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Allergic Inflammation in the Human Lower Respiratory Tract Affected by Exposure to Diesel Exhaust

Marc A. Riedl, David Diaz-Sanchez, William S. Linn,
Henry Gong Jr., Kenneth W. Clark, Richard M. Effros,
J. Wayne Miller, David R. Cocker, and Kiros T. Berhane

A grayscale image of the Earth from space, showing the Western Hemisphere with North and South America visible. The image is partially obscured by a dark red horizontal bar at the bottom.

Includes a Commentary by the Institute's Health Review Committee

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with a Commentary by the HEI Health Review Committee

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CONTENTS

About HEI	v
About This Report	vii
Preface	ix
HEI STATEMENT	i
INVESTIGATORS' REPORT by <i>Riedl et al.</i>	5
ABSTRACT	5
INTRODUCTION	6
SPECIFIC AIMS	6
STUDY DESIGN AND METHODS	7
Diesel Exhaust Delivery System and Exposure Chamber	7
Exposure Atmosphere Monitoring	8
Subject Recruitment and Screening	9
Experimental Protocol	11
Response Assessment	11
Sputum and Blood Assays	11
Physiology and Symptoms	14
STATISTICAL METHODS AND DATA ANALYSIS	15
RESULTS	16
Exposures	16
Sputum Assay Responses in Phase 1	20
Blood Assay Responses in Phase 1	20
Physiologic and Symptomatologic Responses in Phase 1	26
Sputum Assay Responses in Phase 2	29
Blood Assay Responses in Phase 2	33
Physiologic and Symptomatologic Responses in Phase 2	34
DISCUSSION AND CONCLUSIONS	36
Responses to Diesel Exhaust and Inhaled Allergen	36
Diesel Exhaust Characteristics in This Study	
Compared with Others	37
Responses in This Study Compared with Others	38
IMPLICATIONS OF THE FINDINGS	38
ACKNOWLEDGMENTS	39
REFERENCES	39
APPENDIX A. HEI Quality Assurance Statement	41
APPENDICES AVAILABLE ON THE WEB	42
ABOUT THE AUTHORS	42
OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH	42
ABBREVIATIONS AND OTHER TERMS	42

Research Report 165

COMMENTARY <i>by the Health Review Committee</i>	45
INTRODUCTION	45
SCIENTIFIC BACKGROUND	45
Asthma and the Allergic Response	45
Components and Health Effects of DE	46
Components	46
Traffic-Derived Pollution	46
Health Effects	46
TECHNICAL EVALUATION OF THE STUDY	47
Objectives and Specific Aims	47
Study Design	48
Pilot Study and Changes to the Main Study	48
Design of the Main Study	50
Generation and Characterization of Atmospheres	50
Study Population	50
Health Endpoint Measurements	51
Bronchial Reactivity	52
Statistical Methods	52
Key Findings	52
Characterization of Components of the	
Exposure Atmospheres	52
Health Endpoints	53
Summary of Phase 1 and Phase 2 Findings and	
Authors' Interpretations	53
Period Effects	53
THE HEALTH REVIEW COMMITTEE'S EVALUATION	
OF THE STUDY	55
General Comments	55
Strengths and Limitations of the Study	56
Strengths	56
Limitations	56
Comparison of This and Previous Studies by	
These Investigators	56
Routes and Concentrations of Exposure	57
Sources of DEP	57
Airways and Tissues Evaluated at Different Times	57
Major Characteristics of the Participants	57
Other Key Considerations	58
Real-World Level of DE Exposure	58
NO ₂	58
Future Studies of the Effects of DE Exposure	58
Summary and Conclusions	59
ACKNOWLEDGMENTS	60
REFERENCES	60
Related HEI Publications: Particulate Matter and	
Diesel Exhaust	63
HEI Board, Committees, and Staff	65

ABOUT HEI

The Health Effects Institute is a nonprofit corporation chartered in 1980 as an independent research organization to provide high-quality, impartial, and relevant science on the effects of air pollution on health. To accomplish its mission, the institute

- Identifies the highest-priority areas for health effects research;
- Competitively funds and oversees research projects;
- Provides intensive independent review of HEI-supported studies and related research;
- Integrates HEI's research results with those of other institutions into broader evaluations; and
- Communicates the results of HEI's research and analyses to public and private decision makers.

HEI typically receives half of its core funds from the U.S. Environmental Protection Agency and half from the worldwide motor vehicle industry. Frequently, other public and private organizations in the United States and around the world also support major projects or research programs. HEI has funded more than 280 research projects in North America, Europe, Asia, and Latin America, the results of which have informed decisions regarding carbon monoxide, air toxics, nitrogen oxides, diesel exhaust, ozone, particulate matter, and other pollutants. These results have appeared in the peer-reviewed literature and in more than 200 comprehensive reports published by HEI.

HEI's independent Board of Directors consists of leaders in science and policy who are committed to fostering the public-private partnership that is central to the organization. The Health Research Committee solicits input from HEI sponsors and other stakeholders and works with scientific staff to develop a Five-Year Strategic Plan, select research projects for funding, and oversee their conduct. The Health Review Committee, which has no role in selecting or overseeing studies, works with staff to evaluate and interpret the results of funded studies and related research.

All project results and accompanying comments by the Health Review Committee are widely disseminated through HEI's Web site (www.healtheffects.org), printed reports, newsletters and other publications, annual conferences, and presentations to legislative bodies and public agencies.

ABOUT THIS REPORT

Research Report 165, *Allergic Inflammation in the Human Lower Respiratory Tract Affected by Exposure to Diesel Exhaust*, presents a research project funded by the Health Effects Institute and conducted by Dr. Marc A. Riedl of the University of California–Los Angeles and his colleagues. This report contains three main sections.

The HEI Statement, prepared by staff at HEI, is a brief, nontechnical summary of the study and its findings; it also briefly describes the Health Review Committee's comments on the study.

The Investigators' Report, prepared by Riedl and colleagues, describes the scientific background, aims, methods, results, and conclusions of the study.

The Commentary is prepared by members of the Health Review Committee with the assistance of HEI staff; it places the study in a broader scientific context, points out its strengths and limitations, and discusses remaining uncertainties and implications of the study's findings for public health and future research.

This report has gone through HEI's rigorous review process. When an HEI-funded study is completed, the investigators submit a draft final report presenting the background and results of the study. This draft report is first examined by outside technical reviewers and a biostatistician. The report and the reviewers' comments are then evaluated by members of the Health Review Committee, an independent panel of distinguished scientists who have no involvement in selecting or overseeing HEI studies. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, as necessary, to revise their report. The Commentary reflects the information provided in the final version of the report.

PREFACE

HEI's Research on Particles and the Exacerbation of Allergy and Asthma

Particles emitted or formed from gases derived from a number of sources (e.g., diesel- and gasoline-powered vehicles, electric power plants, factories) contribute to the exposure levels of particulate matter (PM) for urban populations. Epidemiologic and experimental studies published in the 1990s suggested that people exposed to diesel exhaust and other particles for a short term would suffer adverse effects on their respiratory and immune systems. In particular, these effects could be more severe in persons with asthma and other allergic diseases. Some studies raised the question of whether various constituents of traffic-generated air pollution (including diesel exhaust particles) might contribute to an increase in symptoms of asthma or rhinitis or might enhance immune responses to allergens. In reviewing this literature, however, the HEI Health Research Committee noted that the epidemiologic studies had not measured ambient concentrations of individual pollutants, and many of the experimental studies had not used relevant exposure routes and particle doses. Thus, the Committee considered that additional research with improved measures of exposure and outcomes was needed to elucidate dose–response relationships.

In 2000, HEI issued Request for Applications (RFA) 00-1, *Effects of Diesel Exhaust and Other Particles on the Exacerbation of Asthma and Other Allergic Diseases*. The RFA sought studies that would provide a better understanding of whether, and by what mechanisms, particles from diesel engines and other sources may contribute to the frequency and severity of asthma attacks and to the exacerbation of allergic responses — especially in susceptible population groups such as children, older people, and people with chronic

diseases. The Committee was interested in research proposals for epidemiologic studies, studies of controlled exposure with humans, and animal studies.

RFA 00-1 was part of a larger HEI effort to study the effects on human health of exposure to current and future diesel particulate emissions and to investigate how the toxicity of diesel particles compares with that of other ambient particles. HEI's earlier efforts included a comprehensive review of the health effects associated with diesel emissions (HEI Diesel Working Group 1995) and a project initiated in 1998 to examine more closely the epidemiologic studies used in quantitative risk assessment (HEI Diesel Epidemiology Expert Panel 1999). Four studies were funded under RFA 00-1; the research project from Marc Riedl of the University of California–Los Angeles is presented in this Research Report. The other three have been published.

Exacerbation of Allergic Inflammation in the Lower Respiratory Tract by Diesel Exhaust Particles Marc A. Riedl, University of California–Los Angeles (original Principal Investigator was David Diaz-Sanchez, now at the U.S. Environmental Protection Agency)

The goal of the study by Riedl and colleagues was to evaluate whether inhalation of diesel exhaust particles by allergic asthmatic individuals would enhance inflammatory or allergic immunologic responses in the lower airways and blood. The study compared the effects of exposure to whole diesel exhaust and to NO₂, which is a major component of the exhaust's gas phase. It is presented in this Research Report 165, *Allergic Inflammation in the Human Lower Respiratory Tract Affected by Exposure to Diesel Exhaust* (Riedl et al. 2012).

The Relationship Between Pollutant Particles in Alveolar Macrophages from Normal Children and Proxy Markers of PM₁₀ Exposure Jonathan Grigg, University of Leicester, United Kingdom

The study by Grigg and colleagues examined whether the detection of PM (which was not specific to diesel exhaust) in macrophages obtained from sputum could be used as a biomarker of PM exposure in children. They also explored the relationship between PM load in macrophages and markers of inflammation in children's lungs. This study was published as Research Report 134, *Black-Pigmented Material in Airway Macrophages from Healthy Children: Association with Lung Function and Modeled PM₁₀* (Grigg et al. 2008).

Health Effects of Diesel Exhaust in Asthmatics: A Real-World Study in a London Street Junfeng (Jim) Zhang, University of Medicine and Dentistry of New Jersey—School of Public Health

The investigators used two locations in London, United Kingdom, to study the effects of a real-world exposure to urban diesel traffic in persons with mild or moderate asthma. The study tested the hypotheses that exposure to diesel exhaust leads to worsening of asthma symptoms, as evidenced by a reduction in lung function and increased oxidative stress and inflammation, and that such effects are dependent on the severity of asthma. Each subject participated in an experimental exposure session and a control exposure session by walking in each site for two hours while portable monitors determined concentrations of selected air pollutants. The exposure site was Oxford Street where motor-vehicle traffic was restricted to taxis and buses, the majority of which were powered by diesel engines; the control site was Hyde Park with no motor-vehicle traffic. The subjects' asthma symptoms, pulmonary function, and indicators of inflammation and oxidative stress were subsequently measured. This study was published as Research Report 138, *Health Effects of Real-World Exposure to Diesel Exhaust in Persons with Asthma* (Zhang et al. 2009).

Fine Airborne Particles and Allergic Diseases

Jack Harkema, Michigan State University

The study by Harkema and colleagues focused on the effect of whole diesel exhaust and concentrated ambient particles on the induction (sensitization phase) and exacerbation (challenge phase) of antigen-induced epithelial remodeling and inflammation in Brown Norway rats. The underlying hypothesis was that inhalation of particles during either sensitization or challenge causes airway remodeling and exacerbates airway inflammation. This study was published as Research Report 145, *Effects of Concentrated Ambient Particles and Diesel Engine Exhaust on Allergic Airway Disease in Brown Norway Rats* (Harkema et al. 2009).

ADDITIONAL HEI STUDIES

While the studies funded under RFA 00-1 were in progress, the U.S. Environmental Protection Agency introduced lower PM emission standards for heavy-duty diesel engines, which are expected to result in lower concentrations of several pollutants in ambient air. As part of an effort to assess how emissions and health effects of diesel engines may be changing with the introduction of technologies developed to meet the new standards, HEI initiated the Advanced Collaborative Emissions Study (ACES) in collaboration with the Coordinating Research Council, a nonprofit organization that directs research on automotive technologies and fuels. This program has several components: detailed studies to characterize the emissions from engines built to meet the 2007 (Coordinating Research Council 2009) and 2010 (currently in progress) emission standards, and a chronic inhalation bioassay in rodents to evaluate the health effects of both short- and long-term exposure to the emissions of a 2007 model-year engine. HEI will publish results from intermediate time points in the chronic exposure animal bioassay during 2012.

REFERENCES

Coordinating Research Council. 2009. Phase I of the Advanced Collaborative Emissions Study. CRC Report: ACES Phase I. CRC, Alpharetta, GA. Available from www.crao.com/reports/recentstudies_2009/ACES%20Phase%20I/ACES%20PhaseI%20Final%20Report%20I5JUN2009.pdf.

Grigg J, Kulkarni N, Pierse N, Rushton L, O'Callaghan C, Rutman A. 2008. Black-Pigmented Material in Airway Macrophages from Healthy Children: Association with Lung Function and Modeled PM₁₀. Research Report 134. Health Effects Institute, Boston, MA.

Harkema JR, Wagner JG, Kaminski NE, Morishita M, Keeler GJ, McDonald JD, Barrett EG. 2009. Effects of Concentrated Ambient Particles and Diesel Engine Exhaust on Allergic Airway Disease in Brown Norway Rats. Research Report 145, Health Effects Institute, Boston, MA.

HEI Diesel Epidemiology Expert Panel. 1999. Diesel Emissions and Lung Cancer: Epidemiology and Quantitative Risk Assessment. Special Report. Health Effects Institute, Cambridge, MA.

HEI Diesel Working Group. 1995. Diesel Exhaust: A Critical Analysis of Emissions, Exposure, and Health Effects. Special Report. Health Effects Institute, Cambridge, MA.

Riedl MA, Diaz-Sanchez D, Linn WS, Gong H Jr, Clark KW, Effros RM, Miller JW, Cocker DR, Berhane KT. 2012. Allergic Inflammation in the Human Lower Respiratory Tract Affected by Exposure to Diesel Exhaust. Research Report 165. Health Effects Institute, Boston, MA.

Zhang J, McCreanor JE, Cullinan P, Chung KF, Ohman-Strickland P, Han I-K, Järup L, Nieuwenhuijsen MJ. 2009. Health Effects of Real-World Exposure to Diesel Exhaust in Persons with Asthma. Research Report 138. Health Effects Institute, Boston, MA.

HEI STATEMENT

Synopsis of Research Report 165

Effects of Controlled Exposure to Diesel Exhaust in Allergic Asthmatic Individuals

INTRODUCTION

Increasingly stringent emission standards and advances in engine technology have resulted in decreasing particulate matter concentrations and gaseous emissions from modern diesel engines. At the same time, diesel engine exhaust (DE) from older engines in the current fleet continues to contribute significantly to traffic-derived ambient particles, an important component of urban air pollution. Epidemiologic and experimental studies published in the 1990s suggested that short-term exposure to either whole diesel emissions or to the particulate fraction (diesel exhaust particles; DEP) may be associated with adverse respiratory and immune system effects in humans. It had been suggested that these effects could be more severe in persons with asthma and other allergic diseases.

Dr. David Diaz-Sanchez, of the University of California–Los Angeles (UCLA), and his colleagues at the University of California–Riverside and Los Amigos Research and Education Institute (LAREI) proposed to evaluate the effects of inhaled DEP on the lower airways and blood of healthy subjects and of allergic asthmatic individuals. The investigators hypothesized that inhalation of DEP would enhance inflammatory or allergic immunologic responses to allergens. HEI's Health Research Committee considered the proposed study to be a logical follow-up to the principal investigator's previous studies of the effects of DEP administered into the upper airways; but it recommended several changes in study design — most importantly, to evaluate the effects of inhalation of whole DE, thus providing the opportunity to study a more realistic concentration and route of exposure to DEP. After discussions with Diaz-Sanchez, the Committee recommended funding a revised proposal to study the effects of DE inhalation.

In the course of the study, Diaz-Sanchez moved to the U.S. Environmental Protection Agency, ending his association with the project; and Dr. Henry Gong, who had been leading the study at LAREI, died. Ultimately, Dr. Marc Riedl at UCLA assumed the role of principal investigator.

APPROACH

For the source of DE, the investigators purchased a 1999 medium-duty diesel pickup truck with a 250-hp turbocharged V-8 engine that used ultra-low-sulfur diesel fuel and had been driven for approximately 60,000 miles. The truck was equipped with a 3-way catalyst, which the investigators decided to remove in order to generate and evaluate the effects of raw diesel exhaust.

In a pilot study, the investigators built a diesel exposure facility at LAREI with a human exposure chamber, characterized the DE generated by the truck, and exposed a small group of healthy (i.e., non-allergic, non-asthmatic) participants to DE and to filtered air. After discussing the pilot data with the Research Committee, the investigators eliminated exposures of healthy non-asthmatic participants from the main study and increased the number of participants who had both allergy and asthma. They also added an exposure to nitrogen dioxide (NO₂), a gaseous component of DE that has the potential to affect some of the same health endpoints.

Thus, the goal of the main study became to evaluate the effects of DE and NO₂ exposure on multiple airway and systemic inflammatory responses in allergic asthmatic individuals in the *absence of* (Phase 1) or *after* (Phase 2) an inhalation challenge with an allergen to which they were sensitive. All participants were non-smokers between the ages of 18 and 50. Phase 1 included 15 subjects with either

mild-intermittent or mild-persistent asthma who were also allergic to at least one of a panel of common aeroallergens. The 15 participants in Phase 2 had the same asthma diagnosis and were sensitive to cat allergen.

Participants in both phases were exposed in random order to DE (at a particle mass concentration of $\sim 100 \mu\text{g}/\text{m}^3$), 0.35 ppm NO_2 , or filtered air (control) for 2 hours while intermittently exercising on a stationary bicycle. To minimize carry-over effects, exposure sessions were separated by at least 4 weeks.

The investigators measured multiple physiologic and pulmonary function endpoints that included specific airway resistance, oxygen saturation, and bronchial reactivity. Bronchial reactivity was measured 1.5 hours after exposure — in Phase 1 subjects were challenged with the bronchoconstrictor methacholine, and in Phase 2 they were challenged with cat allergen. The investigators also assessed several endpoints associated with inflammatory and immunologic responses in the airways (via sputum induction) and blood.

RESULTS

The DE exposure atmospheres were close to the target particle concentration of $100 \mu\text{g}/\text{m}^3$ and contained about 0.35 ppm NO_2 , similar to the concentration used in the NO_2 -only exposures.

Exposure to DE or NO_2 affected only a few inflammatory, immunologic, or physiologic endpoints, and few of the changes occurred in both phases. Several outcome measures were below the limit of detection, and some changes were not in the direction expected for adverse effects; in particular, this was true of some sputum and blood endpoints. Levels in sputum and blood of immunoglobulin E, a key mediator of the allergic immune response, were not affected by either DE or NO_2 exposure in either phase.

DE exposure slightly increased airway resistance in Phase 1 and the numbers of polymorphonuclear cells and eosinophils in sputum in Phase 2. These findings suggested to the investigators that this exposure concentration and duration may be near the lowest levels that might cause airway inflammatory effects in mildly asthmatic individuals. Exposure to NO_2 resulted in only a few changes in Phase 1 (e.g., immunoglobulin M levels in blood increased) and none in Phase 2.

In both Phases 1 and 2, the investigators found that many endpoints, particularly those measured in sputum, showed “period” effects, indicating that a pattern of responses was related to the progression of exposure periods (1st, 2nd, 3rd) rather than to the exposure atmospheres themselves. Such effects have not been reported in other similar studies.

INTERPRETATION AND CONCLUSIONS

The HEI Health Review Committee, which conducted an independent review of the study, noted that the study addressed an important issue — the effects of inhalation exposure to DE, a major component of ambient urban air pollution, on the lower airways and blood of allergic asthmatic individuals. The study had several strong points, including the use of a more realistic concentration and route of exposure than those used in earlier experiments by the same research team; inclusion of an additional control atmosphere (NO_2); evaluation of responses both in the absence of (Phase 1) and after (Phase 2) a post-exposure challenge with cat allergen; and use of minimally invasive techniques for assessing multiple endpoints in lungs and blood — sites that are central to the asthmatic response.

The Committee thought the relative limitations of the study included the evaluation of effects of exposure at only one time point during the late phase of the allergic response (22 hours after exposure) and the use of only one allergen concentration in the challenge protocol in Phase 2. The detection of period effects, particularly for sputum endpoints, may suggest some sort of learning process over the course of the three exposure periods. Whatever their physiologic basis, the period effects complicated assessment of responses to pollutant atmospheres.

The Committee agreed with the authors’ conclusions that the findings were predominantly negative and did not support the original hypothesis that exposure to DE would enhance inflammatory or allergic type responses. The Committee also agreed that caution is needed in extrapolating from these findings. For example, it is possible that individuals with more severe asthma, who are exposed for longer time periods or to higher DE concentrations, or who are exposed to other DE mixtures from other types of diesel engines, fuels, or both, may have different responses.

The lack of effects of DE inhalation in Phase 2 contrasts with the results of previous studies of the effects of DEP administered into the upper airways of allergic individuals by the same team of investigators. Explanations may include differences in the exposures (with much higher concentrations and less realistic routes of exposure in the earlier studies) and likely differences in the composition of the DEP. The times at which tissues were evaluated after challenge and the health status and allergen sensitivity of participants also differed. Furthermore, variations in study design — such as the timing of allergen challenge and the use of exercise versus rest — may also explain why this study did not find changes, which had been found in some earlier studies, in immunologic or inflammatory endpoints after exposure to 0.35 ppm NO₂.

The current study evaluated effects of DE from a 1999 engine with no catalytic converter, which was likely to be representative of a fraction of the vehicle fleet in use at the time. Since 1999, however, tighter emission standards for NO_x and particulate matter

have significantly reduced the overall emissions of DE and its major components from new vehicles. In particular, particle emissions from light- and heavy-duty trucks have been reduced by more than 10-fold through the use of particulate filters (starting with the 2006 and 2007 model years, respectively) and low-sulfur fuels. The concentration of DEP used in the current study (100 µg/m³) may be considered high in the United States and Europe. However, even at this comparatively high level, there appear to be very few biologic effects of exposure to this level of DEP in people with asthma.

For future studies to be relevant to the improvements in engines and fuels, they will need to target much lower concentrations of DEP while also evaluating the gaseous components that will start to dominate the exhaust mixture. HEI's ongoing Advanced Collaborative Emissions Study is among the first to comprehensively study emissions from the new diesel engine and aftertreatment technology, starting with the health effects of exhaust in animals.

Allergic Inflammation in the Human Lower Respiratory Tract Affected by Exposure to Diesel Exhaust

Marc A. Riedl, David Diaz-Sanchez, William S. Linn, Henry Gong Jr., Kenneth W. Clark, Richard M. Effros, J. Wayne Miller, David R. Cocker, and Kiros T. Berhane

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ABSTRACT

To improve understanding of human health risks from exposure to diesel exhaust particles (DEP*), we tested whether immunologic effects previously observed in the human nose also occur in the lower airways. Our overall hypothesis was that cell influx and production of cytokines, chemokines, immunoglobulin E (IgE), and other mediators, which would be measurable in sputum and blood, occur in people with asthma after realistic controlled exposures to diesel exhaust (DE). In Phase 1 we tested for direct effects of DE in subjects with clinically undifferentiated mild asthma. In Phase 2 we tested whether DE exposure would exacerbate response to inhaled cat allergen in subjects with both asthma and cat sensitivity.

The exposure facility was a controlled-environment chamber supplied with DE from an idling medium-duty truck with ultra-low-sulfur fuel and no catalytic converter. We exposed volunteers for 2 hours with intermittent exercise to exhaust with DEP mass concentration near 100 $\mu\text{g}/\text{m}^3$. Exposures to nitrogen dioxide (NO_2) near 0.35 ppm (similar to its concentration in DE) and to filtered air (FA) served as controls. Blood was drawn before exposure

on day 1 and again the next morning (day 2). Sputum was induced only on day 2. Bronchial reactivity was measured ~1 hour after exposure ended. Supplementary endpoints included measures of blood coagulation status, cardiopulmonary physiology, and symptoms. Each phase employed 15 subjects with asthma; 3 subjects participated in both phases. In Phase 1, airway reactivity was measured with inhaled methacholine; in Phase 2, with inhaled cat allergen.

We found little biologic response to DE exposure compared with exposure to control atmospheres. In Phase 1, interleukin 4 (IL-4) in sputum showed an estimated 1.7-fold increase attributable to DE exposure, which was close to statistical significance; airway resistance increased modestly but significantly on day 2 after DE exposure; and nonspecific symptom scores increased significantly during DE exposure. In Phase 2, indicators of airway inflammation in sputum showed a possibly meaningful response: polymorphonuclear leukocytes (PMNs) and eosinophils increased after DE exposure, whereas macrophages decreased. IgE in sputum and the bronchoconstrictive response to cat allergen varied significantly between atmospheres, but not in patterns consistent with our primary hypothesis. Symptom score changes relatable to DE exposure were smaller than those in Phase 1 and not statistically significant.

Controlled exposures, lasting 2 hours with intermittent exercise, to diluted DE at a particle mass concentration of 100 $\mu\text{g}/\text{m}^3$ did not evoke clear and consistent lower-airway or systemic immunologic or inflammatory responses in mildly asthmatic subjects, with or without accompanying challenge with cat allergen. Likewise, these DE exposures did not significantly increase nonspecific or allergen-specific bronchial reactivity. A few isolated statistically significant or near-significant changes were observed during and after DE exposure, including increases in nonspecific symptoms (e.g., headache, nausea) suggestive of subtle, rapid-onset systemic effects. It is possible the lower respiratory

This Investigators' Report is one part of Health Effects Institute Research Report 165, 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. Marc A. Riedl, University of California–Los Angeles, 200 UCLA Medical Practice, Department of Allergy and Immunology, Los Angeles, CA 90095; e-mail: mriedl@mednet.ucla.edu.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award CR-83234701 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.

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

tract is more resistant than the nose to adjuvant effects of diesel particles on allergic inflammation, so that no meaningful effects occur under exposure conditions like these. Alternatively, the experimental conditions may have been near a threshold for finding effects. That is, important lower respiratory effects may occur but may be detectable experimentally with slightly higher DEP concentrations, longer exposures, more invasive testing (e.g., bronchoalveolar lavage), or more susceptible subjects. However, ethical and practical barriers to such experiments are considerable.

INTRODUCTION

The overall goal of this study was to better understand the acute health effects that may result from exposure to DEP — in particular, lower respiratory immunologic responses. Relevant previous research has been reviewed by Viera and colleagues (2009), Riedl (2008), Riedl and Diaz-Sanchez (2005), Nikasinovic and colleagues (2004), Li and colleagues (2003), and Pandya and colleagues (2002). Also, some recently published experimental studies (Nordenhäll et al. 2000; Stenfors et al. 2004) have addressed inflammatory responses to DE observed in the lower respiratory tract, but have not included immunologic assessment; their findings are reviewed briefly in the Discussion and Conclusions section of this report.

The overall body of evidence — from studies in vitro, in laboratory animals, and in the human nose — indicates that DEP can exert direct inflammatory effects at levels relevant to short-term human occupational and ambient exposures. Perhaps more importantly, past evidence shows that DEP can have strong adjuvant effects on the development and intensity of response to inhaled allergens (Diaz-Sanchez et al. 1997, 1999, 2000), which may help to explain the significant spatial and temporal associations of asthma with motor vehicle-related pollution that have often been observed in epidemiologic studies (Venn et al. 2001; Brauer et al. 2002; Lin et al. 2002; Zmirou et al. 2004). In human experimental studies (Diaz-Sanchez et al. 1997, 1999, 2000), adjuvant effects have been found after nasal instillation of 300 µg DEP (comparable to a 24-hour inhaled dose in urban air pollution) along with allergens. Although previous animal experiments have described effects of DEP plus allergen exposure in the lower airway (Inoue et al. 2008; Harkema et al. 2009), to our knowledge no controlled in vivo human DE chamber exposures have investigated the physiologic and immunologic pulmonary effects of DE exposure in conjunction with allergen in subjects with atopic asthma.

The primary goals of this study were to determine (1) whether the immunologic and physiologic changes previously observed in the upper airways of human volunteers after DE exposure also occur in the lower airways, and (2) whether these changes result in exacerbation of asthma in response to allergen. Most importantly, we tested whether exposure to inhaled diluted whole DE preferentially alters lower-airway immunologic responses (cell influx, and production of mediators associated with allergy — including antibodies, cytokines, chemokines, and others — as measured by assays of sputum and blood) in asthmatic volunteer subjects. We also investigated acute physiologic and symptomatic responses likely to result from exposure to inhaled irritants. The experimental DEP concentration was set at 100 µg/m³ particle mass, intended to represent a “worst-case” community exposure level or a moderately high occupational exposure level. We adopted an exposure protocol commonly used in controlled laboratory studies of community air pollution: exposure duration was 2 hours, with intermittent moderate exercise for 15 minutes of each half-hour. Under this protocol, the typical dose of DEP in terms of total mass would be similar to or slightly less than that in the aforementioned nasal-instillation studies (Diaz-Sanchez et al. 1997, 1999, 2000).

Ideally, DEP would have been physically separated from whole DE for controlled exposures in order to verify that any observed responses were caused by inhaled particles rather than accompanying gases. Because that was not feasible, two control conditions were used for comparison with whole DE exposures: a conventional sham exposure to FA with the lowest attainable concentrations of background particulate and gaseous pollutants, and an exposure to filtered air with added NO₂ at a concentration comparable to that in the DE atmosphere. NO₂ was considered to be the most important gaseous component of DE in terms of the relationship between its concentration and inherent toxicity (Blomberg et al. 1997; Barck et al. 2005). Either airway-irritant or immunologic responses to NO₂ might conceivably add to the overall response to DE exposure. Thus, the difference in response between DE and NO₂ exposures would provide the best indication of effects due to DEP.

SPECIFIC AIMS

Specific aims of the project were:

1. Develop a source of DE that would be practical to operate and would produce emissions with characteristics typical of diesel-powered vehicles in current use.

2. Develop a suitable controlled-exposure facility. Modify an existing exposure chamber by adding an interface for the exhaust source (a diesel pickup truck), a dilution system, and monitoring instruments able to characterize the exposure atmosphere in detail.
3. Recruit and medically characterize suitable volunteers.
4. Expose each volunteer to DE and control atmospheres, in random order, with time separation to minimize carryover effects; and characterize their immunologic, physiologic, and symptomatic responses.

The initial strategy for aims 3 and 4 was to compare responses among three groups of 10 subjects each: healthy subjects, asthmatic subjects without cat allergy, and asthmatic subjects with cat allergy. To maximize safety, the first 4 subjects exposed were all healthy. None of them showed a meaningful response when data were examined individually. Accordingly, in consultation with HEI project managers and the Health Research Committee, the strategy was modified: 15 subjects with mild asthma, undifferentiated with respect to cat-allergen sensitivity, were studied in Phase 1; in Phase 2, 15 subjects with mild asthma and known cat allergy were studied (3 subjects participated in both phases). On the basis of available resources and past experience, we determined that a sample of 15 subjects would provide sufficient (better than 80%) statistical power to detect “minimum clinically significant” changes in respiratory-irritant endpoints as statistically significant at $P < 0.05$. No directly comparable past data were available to support statistical power calculations for immunologic measures that would test our primary hypotheses. However, their power was believed to be adequate on the basis of prior nasal-challenge studies, which commonly showed highly significant immunologic responses in similar or smaller numbers of subjects (Diaz-Sanchez et al. 1996, 1997, 2000; Bastain et al. 2003; Riedl and Diaz-Sanchez 2005).

We focused our immunologic investigations on the effects of DEP that have been previously documented in animal models or in the human upper airway (Diaz-Sanchez et al. 1997, 2000; Fujieda et al. 1998): allergic immune responses (e.g., increases in IgE and Th2 cytokines) and inflammatory responses (e.g., cell influx, and increased chemokines and other inflammatory mediators). In addition, we used conventional tests of cardiopulmonary function: bronchial reactivity, spirometry, airway resistance, blood pressure, arterial oxygen saturation (SaO_2), and symptom inventories. These three categories of responses (allergic immune responses, inflammatory responses, and cardiopulmonary function) might be intimately linked; for example, enhanced mediator release might result in rapid

bronchoconstriction, and an elevation in IgE might predispose an individual to greater mediator release and more severe airway responsiveness.

In Phase 1, in which changes in allergic status were not considered, nonspecific bronchial reactivity was measured by conventional methacholine inhalation challenge. In Phase 2, for which all subjects had been prescreened for sensitivity to cat allergen, bronchial reactivity was measured by cat allergen inhalation challenge. In all other ways the protocols for exposure and response measurements were the same in both phases.

In summary, the specific aims focused on two key hypotheses for a population of subjects with mild asthma: (1) realistic exposures to DE provoke clinically meaningful lower-airway and systemic immunologic responses similar to nasal responses observed previously; and (2) DEP can interact with allergens to exacerbate lower-airway allergic inflammation.

STUDY DESIGN AND METHODS

DIESEL EXHAUST DELIVERY SYSTEM AND EXPOSURE CHAMBER

The publication by Sawant and colleagues (2008; Appendix E, which is available on the HEI Web site) describes the exposure facility and procedures in detail.

The DE source was an idling (900 rpm) 250-hp Navistar International T 444E 7.27-L turbocharged V-8 engine in a 1999 medium-duty Ford diesel pickup truck operating on ultra-low-sulfur diesel fuel (typical sulfur concentration 10 ppm by volume, maximum 15 ppm).

The oxidation catalyst originally fitted in the vehicle's exhaust system was removed before characterizing the engine's emissions. We considered it most appropriate to operate the truck without its emissions control catalyst in order to produce emissions characteristic of diesel engines in use without having to factor in any unique characteristics or impacts of the control system. Comparative characterization tests showed that removal of the catalyst increased the mass concentration of particle emissions by 25%–30% and had little effect on measured emissions of gas-phase pollutants (Gong 2002). Characterization tests, following the Federal Test Procedure (FTP)-75 test cycle, were performed at the College of Engineering, Center for Environmental Research and Technology (CE-CERT) vehicle emission research laboratory to confirm that emissions were typical of the chosen engine and model year of the vehicle. These standard emission tests were used to set baseline dilution rates and design constraints appropriate to the exposure facility at Rancho Los Amigos National Rehabilitation Center.

Oxides of nitrogen (NO_x), carbon monoxide (CO), total hydrocarbon, and particulate matter (PM) emissions were in line with those reported by Durbin and colleagues (2003). These species are the most commonly measured pollutants from vehicle exhaust and are appropriate to verify a consistent operational state of the engine over the course of the study.

Additional measurements were conducted for detailed chemical speciation and particle-size distribution, so that the system could be compared rigorously with certification-quality vehicle-testing data and with other laboratories' systems for controlled DE exposure. Both chemical and physical characteristics of the exhaust were verified as matching between the tests at CE-CERT and at the exposure facility. Chemical characterization included polycyclic aromatic hydrocarbons (PAHs), carbonyl compounds, light hydrocarbons, elemental and organic carbon (EC and OC), NO_x , and CO. Physical characterization included PM mass, PM number, and electrical mobility distribution.

To maximize the uniformity of emissions throughout the project, fuel was obtained in barrels from a single source. Periodic engine oil changes were performed in normal fashion by a commercial garage. The vehicle mileage was approximately 60,000, which was deemed sufficiently high to have stable emissions. The truck was driven about 25 km in suburban traffic before each characterization test or exposure to assure complete warm-up and stable operation.

A nozzle inserted into the vehicle's tailpipe collected undiluted exhaust, which was transferred through a short insulated coupling (similar to those used in standard emission test protocols) to a dilution tunnel connected to the exposure chamber. Based on the flows and displacement volumes of the system, the estimated time for dilution was 0.2 seconds. Dilution air, supplied by the laboratory building's climate-control system, was cleaned by permanganate/aluminum oxide chemisorbent (Purafil Inc., Doraville, GA) and high-efficiency particle air (HEPA) filters. The dilution line was in operation for 30 minutes or more, and stable readings on the real-time gas and particle monitoring instruments were verified before beginning an exposure.

The exposure chamber, about 10 m^3 in volume, was constructed of rigid clear plastic panels supported by aluminum framing, and was located inside a laboratory near an exterior wall with a port to the adjacent parking area for the DE source vehicle. A variable-speed blower downstream of the chamber was set to provide air flow of about $2.7 \text{ m}^3/\text{min}$, giving a residence time in the chamber of about 200 seconds. Detailed chemical and physical analyses confirmed that the DEP and associated gases within the chamber were representative of typical nontreated DE streams. A scanning mobility particle sizer (SMPS) (described below) was used to set DEP mass concentration

loading ($100 \text{ }\mu\text{g}/\text{m}^3$) in the exposure chamber, as determined by micro-orifice uniform-deposit impactor (MOUDI) analysis of particle mass versus size range. Background air supplied to the chamber was filtered by passage through potassium permanganate/alumina chemisorbent and activated carbon. For FA exposures, nothing was added. For NO_2 exposures, NO_2 was supplied using a conventional low-flow metering system and gas cylinder containing 5% NO_2 in nitrogen. (A description of the chamber before its modification for DE exposures has been published previously [Weymer et al. 1994].)

EXPOSURE ATMOSPHERE MONITORING

Our initial proposal for the target DEP mass concentration for exposures was $200 \text{ }\mu\text{g}/\text{m}^3$ lasting 1 hour. The HEI Research Committee consulted with us and recommended using $100 \text{ }\mu\text{g}/\text{m}^3$ for 2 hours and the protocol was revised accordingly. Initial pilot tests determined proper adjustments of the exhaust collection and dilution systems to attain the target concentration. However, in subsequent pilot tests we noted that with human subjects in the chamber, the collected PM mass exceeded the intended target. We later determined that this was due to particles released by the subjects during activity in the chamber. Fortunately, the particles emitted by subjects were larger than the particle sizes measured by the SMPS and the target DEP concentration was unaffected.

Detailed descriptions of exposure chamber air monitoring are given in Appendices B and E (Sawant et al. 2008) (both appendices are available on the HEI Web site). Overall particle mass concentration was routinely determined after exposure by low-volume sampling on Teflon filters. Continuous quasi-real-time particle-size distribution measurements were conducted during the exposure using an SMPS that provided electrical mobility and number concentration measurements for particles from 28 to 730 nm, a size range that covered the large accumulation peak of DEP. During two different DE exposures, DEP concentration as a function of particle-size range was documented by sampling with a MOUDI.

In all DE exposures, low-volume samplers with quartz filters collected PM samples for determining OC and EC. As discussed in Appendix B, OC readings are considered to represent total organic-compound mass because the organic-gas sorption artifact typically increases the OC reading by about one-third, which approximately accounts for the proportion of molecular mass contributed by H, N, and O — elements not detected by the OC assay.

Gas-phase carbonyl compounds and PAHs were measured during DE exposures by established techniques: collection with appropriate sampling cartridges or tubes

and analysis by high-pressure liquid chromatography or by gas chromatography–mass spectrometry.

In all exposures, nitric oxide (NO) and NO₂ were measured continuously with a chemiluminescent analyzer and CO was measured with an infrared spectroscopic analyzer (Teledyne-API, San Diego, CA), as in conventional ambient air pollution monitoring.

SUBJECT RECRUITMENT AND SCREENING

Volunteers of age 18 to 50 years were recruited by word of mouth, invitations to eligible participants in previous controlled-exposure studies, and advertisements in local college newspapers and online services. They were required to pass a medical screening examination including medical history, resting 12-lead electrocardiogram (ECG), lung function tests like those to be performed in the exposure studies, skin tests for sensitivity to common local aeroallergens, and inhalation challenge test.

Allergy skin tests were performed using standard epicutaneous (prick) skin testing procedures with the Multitest II applicator (Lincoln Diagnostics, Decatur, IL). A panel of common regional aeroallergens (house dust mite, cat, tree pollen mix, grass pollen mix) were tested with saline and histamine as controls. Skin test results were read at 15 minutes; they were considered positive if a wheal-and-flare reaction was at least 3 mm larger than the response to the saline control.

For each phase, an aerosol challenge was designed to detect early bronchoconstrictive responses in each subject (late allergic responses would be detectable immunologically in the actual exposures). In Phase 1 screening, the challenge agent was aerosolized isotonic saline solution that successively contained 0 (control), 0.075, 0.15, 0.31, 0.62, 1.25, 2.5, 5, 10, and 25 mg/mL of methacholine; each concentration was inhaled in five vital-capacity breaths. Bronchoconstrictive response was measured as the percentage of decrease of forced expiratory volume in 1 second (FEV₁) measured at each concentration compared with response to the saline control. Three minutes elapsed between the last breath of methacholine aerosol and the first FEV₁ test at each level.

In Phase 2 screening, the challenge agent was aerosolized isotonic saline solution with progressively increasing concentrations of cat allergen (Standardized Cat Hair Allergenic Extract, Hollister-Stier Laboratories LLC, Spokane, WA) at 0 (control), 80, 400, 2000, and 10,000 bioequivalent allergen units per mL (BAU/mL), each administered via 1 minute of tidal breathing. Ten minutes elapsed between the last breath of cat allergen and the first FEV₁ test at each level. Thus, the

challenge was designed to detect early bronchoconstrictive response. Exclusion criteria for all subjects included

- inability to exercise on a stationary bicycle or to perform the required test procedures;
- any history of clinically significant chronic cardiorespiratory condition (including rhinitis requiring regular corticosteroid or antihistamine use);
- history of psychiatric disorder or substance abuse;
- occupational exposure to dusts or fumes;
- clinically significant ECG abnormality;
- current smoking or smoking history of more than 1 pack-year; and
- acute respiratory infection in the 4 weeks before any exposure period.

Inclusion criteria for asthmatic subjects were:

- a physician's diagnosis of asthma (however, individuals with a history of oral corticosteroid use within 4 weeks of the screening visit were excluded because corticosteroids were suspected to suppress the immunologic and inflammatory responses of interest);
- a clearly positive response to methacholine challenge (> 20% FEV₁ loss at ≤ 8 mg/mL);
- a positive skin test for sensitivity to one of the common local aeroallergens;
- at screening, FEV₁ of > 65% of predicted normal value (i.e., normal or mildly abnormal); and
- in Phase 2 only, a clearly positive response to inhaled cat allergen (> 15% FEV₁ loss at ≤ 10,000 BAU/mL).

Given these requirements, accepted subjects would be defined clinically as having “mild intermittent” or “mild persistent” asthma.

As explained in the Specific Aims section, the original intention was to study 10 healthy subjects, 10 asthmatic subjects without cat sensitivity, and 10 asthmatic subjects with cat sensitivity. Subsequently, the plan was changed to study 4 healthy subjects first (primarily to verify safety), then 15 allergic asthmatic subjects in Phase 1, then 15 asthmatic subjects with cat allergy in Phase 2. This would provide more statistical power to detect responses in subjects with asthma, whom we expected would be more susceptible to DE exposure. The data for the four healthy subjects are presented in Appendices C and D, which are available on the HEI Web site (www.healtheffects.org).

Table 1 reports individual characteristics of volunteers who passed screening and completed all three experimental exposures. One additional subject was removed from the study before completion because she unexpectedly became pregnant; and another dropped out before

Table 1. Subjects' Characteristics

Subject	Phase ^a	Age	Sex	Height (cm)	Weight (kg)	Ethnic Origin ^b	PC ₂₀ ^c (Phase 1)	Dose / % Decline FEV ₁ ^d (Phase 2)	Medications ^e
1216	1	44	M	171	68	W	0.32		—
1656	1	46	F	180	91	W	0.31		—
2325	2	30	F	165	73	H		400/23	Post DE
2525	1	50	M	178	92	A	0.04		—
2551	2	45	M	162	95	B		10,000/23	PRN, Post FA
2602	1, 2	49	M	180	114	W	— ^f	10,000/51	Reg
2606	1	31	M	180	94	B	2		PRN
2628	1, 2	23	M	175	63	W	— ^g	400/21	—
2641	1	19	M	165	54	W	0.5		—
2642	1	40	M	175	77	W	2 ^h		Reg
2643	1	49	F	168	63	W	8		—
2644	1	33	F	155	101	W	8		PRN
2651	1, 2	27	F	165	68	H	2	80/17	—
2666	1	35	M	185	128	B	0.3		PRN
2669	1	22	M	193	116	W	0.5		PRN
2701	2	54	F	165	127	W		80/15	—
2702	1	48	M	168	56	B	6		—
2703	1	44	F	157	64	W	0.8		— ⁱ
2704	2	19	M	174	66	H		400/32	PRN ^j
2780	2	55	F	157	83	B		400/22	Reg ^k
2847	2	33	F	170	83	H		400/29	—
2871	2	32	F	160	55	H		400/27	—
2876	2	32	F	163	107	A		80/24	—
2878	2	43	M	173	104	H		400/21	—
2879	2	52	M	183	113	B		80/22	Reg
2883	2	29	F	165	78	W		400/33	—
2886	2	19	F	157	52	H		80/25	PRN

^a Subjects who participated in Phase 1 had a positive skin test for one or more aeroallergens; participants in Phase 2 (three of whom also participated in Phase 1) were sensitive to cats; sensitivity was determined with skin tests or inhalation challenge at screening.

^b Ethnic origin: A = Asian, B = black, African-American, H = Hispanic, and W = white not Hispanic.

^c Provocative concentration of methacholine (mg/mL) that reduced FEV₁ by 20% during screening.

^d Data are shown as maximum dose of cat allergen / % decline in FEV₁ during screening.

^e Bronchodilator medication use in proximity to exposures was reported by subjects and was not part of the experimental protocol. (No subject used corticosteroids.) Reg = regular use within 2 weeks before exposure; PRN = occasional use as needed within 2 weeks before exposure; post = between post-exposure testing on day 1 and testing on day 2.

^f PC₂₀ was not measured for this subject during this study; he had a previous bronchial reactivity test in a different study.

^g Challenge was stopped at 8 mg/mL with < 20% decline in FEV₁ due to provoked cough; we considered this response to be positive evidence of bronchial reactivity.

^h FEV₁ fell 11% after control (saline) challenge compared with pre-challenge FEV₁; and it fell > 20% after 2 mg/mL methacholine challenge compared with pre-challenge FEV₁.

ⁱ Reported taking acetaminophen for head and muscle aches post NO₂ exposure. No bronchodilator use reported.

^j Subject reported intercurrent cold symptoms, but no symptoms or use of medications for cold or asthma within 1 week of any exposure.

^k Subject reported sinus symptoms and wheeze overnight after DE exposure and sinus symptoms overnight after FA exposure, but no medication use.

completion to enter a vocational training course at a distant location. Three Phase 1 subjects turned out to have cat sensitivity and also participated in Phase 2.

EXPERIMENTAL PROTOCOL

Subjects were exposed to DE diluted with FA to approximately 100 $\mu\text{g}/\text{m}^3$ particle mass concentration for 2 hours with intermittent (15 minutes in every 30) stationary bicycle exercise in the chamber. They were exposed similarly to filtered air alone as a control and to filtered air containing about 0.35 ppm NO_2 to help determine whether any effects of DE exposure could be attributed to its irritant gas component rather than to particles.

Successive exposures of a given individual were separated by 4 weeks or more to minimize the chance of carry-over effects. Exposures were performed under nominally double-blind conditions. (Because many people can detect the odor of DE at 100 $\mu\text{g}/\text{m}^3$, complete blinding of subjects and staff was impractical.) Each phase of the study employed a crossover design, as is commonly used in clinical drug trials, in which each subject acted as his or her own control. Different sequences of exposure were assigned randomly to individual subjects, with the overall pattern of sequences designed to reveal differences between atmospheres. Thus, if carryover effects (or any time-dependent effects) did in fact occur, they should not confound the effects of the exposure atmospheres.

The target ventilation rate during exercise was 15–20 L/min/ m^2 body surface area. The appropriate ergometer load was set for each subject based on his or her screening exercise test; the subject's exercise ventilation rate was measured with a portable respirometer during the second and fourth exercise periods in each exposure. During exposure the subject's ECG was monitored continuously, and SaO_2 was monitored every 15 minutes via a fingertip pulse oximeter. In Phase 2, depending on scheduling requirements, subjects were exposed one or two at a time. With two at a time, the second subject's schedule was offset by 15 minutes to allow use of the stationary bicycle ergometer and testing equipment in turn.

Table 2 describes the exposure and testing sequence. Exposure protocols were the same in Phases 1 and 2. Protocols for measuring responses were also the same except for the inhalation challenges used to test for increased bronchial reactivity after each exposure. In Phase 1, the methacholine challenge used the same protocol as in the screening exam, except that the maximum concentration was 10 mg/mL rather than 25 mg/mL. In Phase 2, the cat allergen challenge involved 1 minute of breathing aerosolized solution at only one concentration — 20% of the concentration that had decreased FEV_1 by > 15% in each

subject's screening exam — with a 10-minute delay between inhalation challenge and spirometric testing, as was done in screening.

About 1.5 hours after each exposure ended, inhalation challenge with either methacholine (Phase 1) or cat allergen (Phase 2) was performed. A peripheral blood sample for evaluating immunologic and coagulation status was drawn from an antecubital vein before exposure on day 1 and on day 2 (about 22 hours after the exposure ended).

On day 2 only, a sputum sample for evaluating immunologic status and airway inflammation was induced by inhalation of aerosolized hypertonic saline solution (Fahy et al. 1995) after prophylactic administration of a bronchodilator medication. The sputum sample from a baseline test (conducted on a day separated from any exposure by at least 2 weeks) was compared with the sample after filtered air exposure to document any effects of the exposure protocol itself on sputum composition.

A urine sample was collected before exposure and on day 2 (no analyses of urine have been performed in this project; samples have been stored at -80°C for possible future analysis). Physiologic measurements — lung function, vital signs, fraction of exhaled nitric oxide (FENO) and exhaled CO — were recorded before exposure began, immediately after exposure ended, again 2 hours later, and again on day 2. The subject filled out a symptom diary similar to that previously described by Gong and colleagues (2003) at the time of each pre- and post-exposure lung function test, and every 15 minutes during exposure.

Short-acting bronchodilator (4 puffs from an albuterol inhaler) was routinely administered at the conclusion of bronchial reactivity testing, and again before sputum induction on day 2. Subjects were allowed to take their own bronchodilator medications as needed in the interim; any such usage was recorded.

The protocol was reviewed and approved by the institutional review boards of Rancho Los Amigos National Rehabilitation Center and University of California–Los Angeles (UCLA).

RESPONSE ASSESSMENT

Sputum and Blood Assays

All sputum assays were performed by staff at the UCLA Department of Medicine, Division of Clinical Immunology and Allergy. Each collected sputum sample was diluted 1:1 with 0.1% dithiothreitol (Sputalysin 10%, Behring Diagnostics, Somerville, NJ) and homogenized by gentle shaking at 37°C for 30 minutes. After adding a fixed volume of phosphate-buffered saline (PBS), a total cell

Table 2. Exposure Protocol

Clock Time	Time Relative to Exposure	Sequence of Activities
DAY 1		
Before Exposure — Time from Start of Exposure		
07:30	–1:30	Subject arrived in laboratory and rested in clean air Symptom score sheet completed ECG telemetry and pulse oximeter initiated Vital signs (heart rate, blood pressure, respiratory rate, SaO ₂) Cardiopulmonary physical examination 12-Lead ECG at rest
08:20	–0:40	Venous blood drawn (20 mL) FENO measured CO measured
08:30	–0:30	Body plethysmography (sRaw) and spirometry Urine collected
During Exposure		
09:00	0:00	Start of 2-hour exposure with intermittent exercise (15 minutes of every 30) Symptom score sheet completed every 15 minutes SaO ₂ measured every 15 minutes Minute ventilation (V _E) measured during the end of final rest and exercise periods
11:00	0:00	End of exposure Subject rested in clean air Vital signs (including SaO ₂) Body plethysmography (sRaw) and spirometry FENO measured CO measured

(Table continues next page)

count was performed. If a sample contained fewer than 3% columnar epithelial cells (usually meaning that squamous epithelial cells were in the majority), it was considered inadequate to represent lower-airway sputum, and rejected. If accepted, the sample was then centrifuged. The cell pellet was used for differential cell staining and the supernatant for fluid-phase assays. Supernatants were frozen at –80°C between collection and analysis. Differential cell counts (based on ≥ 200 cells/sample) included percentages of alveolar macrophages, lymphocytes, PMNs, and eosinophils by Giemsa staining, as well as eosinophils by Wright staining. The following substances were assayed in the fluid phase: RANTES (Regulated upon Activation,

Normal T-cell Expressed, and Secreted; chemotactic cytokine CCL5); eotaxin; eosinophil cationic protein (ECP); IgG, IgG4, IgA, IgM, and IgE; cat-specific IgE; IL-4, IL-5, IL-8, and IL-12; granulocyte/macrophage colony-stimulating factor (GM-CSF); interferon- γ (IFN- γ); tumor necrosis factor- α (TNF- α); and tryptase. Most assays employed monoclonal antibodies purchased commercially or manufactured at the UCLA laboratory according to manufacturers' instructions or previously reported protocols (Diaz-Sanchez et al. 1994, 1996, 1997, 2000).

Antibody isotype (IgE, IgG, IgG4, IgM, and IgA) levels in supernatants obtained from sputum or serum samples were measured by antigen and isotype-specific enzyme-linked

Table 2 (Continued). Exposure Protocol

Clock Time	Time Relative to Exposure	Sequence of Activities
DAY 1 (Continued)		
After Exposure — Time from End of Exposure		
12:00	+1:00	Symptom score sheet completed
12:30	+1:30	Bronchial reactivity test: methacholine (Phase 1) or cat allergen (Phase 2) challenge with spirometry
1:00	+2:00	Symptom score sheet completed Spirometry FENO measured CO measured
1:10	+2:10	Subject left laboratory with diary to record symptoms overnight
DAY 2		
09:00	+22:00	Subject arrived in laboratory and rested in clean air Diary collected Symptom score sheet completed Vital signs recorded (including SaO ₂) Cardiopulmonary physical examination
09:30	+22:30	Venous blood drawn (20 mL) Urine collected
09:45	+22:45	Body plethysmography (sRaw) and spirometry 12-Lead ECG at rest FENO measured CO measured
10:00	+23:00	Sputum induced Spirometry
10:45	+23:45	Subject left laboratory

immunosorbent assay (ELISA). Briefly, plates were coated overnight with the appropriate anti-Ig antibody at 2 µg/mL. For total IgE, human IgE monoclonal antibody (4.15 and 7.12) and standard (WT IgE) produced in the UCLA Hart and Louise Lyon Immunology Laboratory were used with detection antibody at 1 µg/mL (KPL, Gaithersburg, MD). For human IgA, the standard curve started at 10 µg/mL, and detection antibody was used at 1 µg/mL (Caltag, Burlingame, CA). Human IgG and IgG4 were measured using a standard starting at 10 µg/mL and 1 µg/mL, respectively; and human IgM was measured using a standard starting at 1 µg/mL (Sigma, St. Louis, MO). Detection antibodies for IgG and IgM were used at 1/1000 dilution (Biosource, Camarillo, CA) and

IgG4 at 1/1000 dilution (BD Biosciences, San Diego, CA). Sensitivity of all assays was 1 ng/mL. Cat-specific IgE level was determined using standardized cat hair allergen 10,000 BAU/mL at 1/1000 dilution (Hollister-Stier, Spokane, WA) as coat. The standard used was cat-allergic donor serum starting at 1/50 dilution and secondary antibody at 1 µg/mL (KPL, Gaithersburg, MD). The plates were washed with PBS-Tween and blocked with 1% bovine serum albumin (BSA)-PBS. Assay diluent used was 1% BSA-PBS. Polystyrene microtitre plates (Corning, Lowell, MA) were used for all assays.

Human cytokines IL-4, IL-5, IL-8, IFN-γ, GM-CSF, and TNF-α were measured from sputum supernatants using

commercial BD Opt EIA kits (BD Biosciences, San Diego, CA) following the manufacturer's instructions. The sensitivity of the commercial ELISA kits was 0.2 pg/mL. Human IL-12 was measured using Human IL-12(P70) kit (Anogen, Mississauga, ON, Canada) and sensitivity of the assay was 5 pg/mL. RANTES was measured using commercial Human RANTES kit (Invitrogen, Camarillo, CA) and sensitivity of the assay was 3 pg/mL. ECP was measured using Mesacup ECP kit (MBL, Naka-Ku Nagoya, Japan) and sensitivity of the assay was 0.125 ng/mL. Eotaxin was measured using human eotaxin kit (RD Systems, Minneapolis, MN) and sensitivity of the assay was 5 pg/mL. For tryptase, plates were coated with 0.2 µg/mL human tryptase (Serotec, Raleigh, NC), with standard curve starting at 5 µg/mL and secondary antibody at 1/3000 dilution (Promega, Madison, WI). Soluble intercellular adhesion molecule (sICAM) was measured in serum using commercial human sICAM-1/CD54 (RD Systems, Minneapolis, MN); sensitivity of the assay was 0.096 ng/mL.

Venous blood samples were subdivided, conventionally processed, and submitted to the appropriate laboratory for analysis. Serum samples were frozen at -80°C between collection and analysis. Measurements of markers of coagulability (fibrinogen, factor VII, and vonWillebrand factor [vWF]) were performed by staff at the Vascular Medicine Program, Orthopaedic Hospital, Los Angeles, using standard assays. Other biochemical assays were performed by staff at the UCLA Department of Medicine, Division of Clinical Immunology and Allergy. They included the same immunoglobulins as were measured in sputum, along with IL-6 and ICAM.

In general, frozen blood and sputum samples were thawed and assayed in batches at irregular intervals depending on exposure schedules and other laboratory requirements. For many analytes, maintaining consistent standards from batch to batch was not technically feasible; therefore all samples from any given subject within one phase of the study were assayed in the same batch. Accordingly, between-atmosphere, within-subject statistical comparisons should be biologically meaningful; but comparisons of numerical results between subjects assayed at different times, or between phases, may not be meaningful.

Physiology and Symptoms

Body plethysmography to determine specific airway resistance (sRaw) at functional residual capacity (resting end-expiration) was performed with a MedGraphics Elite DL integrated pulmonary testing system (Medical Graphics Corp., St. Paul, MN). The system was calibrated according to the manufacturer's procedure, using its own internal standards, at the beginning of each day's testing. Forced

expiratory lung function measurements (FVC, FEV_1) were performed with a Jaeger MasterScreen IOS pneumotachograph system (Viasys Inc., Yorba Linda, CA), certified to meet American Thoracic Society (ATS) standards of accuracy, calibrated with a 3-L volumetric syringe by the manufacturer's procedure at the beginning of each day's testing. Blood pressure was measured with an automated sphygmomanometer (Sunbeam Inc., Hattiesburg, MS) and SaO_2 with a fingertip pulse oximeter (Nellcor Inc., Boulder, CO), both employing internal calibration standards.

FENO was measured with a Sievers 280i chemiluminescent analyzer system (GE Analytical Instruments, Boulder, CO), calibrated daily with a Sievers zero-air filter and a certified NO span gas (Scott-Marrin Inc., Riverside, CA). Measurements were performed according to procedures recommended by the ATS (1999, 2005) at 50 mL/sec expiratory flow after a vital-capacity inspiration of unfiltered room air. Ambient NO concentration during the test session was recorded in order to determine whether it had statistically significant influence on the exhaled NO concentration.

Exhaled CO was measured with a Bedfont EC50 Micro III Smokerlyzer (Bedfont Scientific, Rochester, Kent, UK), calibrated at the beginning of each testing day with certified zero and span gases (Air Liquide Inc., Long Beach, CA). With the instrument in continuous monitoring mode, ambient CO concentration was recorded initially, then the subject held his or her breath for 20 seconds before exhaling into the sampling chamber. We did not follow the manufacturer's breath testing procedure (intended to detect smoking-related CO) because it called for zeroing the instrument with ambient air before each test. That would have caused appreciable errors at our location because of its intermittent ambient CO pollution.

A total symptom score and subtotals for respiratory, cardiovascular, and miscellaneous (nonspecific) symptoms were calculated for each time the diary was filled out, using a slight modification of a procedure previously reported (Gong et al. 2003). Table 3 lists the symptoms and scoring options. Changes in average scores were calculated for the exposure period and for the follow-up period (immediately after exposure through day 2) and compared with the pre-exposure value. Thus, a negative score change would represent overall symptom improvement, and a positive change would represent worsening. If a symptom persisted throughout the exposure period, the smallest noticeable increase (e.g., from not present to minimal, minimal to mild, or mild to moderate) would increase the change in average score by one unit. Supplemental questions on the diary form, answered immediately after exposure, determined whether the subject perceived any pollutant odor while in the chamber and whether he or she believed that the exposure had been to DE.

Table 3. Symptom Scoring

Respiratory
Cough
Sputum
Substernal soreness
Shortness of breath
Wheeze
Chest tightness
Pain related to lungs
Sore throat
Nasal discharge, congestion
Cardiovascular
Faintness, dizziness
Fast heartbeat
Irregular heartbeat
Pain related to heart
Miscellaneous
Headache
Fatigue
Stomach upset
Eye irritation
Other
Scoring for Each Symptom at Each Time of Recording
0 Not present
1 Minimal
2 Mild
3 Moderate
4 Severe
5 Incapacitating

STATISTICAL METHODS AND DATA ANALYSIS

Data were analyzed separately for each phase. Summary data for the four healthy subjects and for asthmatic subjects in both phases are presented in Appendix C; individual data for some variables are presented in Appendix D.

Descriptive and hypothesis-testing statistical analyses were performed with SAS software (SAS Institute Inc., Cary, NC). The primary hypothesis-testing tool was a mixed model to test for significant ($P < 0.05$) differences in each response variable across atmospheres within each subject (a crossover design). As in most previous similar studies, the significance level was not corrected to allow for multiple tests. Thus, a few results with $P < 0.05$ would be expected to occur by chance, even if the exposures had no effect. SAS procedure MIXED was chosen as the primary analytical tool because of its easy handling of unbalanced designs, which is required if any data are missing or

unsatisfactory. Responses were expressed as change from before to after each exposure, except for the results of sputum induction and bronchial reactivity tests, which were conducted only after exposures. For variables with markedly skewed data distributions, logarithmic transformations were applied before mixed-model analysis.

Initial analyses used conventional analytical models (commonly used in repeated-measures controlled-exposure studies) with atmosphere treated as a fixed factor (since exposure conditions were similar for all subjects) and subject as a random factor. The key result was the atmosphere effect, that is, the between-atmosphere within-subject contrast; and specifically, the estimated difference in response between exposure to DE and exposure to other atmospheres.

For sensitivity analyses, certain key response variables were reanalyzed using an advanced model (usually used in clinical drug trials; Shen and Lu, 2006; Bing and He, 2010) that could account for time-dependent effects due to the crossover study design: effects of period (1st vs. 2nd vs. 3rd exposure period) and sequence (the particular order in which a subject was exposed to the three atmospheres). In the event of a carryover effect — a change in response to a current test atmosphere caused by a previous test atmosphere — significant differences between sequences should be found. Usually, conclusions from conventional and advanced models concerning effects of DE were similar.

Responses to methacholine challenge could vary markedly between and within subjects, so maximum challenge doses could not be kept consistent and the commonly employed index of response, PC₂₀ — the provocative concentration of methacholine in aerosolized solution necessary to reduce FEV₁ by 20% compared with control — could not be calculated in some instances. To maximize the chance to detect changes in bronchial reactivity, three separate indices were calculated and statistically analyzed. First, reactivity was expressed in terms of the percent change in FEV₁ from just before to just after challenge with the control aerosol (physiologic saline solution).

Second, PC₂₀ was estimated conventionally by linear interpolation between the closest administered doses directly above and below the 20% response level; or by linear extrapolation from the two highest points on the FEV₁-vs.-concentration plot if the maximum methacholine concentration (10 mg/mL) did not reduce FEV₁ by 20%, or if the technician stopped the challenge for safety reasons when a lower dose induced a nearly 20% FEV₁ decline. A categorical variable was included to identify extrapolated PC₂₀ values. Even with extrapolation, PC₂₀ could not be estimated in all instances.

Third, each subject's bronchoconstrictive responses were ranked across atmospheres from 1 for most unfavorable to

3 for least unfavorable, and Friedman rank-sum analysis was used to test for significant differences between atmospheres in place of conventional or advanced mixed models. If maximum methacholine doses differed between exposures, ranking was assigned on the basis of lowest to highest dose required to reduce FEV₁ by at least 20%. If maximum doses were the same in different exposures, ranking was assigned on the basis of largest to smallest percentage of decrease in FEV₁.

Data for serum immunologic variables were log-transformed; then, differences between pre-exposure day 1 and day 2 measurements were calculated as measures of response to exposure. These differences were subjected to conventional mixed-model analyses, first with only atmosphere as a predictor, and then with atmosphere and the pre-exposure value of the variable as predictors. The latter model would assess differences in response to exposure related to preexisting levels of circulating inflammatory mediators. Appendix C contains summary statistics and Appendix D contains individual data for some response variables not presented in detail in the Results section.

RESULTS

EXPOSURES

Tables 4 and 5 present basic exposure monitoring results for Phase 1 and Phase 2, respectively. In general in both phases, exposure variables were controlled close to their targets and were reasonably consistent from minute to minute within exposures (as indicated by the relevant standard deviations) as well as among exposures. CO concentrations averaged well below 1 ppm in FA and NO₂ exposures, but closer to 2 ppm in DE exposures. As described in the Methods section, relatively large particles released from subjects' skin, clothing, and exercise equipment were not entirely controllable. They added appreciably to total particle mass concentration, as determined by Teflon filter sampling, but had little effect on total particle counts or small-particle mass concentrations (as determined by SMPS) because of their size. In the article by Sawant and colleagues (2008; also Appendix E), Figure 5 shows the particle-size

Table 4. Atmosphere Monitoring Data for All Exposure Periods in Phase 1

Measure	Filtered Air	NO ₂	DE
CO, mean ^a (ppm)	0.71 ± 0.54	0.60 ± 0.34	1.67 ± 0.51
CO, SD ^b (ppm)	0.13 ± 0.13	0.13 ± 0.13	0.19 ± 0.15
NO ₂ , mean ^a (ppb)	17 ± 15	357 ± 17	354 ± 41
NO ₂ , SD ^b (ppb)	2 ± 1	9 ± 2	27 ± 17
PM mass by filter, mean (µg/m ³), CE-CERT ^c	—	—	140 ± 17
PM mass by filter, mean (µg/m ³), LAREI ^d	23 ± 14	25 ± 16	127 ± 23
PM mass by SMPS, mean ^a (µg/m ³)	0.8 ± 0.7	0.6 ± 0.2	102 ± 3
PM mass by SMPS, SD ^b (µg/m ³)	0.2 ± 0.1	0.2 ± 0.1	6 ± 2
Particle count, mean ^a (#/mL)	447 ± 410	389 ± 113	122,480 ± 18,010
Particle count, SD ^b (#/mL)	134 ± 143	184 ± 106	8,330 ± 4,980
Temperature, mean ^a (°C)	24 ± 1	24 ± 1	25 ± 1
Temperature, SD ^b (°C)	0.7 ± 0.2	0.7 ± 0.2	0.8 ± 0.2
RH, mean ^a (%)	46 ± 7	46 ± 7	46 ± 8
RH, SD ^b (%)	2 ± 2	2 ± 2	2 ± 1

^a From the continuous (or intermittent) monitoring data for every 2-hour exposure period, a mean ± SD was calculated for each characteristic (temperature, RH) and pollutant (CO, NO₂, PM mass, particle count). From these *means*, a mean ± SD of *means* was calculated to show the consistency of exposure conditions over all the exposure periods.

^b Using the monitoring data described in footnote a: From the *SDs*, a mean ± SD of *SDs* was calculated to show consistency of exposure conditions throughout an individual exposure period.

^c Filters were collected and weighed by CE-CERT staff for DE exposures only.

^d Filters were collected and weighed by EHS-LAREI staff.

Table 5. Atmosphere Monitoring Data for All Exposure Periods in Phase 2^a

Measure	Filtered Air	NO ₂	DE
CO, mean ^b (ppm)	0.56 ± 0.21	0.83 ± 0.14	1.74 ± 0.14
CO, SD ^c (ppm)	0.21 ± 0.24	0.13 ± 0.11	0.14 ± 0.11
NO ₂ , mean ^b (ppb)	17 ± 14	387 ± 21	315 ± 53
NO ₂ , SD ^c (ppb)	3 ± 2	9 ± 6	16 ± 7
PM mass by filter, mean (µg/m ³), CE-CERT ^d	—	—	129 ± 14
PM mass by filter, mean (µg/m ³), LAREI ^e	22 ± 12	22 ± 8	123 ± 16
PM mass by SMPS, mean ^b (µg/m ³)	1.0 ± 0.5	1.0 ± 0.4	104 ± 2
PM mass by SMPS, SD ^c (µg/m ³)	0.3 ± 0.1	0.3 ± 0.1	4 ± 1
Particle count, mean ^b (#/mL)	509 ± 251	523 ± 246	12,9130 ± 11,010
Particle count, SD ^c (#/mL)	161 ± 120	145 ± 62	6,160 ± 1,870
Temperature, mean ^b (°C)	24 ± 1	24 ± 1	24 ± 1
Temperature, SD ^c (°C)	0.7 ± 0.2	0.8 ± 0.2	0.8 ± 0.2
RH, mean ^b (%)	52 ± 11	48 ± 11	46 ± 10
RH, SD ^c (%)	2.6 ± 1.3	2.2 ± 1.2	2.1 ± 1.1

^a Means were calculated for each subject; thus an exposure period with two subjects had twice the weight of an exposure period with one subject.

^b From the continuous (or intermittent) monitoring data for every 2-hour exposure period, a mean ± SD was calculated for each characteristic (temperature, RH) and pollutant (CO, NO₂, PM mass, particle count). From these *means*, a mean ± SD of *means* was calculated to show the consistency of exposure conditions over all the exposure periods.

^c Using the monitoring data described in footnote b: From the *SDs*, a mean ± SD of *SDs* was calculated to show consistency of exposure conditions throughout an individual exposure period.

^d Filters were collected and weighed by CE-CERT staff for DE exposures only.

^e Filters were collected and weighed by EHS-LAREI staff.

distribution in the exposure chamber and demonstrates that this extraneous particle mass is predominantly in particles of 10 µm and larger in aerodynamic diameter (PM₁₀). In this report, Figures 1 and 2 illustrate typical distributions of particle number and particle volume, respectively, compared with particle size for DE exposures. A more detailed description of particle characteristics throughout the course of the project is given in Appendix B.

Table 6 summarizes results of OC and EC assays from DE exposures. Total carbon accounted for nearly three-fourths of the particle mass, and that proportion was consistent throughout all exposures. Typically about two-thirds of the carbon was assayed as OC and one-third as EC. In a separate concurrent study with similar DE exposures, carbon was analyzed by a different method (Fung 1990); its results consistently agreed with the current results showing two thirds as OC.

Monitoring data for selected volatile and semivolatile organic compounds in DE exposures are reported in Table 7 for Phase 1 and Table 8 for Phase 2. The more abundant carbonyl compounds — formaldehyde, acetaldehyde, and acetone — were relatively consistent in concentration across all exposures; the less abundant carbonyls, PAHs, and alkanes were more variable.

As a further check on the stability of the DE generation and monitoring system, the ratio of SMPS particle count to SMPS mass concentration was examined as a function of time. Figure 3 shows the results. There were no abrupt sustained shifts, which rules out any important changes due to mechanical malfunction, engine oil changes, or shifts from one drum of fuel to the next. Even so, there was a significant overall increase with time in particle count per unit mass ($P = 0.02$ for positive linear slope [straight line in Figure 3]), suggesting that the engine tended to emit smaller or less dense particles as it aged. However, the trend was not linear, but was fit much better by a quadratic function (curved line in Figure 3), suggesting an appreciable increase in particle count over the first 2 study years, followed by stabilization, and possibly a downtrend during the final year. The cause of this pattern is unknown.

In summary, exposure conditions were quite consistent throughout the project according to most monitoring data. Number counts, mass concentrations, particle size distributions, and speciation were consistent with current understanding of worst-case ambient DE exposures on heavily traveled freeways.

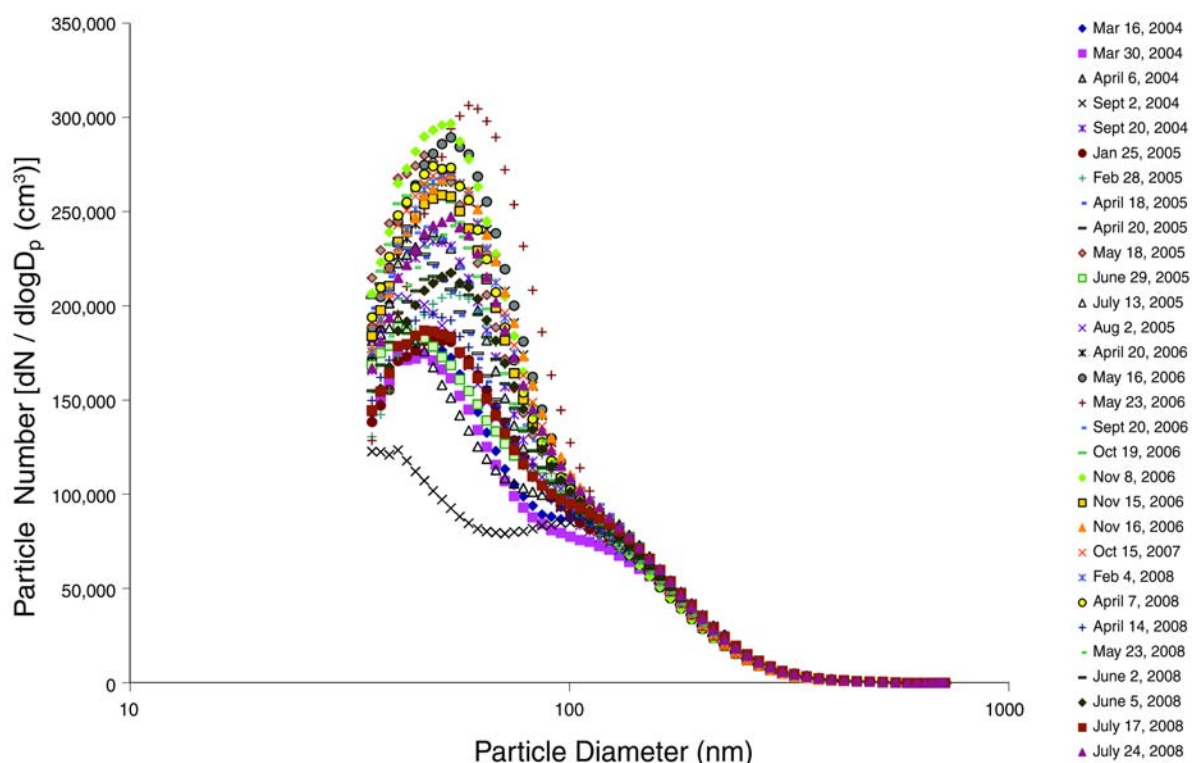


Figure 1. Distribution of particle number vs. particle size in individual DE exposure periods, as determined by SMPS. More complete data are presented in Appendix B.

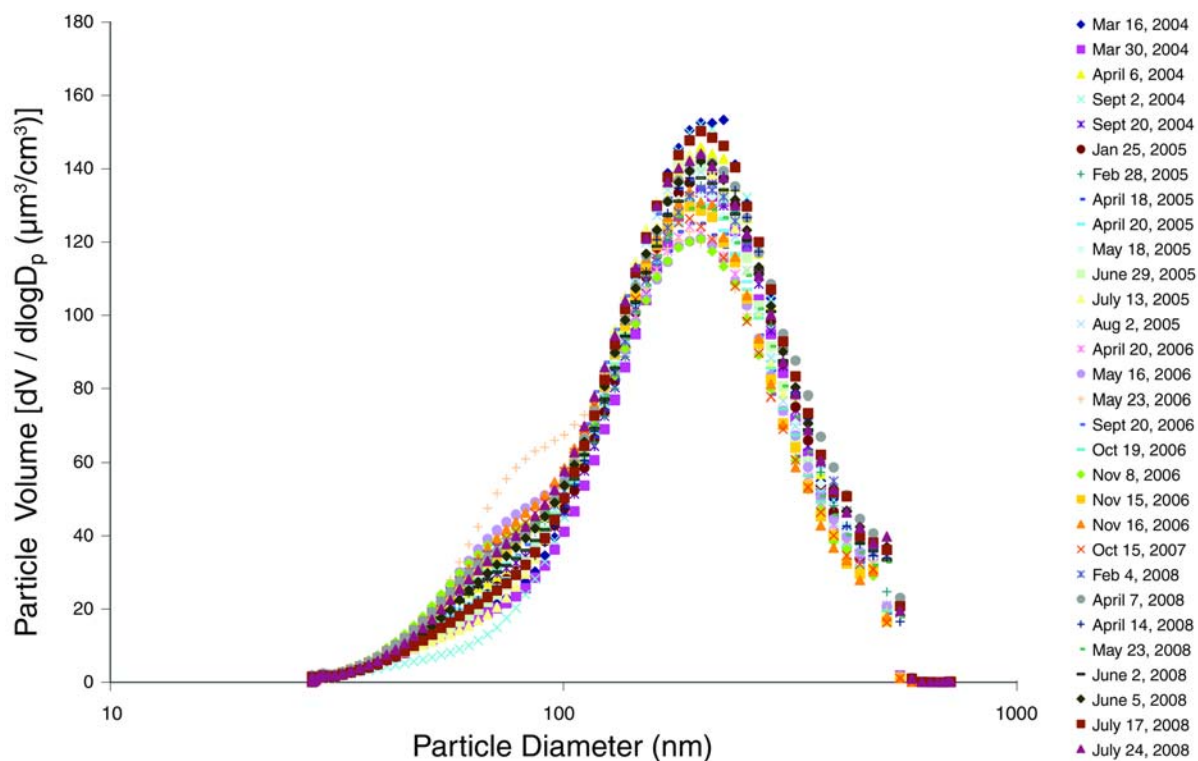


Figure 2. Distribution of particle volume vs. particle size in individual DE exposure periods, as determined by SMPS. More complete data are presented in Appendix B.

Table 6. OC and EC Monitoring Results from DE Exposures in Both Phases

Measure	Phase	<i>n</i> ^a	Mean ± SD ^b
OC (µg/m ³)	1	10	63 ± 6
	2	13	62 ± 6
EC (µg/m ³)	1	10	30 ± 3
	2	13	28 ± 6
Carbon as proportion of total PM mass (%)	1	9	69 ± 9
	2	13	74 ± 9

^a Number of exposure periods with at least one satisfactory sample assay. (Phase 1 had 15 possible exposure periods; Phase 2 had 13 periods because two periods had two subjects.)

^b Means were calculated for each subject with data; thus in Phase 2 an exposure with two subjects had twice the weight of an exposure with one subject. When duplicate assays were available from a given exposure, their mean was used to represent the exposure.

Table 7. Monitoring Results for Selected Organic Compounds During DE Exposures in Phase 1

Compound	<i>n</i> ^a	BDL ^b	Mean ± SD ^c
Formaldehyde (µg/m ³)	14	0	62 ± 10
Acetaldehyde (µg/m ³)	14	0	23 ± 5
Propionaldehyde (µg/m ³)	14	0	5 ± 2
Benzaldehyde (µg/m ³)	14	0	4 ± 3
Butyraldehyde (µg/m ³)	14	0	10 ± 9
Acetone (µg/m ³)	14	0	34 ± 17
Methyl ethyl ketone (µg/m ³)	14	2	4 ± 2
Naphthalene (µg/m ³)	6	0	27 ± 59
Phenanthrene (ng/m ³)	6	0	72 ± 22
Fluorene (ng/m ³)	6	0	117 ± 6
Dodecane (ng/m ³)	6	0	1947 ± 515
Eicosane (ng/m ³)	6	0	606 ± 251

^a Number of subjects (among 15) with assayed samples.

^b Number of assayed samples below detection limit.

^c Excluding samples below detection limit.

Table 8. Monitoring Results for Selected Organic Compounds During DE Exposures in Phase 2

Compound	<i>n</i> ^a	BDL ^b	Mean ± SD ^c
Formaldehyde (µg/m ³)	12	0	55 ± 11
Acetaldehyde (µg/m ³)	12	0	24 ± 6
Propionaldehyde (µg/m ³)	12	0	5 ± 1
Benzaldehyde (µg/m ³)	12	5	3 ± 1
Butyraldehyde (µg/m ³)	12	5	13 ± 7
Acetone (µg/m ³)	12	0	39 ± 13
Methyl ethyl ketone (µg/m ³)	12	0	3 ± 1
Naphthalene (µg/m ³)	11	0	7 ± 14
Phenanthrene (ng/m ³)	11	0	127 ± 70
Fluorene (ng/m ³)	11	0	93 ± 26
Dodecane (ng/m ³)	11	0	1243 ± 1455
Eicosane (ng/m ³)	11	0	254 ± 195

^a Number of subjects (among 15) with assayed samples.

^b Number of assayed samples below detection limit.

^c Excluding samples below detection limit. Means were calculated for all subjects with data; thus an exposure with two subjects had twice the weight of an exposure with one subject.

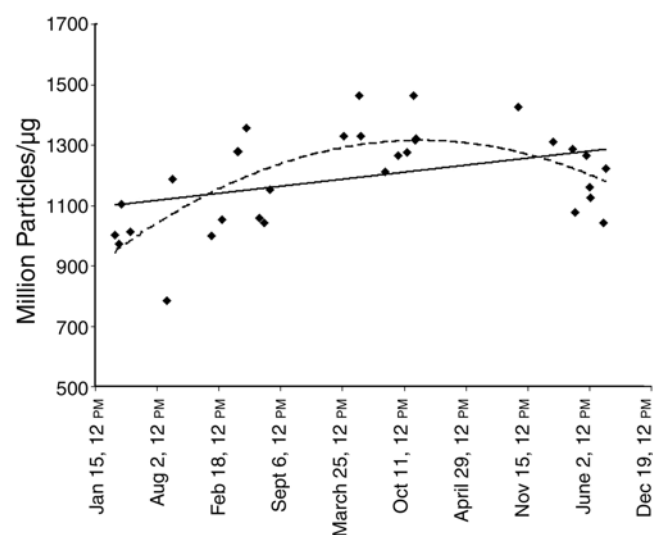


Figure 3. Ratio of SMPS particle count to SMPS mass concentration estimate as a function of time over all DE exposures (March 2004–July 2008). Solid line is best-fit linear trend line; dashed line is best-fit quadratic trend line. Increase over time was significant (see text).

SPUTUM ASSAY RESPONSES IN PHASE 1

For subjects in Phase 1, Table 9 shows summary statistics for cells in sputum by atmosphere; and Table 10 shows individual total and differential cell counts. Conventional and advanced mixed-model analyses showed no statistically significant ($P < 0.05$) or near-significant ($P = 0.05$ – 0.10) variation among DE, NO_2 , and FA exposures for any cell-count variable. Differences between post-FA and baseline cell counts (recorded for sputum inductions separated by 2 weeks from any exposure) were not significant; however, PMNs appeared to increase and macrophages to decrease by about 10%, on average, after FA compared with baseline ($P \sim 0.1$). This might suggest a tendency toward an airway inflammatory response evoked by the exposure protocol itself, even in clean air.

Table 11 shows summary statistics for all biochemical assays of sputum collected after Phase 1 exposures, as estimated from advanced mixed models applied to log-transformed data. Table 12 shows individual data for the species that indicated some response to the exposures. Of 16 species assayed in sputum, only IgG4 varied significantly by exposure atmosphere ($P = 0.04$; see Table 11); its estimated mean decreased after DE (and more so after NO_2) compared with FA, which is inconsistent with our primary hypothesis. IL-4 showed, on average, a 65% increase after DE but a 35% decrease after NO_2 , compared with FA — a near-significant ($P < 0.10$) difference between exposure atmospheres, which is consistent with our primary hypothesis. Tryptase and $\text{TNF-}\alpha$ showed similar proportionate increases after DE compared with FA exposure, but not close to statistical significance; concentrations of these two species were often undetectable. Eotaxin and RANTES showed near-significant

variation between atmospheres, averaging lower after DE compared with other atmospheres, but not in a pattern consistent with the primary hypothesis; they were often undetectable. The only other statistically significant variation was a period effect: an increase of IgM from 1st to 2nd to 3rd exposure period. None of the 16 assayed species differed significantly between post-FA and baseline sputum inductions.

BLOOD ASSAY RESPONSES IN PHASE 1

Table 13 gives summary statistics for biochemical assays of peripheral blood serum for subjects in Phase 1, shown as changes in log-transformed data between pre-exposure day 1 and day 2 (computed as the day-2 value minus the day-1 value [day-2 – day-1 difference]). The tabulated values are based on advanced models with adjustment for period effects (significant for some variables) and sequence effects (never significant). Conventional models showed no significant variation by atmosphere for any variable (data not shown).

When we compared the day-2 – day-1 difference with the day-1 value, some species showed significant negative relationships. Thus, regardless of exposure conditions, subjects with comparatively high levels of these mediators before exposure tended to have lower levels the next day, and vice versa; this suggests a considerable influence of chance variation on the results (i.e., regression toward the mean). IgM, which had the largest proportion of missing data, was the only serum variable to show significant differences between atmospheres. Table 14 shows the relevant individual data. On average, IgM fell after DE exposure but rose after NO_2 exposure compared with FA, which is not consistent with our primary hypothesis.

Table 9. Mean Sputum Cell Counts by Atmosphere in Phase 1^a

Variable ^b	FA	NO_2	DE	Significance
Total cells/ μL	1581 (285)	1583 (290)	1556 (277)	—
Macrophages (%)	39.6 (4.8)	42.4 (5.3)	42.1 (5.3)	—
Lymphocytes (%)	0.6 (0.8)	1.7 (0.9)	2.3 (0.9)	—
PMNs (%)	57.9 (5.0)	52.1 (5.3)	52.6 (5.4)	—
Eosinophils (%)	1.8 (1.4)	3.9 (1.5)	2.9 (1.5)	—
Eosinophils (Wright) (%)	0.9 (0.8)	1.7 (0.9)	1.3 (0.9)	—

^a Means $\pm$ SEs were estimated by advanced model with adjustment for main effects of period (1st vs. 2nd vs. 3rd) and sequence (order of atmospheres). Conventional models produced generally similar estimates. All models failed to show significant effects of atmosphere or period for either untransformed or log-transformed data. Lymphocyte or eosinophil counts of 0 were estimated as 0.5% for statistical purposes.

^b All counts were done with Giemsa stain. Eosinophils were also counted with Wright stain.

Table 10. Individual Data for Sputum Cell Counts in Phase 1^a

Subject	Exposure Period	Atmosphere	Total Cells (1000/ μ L)	Macrophages (%)	Lymphocytes (%)	PMNs (%)	Eosinophils (%)	Eosinophils (Wright) (%)
1216	2	DE	1.74	50	5	43	1	0
1216	1	NO ₂	1.46	62	6	31	1	0
1216	3	FA	1.78	24	0	76	0	0
1656	2	DE	0.38	39	6	55	0	0
1656	3	NO ₂	0.79	37	6	57	0	0
1656	1	FA	0.65	31	3	66	0	0
2525	3	DE	2.97	39	0	57	3	0
2525	2	NO ₂	4.10	40	0	56	4	0
2525	1	FA	3.83	22	0	77	1	2
2602	1	DE	3.38	16	1	83	0	0
2602	3	NO ₂	1.13	16	0	84	0	0
2602	2	FA	1.73	26	1	70	3	0
2606	1	DE	1.57	47	17	23	13	0
2606	2	NO ₂	1.10	62	2	31	5	0
2606	3	FA	1.10	40	2	42	16	10
2628	2	DE	1.16	77	0	19	4	3
2628	3	NO ₂	0.94	29	1	58	12	10
2628	1	FA	0.79	56	0	44	0	0
2641	3	DE	1.42	58	0	32	10	8
2641	1	NO ₂	ND	30	0	50	20	ND
2641	2	FA	0.86	57	0	35	8	4
2642	2	DE	1.13	66	0	34	0	0
2642	1	NO ₂	2.70	73	1	26	0	0
2642	3	FA	2.28	90	0	10	0	0
2643	3	DE	0.35	ND	ND	ND	ND	ND
2643	1	NO ₂	0.28	64	0	36	0	0
2643	2	FA	0.44	24	0	76	0	0
2644	1	DE	1.60	58	0	42	0	0
2644	2	NO ₂	3.15	75	3	22	0	0
2644	3	FA	2.60	56	0	44	0	0
2651	3	DE	0.28	46	0	54	0	0
2651	2	NO ₂	1.40	64	1	35	0	0
2651	1	FA	ND	50	1	49	0	0
2666	2	DE	1.47	28	3	69	0	0
2666	3	NO ₂	ND	ND	ND	ND	ND	ND
2666	1	FA	1.95	35	0	64	0	0
2669	2	DE	2.04	59	3	32	6	4
2669	1	NO ₂	2.74	35	5	51	9	7
2669	3	FA	2.40	68	0	32	0	0
2702	1	DE	0.08	ND	ND	ND	ND	ND
2702	3	NO ₂	0.16	ND	ND	ND	ND	ND
2702	2	FA	0.41	4	2	94	0	0
2703	1	DE	4.28	24	0	76	0	0
2703	2	NO ₂	1.48	27	1	71	1	0
2703	3	FA	1.48	27	2	71	0	0

^a All counts were done with Giemsa stain; eosinophils were also counted with Wright stain. ND = no data. Counts of 0 were estimated as 0.5% for statistical purposes.

Table 11. Means of Sputum Biochemical Assays by Atmosphere in Phase 1^a

Variable ^b	FA	NO ₂	DE	Significance
log ₁₀ (IgA)	1.960 (0.205)	1.914 (0.205)	1.945 (0.205)	—
log ₁₀ (IgE)	−2.344 (0.290)	−2.346 (0.289)	−2.561 (0.290)	—
log ₁₀ (IgG)	0.825 (0.231)	0.798 (0.231)	0.833 (0.231)	—
log ₁₀ (IgG4)	1.157 (0.131)	0.858 (0.131)	1.029 (0.131)	— ^c
log ₁₀ (IgM)	1.435 (0.174)	1.394 (0.174)	1.437 (0.174)	— ^d
log ₁₀ (IL-4)	1.157 (0.304)	0.970 (0.303)	1.376 (0.304)	— ^e
log ₁₀ (IL-5)	0.497 (0.300)	0.311 (0.299)	0.269 (0.300)	—
log ₁₀ (IL-8)	2.099 (0.106)	1.922 (0.106)	2.043 (0.106)	—
log ₁₀ (IL-12)	1.025 (0.236)	1.309 (0.239)	1.000 (0.236)	—
log ₁₀ (IFN-γ)	0.394 (0.209)	0.149 (0.208)	0.390 (0.209)	— ^f
log ₁₀ (TNF-α)	1.357 (0.144)	1.310 (0.143)	1.571 (0.144)	—
log ₁₀ (ECP)	1.187 (0.167)	0.998 (0.166)	1.101 (0.167)	—
log ₁₀ (RANTES)	0.310 (0.178)	0.336 (0.178)	0.120 (0.178)	— ^{e, f}
log ₁₀ (eotaxin)	−0.273 (0.206)	−0.502 (0.208)	−0.500 (0.207)	— ^{e, f}
log ₁₀ (GM-CSF)	0.912 (0.283)	0.759 (0.292)	0.975 (0.283)	—
log ₁₀ (tryptase)	1.338 (0.249)	1.385 (0.254)	1.543 (0.249)	— ^f

^a Means ± SEs of log-transformed data were estimated by advanced model with adjustment for effects of period (1st vs. 2nd vs. 3rd) and sequence (order of atmospheres). Nondetectable concentrations were estimated as one-half the lowest detected concentration for analyses.

^b Measurement units are: ng/mL for immunoglobulins, ECP, and tryptase; pg/mL for others.

^c Atmospheres significantly different ($P = 0.04$). Conventional model produced similar estimates with near-significant effect of atmosphere ($P < 0.10$).

^d Significant period effect ($P = 0.02$): estimated mean increased 25% after 2nd and 50% after 3rd compared with 1st period.

^e Differences between atmospheres were near significance ($P < 0.10$).

^f More than 25% of assays were below quantifiable limits and were estimated as one-half the lowest quantifiable value; therefore means may be appreciably biased.

Table 12. Individual Data for Sputum Biochemical Assays in Phase 1^a

Subject	Exposure Period	Atmosphere	IgG4 (ng/mL)	IL-4 (pg/mL)	Eotaxin (pg/mL)	TNF- α (pg/mL)	RANTES (pg/mL)
1216	2	DE	64.9	<i>0.3</i>	1.154	<i>5.8</i>	41.4
1216	1	NO ₂	13.0	<i>0.3</i>		<i>5.8</i>	36.8
1216	3	FA	295.5	1.6	1.521	<i>5.8</i>	97.7
1656	2	DE	8.4	125.1	<i>-0.974</i>	45.2	1.2
1656	3	NO ₂	9.3	55.6	<i>-0.974</i>	34.3	2.8
1656	1	FA	8.6	184.4	0.005	110.1	<i>0.6</i>
2525	3	DE	18.6	211.0	<i>-0.974</i>	72.4	<i>0.6</i>
2525	2	NO ₂	21.3	17.2	<i>-0.974</i>	58.9	<i>0.6</i>
2525	1	FA	33.0	23.2	<i>-0.974</i>	11.7	<i>0.6</i>
2602	1	DE	18.5	70.3	<i>-0.974</i>	95.3	<i>0.6</i>
2602	3	NO ₂	13.3	18.3	<i>-0.974</i>	<i>5.8</i>	1.2
2602	2	FA	18.4	18.1	<i>-0.974</i>	<i>5.8</i>	1.2
2606	1	DE	9.8	6.2	<i>-0.974</i>	30.5	<i>0.6</i>
2606	2	NO ₂	8.9	153.2	<i>-0.974</i>	113.3	<i>0.6</i>
2606	3	FA	10.5	96.7	0.294	89.3	<i>0.6</i>
2628	2	DE	17.9	69.0	<i>-0.974</i>	95.3	<i>0.6</i>
2628	3	NO ₂	11.9	80.2	<i>-0.974</i>	81.1	<i>0.6</i>
2628	1	FA	14.9	83.6	<i>-0.974</i>	79.7	<i>0.6</i>
2641	3	DE	18.0	123.2	<i>-0.974</i>	108.0	<i>0.6</i>
2641	1	NO ₂	21.4	16.7	<i>-0.974</i>	19.8	<i>0.6</i>
2641	2	FA	31.8	34.3	<i>-0.974</i>	105.0	<i>0.6</i>
2642	2	DE	21.5	94.8	<i>-0.974</i>	97.9	<i>0.6</i>
2642	1	NO ₂	16.3	105.1	<i>-0.974</i>	113.5	<i>0.6</i>
2642	3	FA	20.3	13.8	<i>-0.974</i>	<i>5.8</i>	<i>0.6</i>
2643	3	DE	9.2	175.3	<i>-0.974</i>	222.7	<i>0.6</i>
2643	1	NO ₂	12.0	17.8	<i>-0.974</i>	31.9	2.8
2643	2	FA	9.8	53.4	<i>-0.974</i>	114.6	2.8
2644	1	DE	11.5	106.3	<i>-0.974</i>	52.6	1.2
2644	2	NO ₂	10.8	40.3	<i>-0.037</i>	<i>5.8</i>	<i>0.6</i>
2644	3	FA	10.5	69.4	0.406	44.4	5.5
2651	3	DE	11.3	106.4	<i>-0.974</i>	98.6	<i>0.6</i>
2651	2	NO ₂	11.1	102.3	<i>-0.974</i>	66.3	<i>0.6</i>
2651	1	FA	9.8	127.6	<i>-0.974</i>	97.9	<i>0.6</i>
2666	2	DE	2.0	<i>0.3</i>	0.470	<i>5.8</i>	<i>0.6</i>
2666	3	NO ₂	0.5	<i>0.3</i>	0.371	<i>5.8</i>	3.2
2666	1	FA	19.7	0.5	0.371	<i>5.8</i>	7.7
2669	2	DE	5.0	7.6	0.371	<i>5.8</i>	3.2
2669	1	NO ₂	11.2	<i>0.3</i>	0.243	<i>5.8</i>	7.7
2669	3	FA	5.3	<i>0.3</i>	0.423	<i>5.8</i>	3.2
2702	1	DE	7.0	18.4	0.371	<i>5.8</i>	5.6
2702	3	NO ₂	0.5	<i>0.3</i>	0.371	<i>5.8</i>	75.8
2702	2	FA	5.6	2.8	0.512	<i>5.8</i>	12.1
2703	1	DE	2.3	<i>0.3</i>	0.730	12.3	8.0
2703	2	NO ₂	1.0	<i>0.3</i>	0.162	<i>5.8</i>	12.1
2703	3	FA	1.3	<i>0.3</i>	0.243	<i>5.8</i>	8.0

^a Values in italics are estimates of undetectable concentrations (one-half the lowest quantifiable value).

Table 13. Mean Differences in Serum Biochemical Assays by Atmosphere in Phase 1^a

Variable ^b	FA	NO ₂	DE	Significance
log ₁₀ (IgA)	0.070 (0.101)	0.085 (0.095)	−0.060 (0.095)	— ^c
log ₁₀ (IgE)	0.216 (0.118)	0.018 (0.112)	−0.018 (0.113)	— ^{c, d}
log ₁₀ (IgG)	0.422 (0.323)	−0.483 (0.265)	−0.138 (0.252)	—
log ₁₀ (IgG4)	0.036 (0.096)	0.070 (0.095)	0.147 (0.096)	— ^d
log ₁₀ (IgM)	0.041 (0.046)	0.116 (0.043)	−0.051 (0.043)	— ^{e, f}
log ₁₀ (IL-6)	−0.124 (0.110)	−0.114 (0.105)	−0.101 (0.105)	— ^{d, g}
log ₁₀ (ICAM)	−0.016 (0.039)	−0.018 (0.037)	0.036 (0.037)	— ^{d, f}

^a Means ± SEs of all day-2 − day-1 differences expressed as log-transformed data. Means were estimated by advanced model with adjustment for effects of period (1st vs. 2nd vs. 3rd) and sequence (order of atmospheres). Nondetectable concentrations were estimated as one-half the lowest detected concentration for analyses.

^b Measurement units are ng/mL for immunoglobulins and pg/mL for others.

^c Significant period effect ($P < 0.05$): negative changes and high pre-exposure day-1 values in 1st period, positive changes and low day-1 values in 2nd period.

^d In alternative advanced model, the day-1 value (as a covariate) showed a significant negative relationship with the day-2 − day-1 difference; other effects were not significant.

^e Atmospheres were significantly different ($P < 0.05$): estimates of 20% rise after NO₂ and 20% fall after DE compared with FA exposure.

^f Significant period effect ($P < 0.05$): positive changes in 3rd period.

^g In a separate conventional analysis, the day-1 value (as a covariate) showed a significant period effect ($P < 0.05$): downtrend from 1st to 3rd period.

Table 14. Individual Data for Serum IgM in Phase 1^a

Subject	Exposure Period	Atmosphere	Day 1 IgM (ng/mL)	Day 2 IgM (ng/mL)
1656	2	DE	2579	1342
1656	3	NO ₂	1557	6190
1656	1	FA	915	972
2525	3	DE	214	217
2525	2	NO ₂	198	251
2525	1	FA	211	211
2602	1	DE	375	333
2602	3	NO ₂	342	391
2602	2	FA	289	310
2606	1	DE	692	404
2606	2	NO ₂	401	339
2606	3	FA	317	415
2628	2	DE	217	216
2628	3	NO ₂	208	229
2628	1	FA	238	215

(Table continues next column)

Table 14 (Continued). Individual Data for Serum IgM in Phase 1^a

Subject	Exposure Period	Atmosphere	Day 1 IgM (ng/mL)	Day 2 IgM (ng/mL)
2641	3	DE	353	352
2641	1	NO ₂	358	377
2641	2	FA	350	323
2642	2	DE	340	373
2642	1	NO ₂	461	660
2642	3	FA	461	525
2643	3	DE	388	506
2643	1	NO ₂	620	779
2643	2	FA		301
2644	1	DE	456	424
2644	2	NO ₂	410	474
2644	3	FA	499	832
2651	3	DE	277	280
2651	2	NO ₂	262	263
2651	1	FA	289	284

^a Includes only subjects with satisfactory data.

For Phase 1, Table 15 shows the estimated mean changes in blood coagulation factors — factor VII, fibrinogen, and vWF — between pre-exposure day 1 and day 2. No significant differences by atmosphere were found with a conventional mixed model; nor by atmosphere, period, or sequence with an advanced model. Table 16 shows results from a conventional model that estimated effects of atmosphere, time (day 1 vs. day 2), and their interaction. Only fibrinogen showed significant variation, averaging about 8% lower on NO₂ exposure days than on other days ($P = 0.005$ for main

effect of atmosphere). The atmosphere–time interaction was not significant, which is consistent with the nonsignificant differences in changes between day 1 and day 2 shown in Table 15. In other words, low mean fibrinogen concentrations were present both before and after NO₂ exposure, so the significant variation shown in Table 16 cannot be attributed to the NO₂ atmosphere. It presumably reflects chance variation in unknown intercurrent influences.

Table 15. Mean Differences Between Day-1 and Day-2 Blood Coagulation Factors by Atmosphere in Phase 1^a

Variable	FA	NO ₂	DE	<i>P</i> Value ^b
Factor VII	2.9 (2.8)	1.3 (2.7)	−3.7 (2.7)	0.16
Fibrinogen	2.4 (9.7)	−2.7 (9.3)	2.9 (9.4)	0.78
vWF	2.5 (6.4)	0.3 (6.2)	12.1 (6.2)	0.37

^a Means ± SEs of all day-2 − day-1 differences expressed as log-transformed data. Measurement units are mg/dL for fibrinogen and % normal for the others. Nondetectable concentrations were estimated as one-half the lowest detected concentration for analyses.

^b Tested for atmosphere effect by conventional model with no other predictors.

Table 16. Estimated Means and Significance Tests for Blood Coagulation Factors by Atmosphere and Time of Measurement in Phase 1^a

Atmosphere	Time	Factor VII	Fibrinogen	vWF
DE	Day 1	97.6	318.9	116.1
	Day 2	93.9	321.7	128.2
NO ₂	Day 1	94.1	295.5	117.4
	Day 2	95.4	292.9	117.7
FA	Day 1	89.0	312.9	118.3
	Day 2	91.2	317.8	121.9
<i>P</i> (atmosphere)		0.06	0.005	0.57
<i>P</i> (time)		0.97	0.80	0.13
<i>P</i> (atmosphere × time)		0.43	0.89	0.36

^a Estimated means and significance tests from conventional mixed model applied to all data. Measurement units are mg/dL for fibrinogen and % normal for the others. Nondetectable concentrations were estimated as one-half the lowest detected concentration for analyses.

PHYSIOLOGIC AND SYMPTOMOLOGIC RESPONSES IN PHASE 1

Under our primary hypothesis, increased nonspecific bronchial reactivity, as measured by methacholine challenge, would be the most important physiologic response to DE exposure. Table 17 presents statistical results from methacholine challenges, and Table 18 presents individual data. No measure of bronchial reactivity showed a significant increase after DE exposure, compared with other atmospheres, by advanced mixed-model analysis (Table 17); the overall ranking of responses using the Friedman rank-sum test also did not show significant differences (data not shown). We saw a slight tendency toward increased reactivity after DE exposure, when the model-estimated geometric mean PC₂₀ was about 1.7 mg/mL, compared with 2.5 after FA exposure. As Table 18 indicates, 4 of 15 subjects (2602, 2628, 2643, 2702) never showed FEV₁ losses approaching or exceeding 20% in methacholine challenges; these subjects thus appeared to have had a decrease in bronchial reactivity sometime between their screening exams and their exposures.

Respiratory responses to exposure as measured by FVC, FEV₁, systolic and diastolic blood pressure, or FENO showed no significant differences by atmosphere (data not shown). However, sRaw showed a delayed increase after DE exposure compared with NO₂ or FA exposure. From an advanced model with adjustment for period effect (which was not significant), the estimated mean ($\pm$ SE) increase in sRaw after DE exposure was 14% (11%) at post-exposure testing (not significant) and 55% (16%) on day 2 ($P = 0.012$ for atmosphere effect). Small decreases in mean sRaw were

observed after NO₂ and FA at both times (data not shown). Table 19 shows individual data. Data capture was only 82% for sRaw because some subjects had difficulty performing the necessary breathing maneuvers and a few measurements had to be skipped because of scheduling conflicts with clinical tests requiring the same equipment.

Changes in SaO₂, estimated here using a fingertip pulse oximeter, from pre- to post-exposure did not vary significantly by atmosphere; but changes from pre-exposure to day 2 were close to significant ($P = 0.05$ – 0.08) according to both the conventional and the advanced models. Estimated mean changes were similar with either model: +0.5% after DE, –0.5% after NO₂, and +0.1% after FA. Thus, the apparent effect of DE on SaO₂ was in the favorable direction.

Changes in exhaled CO concentration from pre- to post-exposure tended to be more positive with DE (mean near +0.3 ppm) than with other exposures (means near –0.2 ppm), which is consistent with a slight rise in carboxyhemoglobin (COHb) concentration during DE exposures; this change in COHb reflects the mean increment of approximately 1 ppm in the inhaled CO concentration with DE. This effect did not reach statistical significance in Phase 1, but did in Phase 2 (see the section Blood Assay Responses in Phase 2).

The estimated mean change in total symptom score during exposure, based on the advanced statistical model, was +3.3 during DE, +1.7 during NO₂, and +0.8 during FA. The atmosphere effect was significant ($P = 0.04$), whereas the period and sequence effects were not. On this basis a typical subject had at most one mild symptom increase during FA exposure; two mild or one moderate symptom

Table 17. Means of Spirometric Results for Methacholine Challenge by Atmosphere in Phase 1^a

Variable	FA	NO ₂	DE	Significance
FEV ₁ pre-challenge (L)	3.17 (0.23)	3.21 (0.23)	3.23 (0.23)	—
FEV ₁ control (saline) (% pre-challenge) ^b	98.5 (1.3)	99.4 (1.3)	99.2 (1.3)	—
FEV ₁ maximum dose (% control) ^c	86.2 (2.5)	92.1 (2.6)	86.3 (2.7)	—
log ₁₀ (PC ₂₀)	0.39 (0.22)	0.40 (0.22)	0.23 (0.22)	— ^d
log ₁₀ (PC ₂₀) ^e	0.33 (0.20)	0.41 (0.20)	0.24 (0.20)	—

^a Means $\pm$ SEs from advanced model with adjustment for the effects of period (1st vs. 2nd vs. 3rd) and sequence (order of atmospheres). Nondetectable concentrations were estimated as one-half the lowest detected concentration for analyses.

^b FEV₁ after control (saline) challenge (before methacholine challenges) served as control; reported as a percentage of pre-challenge value.

^c FEV₁ after maximum methacholine dose; reported as a percentage of the control (saline) value.

^d All effects were nonsignificant in a conventional model.

^e In an alternative advanced model including extrapolation as a categorical variable, its effect was significant (estimate = 0.52, $P = 0.03$), indicating higher PC₂₀ in subjects who did not reach 20% loss in FEV₁ at maximum methacholine dose; but atmosphere and period effects remained nonsignificant.

Table 18. Individual Spirometric Data for Responses to Methacholine Challenge in Phase 1

Subject	Exposure Period	Atmo-sphere	Methacholine Dose ^a (mg/mL)	FEV ₁ Pre-Challenge (L)	FEV ₁ Control (saline) (% pre-challenge) ^b	FEV ₁ Maximum Dose (% control) ^c	PC ₂₀ (mg/mL)	PC ₂₀ Estimated by Extrapolation	Rank
1216	2	DE	1.25	2.88	99	58	0.78		2
1216	1	NO ₂	2.5	3.14	95	78	2.24		3
1216	3	FA	0.62	3.08	96	63	0.45		1
1656	2	DE	10	2.77	100	88	32.20	X	3
1656	3	NO ₂	2.5	2.9	99	65	1.74		2
1656	1	FA	1.25	2.69	96	86	2.17	X	1
2525	3	DE	0.15	2.65	100	84	0.19	X	1
2525	2	NO ₂	2.5	2.4	108	78	2.11		2
2525	1	FA	10	2.44	97	102	25.35	X	3
2602	1	DE	10	2.86	101	104			3
2602	3	NO ₂	10	2.53	109	101			2
2602	2	FA	10	2.48	102	98			1
2606	1	DE	0.31	2.52	94	76	0.14		2
2606	2	NO ₂	0.31	2.42	98	83	0.19		3
2606	3	FA	0.31	2.65	97	76	0.12		1
2628	2	DE	10	4.13	105	88	29.78	X	2
2628	3	NO ₂	10	3.83	110	85	14.43	X	1
2628	1	FA	10	4.3	96	92	22.15	X	3
2641	3	DE	2.5	3.63	99	77	2.22		2
2641	1	NO ₂	2.5	3.57	96	82	2.83	X	3
2641	2	FA	2.5	3.32	93	84	5.20	X	1
2642	2	DE	0	3.1	84		0.15	X	1
2642	1	NO ₂	0.075	3.45	88	95	0.30	X	2
2642	3	FA	2.5	3.31	103	84	2.81	X	3
2643	3	DE	10	2.6	100	87	33.50	X	1
2643	1	NO ₂	10	2.69	98	105	50.00	X	3
2643	2	FA	10	2.65	97	91	22.25	X	2
2644	1	DE	5	2.54	105	79	4.79		1
2644	2	NO ₂	5	2.73	93	87	8.18	X	3
2644	3	FA	5	2.7	88	82	5.41	X	2
2651	3	DE	10	3.31	101	82	13.03	X	3
2651	2	NO ₂	5	3.24	102	80	4.74		1
2651	1	FA	10	3.53	96	85	11.41	X	2
2666	2	DE	1.25	3.87	98	55	0.88		2
2666	3	NO ₂	1.25	3.99	95	85	2.05	X	3
2666	1	FA	0.31	3.55	99	75	0.23		1
2669	2	DE	0.075	5.97	97	65	0.04		1
2669	1	NO ₂	0.31	5.76	93	76	0.27		3
2669	3	FA	0.15	5.86	108	64	0.10		2
2702	1	DE	5	3.26	105	96			3
2702	3	NO ₂	10	3.27	104	92			1
2702	2	FA	10	3.17	101	92			2
2703	1	DE	0.31	3	93	76	0.27		1
2703	2	NO ₂	2.5	2.94	100	68	1.42		3
2703	3	FA	0.31	2.57	105	75	0.25		2

^a Individualized dose of methacholine that resulted in a decrease in FEV₁ of > 20%. The maximum dose used was 10 mg/mL.

^b FEV₁ after control (saline) challenge (before methacholine challenges) served as control; reported as a percentage of pre-challenge value.

^c FEV₁ after maximum methacholine dose; reported as a percentage of the control (saline) value.

Table 19. Individual Data for sRaw in Phase 1^a

Subject	Exposure Period	Atmosphere	sRaw Pre-Exposure ^b	sRaw Post-Exposure (% pre-exposure) ^c	sRaw Day 2 (% pre-exposure) ^d
1216	2	DE	19.03	80	—
1216	1	NO ₂	21.25	63	73
1216	3	FA	16.08	83	76
1656	2	DE	7.71	95	62
1656	3	NO ₂	7.23	91	81
1656	1	FA	8.88	89	110
2525	3	DE	4.39	134	186
2525	2	NO ₂	—	—	—
2525	1	FA	4.42	169	83
2602	1	DE	4.67	128	160
2602	3	NO ₂	13.02	105	81
2602	2	FA	12.13	89	121
2606	1	DE	5.4	115	100
2606	2	NO ₂	8.35	105	100
2606	3	FA	5.27	102	91
2641	3	DE	—	—	—
2641	1	NO ₂	5.78	112	125
2641	2	FA	23.2	42	56
2642	2	DE	4.7	197	278
2642	1	NO ₂	10.42	128	—
2642	3	FA	7.04	125	79
2643	3	DE	8.17	121	164
2643	1	NO ₂	3.39	171	152
2643	2	FA	7.29	110	86
2644	1	DE	5.07	148	176
2644	2	NO ₂	6.7	115	89
2644	3	FA	10.77	91	126
2651	3	DE	—	—	—
2651	2	NO ₂	—	—	—
2651	1	FA	4.14	113	85
2666	2	DE	7.08	70	56
2666	3	NO ₂	8.12	69	73
2666	1	FA	6.92	133	99
2669	2	DE	8.45	158	181
2669	1	NO ₂	15.15	80	101
2669	3	FA	22.59	67	51
2702	1	DE	—	—	—
2702	3	NO ₂	10.67	45	49
2702	2	FA	10.22	79	80
2703	1	DE	5.87	70	—
2703	2	NO ₂	5.94	40	55
2703	3	FA	5.34	57	—

^a One subject had no satisfactory data and is not included here. Missing data mean either the subject couldn't perform the necessary breathing measures or the measurement session was missed due to equipment scheduling conflicts.

^b The measurement of sRaw immediately before exposure. Calculation: (lung volume) × (pressure/flow); with units: (L) × [(cm H₂O) / (L/sec)].

^c sRaw immediately after exposure reported as a percentage of the pre-exposure value.

^d sRaw on day 2 reported as a percentage of the pre-exposure value.

increase during NO₂ exposure; and 3 mild, or one mild plus one moderate, or one severe symptom increase during DE exposure. When scores were broken down by symptom categories, respiratory and cardiac symptoms showed no significant variation by atmosphere; thus the excess increase

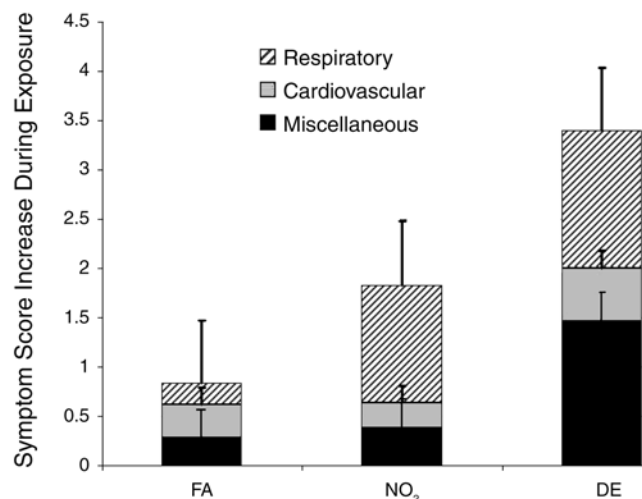


Figure 4. Mean symptom score changes during exposure in Phase 1 subjects. Total bar height represents mean total score change ($\pm$ SE); shaded sections with error flags represent mean ($\pm$ SE) changes for respiratory, cardiac, and miscellaneous symptom categories. An increase of 1 point represents a mild worsening of any one symptom. Analyzed with conventional model.

attributable to DE was most influenced by nonspecific symptoms (i.e., headache, fatigue, eye irritation, stomach upset, and other symptoms not specifically mentioned on the questionnaire).

The estimated mean change in the miscellaneous symptom score during exposure was +1.4 during DE, +0.3 during NO₂, and +0.2 during FA; the atmosphere effect was significant ($P = 0.002$) whereas the period and sequence effects were not. Figure 4 presents the symptom findings graphically. Results from conventional models testing only the atmosphere effect were similar to those from advanced models. Miscellaneous symptom scores were significantly higher when subjects reported that they perceived odor in the chamber, or suspected that DE was present, than when they did not. Subjects exhibited significant, but not highly accurate, abilities to detect DE: 9 of 15 reported it in actual DE exposures, 3 of 15 in NO₂ exposures, and none in FA exposures. Symptoms reported during post-exposure follow-up intervals did not vary significantly by atmosphere.

SPUTUM ASSAY RESPONSES IN PHASE 2

Table 20 shows summary statistics for cells in sputum by atmosphere; and Table 21 shows individual total and differential cell counts from sputum induced on the morning after each exposure. When analyzed with conventional

Table 20. Mean Sputum Cell Counts by Atmosphere in Phase 2^a

Variable ^b	FA	NO ₂	DE	Significance
Total cells/ μ L	1074 (330)	1120 (329)	1156 (330)	—
Macrophages (%)	48.3 (5.0)	49.1 (5.0)	37.9 (5.0)	— ^c
Lymphocytes (%)	2.1 (0.4)	1.4 (0.4)	2.1 (0.4)	—
PMNs (%)	48.7 (5.0)	48.7 (5.0)	58.8 (4.9)	— ^d
Eosinophils (%)	0.9 (1.1)	0.8 (1.1)	1.2 (1.1)	— ^e
Eosinophils (Wright) (%)	1.0 (1.8)	1.1 (1.8)	2.0 (1.8)	— ^f

^a Means $\pm$ SEs were estimated by advanced model with adjustment for effects of period (1st vs. 2nd vs. 3rd) and sequence (order of atmospheres). Counts of 0 were estimated as 0.5% for statistical purposes.

^b All counts were done with Giemsa stain. Eosinophils were also counted with Wright stain.

^c Atmosphere effect was significant ($P = 0.001$); period effect was also significant ($P = 0.002$): estimates were +12 for 1st and +3 for 2nd compared with 3rd period.

^d Atmosphere effect was significant ($P = 0.004$); period effect was also significant ($P = 0.032$): estimates were –12 for 1st and –3 for 2nd compared with 3rd period.

^e No significance with untransformed data. With log-transformed data, atmosphere effect was significant ($P = 0.02$): estimates were 50% higher after DE and 20% higher after NO₂ compared with FA exposure; period effect was also significant ($P = 0.006$): estimates were 50% higher after 2nd period compared with others.

^f No significance with untransformed data. With log-transformed data, atmosphere effect was significant ($P = 0.04$): estimates were 50% higher after DE and 15% higher after NO₂ compared with FA exposure; period effect was not significant.

Table 21. Individual Data for Sputum Cell Counts in Phase 2^a

Subject	Exposure Period	Atmosphere	Total Cells (1000/ μ L)	Macrophages (%)	Lymphocytes (%)	PMNs (%)	Eosinophils (%)	Eosinophils (Wright) (%)
2325	2	DE	1.33	51	3	44	2	3
2325	1	NO ₂	1.92	76	1	21	2	2
2325	3	FA	2.04	64	2	34	0	0
2551	2	DE	0.49	41	2	55	2	2
2551	3	NO ₂	0.57	53	3	44	0	0
2551	1	FA	0.89	53	3	44	0	0
2602	1	DE	2.80	18	2	80	0	0
2602	2	NO ₂	1.60	21	0	79	0	0
2602	3	FA	0.61	37	5	58	0	0
2628	1	DE	1.48	28	2	70	0	0
2628	3	NO ₂	1.34	26	2	72	0	1
2628	2	FA	0.74	29	2	68	1	1
2651	2	DE	0.17	40	2	58	0	0
2651	1	NO ₂	0.96	46	2	52	0	0
2651	3	FA	0.63	24	3	73	0	0
2701	3	DE	1.01	22	4	73	1	2
2701	1	NO ₂	0.88	54	2	44	0	0
2701	2	FA	0.41	27	0	73	0	0
2704	2	DE	1.46	23	4	71	2	0
2704	3	NO ₂	0.73	43	1	56	0	0
2704	1	FA	1.30	47	2	51	0	0
2780	1	DE	0.32	71	4	25	0	0
2780	3	NO ₂	0.32	67	2	31	0	0
2780	2	FA	0.25	86	2	12	0	0
2847	3	DE	0.76	21	1	78	0	0
2847	1	NO ₂	0.19	51	3	46	0	0
2847	2	FA	0.17	52	2	46	0	0
2871	3	DE	1.38	25	0	74	1	0
2871	1	NO ₂	0.58	45	0	55	0	0
2871	2	FA	1.38	33	1	66	0	0
2876	1	DE	0.74	66	5	28	1	1
2876	2	NO ₂	1.12	57	0	40	3	1
2876	3	FA	1.33	56	1	42	1	1
2878	1	DE	0.36	49	0	51	0	0
2878	3	NO ₂	0.89	31	1	68	0	0
2878	2	FA	0.90	33	1	66	0	0
2879	1	DE	1.31	49	0	51	0	2
2879	2	NO ₂	1.76	56	0	44	0	0
2879	3	FA	1.07	52	1	47	0	0
2883	1	DE	4.22	33	1	53	13	29
2883	3	NO ₂	4.60	42	3	45	10	20
2883	2	FA	4.40	36	3	43	18	21
2886	1	DE	0.35	69	1	30	0	0
2886	3	NO ₂	0.12	ND	ND	ND	ND	0
2886	2	FA	0.40	73	2	25	0	0

^a All counts were done with Giemsa stain. Eosinophils were also counted with Wright stain. ND = no data. Counts of 0 were estimated as 0.5% for statistical purposes.

models [data not shown], macrophage, PMN, and eosinophil counts showed near-significant variation by atmosphere; this suggests that an inflammatory response to DE exposure may be present, which would be consistent with our primary hypothesis. Percentages of PMNs and eosinophils increased and the percentage of macrophages decreased after DE exposure, compared with NO₂ and FA exposures. In the advanced mixed-model analyses, both period and atmosphere showed significant ($P < 0.05$) effects on macrophage and PMN percentages. The estimated differences between DE and other atmospheres increased slightly compared with results of the conventional model, in which the atmosphere effect fell slightly short of significance. Macrophages decreased and PMNs increased from 1st to 3rd periods, suggesting more tendency to inflammatory response

in later exposures independent of the atmosphere being presented. In Phase 2 as in Phase 1, the percentages of PMNs were increased and macrophages decreased by roughly 10% after FA exposure compared with baseline sputum inductions separated by 2 weeks from any exposure; the difference did not reach statistical significance.

Table 22 shows summary statistics by atmosphere for sputum biochemical measurements in Phase 2. Table 23 presents individual data for variables that showed possibly meaningful variation among exposure conditions. Estimated means of IgG4 and IL-5 were higher and the mean of RANTES was lower after DE compared with other exposures. None of those differences was close to statistical significance. IgE varied significantly by sequence

Table 22. Means of Sputum Biochemical Assays by Atmosphere in Phase 2^a

Variable ^b	FA	NO ₂	DE	Significance
log ₁₀ (IgA)	0.776 (0.278)	0.927 (0.283)	0.897 (0.278)	— ^c
log ₁₀ (IgE)	−1.335 (0.117)	−1.625 (0.119)	−1.481 (0.116)	— ^d
log ₁₀ (IgG)	0.352 (0.070)	0.269 (0.071)	0.349 (0.070)	—
log ₁₀ (IgG4)	0.360 (0.333)	−0.029 (0.339)	0.489 (0.331)	—
log ₁₀ (IgM)	1.200 (0.195)	1.390 (0.199)	1.300 (0.194)	—
log ₁₀ (IL-4)	1.482 (0.056)	1.480 (0.059)	1.471 (0.055)	— ^e
log ₁₀ (IL-5)	−0.145 (0.338)	−0.111 (0.347)	0.148 (0.334)	— ^f
log ₁₀ (IL-8)	2.018 (0.171)	1.999 (0.172)	1.958 (0.171)	—
log ₁₀ (IL-12)	1.822 (0.156)	1.959 (0.151)	1.915 (0.156)	—
log ₁₀ (IFN-γ)	1.893 (0.217)	1.737 (0.220)	1.704 (0.216)	—
log ₁₀ (TNF-α)	1.578 (0.076)	1.473 (0.078)	1.502 (0.076)	— ^g
log ₁₀ (ECP)	1.018 (0.151)	1.020 (0.153)	1.080 (0.151)	—
log ₁₀ (RANTES)	1.515 (0.102)	1.428 (0.104)	1.406 (0.101)	—
log ₁₀ (eotaxin)	0.994 (0.075)	1.019 (0.076)	1.098 (0.075)	—
log ₁₀ (GM-CSF)	0.129 (0.089)	0.120 (0.087)	0.157 (0.088)	—
log ₁₀ (tryptase) ^h	—	—	—	—

^a Means ± SEs of log-transformed data were estimated by advanced model with adjustment for effects of period (1st vs. 2nd vs. 3rd) and sequence (order of atmospheres). Nondetectable concentrations were estimated as one-half the lowest detected concentration for analyses.

^b Measurement units are ng/mL for immunoglobulins, ECP, and tryptase; pg/mL for others.

^c Period effect near significance ($P = 0.06$): estimate ~55% lower after 2nd and 3rd compared with 1st period.

^d Sequence effect significant ($P = 0.002$). Many samples were below quantifiable limits: nearly all for subjects in certain sequence groups, but few or none for other sequence groups. Thus, the significance reflects overall differences between individuals and does not necessarily imply a carryover effect.

^e Period effect significant ($P = 0.01$): estimate 10% higher after 2nd and 60% higher after 3rd compared with 1st period; results may be biased due to numerous values below quantifiable limits.

^f Period effect significant ($P = 0.03$): estimate 80% lower after 2nd and 4-fold higher after 3rd compared with 1st period; results may be biased due to numerous values below quantifiable limits.

^g Period effect significant ($P = 0.003$): estimate 70% higher after 2nd and 80% higher after 3rd compared with 1st period.

^h Most measurements were below detection limit.

Table 23. Individual Data for Sputum Biochemical Assays in Phase 2^a

Subject	Exposure Period	Atmosphere	IgE (ng/mL)	IgG4 (ng/mL)	IL-5 (pg/mL)	RANTES (pg/mL)
2325	2	DE	0.254	40.61	0.08	11.00
2325	1	NO ₂	0.009	2.02	11.33	33.30
2325	3	FA	0.041	20.49	0.08	72.92
2551	2	DE	0.009	12.34	18.65	68.66
2551	3	NO ₂	0.062	0.004	0.08	11.00
2551	1	FA	0.041	4.07	0.08	41.34
2602	1	DE	0.009	12.60	11.06	31.95
2602	2	NO ₂				
2602	3	FA	0.062	15.39	25.55	11.00
2628	1	DE	0.009	5.01	8.32	11.00
2628	3	NO ₂	0.009	83.34	64.31	11.00
2628	2	FA	0.018	14.97	0.08	11.00
2651	2	DE	0.009	1.50	0.08	11.00
2651	1	NO ₂	0.009	1.37	0.08	11.00
2651	3	FA	0.009	3.84	4.55	48.05
2701	3	DE	0.37	7.53	0.08	11.00
2701	1	NO ₂	0.769	14.24	0.08	11.00
2701	2	FA	1.288	22.31	5.06	11.00
2704	2	DE	0.062	7.03	0.08	11.00
2704	3	NO ₂	0.018	1.86	79.62	33.30
2704	1	FA	0.116	0.23	40.10	109.58
2780	1	DE	0.009	0.007	0.08	22.56
2780	3	NO ₂	0.009	0.004	3.63	76.95
2780	2	FA	0.009	0.004	0.08	33.30
2847	3	DE	0.105	5.50	591.06	11.00
2847	1	NO ₂	0.041	8.42	2.68	11.00
2847	2	FA	0.084	13.45	0.08	11.00
2871	3	DE	0.77	11.42	538.53	33.31
2871	1	NO ₂	0.169	2.74	39.50	55.00
2871	2	FA	0.59	0.14	0.08	11.00
2876	1	DE	0.018	10.98	48.59	60.49
2876	2	NO ₂	0.009	2.01	0.08	41.34
2876	3	FA	0.148	5.06	35.59	80.78
2878	1	DE	0.009	0.72	0.08	67.36
2878	3	NO ₂	0.009	0.004	0.08	90.30
2878	2	FA	0.009	0.39	0.08	48.05
2879	1	DE	0.009	1.44	0.08	134.88
2879	2	NO ₂	0.009	0.92	0.08	114.69
2879	3	FA	0.009	0.82	0.08	62.89
2883	1	DE	0.009	4.96	0.17	61.69
2883	3	NO ₂	0.041	3.57	9.98	76.95
2883	2	FA	0.018	2.48	0.08	64.12
2886	1	DE	0.009	0.004	0.08	41.34
2886	3	NO ₂	0.009	0.004	0.08	22.56
2886	2	FA	0.009	1.24	0.08	11.00

^a Values in italics are estimates of undetectable concentrations (one-half the lowest quantifiable value).

(order of atmospheres), but not by atmosphere or period (1st vs. 2nd vs. 3rd). Although it raises the possibility of a carryover effect, this significant variation appeared to result from chance assignment of individuals with consistently low or undetectable IgE to some sequences but not others, and not from any response to DE. Period effects were significant or near-significant for IgA, IL-4, IL-5, and TNF- α with different patterns.

In Phase 2, unlike phase 1, biochemical measurements showed some significant ($P < 0.05$) differences between sputum inductions at baseline and after exposure to FA: IgE averaged 61% higher and IL-8 69% higher after FA (data not shown). This might be interpreted as an immunologic response to cat allergen inhalation. Conversely, IgA averaged about 60% lower after FA than at baseline, an observation for which there is no clear immunologic explanation and is likely attributable to biologic variability in sputum IgA measurements.

BLOOD ASSAY RESPONSES IN PHASE 2

Table 24 shows summary statistics by atmosphere for serum biochemical measurements in Phase 2 analyzed as changes in log-transformed data between pre-exposure day 1 and day 2. The tabulated values are based on advanced models with adjustment for period and sequence effects.

No significant differences between atmospheres were found. Some variables showed significant period effects or significant negative relationships (or both) between the day-1 values and the day-2 – day-1 differences; these relationships suggest the influence of chance on the results. These significant relationships were also true in Phase 1, but the pattern of statistical significance was somewhat inconsistent between phases. However, in both phases, IgA and IgE were high at the first day-1 measurement compared with those before the 2nd and 3rd exposure periods. Also, IgA changes showed a significant sequence effect in the advanced model when pre-exposure IgA was included as a covariate.

Table 25 shows the estimated mean changes in blood coagulation factors between day 1 and day 2. No significant differences by atmosphere were found using the conventional mixed model. Table 26 shows estimated mean values for each atmosphere and time of measurement (day 1 or 2), along with significance test results from conventional mixed-model analyses. Factor VII showed marginally significant variation with atmosphere, averaging about 3% lower after NO₂ and 6% lower after DE exposures compared with FA ($P = 0.04$ for main effect of atmosphere). Since these differences were present before as well as after the exposures, the significant variation cannot be attributed to the exposure atmospheres and remains unexplained. Fibrinogen and vWF did not vary significantly.

Table 24. Mean Differences in Serum Biochemical Assays by Atmosphere in Phase 2^a

Variable ^b	FA	NO ₂	DE	Significance
log ₁₀ (IgA)	−0.009 (0.023)	−0.010 (0.023)	−0.029 (0.022)	— ^c
log ₁₀ (IgE)	−0.003 (0.015)	−0.018 (0.015)	−0.034 (0.015)	— ^{d, e}
log ₁₀ (IgG)	0.022 (0.126)	0.142 (0.126)	−0.006 (0.123)	— ^e
log ₁₀ (IgG4)	0.225 (0.127)	0.029 (0.128)	−0.074 (0.125)	— ^e
log ₁₀ (IgM)	−0.008 (0.051)	−0.042 (0.051)	−0.030 (0.050)	— ^f
log ₁₀ (IL-6)	−0.136 (0.094)	−0.061 (0.096)	0.020 (0.110)	— ^e
log ₁₀ (ICAM)	−0.029 (0.037)	−0.051 (0.037)	0.016 (0.036)	—

^a Means $\pm$ SEs of all day-2 – day-1 differences expressed as log-transformed data. Means were estimated by advanced model with adjustment for effects of period (1st vs. 2nd vs. 3rd) and sequence (order of atmospheres). Nondetectable concentrations were estimated as one-half the lowest detected concentration for analyses.

^b Measurement units are ng/mL for immunoglobulins and pg/mL for others.

^c Significant period effect ($P < 0.05$): more negative changes in 1st period than in later periods. In an alternative advanced model, day-1 value (as a covariate) showed significant negative relationships with day-2 – day-1 differences; sequence effect was also significant: subjects with FA exposure in 1st period had more positive values in day-2 – day-1 differences than other subjects.

^d Significant ($P < 0.05$) variation in day-1 pre-exposure values by period: high in 1st period.

^e Significant ($P < 0.05$) negative relationship between day-1 values and day-2 – day-1 differences.

^f Significant ($P < 0.05$) variation in day-1 values by sequence: subjects with NO₂ exposure in 1st period averaged lower than subjects with DE exposure in 1st period.

PHYSIOLOGIC AND SYMPTOMOLOGIC RESPONSES IN PHASE 2

Figure 5 graphically illustrates the group mean and individual responses to cat allergen challenge after each exposure. Table 27 presents individual numeric data for responses to cat allergen challenge during screening and after each exposure. Response was expressed as percent change in FEV₁ from pre- to post-challenge, which had a more nearly normal distribution than alternative response metrics. The challenge aerosol solution concentration (20% of the concentration that evoked a > 15% drop in FEV₁ in the screening examination) was 2000 BAU/mL in one subject, 400 BAU/mL in another, and 16 or 80 BAU/mL in the remaining 13 subjects. Neither the challenge concentration nor the pre-challenge FEV₁ was significantly associated with the magnitude of FEV₁ responses, as determined by preliminary conventional or advanced mixed-model analyses (not shown). From a conventional mixed-model, the estimated mean percentage response ($\pm$ SE) was -7 ± 2 in FA, -6 ± 2 in DE (not significantly different from FA), and -1 ± 2 in NO₂ (significantly different from FA or DE, $P < 0.05$).

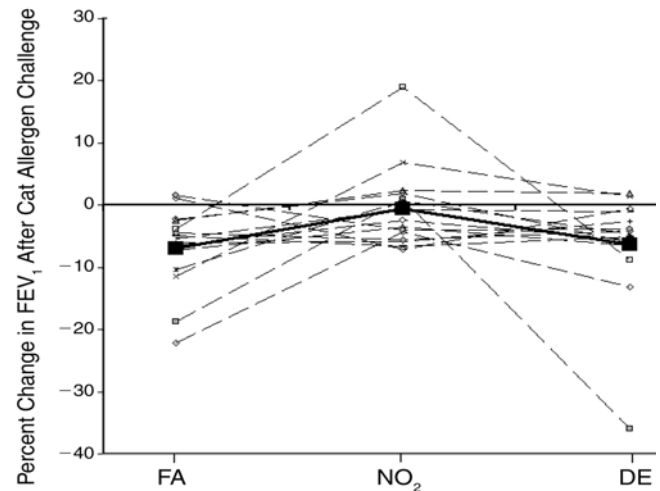


Figure 5. Bronchoconstrictive response to inhaled cat allergen (% change in FEV₁) ~1.5 hours after end of exposure, by atmosphere. Heavy solid line with filled squares is the group mean of responses. Light broken lines with different symbols are individual responses. Difference between NO₂ and the other atmospheres is significant at $P < 0.05$. Analyzed with conventional model.

Table 25. Mean Differences Between Day-1 and Day-2 Blood Coagulation Factors by Atmosphere in Phase 2^a

Variable	FA	NO ₂	DE	<i>P</i> Value ^b
Factor VII	11.3 (10.4)	4.2 (10.4)	5.1 (10.0)	0.87
Fibrinogen	26.8 (19.9)	3.5 (19.9)	6.9 (16.1)	0.67
vWF	14.5 (9.7)	11.3 (9.7)	-8.3 (9.3)	0.21

^a Means $\pm$ SEs of all day-2 - day-1 differences. Measurement units are mg/dL for fibrinogen and % normal for the others. Nondetectable concentrations were estimated as one-half the lowest detected concentration for analyses.

^b Tested for atmosphere effect by conventional model with no other predictors.

Table 26. Estimated Means and Significance Tests for Blood Coagulation Factors by Atmosphere and Time of Measurement in Phase 2^a

Atmosphere	Time	Factor VII	Fibrinogen	vWF
DE	Day 1	144.9	340.1	129.2
	Day 2	150.1	347.0	120.9
NO ₂	Day 1	150.2	335.6	128.5
	Day 2	153.9	343.1	141.4
FA	Day 1	160.0	357.2	123.2
	Day 2	155.9	348.7	126.9
<i>P</i> (atmosphere)		0.04	0.40	0.11
<i>P</i> (time)		0.64	0.81	0.53
<i>P</i> (atmosphere $\times$ time)		0.49	0.68	0.14

^a Estimated means and significance tests from conventional mixed model applied to all data. Nondetectable concentrations were estimated as one-half the lowest detected concentration for analyses. Measurement units are mg/dL for fibrinogen and % normal for others.

Table 27. Individual Spirometric Data for Responses to Cat Allergen Challenge in Phase 2

Subject	Exposure Period	Atmosphere	Cat Allergen Dose ^a (BAU/mL)	FEV ₁ (L)		% Change in FEV ₁	% Change in FEV ₁ at Screening ^b
				Pre-Challenge	Post-Challenge		
2325	2	DE	80	3.36	2.92	−13.1	−9
2325	1	NO ₂	80	3.31	3.17	−4.2	−9
2325	3	FA	80	3.33	2.59	−22.2	−9
2551	2	DE	400	2.06	1.88	−8.7	−10
2551	3	NO ₂	400	2.00	2.38	19.0	−10
2551	1	FA	400	2.05	1.97	−3.9	−10
2602	1	DE	2000	2.77	2.74	−1.1	0
2602	2	NO ₂	2000	2.71	2.69	−0.7	0
2602	3	FA	2000	2.75	2.60	−5.5	0
2628	1	DE	80	4.59	4.40	−4.1	−9
2628	3	NO ₂	80	4.39	4.15	−5.5	−9
2628	2	FA	80	4.31	4.12	−4.4	−9
2651	2	DE	16	3.11	2.99	−3.9	−17 ^c
2651	1	NO ₂	16	3.27	3.14	−4.0	−17 ^c
2651	3	FA	16	3.23	3.28	1.5	−17 ^c
2701	3	DE	16	2.09	2.12	1.4	−16 ^c
2701	1	NO ₂	16	2.05	2.19	6.8	−16 ^c
2701	2	FA	16	2.26	2.00	−11.5	−16 ^c
2704	2	DE	80	3.92	3.72	−5.1	−5
2704	3	NO ₂	80	3.92	3.66	−6.6	−5
2704	1	FA	80	4.53	4.31	−4.9	−5
2780	1	DE	80	1.29	1.23	−4.7	−12
2780	3	NO ₂	80	1.47	1.48	0.7	−12
2780	2	FA	80	1.35	1.21	−10.4	−12
2847	3	DE	80	3.22	2.06	−36.0	−1
2847	1	NO ₂	80	3.17	3.20	0.9	−1
2847	2	FA	80	3.21	2.61	−18.7	−1
2871	3	DE	80	2.91	2.72	−6.5	−8
2871	1	NO ₂	80	2.84	2.74	−3.5	−8
2871	2	FA	80	2.78	2.58	−7.2	−8
2876	1	DE	16	2.82	2.65	−6.0	−24 ^c
2876	2	NO ₂	16	2.61	2.66	1.9	−24 ^c
2876	3	FA	16	2.62	2.56	−2.3	−24 ^c
2878	1	DE	80	3.65	3.63	−0.5	−3
2878	3	NO ₂	80	3.81	3.54	−7.1	−3
2878	2	FA	80	3.69	3.73	1.1	−3
2879	1	DE	16	2.65	2.58	−2.6	−22 ^c
2879	2	NO ₂	16	2.72	2.56	−5.9	−22 ^c
2879	3	FA	16	2.63	2.47	−6.1	−22 ^c
2883	1	DE	80	3.21	3.03	−5.6	−3
2883	3	NO ₂	80	2.87	2.80	−2.4	−3
2883	2	FA	80	3.21	3.00	−6.5	−3
2886	1	DE	16	3.17	3.23	1.9	−25 ^c
2886	3	NO ₂	16	2.86	2.93	2.4	−25 ^c
2886	2	FA	16	2.81	2.74	−2.5	−25 ^c

^a Individualized dose of cat allergen administered by 1 minute of tidal breathing. Dose was 20% of the concentration that provoked > 15% decrease in FEV₁ at screening.

^b Percent change in FEV₁ when the indicated dose of cat allergen was administered during screening.

^c Percent change in FEV₁ after administration of 80 BAU/mL, which was the lowest dose used in screening.

The overall mean change in FEV₁ from before to after challenge was significantly negative ($P < 0.01$), indicating that inhaled cat allergen concentrations were appropriate to induce mild bronchoconstriction as intended. However, some subjects reacted less to the challenges after all three exposures than they had after the comparable dose in screening. An advanced statistical model showed no significant effect of period or sequence, and estimated atmosphere effects were essentially the same as those described above. The comparison of responses to DE and FA exposures does not support the hypothesis that DE exposure at particle mass concentration near 100 $\mu\text{g}/\text{m}^3$ can exacerbate bronchoconstriction from subsequently inhaled cat allergen. The apparently “protective” effect of NO₂ exposure is most plausibly attributed to chance intercurrent influences that reduced subjects' responsiveness to cat allergen on NO₂ exposure days. An alternative explanation is that NO₂ exposure does, in fact, reduce responses to cat allergen, but its “protective” effect is blocked by coexisting DEP. This seems implausible, given that previous studies with different allergen challenges have shown either no effect of NO₂ exposure (e.g., Witten et al. 2005) or exacerbation of allergen response after NO₂ exposure (e.g., Barck et al. 2002).

We found no significant variation by atmosphere in responses measured by conventional FVC and FEV₁, blood pressure, SaO₂, or FENO. Exhaled CO concentration after 20 seconds of breath-holding showed significant ($P < 0.01$) variation by atmosphere, with a positive change pre- to post-DE exposures (mean +0.2 ppm) and negative changes during other exposures (means -0.9 ppm for NO₂, -0.2 ppm for FA). Data capture for sRaw improved to 91% in Phase 2. Change in sRaw after exposure was not significant in Phase 2, in contrast to Phase 1. Miscellaneous symptom score increases were larger during DE than other exposures in Phase 2 as they had been in Phase 1, but the average increase was smaller and differences between atmospheres did not attain significance in Phase 2.

DISCUSSION AND CONCLUSIONS

RESPONSES TO DIESEL EXHAUST AND INHALED ALLERGEN

The current study attempted to address a paucity of data for the effects of DE exposure along with allergen challenge in the lower airway. In either phase of this study, few measures of immunologic status, blood coagulability, or cardiorespiratory physiology showed a response to DE significantly different from the response to FA. In particular,

the immediate bronchoconstrictive response and the delayed immunologic response to inhaled cat allergen were not significantly enhanced after DE exposure. Thus, the results did not strongly support our major hypotheses concerning effects of inhaled DEP. Essentially, we were unable to replicate in the lower airway the inflammatory and allergic adjuvant effects of DEP previously reported in human studies of the upper airway (Diaz-Sanchez et al. 1994, 1996, 1997, 2000). Given existing robust *in vitro* and *in vivo* evidence of pro-inflammatory DEP effects on a variety of cell types (Riedl and Diaz-Sanchez 2005), including bronchial epithelial cells, we must consider the limitations of the current experimental conditions (discussed below) as a likely cause of these findings. However, we cannot exclude possible intrinsic differences in how the upper and lower airways respond to DEP (or in their thresholds of exposure). Such differences between upper- and lower-airway responses to allergen exposure have been reported previously (Sicherer et al. 1997).

A few of the observed effects of DE exposure that were noted as significant might be the result of chance significant findings among many statistical tests. One physiologic measure, sRaw, did show a clinically mild (about 1.5-fold) statistically significant increase the morning after DE exposure, consistent with an unfavorable effect of DE. However, this finding occurred only in Phase 1, which did not include an allergen challenge and was compromised by more-than-usual loss of data. (The loss of data was probably not random given that certain subjects had problems with test performance and equipment availability problems were episodic.) In light of its time course, the increase in sRaw did not appear to be a direct immediate bronchoconstrictive response, such as that which occurs in many people with asthma who exercise while breathing sulfur dioxide or cold air (Utell and Looney 1995). A delayed, immunologically mediated bronchoconstrictive response is plausible. If that is the explanation for the sRaw increase, it is surprising that no consistent increases in mediators were measurable in serum or sputum, and that equal or greater response was not found in Phase 2, when allergen challenge followed DE exposure and data capture improved over that in Phase 1.

Miscellaneous (nonspecific) symptom scores showed a subtle but statistically significant excess unfavorable response to DE exposure in comparison with FA or NO₂ in Phase 1 and a smaller, nonsignificant response in Phase 2. This effect might be psychologically mediated, given that at least some subjects could perceive the odor of DE, or it might reflect physiologic effects of DE exposure not detected otherwise. A possibly relevant finding has been reported by Crüts and colleagues (2008). They performed electroencephalography on

volunteers exposed for 1 hour to DE with 300 $\mu\text{g}/\text{m}^3$ DEP; compared with control subjects exposed to FA, they observed changes that indicated a general cortical stress response, which was detectable after 30 minutes of exposure and persisted at least 1 hour after exposure ended.

The finding of several significant period effects (changes from 1st to 2nd to 3rd exposure periods regardless of the atmosphere) is difficult to interpret. Period effects have not been evaluated in most previous controlled-exposure studies. As with atmosphere effects, a few apparently significant results might have occurred by chance among the many statistical tests. Subjects' psychologic and physiologic responses to the secondary stresses of chamber exposures might have changed appreciably from first to later exposure periods as they gained more experience; this possibility assumes that no effect of the atmospheres overrode the stress responses. Shifts in laboratory standards over time should not have contributed to period effects since all samples from each subject were analyzed at nearly the same time with the same standard. Frozen-storage times for blood and sputum samples were, of course, different according to period, but those differences were relatively small because several months usually elapsed between collection and analysis. In any event, finding period effects serves as a reminder that unmeasured, uncontrolled, time-dependent factors may influence the outcome of any study with human subjects.

Additional data analyses were performed to explore the possibility that the lack of clear immunologic responses to DE exposure was due to low statistical power. (If assays had less precision than originally anticipated, the chosen sample size of 15 subjects might have been too small to detect the expected DE-related changes as statistically significant.) Upper 95% confidence limits — considered to represent the maximum plausible responses to DE in a typical subject — were calculated for two key response measures, IgE and IL-4 in sputum. For simplicity, responses were expressed by subtracting the log-transformed value after FA exposure from that after DE exposure, and expressing the difference as a proportionate change attributable to DE. In Phase 1, the upper 95% confidence limits were a 3.2-fold increase in IgE and a 4-fold increase in IL-4. In Phase 2, assays showed less variability, such that the respective upper limits were 1.1-fold and 1.2-fold. By contrast, in nasal-instillation studies with ragweed allergen accompanied by either DEP or placebo, Bastain and colleagues (2003) found statistically significant and reproducible increases associated with DEP that averaged near 15-fold for IgE and 25-fold for IL-4. (Their subjects' total dose of DEP was similar to or modestly higher than our

subjects' total dose, but was delivered much faster to a much smaller airway surface.)

In summary, results from Phase 1 in our study do not rule out modest immunologic responses to DE; but results from Phase 2 appear to rule out any meaningful IgE or IL-4 responses to DE exposure followed by cat allergen challenge. In any event, results from both phases indicate that responses to lower respiratory inhalation exposure, if any, were much smaller than the responses to nasal-instillation exposure in the study by Bastain.

The limitations of the current study's methods with regard to human subjects should be recognized. Given the ethical considerations of studying effects of DE exposure on subjects with persistent asthma, the subject pool was limited to those with very mild or intermittent disease who might therefore be relatively unreactive to DE. Subjects with more severe asthma, who may be more susceptible, were not included because it would have been unacceptable to discontinue inhaled corticosteroid therapy for a prolonged period (> 12 hours) since this is standard-of-care therapy for patients with persistent disease. Although the effect or duration of effect of inhaled corticosteroid therapy on DE exposure and allergen challenge as used in this study has not been established, it seems likely that such anti-inflammatory therapy might influence the measured immunologic or physiologic endpoints so as to blunt any clear biologic effect of DE exposure. Thus, these results cannot answer the question whether more severely asthmatic and inadequately medicated individuals would be more vulnerable to effects of DE exposure followed by allergen challenge. Moreover, the question may not be answerable by ethically acceptable controlled-exposure studies. In addition, we cannot exclude the possibility that more invasive sampling techniques such as bronchoalveolar lavage or bronchial biopsies, which were not included in our current protocol, might have greater sensitivity to detect inflammatory or immunologic changes after exposures, and so might have shown effects in this study. However, such procedures significantly increase the risks to human subjects and markedly reduce the human subject pool willing to participate.

DIESEL EXHAUST CHARACTERISTICS IN THIS STUDY COMPARED WITH OTHERS

The proportions of DE constituents in different human exposure facilities may differ markedly and complicate the effort to compare biological responses across studies. The following examples are limited to studies with reported DEP mass concentrations near 100 $\mu\text{g}/\text{m}^3$, similar to ours. Sobus and colleagues (2008), characterizing a U.S. Environmental Protection Agency (U.S. EPA) exposure chamber,

found naphthalene concentrations several times lower than ours and phenanthrene concentrations several times higher. One study in a Swedish exposure facility showed CO concentrations similar to ours, but lower levels of NO₂ and formaldehyde (Stenfors et al. 2004). A later study in the same facility showed total carbon modestly lower than ours and EC markedly lower (Behndig et al. 2006). A study in the University of Washington facility showed concentrations of both NO₂ and CO substantially lower than ours (Peretz et al. 2008). Differences in engine design, applied load (if any), fuel, lubricating oil, and dilution-line characteristics all might contribute to these differences. Inconsistencies in analysis might also contribute, especially for trace constituents that are more difficult to analyze, since cross-comparisons of analytical instruments or independent reference standards are uncommon.

RESPONSES IN THIS STUDY COMPARED WITH OTHERS

Inflammation Responses Nordenhäll and colleagues (2000) exposed healthy volunteers to FA and DE with 300 µg/m³ DEP for 1 hour with intermittent exercise. They found increases in sputum neutrophil percentage and sputum IL-6 level at 6 hours after exposure but not at 24 hours. The sputum data at 24 hours appeared to be influenced by the prior collection at 6 hours. We cannot directly compare those results with ours, since we had no collection at 6 hours. Nordenhäll and colleagues found no significant changes in IL-8 or TNF-α levels, which are consistent with our findings.

Stenfors and colleagues (2004) also exposed healthy volunteers to FA and DE with a duration and particle mass concentration similar to ours with no exercise. By bronchial lavage and biopsies 6 hours after the end of exposure, they found increases in airway inflammatory cells, IL-8, and IL-10 after DE exposures. Subsequently, Behndig and colleagues (2006) performed similar exposures of healthy subjects and also found bronchial inflammatory cell responses by lavage 18 hours after exposure ended. Given the differences in experimental protocols, we cannot confirm or rule out that we might have found similar responses in our study.

Pulmonary Responses Stenfors and colleagues (2004) found slight increases in Raw immediately after exposure; but they did not measure pulmonary responses the following day, which is when we found a significant increase in sRaw. They found no significant changes in FVC or FEV₁, which are consistent with our findings and with most other previous DE exposure studies at somewhat higher levels.

Nonspecific Symptoms As mentioned previously, our findings of increases in nonspecific symptoms might be a different manifestation of the general cortisol stress response to DE exposure reported by Crüts and colleagues (2008).

Blood Coagulation Factors Our failure to find significant DE-related changes in blood coagulation factors appears to be consistent with findings elsewhere.

Carlsten and colleagues (2007) exposed healthy young adults to FA and DE at concentrations of 100 or 200 µg/m³ DEP for 2 hours while resting. They evaluated changes in vWF, D-dimer, plasminogen activator inhibitor-1, C-reactive protein (CRP), and platelet count at 3, 6, and 22 hours after exposure. Compared with FA control conditions, they found no significant changes in any of these endpoints that could be attributed to DE exposure and concluded that their data do not support a DE-induced pro-thrombotic phenomenon.

Similarly, Blomberg and colleagues (2005) exposed older adult exsmokers with mild to moderate COPD to FA and DE with a concentration of 300 µg/m³ DEP for 1 hour with intermittent exercise. Their measurements included fibrinogen, CRP, vWF, D-dimer, prothrombin fragment 1 + 2, and clara cell protein at 6 and 24 hours after exposure; they found no significant changes attributable to DE exposure.

Mills and colleagues (2007) exposed men with stable coronary artery disease and prior myocardial infarction to FA and DE with a concentration of 300 µg/m³ DEP for 1 hour with intermittent exercise. They found no direct effect of DE on platelet count, CRP, tissue plasminogen activator (tPA), or plasminogen activator inhibitor-1. However, they observed increased myocardial ischemia (ECG S-T segment depression) during DE exposure, as well as a potentially pro-thrombotic effect 6 hours after exposure (a reduction in acute release of tPA after an intra-arterial infusion of bradykinin).

IMPLICATIONS OF THE FINDINGS

To our knowledge, no other controlled-exposure human study has tested the combined effects of allergen challenge and DE exposure. Several other studies with reasonably similar protocols and sample sizes assessed direct inflammatory responses to DE exposure and have reported small significant effects at DEP concentrations 2 to 3 times higher than ours and at inhaled doses (estimated as the product of concentration, exposure duration, and average ventilation) roughly 1.5 to 2 times higher than ours (Riedl and Diaz-Sanchez 2005). Thus, it is reasonable to assume that our DE atmospheres were below, but close to, a threshold or lowest-observable-effect level that would elicit direct inflammatory effects of DE.

Our results do not suggest that DE markedly exacerbates lower respiratory immunologic or clinical responses to inhaled allergens, but they also do not rule out that possibility in studies with more severe DE exposures or with more susceptible individuals.

ACKNOWLEDGMENTS

Trudy Webb, Marisela Avila, Lisa Valencia, and Kimberly Hudson of the Environmental Health Service, Los Amigos Research and Education Institute, performed subject recruitment, screening, clinical testing, and safety monitoring during exposures. Yaga Szlachcic, M.D. (now Chief, Department of Medicine, Rancho Los Amigos National Rehabilitation Center; and Associate Professor of Cardiology, Keck School of Medicine, University of Southern California), served as Chief of Environmental Health Service after the death of Dr. Henry Gong. Tony Taliaferro, Aniket Sawant, Bethany Warren, Quentin Malloy, and Kathalena Cocker of CE-CERT performed diesel exhaust generation and characterization. Hema Shah and Minna Jyrala of UCLA performed immunologic assays of blood and sputum. Maria Costantini, Ph.D., of the Health Effects Institute, served as Project Manager.

REFERENCES

- American Thoracic Society. 1999. Recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide in adults and children—1999. *Am J Respir Crit Care Med* 160:2104–2117.
- American Thoracic Society. 2005. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide—2005. *Am J Respir Crit Care Med* 171:912–930.
- Barck C, Lundahl J, Halldén G, Bylin G. 2005. Brief exposures to NO₂ augment the allergic inflammation in asthmatics. *Environ Res* 97(1):58–66.
- Barck C, Sandström T, Lundahl J, Halldén G, Svartengren M, Strand V, Rak S, Bylin G. 2002. Ambient level of NO₂ augments the inflammatory response to inhaled allergen in asthmatics. *Respir Med* 96:907–917.
- Bastain TM, Gilliland FD, Li YF, Saxon A, Diaz-Sanchez D. 2003. Intraindividual reproducibility of nasal allergic responses to diesel exhaust particles indicates a susceptible phenotype. *Clin Immunol* 109:130–136.
- Behndig AF, Mudway IS, Brown JL, Stenfors N, Helleday R, Duggan ST, Wilson SJ, Boman C, Cassee FR, Frew AJ, Kelly FJ, Sandström T, Blomberg A. 2006. Airway antioxidant and inflammatory responses to diesel exhaust exposure in healthy humans. *Eur Respir J* 27:359–365.
- Bing D, He X. 2010. Linear Mixed Models in Clinical Trials using PROC MIXED. Paper No. SP-07. Presented at Pharmaceutical Industry SAS Users Group Conference, 2010, Orlando, FL. Available from www.lexjansen.com/pharmasug/2010/sp/sp07.pdf. Last accessed October 24, 2011.
- Blomberg A, Krishna MT, Bocchino V, Biscione GL, Shute JK, Kelly FJ, Frew AJ, Holgate ST, Sandström T. 1997. The inflammatory effects of 2 ppm NO₂ on the airways of healthy subjects. *Am J Respir Crit Care Med* 156(2 Pt 1):418–424.
- Blomberg A, Törnqvist H, Desmyter L, Deneys V, Hermans C. 2005. Exposure to diesel exhaust nanoparticles does not induce blood hypercoagulability in an at-risk population. *J Thromb Haemost* 3:2103–2105.
- Brauer M, Hoek G, Van Vliet P, Meliefste K, Fischer PH, Wijga A, Koopman LP, Neijens HJ, Gerritsen J, Kerkhof M, Heinrich J, Bellander T, Brunekreef B. 2002. Air pollution from traffic and the development of respiratory infections and asthmatic and allergic symptoms in children. *Am J Respir Crit Care Med* 166:1092–1098.
- Carlsten C, Kaufman JD, Peretz A, Trenga CA, Sheppard L, Sullivan JH. 2007. Coagulation markers in healthy human subjects exposed to diesel exhaust. *Thromb Res* 120:849–855.
- Crüts B, van Etten L, Törnqvist H, Blomberg A, Sandström T, Mills NL, Borm PJA. 2008. Exposure to diesel exhaust induces changes in EEG in human volunteers. *Part Fibre Toxicol* 5:4.
- Diaz-Sanchez D, Dotson AR, Takenaka H, Saxon A. 1994. Diesel exhaust particles induce local IgE production in vivo and alter the pattern of IgE messenger RNA isoforms. *J Clin Invest* 94:1417–1425.
- Diaz-Sanchez D, Garcia MP, Wang M, Jyrala M, Saxon A. 1999. Nasal challenge with diesel exhaust particles can induce sensitization to a neoallergen in the human mucosa. *J Allergy Clin Immunol* 104:1183–1188.
- Diaz-Sanchez D, Jyrala M, Ng D, Nel A, Saxon A. 2000. In vivo nasal challenge with diesel exhaust particles enhances expression of the CC chemokines RANTES, MIP-1α, and MCP-3 in humans. *Clin Immunol* 97:140–145.

- Diaz-Sanchez D, Penichet-Garcia M, Saxon A. 2000. Diesel exhaust particles directly induce activated mast cells to degranulate and increase histamine levels and symptom severity. *J Allergy Clin Immunol* 106:1140–1146.
- Diaz-Sanchez D, Tsien A, Casillas A, Dotson AR, Saxon A. 1996. Enhanced nasal cytokine production in human beings after in vivo challenge with diesel exhaust particles. *J Allergy Clin Immunol* 98:114–123.
- Diaz-Sanchez D, Tsien A, Fleming J, Saxon A. 1997. Combined diesel exhaust particulate and ragweed allergen challenge markedly enhances human in vivo nasal ragweed-specific IgE and skews cytokine production to a T helper cell 2-type pattern. *J Immunol* 158:2406–2413.
- Durbin TD, Zhu X, Norbeck JM. 2003. The effects of diesel particulate filters and a low-aromatic, low-sulfur diesel fuel on emissions for medium-duty diesel trucks. *Atmos Environ* 37:2105–2116.
- Fahy JV, Wong HH, Liu JT, Boushey HA. 1995. Analysis of induced sputum after air and ozone exposures in healthy subjects. *Environ Res* 70:77–83.
- Fujieda S, Diaz-Sanchez D, Saxon A. 1998. Combined nasal challenge with diesel exhaust particles and allergen induces in vivo IgE isotype switching. *Am J Respir Cell Mol Biol* 19(3):507–512.
- Fung K. 1990. Particle carbon speciation by MnO₂ oxidation. *Aerosol Sci Technol* 12:122–127.
- Gong H. 2002. Phase I Report to the Health Effects Institute for Phase 1, February 7, 2003: Generating Diesel Exhaust for Human Exposure. Prepared by Cocker DR III and Miller JW, Bourns College of Engineering–Center for Environmental Research and Technology, University of California–Riverside, Riverside, CA.
- Gong H, Sioutas C, Linn WS. 2003. Controlled Exposures of Healthy and Asthmatic Volunteers to Concentrated Ambient Particles in Metropolitan Los Angeles. Research Report 118. Health Effects Institute, Boston, MA.
- Harkema JR, Wagner JG, Kaminski NE, Morishita M, Keeler GJ, McDonald JD, Barrett EG. 2009. Effects of Concentrated Ambient Particles and Diesel Engine Exhaust on Allergic Airway Disease in Brown Norway Rats. Research Report 145. Health Effects Institute, Boston, MA.
- Inoue K, Koike E, Yanagisawa R, Takano H. 2008. Impact of diesel exhaust particles on th2 response in the lung in asthmatic mice. *J Clin Biochem Nutr* 43:199–200.
- Li N, Hao M, Phalen RF, Hinds WC, Nel AE. 2003. Particulate air pollutants and asthma: A paradigm for the role of oxidative stress in PM-induced adverse health effects. *Clin Immunol* 109:250–265.
- Lin S, Munsie JP, Hwang SA, Fitzgerald E, Cayo MR. 2002. Childhood asthma hospitalization and residential exposure to state route traffic. *Environ Res* 88:73–81.
- Mills NL, Törnqvist H, Gonzalez MC, Vink E, Robinson SD, Söderberg S, Boon NA, Donaldson D, Sandström T, Blomberg A, Newby DE. 2007. Ischemic and thrombotic effects of dilute diesel-exhaust inhalation in men with coronary heart disease. *N Engl J Med* 357:1075–1082.
- Nikasinovic L, Momas I, Just J. 2004. A review of experimental studies on diesel exhaust particles and nasal epithelium alterations. *J Toxicol Environ Health B Crit Rev* 7:81–104.
- Nordenhäll C, Pourazar J, Blomberg A, Levin JO, Sandström T, Adelroth E. 2000. Airway inflammation following exposure to diesel exhaust: A study of time kinetics using induced sputum. *Eur Respir J* 15:1046–1051.
- Pandya RJ, Solomon G, Kinner A, Balmes JR. 2002. Diesel exhaust and asthma: Hypotheses and molecular mechanisms of action. *Environ Health Perspect* 110(Suppl 1):103–112.
- Peretz A, Sullivan JH, Leotta DF, Trenga CA, Sands FN, Allen J, Carlsten C, Wilkinson CW, Gill EA, Kaufman JD. 2008. Diesel exhaust inhalation elicits acute vasoconstriction in vivo. *Environ Health Perspect* 116:937–942.
- Riedl MA. 2008. The effect of air pollution on asthma and allergy. *Curr Allergy Asthma Rep* 8:139–146.
- Riedl M, Diaz-Sanchez D. 2005. Biology of diesel exhaust effects on respiratory function. *J Allergy Clin Immunol* 115:221–228.
- Sawant AA, Cocker DR III, Miller JW, Taliaferro T, Diaz-Sanchez D, Linn WS, Clark KW, Gong H Jr. 2008. Generation and characterization of diesel exhaust in a facility for controlled human exposures. *J Air Waste Manag Assoc* 58:829–837.
- Shen D, Lu Z. 2006. Estimate carryover effect in clinical trial crossover designs. Paper No. PO-16. Presented at Pharmaceutical Industry SAS Users Group Conference, 2006, Bonita Springs, FL. Available from www.lexjansen.com/pharmasug/2006/posters/po16.pdf. Last accessed October 24, 2011.

Sicherer SH, Wood RA, Eggleston PA. 1997. Determinants of airway responses to cat allergen: Comparison of environmental challenge to quantitative nasal and bronchial allergen challenge. *J Allergy Clin Immunol* 99:798–805.

Sobus JR, Pleil JD, Madden MC, Funk WE, Hubbard HF, Rappaport SM. 2008. Identification of surrogate measures of diesel exhaust exposure in a controlled chamber study. *Environ Sci Technol* 42:8822–8828.

Stenfors N, Nordenhäll C, Salvi SS, Mudway I, Söderberg M, Blomberg A, Helleday R, Levin JO, Holgate ST, Kelly FJ, Frew AJ, Sandström T. 2004. Different airway inflammatory responses in asthmatic and healthy humans exposed to diesel. *Eur Respir J* 23:82–86.

Utell MJ, Looney RJ. 1995. Environmentally induced asthma (review). *Toxicol Lett* 82-83:47–53.

Venn AJ, Lewis SA, Cooper M, Hubbard R, Britton J. 2001. Living near a main road and the risk of wheezing illness in children. *Am J Respir Crit Care Med* 164:2177–2180.

Viera L, Chen K, Nel A, Lloret MG. 2009. The impact of air pollutants as an adjuvant for allergic sensitization and asthma. *Curr Allergy Asthma Rep* 9:327–333.

Weymer AR, Gong H, Lynness A, Linn WS. 1994. Pre-exposure to ozone does not enhance or produce exercise-induced asthma. *Am J Respir Crit Care Med* 149:1413–1419.

Witten A, Solomon C, Abbritti E, Arjomandi M, Zhai W, Kleinman M, Balmes J. 2005. Effects of nitrogen dioxide on allergic airway responses in subjects with asthma. *J Occup Environ Med* 47:1250–1259.

Zmirou D, Gauvin S, Pin I, Momas I, Sahraoui F, Just J, Le Moulec Y, Brémont F, Cassadou S, Reungoat P, Albertini M, Lauvergne N, Chiron M, Labbé A. 2004. Traffic related air pollution and incidence of childhood asthma: Results of the Vesta case-control study. *J Epidemiol Community Health* 58:18–23.

APPENDIX A. HEI Quality Assurance Statement

The conduct of portions of this study was subjected to independent audit by Mr. David Bush of T&B Systems, Inc. Mr. Bush is an expert in quality assurance for air quality monitoring studies and data management. The audit included reviews of study activities for conformance to the study protocol and reviews of the overall quality of the data. The dates of the audit activities are given below, along with a brief summary of the audit effort and findings.

January 20–22, 2010

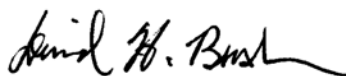
The auditor conducted an on-site audit at the Environmental Health Service at Los Amigos Research and Education Institute (EHS-LAREI) that included a review of the Draft Final Report and the validated project data set the report described. The accuracy and traceability of the data were evaluated by random selection and comparison with original raw data, and several issues identified by the HEI Review Committee were investigated.

In addition, an evaluation of the applicable study laboratories was conducted. An internal independent audit of the laboratories had been conducted previously by Kochy Fung of AtmAA Inc., which had concentrated on analytical procedures and operations at CE-CERT. Review of his audit report revealed only minor issues. Thus, the audit at CE-CERT concentrated on (1) data processing and management with emphasis on quality control data (e.g., blanks, duplicates, and calibrations), and (2) data reduction, validation, and reporting methods. For the UCLA laboratory, which had not been audited earlier for this study, the audit included a review of standard operating procedures used during the assays and of data processing and management. Two issues were mentioned: a problem with how EC and OC data were reported and concern about the use of immunology data across study phases. No other significant issues were noted.

April 2011

The auditor reviewed the Revised Final Report. All recommendations from the audit had been addressed.

A written report of the audit was provided to the HEI project manager, who transmitted the findings to the Principle Investigators. The quality assurance audit demonstrated that the study was conducted by an experienced team with a high concern for data quality.



David H. Bush, Quality Assurance Officer

APPENDICES AVAILABLE ON THE WEB

Appendices B, C, D, and E contain supplemental material not included in the printed report. They are available on the HEI Web site at <http://pubs.healtheffects.org>.

Appendix B. Exposure Atmosphere Monitoring: Details of Methodology and Results

Appendix C. Summary Statistics, Phases 1 and 2

Appendix D. Individual Data

Appendix E. Generation and Characterization of Diesel Exhaust in a Facility for Controlled Human Exposures (Sawant et al. 2008)

ABOUT THE AUTHORS

This project was initiated in 2004 by David Diaz-Sanchez, Ph.D., as principal investigator for immunology (Associate Professor, Clinical Immunology and Allergy, David Geffen School of Medicine, UCLA), in collaboration with Henry Gong, Jr., M.D., as principal investigator for respiratory medicine (Chief of the Environmental Health Service at Los Amigos Research and Education Institute, Downey, California; Professor of Medicine and Preventive Medicine at Keck School of Medicine, University of Southern California; Professor of Medicine at UCLA; and Medical Member of the California Air Resources Board), and J. Wayne Miller, Ph.D. (Adjunct Professor at CE-CERT, University of California–Riverside) and David R. Cocker, Ph.D. (Associate Professor at CE-CERT) as principal investigators for management of vehicle emissions and exposure atmospheres.

In 2007 two events necessitated reorganization of the project: Dr. Gong died, and Dr. Diaz-Sanchez was appointed Chief of the Clinical Research Branch, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina. Marc Riedl, M.D., M.S. (Assistant Professor, Clinical Immunology and Allergy at UCLA), took over Dr. Diaz-Sanchez's role as principal investigator for immunology. Project management was shifted from UCLA to EHS-LAREI: William S. Linn, M.A. (Senior Project Scientist at EHS-LAREI; and Clinical Associate Professor of Preventive Medicine at USC), took over scientific management; and Kenneth W. Clark, M.S. (Research Administrator), took over administrative management. Richard M. Effros, M.D. (Clinical Professor, Internal Medicine, UCLA; and Emeritus Professor, Medical College of Wisconsin), assumed Dr. Gong's role as principal investigator for respiratory medicine. Kiros T. Berhane, Ph.D. (Associate Professor of Biostatistics, Department of

Preventive Medicine, USC), served the project from the outset as statistical consultant and reviewer.

OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH

Sawant AA, Cocker DR III, Miller JW, Taliaferro T, Diaz-Sanchez D, Linn WS, Clark KW, Gong H Jr. 2008. Generation and characterization of diesel exhaust in a facility for controlled human exposures. *J Air Waste Manag Assoc* 58:829–837.

ABBREVIATIONS AND OTHER TERMS

ACES	Advanced Collaborative Emissions Study
BAL	bronchoalveolar lavage
BAU	bioequivalent allergen units
BDL	below detection limit
CE-CERT	College of Engineering, Center for Environmental Research and Technology
CO	carbon monoxide
CO ₂	carbon dioxide
COHb	carboxyhemoglobin
CRP	C-reactive protein
DE	diesel exhaust
DEP	diesel exhaust particles
EC	elemental carbon
ECG	electrocardiogram
ECP	eosinophil cationic protein
EHS-LAREI	Environmental Health Service at Los Amigos Research and Education Institute
ELISA	enzyme-linked immunosorbent assay
FA	filtered air
FENO	fractional exhaled nitric oxide
FEV ₁	forced expiratory volume in 1 second
FVC	forced vital capacity
GM-CSF	granulocyte-macrophage colony-stimulating factor
ICAM	intercellular adhesion molecule
IFN- γ	interferon- γ
Ig	immunoglobulin
IL	interleukin
IR	Investigators' Report
MOUDI	micro-orifice uniform-deposit impactor

NO	nitric oxide	RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted protein
NO ₂	nitrogen dioxide	Raw	airway resistance
NO _x	oxides of nitrogen	SaO ₂	arterial oxygen saturation
OC	organic carbon	SMPS	scanning mobility particle sizer
PAHs	polyaromatic hydrocarbons	sRaw	specific airway resistance
PBS	phosphate-buffered saline	TNF- α	tumor necrosis factor- α
PC20	provocative concentration of methacholine to produce a 20% drop in FEV ₁	tPA	tissue plasminogen activator
PM	particulate matter	U.S. EPA	U.S. Environmental Protection Agency
PMNs	polymorphonuclear leukocytes	UCLA	University of California, Los Angeles
		vWF	vonWillebrand factor

Research Report 165, *Allergic Inflammation in the Human Lower Respiratory Tract Affected by Exposure to Diesel Exhaust*, Marc A. Riedl et al.

INTRODUCTION

Diesel engine exhaust (DE*) is an important component of urban air pollution. Epidemiologic and experimental studies published in the 1990s suggested that people exposed to DE and other particles on a short-term basis may experience adverse effects on their respiratory and immune systems; these effects could be more severe in persons with asthma and other allergic diseases. Although increasingly stringent emission standards and advances in engine technology have resulted in decreasing particulate matter (PM) and gaseous emissions from modern diesel engines, older engines in the current fleet are still significant contributors to traffic-derived ambient PM. The PM, nitrogen oxides (NO_x), and other toxic compounds generated by these older engines remain a public health concern.

Some of the studies from the 1990s raised the question of whether various constituents of vehicle exhaust — including diesel exhaust particles (DEP) — might contribute to an increase in symptoms of asthma or rhinitis or might enhance immune responses to allergens. In reviewing the literature, however, the HEI Health Research Committee noted that many of the experimental studies did not use relevant routes of exposure and particle concentrations, and the epidemiologic studies did not measure ambient concentrations of individual pollutants.

On the Committee's recommendation, HEI issued Request for Application 00-1, *Effects of Diesel Exhaust and Other Particles on Asthma and Other Allergic Diseases*, soliciting studies to examine whether exposure to DEP is associated with alterations in airway inflammation, asthma symptoms, or other allergic responses. Dr. David Diaz-Sanchez, then at the University of California–Los Angeles (UCLA), and

colleagues proposed to evaluate the effects of inhaled aerosolized DEP on the lower airways and in the blood of allergic asthmatic subjects as well as healthy, non-asthmatic subjects. They hypothesized that exposure to DEP would enhance inflammatory or allergic responses, or both.

The HEI Research Committee considered the proposed study to be a logical follow-up to the principal investigator's previous studies (discussed in more detail in the Scientific Background section of this Commentary) but recommended several changes in the study design — specifically, to evaluate the effects of inhalation of whole DE, rather than only DEP, at a realistic exposure level. After discussions with the Committee, Dr. Diaz-Sanchez revised his application to study whole DE. He also proposed to conduct a pilot study to set up a diesel engine and human exposure facility (described in more detail in the Pilot Study and Changes to the Main Study section). The Committee recommended the study for funding, believing that it could be important in understanding the effects of short-term exposure to DE at a realistic concentration and route of exposure in allergic asthmatic individuals. As described in the Technical Evaluation of the Study section of this Commentary, the design of the study changed again after the completion of the pilot study.

This Commentary is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the study and by placing the Investigators' Report (IR) into a scientific and regulatory perspective.

SCIENTIFIC BACKGROUND

ASTHMA AND THE ALLERGIC RESPONSE

Asthma is a chronic obstructive disease of the lungs that is characterized by airway inflammation, mucus hypersecretion, and airway hyperreactivity. Asthma is a heterogeneous disease, but one of the major forms is known as allergic asthma because it can be triggered by an aeroallergen, such as pollen, which results in symptoms that include episodic wheezing, breathlessness, and cough. Stimuli including air pollutants and temperature fluctuations can exacerbate asthmatic responses.

Dr. Riedl's study, "Exacerbation of Allergic Inflammation in the Lower Respiratory Tract by Diesel Exhaust Particles," began in October 2003. Total expenditures were \$1,140,470. The draft Investigators' Report from Riedl and colleagues was received for review in September 2009. A revised report, received in April 2010, was accepted for publication in June 2010. 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 the Review Committee's Commentary.

This document has not been reviewed by public or private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views of these parties, and no endorsements by them should be inferred.

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

The response to an aeroallergen in the airways is generally characterized by the production of immunoglobulin E (IgE), which binds to mast cells; high levels of allergen-specific IgE are often found in people with allergy. The products released by mast cells after binding to IgE are responsible for many of the early phenomena associated with the allergic response, including bronchoconstriction. In humans, IgE and IgG4 (a subtype of IgG) are synthesized by B lymphocytes under the influence of the cytokines interleukin (IL)-4 and IL-13, which are produced by the Th2 subset of CD4⁺ T lymphocytes. Th2 cells also synthesize IL-5, which activates eosinophils — another important type of cell in the allergic response.

Clinical manifestations of the allergic response to an airborne allergen — and hence many asthmatic responses — occur both early (within minutes) and late (within 4 to 8 hours) after exposure. The early phase, mediated by products of activated mast cells in the airways, is characterized by bronchoconstriction and a rapid decline in lung function; the late phase, mediated predominantly by products released by eosinophils and Th2 cells recruited into the airways, is characterized by mucus production and decline in lung function. The changes associated with the late-phase reactions can persist; and repeated exposure to allergens to which a person with asthma is sensitized is thought to result in the development of long-lasting changes in airway structure and function.

COMPONENTS AND HEALTH EFFECTS OF DE

Components

DE is a complex mixture of gaseous and particulate components. The major gases emitted include NO_x (primarily nitric oxide and nitrogen dioxide [NO₂]), carbon dioxide, sulfur compounds, and low-molecular-weight hydrocarbons. DEP generated by the 1999 engine used in the current study and engines of a similar age are composed of a carbon core (predominantly organic carbon [OC]) to which nitrate, sulfate, metals, and organic compounds adhere.

Traffic-Derived Pollution

At the time Request for Applications 00-1 was issued, some epidemiologic studies had found associations between exposure to traffic-related air pollution and enhancement of respiratory conditions — specifically, exacerbation of asthma symptoms and allergic rhinitis (Wjst et al. 1993; Edwards et al. 1994; Weiland et al. 1994; van Vliet et al. 1997). Furthermore, one study had found that children living less than 300 m from a major roadway with heavy truck traffic (an indicator of DE exposure) experienced a greater decrease in lung function than

children living near roadways with primarily automobile traffic (Brunekreef et al. 1997).

In addition to these epidemiologic studies, several studies had evaluated more directly the effects in humans and rodents of exposure to DE and its particulate and gaseous (NO₂) constituents.

Health Effects

Controlled Human Exposure to DE Before the current study got under way, several controlled exposure studies had investigated the effects of short-term exposure (1 to 2 hours) to DE, usually at a PM mass concentration of 300 µg/m³. These studies, summarized in Commentary Table 1, focused on pulmonary function as well as immunologic and inflammatory endpoints in healthy and asthmatic individuals. It is challenging to compare findings across the studies, however, since measurements were made at different time points and in different parts of the airways (e.g., in bronchoalveolar lavage [BAL] fluid and in a biopsy of bronchial tissue). Nonetheless some, but not all of these studies showed small effects on pulmonary function from acute exposure to DE; and all the studies showed some changes in inflammatory markers, although the specific markers that were affected were not consistent from study to study.

Controlled Human Exposure to NO₂ As described in the Technical Evaluation of the Study section of this Commentary, the current study also incorporated an evaluation of the effects of exposure to NO₂, an important component of engine emissions. Before this study, exposure of healthy adults to ≤ 2 ppm NO₂ had found airway inflammatory effects — such as increased numbers of polymorphonuclear cells (PMNs) in BAL fluid — within hours after exposure (e.g., Solomon et al. 2000; Frampton et al. 2002; reviewed in U.S. Environmental Protection Agency [U.S. EPA] 2008). The NO₂ concentrations at which these inflammatory effects were seen were higher than typical ambient concentrations and somewhat higher than the NO₂ concentration evaluated in the current study (0.35 ppm).

Several studies had also assessed the effects of NO₂ exposure in people with asthma who were challenged with allergen. Some studies, but not all, had shown that a short single exposure of individuals with mild asthma to NO₂, at concentrations similar to those used in the current study, could increase both airway responsiveness to allergen and the concentrations of markers of the inflammatory response. For example, Tunnicliffe and colleagues (1994) showed that exposing individuals with mild asthma for 1 hour to 0.1 and 0.4 ppm NO₂ while at rest slightly increased both the early- and late-phase responses

to inhalation of a fixed dose of allergen. In addition, in one of several similar studies conducted in Sweden, Barck and colleagues (2002) showed that exposing mildly atopic individuals with asthma for 30 minutes to 0.26 ppm NO₂, with allergen challenge 4 hours later, resulted in increased numbers of PMNs and levels of eosinophil cationic protein in BAL fluid 19 hours later.

Controlled Rodent and Human Exposures to DEP Several studies have focused on the effects of DEP in the absence of gaseous components. Early studies with rodents suggested that DEP could act as an adjuvant — that is, an enhancer of the antigen-specific antibody response — when administered either intraperitoneally or intranasally at the same time as an allergen such as ovalbumin or Japanese cedar pollen (Muranaka et al. 1986; Takafuji et al. 1987, 1989). In addition, these and subsequent studies with mice showed that co-administration of DEP and allergen specifically enhanced the allergic-type IgE response — rather than other types of immune responses — and enhanced the production of the Th2-associated cytokine IL-4 (Fujimaki et al. 1997; Takano et al. 1997).

In a series of papers published before the current study started, Diaz-Sanchez and colleagues confirmed and extended to humans the effects of intranasal administration of DEP that had been described in rodents (Diaz-Sanchez et al. 1994, 1997, 1999, 2000a,b). As described in more detail in the Health Review Committee's Evaluation of the Study section, the Diaz-Sanchez team worked with at least two samples of DEP that were considered to be similar to each other because they had been collected from the exhaust of light-duty diesel engines in Japan. Intranasal spraying of 300 µg DEP into healthy and mildly allergic human volunteers increased (1) levels of total IgE4 in the nose days later, (2) production of IgE messenger RNA (the mRNA synthesized by the gene coding for the IgE heavy chain), and (3) numbers of IgE-secreting B cells (Diaz-Sanchez et al. 1994). In another study, 300 µg DEP was administered to atopic individuals 24 hours before they were primed and subsequently challenged with the antigen keyhole limpet hemocyanin; the atopic group developed an IgE response that was not detected in the control group, to whom DEP had not been administered (Diaz-Sanchez et al. 1999). This indicated that DEP enhanced primary sensitization, which is the first response of lymphocytes exposed to antigen.

Subsequently the investigators found that intranasal administration of 300 µg DEP into healthy and mildly allergic individuals elevated levels of several markers of inflammation in nasal wash fluid 6 and 24 hours later — increases in total cell counts, lymphocytes, monocyte-macrophages, and neutrophils (but not eosinophils), as

well as increases in levels of eosinophil cationic protein and the chemokines RANTES, MIP-1α, and MCP-3 (but not eotaxin), which are associated with the recruitment of inflammatory cells into tissue (Diaz-Sanchez et al. 2000a).

Of particular relevance for the current study, Diaz-Sanchez and colleagues also reported that DEP administration enhanced several features of the allergic response in allergen-sensitive individuals (Diaz-Sanchez et al. 1997, 2000b). Because individuals in the study were known to have been already sensitized to allergen, DEP exposure thus enhanced a secondary or memory response to the same antigen. In one study, compared with the effects of exposure to allergen alone, the combination of 300 µg DEP and ragweed in ragweed-sensitive individuals increased nasal-wash levels of ragweed-specific IgE (18 hours and 4 days later) and IgG4 (4 and 8 days later), and enhanced the synthesis of mRNAs of the Th2-associated cytokines IL-4, IL-5, and IL-13 (18 hours later) (Diaz-Sanchez et al. 1997). In a second study with participants who were sensitive to house dust-mite, the investigators found that administration of 300 µg DEP immediately before allergen exposure increased nasal levels of histamine (an early phase of the allergic response) and the severity of symptoms (Diaz-Sanchez et al. 2000b). (In an *in vitro* portion of this study, an organic extract of DEP also induced degranulation of a mouse mast cell line.)

Thus, at the time the current study was funded, short-term controlled intranasal exposure of healthy and allergic human volunteers to DEP had been shown to enhance effects associated with allergic sensitization and response and with inflammation in the upper airways (the nose, specifically). Whether similar responses occurred in the lower airways via inhalation, or systemically, had not been addressed; the current study sought to address these key issues.

TECHNICAL EVALUATION OF THE STUDY

OBJECTIVES AND SPECIFIC AIMS

The original objective of the study was to characterize and compare the immunologic and inflammatory responses in the lower airways and blood of asthmatic and non-asthmatic volunteers exposed by inhalation to DE. The investigators hypothesized that exposure to DE would enhance inflammatory and immunologic responses. However, as described below in the Study Design section, the objectives changed after the investigators carried out the pilot study.

Commentary Table 1. Earlier Studies of Controlled Short-Term DE Exposure

Study	Number of Subjects (Healthy Unless Noted)	Exposure Duration and DEP Concentration	Key Findings (Compared with Filtered Air Control)
Rudell et al. 1996	12	1-hour exposures with light exercise - DE passed through a particle trap (PM 1.4×10^6 particles/cm³) - Unfiltered DE (PM 2.6×10^6 particles/cm³)	During exposure: prominent symptoms were irritation of the eyes and nose, unpleasant smell At 30 minutes after each exposure: ↑ airway resistance and sRaw (lung function assessed by whole-body plethysmography) No significant differences between filtered and unfiltered DE for symptoms or lung function
Salvi et al. 1999	15	1-hour exposures with intermittent exercise 300 µg/m³ PM	Immediately after exposure: no change in lung function At 6 hours after exposure - In airway lavage: ↑ histamine and fibronectin; ↑ neutrophils and B lymphocytes - In peripheral blood: ↑ neutrophils and platelets - In bronchial tissue: ↑ neutrophils, mast cells, CD4+ and CD8+ T lymphocytes; upregulation of ICAM-1 and VCAM-1; and ↑ LFA-1+ cells

(Table continues next page)

The specific aims of the study were the following:

1. Develop a source of DE that would be practical to operate and would produce emissions with characteristics typical of diesel-powered vehicles in use at the time.
2. Develop a suitable controlled-exposure facility: modify an existing exposure chamber by adding an interface for the exhaust source (a diesel pickup truck), a dilution system, and monitoring instruments able to characterize the exposure atmospheres in detail.
3. Recruit and medically characterize suitable volunteers.
4. Expose each volunteer to DE and control atmospheres, in random order, with time separation to minimize carryover effects, and characterize their immunologic, physiologic, and symptomatic responses.

STUDY DESIGN

Pilot Study and Changes to the Main Study

In the pilot study, Dr. Diaz-Sanchez's collaborators at the College of Engineering–Center for Environmental Research

and Technology (CE-CERT) at the University of California–Riverside and at Los Amigos Research and Education Institute (LAREI; Downey, California) built an exposure facility at LAREI with a human exposure chamber and characterized the DE generated by a medium-duty diesel truck with 60,000 miles purchased for the study. As data became available during the pilot study and were discussed with HEI's Research Committee, the investigators made the following changes to the main study.

Changes to the Groups Exposed

Summary Only asthmatic, rather than both asthmatic and non-asthmatic participants would be exposed.

Reasons for the Change The investigators had originally proposed to compare responses of healthy non-asthmatic and asthmatic volunteers. Because of costs and the time needed to recruit and expose participants, the investigators agreed to first evaluate the safety of the exposure protocol with only four healthy volunteers. Data from this "safety study" are shown in IR Appendices C and D (available on the HEI Web site). The main study would

Commentary Table 1 (Continued). Earlier Studies of Controlled Short-Term DE Exposure

Study	Number of Subjects (Healthy Unless Noted)	Exposure Duration and DEP Concentration	Key Findings (Compared with Filtered Air Control)
Nightingale et al. 2000	10	2-hour exposures at rest • 200 µg/m³ PM₁₀	At 4 hours after exposure • ↑ level of exhaled CO (maximum at 1 hour after exposure) • In sputum: ↑ neutrophils and myeloperoxidase after exposure • No changes in cardiovascular parameters or lung function Immediately and at 24 hours after exposure: No change in concentration of inflammatory markers in peripheral blood
Salvi et al. 2000	15	1-hour exposures with intermittent exercise • 300 µg/m³ PM	At 6 hours after exposure • ↑ IL-8 gene transcription in bronchial tissue and bronchial wash • ↑ IL-8 and GROα protein expression in bronchial epithelium • Trend toward ↑ IL-5 mRNA gene transcripts in bronchial tissue • No significant changes in gene transcript levels of IL-1β, TNF-α, IFN-γ, and GM-CSF in either bronchial tissue or bronchial wash cells
Nordenhäll et al. 2000	15	1-hour exposures • 300 µg/m³ PM	At 6 hours after exposure • ↑ % neutrophils in sputum • ↑ IL-6 and methylhistamine
Nordenhäll et al. 2001	14 non-smoking volunteers with stable atopic asthma	1-hour exposures • 300 µg/m³ PM	Immediately after exposure: ↑ airway resistance At 6 hours after exposure • ↑ sputum levels of IL-6 • No changes detected in sputum levels of methylhistamine, eosinophil cationic protein, myeloperoxidase, or IL-8 At 24 hours after exposure: ↑ hyperresponsiveness

focus on the responses of subjects who were both asthmatic and allergic. This change allowed the investigators to increase the number of allergic asthmatic subjects from 10 per group to 15.

Changes to the Exposures

Summary Each participant would be exposed to one concentration of DEP (100 µg/m³ in DE), one concentration of NO₂ (0.35 ppm), and filtered air (control) for 2 hours.

Reasons for the Changes The investigators had proposed to expose volunteers for 1 hour via inhalation to DE at a low and a high concentration of DEP, targeted at 20-40

and 200 µg/m³, respectively. After discussions with HEI's Research Committee, and considerations of ethical issues surrounding the use of the higher exposure level, the investigators agreed to a single exposure to 100-µg/m³ DEP for 2 hours, which was considered more environmentally relevant and was comparable to the target diesel concentration in studies that were being conducted by the U.S. EPA and at the University of North Carolina (Sawyer et al. 2008; this latter study also used a 2-hour exposure).

In addition, the investigators also agreed that it was important to evaluate the effects of NO₂ by itself because it is a major gaseous component of DE and has the potential to affect the same health endpoints as whole DE. Thus, the

results of the NO₂-only exposure would help provide information as to whether the effects of DE were due to the particle or the gaseous phase. The NO₂ concentration selected for evaluation, 0.35 ppm, was that measured in the exposure chamber when the exhaust was diluted to approximately 100 µg/m³ DEP.

DESIGN OF THE MAIN STUDY

Building on the results of the Pilot Study, the main study was conducted in two phases. In Phase 1, 15 asthmatic individuals, who were also responsive to one or more allergens, were exposed once each in random order to DE with 100 µg/m³ PM, 0.35 ppm NO₂ alone, and filtered air (control), with no allergen challenge after each exposure. Thus, the goal of Phase 1 was to evaluate the effects of DE and NO₂ on airway and systemic inflammation *in the absence of an allergen challenge*.

In Phase 2, 15 asthmatic individuals, who were also responsive to cat allergen (see Study Population section below), were exposed to the same pollutant atmospheres as in Phase 1, but were challenged 1 hour after exposure with an extract of cat hair. Thus, the goal of Phase 2 was to evaluate the effects of DE and NO₂ on airway and systemic responses — particularly allergic-type responses — *after a cat-allergen challenge*.

In both phases, each study participant was exposed in the exposure chamber while intermittently exercising for 2 hours on a stationary bicycle (15 minutes in every 30). To minimize carry-over effects, exposure sessions were separated by at least 4 weeks. The investigators measured several endpoints associated with inflammatory and immunologic responses in the airways and blood.

In the course of the main study, Dr. Diaz-Sanchez moved to the U.S. EPA, ending his association with the project; and Dr. Gong, who had been leading the study at LAREI, died. Ultimately, Dr. Marc Riedl at UCLA assumed the role of principal investigator.

GENERATION AND CHARACTERIZATION OF ATMOSPHERES

The investigators provide a description of the exposure facility and the characterization of the DE components in the body of the IR, as well as comprehensive details in “Appendix B. Exposure Atmosphere Monitoring” and “Appendix E. Generation and Characterization of Diesel Exhaust in a Facility for Controlled Human Exposures” (Sawant et al. 2008). Both appendices are available on the HEI Web site.

In brief, the DE source was an idling (900 rpm) 250-hp Navistar International T 444E 7.27-liter turbocharged V-8 engine in a used 1999 medium-duty Ford diesel pickup

truck with approximately 60,000 miles, equipped with a 3-way catalyst when purchased, and using ultra-low-sulfur diesel fuel. The truck's emissions were initially characterized on a Federal Test Procedure cycle with and without the catalyst. With the catalyst, PM emissions were about 25% to 30% lower than without the catalyst and the NO_x emissions were higher. For this study, the investigators decided to remove the 3-way catalyst and generate and evaluate raw DE. The raw exhaust was then characterized chemically and physically, as reported in the IR and by Sawant and colleagues (2008).

The exhaust from the tailpipe was delivered to the chamber through a tunnel in which it was diluted with filtered air to the target concentration of 100 µg/m³ DEP. The tunnel passed through a port in the exterior wall of the laboratory.

The emissions were evaluated for PM mass and number and size distribution using a scanning mobility particle sizer (SMPS). The SMPS was used to monitor the particle concentration continuously during the exposure period. The gaseous components characterized included polycyclic aromatic hydrocarbons, carbonyl compounds, light hydrocarbons, elemental carbon (EC), OC, NO_x, and carbon monoxide (CO).

For NO₂ exposures, NO₂ was supplied using a conventional low-flow metering system and gas cylinder containing 5% NO₂ in nitrogen.

STUDY POPULATION

For Phase 1 the investigators recruited 15 asthmatic subjects — diagnosed as having either mild-intermittent or mild-persistent asthma — who were also allergic to at least one of a panel of common aeroallergens. All were non-smokers between the ages of 18 and 50. Further information regarding volunteer recruitment, asthma diagnosis, and inclusion and exclusion criteria can be found in the Study Design and Methods section of the IR. A medical screening examination was performed before acceptance into the study. The examination included pulmonary function testing, a resting electrocardiogram, and allergen skin testing (using a panel of aeroallergens that included house dust-mite, cat allergen, tree pollen mix, and grass pollen mix). Non-specific bronchial reactivity was tested by challenge with increasing concentrations of methacholine.

For Phase 2, the investigators recruited 15 asthmatic subjects who were also allergic to cat hair, as established by skin testing. The criteria for asthma diagnosis, age, and non-smoking status were the same as those applied in Phase 1. Three individuals from Phase 1 were found to have cat allergy and participated in Phase 2 of the study together with 12 additional volunteers. In the screening visit, individuals found to be sensitive to cat allergen were

exposed to increasing concentrations of aerosolized cat allergen solution to establish the dose of allergen to be used after the three exposures. The concentration that reduced forced expiratory volume in 1 second (FEV₁) by more than 15% during this screening procedure was diluted by a factor of five and used for the Phase 2 challenges. Subjects were allowed to take their own bronchodilator medications as needed; any such usage within 2 weeks of an exposure was recorded. No subject used corticosteroids.

HEALTH ENDPOINT MEASUREMENTS

Commentary Table 2, based on Table 2 in the IR, summarizes the categories and some specific endpoints measured at different times before, during, and after the exposures. (The complete results for immunologic and inflammatory endpoints are given later in Commentary Table 3.) Physiologic and pulmonary function endpoints included FEV₁, forced vital capacity (FVC), specific airway resistance (sRaw), bronchial reactivity, and arterial oxygen saturation.

Commentary Table 2. Timing of Health Endpoint Measurements

Category	Endpoint	Timing (– = before start of exposure, + = after end of exposure)
Spirometry	FEV ₁ and FVC	Screening, –30 minutes, end of exposure, +1.5 hours (with challenge), +2 hours, +22.75 hours, +23 hours (after sputum induction)
Body plethysmography	sRaw	–30 minutes, end of exposure, +22.75 hours
Minute ventilation	$\dot{V}_E$	During the end of the last rest and exercise periods
Blood (serum)	Cell numbers and subsets, protein mediators — cytokines, immunoglobulins, and coagulation factors (see Commentary Table 3 for detailed list)	–40 minutes, +22.5 hours
Sputum	Some endpoints were also measured in serum (see Commentary Table 3 for detailed list)	Baseline (at least 2 weeks distant from any exposure), +23 hours
Symptoms	Total and subtotals of respiratory, cardiovascular, and miscellaneous (non-specific)	Symptom score sheet updated at –90 minutes, every 15 minutes during exposure, +1 hour, +2 hours, overnight, +22.75 hours
ECG at rest, vital signs, SaO ₂	Heart rate, blood pressure, respiratory rate	ECG: –90 minutes, +22.75 hours Vital signs: –90 minutes, end of exposure, +22 hours SaO ₂ : every 15 minutes during exposure, end of exposure
Cardiopulmonary physical exam		–90 minutes, +22 hours
Bronchial reactivity	Phase 1. PC ₂₀ and % change in FEV ₁ after the highest methacholine challenge Phase 2. % change in FEV ₁ after the highest cat allergen challenge	+90 minutes
Exhaled NO	FENO	–40 minutes, end of exposure, +2 hours, +22.75 hours
Exhaled CO		–40 minutes, end of exposure, +2 hours, +22.75 hours

Bronchial Reactivity

In Phase 1, all subjects were required to have non-specific airway hyperresponsiveness, defined as a $PC_{20} \leq 8$ mg/mL methacholine (the provocative concentration of methacholine necessary to reduce FEV_1 by 20% compared with a control challenge with saline). However, on repeat bronchial challenges during the study, some subjects did not consistently demonstrate airway hyperresponsiveness and a PC_{20} could not be calculated. For 5 of the 15 participants the airway response to methacholine was highly variable.

To maximize the chance to detect changes in bronchial reactivity, the investigators calculated three separate indices in Phase 1: (1) the percent change in FEV_1 after the control (saline) challenge (just before methacholine challenges began) compared with the pre-challenge measurement; (2) if the maximum methacholine concentration did not reduce FEV_1 by 20%, an estimate of PC_{20} was calculated either by linear interpolation between the closest doses above and below the 20% response level, or by linear extrapolation from the two highest points on an FEV_1 -vs.-concentration plot; and (3) each subject's bronchoconstrictive responses were ranked across atmospheres from 1 to 3 (most to least unfavorable) and a Friedman rank-sum analysis was used to estimate differences in bronchial responsiveness between exposure atmospheres.

In Phase 2, bronchial reactivity was assessed as the percent change in FEV_1 after challenge with a single concentration of cat allergen equal to 20% of the dose that had decreased the FEV_1 by 15% at screening.

STATISTICAL METHODS

Data from Phases 1 and 2 were analyzed separately. Most results were expressed as the percent change in an endpoint between pre-exposure and post-exposure, except for the results of sputum induction and bronchoconstrictor challenges; these were performed only after each exposure and were compared only across the three exposure atmospheres.

The investigators used a mixed-model analysis to test for significant differences in response variables across exposures within each subject. Highly skewed data were log-transformed before analysis. This analytic approach is commonly used in repeated-measures controlled-exposure studies to analyze differences in the response to each exposure for each individual. They used two models: First, for all outcomes they used an unadjusted model — referred to as the conventional model — that treated the atmosphere as a fixed factor and the subject as a random factor. This analysis provided an atmosphere effect; that is, a between-atmospheres within-subject contrast. Second, for some key

response variables (such as IL-4, IgE, and cell numbers) the investigators also fit an adjusted model — referred to as the advanced model — as is commonly employed in drug trials with crossover designs (Jones and Kenward 1989; Tudor and Koch 1994). This richer model added terms for period effects (responses related to a subject's 1st, 2nd, and 3rd exposure periods) and carryover effects (changes in response to the current exposure related to the previous atmosphere).

KEY FINDINGS

Characterization of Components of the Exposure Atmospheres

In general, in both Phases 1 and 2, the concentrations of PM in DE were close to the target concentration of 100 $\mu\text{g}/\text{m}^3$ (see IR Tables 4 and 5) and contained a concentration of NO_2 close to 0.35 ppm. These concentrations were also reasonably consistent from minute to minute within an exposure period, as well as across all exposure periods. In the NO_2 -only exposures, the NO_2 concentrations were consistent with the target of 0.35 ppm.

The particles generated by the diesel engine showed a bimodal distribution (shown in IR Figures 1 and 2): smaller particles (mean aerodynamic diameter approximately 70 nm) dominated particle numbers, and larger particles (mean aerodynamic diameter approximately 250 nm) dominated particle mass. This pattern of both small and large particles remained more or less consistent over the course of the study, with a trend toward an increase in smaller (or less dense particles) as the engine aged (see IR Figure 3). Much larger particles (predominantly of aerodynamic diameter 10 μm and larger) were also detected during the exposures; the investigators determined that these particles were derived from the skin and clothing of participants or from the exercise equipment (Sawant et al. 2008).

CO concentrations ranged from 0.56 to 0.83 ppm in the filtered air and NO_2 exposures, but were higher (approximately 1.7 ppm) in the DE exposures. In all the DE exposures total carbon consistently accounted for 70% to 75% of the particle mass, of which approximately 63% was OC and 30% EC (see IR Table 6). Among the volatile and semivolatile organic compounds characterized in the DE exposures (shown in IR Tables 7 and 8), concentrations across the exposure periods were relatively consistent for some compounds — particularly the more abundant carbonyl compounds formaldehyde and acetaldehyde — but were more variable for others, such as naphthalene and eicosane.

Health Endpoints

Commentary Table 3 shows an overview of all the health endpoint results from Phases 1 and 2. As the investigators note, exposure to DE or NO₂ affected only a few inflammatory, immunologic, or physiologic endpoints. Several outcome measures were below the limit of detection. The changes that were observed are summarized here.

Phase 1 In participants challenged with methacholine, exposure to DE increased sRaw, exhaled CO, IL-4 (in sputum), and miscellaneous symptoms; and it decreased the levels of IgG4 and eotaxin (in sputum) and IgM (in blood).

The increase of approximately 50% in sRaw was significant at 22 hours after exposure, suggesting that DE exposure induced a delayed, clinically mild bronchoconstriction. This finding was based on a limited set of observations, however, because some participants were not able to perform the test satisfactorily. Miscellaneous symptoms (such as headache) increased, but they were not related to respiratory or cardiac effects. The investigators suggest that the increase in exhaled CO after DE exposure is consistent with DE slightly increasing levels of carboxyhemoglobin.

Exposure to NO₂ increased IgM levels in blood as well as miscellaneous symptoms, and decreased oxygen saturation and sputum levels of IgG4, IL-4, eotaxin, RANTES, and fibrinogen.

Phase 2 In the participants challenged with cat allergen, exposure to DE had no effect on measures of airway function including sRaw, which was increased by exposure to DE without allergen challenge in Phase 1. The average changes in FEV₁ after exposure to DE and filtered air were similar (a drop of about 8%), but one individual experienced a decrease of approximately 40% after exposure to DE.

Exposure to DE with allergen challenge also changed the relative proportions of cell types in sputum, which was not observed in Phase 1: PMN numbers increased and macrophage numbers decreased; when analyzed using log-transformed data, eosinophil numbers also increased. These cellular changes in sputum are consistent with a mild inflammatory response. However, they were not accompanied by other typical characteristics of inflammation, such as an increase in total cell number, or changes in levels of cytokines and chemokines or of immunoglobulins. In addition, blood levels of immunoglobulins, cytokines, and inflammatory markers were not affected.

Exposure to NO₂ had few effects: serum factor VII levels decreased, as did the concentration of exhaled CO. Sputum and blood levels of immunoglobulins and cytokines, and blood markers of inflammation, were not affected.

The average change in FEV₁ after exposure to NO₂ was close to zero and thus different from the average changes after DE or filtered air exposures, which are described above. However, the average FEV₁ response to NO₂ appeared to be driven by two individuals who had anomalous increases in FEV₁.

Summary of Phase 1 and Phase 2 Findings and Authors' Interpretations

Most of the immunologic and inflammatory endpoints evaluated were unaffected by exposure to DE or NO₂. Of the changes noted, few occurred in both Phases 1 and 2. The slight increase in airway resistance after DE exposure in Phase 1 and the small increases in sputum PMN and eosinophil numbers in Phase 2 suggested to the investigators that this exposure concentration and duration may be at or near the lowest levels that might cause airway inflammatory effects in this group of volunteers.

Period Effects

After applying the advanced model to data from both Phases 1 and 2, many endpoints, particularly those measured in sputum, showed period effects, which indicate that a pattern of responses depended on whether a participant was in their 1st, 2nd, or 3rd exposure period, rather than on the exposure atmospheres themselves. Such effects have not been reported in other studies. Because samples from all three exposures for each participant were analyzed in a single batch, the investigators ruled out effects due to changes in laboratory technique for analysis of sputum samples over the course of the study. What underlies these effects is not known. The investigators speculate that the participants might have undergone some sort of physiologic or psychological adjustment over the course of the exposures so that they became less stressed at being in the exposure chamber. However, it is not possible to confirm this speculation.

Commentary Table 3. Effects of Exposure to DE and NO₂ (Compared with Filtered Air) on Endpoints in Phases 1 and 2^a

Endpoint	Phase 1		Phase 2	
	DE	NO ₂	DE	NO ₂
Respiratory Physiology				
FVC	=	=	=	=
FEV ₁	= ^b	= ^b	= ^b	= ^b
PC ₂₀	=	=		
sRaw	↑ ^{b, c}	=	=	=
Blood pressure	=	=	=	=
SaO ₂	+ ^d	- ^d	=	=
FENO	=	=	=	=
Exhaled CO	+	=	↑ ^e	↓ ^e
Symptoms				
Total symptom score	↑ ^b	↑ ^b	=	=
Cardiovascular symptoms	=	=	=	=
Respiratory symptoms	=	=	=	=
Miscellaneous symptoms ^f	↑ ^b	↑ ^b	=	=
Sputum^g				
Total cells	=	=	=	=
Macrophages	=	=	↓ ^{b, PE}	= ^{b, PE}
Lymphocytes	=	=	=	=
PMNs	=	=	↑ ^{b, PE}	= ^{PE}
Eosinophils	=	=	+50% ^h	+15% ^h
IgA	=	=	= ^{PE}	= ^{PE}
IgE	=	=	=	=
IgG	=	=	=	=
IgG4	↓ ^b	↓ ^b	=	=
IgM	= ^{PE}	= ^{PE}	=	=
IL-4	↑ ^b	↓ ^b	= ^{PE}	= ^{PE}
IL-5	=	=	= ^{PE}	= ^{PE}
IL-8	=	=	=	=
IL-12	=	=	=	=
IFN-γ	= ⁱ	= ⁱ	=	=
TNF-α	= ⁱ	= ⁱ	= ^{PE}	= ^{PE}
ECP	=	=	=	=
RANTES	- ⁱ	= ⁱ	=	=
Eotaxin	- ⁱ	- ⁱ	=	=
GM-CSF	=	=	=	=
Tryptase	= ⁱ	= ⁱ	No data shown	No data shown

(Table continues next page)

Commentary Table 3 (Continued). Effects of Exposure to DE and NO₂ Compared with Filtered Air on Endpoints in Phases 1 and 2^a

Endpoint	Phase 1		Phase 2	
	DE	NO ₂	DE	NO ₂
Serum^j				
IgA	= PE	= PE	= PE	= PE
IgE	= PE	= PE	= PE	= PE
IgG	=	=	=	=
IgG4	=	=	=	=
IgM	↓ PE	↑ PE	=	=
IL-6	= PE	= PE	=	=
ICAM	= PE	= PE	=	=
Coagulation^k				
Factor VII	=	=	=	=
Fibrinogen	=	=	=	=
vonWillebrand factor	=	=	=	=

^a Summary of data is derived from Phase 1 (Tables 9, 11, 13, 15, 17, Figure 5, and data reported in the text) and Phase 2 (Tables 20, 22, 24, 25, 26, Figure 5, and data reported in the text). For atmosphere effects, arrows up (↑) or down (↓) indicate the direction of statistically significant differences ($P \leq 0.05$); a plus (+) or minus (−) indicates a “close to significant” or “near significant” change as designated by the investigators, with a P value between 0.05 and 0.1; effects with P values > 0.1 are indicated with equal signs (=). PE indicates a significant period effect; that is, a change from 1st to 2nd to 3rd period regardless of the order of atmospheres. Unless otherwise specified, all data were derived from the conventional model.

^b Estimated using the advanced model.

^c Advanced model showed significant differences in Phase 1 at 22 hours after exposure, but not significantly different immediately after exposure (data not shown in IR).

^d In Phase 1, a close-to-significant difference between pre-exposure and day 2 by conventional and advanced models; data not shown in IR.

^e Concentration after a 20-second breath hold; data not shown in IR.

^f Headache, fatigue, stomach upset, eye irritation, other.

^g Sputum analyses in Phases 1 and 2 for cells: no significant differences using either conventional or advanced analyses; all other sputum biochemistry endpoints estimated using the advanced model.

^h Significant when analyzed with log-transformed data, but not when using untransformed data.

ⁱ RANTES, eotaxin, tryptase, TNF- α , and IFN- γ were “often undetectable” in Phase 1.

^j All serum measures are day-2 values minus day-1 pre-exposure values from the advanced model. IgA, IgE, IgM, IL-6, and ICAM showed significant period effects in either Phase 1 or Phase 2.

^k No significant effects for any of the three coagulation endpoints using conventional or advanced models.

THE HEALTH REVIEW COMMITTEE'S EVALUATION OF THE STUDY

GENERAL COMMENTS

The Health Review Committee, which conducted an independent review of the study, thought that the study addressed an important issue: the effects of inhalation exposure to DE and NO₂ (a major component of DE) on the lower airways and blood of allergic asthmatic individuals. The Committee agreed with the authors' conclusions that the study findings were predominantly negative. Inhalation exposure to DE at 100 $\mu\text{g}/\text{m}^3$ PM or to 0.35 ppm NO₂ for

2 hours with intermittent exercise in either Phase 1 or 2 resulted in only a few changes in inflammatory, immunologic, or physiologic status in the lower airways and blood that differed significantly from the results of exposure to filtered air. In addition, the Committee noted that the statistically significant changes detected after exposure to a pollutant atmosphere were generally small, not always in the direction expected for an adverse effect, and of uncertain biologic or clinical significance. They agreed with the investigators that the few changes (airway resistance in Phase 1, small increases in sputum PMN and eosinophil numbers in Phase 2) suggested that this exposure concentration and duration may be near the threshold levels that might cause airway inflammatory effects in mildly asthmatic individuals.

STRENGTHS AND LIMITATIONS OF THE STUDY

The Committee's review of the study highlighted several strong points but also some limitations, which are discussed in detail below.

Strengths

The study had several strong points — most notably, the use of a more realistic concentration and route of exposure to DE than this team had used in their earlier studies. The investigators effectively developed and implemented an exposure system to deliver DE to participants via inhalation with both oral and nasal breathing. Inclusion of intermittent exercise during the exposure period simulated outdoor activity scenarios with increased ventilation rates, which are likely to increase the inhaled amounts of DE. The long interval between exposures (4 weeks) reduced the likelihood of carry-over effects that might result from the previous exposure period or atmosphere. The investigators successfully completed a complex exposure protocol, with an array of measurements that required the cooperation of many asthmatic individuals. The exposure atmospheres were well characterized, and the concentration targets for pollutants were achieved.

The study also included the use of minimally invasive techniques to assess pulmonary function and a suite of relevant inflammatory and immunologic endpoints in sputum and blood. The investigators hypothesized that exposure to particles in the DE would enhance inflammatory or immunologic responses in allergic subjects; the endpoints assessed were appropriate for the hypothesized effects. Of particular interest, the study assessed the effects of exposure to DE and NO₂ on both nonspecific (Phase 1) and allergen-induced (Phase 2) responses. (NO₂ served as an additional control atmosphere; it is a major gaseous component of DE and has the potential to affect the same health endpoints.)

Riedl and colleagues also effectively used a randomized crossover study design, in which each participant acted as his or her own control, in order to focus on the contrasts of interest while controlling for other sources of variability.

Limitations

Lack of an Evaluation of an Exposure–Response

Relationship Exposure to only a single concentration of DEP and NO₂ was an element of the study design agreed upon originally by the HEI Research Committee and the investigators. This was done in part to address ethical issues raised about using a higher concentration of DEP, and in part to allow for the companion NO₂ exposure to test whether the gaseous component of PM might be primarily responsible for the effects of DE in this set of endpoints.

Though this choice was made for understandable reasons, it prevented any assessment of an exposure–response relationship for either pollutant.

In addition, the allergen challenge in Phase 2 was carried out at only one relatively low concentration; therefore the effects of DE along with different concentrations of allergen could not be evaluated (as had been assessed and reported by Diaz-Sanchez et al. 2000b). Use of a low allergen challenge may also have reduced the investigators' ability to identify small changes in biologic responses.

Time Point for Studying Responses As described in more detail in the section Comparison of This and Previous Studies by These Investigators, immunologic and inflammatory endpoints were evaluated at only one time point, which was several hours after allergen challenge and during the late phase of the allergic response.

Period Effects The Committee agreed that the investigators' finding of period effects — that is, changes in endpoints based on whether the exposure period was the 1st, 2nd, or 3rd for a subject — was unexpected and difficult to explain. The investigators' speculation that this might be the result of a reduction in stress over the course of the exposures was not implausible, but the Committee also considered that the period effects might have reflected some sort of learning effect over the course of the study; that is, the participants might have become more experienced at being in the exposure chamber and providing sputum samples over the course of the procedures. Whatever their physiologic basis, the period effects complicated assessment of the effects of the pollutant atmospheres.

Questionable Asthmatic Status The airway response to methacholine in Phase 1 was found to be highly variable in 5 of the 15 participants. Thus, the asthmatic status of these participants is uncertain and had an unpredictable impact on the results from Phase 1.

COMPARISON OF THIS AND PREVIOUS STUDIES BY THESE INVESTIGATORS

In the current study, the predominantly negative findings about inhalation exposure to DE in allergic asthmatic subjects contrast with results from the team's earlier studies with allergic subjects in which they administered DEP (300 µg/m³) intranasally. Those studies showed enhanced features of inflammatory and allergic responses (especially an increase in IgE) in the nose, and in particular, the noses of subjects who were sensitive to ragweed or house dust–mites (Diaz-Sanchez et al. 1994, 1997, 1999, 2000a,b; Gilliland et al. 2004). In the current report, the

investigators consider that differing responses in the lower and upper airways may explain the inconsistencies among the results of their various studies. Although agreeing with the investigators on this point, the Review Committee thought several other factors may help to explain the disparities in findings. These factors are discussed below.

Routes and Concentrations of Exposure

In the current study, participants were exposed via inhalation to 100 $\mu\text{g}/\text{m}^3$ PM in DE over 2 hours, a considerably more realistic concentration and route of exposure than that used in previous studies (300 μg DEP administered intranasally). It is difficult to compare the inhaled dose of DE (and DEP) that reached the airways in the current study with the concentration of DEP delivered to the airways by nasal administration, though it is likely to be, by design, substantially lower. Nonetheless, it is evident that the routes of delivery to the airways are distinct, and it is possible that the airway cells that are first exposed to DE may vary in the two sites. In addition, it is likely that the ratio of DEP concentration to airway surface area in the nasal administration studies is higher than in the current inhalation study. Thus, differences in the exposure route may have had an impact on responses in various studies.

Sources of DEP

All the previously published studies from the Diaz-Sanchez team used at least two distinct samples of DEP collected from light-duty diesel engines in Japan in the 1980s (cited in Diaz-Sanchez et al. 1994, 2000b). In the current study the DE, and hence the DEP, were freshly generated by a 1999 U.S. pickup truck engine using low-sulfur fuels. These sources are very likely to generate DE with dissimilar characteristics and composition of DEP, so the types of biologic effects produced may also vary. In this regard, Kongerud and colleagues (2006) used a standard U.S.-generated DEP (NIST 1650) derived from 4-cycle engines operating under a variety of conditions for 200 hours; NIST 1650 was considered to be representative of DEP emissions from heavy-duty engines (according to the certificate of analysis from the National Institute of Standards and Technology). After intranasal administration to asthmatic or non-asthmatic volunteers, these particles had no effect on levels of cytokines or cells associated with inflammatory or immunologic responses.

Previous studies of human responses conducted by the Diaz-Sanchez team as well as others have indicated that organic components, and particularly polycyclic aromatic hydrocarbons, are responsible for the inflammatory or immunologic effects of DEP exposure (Devouassoux et al.

2002; Lubitz et al. 2010). It is not clear whether levels of potentially toxic components of DEP, such as certain organic compounds, differed between the DE used in the current study and that used in other studies; a detailed characterization of the components of the various types of DEP used would help to clarify this issue.

Airways and Tissues Evaluated at Different Times

The effects in the previous studies were evaluated only in the nose. To capture the changes reflecting the pathophysiology of asthma more accurately, endpoints in the current study were evaluated in the lower airways (via the collection of induced sputum) and in the blood. However, these differences preclude direct comparison among study designs. In addition, in the current study, sputum and blood responses were evaluated at only a single time point, approximately 24 hours after allergen challenge. This 24-hour time point falls outside the late phase of the allergic response, which has generally been defined by the duration of the changes in pulmonary function. However, changes in non-specific bronchial reactivity and airway inflammation can extend for several days. In one of the team's previous studies of responses in allergic volunteers (Diaz-Sanchez et al. 1997), changes in IgE and IgG4 levels were detected in the nose 4 to 8 days after allergen challenge. Thus, it is possible that in the current study some immunologic changes might have occurred at later times than were evaluated. The current study also did not evaluate changes — such as increases in histamine levels — that occur within minutes or in the early phase of the response to allergen challenge. In another study with individuals who were allergic to house dust-mite (see next section), the investigators did observe enhanced early changes in the nasal wash after nasal administration of DEP and challenge with allergen (Gilliland et al. 2004).

Major Characteristics of the Participants

Asthmatic Versus Non-Asthmatic Status A major difference in the characteristics of participants between this and other studies from the Diaz-Sanchez team is that this was the first in which they evaluated asthmatic volunteers. It is difficult to conclude that asthma status per se played a role in the lack of response to DE in the current study — many other features of the participants and study designs also varied. Interestingly, however, studies in Sweden have found inflammatory responses *only* in healthy and not in asthmatic individuals when both groups were exposed to DE (100 $\mu\text{g}/\text{m}^3$ PM for 2 hours) (Stenfors et al. 2004; Behndig et al. 2011).

Allergy to Cat Hair Versus Ragweed or House Dust-Mite

Participants in Phase 2 of the current study had allergic sensitivity to cat hair, which differed from the sensitivities of participants in earlier studies by this team. Participants in previous studies were allergic to either short ragweed (Diaz-Sanchez et al. 1997; Gilliland et al. 2004) or to house dust-mite (Diaz-Sanchez et al. 2000b).

The advantage of using short ragweed in the earlier study was that ragweed was not present in the Los Angeles area when the study was being conducted (Diaz-Sanchez et al. 1997). This ensured that the study subjects had not been recently exposed to the allergen to which they were sensitive, and as a consequence, they had low levels of ragweed-specific IgE (1 ng/mL or less in nasal wash) before they were challenged with ragweed (with or without DEP). This was not the case in the current study, however; participants allergic to cat hair in Phase 2 were not chosen because they had low levels of cat allergen-specific IgE. Although only subjects with no cats in their homes were included in the study, the allergen could have existed in their environments. Thus, it is possible that at least some participants could have already had elevated IgE levels due to a recent exposure, which might also have affected the level of IgE synthesized or measured after allergen challenge in the current study. This possibility is difficult to assess.

Note, though, that the investigators had previously found that exposure to DEP *did* enhance features of the allergic response to house dust-mite, an allergen found in the environment; responses included increases in histamine levels and symptoms as well as a decrease in the amount of allergen needed to provoke an allergic response (Diaz-Sanchez et al. 2000b). Thus, it was not unreasonable for the investigators to consider that DE would enhance responses to another allergen, cat hair, to which participants might have been exposed in their local environment.

Genetic Variability Diaz-Sanchez and collaborators reported previously that expression of variants of genes in the glutathione-S-transferase (GST) family — enzymes that metabolize and detoxify toxic agents — affected the response to DEP exposure in ragweed-sensitive individuals in Los Angeles (Gilliland et al. 2004). Specifically, higher levels of nasal histamine and IgE were detected in individuals who were *GSTM1* null or who expressed the wildtype *GSTP1* allele. The GST genotype of Phase 2 participants in the current study was not evaluated; therefore, it is possible that the relatively small group of 15 subjects, on average, fell into a “low responder” type.

OTHER KEY CONSIDERATIONS

Real-World Level of DE Exposure

Study participants were exposed to the target concentration of 100 $\mu\text{g}/\text{m}^3$ PM in DE for 2 hours with intermittent exercise. Was this a realistic human exposure level? This level of exposure may not be common at all sites in industrialized countries and it exceeds measured ambient concentrations in the developed world. However, some studies conducted in California have indicated that spikes of 50 $\mu\text{g}/\text{m}^3$ black carbon (as an indicator of DE) can be experienced by occupants of vehicles driving along roadways in Los Angeles (Fruin et al. 2004; Sabin et al. 2005). In developing countries, ambient levels of 100 $\mu\text{g}/\text{m}^3$ PM and higher are more common (e.g., in Wuhan and Shanghai, China [Kan et al. 2008, 2010; Qian et al. 2008, 2010]). Even if the level of PM exposure in the current study is considered high, the results indicate that the biologic effects of exposure to this level of DE in people with asthma appear to be very few.

NO₂

In addition to the lack of effects of exposure to DE in the current study, exposure to 0.35 ppm NO₂ alone affected very few of the endpoints measured and had no consistent effects on immunologic or inflammatory endpoints in either Phase 1 or Phase 2. The Review Committee found this somewhat surprising because, as described in the Scientific Background section, some earlier studies had suggested that exposure to NO₂ can enhance responsiveness to allergens, as well as increase the concentrations of markers of the inflammatory response (e.g., Tunnicliffe et al. 1994; Barck et al. 2002). In addition, in its most recent Integrated Science Assessment of the effects of NO₂ for reevaluation of National Ambient Air Quality Standards, the U.S. EPA (2008) concluded that overall, “... studies indicate that NO₂ short-term exposures of less than 1 ppm enhance allergen responsiveness in some allergic asthmatics.” However, not all studies reviewed in the Integrated Science Assessment showed effects of NO₂, and the Assessment speculated that differences in results among studies may be attributed to such factors as the timing of allergen challenge, the use of exercise versus rest, and the evaluation of BAL versus sputum. These differences in experimental conditions may help to explain why exposure to NO₂ in the current study had little or no effect.

FUTURE STUDIES OF THE EFFECTS OF DE EXPOSURE

The diesel engine used in this study was from a 1999 medium-duty truck that operated on California ultra-low-sulfur fuel and without a catalyst. The investigators

removed the catalyst to simulate the emissions from engines in the heavy-duty diesel fleet, which at the study's inception (early 2000s) were not equipped with catalysts (Sawant et al. 2008). However, lower emission standards for NO_x and PM that began to be implemented in 2004 (for light- and medium-duty passenger diesel vehicles) have significantly reduced, and continue to reduce, the overall emissions of DE and its major components — PM, NO₂, and air toxics. In particular, PM emissions from light- and heavy-duty trucks have been reduced by more than 10-fold through the use of particulate filters (starting with the 2006 and 2007 model years, respectively) and low-sulfur fuels (which became widely available outside California in 2006). NO₂ and air toxics emissions are also being reduced: NO₂ through the implementation of Tier 2 and 2010 NO_x emissions standards for heavy-duty vehicles, and air toxics as a result of PM control devices. Thus, although trucks like the one used in this study are still on the road, these changes in air quality standards and technology have already affected the overall emissions of DE and its major components.

To address the impact of changes in diesel technologies, HEI's Advanced Collaborative Emissions Study (ACES), currently under way, is characterizing emissions from and investigating the health effects of exposure to exhaust from a 2007 heavy-duty diesel engine with aftertreatment technology that meets the lower PM standards. Characterization of the emissions from four such engines has shown that PM concentrations are much reduced and the proportion of gaseous components is therefore concomitantly increased (Khalek et al. 2009, 2011). Furthermore, characterization of the exposure atmosphere generated by the engine used for the ACES animal inhalation studies indicates that this engine could not generate PM and NO₂ concentrations that approach those used in the current study while maintaining acceptable temperature and CO levels (Mauderly and McDonald 2012). Similar emission standards have been established in most other industrialized countries. Thus, the DE conditions used in this study would not be representative of the much lower emissions from contemporary heavy-duty engines.

SUMMARY AND CONCLUSIONS

The HEI Health Review Committee, which conducted an independent review of the study by Riedl and associates, thought that it addressed an important issue: the effects of inhalation exposure to DE, a major component of ambient urban air pollution, on health endpoints in the lower airways and blood of allergic asthmatic individuals both in the absence of (Phase 1) and after allergen challenge (Phase 2).

The study has several strengths. Of particular interest, the study used a more realistic concentration and route of DE exposure than those used in previous studies by this team; the effects were also measured in sites (the lungs and blood) that are central to the asthmatic response. An additional strong point of the study is the use of minimally invasive techniques to assess pulmonary function and a suite of inflammatory and immunologic endpoints in sputum and blood. The endpoints assessed were appropriate for the hypothesized responses: that exposure to DE would enhance inflammatory or allergic immunologic responses, or both.

In addition, the investigators effectively developed and implemented an exposure system to deliver DE and NO₂ to participants via inhalation with oral and nasal breathing. They used NO₂, a major gaseous component of DE, as an additional control atmosphere. The exposure atmospheres were well characterized.

Furthermore, the investigators successfully completed a complex exposure protocol, with measurements made at many time points that required the cooperation of many allergic asthmatic individuals. The study also effectively used a randomized crossover study design, in which exposures were given in random order and each participant acted as his or her own control.

Relative limitations of the study design include (1) the use of a single, low concentration of allergen in the challenge protocol in Phase 2, which may have reduced the ability to identify small changes; and (2) evaluation of immunologic and inflammatory endpoints at only one time point during the late phase of the allergic response. In addition, the detection of period effects (responses related to a subject's 1st, 2nd, and 3rd exposure periods) were unexpected and difficult to explain. Whatever their physiologic basis may be, these effects complicated the assessment of responses to the pollutant atmospheres.

The Committee agreed with the authors' conclusions that the findings were predominantly negative. Inhalation exposure to DE at 100 µg/m³ PM or to 0.35 ppm NO₂ alone for 2 hours with intermittent exercise in allergic asthmatic participants in either Phase 1 (no allergen challenge) or Phase 2 (after allergen challenge) resulted in only a few changes in inflammatory, immunologic, or physiologic status that differed significantly from the results of exposure to filtered air. This is an important negative conclusion that does not support the original hypothesis that exposure to DE would enhance inflammatory or allergic-type responses. It should be noted that the concentration of PM used in the current study (100 µg/m³ in DE) may be considered high in the United States and Europe. However, even at this

comparatively high level, the biologic effects of exposure in people with asthma appear to be very few.

The Committee agreed that the study's findings should be extrapolated with caution; it is possible that individuals with more severe asthma may have different responses if they are exposed for longer time periods or to higher DEP concentrations, or if they are exposed to DE mixtures from other types of diesel engines and fuels. The slight increase in airway resistance after DE exposure in Phase 1, and the small increases in sputum PMN and eosinophil numbers in Phase 2, suggest that this exposure concentration and duration may be near the lowest levels that might cause airway inflammatory effects in asthmatic individuals.

The lack of effects of DE inhalation exposure in allergic asthmatic volunteers in Phase 2 contrasts with the results of previous studies of the effects of DEP administered into the upper airways of allergic individuals that were carried out by the same team of investigators. Explanations for the variations in findings may include inhalation — a more realistic route of exposure in the current study — versus intranasal administration; the substantially lower DEP concentration used in this study; and the sources and therefore likely differences in composition of the DEP (derived from different engines). Some variations may have also been related to the tissues evaluated (lower airways and blood vs. the nose); the timing of analysis after allergen challenge (24 hours vs. minutes and up to 8 days); and the participants' health status and allergen sensitivity (asthmatic and allergic to cat hair [Phase 2] vs. non-asthmatic and allergic to ragweed or house dust-mite in earlier studies).

This study did not find changes in immunologic or inflammatory endpoints after exposure to 0.35 ppm NO₂, but some studies using similar exposure concentrations with allergic subjects have found such changes. Dissimilarities in study design — for example, the timing of allergen challenge, the use of exercise versus rest, and the evaluation of BAL fluid versus sputum — may help to explain the differences in the effects of NO₂ exposure.

The current study evaluated effects of DE from a 1999 engine, which is likely to be representative of a fraction of the fleet of vehicles currently on the road in the United States. Since 1999, however, changes in emission standards have resulted in major improvements in emissions, especially with the advent of diesel particle filters for light trucks (2006) and heavy-duty vehicles (2007) and the introduction of new fuels. These changes have already reduced and will continue to significantly lower the overall emissions of DE and its major components — PM, NO₂, and air toxics.

Thus, it is not clear to what extent the results of this study can be extrapolated to the effects of exposure to the substantially lower emissions from current diesel vehicles. To evaluate effects of the new diesel technology, future

studies will need to target much lower concentrations of DEP while also evaluating the gaseous components that will start to dominate the exhaust mixture. HEI's ACES study, soon to be completed, will be among the first to comprehensively study the health effects of the new diesel and aftertreatment technology with animals and will provide information about the composition of exhaust to compare with earlier studies.

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REFERENCES

- Barck C, Sandström T, Lundahl J, Halldén G, Svartengren M, Strand V, Rak S, Bylin G. 2002. Ambient level of NO₂ augments the inflammatory response to inhaled allergen in asthmatics. *Respir Med* 96:907–917.
- Behndig A, Larsson N, Brown JL, Stenfors N, Helleday R, Duggan S, Dove RE, Wilson SJ, Sandström T, Kelly FJ, Mudway IS, Blomberg A. 2011. Proinflammatory doses of diesel exhaust in healthy subjects fail to elicit equivalent or augmented airway inflammation in subjects with asthma. *Thorax* 66:12–19.
- Brunekreef B, Janssen NA, de Hartog J, Harssema H, Knape M, van Vliet P. 1997. Air pollution from truck traffic and lung function in children living near motorways. *Epidemiology* 8:298–303.
- Devouassoux G, Saxon A, Metcalfe DD, Prussin C, Colomb MG, Brambilla C, Diaz-Sanchez D. 2002. Chemical constituents of diesel exhaust particles induce IL-4 production and histamine release by human basophils. *J Allergy Clin Immunol* 109:847–853.
- Diaz-Sanchez D, Dotson AR, Takenaka H, Saxon A. 1994. Diesel exhaust particles induce local IgE production in vivo and alter the pattern of IgE messenger RNA isoforms. *J Clin Invest* 94:1417–1425.

- Diaz-Sanchez D, Garcia MP, Wang M, Jyralla M, Saxon A. 1999. Nasal challenge with diesel exhaust particles can induce sensitization to a neoallergen in the human mucosa. *J Allergy Clin Immunol* 104:1183–1188.
- Diaz-Sanchez D, Jyralla M, Ng D, Nel A, Saxon A. 2000a. In vivo nasal challenge with diesel exhaust particles enhances expression of the CC chemokines RANTES, MIP-1 α , and MCP-3 in humans. *Clin Immunol* 97:140–145.
- Diaz-Sanchez D, Penichet-Garcia M, Saxon A. 2000b. Diesel exhaust particles directly induce activated mast cells to degranulate and increase histamine levels and symptom severity. *J Allergy Clin Immunol* 106:1140–1146.
- Diaz-Sanchez D, Tsien A, Fleming J, Saxon A. 1997. Combined diesel exhaust particulate and ragweed allergen challenge markedly enhances human in vivo nasal ragweed-specific IgE and skews cytokine production to a T helper cell 2-type pattern. *J Immunol* 158:2406–2413.
- Dockery DW, Pope CA III. 1994. Acute respiratory effects of particulate air pollution. *Annu Rev Public Health* 15:107–132.
- Edwards J, Walters S, Griffiths RK. 1994. Hospital admissions for asthma in preschool-children: Relationship to major roads in Birmingham, United Kingdom. *Arch Environ Health* 49:223–227.
- Frampton MW, Boscia J, Roberts NJ Jr, Azadniv M, Torres A, Cox C, Morrow PE, Nichols J, Chalupa D, Frasier LM, Gibb FR, Speers DM, Tsai Y, Utell MJ. 2002. Nitrogen dioxide exposure: Effects on airway and blood cells. *Am J Physiol Lung Cell Mol Physiol* 282:L155–165.
- Fruin SA, Winer AM, Rodes CE. 2004. Black carbon concentrations in California vehicles and estimation of in-vehicle diesel exhaust particulate matter exposures. *Atmos Environ* 38:4123–4133.
- Fujimaki H, Saneyoshi K, Shiraishi F, Imai T, Endo T. 1997. Inhalation of diesel exhaust enhances antigen-specific IgE antibody production in mice. *Toxicology* 116:227–233.
- Gilliland FD, Li YF, Saxon A, Diaz-Sanchez D. 2004. Effect of glutathione-S-transferase M1 and P1 genotypes on xenobiotic enhancement of allergic responses: Randomised, placebo-controlled crossover study. *Lancet* 363:119–125.
- Jones B, Kenward MG. 1989. Design and analysis of crossover trials. Chapman and Hall London, New York.
- Kan H, London SJ, Chen G, Zhang Y, Song G, Zhao N, Jiang L, Chen B. 2008. Season, sex, age, and education as modifiers of the effects of outdoor air pollution on daily mortality in Shanghai, China: The Public Health and Air Pollution in Asia (PAPA) Study. *Environ Health Perspect* 116:1183–1188.
- Kan H, Chen B, Zhao N, London SJ, Song G, Chen G, Zhang Y, Jiang L. 2010. Part I. A time-series study of ambient air pollution and daily mortality in Shanghai, China. In: *Public Health and Air Pollution in Asia (PAPA): Coordinated Studies of Short-Term Exposure to Air Pollution and Daily Mortality in Four Cities*. Research Report 154. Health Effects Institute, Boston, MA.
- Khalek IA, Bougher TL, Merritt PM. 2009. Phase I of the Advanced Collaborative Emissions Study; Coordinating Research Council (CRC) Report ACES-Phase 1. CRC, Alpharetta, GA.
- Khalek IA, Bougher TL, Merritt PM, Zielinska B. 2011. Regulated and unregulated emissions from highway heavy-duty diesel engines complying with U.S. Environmental Protection Agency 2007 emissions standards. *J Air Waste Manage Assoc* 61: 427–442.
- Kongerud J, Madden MC, Hazucha M, Peden D. 2006. Nasal responses in asthmatic and nonasthmatic subjects following exposure to diesel exhaust particles. *Inhal Toxicol* 18:589–594.
- Lubitz S, Schober W, Pusch G, Effner R, Klopp N, Behrendt H, Buters JT. 2010. Polycyclic aromatic hydrocarbons from diesel emissions exert proallergic effects in birch pollen allergic individuals through enhanced mediator release from basophils. *Environ Toxicol* 25:188–197.
- Mauderly JL, McDonald JD. 2012. Advanced Collaborative Emissions Study Phase 3A: Characterization of U.S. 2007-Compliant Diesel Engine and Exposure System Operation. Communication 17. Health Effects Institute, Boston, MA.
- Muranaka M, Suzuki S, Koizumi K, Takafuji S, Miyamoto T, Ikemori R, Tokiwa H. 1986. Adjuvant activity of diesel-exhaust particulates for the production of IgE antibody in mice. *J Allergy Clin Immunol* 77:616–623.
- Nightingale JA, Maggs R, Cullinan P, Donnelly LE, Rogers DF, Kinnersley R, Chung KF, Barnes PJ, Ashmore M, Newman-Taylor A. 2000. Airway inflammation after controlled exposure to diesel exhaust particulates. *Am J Respir Crit Care Med* 162:161–166.
- Nordenhäll C, Pourazar J, Blomberg A, Levin JO, Sandström T, Adelroth E. 2000. Airway inflammation following exposure to diesel exhaust: A study of time kinetics using induced sputum. *Eur Respir J* 15:1046–1051.

- Nordenhäll C, Pourazar J, Ledin MC, Levin JO, Sandström T, Adelroth E. 2001. Diesel exhaust enhances airway responsiveness in asthmatic subjects. *Eur Respir J* 17:909–915.
- Qian Z, He Q, Lin HM, Kong L, Bentley CM, Liu W, Zhou D. 2008. High temperatures enhanced acute mortality effects of ambient particle pollution in the “oven” city of Wuhan, China. *Environ Health Perspect* 116:1172–1178.
- Qian Z, He Q, Lin H-M, Kong L, Zhou D, Liang S, Zhu Z, Liao D, Liu W, Bentley CM, Dan J, Wang B, Yang N, Xu S, Gong J, Wei H, Sun H, Qin Z. 2010. Part 2. Association of daily mortality with ambient air pollution, and effect modification by extremely high temperature in Wuhan, China. In: *Public Health and Air Pollution in Asia (PAPA): Coordinated Studies of Short-Term Exposure to Air Pollution and Daily Mortality in Four Cities*. Research Report 154. Health Effects Institute, Boston, MA.
- Rudell B, Ledin MC, Hammarström U, Stjernberg N, Lundbäck B, Sandström T. 1996. Effects on symptoms and lung function in humans experimentally exposed to diesel exhaust. *Occup Environ Med* 53:658–662.
- Sabin LD, Behrentz E, Winer AM, Jeong S, Fitz DR, Pankratz DV, Colome SD, Fruin SA. 2005. Characterizing the range of children's air pollutant exposure during school bus commutes. *J Expo Anal Environ Epidemiol* 15:377–387.
- Salvi S, Blomberg A, Rudell B, Kelly F, Sandström T, Holgate ST, Frew A. 1999. Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. *Am J Respir Crit Care Med* 159:702–709.
- Salvi, SS, Nordenhäll C, Blomberg A, Rudell B, Pourazar J, Kelly FJ, Wilson S, Sandström T, Holgate ST, Frew AJ. 2000. Acute exposure to diesel exhaust increases IL-8 and GRO- α production in healthy human airways. *Am J Respir Crit Care Med* 161:550–557.
- Sawant AA, Cocker DR 3rd, Miller JW, Taliaferro T, Diaz-Sanchez D, Linn WS, Clark KW, Gong H Jr. 2008. Generation and characterization of diesel exhaust in a facility for controlled human exposures. *J Air Waste Manag Assoc* 58:829–837.
- Sawyer K, Samet JM, Ghio AJ, Pleil JD, Madden MC. 2008. Responses measured in the exhaled breath of human volunteers acutely exposed to ozone and diesel exhaust. *J Breath Res* 2:037019.
- Solomon C, Christian DL, Chen LL, Welch BS, Kleinman MT, Dunham E, Erle DJ, Balmes JR. 2000. Effect of serial-day exposure to nitrogen dioxide on airway and blood leukocyte and lymphocyte subsets. *Eur Respir J* 15:922–928.
- Stenfors N, Nordenhäll C, Salvi SS, Mudway I, Söderberg M, Blomberg A, Helleday R, Levin JO, Holgate ST, Kelly FJ, Frew AJ, Sandström T. 2004. Different airway inflammatory responses in asthmatic and healthy humans exposed to diesel. *Eur Respir J* 23:82–86.
- Takafuji S, Suzuki S, Koizumi K, Tadokoro K, Miyamoto T, Ikemori R, Muranaka M. 1987. Diesel-exhaust particulates inoculated by the intranasal route have an adjuvant activity for IgE production in mice. *J Allergy Clin Immunol* 79:639–645.
- Takafuji S, Suzuki S, Koizumi K, Tadokoro K, Ohashi H, Muranaka M, Miyamoto T. 1989. Enhancing effect of suspended particulate matter on the IgE antibody production in mice. *Int Arch Allergy Appl Immunol* 90:1–7.
- Takano H, Yoshikawa T, Ichinose T, Miyabara Y, Imaoka K, Sagai M. 1997. Diesel exhaust particles enhance antigen-induced airway inflammation and local cytokine expression in mice. *Am J Respir Crit Care Med* 156:36–42.
- Tudor G, Koch GG. 1994. Review of nonparametric methods for the analysis of crossover studies. *Stat Methods Med Res* 3:345–381.
- Tunnicliffe WS, Burge PS, Ayres JG. 1994. Effect of domestic concentrations of nitrogen dioxide on airway responses to inhaled allergen in asthmatic patients. *Lancet* 344:1733–1736.
- U.S. Environmental Protection Agency. 2008. Integrated Science Assessment for Oxides of Nitrogen: Health Criteria (Final Report). EPA/600/R-08/071. Office of Research and Development, Research Triangle Park, NC.
- van Vliet P, Knappe M, de Hartog J, Janssen N, Harssema H, Brunekreef B. 1997. Motor vehicle exhaust and chronic respiratory symptoms in children living near freeways. *Environ Res* 74:122–132.
- Weiland SK, Mundt KA, Rückmann A, Keil U. 1994. Self-reported wheezing and allergic rhinitis in children and traffic density on street of residence. *Ann Epidemiol* 4:243–247.
- Wjst M, Reitmeir P, Dold S, Wulff A, Nicolai T, von Loeffelholz-Colberg EF, von Mutius E. 1993. Road traffic and adverse effects on respiratory health in children. *Br Med J* 307:596–600.

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