5	1 \pm 0.3	-0.6 \pm 0.6	0.3 \pm 0.7

UPASTHMA

Tables 18 and 19 show the results of ECG analyses for UPASTHMA subjects. In the heart rate variability analyses, there were trends toward a decreased SDNN, total power, and very low frequency power with UFP exposure in comparison with air exposure (P values 0.120, 0.098, and 0.073, respectively). A similar trend was observed at high frequency expressed in normalized units. Interestingly, these differences were mostly observed in males, despite absence of gender-related changes in mean heart rate.

Repolarization parameters revealed a marginally significant interaction of UFP, gender, and time ($P = 0.045$) for

effects on QTc duration, with males showing slight shortening of QTc the night and following morning after UFP exposure. Decreased QT variability after UFP exposure when compared to air exposure was shown by the standard deviation of the QT duration and of the QT peak duration (Figure 29). Females mainly contributed to this observation. Although the ANOVA indicated a statistically significant main effect of UFP on T wave complexity ($P = 0.049$, data not shown), the difference occurred at only one time point and appeared to be driven by an outlier value. ST segment analysis in leads II and V5 showed an UFP-associated, nonsignificant trend toward ST depression during the entire 24-hour recording (Figure 30).

Table 19. Cardiac Repolarization Parameters in UPASTHMA Group, Differences from Baseline

ECG Parameter/ Exposure	During Exposure	Hours After Exposure Ended				
		0	3.5	15	21	45
QTc (msec)						
Air	28 ± 9	1 ± 3	10 ± 3	4 ± 8	5 ± 5	5 ± 4
UFP	36 ± 11	5 ± 4	10 ± 4	3 ± 4	4 ± 5	4 ± 6
T amp (µV)						
Air	-77 ± 49	108 ± 15	-16 ± 19	-26 ± 23	-12 ± 12	31 ± 22
UFP	-28 ± 33	93 ± 24	-31 ± 18	-31 ± 27	13 ± 20	-1 ± 26
PCA						
Air	1.5 ± 2.4	-2.5 ± 0.8	7.9 ± 8.7	2.7 ± 2.8	0.2 ± 1.3	-2.6 ± 0.7
UFP	2.4 ± 2.7	-0.8 ± 1.3	1.0 ± 1.3	3.5 ± 1.9	0.6 ± 1.3	-4.4 ± 3.6
PCA variability						
Air	3.3 ± 0.8	-0.4 ± 0.1	39.4 ± 39.2	1.3 ± 0.8	0.2 ± 0.3	0.1 ± 0.3
UFP	4.0 ± 1.1	0.0 ± 0.5	-0.1 ± 0.2	0.1 ± 0.3	-0.3 ± 0.2	-0.3 ± 0.3
ST in L2 (µV)						
Air	-21 ± 8	6 ± 2	3 ± 3	7 ± 3	1 ± 3	2 ± 4
UFP	-23 ± 9	1 ± 3	-5 ± 3	2 ± 3	0 ± 3	-4 ± 5
ST in V2 (µV)						
Air	-12 ± 4	-1 ± 2	-3 ± 3	-20 ± 6	-9 ± 2	-1 ± 2
UFP	-10 ± 5	2 ± 2	1 ± 3	-13 ± 7	-10 ± 4	-1 ± 5
ST in V5 (µV)						
Air	-15 ± 5	2 ± 3	-5 ± 4	6 ± 4	-3 ± 3	6 ± 3
UFP	-15 ± 7	-3 ± 2	-8 ± 4	-4 ± 5	-4 ± 4	2 ± 6

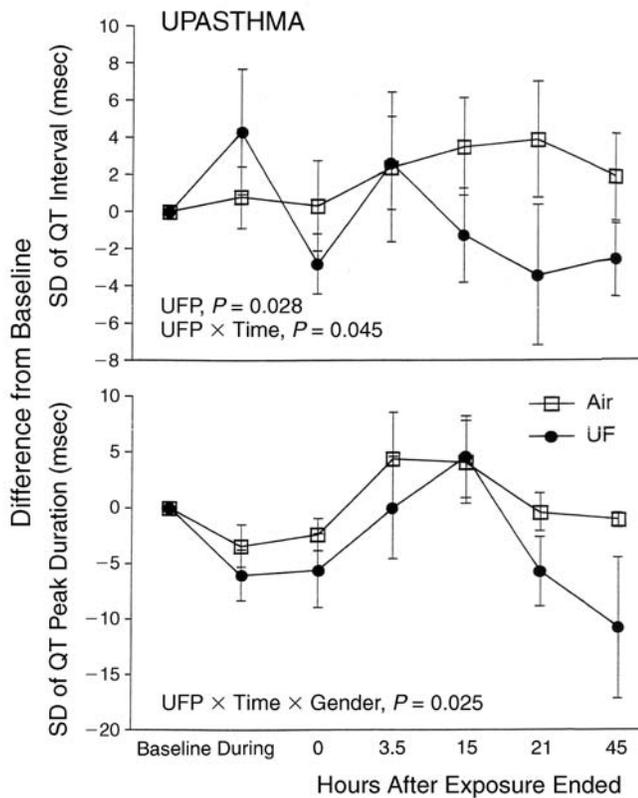


Figure 29. Standard deviations of the QT interval and QT peak duration. UPASTHMA. Differences from baseline. Data represent mean ± SE.

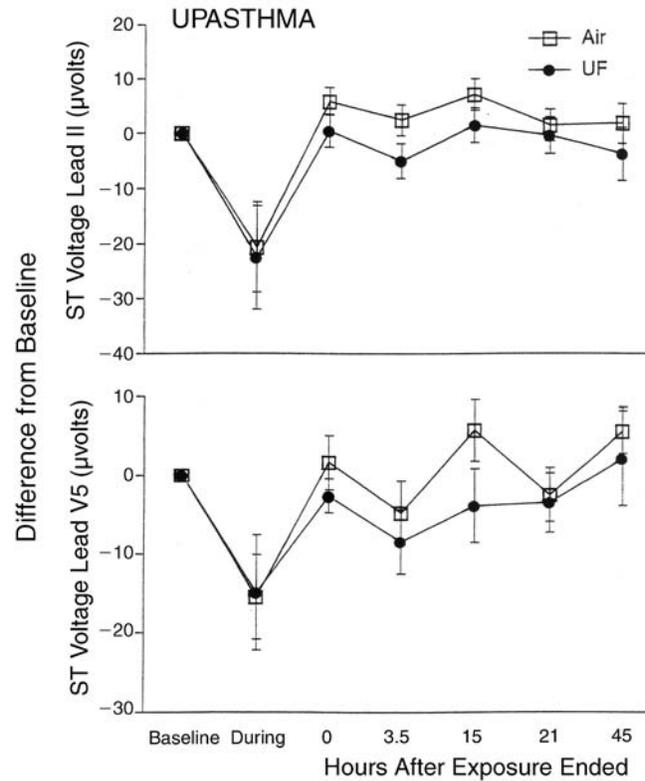


Figure 30. ST segment voltage lead II and ST segment voltage lead V5. UPASTHMA. Differences from baseline. Data represent mean ± SE.

DISCUSSION

These are the first human clinical studies of the respiratory and cardiovascular effects of UFP inhalation. Our choices of particle and exposure system were based on several considerations. First, exposure in a laboratory setting permitted careful and reproducible control of exposure conditions and particle concentrations. Second, elemental carbon is a universal component of ambient combustion particles, and carbon content is often considered a signature of combustion-related particles. Third, because these were the first health studies of UFP exposure, we chose a relatively inert particle composition for safety reasons. Fourth, mouthpiece exposures permitted direct quantitation of respiratory fractional deposition of UFP at rest and during exercise, in both healthy and asthmatic subjects. Fifth, identifying cardiovascular effects resulting from inhalation of a relatively inert particle such as elemental carbon has important implications for understanding potential mechanisms of PM effects.

These carbon UFP studies were undertaken before the technological development of an ambient UFP concentrator. There is no definitive evidence, however, that the concentrated ambient UFP aerosol (with its known variability in size, concentration, and composition) offers a better model for examining possible UFP-induced effects than does a well-controlled single-component UFP aerosol known to be indigenous to urban air pollution (eg, carbon). Collection and resuspension of ambient UFP was not an option because particle collection results in agglomeration with irreversible changes in particle size and surface area.

The key findings of these studies are in three areas: particle deposition, blood leukocytes, and heart rate variability and repolarization.

UFP DEPOSITION

Our data confirm the relatively high predicted respiratory deposition of UFP during breathing at rest, and the findings were in general agreement with results obtained previously (Jaques and Kim 2000; Wilson et al 1985). With exercise, the total respiratory number DF increased about 33% over resting measurements (Table 5), and measured values exceeded predicted values by about 22% for 26-nm particles (Figure 3). One possible explanation for the divergence of these experimental data from model predictions is turbulence. The increased flow demands during exercise move the turbulence-to-laminar flow transition point distally, to smaller-generation airways, enhancing deposition in those airways where laminar flow becomes

turbulent. Prediction models do not account for the effects of increased turbulence in the airways during exercise. Further studies are needed to determine the effects of exercise on regional deposition of UFP, and the effects of varying exercise intensity.

To estimate UFP dose, total particle deposition was calculated using the DF, minute ventilation, and exposure time. For the seven subjects studied at both rest and exercise, minute ventilation during exercise increased 3.3-fold. When combined with the increase in DF, total particle number deposition increased more than fourfold (Table 6). These findings indicate that lung deposition of particles in the ultrafine size range during exercise is greater than expected from changes in minute ventilation, and exceeds estimations using model predictions of deposition. Exercise enhancement of deposition was greatest for the smallest particles.

Predictive models indicate that the regional deposition of UFP in the size range used in this study is predominantly alveolar. Thus, exercise would be expected to substantially increase UFP dose in the alveolar region of the lung through increased intake of particles, increased total DF, and shifting of deposition toward the alveolar region. Exercise may therefore increase the likelihood of alveolar epithelial effects or translocation of particles to the lung interstitium or capillary blood (Nemmar et al 2001, 2002).

We found that subjects with asthma had increased UFP fractional deposition compared with healthy subjects. Patients with chronic obstructive lung disease also have enhanced deposition of fine and ultrafine particles (Anderson et al 1990; Svartengren et al 1991; Brown et al 2002). This is the first study to show enhanced deposition of UFP in subjects with mild asthma. We speculate that the hyperinflation characteristic of even mild asthma may enhance diffusional deposition of UFP in the distal airways and alveoli. These findings indicate an increased airway burden of UFP in asthma and thus may provide a partial explanation for the increased susceptibility of people with asthma to the health effects of PM.

BLOOD LEUKOCYTE EFFECTS

We initially hypothesized that UFP exposure would induce airway inflammation and that this inflammation would be accompanied by increases in expression of surface adhesion molecules on leukocytes. Our findings did not confirm this hypothesis. In contrast, the findings showed reductions in specific leukocyte subsets and adhesion molecule expression associated with UFP exposure when accompanied by exercise. In the UPDOSE group, we

observed small (less than 2%) reductions in the percentage of peripheral blood monocytes in females with exposure to 25 $\mu\text{g}/\text{m}^3$ UFP (Figure 12). We also saw reductions in the percentage of basophils in females with both 10 and 25 $\mu\text{g}/\text{m}^3$ UFP concentrations (Figure 13). These reductions were accompanied by nonsignificant increases in the percentage of PMNs. There were significant reductions in expression of the IgG receptors CD64 and CD32 on blood PMNs. On monocytes, a highly significant, dose-related decrease in ICAM-1 expression was measured immediately after exposure but had resolved by 21 hours after exposure (Figure 20). The percentage of activated lymphocytes (CD25⁺) increased in females with exposure to 25 $\mu\text{g}/\text{m}^3$ UFP (Figure 16).

In the UPASTHMA group, with a single UFP exposure concentration of 10 $\mu\text{g}/\text{m}^3$, we observed reductions in blood basophils (Figure 14), eosinophils (Figure 15), and CD4⁺ T-lymphocytes (Figure 17). Monocytes showed significant reduction in CD11b expression (Figure 21), and PMNs showed reductions in ICAM-1 expression (Figure 22). Eosinophils showed reductions in expression of both CD11b (Figure 24) and CD32.

Thus in both protocols involving exercise (UPDOSE and UPASTHMA), we found effects on blood leukocyte distribution and surface marker expression. These small changes were not generally in the direction consistent with a generalized inflammatory response, in which one would expect an increase in the total leukocyte count and an increase in expression of specific surface markers. For example, proinflammatory cytokines such as tumor necrosis factor and IFN- γ are known to increase the expression of ICAM-1 (Rothlein et al 1988). Indeed, monocytes from the subjects with asthma in these studies showed increased expression of ICAM-1 compared with healthy subjects, a finding consistent with a generalized inflammatory response related to the asthma itself.

Other findings from this study suggest the absence of an inflammatory response to the UFP. No symptoms were associated with UFP exposure, inflammatory cells in induced sputum did not increase, and airway NO production did not increase. In addition, soluble markers of inflammation and leukocyte activation did not increase significantly. Thus, we feel it is unlikely that the leukocyte changes were a response to pulmonary inflammation.

The blood leukocyte changes we observed could result from pulmonary vasoconstriction in response to UFP exposure. The pulmonary capillary bed contains a large population of marginated leukocytes. Reduction in the size of the pulmonary capillaries prolongs the circulation time for leukocytes and thus results in reduced leukocyte

counts in the peripheral blood (Doerschuk 2003). In contrast, exercise increases pulmonary capillary blood volume, washing out marginated leukocytes and resulting in an increase in the peripheral leukocyte count. In 1999 van Eeden and colleagues reported that exercise increased the expression of CD11b on circulating PMNs, presumably by flushing from the marginated pool those cells with higher surface expression of CD11b. If UFP inhalation caused subtle reductions in pulmonary capillary blood flow, one would expect to see reductions in peripheral blood leukocyte subsets. The cells with the longest transit time through the pulmonary capillary bed are likely to be those expressing the highest density of adhesion molecules involved in margination and sequestration (ie, ICAM-1 and CD11b/CD18). In addition, activated leukocytes undergo actin rearrangement with reduced deformability, further increasing transit time through the capillaries. Therefore, we propose that UFP inhalation causes mild pulmonary vasoconstriction with retention of specific leukocyte subsets expressing higher densities of surface adhesion molecules.

If cells are being retained longer in the lung, why did we not see reductions in PMN counts in the peripheral blood? It is possible that retention of PMNs in the lung was partially offset by increases in release of PM from the bone marrow, as has been reported in other studies (Tan et al 2000).

UFP exposure could reduce leukocyte activation and expression of adhesion molecules by three other mechanisms. First, UFP interaction with the endothelium could enhance NO release by endothelial cells. NO has antiinflammatory properties (Lefer 1997): it reduces endothelial expression of adhesion molecules via inhibition of nuclear factor- κB (NF κB) activation and reduces monocyte adhesion to the endothelium (De Caterina et al 1995). Indeed, we observed a late increase in plasma NO₂/NO₃ levels in the UPASTHMA study (Figure 11), but we did not see such a change in the other two studies. Further, increased NO production would not explain the decreases observed in monocytes, basophils, and eosinophils.

Second, if UFP gain access to the pulmonary capillary blood, modulation of cellular functions could result from sequestering soluble cytokines, such as transforming growth factor β , on the surface of the particles, reducing inflammatory effects. Kim and colleagues found that carbon black inhibited fibroblast-mediated collagen gel contraction by binding transforming growth factor β in vitro (Kim et al 2003). Consistent with this possibility, we found marginally significant UFP-associated reductions in plasma IL-6, soluble VCAM-1, and soluble CD40 ligand in healthy subjects, and reductions in soluble E-selectin in

asthmatic subjects. Again, however, this mechanism does not explain the reduction in leukocyte subsets observed in our studies. A third possibility is that circulating UFP were selectively toxic to activated blood leukocytes and induced apoptosis of specific cell subsets.

We observed effects of UFP exposure on lymphocyte subsets. The data confirmed previous observations that people with asthma have a higher percentage of circulating activated T lymphocytes (Corrigan et al 1988; Corrigan and Kay 1990). Lymphocyte expression of CD25 was increased at 3.5 hours after exposure to 25 $\mu\text{g}/\text{m}^3$ UFP in healthy females. CD25 is the α chain of the IL-2 receptor; IL-2 promotes lymphocyte proliferation. IL-2 receptor expression is transcriptionally regulated, and the gene promoter contains several regulatory sequences, including an NF κ B binding site (Weiss and Samelson 2003). Activation of NF κ B appears to play a critical role in IL-2 receptor expression. Our data are consistent with activation of IL-2 receptor gene expression in healthy females. Mechanisms could include UFP interaction with the plasma membrane and activation of intracellular signaling pathways, responses to cytokines released by other cells, or activation of NF κ B by oxidants carried on the surface of the particle. No effect on CD25 expression was detected in the UPASTHMA group, possibly because only the lower concentration (10 $\mu\text{g}/\text{m}^3$) of UFP was used.

In the UPASTHMA group, blood CD4⁺ T lymphocytes decreased immediately after UFP exposure (Figure 17). Previous studies have shown that allergen challenge decreases CD4⁺ lymphocytes in the blood, accompanied by increases in bronchoalveolar lavage fluid (Gerblisch et al 1984), indicating a translocation of T lymphocytes from the blood to the lung. It is tempting to speculate that UFP exposure may have provided a nonspecific stimulus that enhanced allergen processing or presentation in the airway. However, we did not assess alveolar lymphocyte populations with bronchoalveolar lavage and therefore cannot conclude that the observed decrease in CD4⁺ cells represented translocation. Furthermore, we did not see a significant decrease in total lymphocyte numbers in the blood, which argues against translocation.

CARDIAC EFFECTS

This is the first study describing the effects of controlled exposure to UFP on a comprehensive set of ECG parameters related to autonomic regulation of the heart, myocardial substrate, and vulnerability. As expected, young healthy subjects did not show dramatic changes in ECG parameters, but some interesting trends were observed.

Exposure to 10 $\mu\text{g}/\text{m}^3$ UFP at rest was associated with some (not significant) changes in ECG parameters. These changes indicate an increase in parasympathetic tone, which most likely is responsible for a trend toward ST elevation and blunted QTc shortening. Increased variability of T wave complexity after UFP exposure could also be attributed to enhanced parasympathetic response. Heavy breathing, as with exercise, physiologically increases parasympathetic modulation of the heart, and this response seem to be exaggerated by UFP exposure.

In the UPDOSE and UPASTHMA groups, the impact of exercise was seen on most of the ECG variables. As with exposures at rest, the parasympathetic measures of heart rate variability increased during exposure at the lower UFP level (10 $\mu\text{g}/\text{m}^3$), but not at the higher UFP level (25 $\mu\text{g}/\text{m}^3$). This finding may indicate that a threshold response to low level UFP triggers some increase of parasympathetic tone, while higher concentrations might lead to a comparable effect on both the sympathetic and parasympathetic systems. Recovery from exercise showed a blunted response of the parasympathetic system (measured by normalized units of high-frequency components) after exposure to UFP in comparison to exposure to air. This diminished vagal response was not observed 3.5 hours later. An effect of exposure to PM on parasympathetic modulation of the heart was observed by Gold and colleagues (2000), who found reductions in parasympathetic (vagal) tone associated with PM_{2.5} exposure in elderly subjects. Pope and colleagues (1999b) found that elevations of PM₁₀ were associated with increased heart rate and decreased heart rate variability. Animal studies also indicate that concentrated ambient particles (CAPs) induce changes in heart rate variability consistent with increased sympathetic nervous system activity (Godleski et al 2000). Our observations indicate that some effect of UFP on parameters of heart rate variability reflecting control of the heart by the autonomic nervous system is present in healthy subjects although an exact mechanism of these changes is not yet understood.

The repolarization changes in response to UFP exposure with exercise could have a complex mechanism, which remains to be elucidated. Blunted response of vagal modulation on the sinus node does not fully explain the observed blunted response of QTc duration after UFP exposure. It is known that heart rate (sinus node function under the influence of autonomic nervous system) provides only a partial explanation for changes in QT duration (Merri et al 1993). Possibly UFP have an additional effect on repolarization either through a direct effect of the autonomic nervous system on ventricular myocardium

(apart from that on the sinus node) (Merri et al 1993) or by directly affecting ion channel function in the ventricular myocardium through a yet unknown mechanism.

The reduction in QT duration with concomitant increase in T wave amplitude after UFP exposure provides, for the first time, evidence that repolarization is affected by air pollution. These preliminary findings require confirmation in further studies in groups likely to demonstrate more pronounced effects (for example, the elderly and coronary disease patients).

Lengthening of the QTc interval increases the potential for arrhythmias. However, shortening of repolarization is known to be caused by hypoxia and ischemia and to be arrhythmogenic. Calcium, potassium, and chloride channels may contribute significantly to shortening of the action potential duration. For example, the action potential shortening by chloride current activation may perpetuate reentry by shortening the refractory period (Ruiz et al 1996). The other possible explanation for observed QT shortening may be cardiac myocyte functional responses to subtle changes in systemic vascular tone. These changes, in turn, may be related to increased endothelin production and/or reduced NO release by the endothelium in response to particles. Alternatively, UFP may gain access to pulmonary capillary blood, where they could be transported to the heart and cause direct effects on membrane ion channel function.

Exposure of subjects with mild asthma to low-level UFP exposure ($10 \mu\text{g}/\text{m}^3$) shows trends toward decreased heart rate variability, with the changes predominantly in males. There are known gender differences in variety of ECG parameters. For example, heart rate variability is lower and the duration of myocardial repolarization is longer in females than in males. It is interesting that in our study the effect of UFP on these parameters was observed mostly in men, and decreased QT variability was seen mostly in women. These findings further indicate that females and males respond differently to various factors and agents (as has been seen for drugs that affect myocardial repolarization). There is no clear explanation for these patterns in our study, but it is plausible that UFP, even in low concentrations, may decrease autonomic regulation of the heart. In these studies of UFP exposure in healthy subjects, small changes in the ST segment should not be considered indicators of ischemia but rather additional evidence for changes in repolarization morphology.

GENDER DIFFERENCES

Our findings suggest that some effects of UFP may differ by gender. Detecting gender differences was not a primary goal of these studies. However, subject groups included equal numbers of men and women, and the ANOVA included an analysis for gender interactions. In the UPDOSE group, several UFP effects were seen only in females: decrease in oxygen saturation (Figure 5), decrease in blood monocytes (Figure 12) and basophils (Figure 13), and increase in lymphocyte CD25 expression (Figure 16). There were also apparent differences in heart rate variability and cardiac repolarization responses. The UPASTHMA group had fewer gender differences. Plasma NO_2/NO_3 concentration showed a relative increase with UFP in males only (Figure 11). Expression of L-selectin (CD62L) on PMNs was increased in males (Figure 23). The QTc interval, which did not show a main effect of UFP, did show marginally significant gender-related interactions.

Some known gender differences in leukocyte function and cardiovascular responses are based in part on hormonal influences. For example, females have a higher percentage of CD4^+ T cells and a higher $\text{CD4}^+/\text{CD8}^+$ ratio than males. Stimulated blood monocytes from females produce more prostaglandin E_2 (Leslie and Dubey 1994) and less $\text{TNF-}\alpha$ and IL-6 (Schwarz et al 2000) than those from males. There are also gender differences in endothelial function (Chowienczyk and Ritter 2001; Kneale et al 2000; Lieberman et al 1994; Sader and Celemajer 2002), NO production (Best et al 1998; Cicinelli et al 1998; Miller 1999), antioxidant defenses (Massafra et al 2002; Strehlow et al 2003), and risks for cardiovascular disease (Hayward et al 2000). We do not feel these studies have convincingly established or excluded significant gender differences in response to carbon UFP. The female subjects were premenopausal, menstrual phases were not determined, and most were on hormonal contraceptives, which may have affected the responses. The statistically significant gender differences seen in the UPDOSE study did not suggest a consistent physiologic response to UFP and were generally not confirmed in the UPASTHMA study. Future studies of vascular responses to UFP and PM exposure should explore the roles of gender and hormonal influences in determining susceptibility.

COMPARISONS WITH OTHER HUMAN STUDIES OF PM

Evidence from other human studies indicates that exposure to CAPs induces lung inflammation, systemic inflammatory responses, and vascular effects. Human studies of exposure to diesel exhaust have not shown acute effects on

lung function but have shown distal airway inflammation and systemic hematologic effects with increased WBC and platelet counts (Salvi et al 1999). In addition, expression of ICAM-1 was increased in vascular endothelium from bronchial biopsy specimens. Ghio and colleagues (2000) found modest increases in PMNs recovered in bronchoalveolar lavage fluid 24 hours after 2-hour exposures to CAPs (at concentrations up to $311 \mu\text{g}/\text{m}^3$). These investigators found an increased blood fibrinogen concentration in association with CAPs exposure, but no effects on symptoms, lung function, or blood leukocyte differential counts. A separate report (Harder et al 2001) indicated no effects of these exposures on the immune phenotype of cells from bronchoalveolar lavage fluid or on blood lymphocyte subsets. Measurements in this study were done at only one time point (18 hours) after exposure however, and early or delayed changes may have been missed. Gong and colleagues (2003) studied both healthy and asthmatic subjects exposed to CAPs at a concentration of $174 \mu\text{g}/\text{m}^3$ with intermittent exercise. There were no effects on lung function and no evidence for airway inflammation in induced sputum. They did observe small increases in soluble ICAM-1 at 4 and 24 hours after exposure and changes in heart rate variability consistent with enhanced parasympathetic influence on the heart. They observed no changes in blood leukocyte differential counts, fibrinogen, factor VII, or serum amyloid A. Finally, Brook and colleagues (2002) found that resting exposures to a combination of CAPs and ozone resulted in a reduced diameter of the brachial artery, indicating systemic vasoconstriction in response to the exposures.

It is difficult to compare the findings from these studies with our own, in part because of differences in the exposure atmospheres and in the endpoints being measured. The ambient particle concentrators used in these human studies do not concentrate ambient UFP; thus the exposures consisted of concentrated fine particles and ambient (unconcentrated) UFP. In addition, ambient PM represents a complex mixture of chemical species, including organic compounds and metals, which have been hypothesized to be key mediators of PM effects (Ghio et al 1999; Nel et al 2001; Huang et al 2003). In our own studies, mass concentrations were approximately an order of magnitude lower than in the CAPs studies, but particle number and surface area were likely higher. Our particles consisted of elemental carbon without significant content of organic species, metals, oxides, or sulfates. However, even elemental carbon UFP, by virtue of their large surface area, may carry an increased burden of reactive oxygen species in comparison with an equal mass of larger (Li et al 2003). Ambient

UFP have been shown to be potent inducers of heme oxygenase in vitro (Li et al 2000).

In our study, asthmatic subjects did not experience respiratory symptoms or bronchoconstriction after exposure to $10 \mu\text{g}/\text{m}^3$ UFP. This is not surprising because the subjects generally had mild asthma and the exposure concentration was relatively low. It is unlikely that medications masked UFP effects. Subjects did not use bronchodilators during the exposure and observation periods, and most were not on inhaled corticosteroids or long-acting bronchodilators. Possibly a higher exposure concentration would elicit effects or subjects with more severe asthma would show airway effects at this concentration. Comparisons of our effects between the healthy and asthmatic subjects should be interpreted with caution because the groups were not studied concurrently. At the same time, these studies do not provide evidence for increased susceptibility of asthmatic subjects to carbon UFP exposure.

Issues of cost and practicality limited the scope of these studies. Effect sizes were generally small, and the limited sample sizes were likely inadequate to detect significant effects for some endpoints. This is particularly true for the gender comparisons. The subjects were young adults, generally healthy and nonsmokers. The findings may differ for children, the elderly, or people with more severe asthma or other diseases.

In summary, we have developed and validated an exposure system for human studies of inhalation of UFP. We have demonstrated that the total respiratory deposition of UFP is relatively high, consistent with prediction models. However, UFP deposition increased further with exercise and in the presence of mild asthma. Table 20 provides a summary of the findings in these studies. Inhalation of carbon UFP at concentrations up to $25 \mu\text{g}/\text{m}^3$ caused no symptoms, changes in lung function, or evidence for airway inflammation in healthy subjects. Blood leukocyte subsets and adhesion molecule expression did show subtle changes that suggest there may be effects on endothelial function. We also found evidence for effects on heart rate variability and on cardiac repolarization in healthy subjects. Our hypotheses that inhalation of carbon UFP would cause pulmonary inflammation and an acute phase response were not confirmed. If confirmed, however, the finding that these very low mass concentrations of particles have vascular effects will have important implications for future PM regulatory strategies. Further studies are needed to confirm our observations and to determine the role of particle size and surface area in mediating these effects.

Table 20. Summary of UFP Exposure Effects

	UPREST	UPDOSE	UPASTHMA
Respiratory symptoms and pulmonary function	No effects	Decreased \dot{V}_E during exercise	Decreased \dot{V}_E at rest
Vital signs and oxygen saturation	No effects	Decreased oxygen saturation in females	No effects
Airway inflammation	No effects	No effects	No effects
Systemic inflammation	No effects	Decreased soluble CD40 ligand and IL-6 (males)	Decreased L-selectin; increased NO ₂ /NO ₃ concentration (males)
Blood leukocytes	No effects	Decreased monocytes and basophils (females)	Decreased eosinophils and basophils
Lymphocyte subsets and activation	No effects	Increased CD25 ⁺ T cells (females)	Decreased CD4 ⁺ T cells
Blood leukocyte expression of Fc receptors	Increased CD32 (females) on PMNs	Decreased CD23, CD32, CD64 on PMNs; decreased CD32 on monocytes	Decreased CD16 on PMNs; increased CD16 on lymphocytes
Blood leukocyte expression of adhesion molecules	Increased CD54 on monocytes; increased CD49d and CD11a (females) on lymphocytes	Decreased CD49d on PMNs; decreased CD54, CD62L (males), CD49d (females) on monocytes	Decreased CD54 and increased CD62L (males) on PMNs; decreased CD11b and CD54 on monocytes
Markers of blood coagulation	No effects	Decreased fibrinogen (males) and vWf (males)	No effects
Cardiac monitoring	No effects	Decreased QTc interval	Decreased QTc (males), SD of QT interval, and SD of QT peak duration (females)

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 APPENDIX A. HEI Quality Assurance
 Audit Statement

The conduct of this study was subjected to periodic, independent audits by a team from Hoover Consultants. This team consisted of an auditor with experience in toxicology and epidemiology and a practicing board certified physician epidemiologist. The audits included in-process monitoring of study activities for conformance to the study protocol and examination of records and supporting data. The dates of each audit are listed in the table below with the phase of the study examined.

QUALITY ASSURANCE AUDITS

Date, Phase of Study Audited:

September 7–8, 1999 The audit team conducted a phase audit of the UPREST study and observed procedures involving the second exposure for the eleventh subject including blood and sputum sampling, measurement of airway production of NO, processing of biological samples and the exposure itself. The following documentation was examined and/or audited: study protocol dated 12/24/98, subject consent form, summary of work and results dated July 30, 1999, a report describing the preliminary phase of the study (8/15/98–12/31/98) including development of the exposure system and a protocol for the UPDOSE study, subject tracking binder, medical information on each subject, Excel database of medical data derived from hard copy records, Standard Operating Procedures and reports from the University of Rochester Quality Assurance Officer.

March 12–13, 2001 A phase audit of the UPASTHMA study was conducted. Records for the UPDOSE study and the initial subject exposures for the UPASTHMA study were audited. Records included a summary of the work and results for two study periods, the protocol for the UPASTHMA study, Consent Form for UPASTHMA subjects, methods for processing of Holter data and summarized cardiac data. Audit findings from the previous audit were examined to determine that adequate follow-up had occurred.

October 4–5, 2004 Galley proofs of the study report were audited against the data and records from the study.

Written reports of each inspection were provided to the Director of Science of the Health Effects Institute who transmitted these findings to the Principal Investigator.

These quality assurance audits demonstrated the study was conducted by a well-coordinated, experienced team according to the study protocol and standard operating procedures. The report appears to be an accurate representation of the study.



B. Kristin Hoover, Audit Coordinator

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ABBREVIATIONS AND OTHER TERMS

ANOVA	analysis of variance	NO	nitric oxide
CAPs	concentrated ambient particles	NO ₂	nitrite
CD	cell designated	NO ₃	nitrate
DF	deposition fraction	NOS	nitric oxide synthase
DL _{CO}	diffusing capacity for carbon monoxide	PA	partial pressure of NO in the alveoli
DL _{NO}	single-breath diffusing capacity for NO	PBMC	peripheral blood mononuclear cell
ECG	electrocardiography	PBST	phosphate-buffered saline with 0.1% Tween 20
ELISA	enzyme-linked immunosorbent assay	PBSTB	PBST with 1% bovine serum albumin
ELISPOT	enzyme-linked immunospot assay	PCA	principal component analysis
EPA	Environmental Protection Agency (US)	PE	phycoerythrin
FEF ₂₅₋₇₅	forced expiratory flow rate from 25% to 75% of FVC	PHA-p	phytohemagglutinin A
FEV ₁	forced expiratory volume in 1 second	PM	particulate matter
FITC	fluorescein isothiocyanate	PM ₁₀	PM less than 10 µm in diameter
FVC	forced vital capacity	PM _{2.5}	PM less than 2.5 µm in diameter
HF	high frequency power	PMN	polymorphonuclear leukocyte
HRV	heart rate variability	RBC	red blood cell
ICAM-1	intercellular adhesion molecule-1	rMSSD	root mean square of successive differences in N-N intervals
ICRP	International Committee for Radiological Protection	SDNN	standard deviation of NN sinus beat intervals
IFN-γ	interferon-γ	TEOM	tapered element oscillating microbalance
IgE	immunoglobulin E	Th1	subset of CD4 ⁺ T cells that produces IL-2 and IFN-γ
IgG	immunoglobulin G	Th2	subset of CD4 ⁺ T cells that produces IL-4 and IL-5
IL	interleukin [-1, -2, -4, -5, -6, -8]	UFP	ultrafine particles (< 1 µm in diameter)
LF	low frequency power	VCAM-1	vascular cell adhesion molecule-1
MESF	molecules of equivalent soluble fluorochrome	\dot{V}_E	minute ventilation
MPPDep	Multiple Path Particle Deposition Model	\dot{V}_{LNO}	NO production in the alveolar (or lower) airways
mRNA	mRNA	VLA	very late activation antigen
NCRP	National Council on Radiation Protection and Measurements (US)	V _T	tidal volume
NFκB	nuclear factor-κB	$\dot{V}_{U_{NO}}$	NO production in conducting (or upper) airways
NN	normal-to-normal	vWf	von Willebrand factor
		WBC	white blood cell

Ambient particulate matter (PM*) is a complex mixture of solid and liquid particles suspended in air. The size (ranging from approximately 0.005 to 100 μm in aerodynamic diameter), chemical composition, and other physical and biological properties of these particles vary with location and time. This variability derives from differences in the sources of PM. The sources may be natural, the result of geographical conditions, weather, seasonal patterns, or human activities, such as driving vehicles and operating manufacturing or power plants. In addition, reactive species in the atmosphere combine to generate what are known as secondary particles (such as sulfates), which may comprise a significant fraction of total PM.

Although the characteristics of PM differ from place to place, epidemiologic studies in diverse locations have reported that short-term increases in low levels of PM are associated with short-term increases in morbidity and mortality (Health Effects Institute 2001, 2003). People with compromised cardiac or airway function are considered particularly susceptible to the effects of PM (reviewed in US Environmental Protection Agency [EPA] 1996). On the basis of these and other findings, many governmental agencies have set regulatory standards or guidelines for levels of ambient PM. To protect the general population and groups considered most vulnerable to adverse effects from PM, the EPA promulgated National Ambient Air Quality Standards (NAAQS) in 1997 for particles $\leq 2.5 \mu\text{m}$ in aerodynamic diameter (PM_{2.5} or fine particles).

There are several critical questions in PM research:

- Size and chemical composition of particles that may cause harmful human health effects. A key related issue is the toxicity of ultrafine particles—particles <100 nm in diameter. For reasons described in the Scientific Background, some scientists believe that this particle fraction may be particularly toxic (the ultrafine hypothesis of particle toxicity).
- Potential biologic mechanisms of PM effects that may underlie the epidemiologic associations reported between exposure to PM and morbidity and mortality. Currently, PM effects on the respiratory and cardiovascular systems are the focus of research (reviewed in Health Effects Institute 2002).

- Groups of people who may be particularly sensitive to the effects of PM. People with asthma, a chronic disease of the lower airways, are considered to be one of those susceptible groups. Many epidemiologic studies have reported that short-term exposure to particulate pollution exacerbates the symptoms of asthma (eg, Schwartz et al 1993; Lipsett et al 1997) and decreases lung function in people with asthma (eg, Pekkanen et al 1997; Peters et al 1997b).

To address these important PM topics, HEI issued Request for Applications (RFA) 98-1, *Characterization of Exposure to and Health Effects of Particulate Matter*, funding 10 experimental and epidemiology studies. A key component of the RFA was the evaluation of health effects from controlled exposures to ambient particles in healthy and sensitive human participants. In response to the RFA, Dr Mark Frampton and colleagues proposed to study the short-term cardiovascular and airway and systemic inflammatory effects of exposing healthy and asthmatic people to ultrafine carbon particles. They also proposed to measure the deposition of ultrafine particles in the lungs of participants. The HEI Research Committee recommended Frampton's study for funding because few previous studies in humans had investigated the effects of controlled inhalation exposure to ultrafine particles, and these had not evaluated cardiovascular effects (Anderson et al 1992; Kuschner et al 1997).[†]

At the same time that the HEI Research Committee recommended Frampton's study for funding, it also recommended funding a similar controlled exposure study by Dr Henry Gong and colleagues at Rancho Los Amigos Center, California. Gong and his colleagues also proposed to investigate the cardiovascular as well as airway and systemic inflammatory effects of short-term inhalation of particles by healthy and asthmatic people. Gong and colleagues intended to use fine particles concentrated from the ambient air (CAPs) in the Los Angeles area. To facilitate comparisons between the results of the Gong and Frampton studies, HEI organized a workshop before their studies began with both sets of investigators and with Dr Robert Devlin of the Office of Research and Development (EPA, Chapel Hill, North

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

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[†] Dr Frampton's 2-year study, "Health Effects of Exposure to Ultrafine Carbon Particles in Healthy Subjects and in Subjects with Asthma," began in August 1998. Total expenditures were \$1,123,049. The draft Investigators' Report from Frampton and colleagues was received for review in July 2003. A revised report, received in January 2004, was accepted for publication in February 2004. During the review process, the HEI Health 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 Commentary.

Carolina). Dr Devlin, who had initiated a human CAPs exposure study in his laboratory but had not yet published the results, discussed the parameters he was measuring in his study. As a result of this meeting, Gong and Frampton agreed to use similar exposure protocols and to evaluate a similar set of health endpoints in their HEI studies. Gong's study was recently published (Gong et al 2003). This Commentary includes a section that compares the findings of the Frampton and Gong studies and those of the EPA researchers and their collaborators (Ghio et al 2000; Harder et al 2001; Holgate et al 2003).

SCIENTIFIC BACKGROUND

PM AND CARDIOVASCULAR RESPONSES

Before the current study started, plausible biological mechanisms to explain the epidemiologic associations between increases in daily PM levels and short-term increases in morbidity and mortality were scarce. Controlled-exposure studies in laboratory animals, particularly in rodents with features induced to model human disease, had shown that PM could induce inflammation in the airways. In rats with pulmonary hypertension and right-heart hypertrophy, which had been induced with the compound monocrotaline, intratracheal instillation of high concentrations of residual-oil fly ash (a highly toxic, combustion-generated, urban particulate rich in metals) generated inflammation in the airways and led to death of some animals (Costa et al 1994; Killingsworth et al 1997).

In addition, a preliminary study (Gordon et al 1998) of rats that inhaled fine particles (concentrated approximately sixfold from ambient air in New York City) showed no changes in lung inflammatory markers, but small changes in the distribution of peripheral blood leukocytes (an increased percentage of neutrophils and a decreased percentage of lymphocytes). This finding suggested that exposure to CAPs might induce inflammatory effects detectable in the circulation (Gordon et al 1998).

In 1995, Seaton and colleagues proposed that particle deposition in the airways would lead to a systemic inflammatory response, followed by an acute phase response (Seaton et al 1995). The acute phase response is part of the body's defenses against infectious and other noxious agents. Within hours of exposure, the body produces proteins in the circulation that include serum amyloid A, C-reactive protein, and fibrinogen. Because fibrinogen binds to platelets and contributes to their aggregation, Seaton and colleagues proposed that particle deposition in the airways would lead to a transient increase in the coagulability

of blood. This, in turn, would result in an increased risk of thrombus formation and possible cardiovascular consequences. Subsequently, Peters and colleagues (1997a) found an increase in plasma viscosity associated with exposure to PM in Augsburg, Germany.

In the current study, Frampton and colleagues measured many parameters associated with the inflammatory and coagulation pathways. Sidebar 1 describes how these parameters are associated with cardiovascular responses and asthma. Prior to Frampton's work, some studies had also suggested that animals exposed to PM undergo changes in cardiac rhythm (namely, heart rate variability [HRV] (fluctuations in the rhythm of the heart beat) and repolarization (the time between electrical stimuli governing contraction and relaxation of the heart) (Campen et al 1997; Godleski et al 1997; Watkinson et al 1998). Godleski and colleagues found that a small number of dogs exposed intratracheally to a CAPs aerosol (concentrated up to 60 times more than ambient fine particle levels in Boston air) showed cardiac electrophysiologic changes (Godleski et al 1997). Watkinson and colleagues showed that intratracheally instilled residual-oil fly ash generated arrhythmias in monocrotaline-treated rats (Watkinson et al 1998).

TOXICITY OF ULTRAFINE PARTICLES

Particles of different sizes have been associated with health effects. Some scientists have hypothesized that ultrafine particles—emitted in high numbers by combustion engines and the dominant contributor of particle number in PM_{2.5}—may be especially toxic. This hypothesis is based on initial findings (reviewed in Utell and Frampton [2000], Frampton [2001], and Oberdörster [2001]) that suggest:

1. Smaller particles may penetrate deeper into the airways than larger particles do and reach the alveolar regions of the lungs.
2. Smaller particles have a greater total surface area than larger particles of the same mass. Thus, they may present a larger surface area for interacting with airway tissue or for transporting toxic material associated with the particle surface into the airways.
3. *In vitro*, smaller particles are not as effectively phagocytosed (ie, ingested for removal by cells of the innate immune response) as larger particles are.
4. *In animals*, ultrafine particles instilled intratracheally are more effective than fine particles in inducing airway inflammatory responses.
5. Smaller particles may move rapidly to tissues outside the airways (Nemmar et al 2001, 2002).

SIDEBAR 1. INFLAMMATION IN CARDIOVASCULAR RESPONSES AND ASTHMA

Foreign agents such as particles, bacteria, or viruses that enter the tissues trigger an inflammatory response, a cascade of events that activates multiple cell types throughout the host and leads to elimination of the foreign agent (Health Effects Institute 2002). Inflammation in the airways manifests as an increase in numbers of certain types of leukocytes (white blood cells)—such as macrophages, neutrophils, eosinophils, or lymphocytes—in lung tissue or lung fluids. The types of leukocytes that are found depend on several factors, including time after induction of the response and initial state of the host's airways (eg, healthy vs inflamed).

One early characteristic of the inflammatory response is the induction or increase in expression of molecules such as lymphocyte function-associated antigen-1 (LFA-1 or CD11aCD18) and very late antigen 4 (VLA-4 or CD49dCD29) on the surface of neutrophils and macrophages (see Sidebar 2). These phagocytic cells are involved in the first lines of the body's defenses. Sidebar 2 explains the standard nomenclature for surface molecules expressed by leukocytes.

Endothelial cells—the cells at the interface of blood vessels and tissues—are also activated by the inflammatory response. As a consequence, they increase the expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E- and P-selectins. These molecules are the ligands for adhesion molecules, such as LFA-1, expressed by the activated leukocyte. Through these paired interactions, the adhesion of leukocytes to endothelial cells is increased in the inflammatory response. As a consequence of activation and increased adhesive properties, neutrophils or macrophages move from the blood, through the endothelial cell layer, and into the tissue.

Inflammatory responses that start in the airways may also be detected in the circulation. After leukocytes and endothelial cells have been activated, many of the surface molecules induced (including VCAM-1 and ICAM-1) are shed or secreted into the plasma, where the soluble forms of these molecules can be measured and used as markers of inflammation. In addition, during migration of cells into the lung and subsequent stages of the inflammatory process, activated leukocytes and the airway epithelial and endothelial cells release cytokines and chemokines, which are mediators that facilitate communication among cells. These mediators may be found in the lungs and in the circulation. Among these are the proinflammatory cytokines interleukin [IL] 6 and 8. IL-8 is chemotactic for phagocytic cells, including neutrophils; that is, it induces these cells to move down a concentration gradient toward the IL-8 source. IL-6 stimulates the liver to secrete the acute-phase proteins, which appear in the circulation within 6 to 24 hours. One of the acute-phase proteins, fibrinogen, is involved in platelet aggregation, which affects the cardiovascular system. Platelet aggregation also releases CD40 ligand (CD40L) into the circulation, where the soluble

form of the molecule, sCD40L, can be measured. Proinflammatory cytokines also induce the expression of nitric oxide (NO) synthase in airway cells; the synthesis of NO by this enzyme is thought to play a role in inflammatory diseases of the airways (Barnes 1996).

Because coronary heart disease is now understood to involve inflammation in blood vessels, molecules associated with the inflammatory and acute phase responses (C-reactive protein, fibrinogen, and sCD40L in particular) are currently used or being considered as risk factors for coronary heart disease and other cardiovascular events (Freedman 2003; Tall 2004).

Chronic airway inflammation is a characteristic of asthma. Other features of asthma are the presence of eosinophils in the airways and a skewing of the immune response of T lymphocytes toward what is known as the *Th2 pattern*. This immune response involves the production of cytokines, including IL-4, IL-5, and IL-13, by the Th2 subset of CD4⁺ T lymphocytes.

SIDEBAR 2. NOMENCLATURE FOR CELL DESIGNATED NUMBERS

To standardize nomenclature of molecules expressed on the surface of cells in many different species, molecules have been assigned cell designated (CD) numbers (eg, CD4 or CD19). Some two-chain molecules have two CD designations (eg, CD11aCD18). Currently, more than 250 molecules have been given such CD designations. This nomenclature highlights the fundamental similarities from species to species in the structure and function of molecules with the same CD designation. Some surface molecules have a limited cellular distribution. For example, CD3 is expressed only on T lymphocytes and natural killer cells, and CD154 (also known as CD40 ligand [CD40L]) is expressed primarily on platelets and activated T lymphocytes. Other molecules may be expressed on multiple cell types: CD54 (also known as intercellular adhesion molecule-1 [ICAM-1]) is expressed on all leukocytes.

Many cell surface-associated molecules are still commonly known by their original nonstandard names and abbreviations. For example, both intercellular adhesion molecule-1 and ICAM-1 are used for CD54. Table 1 of the Investigators' Report lists the CD designation, the commonly accepted name, and the function of each molecule evaluated in the current study. To be compatible with Frampton and colleagues' Investigators' Report, we use the nonstandard names in this Commentary.

Few studies have compared the effects of exposure to ultrafine and fine particles via inhalation, the normal physiologic route. The results of recent studies suggest that properties other than size, such as solubility, are likely to play an important role in particle effects (Leikauf et al 2001; Hahn et al 2004).

Prior to the current study, some epidemiologic studies had described associations between increases in respiratory effects and exposure to ultrafine particles in children with asthma (Pekkanen et al 1997; Peters et al 1997b). However, these studies found no greater effect for ultrafine particles than for fine particles, so did not establish whether ultrafine particles have a larger independent effect. In addition, during the early stages of the current study, Wichmann and colleagues (2000) reported that mortality was associated with ultrafine

particle levels in a German city, Erfurt. The association reported between ultrafine particles and mortality, however, was not greater than associations reported for other components of the PM mix. Few studies had evaluated the effects of controlled human inhalation exposure to ultrafine particles: Anderson and colleagues (1992) found little or no effect on respiratory function or symptoms immediately after 15 healthy and 15 asthmatic volunteers had inhaled 100 µg/m³ ultrafine sulfuric acid aerosol for 2 hours with intermittent exercise. In 6 healthy volunteers, Kuschner and colleagues (1997) found little or no effect on cell numbers and cytokine concentrations in bronchoalveolar lavage fluid, pulmonary function, or peripheral blood neutrophil concentrations 18 to 20 hours after inhaling a high concentration of fine and ultrafine magnesium oxide particles.

In addition, few studies had examined the deposition of ultrafine particles in the airways during spontaneous breathing or exercise (Anderson et al 1990). Frampton and colleagues' study examined the short-term effects of inhaling ultrafine particles on airway deposition and cardiovascular and respiratory parameters in healthy and asthmatic individuals.

OBJECTIVES

The objectives of the study were:

1. Develop a facility for controlled inhalation exposures of humans to ultrafine particles.
2. Measure the pulmonary fractional deposition of ultrafine carbon particles in healthy and asthmatic people.
3. Evaluate airway and systemic responses in healthy and asthmatic individuals who were exposed to 10 or 25 $\mu\text{g}/\text{m}^3$ ultrafine carbon particles by inhalation for 2 hours. The parameters to be measured included: respiratory function; airway inflammation; expression of blood leukocyte and endothelial adhesion molecules; markers of the systemic acute phase response and blood coagulability; and cardiac electrical activity and repolarization.

The investigators hypothesized that exposure to ultrafine particles would induce airway inflammation by activating circulating leukocytes and vascular endothelium. In turn, this would result in a systemic acute phase response and a transient increase in coagulability (hypercoagulability). Induction of a hypercoagulable state might be associated with changes in levels of circulating cardiovascular mediators that might in turn lead to changes in HRV and repolarization. Frampton and colleagues also hypothesized that induction of inflammation might lead to cardiac electrophysiologic changes mediated via the autonomic nervous system (the branch of the nervous system that controls peripheral organs). People identified from epidemiologic studies as being susceptible to particle effects (such as people with asthma) would be more affected than healthy people.

Frampton and colleagues further hypothesized that deposition of ultrafine particles would be greater in people with asthma compared with healthy individuals. The investigators also postulated that exposure to ultrafine particles might skew the immune response in people with asthma further toward Th2 cytokine production.

STUDY DESIGN

EXPOSURE PROTOCOLS

The investigators exposed healthy and asthmatic volunteers (lifetime nonsmokers, aged 18–40 years) by mouthpiece for 2 hours/session to ultrafine carbon particles and filtered air (ambient air passed through charcoal and high efficiency particle filters). Participants wore noseclips during the exposure. They were allowed to remove the mouthpiece for 10 minutes after 1 hour of exposure. The investigators performed three substudies, each with equal numbers of male and female participants:

1. *UPREST*: 12 healthy participants exposed at rest to 10 $\mu\text{g}/\text{m}^3$ ultrafine carbon particles and to filtered air. Exposures were separated by at least 2 weeks.
2. *UPDOSE*: 12 healthy participants exposed to 10 and 25 $\mu\text{g}/\text{m}^3$ ultrafine carbon particles and to filtered air. Exposures were separated by at least 2 weeks. Participants exercised intermittently on a stationary bicycle—for the first 15 minutes of each half hour of exposure at an intensity adjusted to increase the minute ventilation to approximately 20 L/min/ m^2 body surface area. For safety reasons, the investigators restricted randomized exposures so that each participant received the 10 $\mu\text{g}/\text{m}^3$ exposure before the 25 $\mu\text{g}/\text{m}^3$ exposure.
3. *UPASTHMA*: 16 asthmatic participants exposed to 10 $\mu\text{g}/\text{m}^3$ ultrafine particles and to filtered air with intermittent exercise (as in the UPDOSE protocol). Exposures were separated by at least 3 weeks. The severity of asthma in the study participants was consistent with the US National Institutes of Health (NIH) classification of mild intermittent, mild persistent, or moderate persistent asthma (NIH 1997). Originally, the investigators proposed a dose–response study using 10 and 25 $\mu\text{g}/\text{m}^3$ ultrafine particles that paralleled the UPDOSE protocol. Because Frampton and colleagues thought that asthmatic participants might have severe responses to the higher level of particles, they used only the lower exposure concentration but increased the number of participants in an attempt to enhance their ability to detect effects at the lower exposure level.

Each substudy required 5 to 7 visits for each subject. The first visit was a screening that included a physical examination, exercising on a stationary bicycle to measure minute ventilation, and sputum induction. On the next visit, at least a week later, the investigators measured blood pressure, pulmonary function by spirometry, heart rate, oxygen saturation by pulse oximetry, and levels of exhaled NO. They also took a blood sample, attached a 12-lead Holter heart monitor

(worn until the following day), and took a resting 10-minute electrocardiogram recording. In addition, they asked each person to fill out a symptom questionnaire.

Subjects were then exposed to particles or filtered air for 2 hours. The investigators measured the same set of physiologic, cardiovascular, and inflammatory parameters immediately after exposure and 3.5 hours later. The participants remained in the Rochester Clinical Research Center until the latter measurements had been made. Study participants were discharged and given an activity diary. They returned in the morning of the day after exposure and the investigators made the same set of measurements they had taken previously. In addition, the investigators induced sputum in the volunteers; that is, at a timepoint 22 hours after exposure.

Frampton and colleagues made an identical set of measurements when the study participants returned for subsequent exposures. In the UPASTHMA substudy, participants returned for an additional set of measurements 2 days after exposure; this set also included a 10-minute ECG recording. The investigators also asked the test subjects to assess the severity of their symptoms on a scale of 0 to 5 after exposure.

PARTICLE GENERATION AND EXPOSURE SYSTEM

Frampton and colleagues generated ultrafine carbon particles (count median diameter approximately 25 nm) from the spark discharge of graphite electrodes; they added filtered air to the particles to achieve the target concentrations of 10 and 25 $\mu\text{g}/\text{m}^3$. Analysis of particle composition indicated that the spark discharge generated organic carbon species in addition to elemental carbon, as had been described by others (McDonald et al 2001). Using time of flight mass spectrometry, the investigators determined that 96% of the particles emerging from the spark generator consisted of elemental carbon. Thus, they concluded that the organic carbon compounds detected might have been artifacts that resulted from collecting particles on Teflon filters.

Frampton and colleagues used condensation particle counters and an electrical detection mobility analyzer to determine the distribution of particle sizes and particle number, surface area, and volume concentrations in the inspired and expired aerosols. The investigators measured particle mass concentrations using a tapered element oscillating microbalance.

PARTICLE DEPOSITION

The investigators measured inspired and expired particle concentrations continuously every 5 seconds during the

exposure. They calculated a total respiratory deposition fraction for number and mass concentration using the equation:

$$\text{Deposition Fraction} = \frac{\text{Inspired Concentration} - \text{Expired Concentration}}{\text{Inspired Concentration}}$$

Frampton and colleagues calculated the deposition fraction for total ultrafine particles as well as for each of 8 size bins, ranging from 7.5 to 75.0 nm count median diameter (midpoints 8.7–64.9 nm). The investigators compared the deposition fractions they calculated with theoretical deposition fractions generated using three different models: (1) the International Committee for Radiological Protection model; (2) the National Committee for Radiological Protection model; and (3) the Multiple Path Particle Deposition Model.

MEASUREMENT OF BIOLOGICAL FUNCTION

Pulmonary Function

The investigators measured forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV_1) by spirometry, lung volumes by plethysmography, and diffusing capacity for carbon monoxide (DL_{CO}). They also made continuous measurements of respiratory rate and minute ventilation.

Airway Nitric Oxide

Frampton and colleagues separately calculated NO production in the conducting (upper) airways and in the alveolar (lower) airways. They also measured NO concentrations in exhaled breath.

Blood Markers of Coagulation, Inflammation, and the Immune Response

Frampton and colleagues measured multiple soluble markers in blood. These included the cytokine IL-6; the coagulants factor VII and von Willebrand factor (vWf); and components of the acute phase response, fibrinogen, and serum amyloid A. They also measured several molecules shed from cells into blood: the soluble forms of ICAM-1 (sICAM-1), L-selectin (sCD62L), P-selectin (sCD62P), E-selectin (sCD62E), VCAM-1 (sVCAM-1), and CD40 ligand (sCD40L). In asthmatic participants they also measured the number of lymphocytes expressing the cytokines interferon gamma ($\text{IFN-}\gamma$) and IL-4, which are associated with the Th1 and Th2 subsets of CD4^+ T cells, respectively.

Using fluorescence-activated cell sorting (FACS), Frampton and colleagues also determined the expression of several proteins on the surface of different sets of leukocytes (described in Table 2 of the Investigators' Report). This technique allowed the investigators to quantify both

the number of cells expressing a particular molecule and the level of expression of that molecule on the cell. Because some of these cell surface molecules are expressed only after a cell is activated, this analysis also provided some assessment of cell activation. The following cells and surface-associated molecules were evaluated:

- T lymphocytes: CD3, CD4, CD8, CD25, and CD45RO;
- B lymphocytes: CD19;
- Natural killer (NK) cells: CD56;
- Adhesion molecules and components of adhesion molecules: CD11a, CD11b, CD18, CD49d, CD54, and CD62L expressed on multiple cell types;
- Receptors for the Fc portion of specific immunoglobulin molecules (Fc receptors): CD16, CD32, and CD64, expressed on multiple cell types.

Sputum Induction

Sputum was induced as part of screening and 22 hours after each exposure. The investigators measured differential cell counts in the sputum plug and assayed the cell-free supernatant for IL-6 and IL-8.

Cardiac Monitoring

Frampton and colleagues obtained a continuous 12-lead ECG Holter recording for each participant from before the start of exposure until the following morning. During each continuous ECG recording, the investigators also took 5-minute supine resting ECG readings before exposure, immediately after, and 3.5 and 21 hours after exposure. (For UPASTHMA, subjects also returned 45 hours after exposure for an additional ECG monitoring session.) In addition, 5-minute ECG segments were identified during the final 15 minutes of exposure and during the night (2 AM) after the exposure.

From the ECG readings, the investigators assessed multiple HRV parameters. These included the high frequency power (HF, 0.15–0.40 Hz), low frequency power (LF, 0.04–0.15 Hz), and LF/HF ratio; the standard deviation of normal-to-normal sinus beat intervals (SDNN) and root mean square of successive differences in normal-to-normal intervals (rMSSD). They measured beat-to-beat RR intervals, ST segment levels, T wave amplitude, and T wave complexity. They also measured QTc, a measurement of the time between the start of the depolarization of the ventricles of the heart (an electrical stimulus that contracts the heart to pump blood) and the end of the repolarization of the ventricles (the electrical stimulus that relaxes the heart), corrected for heart rate. In addition, Frampton and colleagues quantified arrhythmias for the entire recording period.

DATA MANAGEMENT AND STATISTICAL METHODS

UPREST and UPASTHMA

These studies utilized a two-period crossover design in which each subject was exposed to both ultrafine particles and filtered air. The investigators used repeated measures analysis of variance (ANOVA) as their standard analysis for continuous endpoints. In the analysis, order of presentation and gender were treated as between-subject factors, and treatment, period, and time (when repeated measurements were made after each exposure) were considered within-subject factors. The analysis included tests for period and carryover effects. When carryover effects were highly significant, first period data were examined separately. For some endpoints, repeated measurements were made at uniform intervals after each exposure. In these cases the ANOVA included tests for an effect of time as well as interactions with other effects in the model.

Each ANOVA included an examination of residuals as a check on the required assumptions of normally distributed errors with constant variance. If these assumptions were not satisfied, data transformations (for example, square-root transformation for cell counts) were considered. In the special case that only one measurement was made in each period, the ANOVA simplified to three different *t* tests, for treatment, period, and carryover effects. In this case, non-parametric tests such as the Wilcoxon rank sum test were used if the observations were not normally distributed.

UPDOSE

The study utilized a three-period crossover design in which each subject received both low (10 $\mu\text{g}/\text{m}^3$) and high (25 $\mu\text{g}/\text{m}^3$) concentrations of ultrafine particles and filtered air. Because each subject received the low level exposure before the higher level, there were three possible exposure sequences depending on where in the sequence the air exposure was placed. Equal numbers of subjects were randomly assigned to each sequence. The statistical analysis was based on the usual ANOVA model for crossover designs; that is, the ANOVA included effects for treatment (three levels), and period (three levels). Because of the nature of the design, carryover effects were limited to having been previously exposed (in the period just before the present one) to 10 $\mu\text{g}/\text{m}^3$ (all subjects) and 25 $\mu\text{g}/\text{m}^3$ (four subjects). A random subject effect was included in the model. Interactions between subject and both period and treatment were also included as random effects. Because the data were not balanced for this model, parameters for the model were estimated by the method of maximum likelihood. Residuals were examined as a check on the assumptions, and when necessary, data transformations were

considered. In some cases a logarithmic or square root transformation was necessary to stabilize the variance.

For all the substudies, a *P* value of 5% was considered statistically significant. The HEI Review Committee thought that the investigators' approach to assessing carryover effects was appropriate for estimating the main effects of exposure. However, the report did not discuss the number of study participants who manifested carryover effects or the magnitude of the effects. The investigators did note that "the pattern of carryover effects did not indicate any systematic persistence in the second period."

RESULTS

DEPOSITION OF ULTRAFINE PARTICLES

Based on particle number, the average fractional deposition in the lower airways for ultrafine particles was approximately 0.65 for all resting healthy volunteers (Tables 5 and 6); that is, 65% of the inhaled particles were deposited in the airways. The resting value for asthmatic participants was slightly higher, 0.76. From these values, the investigators calculated that the total number of particles deposited in asthmatic participants was 50% greater than the number of particles deposited in healthy participants. With exercise, the deposition fraction increased to approximately 0.85 for healthy and asthmatic participants. The investigators further calculated that four times as many particles were deposited in the airways of exercising participants—both healthy and asthmatic—than in the airways of resting participants. Similar exercise-induced increases in particle deposition were found when particle mass was used as the measure. The deposition fraction was the same in men and women and after exposures at 10 or 25 $\mu\text{g}/\text{m}^3$ particles.

In all groups—healthy or asthmatic, at rest or during exercise—deposition fractions were greater for particles of smaller diameter. For example, based on particle number, the deposition fraction at rest was 0.80 for ultrafine particles in the range 7.5 to 10.0 nm but 0.55 for particles in the range 56.2 to 75 nm.

HEALTH EFFECTS ASSOCIATED WITH EXPOSURE TO ULTRAFINE PARTICLES

Overall, few changes were detected at different measurement times (0, 3.5, and 21 hours [and 45 hours in asthmatic participants only]) after exposure to ultrafine particles in any protocol. Changes in some parameters differed from baseline only at a single timepoint. In addition, as was found in the study by Gong and colleagues, the investigators

noted some changes after the control exposures, which may have been the result of exercise. In some important examples listed below, the changes reported as a consequence of the control exposure were greater than changes after particle exposure.

The following paragraphs, the Investigators' Report Table 20, and the Commentary Table summarize the main study findings. The Commentary Table also compares the findings of this study with those of the HEI controlled-exposure study by Gong and colleagues (2003) and of the studies by the EPA group.

- No effects were found in healthy resting volunteers (ie, UPREST) on any inflammatory, cardiovascular or pulmonary function parameter measured.
- Some small changes were noted in exercising healthy and asthmatic volunteers (ie, UPDOSE and UPASTHMA). Of the changes associated with exposure to ultrafine particles, many were decreases compared to air exposure. When responses were analyzed separately by sex, some exposure-associated effects were greater in one sex than the other. The key results are described in the following paragraphs.

Airway Inflammation

No effects on markers in induced sputum were detected in either exercising healthy or asthmatic volunteers. Parameters examined included total cell numbers, levels of leukocyte subpopulations, and levels of IL-6 and IL-8. NO production in the airways was not affected.

Systemic Inflammation, Coagulation and Cardiovascular Measures

Among soluble markers in healthy participants, the control exposure increased sCD40L levels more than exposure to ultrafine particles (Figure 9). Compared to baseline values, the control exposure increased levels by approximately 50% 21 hours after exposure. The control exposure increased IL-6 levels more than ultrafine particle exposure, particularly in men at 3.5 hours after exposure (Figure 7). At this 3.5-hour timepoint, IL-6 levels in men in the control exposure group were approximately double the baseline value. Fibrinogen and von Willebrand factor levels in healthy men decreased after exposure to 25 $\mu\text{g}/\text{m}^3$ particles (Figure 25); no effects were noted at 10 $\mu\text{g}/\text{m}^3$ or at either exposure level in women.

In asthmatic participants after ultrafine particle exposure, the soluble marker plasma nitrite/nitrate (a measure of endogenous NO production) showed a small increase (5–10%) over time, whereas levels decreased in air-exposed

Commentary Table. Controlled Exposure of Humans to Particles: Current Study, HEI Research Report 118 (Gong et al), and EPA Studies

	Frampton	Gong	EPA ^a
Participants	24 healthy and 16 with mild to moderate asthma (18–40 yr)	12 healthy and 12 with mild asthma (18–45 yr)	38 healthy (18–40 yr)
Methods	Each person exposed to particles and filtered air	Each person exposed to particles and filtered air	Each person exposed to particles (n = 30) or filtered air (n = 8)
Type of particles	Ultrafine carbon particles (7–75 nm, 25 nm median) generated in laboratory	Fine CAPs (average 174 µg/m ³ , range 99–224 µg/m ³) from Los Angeles area air; particles 0.6–1.0 µm preferentially concentrated	Fine CAPs from Research Triangle Park air (range 23–311 µg/m ³); particles 0.6–1.0 µm preferentially concentrated
Exposures	Inhalation via mouthpiece for 2 hr <i>Healthy resting:</i> 10 µg/m ³ <i>Healthy exercise:</i> 10 then 25 µg/m ³ <i>Asthma exercise:</i> 10 µg/m ³	Inhalation, exercise for 2 hr	Inhalation, exercise for 2 hr
Timing of measurements	Immediately before, immediately after, 3.5 hr after, the day after and 2 days after exposure for asthma	Immediately before, immediately after, 3.5 hr after, and the day after exposure (and 2 days after exposure for asthma)	Blood: before and 18 hr after exposure Bronchoscopy and lavage: 18 hr after exposure Respiratory function: before and after exposure
Summary of Findings			
1. Airway inflammation	<i>Healthy and asthma exercising:</i> No changes <i>Healthy resting:</i> No changes ^b	<i>Healthy and asthma:</i> Few changes; ↓ 50% in columnar epithelial cells in induced sputum. No changes in sputum IL-6 or IL-8	<i>Healthy exercising:</i> 3 × ↑ in %PMN in bronchial wash but no change in total cell number; IL-6 or IL-8; or VCAM-1 or P-selectin in tissue biopsy ↓ CD3 ⁺ cells in tissue biopsy Not measured
2. Systemic inflammation	Few changes	Few changes	
a) Soluble markers	<i>Healthy exercising:</i> smaller ↑ in sCD40L and IL-6 with UF compared to air (about ↑ 50%) <i>Asthma male exercising:</i> small ↑ NO	<i>Healthy and asthma:</i> small ↑ in sICAM-1. No change in serum amyloid A	
b) Leukocytes	<i>Healthy:</i> No difference between air and particles on total cell number (all exposures ↑). <i>Healthy female:</i> ↓ about 10% monocytes at 21 hr, ↓ 50% basophils at all times; 10% ↑ in CD25 ⁺ T cells at 3.5 hr. <i>Asthma:</i> ↓ 50% basophils at 0 and 3.5 hr, and ↓ 10% CD4 ⁺ T cells immediately after exposure. <i>Adhesion molecules and Fc receptors:</i> Some changes in expression on different cells, mostly ↓; some differences between healthy and asthma <i>Healthy male:</i> small ↓ fibrinogen and vWf	<i>Healthy and asthma:</i> No changes in total cell number or differential cell counts	No effect on numbers of total cells or leukocyte subsets
3. Coagulation		<i>Healthy and asthma:</i> No change in fibrinogen or vWf. ↑ 5%–10% in factor VII	Small ↑ in fibrinogen
4. Cardiac electrophysiology	<i>Healthy and asthma:</i> little or no change in HRV <i>Healthy:</i> smaller ↑ in QTc interval after particles than air <i>Asthma female:</i> ↓ SD of QT interval and SD of QT peak duration	<i>Healthy and asthma:</i> No change in HF or LF HRV; ↑ normalized HF leading to ↓ LF/HF ratio. No change in QTc	Not measured
5. Cardiac function		<i>Healthy and asthma:</i> small ↓ in heart rate	Not measured
6. Pulmonary function	<i>Healthy and asthma:</i> No change in FEV ₁ or FVC; ↓ minute ventilation	No change in FEV ₁ or FVC	No change in FEV ₁ or FVC or others

Asthma = subjects with asthma; healthy = healthy subjects.

^a EPA references: Ghio et al 2000; Harder et al 2001; Holgate et al 2003.

^b The Frampton study reported no changes in healthy resting participants in any of the categories of parameters described in this table. Thus, the remainder of this column refers only to exercising participants.

individuals (Figure 11). This pattern was driven by changes in men, as levels did not change in women.

Among healthy participants, total leukocyte numbers increased after all exposures, including air, with an increase in the percentage of polymorphonuclear leukocytes (PMNs) and a decrease in other leukocyte types (data not shown). Monocyte levels decreased in all groups after exposure, from approximately 8% of total leukocytes at baseline to 6% to 7% 3.5 hours after exposure. Levels returned to baseline by 21 hours, except in healthy women exposed to 25 $\mu\text{g}/\text{m}^3$ ultrafine particles (Figure 12). Basophils at 0 and 3.5 hours after exposure to 10 or 25 $\mu\text{g}/\text{m}^3$ particles decreased in women from a baseline level of approximately 1% of total leukocytes to approximately 0.5% total leukocytes. Even at 21 hours after exposure, basophil levels were still below baseline in ultrafine particle-exposed women. Lymphocyte assessments in women revealed that approximately 10% more T cells (ie, CD3⁺) expressed CD25 3.5 hours after ultrafine particle exposure than at baseline. Levels returned to baseline by 21 hours after exposure. Expression of some adhesion molecules and Fc receptors on monocytes and PMNs decreased after exposure to ultrafine particles (summarized in the Investigators' Report Table 20). Some changes were noted in only men or only women.

Among asthmatic participants, total leukocyte numbers were not affected by exposure to ultrafine particles. Basophil levels decreased approximately 50% (from a baseline level of 0.8% total leukocytes) at 0 and 3.5 hours after exposure to ultrafine particles (Figure 14). Immediately after exposure to ultrafine particles, CD4⁺ T lymphocytes decreased from 46% to 41% as a percentage of total lymphocytes. The investigators stated that no other lymphocyte subset was affected. No changes were found after exposure to ultrafine particles in the numbers of lymphocytes expressing the cytokines IFN- γ and IL-4. Expression of some adhesion molecules and Fc receptors on leukocytes changed after exposure to ultrafine particles (summarized in Table 20). Most of these changes were decreases in expression.

Pulmonary Function and Symptoms In healthy participants, exposure to ultrafine particles was associated with decreased minute ventilation during exercise and, in women only, decreased oxygen saturation; asthmatic participants at rest also showed decreased minute ventilation. In people with asthma, symptoms, vital signs, and oxygen saturation were not affected by exposure to ultrafine particles.

Cardiac Electrophysiologic Changes Exposure to ultrafine particles in healthy and asthmatic participants was associated with little if any change in measures of HRV

(Tables 16 and 18, and Figure 27). Some small changes were noted in measures of cardiac repolarization (Tables 17 and 19, and Figures 28 and 29). In healthy participants, exercise during exposure to either air or ultrafine particles decreased the QT interval and increased QTc. However, ultrafine particle exposure decreased the QT interval slightly more than did air exposure (Figure 28). The increase in QTc with ultrafine particle exposure was less than during air exposure (Figure 28). Over the next 21 hours, QTc values returned close to baseline levels after exposure to ultrafine particles but remained above baseline in people exposed to air. Thus, at all times the QTc was approximately 20 milliseconds less in people exposed to ultrafine particles than air. In asthmatic volunteers in the hours after exposure, the standard deviation of the QT interval and QT peak duration were slightly lower after ultrafine particle exposure than after air exposure (Figure 29). The investigators state that this pattern was driven mainly by responses in women.

DISCUSSION

This is an innovative and complex study. It is the first to evaluate cardiovascular and respiratory responses at multiple timepoints in healthy and asthmatic individuals before, during, and after exposure via inhalation of ultrafine particles. Moreover, Frampton and colleagues carried out a technically demanding protocol that required cooperation from volunteers on multiple visits to the Rochester research facility in order to make intrusive measurements of cardiovascular function.

The particles studied were relevant to normal human exposures: although generated in the laboratory, the particles were composed of elemental carbon, a major component of urban PM. The concentrations of ultrafine particles used in the study—10 and 25 $\mu\text{g}/\text{m}^3$, which correspond to 2 and 7×10^6 particles/cm³ (Table 4)—were much higher than average levels of ultrafine particles found in ambient urban air, however. For example, in Erfurt, Germany (Wichmann et al 2000) the daily average mass concentration of particles 50 nm in diameter was approximately 0.1 $\mu\text{g}/\text{m}^3$. In the Los Angeles area (Hughes et al 1998), the daily average mass concentration for all ultrafine particles was 1 $\mu\text{g}/\text{m}^3$. In a recent study of traffic-related PM exposures, Zhu and colleagues (2002) found the highest number of particles, dominated by small particles in the ultrafine range, to be approximately $1.5 \times 10^5/\text{cm}^3$ 30 m downwind of a freeway.

Frampton and colleagues' findings indicate that in healthy resting people a 2-hour exposure via mouthpiece to 10 $\mu\text{g}/\text{m}^3$ inhaled ultrafine carbon particles had no effect on inflammation in the airways or in the circulation, coagulation

pathways, or cardiac electrophysiologic parameters measured up to 21 hours later. In addition, the investigators did not find evidence of pulmonary inflammation or an acute phase response in exercising healthy or asthmatic volunteers. Thus, as the investigators appropriately note, these findings do not support their original hypothesis that exposure to ultrafine particles would induce pulmonary inflammation and an acute phase response.

The investigators did find some changes associated with particle exposure in exercising asthmatic and healthy participants. The changes in healthy and asthmatic participants were generally similar, suggesting that for these exposures asthmatic participants are not more susceptible than healthy participants. Some of these changes were more prominent in one sex than the other. As the investigators appropriately note, some cardiovascular and leukocyte responses differ between the sexes (Sader and Celermajer 2002), but the current study findings have not “convincingly established or excluded significant gender differences in responses to carbon ultrafine particles.”

The fact that the investigators detected small changes in cardiac repolarization but found no significant changes in HRV parameters suggests that ultrafine particles induce few changes in electrophysiologic parameters in either healthy or asthmatic participants. As the investigators appropriately note, the small changes detected in cardiac repolarization parameters such as QTc have uncertain biological or clinical significance. For example, changes in QTc associated with ultrafine particle exposure rapidly returned to baseline, but changes after air exposure did not. In addition, Frampton and colleagues found that QTc increased approximately 20 milliseconds less in exercising healthy people exposed to ultrafine particles than in those exposed to air. Generally, prolongation of the interval for more than 30 milliseconds is thought to be clinically relevant.

Among the key effects Frampton and colleagues detected were small changes in leukocyte subsets in the circulation: an apparent redistribution of cell types (because the total number of circulating leukocytes did not change) and changes in expression of activation markers expressed on specific leukocyte populations. Interestingly, the majority of changes that Frampton and colleagues detected in circulating cell numbers were *decreases*—specifically, decreases in numbers of basophils (healthy females and all asthmatic participants), monocytes (all healthy participants), and CD4⁺ T cells (asthmatic participants)—rather than increases that are generally associated with inflammatory responses. Some of the decreases in cell numbers in the circulation represented substantial changes in a particular leukocyte subset. For example, the number of basophils decreased in the circulation by approximately

50% in healthy and asthmatic populations after exposure to ultrafine particles, and CD4⁺ T cells decreased by approximately 12% in asthmatic participants. The investigators did find a small compensatory increase in circulating PMN number, a feature of systemic inflammatory responses to PM in other systems (eg, Salvi et al 1999, healthy humans exposed to diesel exhaust; Gordon et al 1998, rats exposed to CAPs; Clarke et al 2000, dogs exposed to CAPs).

Exposure to ultrafine particles was also associated with decreased expression of adhesion molecules on the surface of certain leukocytes in the blood (markers of activation). Generally, however, increased expression of adhesion molecules on leukocytes and vascular endothelial cells is considered a feature of activation in the inflammatory response (discussed in the Scientific Background). The investigators speculate on how particle exposure in exercising participants may be associated with decreases in leukocyte numbers and decreased expression of activation markers in circulating leukocytes. They propose that exposure to ultrafine particles induces mild constriction of blood vessels and activation of endothelial cells in the pulmonary vascular bed. Further, because exercise has been shown to release PMN into the circulation from the margins of blood vessels in the lung (eg van Eeden et al 1999), Frampton and colleagues suggest that particle exposure in exercising participants inhibits the outflow of activated leukocytes from the lung into the circulation. This interesting idea merits further study. However, the investigators did not evaluate the vascular bed of the lungs directly and so cannot confirm this interpretation. Vasoconstriction of small pulmonary arteries has been described to occur in healthy and bronchitic rats exposed to Boston-derived CAPs (Batalha et al 2002).

At face value, the biological significance of particle exposure rapidly depleting from the circulation the particular cell types reported—and in high numbers—is not clear. Basophils, whose circulating level dropped by 50%, are involved in the immunologic response to allergens (agents that trigger allergic responses) and have not previously been linked with *in vivo* effects of particle exposure. CD4⁺ T cells are activated by exposure to antigens, but generally only a tiny fraction of the total CD4⁺ T cell population (1 cell in 10⁵) is activated by a particular antigen. Thus, the particle exposure-associated depletion of approximately 12% of the total CD4⁺ T cell pool from blood is surprising.

As the investigators discuss, other explanations for the findings are possible; first, ultrafine particle exposure may result in the release of anti-inflammatory cytokines (such as transforming growth factor β) that interact with the surface

of particles and prevent or decrease an inflammatory response in the blood. Second, ultrafine particles might be selectively toxic to cells activated in the circulation and lead to the removal of these cells by apoptosis. An alternative possibility is that the changes measured are simply random; that is, that the investigators measured many different parameters, and by chance some of these measurements differed from baseline values. The chance nature of these positive findings may also explain why some of the findings were more prominent in one sex than the other.

One of the initial hypotheses of the study was that exposure to particles would change the pattern of cytokines synthesized by CD4⁺ T cells to a more Th2-like or allergic pattern (decreased IFN γ and increased IL-4, leading to immunoglobulin E synthesis). Skewing of responses to a Th2 pattern has been found in rodents exposed to diesel exhaust, another type of PM emission (van Zijverden et al 2000; Steerenberg et al 2003). The fact that the investigators did not find such changes suggests that the ultrafine carbon emissions may lack the components that activate Th2 CD4⁺ T cells and hence the type of immunoglobulin synthesized by B cells. Alternatively, it may indicate that ultrafine particle exposure-related changes in Th1 and Th2 cytokines cannot be detected by measuring the number of cytokine-producing cells in blood. As Frampton and colleagues reported exposure-associated activation of CD4⁺ T cells in blood in the current study, further studies of particle effects on CD4⁺ T cells and the cytokines they produce may be worth pursuing.

PARTICLE DEPOSITION

Apart from an early study examining a very small number of people with asthma (Anderson et al 1990), this is one of the first studies to examine total deposition of ultrafine particles in the lower airways of asthmatic volunteers. Thus, the investigators' finding that the deposition fraction for ultrafine particles was greater in resting asthmatic participants than resting healthy participants is novel. It suggests that at identical particle exposure levels asthmatic people may be exposed to a greater lung dose of ultrafine particles than healthy people (approximately 50% greater in this study). Their finding that the number and mass of particles deposited in exercising healthy and asthmatic participants increased approximately four times compared to levels deposited in resting participants, suggests that exercise does increase the dose of particles to the lung. Exercising healthy participants showed a limited number of effects of exposure to ultrafine particles, whereas resting healthy participants in this study showed no effects of exposure, which may be the result of the number or mass of particles taken in by the exercising individuals

exceeding some threshold. The investigators' finding that even within the ultrafine particle range smaller particles have increased deposition compared to larger particles is interesting and suggests that smaller particles preferentially deposit in the respiratory system.

One of the key features of the study design was that participants were exposed by mouthpiece to inhaled ultrafine particles. Under normal conditions, people inhale particles through the nose as well as by the mouth although exercise increases mouth breathing. Data suggest, however, that a minimal fraction of inhaled ultrafine particles in the size range used in the current report deposit in the nose (eg Heyder et al 1986). Nonetheless, some ultrafine particles inhaled via the nose are likely to be trapped there and not deposit in the lower airways. Thus the number or mass of ultrafine particles depositing in the lower airways of participants in this study might have been higher than the number or mass of particles depositing in the lower airways of people exposed to an identical concentration of particles via the nose as well as the mouth.

COMPARISON WITH RESULTS OF THE GONG STUDY AND OTHER CONTROLLED HUMAN EXPOSURE STUDIES

As described in the Introduction, before their studies started Frampton and Gong discussed designs, exposure protocols, and endpoints to determine areas of overlap that would allow some comparisons to be made when the studies were completed. The top section of the Commentary Table shows that Frampton and Gong used similar study designs and exposure protocols and investigated similar sets of endpoints. Both studies used a crossover design (ie, using the individual participant as his or her own control) to evaluate cardiovascular and respiratory effects of a single 2-hour exposure to particles and filtered air in a small number of exercising healthy and asthmatic participants. Frampton and colleagues' study also compared effects in the absence of exercise and evaluated the effect of an additional concentration of particles.

The particles used in the two studies differed. Gong evaluated fine particles concentrated from ambient air in the Los Angeles area to a target concentration that represented a worst-case scenario exposure (target 200 $\mu\text{g}/\text{m}^3$, actual 174 $\mu\text{g}/\text{m}^3$). As described above, Frampton evaluated ultrafine carbon particles at levels higher than those measured in urban air.

Despite the efforts to use similar protocols, comparing results of the Frampton and Gong studies is challenging: the studies involved small numbers of participants exposed to different types of particles in different locations. Nonetheless, some common themes are visible in the results of the two

studies. The first is that results of both studies indicate that few cardiovascular, respiratory, or cardiac electrophysiologic endpoints change in exposed healthy volunteers. Second, inflammatory effects in the airways are minimal. Third, changes in cardiovascular, respiratory, and cardiac electrophysiologic endpoints in asthmatic participants do not appear to differ greatly from those measured in healthy participants. These general conclusions may have important implications for designing future studies of particle effects: What kinds of particles should be studied? What kinds of clinical conditions may give qualitatively and quantitatively different responses from those in healthy people? Should other endpoints be examined, and if so, what is the rationale for choosing them?

The results of the Frampton and Gong studies, which indicate that CAPs and ultrafine particles induced few inflammatory, electrophysiologic, or vascular responses, parallel those of CAPs studies in healthy adults that used similar levels and durations of exposure in the Eastern United States (Ghio et al 2000; Harder et al 2001; Holgate et al 2003). These studies are also summarized in the Commentary Table. Ghio and colleagues (2000), at the EPA research facility in North Carolina, found that CAPs induced a mild inflammatory response in healthy participants exposed to 20 to 300 $\mu\text{g}/\text{m}^3$ CAPs for 2 hours while exercising intermittently (the same pattern as participants in the studies of Gong and Frampton). They found no changes in many endpoints, including symptoms, lung function, blood leukocyte differential counts, or levels of fibrinogen, IL-6, and IL-8 in bronchoalveolar lavage fluids. They did detect increases in lavaged PMN numbers and blood fibrinogen levels 18 hours after CAPs exposure (Ghio et al 2000).

By evaluating additional blood and lavage fluid parameters for the participants that Ghio and colleagues originally studied, Harder and colleagues (2001) found that CAPs exposure did not affect expression of several cell surface markers associated with activation of different cell types. This suggested that CAPs did not affect immune defenses in the airways or blood of exposed individuals (Harder et al 2001). In a further study of some of the participants in the EPA CAPs studies (Holgate et al 2003), bronchial tissues did not show any CAPs-related changes in the parameters measured, which included numbers of neutrophils, distribution of T lymphocyte subsets, and expression of adhesion molecules by vascular endothelium.

Frampton and colleagues did detect changes in circulating T lymphocyte subsets—specifically, decreased numbers of circulating CD4+ T cells in healthy participants and increased expression of the activation marker CD25 in women. In the EPA CAPs studies, T lymphocyte changes were not detected in the circulation (Harder et al 2001) or in

the airways (Holgate et al 2003). Frampton and colleagues also detected changes in the levels of some adhesion molecules, such as ICAM-1, expressed on the surface of certain leukocytes. Gong and colleagues did not measure cell-associated ICAM-1 but found small increases in levels of soluble ICAM-1. Holgate and colleagues did not find any change in expression of adhesion molecules (including ICAM-1) expressed by bronchial tissue vascular endothelium.

Effects on vascular parameters, in particular acute phase reactants, differ among the studies. For example, the Ghio, Frampton, and Gong studies all measured fibrinogen levels: after CAPs exposure Ghio and colleagues (2000) detected increases in blood levels, but at a similar time after exposure Gong and colleagues (2003) found no changes; after exposure to ultrafine particles, Frampton and colleagues detected small decreases in healthy men.

Similarly, both Frampton and Gong measured HRV and repolarization parameters QT and QTc. Gong found a small increase in normalized HF, and thus a decrease in the LF/HF ratio, an indication of a small change in parasympathetic influence on the autonomic nervous system. Frampton found few or no exposure-related effects on HRV parameters. Gong found that QTc did not change after exposure in healthy or asthmatic participants, whereas Frampton found smaller increases in the QTc interval associated with ultrafine particle exposure than with air exposure in healthy and asthmatic men. Other human CAPs exposure studies have also reported electrophysiologic and vascular changes: Devlin and colleagues (2003) reported decreased HRV in elderly people exposed to CAPs and Brook and colleagues (2002) in Toronto, Canada, found that a combination of CAPs and ozone reduced the diameter of the brachial artery (that is, were vasoconstrictive) in healthy adults exposed at rest, but they did not examine effects of the pollutants separately or evaluate possible effects on systemic or airway inflammation.

As described above, however, comparing these sets of results is challenging. The PM components responsible for effects may vary from place to place. In addition, the timing of measurements made is also critically important. In the Gong and Frampton studies, measurements were made at several timepoints, whereas in the EPA studies measurements were made only at 18 hours after exposure.

CONCLUSION

In summary, Frampton and colleagues did not detect airway or systemic effects after 12 healthy people at rest inhaled 10 $\mu\text{g}/\text{m}^3$ ultrafine carbon particles (median diameter approximately 25 nm) via a mouthpiece for 2 hours.

The investigators found few ultrafine particle-associated airway, systemic, or cardiac electrophysiologic changes at different times after similarly exposing 12 healthy volunteers to 10 or 25 $\mu\text{g}/\text{m}^3$ particles and 16 mildly asthmatic volunteers to 10 $\mu\text{g}/\text{m}^3$ particles, with intermittent exercise for both groups. For example, they found no changes in lung function, airway inflammation, total blood leukocyte differential counts, or the levels in blood of fibrinogen or serum amyloid A.

They did find that the numbers of some leukocytes changed in the circulation: decreases in basophils and monocytes in healthy women, and decreases in basophils and CD4^+ T cells in asthmatic participants. Levels of the activation marker CD25 also increased on T cells from healthy women and levels of expression of some adhesion molecules decreased on different sets of leukocytes in healthy and asthmatic participants. The investigators speculate that these changes signify that exposure to ultrafine particles activates some sets of leukocytes and vascular endothelium; activated leukocytes then migrate to and are retained in the vascular bed of the lung. This is an interesting idea that is worth studying. The investigators also found a few cardiac electrophysiologic changes—particularly in measures of cardiac repolarization—in healthy and asthmatic participants, but they were of small magnitude.

The clinical significance of any of the ultrafine particle-associated changes is not clear. The changes observed suggest, however, that in this small set of healthy and mildly asthmatic participants, the effects of exposure 10 or 25 $\mu\text{g}/\text{m}^3$ ultrafine carbon particles for 2 hours are small. The paucity of effects in the current study is consistent with the results of studies conducted in North America of human exposures to concentrated fine particles. Consequently, the results of the current study do not lend support to the hypothesis that ultrafine particles are more toxic than other components of the broader particle mix. Conducting controlled exposures with ultrafine particles of different sizes and composition; with different susceptible populations (such as those with cardiovascular disease); using a larger number of participants; longer exposure durations and higher concentrations; and measuring different endpoints would increase the statistical and scientific strength of future studies, and thus provide a stronger test of the ultrafine hypothesis.

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