RESEARCH REPORT

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Mechanisms of Morbidity and Mortality from Exposure to Ambient Air Particles

John J Godleski, Richard L Verrier, Petros Koutrakis, and Paul Catalano

Includes the Commentary of the Institute's Health Review Committee

HEALTH
EFFECTS
INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate matter) and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 200 projects at institutions in North America and Europe and has published over 200 research reports.

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

HEI STATEMENT

Synopsis of Research Report 91

Effects of Concentrated Ambient Particles on the Cardiac and Pulmonary Systems of Dogs

INTRODUCTION

Epidemiology studies have indicated that short-term exposure to low-level increases in particulate matter is associated with an increase in morbidity and daily mortality, particularly in individuals with cardiopulmonary conditions. A plausible biologic mechanism linking low-level particle exposure and pathophysiologic effects has not been established, however. Assessing the effects of particulate matter in appropriate animal models is important to learning how particulate matter may exert adverse health effects. The Health Effects Institute funded the study described in this report as part of a research program to reduce this information gap.

APPROACH

Dr John Godleski and colleagues at Harvard School of Public Health conducted an exploratory study to test the effects of particulate matter exposure in dogs, which share many features of the human cardiovascular system. The investigators hypothesized that particulate matter might affect the animals' cardiac function, leading to arrhythmia, and might induce inflammatory responses and changes in pulmonary mechanical measurements. To maximize possible effects, they used a device to concentrate particles up to 30 times their level in ambient Boston air and exposed dogs to these concentrated ambient particles (CAPs) via inhalation. The investigators physically and chemically identified components of the CAPs and tested their effects in 12 dogs, in 6 of which they also induced a coronary occlusion to simulate human coronary artery disease. They evaluated the effects of CAPs on electrocardiographic (ECG) wave patterns and performed a sophisticated analysis of each dog's ECG to measure possible effects of particulate matter on other electrical properties of the heart: heart rate variability, which is influenced by the involuntary nervous system, and T wave alternans, a change in the heart beat pattern. These two are among the measures currently used to predict further heart problems in humans with cardiovascular disease, but they have not been established as predictive parameters in normal humans or other species. The investigators also assessed whether changes in respiratory parameters or inflammatory responses were associated with CAPs exposure.

RESULTS AND INTERPRETATION

The most biologically and clinically significant finding was that in dogs with induced coronary occlusion, CAPs affected one of the major ECG signs of myocardial ischemia in humans, known as *elevation of the ST segment*. CAPs-exposed animals showed a shortened time to ST segment elevation and an increased magnitude of the ST segment compared to controls. These findings suggest what may be a plausible mechanism to explain PM's effects on individuals with cardiopulmonary conditions: exposure to particulate pollution may make patients with ischemic heart disease more susceptible to developing serious cardiac effects. If substantiated in larger groups of animals, the evidence may help to explain the previously described association between increased particulate pollution and cardiopulmonary morbidity and mortality. Animals with an induced coronary occlusion also showed other changes in cardiac and respiratory parameters after exposure to CAPs.

The investigators also reported that normal dogs showed CAPs-induced changes in heart rate variability and average heart rate (which fluctuated widely from day to day during the course of the study), decreases in T wave alternans, and changes in respiratory parameters such as breathing rates and air flow rates. They did not identify whether the variability in responses was due to day-to-day fluctuation of a specific component of the particulate mixture. In addition, the investigators reported that CAPs had little or no effect on inflammatory mediators, suggesting that changes in cardiac and pulmonary responses occurred in the absence of significant airway inflammation.

The investigators interpreted their findings to indicate that CAPs influenced the nervous system's control of the normal dog's heart but did not necessarily induce arrhythmia. This interpretation may be reasonable, but the statistical approach the investigators used to identify changes in heart rate variability is not clearly applicable to the small number of dogs tested. In addition, it is not apparent whether it is appropriate to extrapolate these results to humans because the human and dog cardiovascular systems differ in some critical features. Furthermore, the clinical significance of changes in heart rate variability or T wave alternans in normal dogs, or in humans who do not have preexisting heart disease, is currently unknown.

Because Godleski and colleagues tested only a small number of animals, confirmation of the findings both in animals with impaired cardiac function and in normal animals is required in larger studies. Studies of effects in humans, such as those currently underway at HEI, are also expected to provide information about the possible effects of particulate matter on the heart.

This Statement, prepared by the Health Effects Institute, is a summary of a research project sponsored by HEI from 1995 to 1999. This study was conducted by Dr John J Godleski of Harvard School of Public Health, Boston, Massachusetts. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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HEALTH EFFECTS INSTITUTE

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STATEMENT Health Effects Institute

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

PREFACE

INVESTIGATORS' REPORT

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

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COMMENTARY Health Review Committee

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

RELATED HEI PUBLICATIONS

PREFACE

In 1994, HEI initiated a research program to investigate the complex issues associated with the health effects of exposure to particulate matter (PM)* in the air. This program was developed in response to growing concern about the potential public health significance of reported associations between daily fluctuations in levels of PM and changes in daily morbidity and mortality in time-series epidemiology studies. These results were questioned for a variety of reasons, including the lack of support from experimental studies and the lack of a mechanism to explain how such effects would occur. To address these issues HEI undertook two research initiatives in 1994: (1) the Particle Epidemiology Evaluation Project (Samet et al. 1995, 1997), which evaluated six of the time-series epidemiology studies that had reported effects of PM on mortality; and (2) a program of toxicologic and epidemiologic studies (funded from RFA 94-2, *Particulate Air Pollution and Daily Mortality: Identification of Populations at Risk and Underlying Mechanisms*), which aimed to understand better how PM might cause toxicity and what factors might affect susceptibility. In all, HEI has issued five requests for research on PM and funded 34 studies or reanalyses over the last five years.

This Preface provides general regulatory and scientific background information relevant to studies funded from RFA 94-2, including the study by John Godleski, which is described in the accompanying Report and Commentary. All of the studies from RFA 94-2 have been completed and are either under review by HEI or have been published. The *HEI Program Summary: Research on Particulate Matter* (Health Effects Institute 1999) provides information on studies funded since 1996.

BACKGROUND

Particulate matter (PM) is the term used to define a complex mixture of anthropogenic and naturally occurring airborne particles. The size, chemical composition, and other physical and biological properties of PM depend on the sources of the particles and the changes the particles undergo in the atmosphere. In urban environments, these particles derive mainly from combustion, including mobile sources such as motor vehicles and stationary sources such as power plants. The most commonly used descriptor of particle size is *aerodynamic diameter*. Based on this parameter, ambient particles tend to fall into three size classes (often defined as modes): ultrafine or nuclei mode (particles less than 0.1 μm in diameter); fine or accumulation mode (particles between 0.1 and 2.5 μm in diameter), and coarse (particles larger than 2.5 μm in diameter). Fine and ultrafine particles are dominated by emissions from combustion processes while coarse particles are mostly generated by mechanical processes from a variety of noncombustion sources. Generally, the ultrafine and fine fractions are composed of carbonaceous material, metals, sulfate, nitrate and ammonium ions. The coarse fraction is composed mostly of mechanically generated particles and consists of insoluble minerals and biologic aerosols, with smaller contributions from primary and secondary aerosols and sea salts (US Environmental Protection Agency [EPA] 1996).

A number of early epidemiologic studies indicated that human exposure to high concentrations of PM, such as London fog, had deleterious effects (such as increased number of deaths), particularly in children, the elderly, and those with cardiopulmonary conditions (Firket 1931; Ciocco and Thompson 1961; Logan 1953; Gore and Shaddick 1968). Because of this apparent relation to increased mortality, the EPA has regulated the levels of ambient PM since 1971, when the Clean Air Act was first promulgated. This act authorized the EPA to set National Ambient Air Quality Standards (NAAQSs) for a number of potentially harmful air pollutants (including PM) in order to protect the health of the population, particularly those thought to be sensitive.

The first NAAQS for PM was based on controlling total suspended PM or particles up to 40 μm in diameter. In 1978, the standard was revised to regulate inhalable particles, or particles than can deposit in the respiratory tract and therefore have greater potential for causing adverse health effects. These are particles with an aerodynamic diameter of 10 μm or less (PM_{10}). More recent epidemiologic studies, published in the early 1990s, indicated a relatively consistent association between small short-term increases in PM levels and increases in both mortality and morbidity from respiratory and cardiovascular diseases (reviewed by the Committee of the Environmental and Occupational Health Assembly, American Thoracic Society 1996).

Some studies also suggested that long-term exposure to low levels of PM is associated with adverse effects (Dockery et al 1993; Pope et al 1995). These latter studies

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

Table 1. Current NAAQSs for PM (set in 1997)

	PM_{10}	$PM_{2.5}$
Daily Standard	150 μg/m^3	65 μg/m^3
Annual Standard	50 μg/m^3	15 μg/m^3

also pointed to a possible role of fine particles (less than 2.5 μm in aerodynamic diameter or $PM_{2.5}$). In 1997, the EPA considered the evidence for the effects of fine particles sufficient to promulgate a fine particle standard while retaining the PM_{10} standard (US Environmental Protection Agency 1997) (see Table 1). The next review of the PM NAAQS is scheduled to be completed by the year 2002. Scientific information for that review must be peer reviewed and available by the late spring of 2000.

RESEARCH PROGRAM FROM RFA 94-2

The wealth of epidemiologic data published in the early 1990s suggested an association between PM and health effects, but aspects of these fundings were not well understood. Problems involved uncertainties in the exposure estimates, confounding by weather or other factors, the role of copollutants, and the mechanisms by which particles may cause effects. Moreover, although the epidemiologic findings were consistent across different communities exposed to distinct mixes and levels of pollutants, they were not well supported by either human chamber studies or animal inhalation studies aimed at delineating pathologic changes that might result in death. Failure of the experimental studies to provide support for the epidemiologic findings was attributed to insufficient statistical power, use of particles not representative of ambient particles, or use of animals not representative of the individuals susceptible to increased mortality.

By the mid 1990s, it became apparent that the research to advance our understanding of the association between exposure to particles and daily mortality found in the epidemiologic studies needed to focus on identifying (1) susceptible populations, (2) mechanisms by which particles may lead to increased mortality, and (3) characteristics of the particles responsible for the effects. It was recognized that both epidemiologic and experimental studies would be required.

The HEI program initiated in 1994 was aimed at addressing these research needs. Six epidemiologic and toxicologic studies were funded through RFA 94-2, and three additional studies were added through the preliminary application process. As a group, the five epidemiologic studies investigated: (1) social and medical factors that might increase the risk of mortality when particulate pollution increases (Mark Goldberg of the National Institute of Scientific Research, University of Quebec); (2) components of particulate pollution that might account for its effect on mortality (Morton Lippmann of the New York University School of Medicine and Erich Wichmann of the GSF Insitute of Epidemiology and Ludwig Maximilian University); and (3) cause of death (Harvey Checkoway of the University of Washington and Mark Goldberg) or possible pathophysiologic mechanisms that might lead to death in people exposed to particulate air pollution (Douglas Dockery of Harvard School of Public Health [see Dockery et al. 1999]).

The four experimental studies tested the hypothesis that older animals or animals with preexisting lung or heart disease or respiratory infections are more sensitive to the acute effects of particles than healthy animals. They investigated possible mechanisms leading to mortality such as inflammation, changes in immune response, or changes in cardiac and respiratory function. Three of these studies used for the first time concentrated ambient particles (CAPs) (John Godleski of Harvard School of Public Health, and Terry Gordon and Judith Zelikoff of New York University School of Medicine). In these CAPs studies, particles in the range of about 0.1 to 2.5 μm are concentrated while those greater than 2.5 μm are removed and those under 0.1 μm remain at the ambient concentration. CAPs exposures represent a significant fraction of ambient PM and provide a reasonable approach to mimicking the exposure to PM in epidemiology studies. The fourth experimental study (Günter Oberdörster of the University of Rochester School of Medicine and Dentistry) focused on evaluating the effects of different ultrafine particles that have been hypothesized to be more toxic than fine particles.

CONTINUING RESEARCH

Many of the key questions identified in the early 1990s are still relevant and much research is ongoing to address them. The research strategies have evolved, however, as results from previous studies have provided insights into which animal models and which endpoints may be the most helpful to evaluate. In addition, advances in exposure assessment and statistical methods have pointed to new approaches for conducting epidemiologic studies. Since RFA 94-2, HEI has funded a number of research projects that build on the new findings and approaches. These studies will be completed over the next two years (2000–2002).

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INVESTIGATORS' REPORT

Mechanisms of Morbidity and Mortality from Exposure to Ambient Air Particles

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ABSTRACT

The studies reported here assessed pathophysiologic mechanisms that result from exposure to concentrated ambient particles (CAPs)* in animals with and without cardiopulmonary compromise. These studies were carried out to determine the biologic plausibility of epidemiologic observations of increases in particulate air pollution associated with increases in human morbidity and mortality.

Dogs were exposed two at a time to CAPs or filtered air via tracheostomy for six hours per day on three consecutive days. The electrocardiogram (ECG) and breathing pattern were recorded continuously, and indicators of inflammation were also assessed.

In one experimental design, normal dogs were exposed in pairs to CAPs and subsequently to filtered air or to filtered air and subsequently CAPs (the double CAPs/double sham design). Comparisons were made between the CAPs measurements and each dog's own sham responses. In another design, one dog was exposed to CAPs while the chambermate received a sham exposure; these experiments were followed by crossover of the protocol the subsequent week (the crossover design). Comparisons were made between the CAPs exposure and both the chambermate's sham and each dog's own sham responses.

The crossover experiments were conducted in normal animals and in animals who had undergone balloon occlusion of the left anterior descending (LAD) coronary artery to induce myocardial compromise. The effects of CAPs in animals with induced chronic bronchitis were part of the original specific aims; because these studies were not fully pursued, the results are presented only in Appendix A.

In normal dogs, analyses of all double CAPs and crossover studies revealed low frequency (LF) and high frequency (HF) powers for heart rate variability (HRV) that were significantly higher for CAPs exposure compared to sham exposure. Variation in day-to-day exposure concentrations, aerosol composition, and pathophysiologic responses were also found. The crossover design, continuous measures of aerosol mass, and biologic responses were incorporated in the development of a statistical model that allowed isolation of changes associated with CAPs from changes due to animal variations. Comparison of individual exposures with this model revealed a range from no response in any measured parameter to statistically significant changes in cardiac autonomic balance, pulmonary air flow, and breathing pattern. On days in which dogs showed statistically significant changes in responses, the findings were consistent in both cardiac and respiratory parameters. Days associated with significant increases in LF and HF HRV, LF/HF HRV ratio, and heart rate standard deviation (HR SD) were also associated with decreases in average heart rate. These same days had decreases in respiratory frequency, tidal volume, minute volume, and peak flows with corresponding increases in respiratory cycle times and enhanced pause (Pau_{enh}), a measure of bronchoconstriction. These cardiac and respiratory changes suggest an effect mediated via both the sympathetic nervous system and the vagus nerve. Alternatively, days associated with increased heart rate had decreases in the HR SD; decreases or no change in HF and LF HRV; increases in respiratory flows and volumes; and decreases in breathing cycle times, all suggesting only sympathetic nervous system mediation.

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

This Investigators' Report is one part of Health Effects Institute Research Report Number 91, 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 John J Godleski, Harvard School of Public Health, Department of Environmental Health, 665 Huntington Avenue, Boston MA 02115.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award R824835 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.

When all data from the crossover design experiments were assessed with this model, the heart rate and respiratory rate were significantly decreased in relation to both cumulative and actual exposure and the LF HRV, LF/HF HRV ratio, HR SD, and all other respiratory parameters were significantly increased ($p < 0.0001$ for all). When cardiac data were grouped by days in which the air mass trajectory came from the north or northwest (versus west, south, east, or northeast), significant increases in HR SD and HF HRV and significant decreases in average heart rate were associated with the northwest trajectory. Within this dichotomy, neither respiratory parameters nor any exposure assessment parameters exhibited significant differences. When T wave alternans results were compared in CAPs and sham exposures of normal animals, a statistically significant decrease in T wave alternans was observed for CAPs exposure, consistent with increased vagally mediated activity in these animals. In studies with double CAPs and double sham exposures, bronchoalveolar lavage (BAL) fluid neutrophil percentages in CAPs-exposed animals were increased over sham (≈ 15% versus ≈ 5%, respectively; $p = 0.05$). Other BAL parameters showed no differences between CAPs and sham exposure. Also, comparing CAPs and sham exposures, no differences in peripheral white blood cell (WBC) count, differential, or fibrinogen were detected.

In dogs that underwent coronary occlusion, differences in responses to CAPs and sham exposures were observed again. With CAPs exposure, HF increased and HF surges occurred. Overall, the influence of HF differences between CAPs and sham exposures in this protocol was more striking (compared with the normal dogs) with significant increases observed on most CAPs exposure days. The most statistically and biologically significant difference in compromised dogs was the timing of ST segment elevation after exposure. With CAPs exposure, ischemia-induced ST segment elevation began almost 1 minute sooner than in the sham-exposed dogs.

In summary, normal dogs exposed to CAPs exhibited considerable daily variability in both exposure and response parameters. Our statistical methods for assessment of health effects in these animals allowed us to gain insights into biological responses to inhaled CAPs. In normal dogs, statistically significant cardiopulmonary effects (specifically responses mediated by the autonomic nervous system) were associated with most CAPs exposures. Biologically modest increases in pulmonary inflammation were observed. In dogs with coronary occlusion, exposure to CAPs significantly advanced the time of ST segment change and, by implication, the repolarization changes associated with enhanced propensity to life-threatening arrhythmia.

Overall, these studies indicate that inhalation of ambient particles may produce responses in the cardiac and respiratory systems that form the basis of plausible mechanisms by which exposure to increased levels of particulate could lead to fatal outcomes in predisposed individuals. The findings of this report indicate need for continued research on the cardiac responses to inhaled particles.

INTRODUCTION

OBJECTIVE

Epidemiologic studies have shown that exposures to particulate air pollution are associated with increased cardiorespiratory morbidity and mortality (Fairley 1990; Pope et al 1992, 1995; Schwartz 1991; Schwartz and Dockery 1992a,b; Dockery et al 1993; Ostro 1993; Spix et al 1993; Dockery and Pope 1994; Schwartz 1994a,b,c,d; Burnett et al 1995; Schwartz and Morris 1995). The studies of this report focus on the biologic plausibility of these epidemiologic observations and discovery of mechanisms by which increases in morbidity and mortality associated with ambient air particles might be explained. The following concepts and assumptions are fundamental to our approach.

1. Ambient particles, unaltered by collection or experimental exposure techniques, should be used for exposures.
2. Animal studies should model human populations, and adverse effects assessed should include those related to the increased morbidity and mortality identified epidemiologically.
3. By concentrating ambient airborne particles for use in exposures, the increased dose that is delivered to experimental animal populations should be sufficient for observation of biologic responses.

On the basis of these concepts, our studies utilized the Harvard ambient particle concentrator (HAPC), a recently developed device that can increase ambient particle concentrations up to several hundred micrograms per cubic meter without changing their physical or chemical characteristics; a typical northeastern US urban aerosol, composed mostly of transported sulfur-containing acidic particles during the summer and local combustion product particulates in winter; animal models of human cardiac and pulmonary diseases that have been developed or used in our laboratory to elucidate mechanistic effects; and

established cardiac and pulmonary physiologic methods to define mechanisms of acute morbidity and mortality and to test mechanistic hypotheses of adverse effects.

The overall goal of these studies is to determine whether inhalation of urban particles produces or enhances an inflammatory response in the lung and to determine the extent to which this response has systemic effects manifested in subtle ECG patterns associated with fatal cardiac outcomes. In addition, these studies explore the possibility that cardiac responses to inhaled particle exposures may occur without significant pulmonary inflammation, especially via mechanisms mediated by the autonomic nervous system. These studies are based on the hypothetical mechanisms illustrated in Figure 1. Although these studies do not test every hypothetical step, the principles of an inflammatory mechanism outlined on the right side of the figure, and neurally mediated mechanisms outlined on the left, are tested.

RATIONALE

Human Epidemiologic Findings Associated with Increased Particulate Air Pollution

Mortality associated with air pollution was recognized in the winter fog episodes in the Meuse Valley in Belgium in 1930, in Donora, Pennsylvania, in 1948, and in London

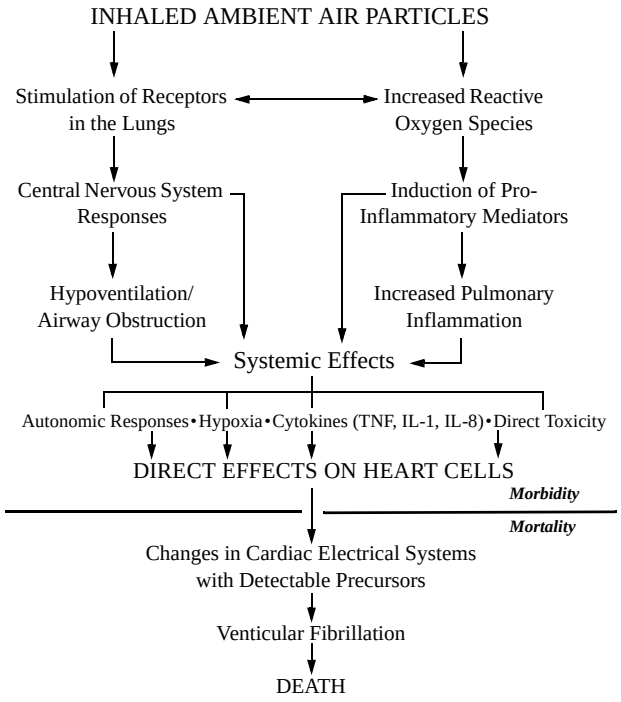


Figure 1. Hypothetical mechanisms by which inhaled ambient particles might result in death.

in 1952. The Harvard six cities study (Ferris et al 1979), a prospective epidemiology study of the effects of sulfur dioxide and respirable particles, suggested that increased respiratory symptoms such as bronchitis, chronic cough, and chest illness are associated with higher concentrations of ambient particles (Dockery et al 1989). Other studies report an association between increased daily mortality and ambient particle concentration levels in three US cities: New York (Schimmel and Murawski 1976), Philadelphia (Wyzga 1976), and Los Angeles (Kinney and Özkaynak 1991). Despite substantial differences in the chemical composition of particles in these cities, mortality increased with increased particle levels.

The toxic component or components responsible for increased mortality have not been determined from the epidemiologic studies. During air pollution episodes, pollutant concentrations (particulate and gaseous), temperature, and relative humidity are usually correlated. Schwartz (1994a) found that hospital admissions for chronic obstructive pulmonary disease and pneumonia correlated with increased levels of ozone and particulate matter (PM) less than 10 μm in aerodynamic diameter (PM_{10}). However, controlling for one pollutant did not affect the magnitude of the association with the other pollutant. Persons with preexisting disease are most susceptible to the effects of small increases in particulate air pollution (Utell and Samet 1993; Schwartz 1994a), but if only the severely ill are dying, one would expect a slight decrease in death rates immediately after the initial increase that follows the pollution shift. This corresponding mortality decrease has never been observed. Furthermore, deaths of people outside the hospital have increased as well. Studies reported by Dockery and colleagues (1993) in the *New England Journal of Medicine* show a definite dose-response relation between particulate exposure and mortality.

Toxicologic studies of human exposures to single pollutants at higher than ambient concentrations have not revealed impressive effects (Hackney et al 1989; Anderson et al 1992; Utell and Samet 1993). Other studies report changes in clearance or nonspecific airway reactivity following sulfuric acid exposures (Utell et al 1983a,b; Frampton et al 1992). No study, however, has demonstrated pathological changes that might result in death. Some investigators have demonstrated greater decrements in respiratory function among certain persons with asthma exposed to acidic particles (Koenig et al 1989). In addition, studies with animals have reported modest changes in clearance of particles from the lungs (Gearhart and Schlesinger 1989) or in airway responsiveness (Schlesinger 1990). In a large chronic study of canines by Heyder and colleagues (1992, 1994), inhalation of neutral or acid sulfite at levels of

0.6 mg/m^3 produced modest effects in parameters of particle clearance. A battery of BAL, pulmonary function, and morphometric measurements were unrevealing (Heyder et al 1992, 1994). Long-term exposure of animals to respirable particles has not produced lethal consequences unless particle concentrations were excessive, resulting in overload conditions (Morrow 1991; Heinrich 1994; Mauderly 1994). Ours is the first comprehensive study in which large animal subjects are exposed by inhalation to unaltered, concentrated ambient air particulates to assess the health effects of that complex mixture.

Ambient Particles and the Harvard Ambient Particle Concentrator

A particle concentrator that can increase the concentration of ambient air particles so that inhalation studies may be performed was developed in our department (Sioutas et al 1995a,b, 1997). This system concentrates ambient particles by a factor of roughly 30 to achieve concentrations in the range of hundreds of micrograms per cubic meter without affecting their size distribution or chemical composition (Sioutas et al 1997). In our studies, dogs were exposed to a typical northeastern US urban aerosol (Boston MA). The physical, chemical, and biological characteristics of the aerosol exposures were determined, enabling us to investigate response as a function of specific aerosol chemical properties.

Animal Models of Disease in Environmental Research

Laboratory-based air pollution research has been driven by the concept of finding effects at the lowest possible airborne concentrations (so that data will be usable for developing air quality standards and pollution control strategies) as well as by the principle that better models and more sophisticated measurements of effects will yield the most useful information. The epidemiologic findings associate air particulate levels with mortality in large populations, which is perhaps the least sophisticated measurement of biologic effects. To increase the sensitivity of this measurement in studies with laboratory animals, higher than ambient concentrations of particles are needed in addition to animal models of disease. Mortality is unlikely to be observed in studies involving the acute exposure of a limited number of normal laboratory animals. The representativeness of humans or animals used in experimental studies is important because the persons affected in the epidemiologic studies are likely to have included those compromised by age, disease, or other factors. Humans and animals in experimental studies need to represent groups sensitive for these effects. Both chronic bronchitis and coronary heart disease have been identified as important preexisting conditions (Dockery et al 1989, 1993; Schwartz 1994a; Schwartz and Morris 1995) and are used in these studies as models of disease to increase the responsiveness of animals to the inhaled particles.

Potential Mechanisms of Morbidity and Mortality

Many epidemiologic studies associate preexisting inflammatory lung diseases with adverse effects of airborne particulates. Recent investigations suggest a primary pathogenetic role for inflammation in exacerbations of pulmonary disease and focus upon the presence of neutrophils in symptomatic chronic airway disease (Mullen et al 1985; Thompson et al 1989). The hypothesis, that airway function is modulated by inflammatory cells activated and sequestered within the lung, has become especially attractive in relation to both asthma and chronic bronchitis. Two studies (Hutson et al 1990; Pauwels et al 1990) have shown that neutrophil accumulation and increased airway responsiveness in asthma are correlated. The presence of pulmonary inflammation is a clear starting place to assess changes associated with morbidity and mortality, and it is one for which there is evidence of acute changes with inhalation of particles. Thus, in Figure 1, emphasis is placed upon initial responses of increased reactive oxygen species as stimulators of inflammation (Shi et al 1996a,b); they may also stimulate neural receptors to initiate autonomic responses.

The focus of our studies on interleukin-8 (IL-8) and platelet factor-4 family cytokines as important mediators of inflammation is based upon the biologic properties of these peptides (reviewed by Baggiolini and colleagues 1989). The IL-8 gene has been cloned and sequenced in humans (Lindley et al 1988; Peveri et al 1988) and in dogs (Ishikawa et al 1993). Cross reactions of dog IL-8 with antibodies specific for human IL-8 have been reported, as have canine responses to recombinant human IL-8 reagents (Thomsen et al 1991; Jorens et al 1992; Meurer et al 1993; Zwahlen et al 1994). Interleukin-8 is primarily a neutrophil chemotactic factor that induces activation of the motile apparatus and directional migration of neutrophils, expression of surface adhesion molecules, release of stored enzymes, and production of reactive oxygen metabolites (Peveri et al 1988; Thelen et al 1988; Detmers et al 1990; Kunkel et al 1991). Interleukin-8 has limited reactivity with cell types other than neutrophils and appears to be relatively resistant to inactivation by plasma proteases (Peveri et al 1988). These features, plus the fact that it is a product of many cell types including lung macrophages (Strieter et al 1990), pulmonary epithelial cells (Standiford et al 1990) and fibroblasts, make this cytokine very likely

to play a role in exacerbations of chronic bronchitis associated with noxious stimuli (Lloyd and Johnston 1993).

A number of expression studies from our laboratory illustrate the role of IL-8 family cytokines, the cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF) in response to environmental agents (Kobzik et al 1993; Frevert et al 1995a,b). In the studies reported here, we examined the roles of IL-8, TNF, and IL-1 in morbid responses to air particles in the lung. We also studied the extent to which systemic inflammation occurs as indicated by the WBC count and cell differential of the peripheral blood. The potential role of cytokines in direct effects on cardiac cells, as hypothesized in Figure 1, is discussed in the next section.

Cardiac Responses to Inhaled Particles

Sudden cardiac death, which claims more than 350,000 lives annually in the United States, results from abrupt disruption of heart rhythm, primarily in the form of ventricular fibrillation. Death occurs not from extensive cardiac injury but rather from transient triggers that impinge on the electrically unstable heart (Lown and Verrier 1976; Corbalan et al 1976; Corr et al 1986; Zipes and Miyazaki 1990). Identification of individuals at risk for sudden cardiac death is a major objective in cardiology. Electrical instability can be triggered by autonomic mechanisms or by inflammatory mechanisms (Figure 1). Quantifications of HRV and T wave alternans are novel approaches to defining vulnerability to fatal cardiac events. Consistent changes in HRV and the occurrence of T wave alternans have been observed prior to fibrillation under diverse conditions, including coronary artery occlusion, hypothermia, Prinzmetal's vasospastic angina, and the long QT syndrome (Schwartz and Malliani 1975; Rozanski and Kleinfeld 1982; Schwartz 1985; Smith et al 1988; Schwartz et al 1991; Rosenbaum et al 1994). T wave alternans is a measure of beat to beat fluctuation in the magnitude and shape of the T wave. The magnitude of this fluctuation provides a means for assessing vulnerability to ventricular fibrillation. Another useful ECG assessment is the ST segment, the portion of the electrocardiographic wave form between the S and T waves. When the level of this segment is either elevated or reduced below baseline, it provides an indication of heart injury or inadequate oxygen supply. The QT interval, the duration between the Q and T waves, provides a measure of altered cardiac repolarization. Individuals who have congenitally prolonged QT intervals have a marked risk for cardiac death due to arrhythmia.

Early studies showed that psychological stress may have profound influences upon ventricular arrhythmias during myocardial infarction in the conscious dog (Corbalan et al 1974). Recent studies have solidified the importance of T wave alternans as an index of vulnerability under a variety of conditions (Verrier and Nearing 1994), including ischemia (Nearing et al 1994). Adam and coworkers (1984) showed a correlation in overall energy of the T wave and the ventricular fibrillation threshold during coronary artery occlusion and hypothermia in dogs. In the studies reported here, we employ newly developed signal processing techniques that permit simultaneous tracking of rapid changes in autonomic nervous system activity by HRV assessment and of cardiac vulnerability by complex demodulation and T wave alternans (Nearing et al 1991, 1994). These techniques are critically important in determining involvement of autonomic nervous system mechanisms in response to particle inhalation.

The relation of local or systemic inflammation, as mediated via chemokines and cytokines, to HRV and T wave alternans has not been studied. It is now well known, however, that levels of TNF (Basaran et al 1993; Strieter et al 1993; Vaddi et al 1994), IL-1 (Blum et al 1994), and IL-8 (Abe et al 1993) increase during cardiac ischemia and myocardial infarction. IL-1 can also activate the sympathetic nervous system (Haefeli et al 1993). Similarly, myocardial dysfunction in sepsis is related to elevated TNF (Cunnion and Parillo 1989; Giroir et al 1994). These cytokines are also correlated with myocardial dysfunction in long-standing heart failure (Mann and Young 1994). In vitro effects of cytokines on isolated myocardial cells have been studied in a number of systems. Decreased contractility resulted from the presence of TNF (DeMeules et al 1992; Finkel et al 1992; Yokoyama et al 1993); IL-1 prolonged action potential duration (Li and Rozanski 1993). Both IL-1 and TNF induced arrhythmias in cardiac cells in vitro (Weisensee et al 1993). Thus, relations of the cytokines and chemokines shown in Figure 1 with myocardial abnormalities have been observed. Our direct study of inflammation as a result of CAPs inhalation and our sophisticated analyses of potential harbingers of fatal cardiac arrhythmia focus directly on the question of death resulting from inhaled urban particles.

HRV, the primary measure of cardiac activity analyzed in these studies, has been shown to have implications in survival and to be a very useful measure of sympathetic and parasympathetic influences on the heart (Ewing et al 1981; Lombardi et al 1987; Dougherty and Burr 1992; van Ravenswaaij-Arts et al 1993; Bigger and Schwartz 1994). Neural control of the heart is very important in the cardiac response to many stimuli (Levy and Warner 1985; Janse 1990). Figure 2 outlines current concepts of autonomic influence on the heart and the implications for mortality and morbidity. Briefly, influences of the sympathetic nervous system increase heart rate and decrease HRV. When

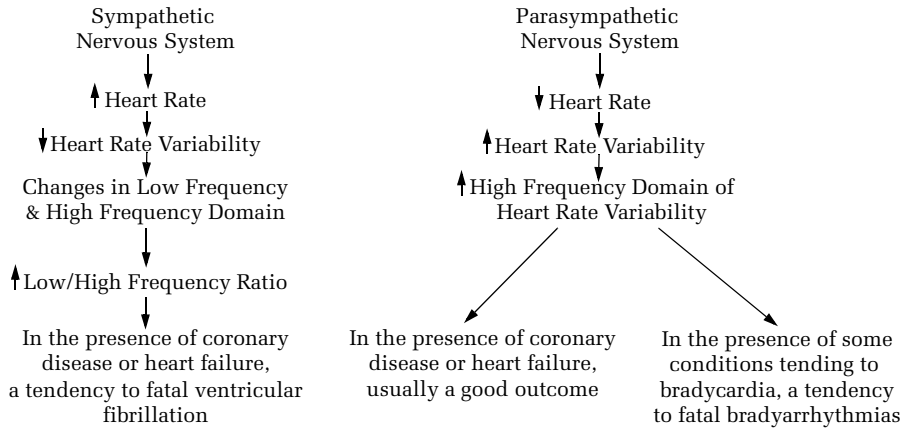


Figure 2. Influences of the autonomic nervous system on cardiac pathophysiology.

this decrease in HRV occurs in the presence of coronary heart disease (De Ferrari et al 1991; Mortora et al 1997), myocardial infarction (Adamson et al 1994; Moser et al 1994), or congestive heart failure (Kollai et al 1994; Butler et al 1995), the tendency toward a poor outcome increases, specifically toward tachyarrhythmias and ventricular fibrillation (Lown and Verrier 1976; Lombardi et al 1983; Magi et al 1983; Valkama et al 1995; van den Berg et al 1997). Indeed, therapy is often directed toward decreasing the sympathetic influence on the heart as a means to increase survival. This decrease of sympathetic influence results in parasympathetic predominance, which is characterized by decreased heart rate and increased HRV; and in the presence of coronary disease, these changes favor a good outcome (Moser et al 1994; Tsuji et al 1994). However, markedly increased parasympathetic stimulation in the presence of conditions such as long QT syndrome (either congenital or acquired) (Viskin et al 1996), poisonings with various materials (Ludomirski et al 1982), and other conditions (Blattberg and Levy 1969; Deutschman et al 1994) can worsen cardiac status and result in a fatal bradyarrhythmia. Techniques for measuring HRV thus have the potential to provide critical insight in studies of arrhythmogenesis due to particle inhalation and responses to inhalation of CAPs.

Among inhaled particle effects on the heart mediated by neural mechanisms, as outlined on the left side of Figure 1, the pulmonary chemoreflex is the one known response most likely to be involved. This response is mediated by irritant activation of sensory nerves in the laryngeal, tracheobronchial, or alveolar regions of the lungs and consists of apnea followed by tachypnea together with bradycardia and systemic hypotension (Coleridge and Coleridge 1994). Pulmonary chemoreflexes, initiated from sensory nerves in the bronchial and alveolar regions of the lungs (Coleridge and Coleridge 1984), are activated by nonspecific effects of sensory nerves. At low concentrations of irritant, these impulses are transmitted centrally via the vagal nerves (Coleridge et al 1993) and centrally processed in the medulla and the cerebral cortex (Coleridge and Coleridge 1994; Jansen et al 1995). The efferent neural responses include changes in breathing frequency: apnea at low doses of irritants and rapid shallow breathing at higher doses. Neural drive to the heart causes a parasympathetically induced decrease in heart rate. The release of acetylcholine from efferent nerves in the systemic vasculature may cause dilation of the vessels in the bronchi, skeletal muscle, and myocardium (Coleridge and Coleridge 1994). This vasodilation, together with decreased heart rate, may result in a marked decrease in systemic arterial pressure (reviewed by Yeates 2000). Thus, in addition to the parasympathetically induced bradyarrhythmia, vasodilation or other potent effects of acetylcholine may play an important mechanistic role as well.

Potential mechanisms by which sympathetic stimulation might occur from the inhalation of particles are less clear. Nonspecific sympathetic stress responses have been described (Gilman et al 1990). Increasing sympathetic tone with cardiac disease has the potential to exacerbate tachyarrhythmias and ventricular fibrillation (Figure 2).

SPECIFIC AIMS

TO ASSESS MORTALITY AND MORBIDITY FROM EXPOSURE TO CONCENTRATED AMBIENT PARTICLES USING NORMAL ADULT DOGS

Dogs were exposed, two at a time, for six hours per day on three consecutive days. Dogs were chosen as the laboratory animal because their cardiac responses, especially T

wave alternans, are very well characterized and are strikingly similar to those of humans. To determine whether mortality and morbidity are related to CAPs exposure, we used two different experimental designs. In the first, both dogs were CAPs- or sham-exposed at the same time; BAL and biopsies were performed after the third day of exposure (subsequently referred to as the double CAPs/double sham design). This design required longer intervals between exposures and was used primarily for assessment of pulmonary inflammation. It allowed for comparison of repeated measures on the same dog but lacked a contemporaneous control. In the second design, one dog received a CAPs exposure while the chambermate received a sham exposure; in the following week the dogs were switched and each received the other exposure (subsequently referred to as the crossover design). With this design, the CAPs-exposed dog could be compared to both the sham-exposed chambermate as well as to its own sham exposure.

These protocols allowed us to monitor several parameters: (1) the ECG continuously, including HRV patterns and the specific pattern of T wave alternans that is a known precursor of fatal ventricular fibrillation; (2) pulmonary mechanical function throughout the daily exposure to detect subtle changes in breathing associated with dyspnea or increased stress; and (3) BAL and transbronchial (TB) biopsy parameters at the end of the third day of exposure to detect evidence of a pulmonary inflammatory response. We attempted to define cellular and exudative inflammatory responses; the proinflammatory chemokine, IL-8, produced by macrophages and lung structural cells, as the primary mediator of acute inflammation; and the roles of IL-1 and TNF, two well-known proinflammatory cytokines with systemic effects. Pathologic changes at death were assessed in all animals with emphasis on morphologic quantification of pulmonary and cardiac pathologic changes.

Hypotheses tested in this specific aim were:

- Normal dogs will not die from exposure to CAPs, but they may have changes in HRV or T wave alternans associated with fatal arrhythmias.
- In comparison to filtered-air sham-exposed dogs, CAPs dogs will have statistically significant changes in pulmonary mechanical measurements including rate, volume, and flow parameters.
- Changes in BAL and TB biopsy parameters indicative of inflammation (including increases in neutrophils and measurements of proinflammatory cytokines and chemokines) are expected to be significant.

TO DEFINE ROLE OF PREEXISTING INFLAMMATION IN INCREASED MORTALITY AND MORBIDITY FROM CONCENTRATED AMBIENT PARTICLES USING DOGS WITH CHRONIC BRONCHITIS

Our hypothesis was that in the presence of preexisting inflammation, responses to inhaled particles are potentiated and result in increased pulmonary inflammation, release of cytokines that exert systemic effects, and changes in cardiac rhythm associated with fatal arrhythmia. Exposure and evaluation protocols were the same as described for specific aim 1. The disease model of chronic bronchitis in the dog was produced by high-dose exposure to sulfur dioxide for 4 to 6 weeks and has been used and characterized in our laboratory. After consultation with Health Effects Institute personnel, this specific aim was not fully pursued in these studies. Therefore, findings of this specific aim are not presented in this report.

TO DEFINE ROLE OF CORONARY ARTERY DISEASE IN INCREASED MORTALITY AND MORBIDITY FROM AMBIENT PARTICLES

Our hypothesis was that cardiac mechanisms are the cause of death from exposure to airborne particulates and that alterations in cardiac rhythm due to exposure are potentiated in the presence of cardiac ischemia. Exposure protocols used the design and evaluation protocols described in the first specific aim. Cardiac ischemia was produced by mechanical occlusion of the LAD coronary artery.

METHODS IN NORMAL DOGS

SUMMARY OF PROTOCOL

1. Dog is purchased from an approved supplier.
2. Initial veterinary workup included complete blood count (CBC) and differential WBC, veterinary physical exam, blood chemistry profile, microfilaria (heartworm) screen, and stool test for ova and parasites (all had to be within normal limits to proceed).
3. Initial behavioral assessment involved finding a compatible chambermate among several available dogs.
4. Dog had surgery for tracheostomy, baseline BAL and TB biopsies.
5. Two-week postoperative recovery included daily veterinary care of surgical sites, monitoring temperature, and clinical assessment. Sutures were removed. Dogs began wearing denim jackets to protect surgical sites. These were worn throughout all subsequent events.

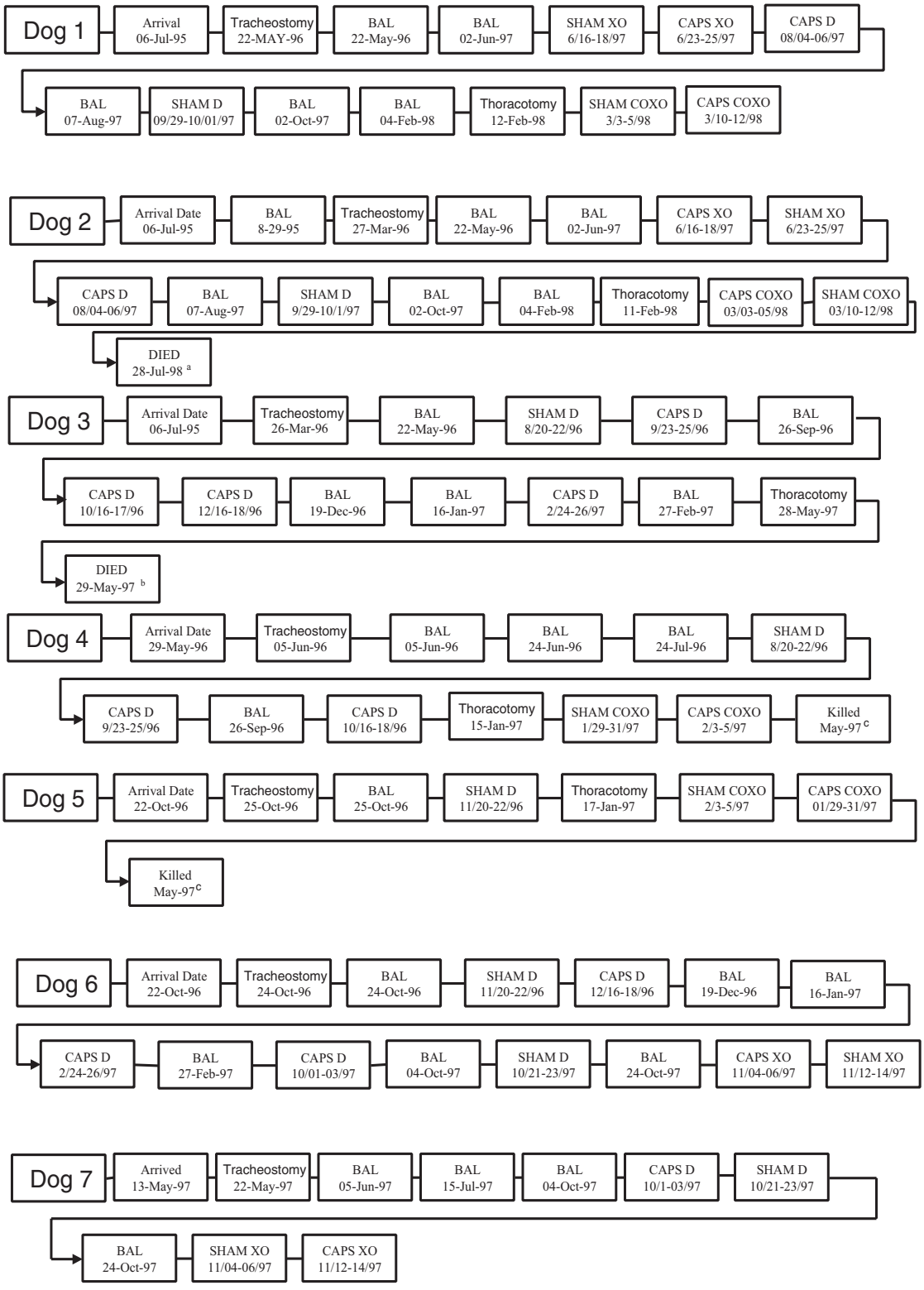
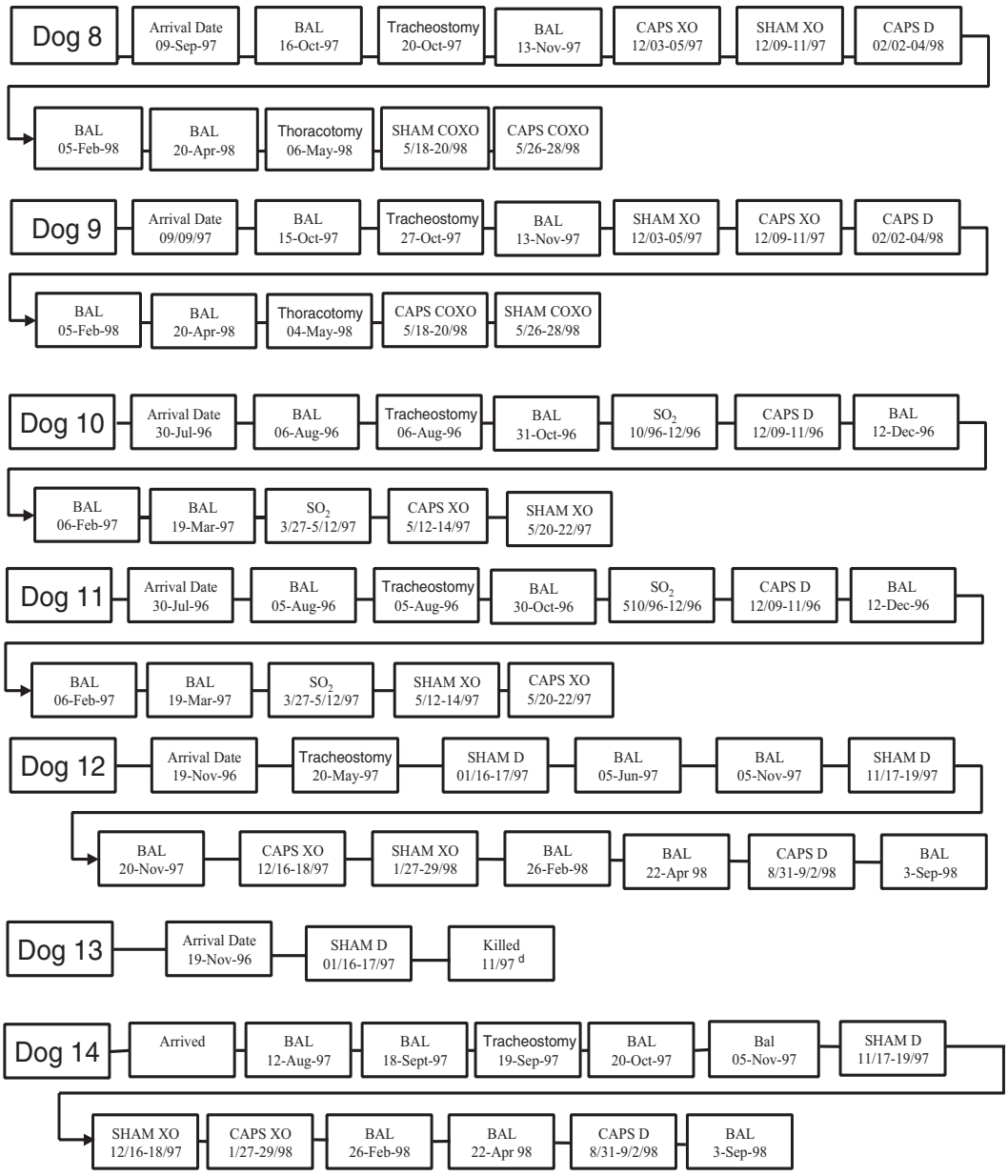


Figure 3. Timeline for each dog used in these studies. Exposure code: D = double; XO = crossover; COXO = coronary occlusion crossover.



[a]This dog died unexpectedly at night four months after the last experiment on a day when ambient outdoor pollution was very high. There was no evidence of respiratory abnormality at autopsy, and no evidence of any acute myocardial infarction. This dog was presumed to have died of arrhythmia. No specific evidence relates this dog's death to either the previous exposure or any particular condition or event on the date of death.

[b]This dog died postoperatively after thoracotomy. During thoracotomy, there was concern that a solution had been mislabeled, and this solution may have caused acute lung injury, which was the cause of death.

[c]These dogs were killed after coronary occlusion studies. Autopsy revealed chronic inflammatory reactions associated with implanted hardware. Based on these findings, subsequent occlusion studies implanted only the balloon occluder.

[d]This dog appeared healthy on arrival and during initial studies. However, it began to lose weight and failed to thrive. Complete veterinary assessment and autopsy did not uncover the cause of this dog's problem. It was not used in any experiments as soon as weight loss was noted.

Figure 3 *(continued)*. Timeline for each dog used in these studies. Exposure code: D = double; XO = crossover; COXO = coronary occlusion crossover.

6. Weeks 3 through 5: Chamber training began and involved bringing dogs to the exposure lab, acclimating them to each other, to the chamber environment, and to the chamber equipment, and connecting them to equipment for progressively longer time periods.
7. Week 5: Temporary needle electrodes placed for ECG recording.
8. Week 6: Exposure to concentrated air particles on three consecutive days, six hours per day. The crossover exposure protocol was 1 dog sham, 1 dog CAPs, and subsequent week crossover. The double CAPs/double sham exposure protocol was both dogs sham or both dogs CAPs, with randomization into these protocols (that is, sham versus CAPs).
9. Exposure assessed in relation to exposure substance.

Particles

- Fine mass concentration for ambient and concentrated particles less than 2.5 μm in aerodynamic diameter ($PM_{2.5}$) by gravimetric analysis.
- Sulfate concentration for ambient and concentrated particles by ion chromatography (IC)
- Nitrate concentration for concentrated particles by IC
- Trace metals concentrations for concentrated particles by x-ray fluorescence (XRF)
- Elemental carbon (EC) and organic carbon (OC) concentrations for concentrated particles by thermal-optical reflectance
- Endotoxin analysis for concentrated particles by KLARE limulus amoebocyte assay
- Continuous (5-minute average) nonvolatile fine mass for concentrated particles using a tapered element oscillating microbalance (TEOM)
- Continuous (5-minute average) BC, a surrogate for EC, for concentrated particles by aethalometer
- Particle size distribution for ambient particle mass by micro-orifice impactor using gravimetric analysis
- Particle strong acidity for ambient particles using potentiometric techniques

Gases

- Carbon monoxide using TECO 48 nondispersive infrared monitor for ambient air

Other Parameters

- Chamber temperature and relative humidity
- Concentrator operational pressures and flows
- Meteorological data (ambient temperature, pressure, dew point, relative humidity, wind speed and direction, air mass trajectory)

10. Electrocardiographic data collected continuously during each CAPs or sham exposure at V_4 and V_5.
11. Electrocardiographic data analyses: heart rate variability; 1-minute average morphology T wave variability; and T wave alternans complex demodulation and special techniques.
12. Video monitoring of dogs during CAPs or sham exposures to determine position, activity and behavior of dogs at all times during exposures. Data correlated to ECG and pulmonary function analyses to eliminate changes not related to the exposure.
13. Pulmonary parameter data collected continuously during each CAPs or sham exposure. Parameters obtained: frequency, tidal volume, minute volume, flow rates, pause, and durations.
14. Blood studies before and after exposures: CBC and WBC differential; fibrinogen and other coagulation factors.
15. BAL and biopsy after the third day of exposure of double CAPs or double sham protocols only.
16. Repetitions of CAPs and sham exposures (× 2 or 3) on each animal after at least 3 weeks from previous exposure.

EXPERIMENTAL DESIGN

Tracheostomized dogs (n = 14) were exposed two at a time in the same chamber for six hours a day on three consecutive days. Three exposure protocols were used: (1) One dog was CAPs-exposed while the other dog was sham-exposed to filtered air; (2) both dogs were CAPs-exposed; and (3) both dogs were sham-exposed. All dogs went through each of these protocols at least once. Therefore, all dogs received multiple exposures to concentrated air particles. Figure 3 is a complete timeline for each dog used in these experiments. The timeline shows the date of arrival at Harvard School of Public Health, the date of tracheostomy, the sequence and dates of each exposure protocol applied to that dog, the dates of BAL procedures, and other pertinent information in relation to each dog.

The HAPC delivered CAPs to fully awake dogs via a tracheostomy tube. Figure 4 illustrates the concentrator, its connection to the exposure chamber, instrumentation for characterization of the exposures, and the parameters measured. Figure 4 illustrates two dogs in the chamber with one dog exposed to CAPs and the other to filtered air. Dogs were exposed in repeated three-day units in both the

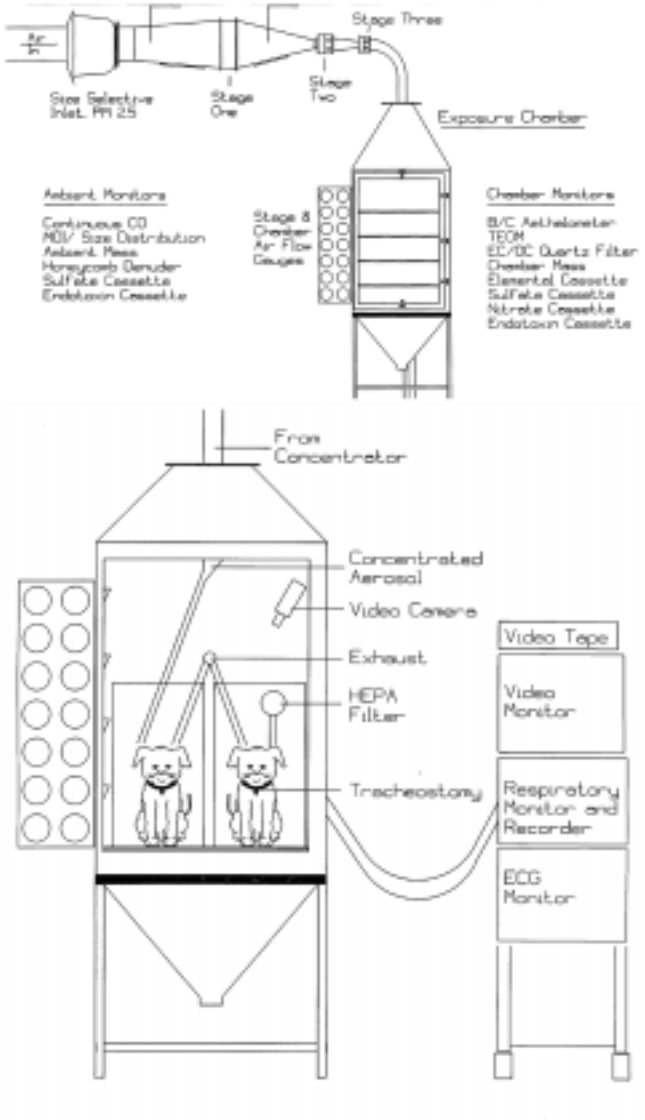


Figure 4. Harvard ambient particle concentrator, inhalation chamber, and particle monitoring methods (upper panel) and dogs in the chamber connected to the concentrator and physiologic monitors (lower panel).

double CAPs/double sham and crossover protocols. Assessment of the same dog in multiple exposures assured that the responses observed were the result of the exposure and not a peculiarity of the dog.

Studies using the crossover design compared exposure parameters to cardiac responses measured by electrocardiography and to pulmonary responses related to breathing parameters, including frequency, volumes, and flows. Studies using the double CAPs/double sham included exposure assessment and the cardiac and pulmonary measures noted above in addition to BAL to assess inflammation in the cells and fluid. Samples for peripheral WBC count and plasma fibrinogen level were taken before and after exposure on all three days.

PROCEDURES

Harvard Ambient Particle Concentrator

We developed the HAPC to concentrate respirable particles from ambient air. One of the major advantages of the HAPC system is that particles remain airborne throughout the concentration process, which allows delivery directly to an inhalation exposure system (Sioutas et al 1995a,b, 1997). The HAPC utilizes a series of virtual impactors that separate particles according to their aerodynamic size. Figure 5 illustrates the slit design used in the HAPC. A jet of particle-laden air is accelerated into a jet collection probe, which is slightly larger in diameter than the acceleration jet nozzle. Fine particles have enough momentum to cross the air streamlines and enter the jet collection probe, whereas ultrafine particles and gases follow the deflected air streamlines around the collection probe.

To keep the fine particles suspended in the collection probe, a fraction of the total flow is permitted to pass through the collection probe with the particles. This fraction of the airflow, typically 10% to 20% of the total, is termed the *minor flow*. The concentration of fine particles in the minor flow is enriched compared to ambient air by a factor of Q_{tot}/Q_{min}, in which Q_{tot} is the total flow entering the virtual impactor and Q_{min} is the minor flow. The minor flow of the HAPC virtual impactors, which have a 50% cutpoint of 0.1 μm, contains essentially all of the ambient mass because the mass fraction of particles smaller than 0.1-μm aerodynamic diameter in ambient air is negligible (Whitby et al 1972; McMurry and Zhang 1990; Hitzenberger and Puxbaum 1993). Ultrafine particles, defined as

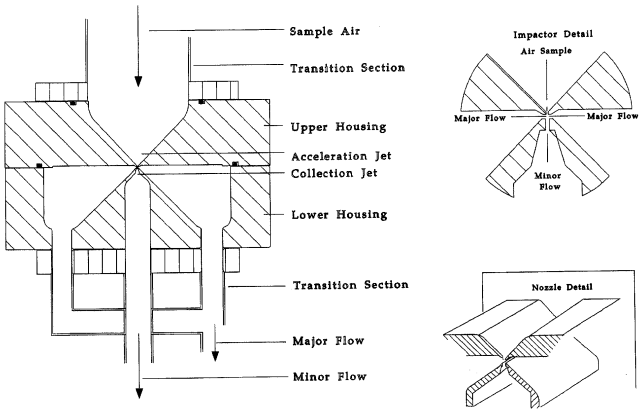


Figure 5. Slit virtual impactors used in the concentrator. (Adapted from Sioutas et al 1995b.)

particles with diameters smaller than 0.1 μm, are not likely to be concentrated by the HAPC system. However, although the ultrafine particles are not significantly enriched by the HAPC, neither are they excluded from the minor flow.

Like ultrafine particles, ambient gases are neither enriched by the HAPC nor excluded from the HAPC minor flow. The specificity of the HAPC minimizes potential synergistic effects with ambient gases in the exposure studies. The concentrations of nonreactive ambient gases such as CO are not altered by the HAPC; however, reactive components (such as ozone and nitric acid) are likely to be lost on the internal surfaces of the concentrator. In the case of nitric acid, loss may disturb the gas-particle equilibrium and possibly loss of particulate ammonium nitrate. Qualitatively, we estimate this problem to be minimal because residence time within the concentrator is short; in addition, volatilization should be slow enough to make exposure losses negligible. The relatively low particulate nitrate concentrations typical of northeastern US ambient air also reduce the potential impact of this process.

The ambient outdoor intake of the HAPC is mounted through the window of the exposure laboratory and is positioned approximately 1 m from the building. During the studies reported here, no particle sources were identified in the immediate vicinity of the inlet, which was approximately 100 m from Huntington Avenue. The HAPC consists of a preselective conventional impactor inlet, which removes particles larger than 2.5 μm in diameter, and a series of three virtual impactors, which gradually increase the concentration of airborne fine particles (Figure 3). The intake sampling flow rate is 5,500 L/minute. The size-selective inlet is a modified version of the Sierra-Anderson Hi-Vol PM_{10} inlet, which was designed to have a cutpoint of 10 μm at 1,100 L/minute. Increasing the average jet velocity of the inlet without altering the operating parameters of the impactor decreases the impactor cutpoint. The effective cutpoint of the inlet operating at 5,500 L/minute is approximately 2.8 μm (Sioutas et al 1997). Measurements of ambient particle size in our experiments indicate that nearly all of the ambient aerosols in Boston have a mass median aerodynamic diameter (MMAD) considerably below 2.5 μm and most are well below 1 μm.

From the inlet, the ambient aerosol is drawn through stainless steel ducts before it enters the first stage virtual impactors. The ducts have an inside diameter of 12 inches to minimize particle loss and maintain operating pressure. The first virtual impaction stage consists of five slit-nozzle virtual impactors, arranged in parallel, that operate at an intake flow rate of 1,100 L/minute each (5,500 L/minute total). As the minor-to-total flow ratio is 0.2, the concentrated flow of each impactor is 220 L/minute. The minor (concentrated) flows of each impactor in the first stage are combined and drawn through a plenum that serves as a transition to the second stage of the concentrator.

In the second stage of the HAPC, a single slit-nozzle virtual impactor, identical to those of the first stage, operates at an intake flow rate of 1,100 L/minute. The concentrated flow from this stage is drawn through a transition piece and finally through a third virtual impaction stage at an inlet flow rate of 220 L/minute. The minor/total flow ratio of the third stage is slightly lower than 0.2 so that the concentrated aerosol exits the third (final) stage at 45 L/minute. This provides a sufficient supply of concentrated particles for both characterization of the aerosol and animal exposure. From the third stage, concentrated particles are drawn through the tubes connected to dog tracheostomies and then ultimately to a pump maintaining the 45 L/minute flow. The dogs are inside an exposure chamber that operates at the same negative pressure as the concentrator output of the minor flow of stage three. The pressure at which the dogs breathe is similar to modest altitude elevation.

The concentrator operates optimally at 2.5 inches of water pressure drop per stage, or 7.5 inches of water for all three stages. In this study the negative pressure ranged from 7.5 to 25 inches of water, depending on the alignment of the slits of the virtual impactors and other parameters. Alignment is absolutely critical for optimal operation of the HAPC. In these studies, we designed a measuring gauge that limits misalignment of the slits to a maximum of 0.001 inch. When the slits are misaligned, the operating negative pressure increases, the concentration factor decreases, and excessive particle losses result from impaction of particles on the edges of the collection slit nozzle. (For this reason, some of the early exposures have mass concentration enrichment factors less than 20.) By improving the reproducibility of the alignment, operating negative pressures can be kept consistently low and the ease of maintenance and cleaning of the impactor stages improves.

Conditions other than misalignment can also result in increased particle losses in the collection slit nozzle, thus in larger negative operating pressures and lower concentration efficiencies. In particular, the combination of high ambient particle concentration with high relative humidity can lead to excess particle losses by deposition in the collection slit. Obstruction of the collection slit then increases the negative operating pressure of the stage three minor flow. The HAPC minor flow pressures thus were monitored carefully in these studies as they are the best indicators of concentration enrichment efficiency.

Physical and Chemical Characterization of Ambient Particle Exposures

A number of integrated and continuous sampling techniques were used to determine the concentration and composition of both ambient and concentrated particles (Table 1). For all integrated samples, the volumes were determined from flow measurements made using calibrated rotameters and sampling durations. Sampling flow rates for continuous instruments and dynamic zeroes were measured on a regular basis for QA/QC purposes.

Ambient Measurements Between the 2.5-μm size-selective inlet and the first stage of the concentrator, a small fraction of the ambient air is diverted to a manifold so that ambient air particles can be characterized. Physical and chemical characteristics were measured.

1. Fine particle mass ($PM_{2.5}$) and sulfate concentrations (both from the same filter) were assessed. Ambient fine mass was sampled on a 47-mm Teflon filter contained in a plastic filter-holder assembly. Samples were collected for the duration of exposure (usually 6 hours) at a sampling flow rate of 30 L/minute. Precise weighing of the filters before and after sampling yielded the net mass collected on the filter. Mass concentration was then determined from the net mass collected and the total volume of air sampled. Teflon filters used to collect the fine mass component were

Table 1. Summary of Physical and Chemical Characterization Methods Used for Ambient and Concentrated Particles for Animal Exposure Studies

Sampling Airstream	Sample Type	Parameter or Species	Sampling Technique	Analytical Method	Date Started	Estimated Precision	Estimated LOD	Reference (or Instrument)
Ambient air particles	Integrated	Mass concentration	Teflon filter	Gravimetric analysis	8/96	5%	2.4 μg/m^3	Cahn 31 or Mettler MT5 microbalance
		Sulfate	Teflon filter	Ion chromatography	8/96	5%	0.3 μg/m^3	Koutrakis et al 1988a, 1993
		Ammonium	Teflon filter	Ion chromatography	1/98	7.5%	0.2 μg/m^3	Koutrakis et al 1988a, 1993
		Particle size distribution	Micro-orifice impactor	Gravimetric analysis	10/96	10%	0.8 μg/m^3 per stage	Marple et al 1991
		Particle strong acidity (H^+)	Teflon filter	Potentiometry	8/97	7%	8 nmol/m^3	Koutrakis et al 1988b, 1993
Ambient air gases	Continuous	Carbon monoxide	Direct	Nondispersive infrared	6/97	0.1 ppm	0.1 ppm	TECO model 48
Concentrated particles	Integrated	Mass concentration	Teflon filter	Gravimetric analysis	8/96	5%	24 μg/m^3	Cahn 31 or Mettler MT5 Microbalance
		Soluble ions (SO_4^{2-}, NO_3^-, NH_4^+)	Teflon filter w/coated glass fiber for NO_3^-	Ion chromatography	8/96 SO_4^{2-} 3/98 NO_3^- 3/98 NH_4^+	5% SO_4^{2-} 4% NO_3^- 7.5% NH_4^+	3 μg/m^3 SO_4^{2-} 0.8 μg/m^3 NO_3^- 2 μg/m^3 NH_4^+	Koutrakis et al 1988a, 1993
		Elemental and organic carbon	Quartz fiber filters	Thermal and optical reflectance	9/97	3.1% OC 6.4% EC	5.3 μg/m^3 OC 1.8 μg/m^3 EC	Chow et al 1993
		Trace metals	Teflon filter	X-ray fluorescence	8/96	see Table 2	see Table 2	Dzubay and Stevens 1975
		Endotoxins	Nucleopore filter	KLARE limulus amoebocyte assay	Resumed 2/98	15.2%	0.26 μg/m^3	Milton et al 1992
	Continuous	Nonvolatile fine mass concentration	Direct	Tapered element oscillating microbalance	4/97	5–10% for 5-minute average	7 μg/m^3 for 5-minute average	Patashnick and Rupprecht 1991
		Black carbon	Direct	Aethalometer	5/97	5–8% for 5 minutes	500 ng/m^3 for 5 minutes	Hansen 1984

weighed before and after sample collection on an electronic microbalance (Cahn model C-31, Orion Research, Boston MA) or model MT5 C-31 (Mettler Toledo, Columbus OH). Standard operating procedure for this project specified that all filters be weighed twice before and twice after exposure to maximize the sensitivity of the gravimetric determination. In order to assure consistent values for mass, the filters were equilibrated in a room with controlled temperature (70 ± 5°F) and humidity (40 ± 5% relative humidity) both before (24 hours) and after (48 hours) sampling. To eliminate the effects of static charge, the Teflon filters were passed over ^{210}Po sources (alpha rays) immediately before each weighing. The limit of detection (LOD) for gravimetric analysis of ambient mass, based on reproducibility of weighing and field blanks, is 2.4 μg/m^3 for a 6-hour sample at 30 L/minute. Precision of the measurement is within 5%. Accuracy also is expected to be within 5% on the basis of flow measurement and mass measurement uncertainties.

After gravimetric analysis, these filters were then extracted and analyzed by IC using a conductivity detector for sulfate. Although the analytical method also was used for nitrate, the nitrate concentrations determined for this filter are not reported because the nitrate is likely to be volatilized from the Teflon filter during sampling. The analytical method was described in detail elsewhere (Koutrakis et al 1988a). The LOD for ambient sulfate analysis was 0.3 μg/m^3 with a precision of 5% on the basis of results from previous field studies.

2. Particle size distribution (30 L/minute) was characterized as MMAD and geometric standard deviation (GSD). The particle size distribution of ambient fine particle mass was sampled using a multiple orifice impactor (Marple et al 1991). Within the sampler, particles ranging from 15 to 0.1 μm were separated sequentially by a set of eight impaction stages with successively smaller jet diameters. Particles larger than 15 μm were removed on the first (filterless) stage, but not analyzed. The second stage collected particles from 3.2 to 15 μm; the third, 1.8 to 3.2 μm; the fourth, 0.56 to 1.8 μm; the fifth, 0.29 to 0.56 μm; the sixth, 0.18 to 0.29 μm; and the seventh, 0.09 to 0.18 μm. The last (eighth) stage was an after filter to collect all particles smaller than the collection limit of the seventh stage (0.094 μm). Because the HAPC provides a size cut of approximately 2.5 μm, particles collected on the first two stages were not analyzed. The use of multiple jets for the impaction stages decreased the pressure drop across each stage to a minimum, which reduced evaporation of water from hygroscopic particles and limited distortion of the size distribution of the ambient particles to no more than about 3%.

   Particles accumulated on 37-mm Teflon filter substrates mounted on the collection surface of each stage. Sampling was conducted for 18 hours (all 3 days of exposure) at a flow rate of 30 L/minute so that mass sufficient for gravimetric analysis, and perhaps x-ray fluorescence or other analysis at a later date, would exist on archived filters. On the basis of reproducibility of gravimetric analysis and results from field blanks, the LOD for gravimetric analysis was 0.8 μg/m^3 for each stage, sampling for 18 hours at 30 L/minute.

3. The strong acidity of ambient fine mass was measured using a honeycomb denuder filter pack system (Koutrakis et al 1993; Sioutas et al 1994) that includes an impactor inlet, two glass honeycomb denuders, and a filter pack with a Teflon filter. Air flows at 30 L/minute through the impactor inlet, which removes any large particles that have penetrated the HAPC size-selective inlet. These large, typically alkaline, particles must be removed because they could neutralize the acidic fine particles, causing an underestimation of fine-particle H^+. For the same reason, the air is routed through two honeycomb diffusion denuders coated with citric acid to collect ammonia. Fine particles pass through the denuders with negligible losses as they have both much smaller rates of diffusion to the walls than the gases and much less momentum than the large particles (Sioutas et al 1994). These fine particles are then collected on a Teflon filter.

   After the sampling period, Teflon filters are removed from the filter pack in an ammonia-free hood (to prevent neutralization of the collected fine particle acidity) and stored in an ammonia-free chamber until analysis. Samples are analyzed potentiometrically using a pH meter with a semimicro-combination electrode. The analysis is described in detail elsewhere (Koutrakis et al 1988a). The LOD for ambient particle strong-acidity measurement for 6-hour samples at 30 L/minute is 8 nmol/m^3 with a precision of 7%, on the basis of results from previous field studies.

   Filter extracts were subsequently analyzed for ammonium ion using IC with a conductivity detector. The analytical method is described in detail elsewhere (Koutrakis et al 1988b). The LOD for ambient particle ammonium measurement for a 6-hour sample collected at 30 L/minute was assumed to be 0.2 μg/m^3

with a precision of 7.5% based on results from previous field studies.

4. Ambient CO was measured using a continuous nondispersive infrared (IR) analyzer (TECO model 48), a US Environmental Protection Agency (EPA)–designated reference method for CO. The measurement principle is based on the characteristic infrared absorption of the CO molecule. The CO concentration is determined by comparison of the IR absorbance of ambient air to a reference cell. The CO monitor samples at 2 L/minute; instrument specifications list the LOD of the instrument at 0.2 parts per million (ppm) and precision as ± 0.1 ppm. On the basis of external instrument calibration, the CO monitor demonstrated an accuracy of 3% to 5% for all exposure studies.

Concentrated Particles Measurements Of the 45 L/minute available from the HAPC output flow containing concentrated fine particles, 12 L/minute were diverted to a manifold for analytical measurements. The remaining 33 L/minute were available for animal exposures (Figure 4). The physical and chemical measurements on concentrated particles included both integrated and continuous measurements.

1. Fine particle mass, sulfate, and nitrate concentrations were collected by and determined from one filter pack. Concentrated fine mass ($PM_{2.5}$) was sampled on a 47-mm Teflon filter contained in a plastic filter holder assembly. A carbonate-coated glass-fiber filter downstream of the Teflon filter collected nitrate volatilized from the collected fine particles. Samples were collected for the duration of exposure at a sampling flow rate of 3.0 L/minute. Concentrated particle mass collected on the Teflon filter was analyzed gravimetrically using the procedure described previously for analysis of ambient particle mass. The expected LODs for concentrated fine mass concentration were greater than for ambient mass concentration because the sample volume was smaller. The LOD for gravimetric analysis of concentrated fine mass, on the basis of reproducibility of weighing and field blanks, was 24 $\mu g/m^3$ for a 6-hour sample at 3.0 L/minute. Precision of the measurement was within 5%. Accuracy also was expected to be within 5% on the basis of flow and mass uncertainties.

   After gravimetric analysis, the Teflon filters were extracted and analyzed by IC using a conductivity detector for sulfate and nitrate. The filter pack's backup carbonate-coated glass-fiber filter was also extracted and analyzed for nitrate. The concentrated particle nitrate determinations from both filters provided the total nitrate concentration. Use and analysis of the backup filter allowed for correction of nitrate volatilization losses from the Teflon filter during sampling. The analytical method is described in detail elsewhere (Koutrakis et al 1988a). The LOD for concentrated particle sulfate analysis was 3 $\mu g/m^3$ with a precision of 5% on the basis of results from previous field studies. For concentrated particle nitrate, the LOD was estimated at 2 $\mu g/m^3$ with a precision of 4% on the basis of results from previous field studies.

2. Trace metal concentrations were sampled on a Teflon filter in parallel with the filter pack used for fine mass, nitrate, and sulfate and analyzed by XRF. With this method, x-rays are produced by ejection of an inner-shell electron from an atom in the sample, thereby creating a vacancy in the inner atomic shell. A higher-energy electron drops into the lower-energy orbital and releases a fluorescent x-ray to remove excess energy (Jaklevic et al 1977). The frequency of the x-ray, which is characteristic of the emitting element, unambiguously identifies the element. The intensity of the fluorescent x-ray at a given frequency is proportional to the number of atoms of the specific element present. The element's concentration can be determined by comparing that intensity directly with standards (Dzubay and Stevens 1975). A grounded anode diffraction-type x-ray tube with a molybdenum anode is the excitation source for XRF, and a high-resolution SiLi detector with pulsed optical feedback detects the x-rays, providing high count-rate capabilities.

   This method required no extensive sample preparation and was nondestructive and indifferent to the oxidation state of the element. The LODs estimated for measured 6-hour trace element concentrations are shown in Table 2. X-ray fluorescence analysis was performed by Chester LabNet (Tigard OR). Excitation conditions and count times for the protocol are also included in Table 2.

3. Elemental and organic carbon fine particle concentrations were collected as integrated 6-hour samples using two prefired quartz-fiber filters mounted in a honeycomb denuder filter pack. Quartz-fiber filters have a tendency to adsorb gas-phase organics, which causes the OC measurement to include adsorbed as well as aerosol-phase carbon. To reduce this artifact, an activated carbon paper denuder is used upstream of the quartz-fiber filters. Gas-phase organics are removed by the denuder, and particulate organics are subsequently trapped by the first quartz-fiber filter. The second, downstream, quartz-fiber filter traps OC that volatilizes from the first filter during sampling. The OC value measured on this downstream filter

Table 2. Limit of Detection and Precision of Trace Element Concentrations Analyzed by X-ray Fluorescence

Element	LOD[a] (μg/m^3)	Precision[b] (%)	Concentration[c] (μg/m^3)	Excitation Condition[d]
Na	0.249	11.3	1.17 ± 3.43	0
Al	0.049	18.6	1.46 ± 2.17	0
Si	0.037	6.4	4.66 ± 4.76	0
S	0.045	2.9	20.6 ± 18.7	1
Cl	0.051	4.5	2.04 ± 7.38	1
K	0.018	5.7	1.46 ± 1.21	1
Ca	0.016	2.5	2.06 ± 2.17	1
Ti	0.014	5.2	0.25 ± 0.39	2
V	0.013	11.5	0.098 ± 0.122	2
Cr	0.008	33.6	0.009 ± 0.010	2
Mn	0.012	8.2	0.091 ± 0.067	3
Fe	0.010	4.3	3.47 ± 2.93	3
Ni	0.006	11.6	0.07 ± 0.08	3
Cu	0.008	8.7	0.11 ± 0.07	3
Zn	0.006	4.6	0.38 ± 0.34	3
As	0.010	51.4	0.016 ± 0.020	4
Se	0.008	23.2	0.017 ± 0.024	4
Br	0.008	5.7	0.07 ± 0.07	4
Cd	0.058	—[e]	0.020 ± 0.027	5
Pb	0.021	10.9	0.14 ± 0.09	4
Ba	0.286	25.8	0.71 ± 0.33	4

[a] LODs 2Σ interference-free detection limits supplied by Chester LabNet, based on six-hour sampling at 3.0 L/min.

[b] Precision percentage based on replicate analyses.

[c] Mean ± SD of 104 total samples analyzed by XRF.

[d] Excitation Conditions: 0 = 240 sec livetime, Rh target, cellulose prefilter, voltage 7.5 kV, and 0.25 mA current.
1 = 720 sec livetime, Ti target, cellulose prefilter, voltage 25 kV, and 3.0 mA current.
2 = 720 sec livetime, Fe target, cellulose prefilter, voltage 35 kV, and 3.0 mA current.
3 = 480 sec livetime, Ge target, cellulose prefilter, voltage 35 kV, and 3.0 mA current.
4 = 960 sec livetime, Rh target, Rh prefilter, voltage 35 kV, and 1.50 mA current.
5 = 480 sec livetime, Rh target, W prefilter, voltage 55 kV, and 0.75 mA current.

[e] Cd was not above LOD in any of the replicate analyses.

allows a correction to be made of the particulate EC and OC measurements.

The Desert Research Institute analyzed EC and OC with a thermal and optical reflectance method (Johnson et al 1981; Chow et al 1993) that utilizes preferential oxidation of OC and EC compounds under different temperature and oxidation conditions to determine OC and EC from a small sample taken from the first quartz-fiber filter. Organic carbon compounds volatilize from the sample deposit in a helium atmosphere at low temperatures; EC remains on the filter under these conditions. These volatilized compounds, passed through heated manganese dioxide, are oxidized to carbon dioxide (CO_2). The CO_2 is reduced to methane (CH_4) when it flows through a hydrogen-enriched nickel catalyst. Finally, CH_4 equivalents are determined by a flame ionization detector (FID). Elemental carbon is removed from the filter at higher temperatures under more oxidizing conditions; subsequent analysis is identical to that for OC. Pyrolysis of OC compounds to EC is corrected by changing the optical reflectance of the filter during analysis. Without this correction, the OC fraction of the sample would be underestimated, and the EC fraction would include some pyrolyzed OC. The method is described in detail elsewhere (Chow et al 1993).

Samples were maintained at temperatures below 0°C before and after sampling and during shipping. On the

basis of field blanks, the LOD is 5.3 μg/m^3 for OC and 1.8 μg/m^3 for EC. Precision estimates, determined on the basis of root mean square error of replicate analyses, were 3.1% for OC and 6.4% for EC. Uncertainties for the method were below 10% for EC, OC, and total carbon (TC).

4. Endotoxin samples were collected using endotoxin-free filter packs prepared in Dr Donald Milton's laboratory at Harvard School of Public Health. The filter pack consists of a 2-μm Nucleopore filter and backing pad in a filter cassette. Analysis is performed using the KLARE Limulus Amoebocyte Assay of Milton and colleagues (1992).

   During January and February 1997, both ambient and concentrated particles were sampled for endotoxins. When analysis of these samples did not detect any endotoxins, sampling was discontinued. When sampling for endotoxins resumed in February 1998, concentrated particles only were sampled and analyzed.

5. Concentrations of CAPs nonvolatile fine mass were measured continuously (5-minute average) with a TEOM during exposures. The Patashnick and Rupprecht (1991) TEOM method provides continuous measurement of $PM_{2.5}$, and is designated by the EPA as an equivalent method for measurement of 24-hour mean PM_{10} for compliance purposes. This method is more sensitive than other EPA-approved continuous PM_{10} methods, does not use any radioactive sources, and provides a direct measurement of the particulate mass collected on the filter.

   The TEOM samples concentrated particles at a flow rate of 1.0 L/minute on a filter heated to 50°C. The filter, which is attached to the tip of a hollow, tapered, oscillating glass rod, accumulates the particles. Change of frequency of the oscillation is directly related to the change in mass on the filter over time. The method is fully described elsewhere (Patashnick and Rupprecht 1991). The TEOM filter is heated to eliminate interference from particle-bound water and to provide a stable and reproducible measurement of ambient mass without the semivolatile component.

   When the presence of semivolatile particulate mass in the atmospheric aerosol introduces difficulty and uncertainty in measuring atmospheric PM, even traditional integrated measurements of particulate mass can substantially underestimate the actual mass. Semivolatile or volatile components, such as liquid water associated with hygroscopic material (particle-bound water), ammonium nitrate in equilibrium with nitric acid and ammonia, and semivolatile organic compounds (SVOC), may account for a substantial fraction of the total particulate mass observed in certain locations. The amount of material in the particulate (that is, condensed) phase changes with the temperature and relative humidity and will decrease to maintain the equilibrium pressure of the gas phase components if the gas phase concentration decreases.

   The LOD of the TEOM for a 5-minute average mass measurement at a flow rate of 1 L/minute was approximately 7 μg/m^3 (based on three times the standard deviation of 5-minute concentration averages on zero air). The relative precision for a 5-minute measurement was estimated at less than 10%. Estimated accuracy of the method for measuring nonvolatile mass, on the basis of flow and mass transducer uncertainties, is within 5%.

6. Black carbon concentration, a surrogate of EC, was measured in real time with a model AE-14 aethalometer. The method is based on the optical attenuation of light by EC particles collected on a quartz-fiber filter tape at a sample flow rate of 0.75 L/minute. The light source is an incandescent bulb with an effective center wavelength of 820 nm. The overall optical attenuation decreases exponentially with filter loading and is calculated from the decrease in light transmission measured at the end of the cycle. The optical transmission of an unexposed portion of the filter is measured at the end of each cycle to control for drift in instrument parameters. The change in light attenuation from one measurement cycle to the next is reported as a linearized and scaled *attenuation unit*. The mean BC concentration during the measurement cycle then is determined from the attenuation units and sample volume data, using an internal, empirically derived conversion factor (19.2 m^2/g). Black carbon data from this instrument have agreed well with EC data in comparisons (Hansen and McMurry 1990; Allen et al 1999). The principle of the method is described in detail elsewhere (Hansen et al 1984).

   The LOD of the aethalometer for a 5-minute average BC measurement at a flow rate of 0.75 L/minute was approximately 500 ng/m^3, on the basis of three times the standard deviation of 5-minute concentration averages for zero air. The estimated relative precision observed for a 5-minute measurement was less than 10%. Estimated accuracy of the method for measuring EC, on the basis of comparison with integrated EC samples, was approximately 50% for an 8-hour average (R^2 = 0.96).

7. Continuous (hourly) weather data (including wind speed, direction, barometric pressure, outside temperature, and relative humidity) were obtained from

the National Weather Service at Logan Airport (Boston MA) via Weather Services Corporation (Lexington MA). Trajectory data were obtained from the National Oceanic and Atmospheric Administration Air Resources Laboratory archives, which include the ETA Data Assimilation System (EDAS). At the laboratory's Web site (www.arl.noaa.gov), the Hysplit model was used to determine air mass trajectories to Boston for the preceding 96 hours.

Animals and Their Preparation

Purchase of Dogs All dogs were purchased from Butler Farms (USDA #21-A-003; Clyde, NY), a US Department of Agriculture–approved breeding facility for purpose-bred dogs for research. The dogs, retired breeders less than 5 years old, were mongrel intact females weighing between 14 and 17 kg. The dogs were vaccinated for canine distemper, parvovirus, adenovirus type 2, hepatitis, parainfluenza, leptospirosis, and rabies; they also were dewormed routinely and received a clinical exam, an ophthalmic exam, and an ECG at Butler Farms. We received a medical record for each dog. The dogs were shipped from Butler Farms to Harvard School of Public Health by air.

All protocols that involved the dogs in this study were reviewed and approved by the Harvard Medical Area Standing Committee on Animals.

Initial Clinical Evaluation of Dogs Dogs were evaluated for their suitability for the study with respect to size, weight and body condition, temperament, and health. Suitable dogs were between 16 and 18 inches tall at the shoulder. Dogs that appeared thin were monitored for weight gain for two to three weeks; if they failed to gain weight during this period, they were not used in the study. Dogs chosen for this study were good natured and tolerated extensive manipulation by humans; they could not be excessively aggressive toward other dogs because they lived and worked in pairs. The comprehensive veterinary physical exam (Jones 1994) included body temperature, cardiac auscultation, heart rate and rhythm, pulse, respiratory rate, auscultation of lung fields, peripheral lymph node palpation, and abdominal palpation; if any serious abnormalities were detected (such as evidence of respiratory, cardiac, neoplastic, or systemic disease) the animal was eliminated from the study.

Initial Laboratory Evaluation of Dogs Clinical diagnostic laboratory studies were carried out through Tufts Veterinary Diagnostic Laboratory (Grafton MA) on all dogs upon entry into the study: (1) CBC and differential; (2) chemistry profile, including glucose, blood urea nitrogen, creatinine, calcium, phosphorous, total protein, albumin, globulin, albumin/globulin ratio, sodium, chloride, potassium, carbon dioxide, anion gap, total bilirubin, alkaline phosphatase, γ-glutamyltransferase, alanine aminotransferase, aspartate aminotransferase, cholesterol, triglycerides, and amylase; (3) microfilaria screen; (4) occult heartworm test; and (5) fecal examination for ova and parasites. Abnormalities in the blood count and chemistry were assessed clinically, and the test or tests were repeated to rule out laboratory error. Treatable abnormalities with no implications for the experimental studies were treated. Serious abnormalities or those with direct or indirect implications for the experimental studies caused elimination of the animal from the study. For example, a positive occult heartworm test resulted in retesting to rule out the possibility of a false positive result, but a second positive test eliminated the animal from the study. If the fecal study revealed parasitic ova, the dog would be treated with the appropriate anthelmintic medication.

Behavioral Assessment All experiments were performed using pairs of dogs, and each pair was housed in a single run. Therefore, these pairs had to be compatible both in the runs and in the exposure chamber. Dogs were placed in pairs at or shortly after their arrival at Harvard School of Public Health facility. When possible, dogs of similar size and general temperament (for example, very excitable versus calm, rough versus gentle, active versus quiet) were placed together. The behavior of the dogs in the runs was evaluated by the project veterinarian and veterinary technician. Dogs were judged to be incompatible and were regrouped if one dog was excessively aggressive or fearful in interactions with its partner. Signs of aggression included outright fighting, offensive growling, snapping, aggressive body posture (rigid stance, stiff wagging of tail tip, erect or arched neck), and piloerection. Signs of fear included tail between the legs, belly-up position, submissive urination, low or cowering body position, trembling, avoidance behavior, averted gaze, and ears back against the head (Overall 1997). Mild levels of aggression were tolerated, but continuous high levels of aggression or fear warranted regrouping of dogs. A mild level of aggressive behavior was normal during the first weeks while the pairs established a dominance hierarchy. After the dogs were acclimated in the runs, chamber training began and their behavior was evaluated in the chamber. No aggression or fear was accepted in this setting because the associated autonomic nervous system stimulation could interfere with the experimental results.

Chamber Training Dogs were gradually acclimated to the exposure chamber over the 2 to 4 weeks before their

first exposure. Dog comfort determined the rate of training. Subjective evaluation of dog comfort was made by a veterinarian. Trembling, panting, restlessness, a hunched body position, and a fearful or worried expression of the eyes were all used as indicators of discomfort and reason for attention. Time spent sleeping, relaxed body posture and facial expression, and greeting behavior toward caretakers outside the chamber were interpreted as indicators of dog comfort. Pairs of dogs remained together throughout in training sessions and living quarters. At all steps in the training process, the dogs were rewarded with food treats and attention.

Initially dogs were brought to the inhalation laboratory in the dog carrier and allowed to interact with each other and with their caretakers. The length and number of these sessions varied: usually two or three sessions lasted one to four hours. Next, the dogs were placed in the chamber for one to two hours; the chamber time was gradually extended up to six hours. Then, a tracheostomy tube (6 or 7 mm inner diameter depending on size of animal and of tracheostomy stoma) was set in place for one to two hours during a chamber session without inflating the cuff. In the next stage, the cuff of the tracheostomy tube was inflated and left in place for four to six hours during a training session. The next advance in training was to turn on the minor flow pump and attach the inspiratory and expiratory breathing tubes to the tracheostomy tubes. The breathing tubes were fastened around the dog's neck and taped together in the back such that the two tubes encircled the neck. The animals were kept in the chamber for two to three hours; the time was increased gradually to a full six hours. All steps were repeated until the dogs appeared completely relaxed in this setting.

Surgical Procedures

In normal dogs, procedures include tracheostomy, BAL and biopsy, and placement of electrodes.

Anesthesia Dogs were fasted a minimum of ten hours prior to anesthetic induction. Premedication and induction anesthesia, an intramuscular mixture of atropine (0.04 mg/kg), ketamine (10 mg/kg), and xylazine (1.5 mg/kg), was administered either in the dorsolumbar musculature or in the semitendinosus muscle. The induction agent had taken effect when the dog reached a level of deep sedation or light surgical anesthesia. Heart and respiratory rates, as well as anesthetic depth, were monitored at five-minute intervals during this period. In tracheostomized dogs, the endotracheal tube was inserted through the tracheostomy stoma; in untracheostomized dogs, it was inserted through the oral cavity and larynx. The endotracheal tube cuff was inflated. Anesthesia was maintained with halothane gas administered via a semiclosed rebreathing system (using an OHIO Heidbrink Kinet-o-meter anesthesia machine (Ohio Medical Products, Madison WI). Oxygen flow was set at 800 to 1,000 mL/minute until the animal reached the desired level of anesthesia and then was reduced to 400 to 500 mL/minute for maintenance. The dog was maintained at the appropriate level of anesthesia by adjusting the percentage of inhaled halothane (between 0% and 4%). At 5-minute to 15-minute intervals, several deep breaths were administered. Paralube ophthalmic ointment was put in both eyes.

During all general anesthesia procedures, the following parameters were monitored in each dog: heart rate by pulse oximeter (Nellcor N-20 PA, Puritan Bennet, Pleasanton CA); hemoglobin oxygen saturation by the pulse oximeter and maintained above 90%; ECG via three needle electrodes inserted in the left front, right front, and left rear limbs with tracings displayed continuously; mucous membrane color and capillary refill time (maintained under 3 seconds); palpebral reflex; eyeball position; jaw tone; movement; and rectal temperature (the dogs were routinely kept on a warm water heating pad during anesthesia). Perioperative analgesia (butorphanol 0.2 mg/kg) was given intramuscularly as needed. A perioperative antibiotic, enrofloxacin (Baytril), was given intramuscularly in the dorsolumbar or semitendinosus muscles at the outset and every two hours during tracheostomies, BAL and biopsy. Intravenous fluids (lactated Ringer's solution, 10 mL/kg/hour) were administered via an intravascular catheter in a cephalic vein during procedures lasting more than 1.5 hours.

Recovery Dogs that had been on halothane anesthesia were allowed to breathe 100% oxygen for 5 to 15 minutes. The endotracheal tube was removed as soon as the dog began to swallow. The animals remained on a heating pad or were covered warmly until their rectal temperature returned to 100°F or until they stood up and began to wander.

Bronchoalveolar Lavages and Transbronchial Biopsies These procedures were performed before the tracheostomy, between the tracheostomy and the first exposure, and before and after double CAPs or sham exposures. Bronchoalveolar lavage was performed with the dog under full anesthesia. When the dog was heavily sedated, an endotracheal tube was inserted through the mouth and larynx or directly through the tracheostomy stoma (if one was present). Halothane gas was administered at a concentration that maintained the animal at a light surgical level of anesthesia. The dog was given intramuscular butorphanol (0.2 mg/kg) in the dorsolumbar or the semimembranosus/

semitendinosus muscles before the onset of lavage to suppress the cough reflex. The bronchoscope (Olympus BF type 1T with a light source Olympus CLE-3 from Olympus Optical Company, Tokyo, Japan) was inserted through the endotracheal tube and into the right mainstem bronchus and into the right lung until it became wedged in a small airway. Dulbecco 1X phosphate-buffered saline (50 mL) (Sigma Chemical, St Louis MO) without calcium or magnesium ions was used for each lavage.

The bronchoscope was advanced and retracted only slightly (several millimeters) during this procedure so that the same position was maintained in the lungs throughout the lavages. Lavage fluid was aspirated from the lung into the vacuum trap on the bronchoscope and was transferred into a 50-mL conical centrifuge tube. The first lavage was kept separate; subsequent lavages were combined. A sample of the first lavage was submitted to the Tufts Veterinary Diagnostic Laboratory for aerobic microbial culture and sensitivity testing. As soon as the first lavage was completed, the dog was given an intramuscular dose of enrofloxacin antibiotic (2.5 mg/kg) in the dorsolumbar muscles. Lavage was repeated six to nine times until a minimum of 200 mL of lavage fluid was retrieved. The second and subsequent lavages were pooled in 50-mL centrifuge tubes and kept on ice until they were processed. The total volume of fluid retrieved was recorded. The bronchoscope was not withdrawn between lavages.

For biopsies, the bronchoscope was reinserted to the level of the carina. A serrated, radial-jaw biopsy forceps (Microinvasive, Boston Scientific Corporation, Watertown MA) was passed down the instrument channel just beyond the end of the scope. The radial jaws were opened and a piece of mucosa grasped from a point just distal to the carina in the left mainstem bronchus. The biopsy was repeated until two or three specimens (1 to 2 mm^3) were obtained from the airway. After adequate airway specimens were obtained, the scope was advanced down the right mainstem bronchus, and as far into the small airways of the right lung as possible; the biopsy procedure was repeated at this location until two or three samples of the lower airway and parenchyma were obtained.

Tracheostomies These procedures were performed no sooner than two weeks after arrival of the dogs at our facility, and no later than three weeks before the first exposure sessions. Chronic tracheostomies in dogs facilitate exposure to both gaseous and particulate air pollution, permit continuous monitoring of breathing parameters during an exposure, and furnish access for BAL without upper airway trauma. Chronic tracheostomies were surgically created in each dog by the method of Dalgard and colleagues (1979) as further refined by Nelson (1993). Drazen and associates (1982) have reported that tracheostomy per se does not affect airway mechanics.

The tracheostomies were performed under general anesthesia as described above. After the animal reached a state of heavy sedation or light anesthesia, the surgical site (centered on the ventral midline of the neck and extending from 1 to 2 cm cranial to the larynx to a point just past the thoracic inlet, and 10 to 15 cm laterally on either side of the midline) was prepared for surgery. A transoral endotracheal tube was inserted. The skin was opened and blunt dissection exposed the trachea and its lateral walls so that it could be elevated to the skin. The medial edges of the sternohyoid muscles were sutured to the fascia of the dorsolateral-lateral aspect of the trachea to decrease tension on the skin-to-mucosa anastomosis and thereby decrease the tendency for dehiscence. An H-shaped incision extending the full thickness of the tracheal wall was made centrally between the fourth and fifth tracheal rings. The two tracheal wall flaps then were opened outward, the skin flaps opened inward, and the skin sutured to the mucosa around the entire tracheostomy. A jacket with a high neck was placed on the dog to protect the tracheostomy site. The tracheostomy healed completely within two weeks with a permanent stoma that required minimal maintenance. For exposures, a modified, cuffed tracheostomy tube was inserted for monitoring respiratory rate and air flow.

Electrocardiogram Monitoring Modified needle electrodes (Grass Instruments, Braintree MA) were implanted to provide low noise signals at fixed locations to localize changes in atrial and ventricular activity. Four rectangular areas centered over the proposed sites of electrode placement were clipped and shaved. The clipped areas extended at least 5 cm in all directions from the insertion sites to allow space for suturing of the two electrode loops. Electrodes were cold sterilized in 3.4% alkaline glutaraldehyde solution in preparation for the procedure.

The six subcutaneous modified needle electrodes were placed in the following locations: (1) left arm (LA): left 3rd to 4th intercostal space at same level as the left leg electrode; (2) left leg (LL): left 12th intercostal space between the costochondral junction of the 13th rib and the costal arch; (3) V_4: left 5th intercostal space 3.5 to 4 inches above the edge of the sternum; (4) V_5: left 6th intercostal space 3 inches above the edge of the sternum; (5) right arm (RA): right 3rd to 4th intercostal space at same level as the right leg electrode; and (6) right leg (RL): right 12th intercostal space between the costochondral junction of rib 13 and the costal arch.

Electrodes were inserted subcutaneously the full length of the electrode needle. The shaft of the electrode was sutured to the skin with one interrupted suture, and the proximal loop of the electrode was secured with 2 to 3 sutures. The distal electrode loop was positioned cranially or caudally to the needle such that the electrode wire formed a slight arc; this served as a tension relief device to minimize movement of the needle when the electrode wire was tensed. Electrode signal quality was assessed for functionality. The six electrode wires were gathered together over the dog's back and wrapped in a gauze, which in turn was wrapped with cloth tape. A snug-fitting vest, prepared for the dog from a 6-inch cotton orthopedic stockinet, covered and helped to protect the electrode insertion sites. The electrode wires were passed through a small hole in the stockinet and secured with cloth tape over the animal's back, where they were covered by its jacket. The denim jacket with Velcro closures was placed on the dog, and several rounds of black electrical tape were applied to fully secure the jacket.

The electrodes were kept in place for 1 to 2 weeks, depending on the length of the experiment, and the dogs were evaluated daily for evidence of excessive tissue inflammation and behavioral indicators of electrode-associated discomfort. The decision to remove and replace an electrode before the end of an experiment was largely subjective and was made by the veterinarian. Some mild discomfort and itching were tolerated. If electrodes were removed, they were later replaced slightly offset from their original position.

Cardiac Monitoring and Analysis Methods

Animals in earlier three-day experiments in this study were monitored for six hours per day with a personal computer (PC)–based acquisition system (DAS-20, Keithly, Cleveland OH). Subsequently, electrocardiograms were recorded using the Marquette 8500 Holter monitor (Marquette Medical Systems, Milwaukee WI). This monitor can record two ECG channels, which correspond to the V_4 and V_5 leads, and can capture the ECG signal throughout the six-hour exposure; it uses one 60-minute normal bias audio tape for each 24-hour recording. Holter recordings have an effective sampling frequency of 128 Hz, whereas ECGs recorded on the PC were sampled at 500 Hz.

The digitally stored ECGs from the Holter monitor or the PC system were analyzed on the Marquette Medical Systems (MARS) Unity workstation. The MARS workstation data processor records the timing and morphology of the beats. The processed data were examined for unlabeled or mislabeled beats as well as regions of noise and artifact. In all studies, an average of 59.75 ± 1.09 (SD) of every 60 seconds of data collected were used for analysis. After correction, MARS software facilities were used to export beat timing and annotation information for analysis.

Tracking autonomic activity by HRV was the primary cardiac analysis in these studies. Heart rate variability was extracted from the ECG data by custom PC-based software. All HRV measures were computed on a minute-to-minute basis from a rate tachogram (Berger et al 1986) constructed from acceptable normal-to-normal (NN) heart beat intervals; intervals containing noise or artifact regions or abnormal beats were excluded from analysis. Tachogram gaps were set to the mean tachogram rate over all available intervals to avoid spurious variance that would result from interpolation, and all variance measures were appropriately scaled for the available tachogram duration. Frequency measures of HRV (Ten Voorde et al 1994) were estimated by computing the tachogram periodogram, scaling the periodogram by applying the Parseval identity, and summing the appropriate coefficients in frequency regions of interest. Spectral measures included LF power (0.05–0.15 Hz), HF power (0.15–1.00 Hz), and LF-to-HF ratio (LF/HF). The HF power region was extended beyond its usual upper bound to accommodate the range of respiratory frequencies and consequent sinus arrhythmia observed in canines.

Parasympathetic activity was evaluated by finding the HF value. The LF value represents the combined influence of the sympathetic nervous system and other components. To assess the autonomic balance between sympathetic and parasympathetic nervous system activity, the LF/HF ratio is employed (Pagani et al 1986). Variation in breathing pattern at the time of measurement can affect HRV; since respiration was monitored continuously, it was possible to evaluate and control for interactions between altered respiratory activity and measurement of HRV.

The digital ECG also provided assessment of T wave alternans. Complex demodulation of the T wave provides a quantitative measure of vulnerability to ventricular fibrillation (Nearing et al 1994) under diverse conditions including intrinsic autonomic tone, sympathetic and parasympathetic stimulation, and adrenergic blockade (Verrier and Nearing 1994). Electrocardiographic data, recorded throughout the exposure, were analyzed by the method of complex demodulation (Nearing et al 1991, 1994; Nearing and Verrier 1993). Using the digital ECG data, the periods from 60 to 290 msec following the R wave of each successive beat were divided into bins 10-msec wide. To derive a single time series, X(n), the area between the ECG and the isoelectric baseline were computed for each 10-msec interval. Then successive beats were sequenced into a time series for each of the 10-msec bins: {X(n), n = 1, 2, . . . N}.

The R-R interval was employed to sort out and remove premature beats that could introduce artifact. A 16th-order Butterworth filter was used for both detrending and demodulating to remove large low-frequency variations in T wave area and to leave a cleaner signal for spectral estimation. Estimates of the magnitude of beat-to-beat alternation in the amplitude of each of these time series were derived using complex demodulation, an harmonic analysis that provides a continuous measure of oscillation with slowly changing amplitude and phase. It detects features that might be missed or misrepresented by standard Fourier analysis methods which assume data stationarity.

An additional cardiac analytical technique used in these studies displays ECG morphology in three dimensions. This technique provided a topographic visualization of the ECG across the entire duration of the experiment. The investigator's expertise essentially functioned as a powerful pattern-recognition tool to detect subtle changes in morphology evolving against a background of reference ECG signals. Median templates were used to characterize the morphology of the ECG signal according to such features as beat-to-beat interval lengths, general shape patterns, and waveform height threshold.

Respiratory Monitoring and Analysis of Rate, Breathing Volumes, and Air Flows

Breathing patterns were measured by monitoring airflow through the dog's tracheostomy tube (Figure 4). The tracheostomy tube was fitted with a minimal flow restrictor; pressure differential across this restrictor was recorded by pressure transducers. Thus, each breath throughout the six hours of exposure was available for analysis. A complete hardware and software system (BUXCO Electronics, Troy NY) collected and processed the signals generated by the transducers. The start and end of inspiration and expiration were recorded on the pressure-time traces. The data were stored on a PC and analyzed by the BUXCO software. The BUXCO program computed all the breathing parameters on a breath-by-breath basis and reported the average of each minute (Hamelmann et al 1997; Tepper et al 1997). Figure 6 illustrates the analysis of a breath by the BUXCO system. The breathing parameters were time for inspiration (*T*I), time for expiration (*T*E), peak inspiratory (PIF) and expiratory (PEF) flows, tidal volume (*V*T), relaxation time (*T*rel)—defined as the time of decay of the expiration volume (area under pressure versus time curve for expiration) to 30%, minute ventilation ($\dot{V}$), breathing frequency (f), end inspiratory (EIP) and end expiratory (EEP) pauses, pause (Pau), and enhanced pause (Pau_{enh}). The parameter Pau was *T*E/*T*rel – 1, and Pau_{enh} was Pau × PEF/PIF. The derived data also indicated periods of apnea in the animals

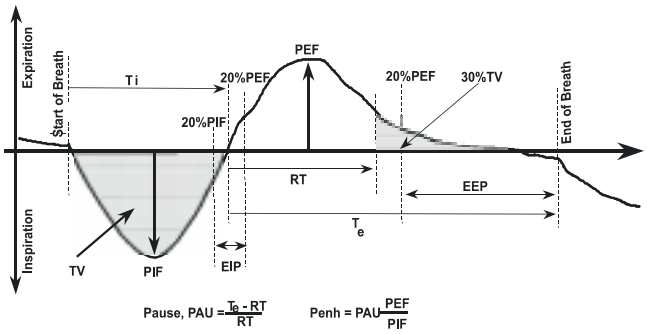


Figure 6. A breath analysis and definition of respiratory parameters determined from the recorded breath. (Adapted from Hamelmann et al 1997.)

during the exposure period. Flow and volume calibrations were done each day. Dogs used early in these studies had breathing monitored via a Respitrace system, which provided only data on respiratory frequency.

Bronchoalveolar Lavage and Transbronchial Biopsy

At the end of the third day of exposure in the double CAPs/double sham protocols, BAL was performed on the animals as described in the section on surgical procedures. The aspirated fluid was quantified in graduated cylinders. Duplicate, well-mixed samples (100 µL) of the BAL return were cytocentrifuged onto microscope slides (Cytospin 2; Shandon Southern Instruments, Sewickley PA), air-dried, and stained with Wright Giemsa stain (VWR Stat Stain, Brisbane CA). From these slides, a differential count of 400 cells was performed using standard morphologic criteria (Rebar et al 1980; Muggenberg 1980). The remaining BAL fluid was strained through sterile coarse cotton gauze to remove mucus and centrifuged at 400 × *g* for 8 minutes at 15°C. The resulting cell pellet was resuspended in medium M199 (Sigma, St Louis MO). An aliquot of this cell suspension was counted in a hemocytometer after dilution in 28.6% acetic acid (Unopette® model #5856, Becton Dickson Laboratories, Rutherford NJ). We confirmed that the percentages of (1) cells identified as alveolar macrophages by the Wright Giemsa stain and (2) cells staining positively with alpha naphthyl acetate esterase stain (Sigma) are highly correlated ($r = 0.971$, $p < 0.001$). Therefore, the only results reported are of cell types identified by Wright Giemsa stain. Fluid from the first lavage was centrifuged briefly (8,000 rpm, 1 minute, 25°C), and the supernatant and cells were stored separately at –4°C. Remaining cells recovered by BAL are lysed in guanidine thiocyanate and stored at –70°C for RNA isolation.

Acellular BAL fluid was used for total protein and β-*N*-acetyl glucosaminidase (β-*N*-AG) measurements. Total protein levels were measured as an indicator of pulmonary vascular permeability (increased levels indicating increased

permeability). Bradford's method (1976) was used to measure protein, employing protein detection reagents from a commercially available kit (Pierce, Rockford IL). All other reagents were obtained from Sigma. The presence of β-*N*-AG, a macrophage lysosomal enzyme, determined by the method of Selliger and coworkers (1960), was measured to indicate macrophage activity and cytotoxicity. All reagents were obtained from Sigma. Biopsy tissue was snap frozen for use in immunocytochemical studies.

Immunocytochemical Identification of Cytokines

For cells in suspension, cytocentrifuge preparations were made (800 rpm × 5 minutes) of 0.1-mL aliquots containing between 1 and 5×10^4 cells in buffer. Transbronchial biopsy tissue was surrounded with OCT embedding compound (Sakura Finetek, Torrance CA) and snap frozen in liquid nitrogen. Cryostat sections were cut 8 μm thick. Both cytospin slide preparations and cryostat sections were allowed to air dry completely at room temperature and were either used immediately for immunolabeling or wrapped in aluminum foil and stored at −70°C. Both polyclonal rabbit-antihuman TNFα and IL-1 β (Genzyme, Cambridge MA) have been used previously on paraffin sections of canine tissue, but rabbit antibodies resulted in excessive nonspecific staining on cytospins and frozen sections (Pelletier et al 1993; Pickvance et al 1993; Day 1996). For our studies, antibodies raised in goat (R+D Systems, Minneapolis MN) to human IL-1 β, IL-8 and TNFα were used with affinity-purified goat-antidog myoglobin (Bethyl Laboratories, Montgomery TX) as the positive staining control. Staining was revealed using Vector (Burlingame CA) elite reagents.

The general immunolabeling protocol used in this study is an immunoperoxidase technique based on the avidin-biotin complex method (Hsu et al 1981). Sections or cytospins then were fixed in buffered 2% paraformaldehyde at 4°C and then immersed in methanol at −20°C. Sections then were serially incubated in (1) a 10% serum solution (serum from the animal source of the secondary antibody) to block nonspecific binding sites; (2) a primary specific or control antibody; (3) a secondary biotinylated antibody; and (4) an avidin-biotin-peroxidase complex. After each incubation, sections were washed in buffer. Antibody binding was detected by incubation with a diaminobenzidine–hydrogen peroxide substrate solution. Slides were counterstained with hematoxylin, dehydrated through graded alcohols and xylene, and mounted with coverslips. Controls included nonspecific antibody or antisera at the same concentration as specific antibody. Buffer alone was used to control for nonspecific binding of the secondary reagents. Replicate slides were used.

Peripheral Blood White Blood Cell Count

Blood was drawn before and after each CAPs or sham exposure for CBC and fibrinogen studies. The CBC samples were collected with EDTA, stored at 4°C, and sent to Tufts Veterinary Diagnostic Laboratory for analysis, including the total WBC count and differential. Blood for fibrinogen analysis, obtained with the first venipuncture attempt, was collected with sodium citrate and immediately transported to Dr Geoffrey Tofler's laboratory at Beth Israel Deaconess Medical Center for analysis. Plasma fibrinogen levels were determined using the von Clauss (1957) method, a functional assay for thrombin-clottable fibrinogen. Briefly, dilute plasma was mixed with a thrombin solution of constant high concentration, and the clotting time of the mixture was measured. Fibrinogen concentration, which was inversely proportional to the clotting time, was determined from a standard curve. The ST4 coagulation instrument (Diagnostica Stago, Asnières, France) was used to measure clotting time.

DATA ANALYSES AND INTERPRETATION

Integrated exposure measurement data are presented in this report as descriptive measurements on individual days. Any manipulations of these data are described in relation to the individual measurements. Continuous measurements of mass carbon and BC are used in the statistical models described below.

Statistical Analysis of Response Parameters

The studies reported here were designed so that data could be analyzed using a highly simplified analysis of response parameters or complex modeling of the data. In the simple approach, mean responses over the six hours of exposure were determined for all cardiac and respiratory parameters. All data for each dog was dichotomized into CAPs or sham exposures, and all dogs were compared for their individual overall response to CAPs or sham exposure using the paired *t* test.

Integration and Analyses of Exposure Data and Animal Response Parameters

A more complex approach to data analysis was also used. These analyses took advantage of the continuously collected data as well as the crossover design. Because repeated measurements in the same animals provided each animal with its own control, we used methods appropriate for normally distributed data with raw values averaged over a maximum of five minutes. Data from dogs exposed to CAPs are compared both to data from themselves as filtered air sham and to their chambermate's

aged over a maximum of five minutes. Data from dogs exposed to CAPs are compared both to data from themselves as filtered air sham and to their chambermate's sham data. Chambermates often tend to behave similarly over a six-hour exposure. For instance, a steady decrease in a particular monitored response for both sham and CAPs subjects across a six-hour exposure period reflects a chamber effect. Common nonlinear trends across an exposure period also could represent similar responses to environmental stimuli or feedback between chambermates. When a response exhibits the same trend for both sham and CAPs subjects, such trends are a nuisance. We controlled for these trends by fitting semiparametric, or partial linear, models (Speckman 1988; Hastie and Tibshirani 1990; Hobert et al 1997) that assume a day-specific smooth function over time common to sham and CAPs subjects. These curves represent a "day" effect, which can be estimated as a result of the orthogonality of the day, subject, and CAPs effects in the crossover design. The semiparametric model assumes that these latter two factors, subject and CAPs, affect a physiologic response in a linear fashion.

Specifically, let y_{ijt} be the response of interest at time t, $t = 1, \ldots, T_j$, for subject i, $i = 1, \ldots, I$ on day j, $j = 1, \ldots, J$. The form of the general semiparametric model equation is

$$y_{ijt} = x_{ijt} + f_j(t) + \quad _{ijt} \tag{1}$$

in which x_{ijt} is $1 \times p$ row vector of fixed covariates, is a column vector of unknown regression parameters, and the $\{f_j(\bullet)\}$ are arbitrary smooth functions. The error terms $_{ijt}$ are assumed to be normally distributed with marginal mean and variance 0 and ^{2}, respectively. The smooth functions $\{f_j(\bullet)\}$ control for day-to-day variation in both magnitude and trend across a six-hour exposure period. The parametric portion of model (1) estimates the effect of inhaled CAPs, relative to sham, for a particular day while controlling for subject differences. This implies a parametric term

$$x_{ijt} \quad = \quad _i^S + \quad _j^C \, x_{ijt}^{PM} \tag{2}$$

in which x_{ijt}^{PM} is the $PM_{2.5}$ exposure dose inhaled by subject i on day j at time t. This exposure dose has been calculated in two ways: by the cumulative amount of $PM_{2.5}$ inhaled by subject i on day j before time t; and by the actual or instantaneous $PM_{2.5}$ concentration for subject i on day j at time t. Dose in both cases uses the continuous data of inhaled minute volume and mass concentration in the air to determine exposure dose. A deposition fraction is not included in the calculation. The coefficients $\{ \quad _j^C\}$ are the parameters of interest, representing the within-dog effect of inhaled $PM_{2.5}$ on each day. Each is based on the performance of two dogs in the crossover experiment. The terms $\{ \quad _i^S\}$ specify a subject-specific curve intercept. Identifiability requires a constraint such as $\{ \quad _i^S\} = 0$ for one subject in each pair, so that the nonzero $\{ \quad _i^S\}$ coefficients represent a shift in the intercept of the curve for subject i relative to its chambermate. This model assumes that subject differences remain constant across a day.

Given that model (1) is correct, and assuming that all observations are independent, fitting the model produces consistent estimates $\hat{}$ and $\{\hat{f}_j(\bullet)\}$ for and $\{f_j(\bullet)\}$, respectively. The standard errors, however, are likely to be incorrect because observations for a given subject on a given day are correlated even after allowing for the time trends via $\{f_j(\bullet)\}$. Thus, for each (i,j) combination, errors $_{ijt}$ are assumed to follow a first-order autoregressive (AR1) process; that is, the errors satisfy

$$_{ijt} = \quad _{ijt(t-1)} + \quad _{ijt} \tag{3}$$

where the vectors $_{ij} = (\ _{ij1}, \ _{ij2}, \quad , \ _{ijTj})$ consist of independent, mean zero normal random variables with variance $\mathrm{Var}(\ _{ijt}) = \mathrm{Var}(y_{ijt} | y_{ij1}, \quad , y_{ij(t-1)}) = (1 - \quad)\ ^2$.

Model (1) with AR1 error assumption (3) was fit using a hybrid iterative algorithm that combines ideas behind semiparametric additive models (Hastie and Tibshirani 1990) and transitional regression models (Brumback et al 2000). In particular, the backfitting algorithm that was employed used the smooth functions $\{f_j(\bullet)\}$ estimated using locally weighted regression (LOESS) with tri-cube-weight function (Cleveland 1979); see Coull et al (2000) for details. All semiparametric analyses were performed using the statistical software package S-Plus (Chambers and Hastie 1993).

For both healthy dogs and coronary occlusion crossovers, the model was fit separately to each respiratory and cardiac response. In the current study, December 3, 1997, is not included in the analysis because the cardiac data for that day contained considerable artifact resulting from a poor connection; this was the only unusable day in over 100 days of analytical data. The fits for the 23-day healthy-dog crossover (8 dogs) were based on $n_1^H = 3{,}038$ observations (H = healthy) for each respiratory response and $n_2^H = 2{,}987$ observations for each cardiac response. The fits for the 12-day coronary occlusion crossover (4 dogs) were based on $n^{CO} = 1{,}556$ observations (CO = coronary occlusion) for each respiratory and cardiac response. Each day-specific LOESS smoothing was estimated using a span of one third; a sensitivity analysis demonstrates that the estimated CAPs effects are robust to this choice of span. For both the healthy dog and coronary occlusion crossovers, initial inspections of residual plots, based on the

In addition to model (2) that specifies a different CAPs effect for each day, alternative models that estimate overall CAPs effects (averaged over days) were fit to each crossover data set. In particular, the model

$$y_{ijt} = \beta_i^S + \beta^C x_{ijt}^{PM} + f_j(t) + \varepsilon_{ijt} \quad (4)$$

where the errors ε_{ijt} follow first-order autoregressive process (3), specifies overall CAPs effect β^C. As in the heterogeneous effect case, separate model fits were obtained using two different characterizations of exposure dose: the cumulative amount of $PM_{2.5}$ inhaled by suject *i* and day *j* before time *t* and the actual instantaneous $PM_{2.5}$ concentration for subject *i* on day *j*.

For both models (2) and (4), an approximate *F* test (Hastie and Tibshirani 1990) was used to test the null hypothesis that a particular regression coefficient is zero in the presence of the other terms in the model. Correct standard errors then can be obtained using the equivalence between a partial *F* statistic and a squared Wald *t* statistic (Myers 1990).

We investigated the effects of elemental composition on biologic response by relating the CAPs composition of groups of elements to estimated $PM_{2.5}$ coefficients $\{\hat{\beta}_j^C\}$ for the semiparametric model fit to the healthy dog crossover data. Specifically, for the 23 days in the crossover experiment, a principal components analysis with five components and varimax rotation was conducted on 22 elemental concentrations (SAS Institute 1990). Each element concentration was summed into one of five groups on the basis of its largest rotated factor loading, and each group sum was converted to a proportion of total elemental concentration. Only chromium is not assigned to a factor.

The resulting elemental group proportions were related to estimated daily cumulative and instantaneous CAPs effect using the first-order polynomial Scheffe mixture model (Cornell 1990)

$$\beta_j^C = \alpha_1 P_{1j} + \alpha_2 P_{2j} + \alpha_3 P_{3j} + \alpha_4 P_{4j} + \alpha_5 P_{5j} + \varepsilon_j \ , \quad (5)$$

in which P_{kj} is the proportion of the total elemental concentration resulting from element group *k* on day *j*.

Bronchoalveolar Lavage Analysis

We subjected mean differential cell count percentages (macrophages, polymorphonuclear leukocytes, lymphocytes, and eosinophils) and protein levels (total BAL protein and β-*N*-AG) to analysis of variance (ANOVA) with post hoc Bonferroni (Glantz 1997) comparison of individual groups. Comparisons were performed on means calculated from baseline, postsham exposure, and post-CAPs exposure BAL measurements. Bronchoalveolar lavage data for three individual dogs (Dogs 1, 6, and 7) were subsequently analyzed by repeated measures ANOVA to determine if there was an effect of CAPs exposure. Differences between groups were determined by Bonferroni post hoc analysis. Values at baseline, after sham exposure, and after CAPs exposure were compared.

Blood data were separated into two treatment groups (sham and CAPs) with multiple treatments per group (three days of exposure) for analysis of whole blood cell counts and fibrinogen assays. Repeated measure ANOVA tests were performed on combined data from all sham-exposed and CAPs-exposed animals. Mean differential cell count percentages (polymorphonuclear leukocytes, lymphocytes, monocytes, and eosinophils), total WBC counts (per microliter blood), and fibrinogen levels were analyzed; and post hoc Bonferroni comparison of individual daily means also were performed. Subsequent repeated measure ANOVA tests with post hoc Bonferroni analyses were performed on pairs of dogs simultaneously treated in double CAPs/double sham exposure regimens. Crossover design exposure studies also were tested to determine potential CAPs effects. Blood parameters from individual days of exposure were assessed against mean baseline measurements to measure toxicity of specific CAPs exposures during the three-day exposure regimens.

STUDIES IN DOGS WITH CORONARY OCCLUSION

SUMMARY OF PROTOCOL

This protocol was an extension of that used for the studies in normal dogs, in which the dogs were well trained, and their normal response patterns were established. The protocol for coronary occlusion included cardiac surgery, a 2-week recovery period, the occlusion protocol, and the crossover design used for the exposures (Figure 7). Assessments included the same continuous cardiac and respiratory measurements and analyses as described for normal dogs.

EXPERIMENTAL DESIGN

For coronary artery occlusion studies, the dogs underwent thoracic surgery for implantation of a balloon occluder on the LAD artery (Figure 8). The first two dogs to enter this protocol also had a coronary artery flow probe on the LAD, an epicardial thermistor for temperature measurement, and for recording ECG, bipolar electrodes overlying the myocardium supplied by the LAD and overlying

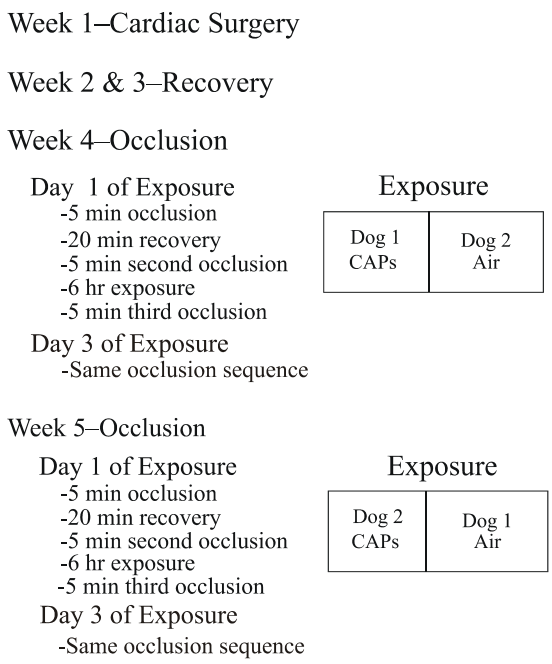


Figure 7. Coronary occlusion study design.

the left atrial appendage. The most critical device, the balloon occluder, was implanted in all dogs on this protocol, but the flow probe was omitted when space along the coronary artery was insufficient or if its presence would adversely affect the balloon. The thermistor and pericardial ECG leads were omitted in later studies because they increased chronic inflammatory reactions in the pericardium.

On the day of occlusion and exposure, blood was drawn for baseline, postexposure and postocclusion cardiac enzymes (including lactate dehydrogenase [LDH] and creatine phosphokinase [CPK], analyzed at Tufts Veterinary Diagnostic Laboratory) as well as for CBC and fibrinogen analyses. The ECG and blood flow in the LAD were recorded continuously. Occlusion of blood flow in the LAD for 5 minutes should produce ischemia, but not frank infarction. During this period of the study, the Doppler flow probe monitor was used to verify decreases in coronary artery flow, and/or the ECG monitor was used to verify increases in heart rate and ST segment elevation along with changes in T wave morphology typical of ischemia. After the occlusion was opened, the cardiac parameters returned to baseline immediately. After a 20-minute interval, a second 5-minute occlusion was carried out. The CAPs or sham exposure began when the second occlusion was released. Immediately after exposure, another 5-minute occlusion was carried out.

PROCEDURES

Cardiac Surgery

Thoracotomies were performed for implantation of a vascular occluder and a Doppler flow probe around the LAD. The surgery was done 10 to 14 days before the start of the coronary occlusion protocols. Anesthesia was induced with an intramuscular mixture of atropine (0.04 mg/kg), ketamine (10 mg/kg), and xylazine (1.5 mg/kg) followed by maintenance with isofluorane. An anesthesia machine (VMC, Matrix Medical, Colonial Medical Supply Company, Franconia NH) was attached to a ventilator (model 2000 Hallowell EMC, Pittsfield MA) to provide breathing when the pleural cavity was entered. The percentage of inhaled isofluorane was adjusted (between 0.5% and 2%) to maintain the dog at a surgical level of anesthesia. Left lateral intercostal thoracotomy through the fourth or fifth intercostal space was performed using routine methods as outlined by Nelson (1983).

A vertical incision (4 to 5 cm long) centered over the ventral border of the left atrial appendage was made through the parietal pericardium. The LAD was identified in the paracoronal intervertebral groove just caudal and slightly medial to the great coronary vein. A length of the LAD (1 to 2 cm) was bluntly dissected just ventral to the tip of the left atrial appendage (see Figure 8) with care to maintain the adventitia intact so that adipose tissue was not removed. These precautions precluded any apparent deficit in the coronary vasoconstrictor response to neurogenic stimuli (Varley 1967; Vatner et al 1971; Vatner and McRitchie 1975; Denn and Stone 1976; Billman and Randall 1981). An epoxy-cuff pulsed-Doppler probe (Craig J Hartley, Baylor College of Medicine, Houston TX) with a

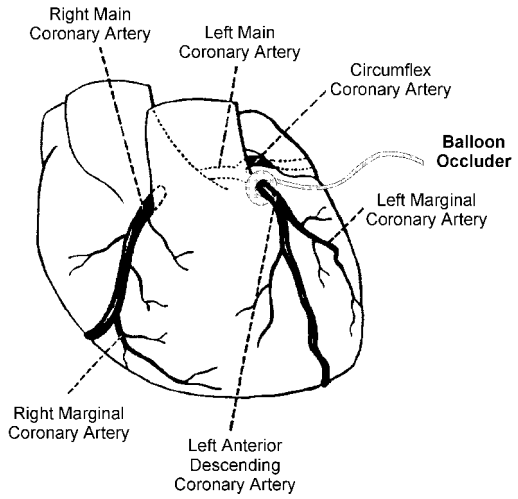


Figure 8. Surgical implantation of balloon occluder around a coronary vessel.

2.5 or 3.0 mm inner diameter was placed around the dissected portion of the LAD and secured with its umbilical tape ties. The inflatable cuff of a vascular occluder (In Vivo Metric OC3.5, Healdsburg CA) was placed around the LAD just downstream from the flow probe and secured by tying a 2–0 silk suture through its two eyelets. The Doppler flow probe was tested for audible flow through the LAD. The vascular occluder was tested by injecting normal saline with a 1-mL syringe until flow through the LAD ceased. The volume needed to completely occlude the LAD was recorded, and the occluder deflated. This procedure was repeated one or two times to verify the volume of saline required for complete occlusion and to assure the absence of leaks in the balloon cuff.

The pericardium and thorax were closed to minimize inflammatory reaction and obtain a secure anastomosis. After a stab incision was made through the pericardium cranial to the main pericardial incision, the Doppler flow probe wire and vascular occluder tubing were passed through the incision to exit the pericardial sac. The pericardial incision was closed with a simple continuous or Ford interlocking pattern of 3–0 or 4–0 nylon. An intercostal nerve block with up to 1.5 mg/kg of bupivicaine with epinephrine (1:200,000) was achieved by injecting small volumes (0.5 to 1.0 mL) into 2 to 3 intercostal spaces cranial and caudal to the thoracotomy incision. A large hemostatic forceps was used to pass the vascular occluder tubing and the flow probe wire through the connective tissue and musculature of the third intercostal space. A chest tube was inserted and secured to the skin with a "Chinese finger cuff" suture pattern. The thoracic cavity was thoroughly lavaged with warm saline. The ribs were approximated and the muscles apposed in routine fashion (Nelson 1983).

The flow probe wire and the vascular occluder tubing were tunneled subcutaneously from the third intercostal space, exited through a stab incision in the skin of the cervicothoracic area, and secured to the skin with multiple sutures. The thoracic subcutaneous layer was closed with either a simple continuous or Ford interlocking pattern of 3–0 Dexon and the skin closed with surgical staples.

During this procedure, a three-way stopcock was fitted to the end of the thoracostomy tube. Any air or fluid in the pleural space was evacuated using a 60-cc syringe with the dog in both right and left lateral recumbency. Evacuation was repeated multiple times during the recovery period. The dog was weaned off the ventilator and allowed to breathe spontaneously; when the animal began to swallow, the endotracheal tube was removed. The thoracostomy tube was removed when the animal was breathing spontaneously with an approximately normal tidal volume, and no more air or fluid could be aspirated from the thoracic cavity. The chest tube was usually removed within 3 to 4 hours of the cessation of ventilation. All dogs were examined daily by veterinarians and skin sutures were removed 10 to 14 days postoperatively.

Coronary Occlusion and Analyses

After the dogs had healed, exposure experiments were carried out using the crossover design. Coronary occlusion was produced in the dog by inflating the balloon occluder with the predetermined volume of saline. The occlusion was maintained for 5 minutes and then slowly released; 20 minutes later, a second occlusion was induced. Throughout these procedures, the dogs were monitored continuously with the Holter unit, as described for the studies with normal dogs. (Two occlusions were needed because the first, a conditioning occlusion, tended to have different effects from all subsequent occlusions. Thus, comparisons were made between the second and subsequent occlusions.)

The 6-hour CAPs or sham exposure was followed by a third 5-minute occlusion at the end of exposure. During the three occlusions, the Doppler flow probe was used to verify the absence of flow in the vessel and/or the ECG was monitored for changes characteristically indicative of ischemia. When the occlusions were released, the volume of returned saline was noted to assure that the occluder did not leak. In those dogs that did not have a Doppler flow probe implanted, the ECG changes produced during balloon inflation were sufficiently dramatic to leave little doubt that occlusion had been produced. The ECG analyses in relation to the coronary occlusion studies included continuous HRV measurement, three-dimensional graphic analysis of ECG, T wave alternans quantification, analysis of time to onset of ST segment changes that indicate ischemia, and the degree of change in T wave and ST segment amplitude. The ECG tracings of CAPs and sham animals were compared using the statistical approaches described for normal animal studies.

RESULTS

The timeline for each dog details the sequence of protocol events for each of the 14 dogs used in the experiments (Figure 3). Data on 179 exposure days are included in this report: 101 were CAPs exposures and 78 were sham exposures. Normal dogs were used in 71 CAPs exposure days and 54 sham exposure days.

Results from studies with normal dogs (specific aim 1) are grouped either as all normal dog studies or separated

by the double sham and double CAPs or crossover design protocols. Specific aim 2, using dogs with chronic bronchitis, was not fully pursued; these data are reported only in Appendix A. The data include 12 CAPs exposure days and 6 sham days.

Results related to specific aim 3, using dogs with cardiac ischemia caused by coronary artery occlusion, are presented in the second part of the results section and include 18 CAPs and 18 sham exposure days.

For all protocols, the descriptive exposure data are followed by descriptive biologic response data, then integration of exposure data and biologic response. Tables 3, 8, and 19 (in addition to Figure 3), can be used to understand relations among dogs, dates, and exposure protocols as well as to see specific exposure measurements for specific days.

NORMAL DOGS

Exposure Parameters and Exposure Assessment

Crossover Design Studies Exposure data for 24 days of study in the crossover design protocol were collected during both summer and winter months in 8 different dogs (see Table 3). The particle size, MMAD, of the CAPs exposure aerosol ranged from 0.23 to 0.34 μm; the GSD ranged from 1.7 to 2.9. Data for mass (from the TEOM measurements) and BC (from aethalometer measurements) are presented as 6-hour averages (Table 3). These data were collected continuously, however, and statistical analyses presented later in the report used all the data, not just the tabulated averages. The continuous and integrated mass measurements differed modestly (Table 3). The CAPs mass

Table 3. Exposure Parameters for Crossover Design Studies with Normal Dogs[a]

Date	Animal (n = 8 total)	MMAD ± GSD (μm)	Black Carbon (Mean ± SD μg/m^3)	TEOM $PM_{2.5}$ (Mean ± SD μg/m^3)	Mass CAPs (μg/m^3)	SO_4 CAPs (μg/m^3)	Mass Ambient (μg/m^3)	SO_4 Ambient (μg/m^3)	Mass Concentration Factor	SO_4 Concentration Factor	H^+ Acidity (nEq/m^3)
6/16/97	2	0.34 ± 2.0	4.7 ± 1.8	93.5 ± 17.2	112.5	14.9	5.5	0.7	20.5	21.3	NA[b]
6/17/97	2	0.34 ± 2.0	24.2 ± 6.6	726.4 ± 190.2	799.3	217.3	23.9	6.3	33.4	34.5	NA
6/18/97	2	0.34 ± 2.0	28.0 ± 6.9	828.0 ± 88.2	1055.8	206.6	28.3	6.0	37.3	34.4	NA
6/23/97	1	0.27 ± 2.2	2.4 ± 0.8	135.4 ± 17.4	154.6	34.9	6.1	1.2	25.3	29.1	NA
6/24/97	1	0.27 ± 2.2	3.7 ± 1.0	114.1 ± 15.2	121.6	11.7	6.7	0.5	18.1	23.4	NA
6/25/97	1	0.27 ± 2.2	14.5 ± 5.8	666.0 ± 301.8	691.2	230.1	23.7	7.1	29.2	32.4	NA
11/4/97	6	0.25 ± 2.4	7.4 ± 2.1	228.3 ± 64.8	310.7	61.7	14.7	2.1	21.16	28.92	1
11/5/97	6	0.25 ± 2.4	5.4 ± 1.4	184.3 ± 15.6	239.0	105.7	7.9	2.8	30.30	38.17	6
11/6/97	6	0.25 ± 2.4	8.3 ± 0.9	330.8 ± 24.2	421.2	117.6	9.2	2.7	45.58	42.85	3
11/12/97	7	0.23 ± 2.7	7.5 ± 2.2	172.3 ± 48.2	190.2	60.5	7.3	2.2	26.08	27.64	0
11/13/97	7	0.23 ± 2.7	3.2 ± 0.8	69.1 ± 10.6	101.6	23.0	3.8	0.9	26.83	26.49	1
11/14/97	7	0.23 ± 2.7	6.7 ± 2.1	89.9 ± 27.1	102.5	15.2	5.8	1.3	17.62	11.54	−2
12/3/97	8	0.29 ± 1.7	6.4 ± 5.6	95.2 ± 32.7	93.7	10.0	3.2	0.5	29.29	22.17	−5
12/4/97	8	0.29 ± 1.7	24.1 ± 2.4	637.1 ± 116.2	785.1	215.3	17.9	4.5	43.81	47.42	−1
12/5/97	8	0.29 ± 1.7	17.0 ± 4.8	359.8 ± 80.1	443.2	131.8	12.2	4.1	36.42	32.46	−1
12/9/97	9	0.23 ± 2.3	25.1 ± 10.3	378.3 ± 160.5	460.1	58.0	20.5	2.9	22.42	19.97	−3
12/10/97	9	0.23 ± 2.3	16.3 ± 4.4	380.2 ± 54.8	435.3	100.8	14.8	3.2	29.51	31.18	−2
12/11/97	9	0.23 ± 2.3	5.0 ± 2.2	108.2 ± 12.2	168.5	30.2	9.0	1.8	18.62	16.86	0
12/16/97	12	0.26 ± 2.2	28.3 ± 7.1	551.3 ± 48.3	674.4	102.7	28.5	5.9	23.70	17.3	−1
12/17/97	12	0.26 ± 2.2	13.4 ± 5.9	285.7 ± 34.5	332.4	67.1	13.2	3.5	25.19	19.4	−2
12/18/97	12	0.26 ± 2.2	5.7 ± 3.6	97.3 ± 36.2	119.3	23.2	4.9	1.3	24.49	18.1	−5
1/27/97	14	0.28 ± 2.9	15.7 ± 12.0	279.3 ± 140.2	442.4	113.8	14.7	3.6	30.09	32.1	−1
1/28/98	14	0.28 ± 2.9	6.7 ± 1.4	217.2 ± 11.3	284.5	49.0	9.5	1.6	29.86	30.3	−8
1/29/98	14	0.28 ± 2.9	6.5 ± 2.0	149.0 ± 43.0	184.0	43.3	5.4	1.2	34.20	37.3	−2

[a] For each pair of dogs (2 and 1, 6 and 7, 8 and 9, 12 and 14), one dog was sham-exposed on the same day the other dog was exposed to CAPs.

[b] NA = Not Available.

Table 4. Elemental Analysis Parameters for Crossover Design Studies with Normal Dogs[a]

Date	Mass CAPs	Na	Al	Si	S	Cl	K	Ca	Ti	V	Cr	Mn	Fe	Ni	Cu	Zn	As	Se	Br	Cd	Pb	Ba
6/16/97	112.5	0.000	5.677	10.466	5.806	0.000	1.960	2.417	0.394	0.030	0.005	0.120	5.252	0.022	0.092	0.291	0.014	0.007	0.042	0.056	0.424	0.164
6/17/97	799.3	0.000	1.065	5.945	68.992	0.000	3.722	3.740	0.339	0.129	0.011	0.100	4.716	0.071	0.112	0.617	0.000	0.031	0.255	0.000	0.511	0.165
6/18/97	1055.8	0.000	0.620	6.266	66.042	0.000	4.781	2.173	0.311	0.278	0.001	0.166	5.096	0.130	0.249	0.972	0.093	0.057	0.434	0.040	0.386	0.206
6/23/97	154.6	0.000	1.810	4.045	13.063	0.000	1.074	1.442	0.212	0.000	0.001	0.084	2.547	0.000	0.091	0.194	0.036	0.005	0.008	0.071	1.030	0.297
6/24/97	121.6	0.888	1.049	3.302	4.617	0.000	1.064	1.618	0.188	0.000	0.008	0.092	2.901	0.001	0.091	0.234	0.007	0.000	0.015	0.000	0.561	0.080
6/25/97	691.2	0.000	1.599	6.429	78.701	0.000	1.407	1.250	0.282	0.059	0.003	0.068	3.323	0.054	0.140	0.439	0.026	0.035	0.098	0.074	0.574	0.209
11/04/97	310.7	0.000	2.619	9.013	19.861	0.240	1.788	8.238	0.305	0.032	0.020	0.140	6.351	0.022	0.161	0.366	0.015	0.019	0.117	0.000	1.090	0.121
11/05/97	220.9	0.000	0.529	2.937	27.517	0.000	0.863	1.543	0.241	0.025	0.021	0.136	4.927	0.032	0.157	0.352	0.000	0.022	0.047	0.000	0.747	0.146
11/06/97	407.3	0.000	1.508	6.808	44.399	0.000	2.523	3.252	0.364	0.146	0.034	0.198	5.693	0.092	0.154	1.049	0.024	0.027	0.086	0.000	0.943	0.163
11/12/97	187.6	0.000	1.006	2.906	19.098	0.000	1.006	1.172	0.164	0.074	0.011	0.096	4.383	0.071	0.188	0.310	0.000	0.002	0.062	0.000	0.792	0.139
11/13/97	101.6	0.000	1.375	3.553	7.555	0.000	0.952	1.429	0.171	0.053	0.004	0.066	2.644	0.042	0.099	0.240	0.021	0.009	0.022	0.029	0.382	0.125
11/14/97	102.5	11.847	0.539	1.434	5.669	8.963	0.682	1.789	0.155	0.240	0.003	0.080	1.669	0.207	0.074	0.643	0.010	0.018	0.061	0.026	0.570	0.111
12/03/97	128.1	2.794	2.189	5.555	4.722	7.063	1.154	2.141	0.251	0.044	0.028	0.086	6.691	0.068	0.201	0.313	0.000	0.004	0.011	0.000	1.020	0.137
12/04/97	443.2	0.000	0.000	2.774	67.632	0.000	2.310	1.147	0.284	0.207	0.025	0.142	4.878	0.156	0.217	0.885	0.031	0.145	0.299	0.000	0.778	0.284
12/05/97	785.1	0.000	0.000	1.658	47.511	0.000	1.296	1.088	0.223	0.121	0.030	0.139	4.591	0.126	0.170	0.586	0.031	0.055	0.072	0.038	0.552	0.181
12/09/97	460.1	1.412	5.127	12.293	20.938	2.331	3.413	6.249	0.780	0.445	0.031	0.236	12.201	0.351	0.263	1.406	0.042	0.016	0.110	0.013	0.888	0.448
12/10/97	433.1	0.000	1.518	6.957	32.823	0.000	2.658	2.811	0.395	0.289	0.020	0.225	6.383	0.236	0.209	0.793	0.031	0.022	0.091	0.000	1.066	0.265
12/11/97	166.2	0.000	1.239	3.714	9.788	0.000	1.316	1.471	0.245	0.152	0.004	0.099	2.746	0.133	0.110	0.399	0.055	0.002	0.044	0.085	1.030	0.190
12/16/97	674.4	0.000	3.077	10.219	34.426	1.156	3.745	3.697	0.566	0.557	0.021	0.248	10.209	0.483	0.350	1.647	0.055	0.061	0.197	0.000	1.577	0.475
12/17/97	332.4	0.000	1.731	6.425	20.380	0.000	1.931	2.420	0.230	0.081	0.026	0.172	6.151	0.098	0.192	0.716	0.058	0.111	0.099	0.000	1.321	0.229
12/18/97	119.3	0.000	0.601	2.335	7.197	0.000	0.957	1.063	0.154	0.039	0.000	0.086	2.607	0.048	0.146	0.309	0.028	0.000	0.008	0.000	0.729	0.077
1/27/98	442.4	6.755	4.499	14.346	40.428	21.222	3.535	7.050	0.600	0.361	0.025	0.244	11.571	0.264	0.257	1.138	0.038	0.015	0.211	0.000	1.032	0.323
1/28/98	284.5	23.683	0.562	3.585	18.628	60.074	2.557	4.117	0.211	0.052	0.006	0.108	4.215	0.029	0.119	0.501	0.004	0.022	0.168	0.015	0.369	0.158
1/29/98	184.0	8.141	0.690	4.766	15.253	14.403	1.673	2.039	0.293	0.103	0.012	0.089	3.891	0.102	0.116	0.360	0.005	0.007	0.185	0.002	0.720	0.109

[a] All values are in $\mu g/m^3$.

Table 5. Composition of Concentrated $PM_{2.5}$ During 18 Days of Crossover Design Studies with Normal Dogs[a]

Species	Mean Concentration ($\mu g/m^3$)	Fraction of Fine Mass (Mean ± SD in %)
$(NH_4)_2SO_4$	101.5	30.9 ± 11.0
Organic carbon	85.2	28.0 ± 11.4
Elemental carbon	21.9	7.1 ± 2.7
Sum of all trace elements	41.4	16.3 ± 11.7
Unexplained	71.9	17.8 ± 17.2
Total $PM_{2.5}$	321.9	100.0

[a] Concentrations of EC and OC not available for June 1997 exposures.

column presents data gravimetrically determined from the mass collected on filters over the 6-hour exposure. Data are also included on concentrated mass and sulfate as well as the ambient levels of mass, sulfate, and particulate acidity. From these latter measures, the degree of concentration achieved each day by the HAPC is calculated. Concentrated ambient particle mass concentrations ranged from about 100 $\mu g/m^3$ to just over 1,000 $\mu g/m^3$; concentration factors, from 18 to 45. Endotoxin data were often below the LOD. (Data for endotoxin are not shown.) Table 4 presents the elemental analysis data for 21 elements over the 24 days of exposure.

Average composition of the concentrated $PM_{2.5}$ during the crossover exposures in normal dogs was determined for the concentrations of sulfate, trace elements, EC, and OC measured over the same exposure periods. The mass of each was determined, and the fraction of total mass of each chemical species was calculated (Table 5). The four species listed comprised an average of 82.2% of the total fine

Table 6. Rotated Factor Pattern from Principal Component Analysis of Crossover Design Study Data

Element	Component 1[a]	Component 2[a]	Component 3[a]	Component 4[a]	Component 5[a]
Al	0.2293	0.8917[b]	−0.1860	−0.1110	0.1128
As	0.6337[b]	0.0163	0.3583	−0.2506	0.3782
Ba	0.6787[b]	0.1335	−0.2412	−0.2880	−0.3254
BC	0.5256	0.1628	0.7749[b]	−0.0990	−0.0947
Br	0.1838	0.0827	0.8974[b]	0.2140	−0.0190
Ca	0.1249	0.7871[b]	0.0733	0.2398	−0.3226
Cd	−0.0536	−0.0542	−0.0872	−0.1613	0.8805[b]
Cl	−0.0499	0.0697	0.0079	0.9491[b]	−0.0386
Cr	0.3639	0.2349	0.1332	−0.1958	−0.7490
Cu	0.7451[b]	0.2563	0.3974	−0.1061	−0.2741
Fe	0.5686	0.6989[b]	0.1928	0.0044	−0.3425
K	0.3818	0.4795	0.7095[b]	0.1490	−0.0127
Mn	0.6838[b]	0.4821	0.2226	−0.0553	−0.3837
Na	−0.0495	−0.0460	−0.0689	0.9787[b]	−0.0061
Ni	0.8913[b]	0.2352	0.1394	0.0722	−0.1096
Pb	0.8089[b]	0.3728	0.2002	−0.0906	0.0211
S	0.0254	−0.0126	0.8790[b]	−0.1879	−0.0554
Se	0.3346	−0.3399	0.5894[b]	−0.1748	−0.2950
Si	0.3256	−0.9066[b]	0.1304	−0.0548	−0.0490
Ti	0.5352	0.7710[b]	0.1969	−0.0188	−0.1135
V	0.8412[b]	0.3016	0.2876	0.0999	−0.0553
Zn	0.7791[b]	0.3210	0.4076	0.0598	−0.2246

[a] Component 1: arsenic, barium, copper, manganese, nickel, lead, vanadium and zinc.
Component 2: aluminum, calcium, iron, silicon, and titanium.
Component 3: BC, bromine, potassium, sulfur and selenium.
Component 4: sodium and chlorine.
Component 5: cadmium.

[b] Indicates assignment of an element to an elemental group (ie, these had the highest correlation among all measured elements).

mass. The average composition for concentrated fine particle mass during the dog crossover exposure study was calculated for 18 rather than 24 days because EC and OC concentrations were unavailable for the June 1997 crossover. Ammonium sulfate constituted the largest fraction of the fine mass, accounting for an average of 30.9%. Organic carbon accounted for an average of 28.0%, and EC contributed an additional 7.1%. Overall, the more abundant trace elements (Fe, Si, Cl, and others) constituted 16.3% of the $PM_{2.5.}$ The unexplained fraction of the mass, an average of 17.8%, consisted largely of ammonium, nitrate, carbonate, and traces of particle-bound water.

Five components were evident from the principal component analysis of concentrations of chemical species (Table 6). The first component included arsenic, barium, copper, manganese, nickel, lead, vanadium and zinc. The second component included aluminum, calcium, iron, silicon, and titanium. The third component incorporated black carbon (BC), bromine, potassium, sulfur and selenium. The fourth component was sodium and chlorine. Cadmium alone was the fifth component, but levels were frequently below the limit of detection (LOD) (see Table 2).

Weather data of the crossover experiments (Table 7) include average ambient temperature, dew point, relative humidity, barometric pressure, wind speed, and wind direction at Boston's Logan Airport at the time the experiments were done. Trajectory, an overall assessment of the 96 hours preceding noon of the day of the experiment, and local wind direction showed a weak correlation.

Double CAPs/Double Sham Studies The double CAPs studies overall had lower CAPs mass exposure concentrations (mean 195.5 compared to 363.5 in the crossover studies)(Table 8). As in the crossover studies, the double CAPs study dogs experienced considerable day-to-day variations in mass, sulfate, and elements even within the

Table 7. Mean Boston Weather Data During Crossover Design Studies with Normal Dogs

Date[a]	Temperature (°F)	Dewpoint (°F)	Relative Humidity (%)	Barometric Pressure (mm Hg × 100)	Wind Speed (mi/hour)	Wind Direction (degree)	Wind Trajectory Pattern
6/16/97	67.43	49.29	52.43	3000.14	16.00	120	S
6/17/97	68.00	62.20	81.60	2985.80	11.80	110	S
6/18/97	67.71	61.57	80.86	2993.43	11.00	80	SW
6/23/97	81.57	54.57	39.57	2994.43	14.57	320	NW
6/24/97	74.43	55.43	51.57	3003.57	11.29	270	NW
6/25/97	78.43	66.71	67.71	2985.29	11.43	130	NW
11/4/97	57.14	50.57	78.71	2997.86	7.43	360	SW
11/5/97	52.57	33.86	49.29	3037.29	11.57	280	SW
11/6/97	51.29	41.29	68.57	3044.00	14.43	70	SW
11/12/97	40.57	23.86	51.57	2987.14	15.00	240	NW
11/13/97	38.43	12.14	33.71	3015.71	10.71	320	NW
11/14/97	35.14	30.57	84.14	2974.67	24.57	50	NW
12/4/97	46.71	40.57	79.43	2956.14	7.43	260	NW
12/5/97	41.29	38.57	90.43	2943.43	6.86	290	NW
12/9/97	38.50	33.67	82.50	3000.33	4.83	70	NW
12/10/97	37.57	27.71	67.29	2986.86	5.00	360	NW
12/11/97	29.43	21.43	71.71	3005.29	11.14	360	NE
12/16/97	36.86	25.57	63.71	2999.14	3.43	360	N
12/17/97	47.86	27.86	46.00	2978.00	8.71	240	W
12/18/97	39.86	21.71	49.14	3000.57	11.14	320	W
1/27/98	27.57	20.00	70.29	3050.29	8.33	90	NW
1/28/98	35.86	33.43	90.57	3001.43	21.14	50	NW
1/29/98	39.88	32.75	74.88	2971.12	13.62	360	NW

[a] Since biologic data for 12/3/97 were not usable, no weather data are shown here.

consecutive 3-day exposures (Table 9). The days in this study also tended to have lower concentration factors than days for the crossover studies. Differences are related partly to the fact that some of these studies were carried out before optimization of the HAPC slit alignment. The corresponding biologic data, as CAPs versus sham differences, were used to assess both cardiorespiratory parameters and inflammation.

Cardiopulmonary Responses Normal canine subjects in both double and crossover studies showed considerable HF influence in HRV. Figure 9 illustrates a Fourier-transformed HRV spectrum of a normal resting dog. The marked degree of HF influence evident in this example suggests the importance of this parameter as a canine response element of cardiac physiologic balance and vagally mediated influence on the heart. The effect of CAPs exposure on HRV is evident when the data from crossover and double CAPs and double sham experiments are pooled and reported as each dog's re- sponse to CAPs or sham exposure (Table 10). These data do not take the concentration of CAPs or any composition data into consideration; there are

Table 8. Exposure Parameters for Double CAPs/Double Sham Exposure Studies with Normal Dogs

Date	Animal (n = 12 total)	MMAD ± GSD[a] (μm)	Mass CAPs (μg/m^3)	SO_4 CAPs (μg/m^3)	Mass Ambient (μg/m^3)	SO_4 Ambient (μg/m^3)	Mass Concentration Factor	SO_4 Concentration Factor
8/20–22/96	3, 4		Sham					
9/23/96	3, 4	X	106.1	13.2	5.4	0.7	19.6	19.1
9/24/96	3, 4	X	236.2	31.8	7.9	1.4	29.9	22.2
9/25/96	3, 4	X	238.4	74.1	9.7	2.7	24.6	27.0
10/16/96	3, 4	0.19 ± 3.7	369.0	119.6	38.9	13.5	9.5	8.9
10/17/96	3, 4	0.19 ± 3.7	155.0	43.1	15.2	3.7	10.2	11.6
11/20–22/96	6, 5		Sham					
12/16/96	6, 3	0.26 ± 3.3	167.8	53.7	12.3	5.7	13.6	9.4
12/17/96	6, 3	0.26 ± 3.3	85.8	11.6	4.2	1.5	20.4	7.7
12/18/96	6, 3	0.26 ± 3.3	172.7	70.3	11.7	4.2	14.8	16.7
1/16–17/97	12, 13		Sham					
2/24/97	6, 3	0.24 ± 1.7	145.1	36.0	6.6	2.2	22.0	16.3
2/25/97	6, 3	0.24 ± 1.7	60.1	15.3	3.9	1.1	15.4	13.7
2/26/97	6, 3	0.24 ± 1.7	180.5	34.9	8.7	2.8	20.7	12.6
8/4/97	1, 2	X	150.9	53.8	15.7	5.3	9.6	10.1
8/5/97	1, 2	X	108.2	31.6	10.0	2.7	10.8	11.8
8/6/97	1, 2	X	124.8	28.5	12.2	2.8	10.2	10.3
9/29–10/1/97	1, 2		Sham					
10/1/97	6, 7	0.20 ± 3.1	50.1	6.6	3.9	0.4	13.0	17.0
10/2/97	6, 7	0.20 ± 3.1	84.6	11.2	7.5	0.6	11.3	19.2
10/3/97	6, 7	0.20 ± 3.1	235.4	38.0	15.4	2.5	15.3	15.1
10/21–23/97	6, 7		Sham					
11/17–19/97	14, 12		Sham					
2/2/98	8, 9	0.27 ± 2.1	627.0	155.7	28.0	6.3	22.4	24.5
2/3/98	8, 9	0.27 ± 2.1	504.0	133.3	17.6	4.9	28.6	27.5
2/4/98	8, 9	0.27 ± 2.1	266.6	99.2	11.4	3.6	23.4	27.7

X = Not measured.

Table 9. Elemental Analysis Parameters for Double CAPs/Double Sham Exposure Studies with Normal Dogs[a]

Date	Mass CAPs	Na	Al	Si	S	Cl	K	Ca	Ti	V	Cr	Mn	Fe	Ni	Cu	Zn	As	Se	Br	Cd	Pb	Ba
09/23/96	106.1	0.000	0.266	1.901	3.320	0.030	0.633	2.931	0.113	0.054	0.000	0.031	1.343	0.032	0.068	0.190	0.000	0.007	0.017	0.043	0.085	0.776
09/24/96	236.2	1.894	13.704	22.093	9.016	0.000	3.230	7.303	3.901	0.132	0.030	0.187	8.470	0.104	0.114	0.508	0.016	0.007	0.017	0.000	0.094	0.759
09/25/96	238.4	0.000	0.178	1.823	25.500	0.000	0.516	1.412	0.164	0.023	0.004	0.043	1.580	0.029	0.076	0.232	0.000	0.011	0.028	0.000	0.089	0.536
10/16/96	369.0	0.000	0.724	5.882	73.778	0.000	1.864	1.306	0.232	0.019	0.008	0.093	3.145	0.012	0.095	0.606	0.000	0.047	0.116	0.015	0.206	0.094
10/17/96	155.0	0.000	0.710	3.866	19.253	0.000	1.057	1.390	0.136	0.009	0.000	0.080	2.297	0.021	0.085	0.265	0.024	0.022	0.012	0.028	0.077	0.583
12/16/96	167.8	0.000	0.105	1.208	17.000	0.000	0.375	0.696	0.089	0.252	0.000	0.038	1.418	0.187	0.084	0.634	0.000	0.000	0.093	0.038	0.125	1.139
12/17/96	85.8	1.778	0.189	0.508	3.400	2.253	0.296	0.238	0.061	0.063	0.000	0.028	0.603	0.049	0.039	0.133	0.000	0.009	0.014	0.000	0.110	0.582
12/18/96	172.7	0.000	0.004	0.885	22.630	0.000	0.316	0.231	0.055	0.069	0.001	0.000	0.782	0.063	0.067	0.160	0.000	0.017	0.048	0.042	0.132	0.635
02/24/97	145.1	0.000	0.574	2.884	12.838	0.000	0.738	1.132	0.147	0.021	0.000	0.052	2.250	0.017	0.057	0.159	0.000	0.000	0.025	0.000	0.068	0.466
02/25/97	60.1	0.000	0.676	3.244	6.246	0.079	0.759	0.946	0.145	0.034	0.007	0.077	2.382	0.028	0.080	0.121	0.028	0.003	0.031	0.014	0.049	0.208
02/26/97	180.5	0.000	0.736	2.564	4.577	0.000	0.617	0.500	0.107	0.009	0.005	0.039	1.421	0.022	0.043	0.146	0.000	0.013	0.009	0.091	0.070	0.634
08/04/97	150.9	1.163	0.474	1.496	18.709	0.000	0.654	1.256	0.083	0.434	0.000	0.019	1.050	0.242	0.019	0.213	0.000	0.003	0.024	0.016	0.075	0.289
08/05/97	108.2	0.000	0.304	1.376	11.078	0.000	0.421	0.728	0.074	0.107	0.007	0.043	1.255	0.065	0.038	0.157	0.000	0.003	0.027	0.000	0.077	0.547
08/06/97	124.8	0.000	0.881	2.642	10.129	0.000	0.812	1.210	0.132	0.086	0.003	0.071	1.818	0.051	0.058	0.152	0.005	0.016	0.037	0.000	0.057	0.287
10/01/97	50.1	0.079	0.284	1.319	1.920	0.079	0.274	1.294	0.094	0.012	0.000	0.032	1.206	0.008	0.054	0.106	0.000	0.000	0.000	0.000	0.046	0.517
10/02/97	84.6	0.000	0.808	2.077	3.471	0.411	0.721	1.324	0.118	0.009	0.000	0.061	1.719	0.004	0.063	0.107	0.000	0.000	0.015	0.026	0.068	0.540
10/03/97	235.4	0.000	1.088	3.960	12.104	0.000	1.424	1.671	0.229	0.136	0.015	0.124	4.178	0.094	0.230	0.542	0.013	0.033	0.076	0.000	0.264	0.954
02/02/98	627.0	1.831	2.519	11.034	50.739	4.378	3.138	3.960	0.587	0.458	0.031	0.168	10.508	0.225	0.294	0.917	0.060	0.033	0.175	0.066	0.326	1.175
02/03/98	504.0	1.142	1.551	8.226	43.551	0.000	2.685	2.481	0.460	0.127	0.028	0.192	7.849	0.113	0.220	0.716	0.025	0.097	0.216	0.000	0.281	0.843
02/04/98	266.6	3.499	0.875	3.790	32.721	1.662	1.888	2.217	0.245	0.039	0.006	0.126	3.401	0.040	0.121	0.629	0.026	0.015	0.103	0.001	0.152	0.519

[a]All values are in $\mu g/m^3$.

Table 10. Cardiac Measurements for All Normal Dogs in Crossover and Double CAPs/Double Sham Studies: Each Dog's Responses

	Number of Days		LF Power of HRV (beats/min^2/Hz)		HF Power of HRV (beats/min^2/Hz)		LF/HF Ratio		Mean HR (beats/min)		HR SD (beats/min)	
Animal	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs
1	6	6	35.10	40.93	196.38	327.49	0.17	0.15	83.50	71.29	25.86	20.90
2	6	6	24.87	62.81	77.44	205.72	0.33	0.35	76.08	74.51	20.63	17.92
3	3	11	24.46	24.92	200.23	270.84	0.33	0.16	92.63	82.90	16.29	18.54
4	3	6	42.91	58.00	175.27	323.55	0.29	0.22	81.77	72.23	16.29	21.21
6	9	12	59.51	62.82	439.80	454.25	0.21	0.22	89.69	88.28	24.83	24.96
7	6	6	60.20	63.42	499.06	362.86	0.21	0.27	89.10	96.95	25.37	20.75
8	3	6	28.82	38.89	186.81	203.52	0.26	0.29	64.45	59.89	16.30	17.31
9	3	6	80.27	69.28	353.25	480.96	0.29	0.20	81.12	78.76	22.78	25.41
12	9	6	39.37	43.73	244.36	246.34	0.19	0.20	68.05	75.38	18.82	19.23
14	6	6	29.90	40.63	188.08	237.64	0.25	0.28	76.10	71.18	16.22	18.24
Mean			42.54	50.54	256.07	311.32	0.25	0.23	80.25	77.14	20.34	20.45
Paired *t* test			$p = 0.04$		$p = 0.04$		$p = 0.21$		$p = 0.09$		$p = 0.50$	

no controls for any interaction of the heart rate or respiratory rate in this analysis of Table 10. Nevertheless, HF and LF both are increased significantly with CAPs exposure (LF sham = 42.54, LF CAPs = 50.54, $p = 0.04$; HF sham = 256.07, HF CAPs = 311.32, $p = 0.04$). Heart rate generally decreases, but not significantly ($p = 0.09$). Respiratory data are provided in Tables 11 and 12. Considerable changes occurred with CAPs exposure, but none reach statistical significance in this simple analysis.

The data presented in Tables 10, 11, and 12 also do not take into consideration the fact that the response may change consistently during an experiment. Indeed, frequently the parameters increased or decreased with the duration of exposure. In one representative double CAPs/double sham study, both high and low frequency increases were observed during a CAPs exposure (Figure 10), but the same dog receiving a sham exposure had both a lower response and no increase over time for either of these parameters. Both HF and LF responses for Dog 7 in the upper panels of Figure 10 increased during the CAPs exposure, compared to this dog's sham exposure, in addition to increasing over time. Dog 6 in the lower panel also showed an increase in both HF and LF for the CAPs response compared to the sham response, but to a lesser degree. Also, for Dog 6, an increase in the CAPs exposure occurred in both HF and LF over time, but this increase over time is not present in the sham exposure.

Figure 11 shows that the Dog 7 heart rate is higher in the CAPs exposure, consistent with the HF and LF data for this dog in Table 10, where the mean heart rate from six CAPs exposures for this dog is higher than that from six sham exposures. Table 10 also demonstrates that this was not the predominant response; only 2 of 10 dogs had an increase in average heart rate with CAPs exposure. Note that Dog 6 in Figure 11 shows essentially no difference in heart rate response to CAPs or sham exposure. For this dog, the data in Table 10 are means of 12 CAPs and 9 sham exposures, and the net results showed no biologic difference. Assessment of the change in heart rate over time, however,

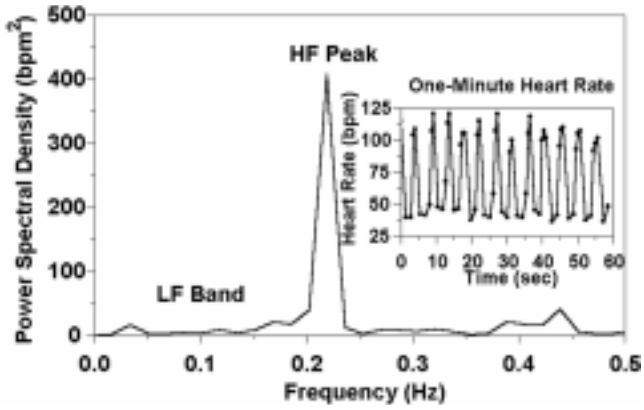


Figure 9. Fourier transformed spectrum of the typical resting pattern of canine HRV. Note dominance of the HF influence.

Table 11. Respiratory Measurements for All Normal Dogs in Crossover and Double CAPs/Double Sham Studies: Each Dog's Responses

	f (breaths/min)		V_T (mL)		$\dot{V}$ (mL/min)		PIF (L/min)		PEF (L/min)	
Animal	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs
1	39.01	34.79	116.21	329.14	3414.74	7326.30	221.40	530.49	234.27	477.46
2	41.90	31.39	121.92	380.23	3785.72	8264.83	261.66	744.56	258.77	531.97
4[a]	62.10	32.95								
6	32.97	41.07	137.75	144.84	3674.61	4022.88	311.37	293.60	178.99	248.53
7	32.49	26.92	107.51	79.15	3563.99	1997.61	299.67	144.32	259.38	156.84
8	26.38	32.42	56.18	66.98	1446.95	2199.28	114.78	154.48	144.94	162.25
9	57.13	36.66	120.94	59.53	7475.40	2195.18	385.99	177.49	371.79	182.26
12	60.28	83.27	83.23	29.57	3053.13	2144.58	219.28	131.84	192.64	126.98
14	63.56	71.70	50.52	66.92	2806.29	4427.46	165.21	263.62	175.22	315.23
Mean	46.20	43.46	99.28	144.55	3652.60	4072.27	247.42	305.05	227.00	275.19
Paired t test	$p = 0.31$		$p = 0.16$		$p = 0.36$		$p = 0.26$		$p = 0.22$	

[a] Respitrace (Ardsley NY) system used to monitor breathing pattern.

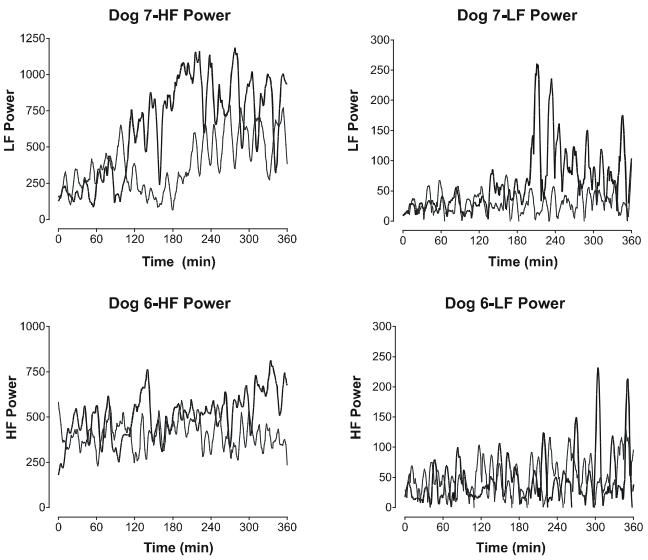


Figure 10. HF and LF patterns of the same dogs during 6-hour double CAPs (10/1/97, darker line) and double sham (10/23/97, lighter line) exposures.

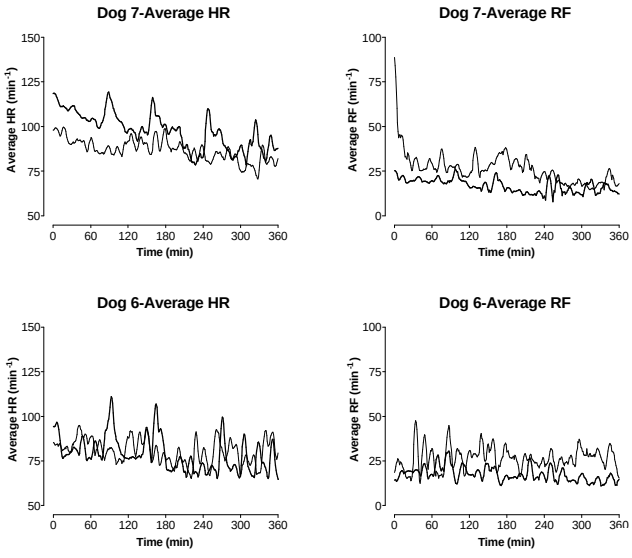


Figure 11. Heart rate and respiratory frequency patterns for the same dogs exposed in double CAPs (10/1/97, darker line) and double sham (10/23/97, lighter line) experiments.

Table 12. Respiratory Measurements for All Normal Dogs in Crossover and Double CAPs/Double Sham Studies: Each Dog's Responses

	EIP (msec)		T_I (sec)		EEP (msec)		T_E (sec)		Pau_{enh}		T_{rel} (sec)		Pau	
Animal	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs
1	37.90	50.66	1.12	1.08	1098.75	1079.50	1.99	2.07	4.98	1.98	0.62	0.84	2.75	1.82
2	41.51	51.13	0.93	0.91	918.12	1521.66	1.64	2.66	2.36	1.78	0.56	0.90	2.19	2.22
6	112.72	389.90	0.82	1.16	435.24	277.60	1.85	1.57	0.99	0.53	1.10	1.19	1.15	0.55
7	53.20	60.24	0.74	1.06	1029.66	1160.18	1.84	1.90	3.31	4.36	0.79	0.76	2.34	2.75
8	43.46	64.81	1.18	1.00	1188.55	1300.16	1.80	1.98	7.33	6.31	0.47	0.58	3.95	3.78
9	47.04	80.00	0.60	0.87	680.28	1247.82	1.23	1.81	3.75	4.75	0.44	0.56	2.64	3.23
12	54.26	39.82	0.72	0.48	1080.19	325.08	1.92	0.76	2.10	2.41	0.80	0.28	1.89	1.75
14	52.32	48.00	0.54	0.58	207.93	429.01	0.74	0.86	1.99	3.74	0.31	0.30	1.52	2.23
Mean	55.30	98.07	0.83	0.89	829.84	917.63	1.63	1.70	3.35	3.23	0.64	0.68	2.30	2.29
Paired *t* test	$p = 0.12$		$p = 0.23$		$p = 0.29$		$p = 0.37$		$p = 0.42$		$p = 0.34$		$p = 0.48$	

showed that both CAPs and sham exposures were associated with decreases over time in both dogs (albeit a greater decrease with CAPs exposure). Again, this response was stronger in Dog 7.

The dogs' respiratory rates under CAPs exposure compared to sham exposure decreased over time (Figure 11, right panel). For this parameter in Table 12, 5 of 9 dogs had lower respiratory rates with CAPs exposure, and overall CAPs and sham exposures showed no significant difference in rate, although an apparent relation existed with time, which is more noticeable in Dog 7. Thus, it is apparent that responses may change over the duration of the experiment. Because continuous response and exposure data were collected, optimal analysis required their use as described in the statistical methods. Furthermore, these two examples (Dog 6 and Dog 7) show the similarity as well as the differences among dogs given the same exposures. The similarity in responses to the CAPs exposure may represent a similar effect of the exposure on both dogs, but the double exposure protocol does not provide adequate support for this interpretation. Thus, we deemed the crossover design protocol necessary for optimal analysis of cardiopulmonary responses.

The crossover HF data for four pairs of dogs (Figures 12 through 15) illustrate the response of each pair of dogs on each day of exposure, and each figure is a full representation of the crossover. The top portion of each panel shows the concentration of CAPs over the period of exposure, illustrating that concentration often varied throughout the day.

Considerable day-to-day variation and complexity were evident in dog heart and lung responses to CAPs and exposures. On some days, no response to CAPs exposure concentrations was evident. In contrast, data from other days showed considerable HF response to CAPs, far above the sham dog's level, and increasing throughout the exposure period. At the same time, the sham dog's HF remained relatively constant, but different from the CAPs dog's sham pattern. For example, dog 1, regardless of exposure, had a higher level of the HF domain than its chambermate (Figure 12). On days of sham exposure, the HF was relatively flat, whereas with CAPs exposure, HF increases on 2 of the 3 days. Dog 2 had an increasing response to sham exposure on June 23 and June 24, but the slope does not appear to be as great as that of her chambermate exposed to CAPs.

The pulmonary response parameter PEF, an indicator of bronchoconstriction or marked change in breathing pattern, ranged from no change in PEF throughout a 6-hour exposure to distinctive changes in PEF, with an increase or decrease in this parameter throughout a CAPs exposure period (Figures 16 through 19). Clearly, these responses are complex, and the need to have an approach to assess both dog-to-dog and day-to-day variability in CAPs toxicity is apparent. Our statistical approach in the crossover design compares the CAPs-exposed dog to the sham-exposed chambermate as well as to its own sham exposure. In addition, continuous measures of exposure are used in the model as well as the minute volume to provide

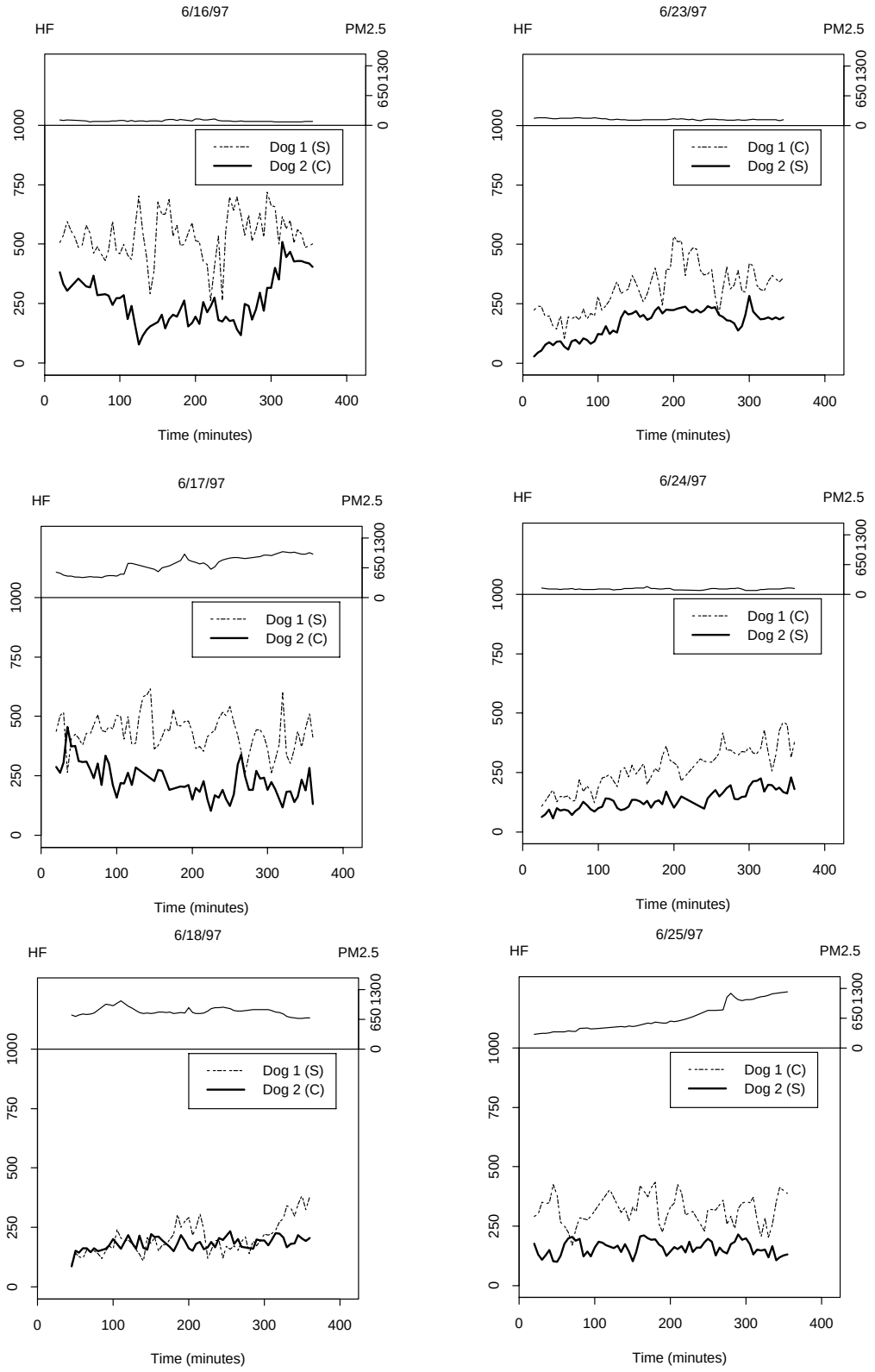


Figure 12. HF patterns of CAPs (C) and sham (S) exposures observed in June 1997 for dogs 1 and 2 on all crossover days. The CAPs mass concentration is shown in the upper panel for each day. These patterns can be compared with the pairs shown in Figures 13 through 15.

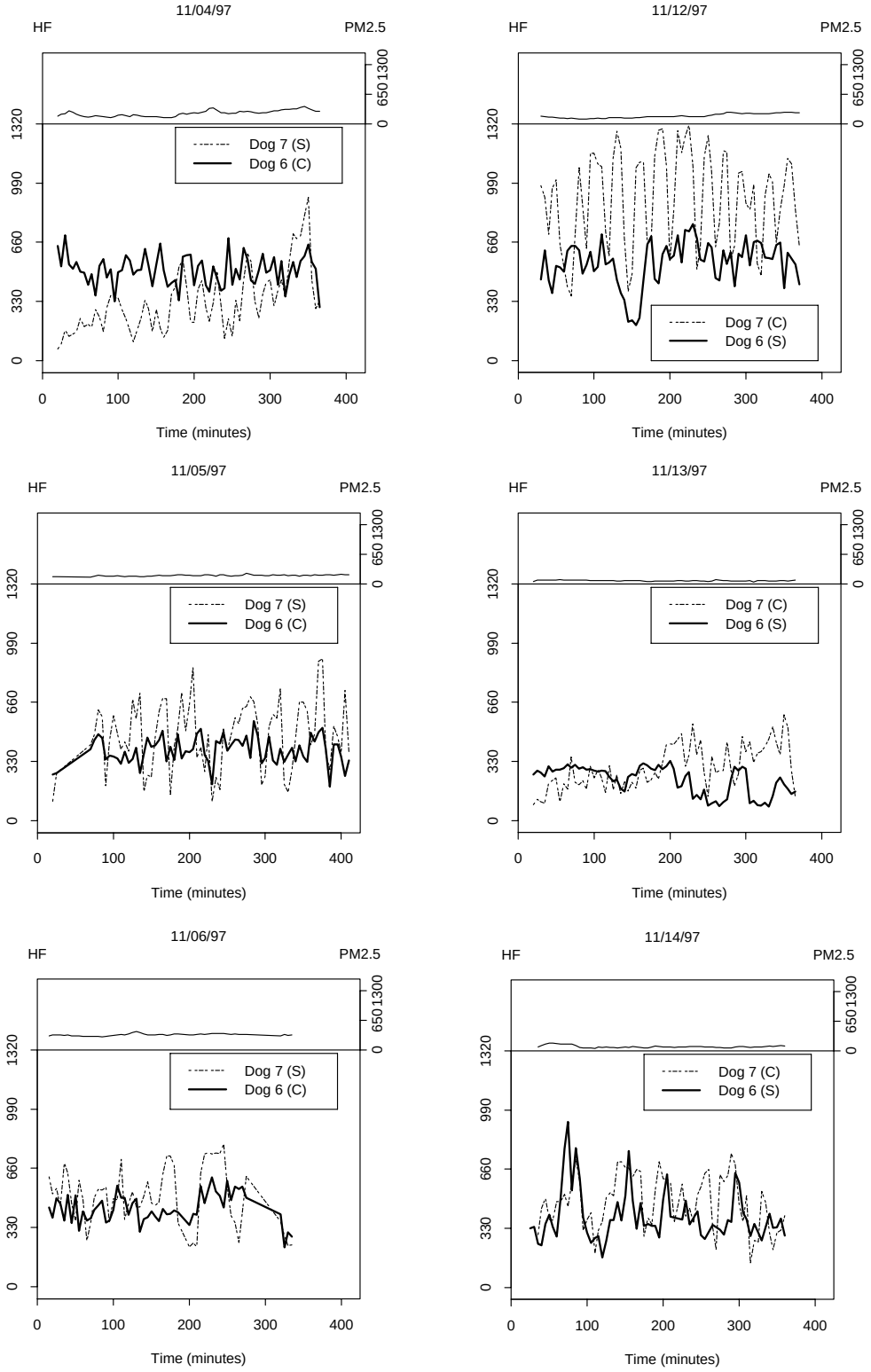


Figure 13. HF patterns of CAPs (C) and sham (S) exposures observed in November 1997 for dogs 6 and 7 on all crossover days. The CAPs mass concentration is shown in the upper panel for each day. These patterns can be compared with the pairs shown in Figures 12, 14, and 15.

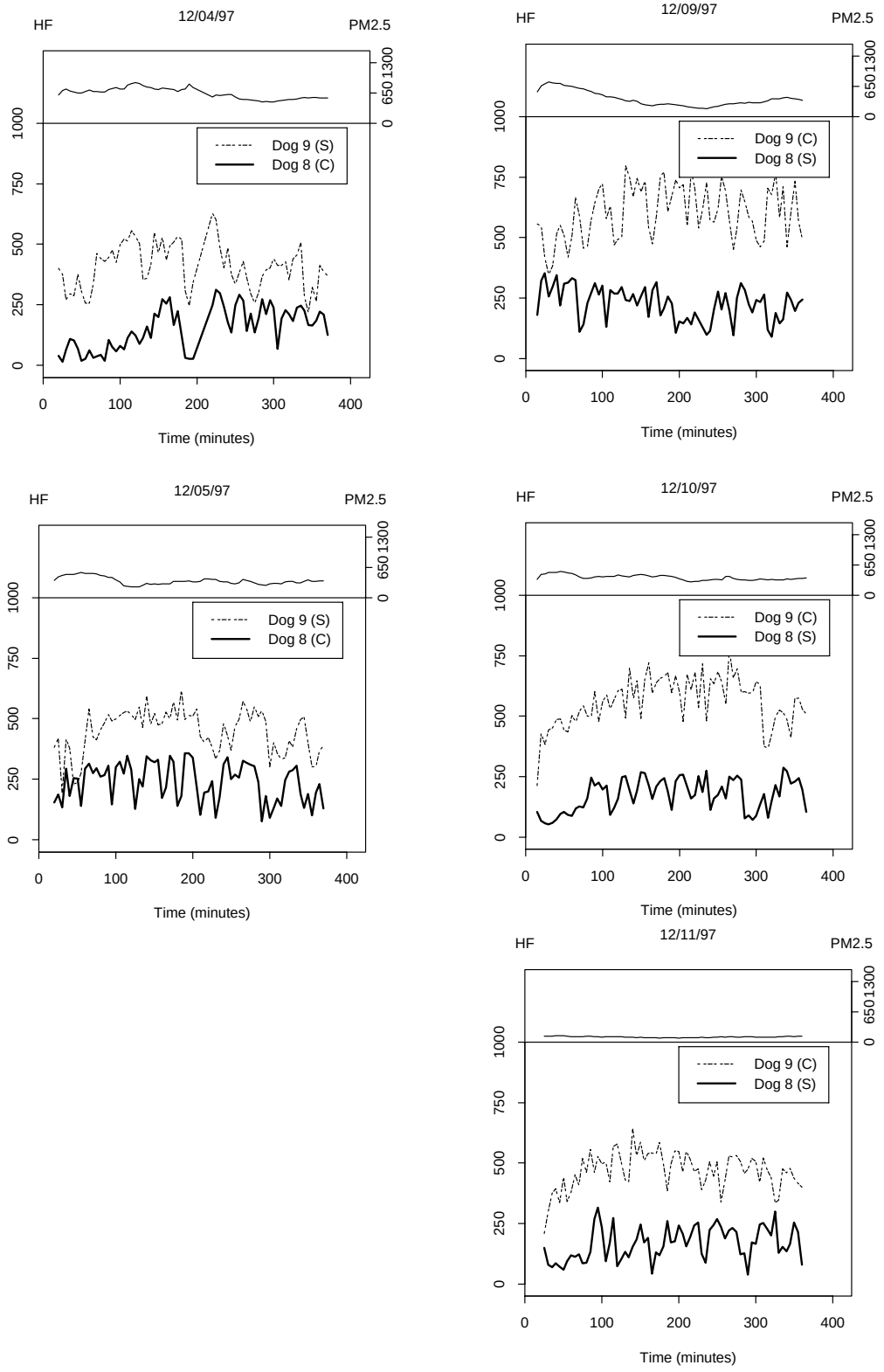


Figure 14. HF patterns of CAPs (C) and sham (S) exposures observed in December 1997 for dogs 8 and 9 on all crossover days. The CAPs mass concentration is shown in the upper panel for each day. These patterns can be compared with the pairs shown in Figures 12, 13, and 15.

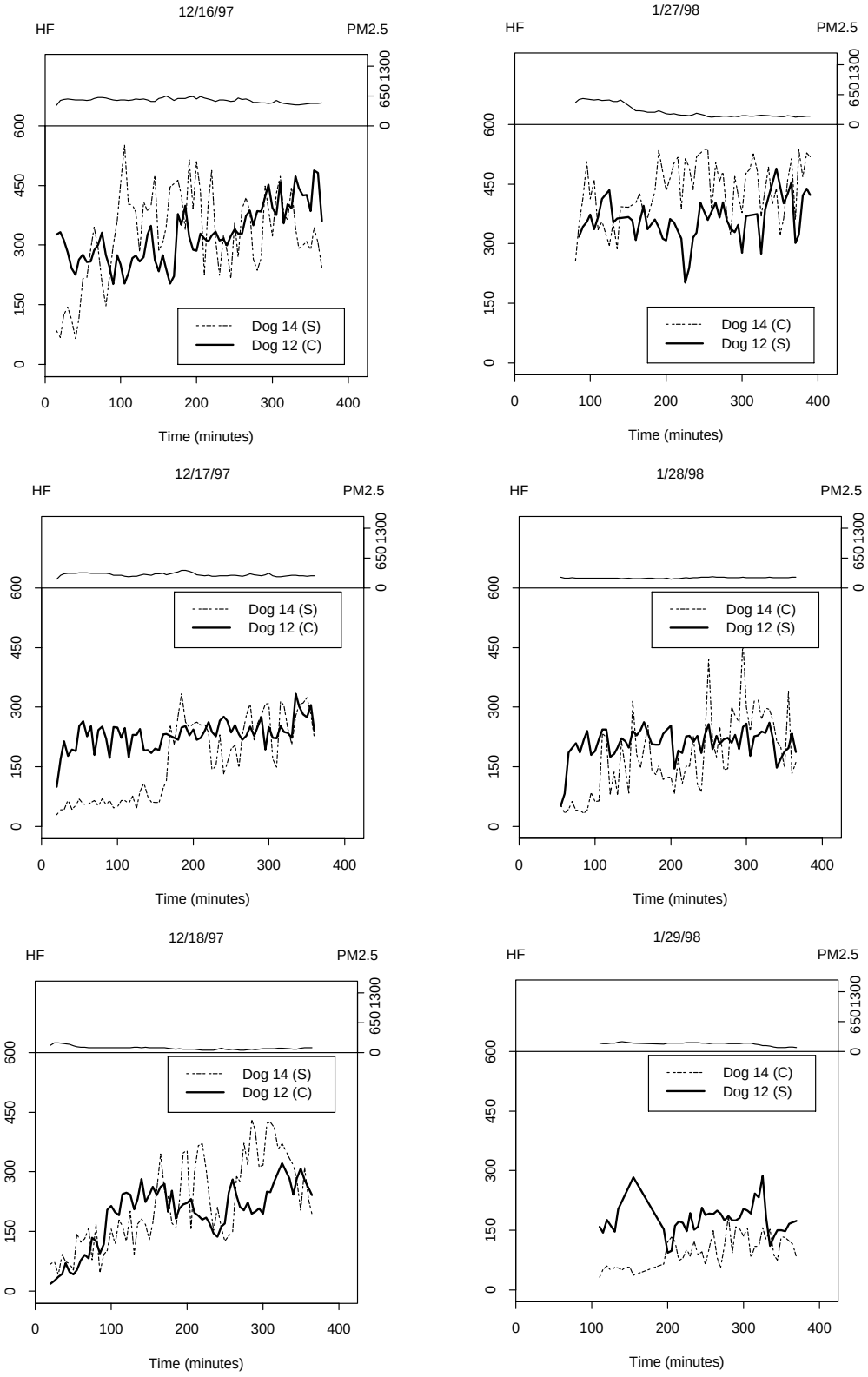


Figure 15. HF patterns of CAPs (C) and sham (S) exposures observed in December 1997 and January 1998 for dogs 12 and 14 on all crossover days. The CAPs mass concentration is shown in the upper panel for each day. These patterns can be compared with the pairs shown in Figures 12 through 14.

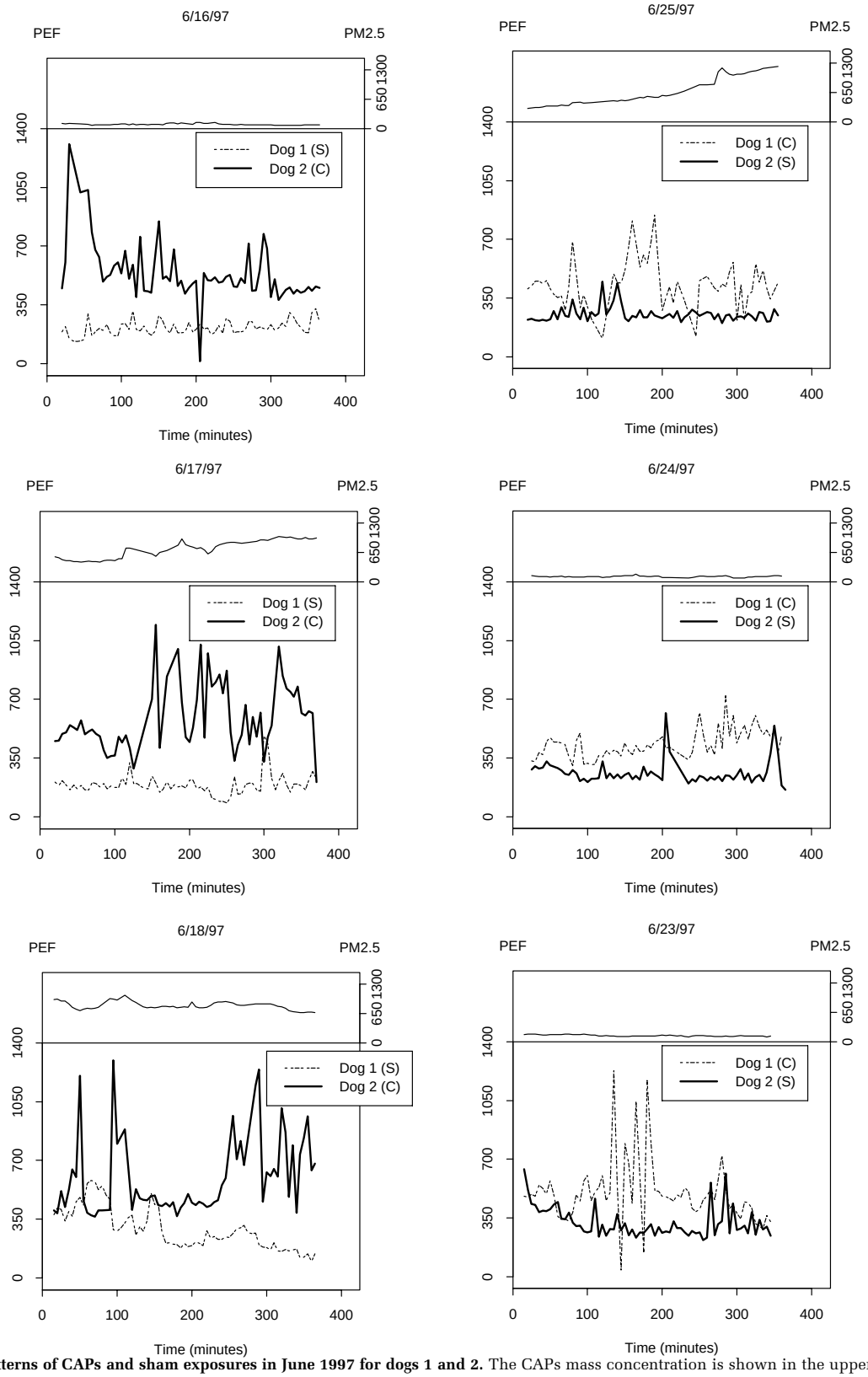


Figure 16. PEF patterns of CAPs and sham exposures in June 1997 for dogs 1 and 2. The CAPs mass concentration is shown in the upper panel for each day. These patterns can be compared with the pairs shown in Figures 17 through 19.

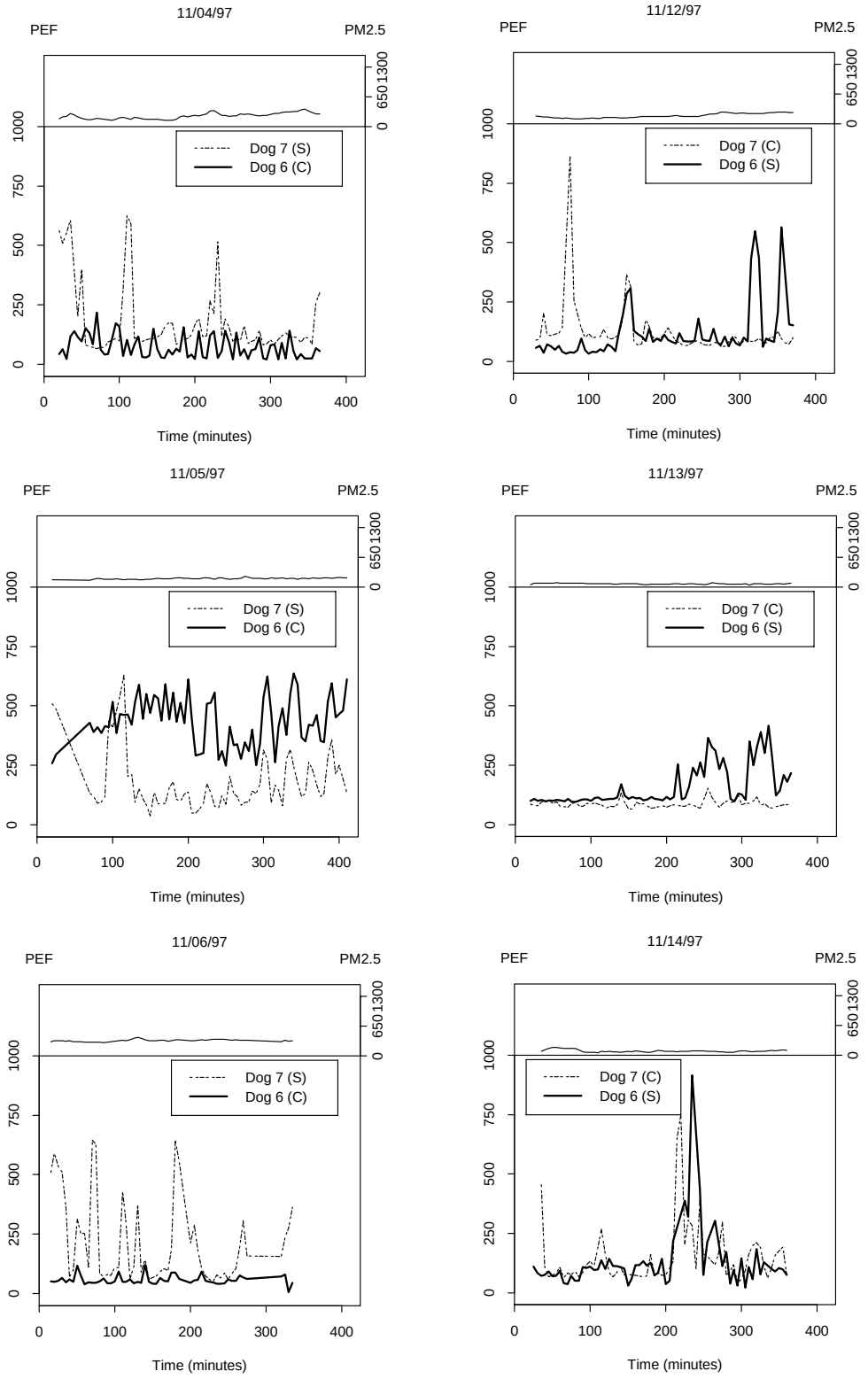


Figure 17. PEF patterns of CAPs and sham exposures in November 1997 for dogs 6 and 7. The CAPs mass concentration is shown in the upper panel for each day. These patterns can be compared with the pairs shown in Figures 16, 18, and 19.

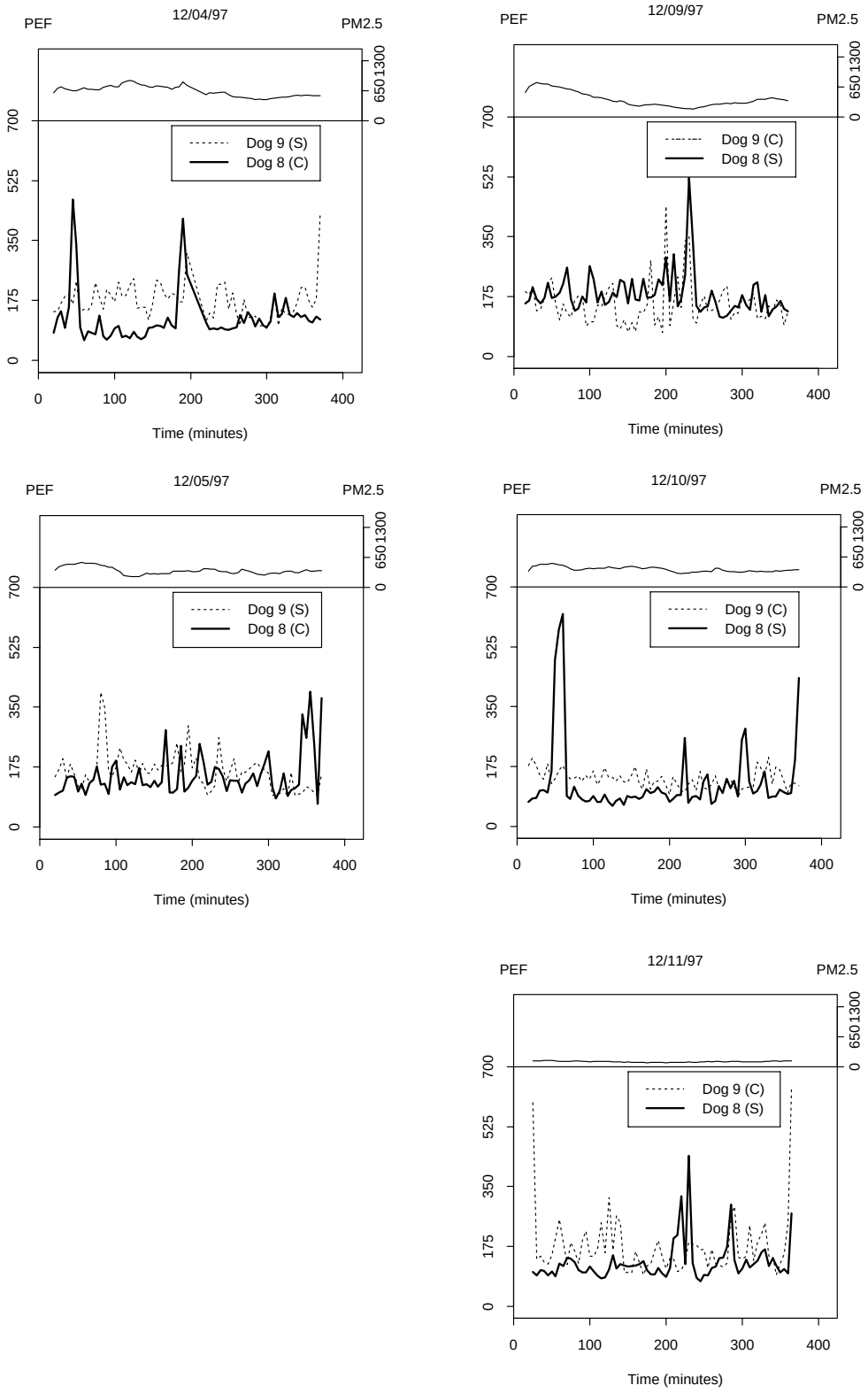


Figure 18. PEF patterns of CAPs and sham exposures in December 1997 for dogs 8 and 9. The CAPs mass concentration is shown in the upper panel for each day. These patterns can be compared with the pairs shown in Figures 16, 17, and 19.

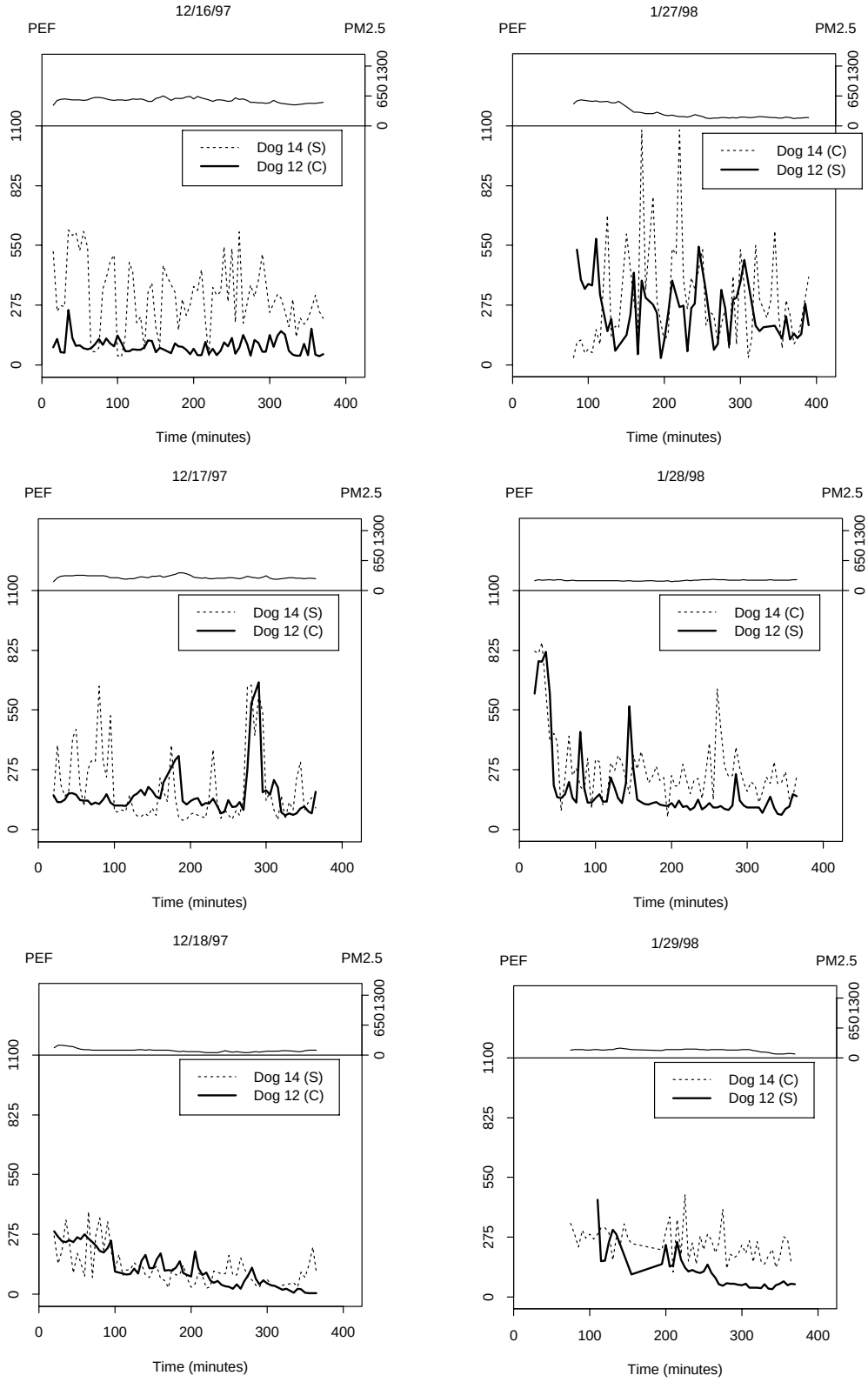


Figure 19. PEF patterns of CAPs and sham exposures in December 1997 and January 1998 for dogs 12 and 14. The CAPs mass concentration is shown in the upper panel for each day. These patterns can be compared with the pairs shown in Figures 16 through 18.

a cumulative exposure dose. The estimated regression coefficient $\{\hat{\beta}_j^C\}$ is a measure of the magnitude of response over time, response in relation to exposure, and response controlling for known confounding factors (such as the effect of respiratory frequency on HRV parameters). Figure 20 shows how the statistical comparisons are made using an example from Figures 12 through 15.

The estimated regression coefficients $\{\hat{\beta}_j^C\}$ for each response in the healthy dog crossover experiments are ordered with respect to size and direction of biologic response (Tables 13 through 15): bold type indicates that

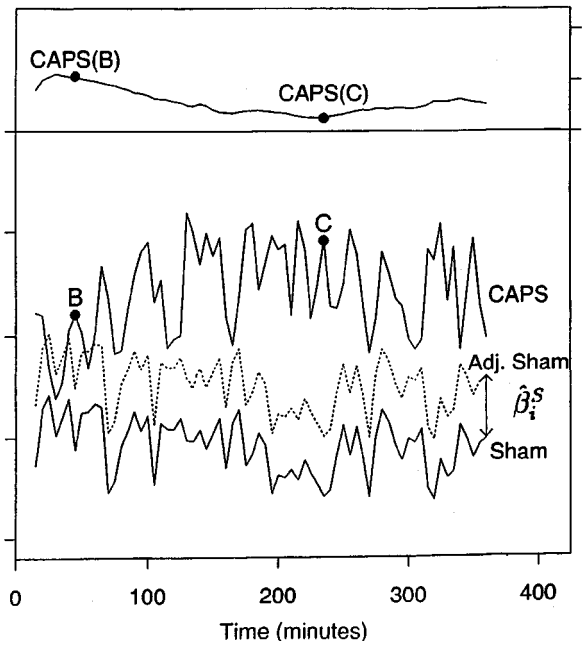


Figure 20. Day-specific response comparisons [see equation (2)]. For exposure day *j*, note the differences in slope of adjusted sham compared to CAPs as well as in relation to the function of CAPs (B) and differences of C–B as a function of CAPs (C)–CAPs (B). Upper panel shows CAPs mass concentration trend.

Table 13. Cumulative Effects of $PM_{2.5}$ on Cardiac Parameters by Day for Crossover Design Studies with Normal Dogs[a]

LF Power of HRV		HF Power of HRV		LF/HF Ratio		Mean HR		HR SD	
Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$
6/16/97	**−0.0404**	12/18/97	**−0.0433**	6/16/97	**−0.0437**	11/13/97	**−3.4353**	12/18/97	**−0.0183**
11/6/97	**−0.0388**	12/11/97	**−0.0285**	1/27/98	**−0.0400**	11/12/97	**−2.1754**	11/6/97	**−0.0088**
11/5/97	**−0.0114**	12/16/97	**−0.0242**	11/6/97	**−0.0395**	11/14/97	**−1.3672**	12/16/97	**−0.0081**
12/4/97	−0.0056	12/17/97	**−0.0104**	1/29/98	−0.0163	12/5/97	**−0.5194**	12/11/97	−0.0080
12/18/97	−0.0039	12/9/97	**−0.0061**	11/5/97	**−0.0099**	12/11/97	**−0.5106**	12/17/97	**−0.0039**
6/17/97	**−0.0022**	11/6/97	−0.0051	12/4/97	−0.0071	11/4/97	**−0.4785**	6/16/97	**−0.0033**
1/29/98	−0.0003	11/5/97	**−0.0033**	6/17/97	**−0.0025**	12/4/97	**−0.2917**	11/5/97	**−0.0030**
12/17/97	−0.0001	6/23/97	−0.0026	6/18/97	0.0005	6/24/97	**−0.2142**	12/9/97	−0.0019
6/18/97	**0.0010**	12/10/97	−0.0016	12/5/97	0.0039	6/23/97	**−0.1675**	12/10/97	−0.0018
1/27/98	0.0012	6/24/97	−0.0012	1/28/98	0.0043	12/9/97	**−0.1391**	6/17/97	−0.0002
6/25/97	**0.0061**	6/16/97	−0.0002	6/25/97	**0.0061**	1/28/98	**−0.1080**	6/23/97	0.0000
12/10/97	0.0117	6/17/97	−0.0001	12/17/97	0.0108	1/29/98	−0.0857	6/18/97	**0.0004**
12/16/97	**0.0118**	11/4/97	−0.0001	6/23/97	**0.0156**	6/25/97	**−0.0434**	6/25/97	**0.0007**
12/9/97	**0.0124**	6/25/97	0.0004	12/10/97	0.0161	6/18/97	**−0.0262**	12/4/97	0.0008
6/23/97	**0.0124**	6/18/97	**0.0008**	6/24/97	**0.0204**	1/27/98	−0.0149	6/24/97	**0.0019**
1/28/98	**0.0148**	12/4/97	0.0009	12/9/97	**0.0214**	6/17/97	0.0099	11/4/97	**0.0051**
6/24/97	**0.0189**	1/29/98	**0.0112**	11/4/97	**0.0319**	12/10/97	0.0243	1/28/98	**0.0056**
12/5/97	**0.0209**	1/28/98	**0.0112**	12/18/97	**0.0339**	11/5/97	**0.1131**	1/29/98	**0.0063**
11/4/97	**0.0334**	12/5/97	**0.0168**	12/16/97	**0.0374**	12/16/97	**0.1198**	12/5/97	**0.0097**
12/11/97	**0.1050**	11/14/97	**0.0249**	11/13/97	0.0534	6/16/97	**0.1593**	1/27/98	**0.0144**
11/14/97	**0.1057**	1/27/98	**0.0387**	11/14/97	**0.0829**	12/17/97	**0.1940**	11/14/97	**0.0239**
11/13/97	**0.1286**	11/12/97	**0.0576**	12/11/97	**0.1336**	12/18/97	**0.4958**	11/12/97	**0.0395**
11/12/97	**0.2537**	11/13/97	**0.0844**	11/12/97	**0.2021**	11/6/97	**0.6218**	11/13/97	**0.0469**

[a] All measurements were controlled for *f* and $\dot{V}$, and all except mean HR were controlled for heart rate. All data except mean HR are presented as log values. Boldface type indicates significance at $p < 0.05$.

Table 14. Cumulative Effects of $PM_{2.5}$ on Pulmonary Parameters by Day for Crossover Design Studies with Normal Dogs[a]

f		V_T		$\dot{V}$		PIF		PEF	
Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$
11/13/97	**−0.2549**	11/13/97	**−0.0931**	11/13/97	**−0.3205**	11/13/97	**−0.2504**	11/13/97	**−0.2470**
11/12/97	**−0.1253**	12/16/97	**−0.0445**	11/12/97	**−0.1043**	11/12/97	**−0.0514**	11/12/97	**−0.0698**
12/11/97	**−0.0433**	11/14/97	**−0.0121**	12/16/97	**−0.0557**	12/16/97	**−0.0510**	12/16/97	**−0.0371**
11/6/97	**−0.0269**	1/27/98	**−0.0121**	11/6/97	**−0.0340**	11/6/97	**−0.0284**	11/6/97	**−0.0352**
12/10/97	**−0.0212**	12/9/97	**−0.0099**	11/14/97	−0.0241	12/11/97	−0.0235	11/4/97	**−0.0350**
12/5/97	**−0.0198**	11/4/97	−0.0078	12/10/97	**−0.0214**	11/4/97	**−0.0219**	11/14/97	**−0.0286**
11/14/97	−0.0192	11/6/97	−0.0067	11/4/97	**−0.0205**	12/18/97	−0.0131	12/9/97	**−0.0227**
12/16/97	**−0.0170**	12/17/97	−0.0064	12/11/97	−0.0168	12/10/97	**−0.0127**	12/11/97	−0.0218
6/24/97	**−0.0145**	12/18/97	−0.0062	12/4/97	**−0.0124**	11/14/97	−0.0111	12/18/97	**−0.0166**
11/4/97	−0.0081	12/4/97	−0.0041	12/9/97	−0.0081	12/4/97	**−0.0072**	12/10/97	**−0.0123**
1/28/98	**−0.0078**	12/10/97	−0.0035	12/5/97	−0.0068	12/9/97	−0.0052	12/4/97	0.0007
12/4/97	**−0.0068**	1/29/98	0.0005	1/27/98	−0.0047	12/17/97	−0.0035	6/18/97	**0.0020**
6/23/97	**−0.0064**	6/17/97	**0.0018**	12/18/97	−0.0032	1/27/98	−0.0015	6/17/97	**0.0029**
12/18/97	−0.0053	6/18/97	**0.0031**	6/18/97	**0.0008**	12/5/97	−0.0013	12/17/97	0.0033
11/5/97	**−0.0026**	6/25/97	**0.0077**	6/17/97	**0.0036**	6/18/97	**0.0018**	1/27/98	0.0034
6/18/97	**−0.0019**	12/11/97	0.0136	6/25/97	**0.0059**	6/17/97	**0.0034**	6/25/97	**0.0051**
6/25/97	−0.0009	12/5/97	**0.0157**	12/17/97	**0.0087**	6/25/97	**0.0065**	1/28/98	**0.0133**
12/9/97	−0.0008	6/16/97	**0.0161**	1/28/98	**0.0126**	11/5/97	**0.0099**	6/16/97	**0.0137**
6/17/97	**0.0022**	11/5/97	**0.0178**	11/5/97	**0.0128**	6/23/97	**0.0198**	6/23/97	**0.0160**
12/17/97	**0.0071**	1/28/98	**0.0209**	6/23/97	**0.0175**	1/28/98	**0.0198**	11/5/97	**0.0223**
1/27/98	**0.0099**	11/12/97	0.0221	6/24/97	**0.0234**	6/16/97	**0.0269**	12/5/97	**0.0231**
6/16/97	**0.0138**	6/23/97	**0.0272**	6/16/97	**0.0297**	6/24/97	**0.0293**	6/24/97	**0.0238**
1/29/98	**0.0548**	6/24/97	**0.0377**	1/29/98	**0.0440**	1/29/98	**0.0323**	1/29/98	**0.0408**

[a] All data are presented as log values. Boldface type indicates significance at $p < 0.05$.

the p value associated with the t test for an individual regression coefficient is less than 0.05. Applied to the cardiac responses, model (1) controls for respiratory performance by including log respiratory frequency and log minute volume in a parametric term (2). For cardiac responses, average heart rate per animal also is included in (2) except in assessment of average heart rates. Note that no correction for multiple tests has been employed in this setting and that on certain days exposure to CAPs has considerable influence on all cardiac parameters. On days with strongly positive responses in the parameters HF, LF, and LF/HF, such as November 12 or 13, heart rate decreased and the respiratory response was characterized by decreasing frequency, minute volume, and air flows. In addition, time of inspiration and expiration, pause, and Pau_{enh} all increased. Taken together, these and the other days with similarly significant responses can be interpreted as days in which changes in breathing pattern were consistent with a noxious response; similarly, the considerable cardiac effect as mediated by the vagus nerve indicates a

Table 15. Cumulative Effects of $PM_{2.5}$ on Pulmonary Parameters by Day for Crossover Design Studies with Normal Dogs[a]

EIP		T_I		EEP		T_E		Pau_{enh}	
Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$
12/16/97	**−0.0177**	1/29/98	**−0.0422**	1/29/98	**−0.1177**	1/29/98	**−0.0654**	11/4/97	**−0.0892**
12/17/97	**−0.0106**	12/9/97	**−0.0169**	6/16/97	**−0.0403**	12/17/97	**−0.0157**	12/11/97	**−0.0678**
1/29/98	**−0.0083**	6/16/97	**−0.0120**	12/17/97	**−0.0363**	6/16/97	**−0.0133**	11/12/97	**−0.4051**
11/6/97	−0.0001	6/17/97	**−0.0025**	11/5/97	**−0.0180**	1/27/98	**−0.0133**	12/9/97	**−0.0420**
12/4/97	0.0005	12/17/97	−0.0023	12/11/97	−0.0122	11/5/97	**−0.0065**	11/14/97	−0.0389
6/17/97	0.0006	1/27/98	−0.0011	12/9/97	−0.0082	6/17/97	**−0.0017**	6/16/97	**−0.0327**
12/10/97	0.0006	6/25/97	0.0004	11/4/97	−0.0075	12/18/97	−0.0010	1/29/98	**−0.0250**
6/18/97	**0.0009**	6/18/97	**0.0015**	6/17/97	**−0.0069**	6/25/97	**0.0018**	12/18/97	−0.0161
12/11/97	0.0025	12/4/97	**0.0046**	6/25/97	**0.0049**	6/18/97	**0.0029**	6/23/97	**−0.0146**
6/25/97	**0.0030**	1/28/98	**0.0046**	12/10/97	0.0058	12/9/97	0.0046	6/24/97	**−0.0132**
1/27/98	0.0035	12/10/97	**0.0061**	6/18/97	**0.0060**	11/4/97	0.0068	6/25/97	**−0.0054**
11/4/97	0.0044	6/23/97	**0.0061**	1/27/98	0.0066	12/4/97	**0.0076**	1/28/98	−0.0054
12/5/97	0.0067	6/24/97	**0.0079**	12/18/97	0.0153	6/23/97	**0.0077**	12/17/97	−0.0006
12/9/97	**0.0071**	11/4/97	**0.0087**	6/23/97	**0.0167**	1/28/98	**0.0116**	6/17/97	**0.0015**
6/23/97	**0.0080**	12/18/97	**0.0096**	12/4/97	**0.0190**	6/24/97	**0.0171**	11/6/97	0.0024
6/24/97	**0.0102**	12/16/97	**0.0096**	11/6/97	**0.0227**	12/10/97	**0.0181**	6/18/97	**0.0046**
6/16/97	**0.0103**	11/14/97	0.0116	1/28/98	**0.0304**	12/16/97	**0.0211**	1/27/98	0.0079
1/28/98	**0.0123**	11/5/97	**0.0123**	6/24/97	**0.0318**	11/6/97	**0.0233**	11/5/97	**0.0159**
12/18/97	**0.0204**	11/6/97	**0.0164**	11/14/97	0.0414	12/11/97	**0.0248**	12/10/97	**0.0206**
11/5/97	**0.0271**	12/5/97	**0.0278**	12/16/97	**0.0534**	11/14/97	**0.0264**	12/4/97	**0.0350**
11/12/97	**0.0976**	12/11/97	**0.0474**	12/5/97	**0.0598**	12/5/97	**0.0265**	12/16/97	**0.0640**
11/14/97	**0.1132**	11/12/97	**0.0920**	11/12/97	**0.0607**	11/12/97	**0.1429**	12/5/97	**0.0705**
11/13/97	**0.3270**	11/13/97	**0.1963**	11/13/97	**0.4034**	11/13/97	**0.2635**	11/13/97	**0.1525**

[a] All data are presented as log values. Boldface type indicates significance at $p < 0.05$.

slowing of the heart and an effect on HRV independent of the respiratory rate changes alone. A negative $\{\hat{\beta}_j^C\}$ indicates a decrease in the measured parameter over the 6-hour CAPs exposure relative to sham.

The data in Tables 13, 14, and 15 are very important in understanding day-to-day variation. Days of lesser and greater response are not directly related to the total exposure dose as measured by mass (Table 3). Table 16 shows the magnitude, direction, and significance of the overall responses. When all data are used, both the number of responses and the magnitude of the response were driving the overall result. If the individual day data reflected only random effects, the overall results would cancel out and no net effect would be discernible. As shown in Table 16, however, highly significant decreases in respiratory rate and cardiac rate occurred for both cumulative and actual dose assessments. At the same time highly significant increases in LF, LF/HF ratio, and HR SD were observed along with increases in all the remaining respiratory parameters. Taken together these data support the concept

Table 16. Effects of $PM_{2.5}$ on Cardiac and Respiratory Parameters in All Normal Dogs in Crossover Studies[a]

	Using Cumulative Exposure Dose			Using Actual Exposure Dose		
Response	$\hat{\beta}^C$	SE	p Value	$\hat{\beta}^C$	SE	p Value
log LF	1.9×10^{-3}	3.0×10^{-4}	< 0.0001	6.0×10^{-4}	6.7×10^{-5}	< 0.0001
log HF	4.0×10^{-4}	2.0×10^{-4}	< 0.06	4.5×10^{-5}	3.8×10^{-5}	NS
log LF/HF ratio	1.6×10^{-3}	4.0×10^{-4}	< 0.0001	5.0×10^{-4}	7.7×10^{-5}	< 0.0001
Mean HR	-2.4×10^{-1}	4.8×10^{-3}	< 0.0001	-6.7×10^{-3}	1.0×10^{-3}	< 0.0001
log HR SD	3.0×10^{-4}	9.0×10^{-5}	< 0.0001	7.4×10^{-5}	1.6×10^{-5}	< 0.0001
log V_T	4.2×10^{-3}	4.0×10^{-4}	< 0.0001	5.0×10^{-4}	9.0×10^{-5}	< 0.0001
log $\dot{V}$	2.3×10^{-3}	4.0×10^{-4}	< 0.0001	7.0×10^{-5}	1.0×10^{-4}	NS
log f	-1.3×10^{-3}	3.0×10^{-4}	< 0.0001	-4.0×10^{-4}	7.0×10^{-5}	< 0.0001
log EEP	3.3×10^{-3}	7.0×10^{-4}	< 0.0001	9.0×10^{-4}	1.0×10^{-4}	< 0.0001
log EIP	2.0×10^{-3}	4.0×10^{-4}	< 0.0001	5.0×10^{-4}	7.0×10^{-5}	< 0.003
log T_E	2.0×10^{-3}	4.0×10^{-4}	< 0.0001	5.0×10^{-4}	8.0×10^{-5}	< 0.0001
log T_I	9.0×10^{-4}	3.0×10^{-4}	< 0.01	2.0×10^{-4}	8.0×10^{-5}	< 0.003
log PEF	3.2×10^{-3}	4.0×10^{-4}	< 0.0001	2.0×10^{-4}	9.6×10^{-5}	< 0.02
log PIF	3.1×10^{-3}	4.0×10^{-4}	< 0.0001	3.0×10^{-4}	8.6×10^{-5}	< 0.001
log Pau_{enh}	3.0×10^{-3}	9.0×10^{-4}	< 0.001	1.0×10^{-4}	3.0×10^{-4}	NS

[a] Each response was calculated from 23 days. NS = Not significant.

that perturbations occurred in both sympathetic and parasympathetic influences on the heart and lung.

Tables 13, 14, and 15 also demonstrate the day-to-day variability in $PM_{2.5}$ effect on respiratory and cardiac performance. Data from the principal component analysis are presented in Table 6. Table 17 shows the constructed elemental groupings for each date based on the findings in Table 6. Table 18 shows the compositional effect estimates $\ddot{\alpha} = (\ddot{\alpha}, \ldots \ddot{\alpha}_k)$ and associated p values from model (5). At $p < 0.05$, the estimates $\ddot{\alpha}_k$ and associated p values indicate that few significant associations were found. These were with factor 4. Factor 2 had associations closest to the overall effects (ie, decreased respiratory frequency and average heart rate, and increased end expiratory pause). Although these were relatively close, they were not statistically significant. Group 5 is not included in Table 18 because levels were often below the LOD for cadmium. Other models and approaches can be applied to these data, and this is an area of continued investigation.

The potential role of transported constituents was analyzed in relation to trajectories of air masses coming to Boston on each day of the study. Table 7 lists the trajectory of the air mass for each day of the crossover design experiments. If the $\{\hat{\beta}_j^C\}$ estimates from Tables 13, 14, and 15 are dichotomized by trajectory and those from the north and northwest are compared to those of the other directions, significantly different patterns appear. Figure 21 illustrates the differences for the $\{\hat{\beta}_j^C\}$ of average heart rate (NW = −0.60 versus other = 0.06; $p = 0.02$). The HF data and HR SD have significant increases when the air mass trajectory is from the northwest. This suggests an association with an increase in vagal activity; perhaps aerosols coming from the northwest of Boston have a pulmonary irritant effect. Viewed from the opposite perspective, the significant decrease in HF and HR SD with a corresponding increase in heart rate (Figure 21) when aerosol came predominantly from the continental United States suggests that sympathetic effects may provide a basis for the significantly different responses in positive and negative directions in Table 13. When exposure measurements were dichotomized as described, comparisons show no statistical differences using a two-tailed heteroscedastic t test at the $p < 0.05$ level. The nickel concentration difference is NW = 0.080, other = 0.072, $p = 0.068$; the lead concentration

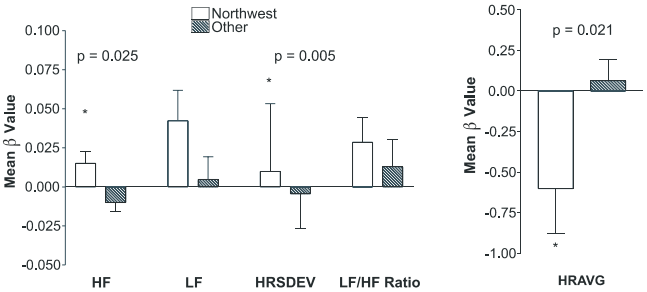


Figure 21. Comparison of northwest trajectories versus other trajectories on various cardiac parameters.

Table 17. Constructed Elemental Group Proportions for Each Date for Crossover Studies in Normal Dogs

Date	Component 1[a]	Component 2[a]	Component 3[a]	Component 4[a]	Component 5[a]
6/16/97	0.030517	0.638100	0.329920	0.000000	0.001468
6/17/97	0.014857	0.137780	0.847360	0.000000	0.000000
6/18/97	0.021320	0.124380	0.853950	0.000000	0.000348
6/23/97	0.060970	0.353950	0.582580	0.000000	0.002497
6/24/97	0.052184	0.443860	0.460450	0.043510	0.000000
6/25/97	0.014369	0.117910	0.867040	0.000000	0.000679
11/4/97	0.033621	0.458150	0.504080	0.004150	0.000000
11/5/97	0.034946	0.232740	0.730710	0.000000	0.000000
11/6/97	0.036548	0.232740	0.730710	0.000000	0.000000
11/12/97	0.042817	0.247180	0.710000	0.000000	0.000000
11/13/97	0.046789	0.417530	0.534360	0.000000	0.001324
11/14/97	0.046624	0.134650	0.316480	0.501610	0.000632
12/4/97	0.025413	0.085470	0.889110	0.000000	0.000000
12/5/97	0.025259	0.100230	0.874010	0.000000	0.005057
12/9/97	0.043376	0.389630	0.527060	0.039800	0.000134
12/10/97	0.042614	0.247210	0.710180	0.000000	0.000000
12/11/97	0.077954	0.338460	0.580520	0.000000	0.003067
12/16/97	0.053361	0.274810	0.660390	0.011440	0.000000
12/17/97	0.051449	0.304190	0.644360	0.000000	0.000000
12/18/97	0.066204	0.306110	0.627690	0.000000	0.000000
1/27/98	0.028208	0.293750	0.462150	0.215890	0.000000
1/28/98	0.010647	0.100800	0.223040	0.665390	0.000116
1/29/98	0.027007	0.196450	0.397290	0.379220	0.000337

[a] Component 1: arsenic, barium, copper, manganese, nickel, lead, vanadium and zinc. Component 2: aluminum, calcium, iron, silion, and titanium. Component 3: BC, bromine, potassium, sulfur and selenium. Component 4: sodium and chlorine. Component 5: cadmium.

Table 18. Linear Regression Analysis of Daily Elemental Concentration Proportion versus $\hat{\beta}_j^C$ for Crossover Studies in Normal Dogs[a]

Cardiac or Respiratory Parameter	Factor 1 (Ni)		Factor 2 (Si)		Factor 3 (S)		Factor 4 (Cl)	
	$\hat{\alpha}_k$	p Value	$\hat{\alpha}_k$	p Value	$\hat{\alpha}_k$	p Value	$\hat{\alpha}_k$	p Value
log LF	−0.0076	0.8654	−0.0018	0.7306	0.0012	0.5627	0.0077	0.0402
log HF	0.0272	0.1663	−0.0013	0.5372	−0.0004	0.6489	−0.0040	0.0140
log LF/HF ratio	−0.0368	0.4177	−0.0009	0.8642	0.0017	0.4129	0.0119	**0.0031**
Mean HR	−0.2754	0.6370	0.0043	0.9480	0.0057	0.8308	−0.0390	0.3980
log HR SD	0.0050	0.5994	−0.0001	0.9465	−0.0001	0.8394	−0.0004	0.5808
log T_I	0.0765	0.0636	−0.0052	0.2549	−0.0010	0.5845	−0.0036	0.2580
log T_E	0.0244	0.5954	−0.0024	0.6520	0.0007	0.7398	−0.0032	0.3730
log PIF	−0.0557	0.4531	0.0115	0.1803	−0.0004	0.9079	−0.0028	0.6280
log PEF	0.0068	0.9299	0.0031	0.7265	−0.0004	0.9100	−0.0049	0.4258
log V_T	−0.0263	0.7037	0.0093	0.2453	−0.0003	0.9190	−0.0055	0.3188
log f	0.0016	0.8107	−0.0076	0.1049	0.0013	0.4576	0.0032	0.3086
log $\dot{V}$	−0.0742	0.3494	0.0127	0.1668	−0.0004	0.9162	−0.0011	0.8569
log EIP	−0.0093	0.8701	0.0071	0.2798	0.0001	0.9634	0.0036	0.4239
log EEP	0.1131	0.1940	−0.0117	0.2376	−0.0009	0.8230	−0.0073	0.2834
log Pau_{enh}	**0.1604**	**0.0085**	**−0.0232**	**0.0015**	0.0005	0.8574	−0.0096	0.0376

[a] Values are estimated regression coefficients ($\hat{\alpha}_k$) and p values. Boldface type indicates significance at $p < 0.05$.

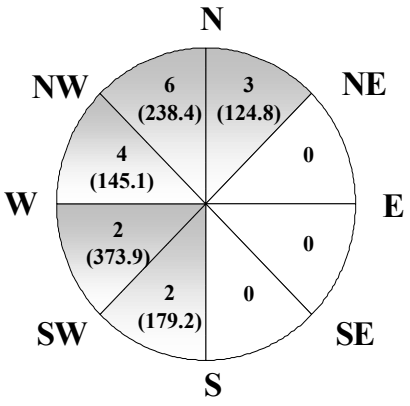


Figure 22. Air mass trajectory for each double CAPs exposure day. Numbers represent days from that trajectory and mean mass concentration in parentheses.

difference is NW = 0.066, other = 0.162, $p = 0.088$; the vanadium difference is NW = 0.082, other = 0.101, $p = 0.148$; the OC/total carbon fraction is NW = 27.6, other = 43.9, $p = 0.127$; the EC/total carbon fraction is NW = 11.5, other = 14.9, $p = 0.078$. Sodium and chlorine are also within $p = 0.10$. Of these differences, which are not statistically significant but close, only nickel has an increase from the northwest direction. All the others are decreased from that direction.

Figure 22 shows the trajectory patterns in the double CAPs exposures. Again, the predominant direction is northwest and north-northwest. An example of a north-northwest trajectory on a day with an increased HF response in the crossover protocol is shown in Figure 23. A day with significantly negative responses (from Tables 13, 14, and 15) and a southwesterly trajectory is shown in Figure 24.

Electrocardiogram morphology was studied using three-dimensional plots of one-minute ensemble ECG averages throughout the exposure. The morphology revealed by this method in a sham-exposed animal is illustrated in the upper panel of Figure 25. This example, which was a predominant

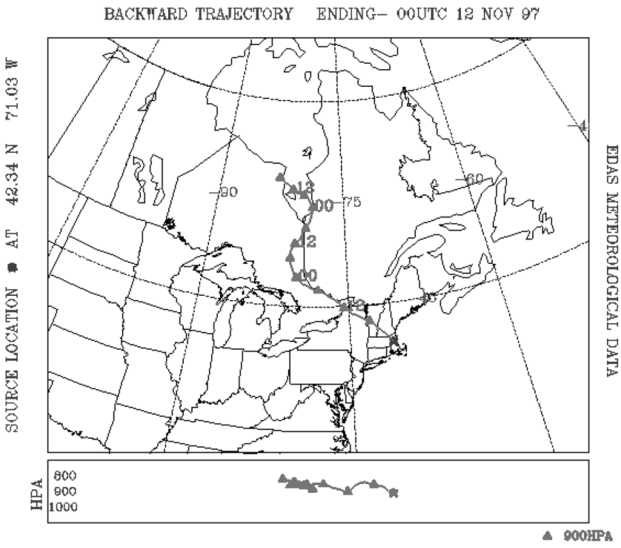


Figure 23. North northwest trajectory pattern associated with increased cardiopulmonary effects mediated by the vagus nerve. Data are from 11-12-97 and projected backward 96 hours. Exposure concentration was 190.2 mg/m^3 and sulfate was 60.5 mg/m^3. (From the Air Resources Laboratory of the US Oceanic and Atmospheric Administration.)

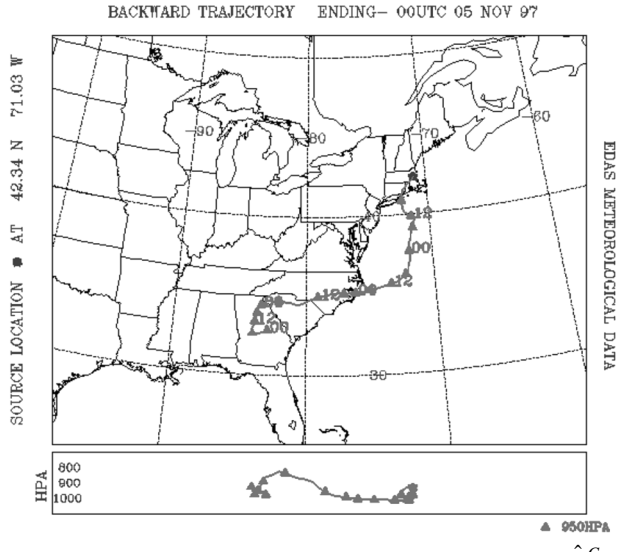
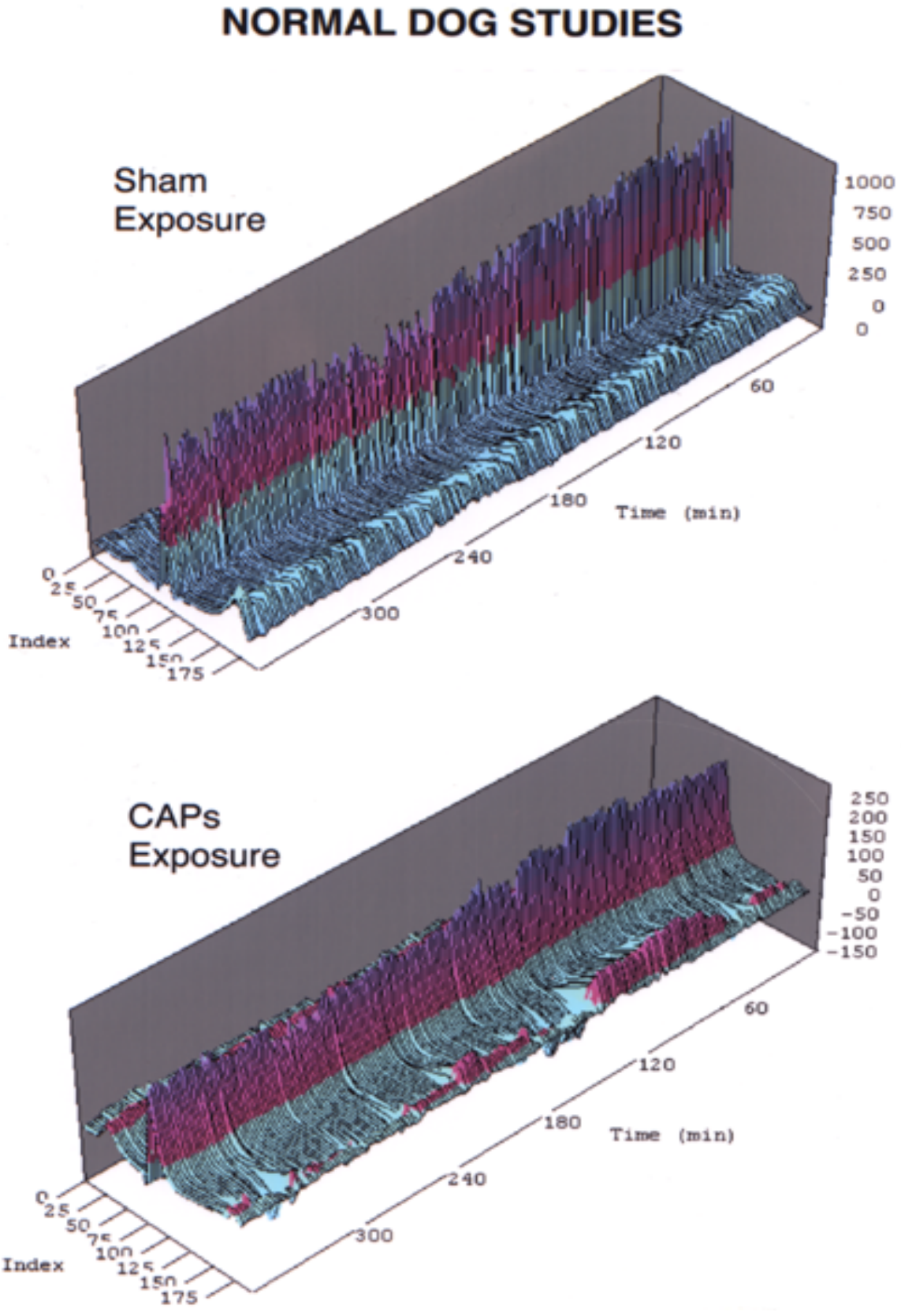


Figure 24. Southerly trajectory pattern associated with essentially $\{\hat{\beta}_j^C\}$ no response in the animals. Data are from 11-5-97. A day with a mass level of 239 mg/m^3 and sulfate level of 105.7 mg/m^3. The amount of sea salt in the elemental analysis is surprisingly small despite the trajectory pattern. Data are projected backward 96 hours. (From the Air Resources Laboratory of the US Oceanic and Atmospheric Administration.)


NORMAL DOG STUDIES
Sham
Exposure
1000
750
500
250
0
0
60
120
180
Time (min)
240
300
0
25
50
75
100
125
150
175
Index
CAPs
Exposure
250
200
150
100
50
0
-50
-100
-150
60
120
180
Time (min)
240
300
0
25
50
75
100
125
150
175
Index

sham pattern, shows very little variation in the ECG pattern throughout the six-hour sham exposure. In dogs exposed to CAPs, the ECG morphology (lower panel) showed considerable variation in T wave morphology. During CAPs exposure in this dog, T waves occasionally inverted. Some of the T wave change can be explained by the dog changing position as shown in the video analysis; however, in pilot comparisons of video analysis and ECG morphology, much of this T wave change cannot be so explained. Overall, there were no differences in movement between CAPs-exposed and sham-exposed dogs. Complete video analysis has been done, but correlation to ECG morphology requires manual measurements and is not yet completed.

T wave alternans analyses from dogs with significantly increased LF and dogs with no change in LF were carried out on the full six hours of exposure for two days randomly selected from the 23 days of the crossover study. Figure 26 illustrates two of these analyses. A prominent peak in T wave alternans near the end of the exposure in one dog corresponds to an increase in the LF/HF ratio. The other dog illustrated has T wave alternans measurements at background levels.

In all 23 days of crossover design experiments, three five-minute periods of the exposure were analyzed to determine the level of T wave alternans in both the CAPs and sham exposed dogs (n = 130 assessments). These periods were selected to correspond to the highest LF/HF ratio during the exposure (CAPs or sham) on the basis of results from the studies illustrated in Figure 26. The first and last hours of exposure were eliminated from the selection to eliminate factors not related to exposure. In comparing the level of T wave alternans in relation to CAPs or sham exposure using the Student t test, a significant ($p < 0.05$) decrease in T wave alternans was associated with CAPs exposure (CAPs = 34.9 ± 2.8; sham = 46.5 ± 2.8). Within the CAPs data, T wave alternans levels were not different when data are dichotomized by trajectory.

Assessment of Inflammation

Bronchoalveolar Lavage Results Analysis of BAL samples from normal dogs exposed to CAPs produced evidence of mild pulmonary inflammation. Preexposure baseline, sham exposure, and CAPs exposure BAL data were compared for all dogs; cell differentials and biochemical analyses of lavage fluid were employed as biomarkers of inflammation. The percentage of BAL polymorphonuclear leukocytes was elevated in the CAPs-exposed animals (p = 0.05; Figure 27). Bronchoalveolar macrophage percentages were decreased in CAPs-exposed animals, but not significantly (p = 0.14; Figure 27); lymphocyte and eosinophil differentials were not affected by CAPs exposure (Figure 27). Biochemical analysis of total BAL protein and β-*N*-AG indicated increased levels that were not quite significant (BAL protein: p = 0.08; β-*N*-AG: p = 0.06; Figure 28). Complete BAL data sets from three dogs also were analyzed to seek any evidence of individual effect of CAPs treatment (baseline versus sham versus CAPs). Repeated measures ANOVA indicated no significant differences in the percentage of macrophages or neutrophils retrieved by BAL. Total protein levels also were not significantly elevated after CAPs exposure.

Blood Analyses Results Analyses of WBC total counts, differential percentages, and fibrinogen levels in whole blood did not reveal any significant effect of CAPs versus sham exposure. Daily means for each parameter were assessed against a preexposure baseline for both sham-exposed and CAPs-exposed dogs. CAPs-exposed animal results subsequently were compared to sham-exposed animal results. Individual exposure days (CAPs) were compared to baseline levels to determine daily effects. Polymorphonuclear leukocytes, lymphocytes, monocytes, and eosinophils were not significantly affected by either exposure (Figure 29). Total blood WBC counts in CAPs-exposed dogs also were unchanged compared to baseline levels or levels in sham-exposed dogs (Figure 30). Fibrinogen levels increased with each day of sham and CAPs exposure, but these changes were not significant (Figure 30).

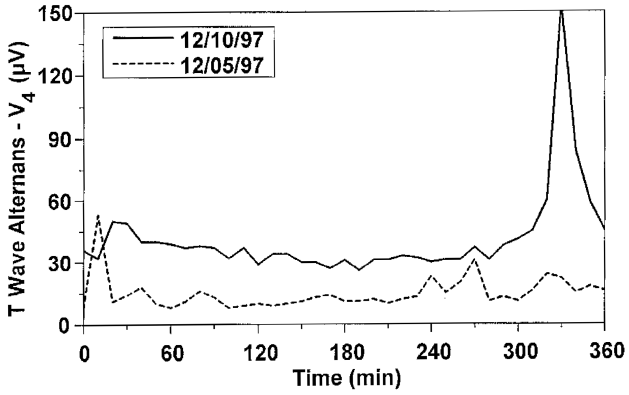


Figure 26. Continuous assessment of T wave alternans over two days of exposure representing a day of relatively low HF (12-10-97) and higher HF (12-5-97). Note that T wave alternans is relatively flat throughout the period but increases dramatically near the end of exposure on 12-10-97.

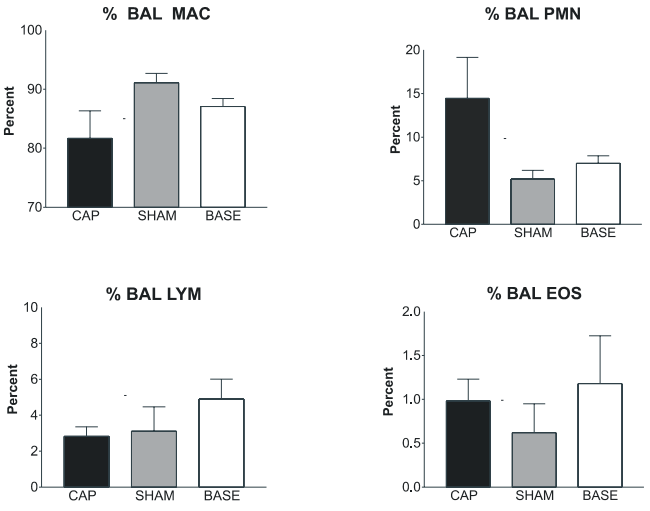


Figure 27. Biomarkers of inflammation due to inhalation of CAPs. Results were obtained from analyses performed on all BAL samples recovered from animals prior to treatment (BASE; n = 24), after 3 days of sham exposure (sham; n = 10), and after 3 days of CAPs inhalation exposure (CAPs; n = 12). Panels represent differential analyses of cells recovered by BAL. The percentage of BAL PMN with CAPs exposure was higher than sham (p = 0.05). MAC = macrophages; PMN = polymorphonuclear leukocytes; LYM = lymphocytes; EOS = eosinophils.

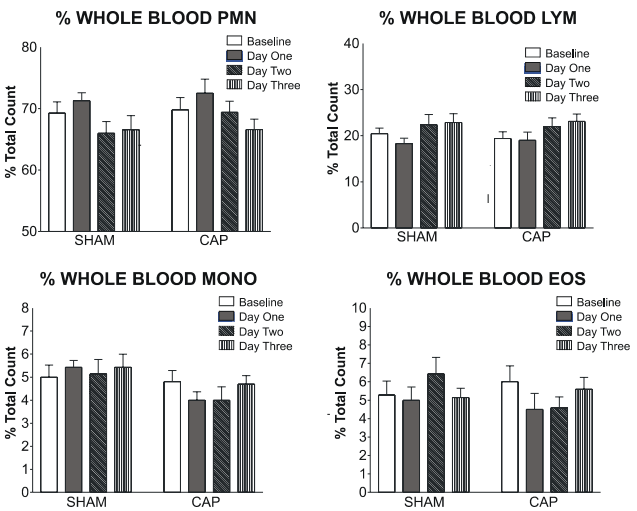


Figure 29. Biomarkers of alterations in peripheral blood after inhalation. Animals were exposed to either ambient air (n = 7) or CAPs (n = 10) for 3 days. Analyses were performed on all blood samples recovered from animals prior to treatment (Baseline), after one day of exposure (Day One), after two days of exposure (Day Two), or after three days of exposure (Day Three). All differential analyses were made on cells obtained from whole blood samples. PMN = polymorphonuclear leukocytes; LYM = lymphocytes; MONO = mononuclear cells; EOS = eosinophils.

Blood data from pairs of dogs that had been treated identically were assessed as subsets to determine whether specific CAPs exposures induced alterations. Cell counts, differential percentages, and fibrinogen levels in CAPs-exposed pairs of animals indicated no significant changes when compared to data observed after three days of sham exposure. Likewise, dogs involved in crossover design studies were not affected when CAPs and sham exposures were compared.

On the basis of physiologic measurements in which changes correlated with particular trajectories, CAPs trajectory was analyzed as a differential effector of BAL parameters. Analysis of BAL samples from normal dogs exposed to CAPs arriving from the northwest versus other directions did not indicate significant differences in toxicity (data not shown). Preexposure baseline, sham exposure, and directional CAPs exposure BAL data were compared for all dogs; cell differentials and biochemical analyses of lavage fluid were employed as biomarkers of inflammation. The percentages of BAL macrophages and polymorphonuclear leukocytes were not significantly altered in any of the CAPs-exposed groups, although on days with northwest trajectories the animals did have higher mean percentages of neutrophils. Biochemical analysis of total BAL protein and β-*N*-AG also indicated increased levels associated with CAPs on days with northwest trajectories, although this was not statistically significant.

Analyses of Transbronchial Biopsies Representative frozen sections of bronchial and transbronchial biopsies were assessed using immunocytochemistry and light microscopy. These biopsies had minimal chronic inflammation, no neutrophil infiltration, and no evidence of positive staining for

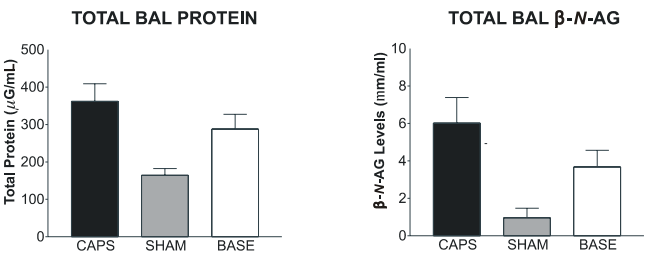


Figure 28. Biochemical analyses of acellular supernatants from BAL. Each value is the mean ± SE. No significant differences were found.

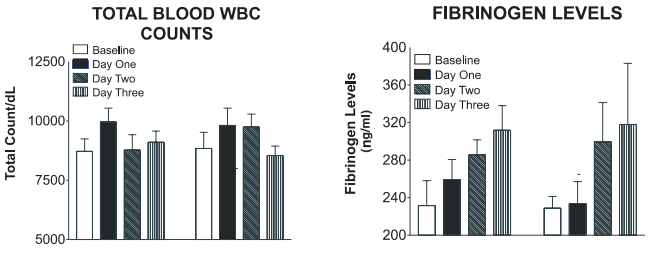


Figure 30. Total blood WBC counts and fibrinogen measurements after sham and CAPs exposures. Each value represents the mean ± SE. No significant differences were found. Analyses were performed on all blood samples recovered from animals prior to treatment (Baseline), after one day of exposure (Day One), after two days of exposure (Day Two), or after three days of exposure (Day Three).

IL-8, IL-1, or TNF. In view of totally negative results in a pilot study of representative biopsies, a full assessment of all biopsy tissue was not pursued. Lack of evidence of acute inflammation in the biopsies was thought to result from the modest acute inflammatory BAL findings and the small volume of tissue sampled by these biopsies.

DOGS WITH CORONARY OCCLUSION

Exposure Parameters

Exposure parameters in the coronary occlusion studies show considerable variation in the mass and sulfate concentration in all these experiments (Table 19). Concentration factors are lower in earlier studies and considerably higher in later studies. Endotoxin concentrations in concentrated aerosol had a mean ± SE of 14.27 ± 4.7 μg/m^3. Of 18 endotoxin determinations, 6 were below the LOD. Table 20 lists the weather data for the coronary occlusion studies.

Cardiopulmonary Responses

Coronary occlusion caused visible changes in the ST segment and T wave. Figure 31 illustrates the degree of this change in ST-segment elevation and the height of the T wave in a three-dimensional plot of the ECG before, during, and after occlusion. Clearly, considerable change takes place during the occlusion but resolves immediately to baseline after the occlusion. Despite the variations in CAPs concentrations, and often relatively low concentrations

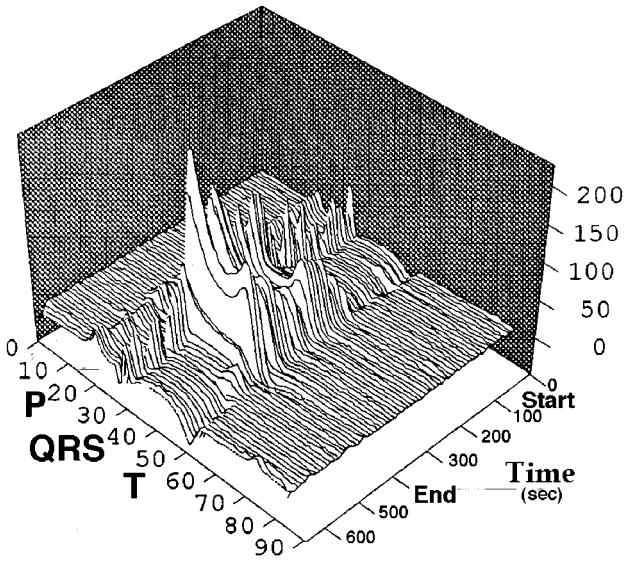


Figure 31. ST and T wave changes during occlusion. Note the latent period from the start of occlusion to T wave elevation, the height of the ST segment, and the abrupt decline to normal with release of the occlusion.

Table 19. Exposure Parameters for CAPs and Sham Exposure Studies of Dogs with Coronary Occlusion[a]

Date	Animal (n = 6 total)	MMAD ± GSD (μm)	Mass CAPs (μg/m^3)	SO_4 CAPs (μg/m^3)	Mass Ambient (μg/m^3)	SO_4 Ambient (μg/m^3)	Mass Concentration Factor	SO_4 Concentration Factor
1/29/97	5	0.05 ± 4.7	71.8	14.0	4.6	0.9	15.6	15.6
1/30/97	5	0.05 ± 4.7	101.4	21.1	8.6	1.7	11.8	12.4
1/31/97	5	0.05 ± 4.7	182.0	105.7	27.4	7.0	6.6	15.1
2/3/97	4	0.10 ± 4.0	182.9	57.6	19.3	5.6	9.5	10.3
2/4/97	4	0.10 ± 4.0	75.6	32.1	8.1	3.3	9.3	9.7
2/5/97	4	0.10 ± 4.0	90.8	27.3	7.3	2.1	12.4	13.0
3/3/98	2	0.28 ± 1.9	550.3	238.5	14.4	5.3	38.2	44.6
3/4/98	2	0.28 ± 1.9	431.3	214.2	10.9	4.6	39.5	46.6
3/5/98	2	0.28 ± 1.9	257.0	86.1	9.7	3.1	26.6	28.0
3/10/98	1	0.27 ± 2.0	136.0	43.6	4.2	1.2	32.1	36.3
3/11/98	1	0.27 ± 2.0	182.2	53.6	5.3	1.2	34.1	46.5
3/12/98	1	0.27 ± 2.0	188.8	52.3	5.4	1.5	35.0	34.2
5/18/98	9	0.23 ± 2.6	191.7	38.0	6.6	1.0	29.0	37.3
5/19/98	9	0.23 ± 2.6	228.1	33.4	6.9	1.0	33.1	34.6
5/20/98	9	0.23 ± 2.6	223.0	34.0	7.7	1.0	29.1	32.7
5/26/98	8	0.24 ± 1.8	557.9	93.3	14.6	2.9	38.2	32.7
5/27/98	8	0.24 ± 1.8	425.0	57.7	9.6	1.4	44.1	41.6
5/28/98	8	0.24 ± 1.8	741.2	155.5	16.5	4.3	44.8	36.6

[a] For each pair of dogs (5 and 4, 2 and 1, 9 and 8), one dog was sham-exposed on the same day the other dog was exposed to CAPs.

(100 μg/m^3), the existence of coronary occlusion changed the autonomic balance of cardiac effects. One representative Fourier-transformed HRV spectral pattern shows the LF and HF influence on the heart after a coronary occlusion (Figure 32). In comparison to Figure 9, considerable LF influence is evident in both the CAPs and sham dogs. With this change in autonomic balance, sensitivity of the responses to CAPs exposure markedly increased. Tables 21, 22, and 23 list the mean effect of CAPs or sham exposure on cardiac and respiratory parameters in these crossover design studies; HF is significantly increased and respiratory frequency is significantly decreased.

Tables 24, 25, and 26 show the data as $\{\hat{\beta}_j^C\}$ values on individual days. Data from the two dogs from coronary occlusion studies in 1997 could not be analyzed in this way because the respiratory data for those animals was collected with an earlier system that produced reliable data only for the respiratory frequency parameter. Note that, despite lesser exposure concentrations, HF increases were predominantly positive and the majority were significant ($p < 0.05$). Respiratory parameters also showed considerable changes with decreases in frequency associated with decreased minute volume and peak flows, and corresponding increases in inspiration and expiration time and Pau_{enh}. The crossover design data for the HF parameter for four dogs are shown in Figures 33 and 34. Figures 35 and 36 illustrate the crossover data for PEF in the coronary occlusion studies.

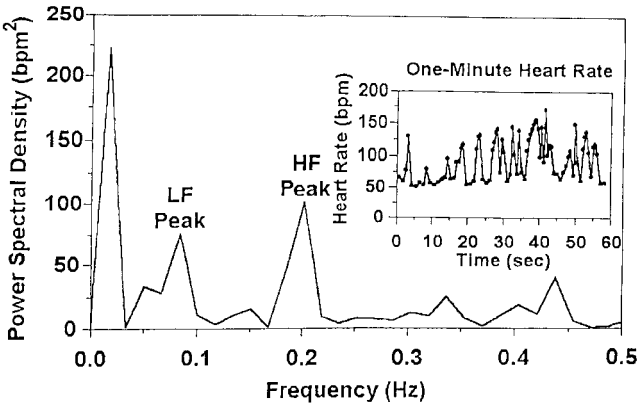


Figure 32. Fast Fourier transformed HRV spectrum of the typical resting pattern of a dog after coronary occlusion. Note the increase in LF influence compared to Figure 9.

As shown in the design schematic, one dog is exposed to CAPs while her chambermate receives filtered air. In Figure 37, HF is plotted over the time of exposure on three consecutive days with occlusion on the first and third day ($n = 2$) or third day alone ($n = 4$). On all three of these days of exposure with these dogs, the dog exposed to CAPs (Dog 5)

Table 20. Mean Boston Weather Data During CAPs and Sham Exposure Studies of Dogs with Coronary Occlusion

Date	Temperature (°F)	Dewpoint (°F)	Relative Humidity (%)	Barometric Pressure (mm Hg × 100)	Wind Speed (mi/hour)	Wind Direction (°N)	Wind Trajectory Pattern
1/29/97	26.43	2.57	32.14	3045.43	14.00	280	NW
1/30/97	20.57	11.57	65.57	3052.71	8.43	20	NW
1/31/97	23.71	23.00	97.14	2995.00	7.29	340	NW
2/3/97	37.83	35.83	91.50	3024.17	6.33	230	NW
2/4/97	32.67	24.17	69.67	3062.33	12.67	60	NW
2/5/97	39.29	37.43	92.29	2982.14	7.57	310	NE
3/3/98	38.00	36.29	92.29	2948.43	9.00	10	SW
3/4/98	47.43	28.71	46.86	2971.43	8.29	290	NW
3/5/98	37.00	34.29	90.00	2993.00	10.71	20	NW
3/10/98	49.43	37.00	62.00	2950.57	15.29	280	W
3/11/98	29.00	7.67	34.50	2995.17	18.17	310	NW
3/12/98	25.00	3.00	35.43	3001.29	19.29	250	NW
5/18/98	80.14	45.43	31.14	2985.14	14.29	320	NW
5/19/98	66.14	49.57	56.71	2979.71	15.86	330	SE
5/20/98	73.71	52.00	47.29	2976.71	15.29	200	SW
5/26/98	73.14	53.57	51.71	2973.86	8.00	150	NW
5/27/98	66.75	44.00	44.63	3003.88	12.75	120	W
5/28/98	79.86	52.14	39.00	2998.14	8.57	310	NW

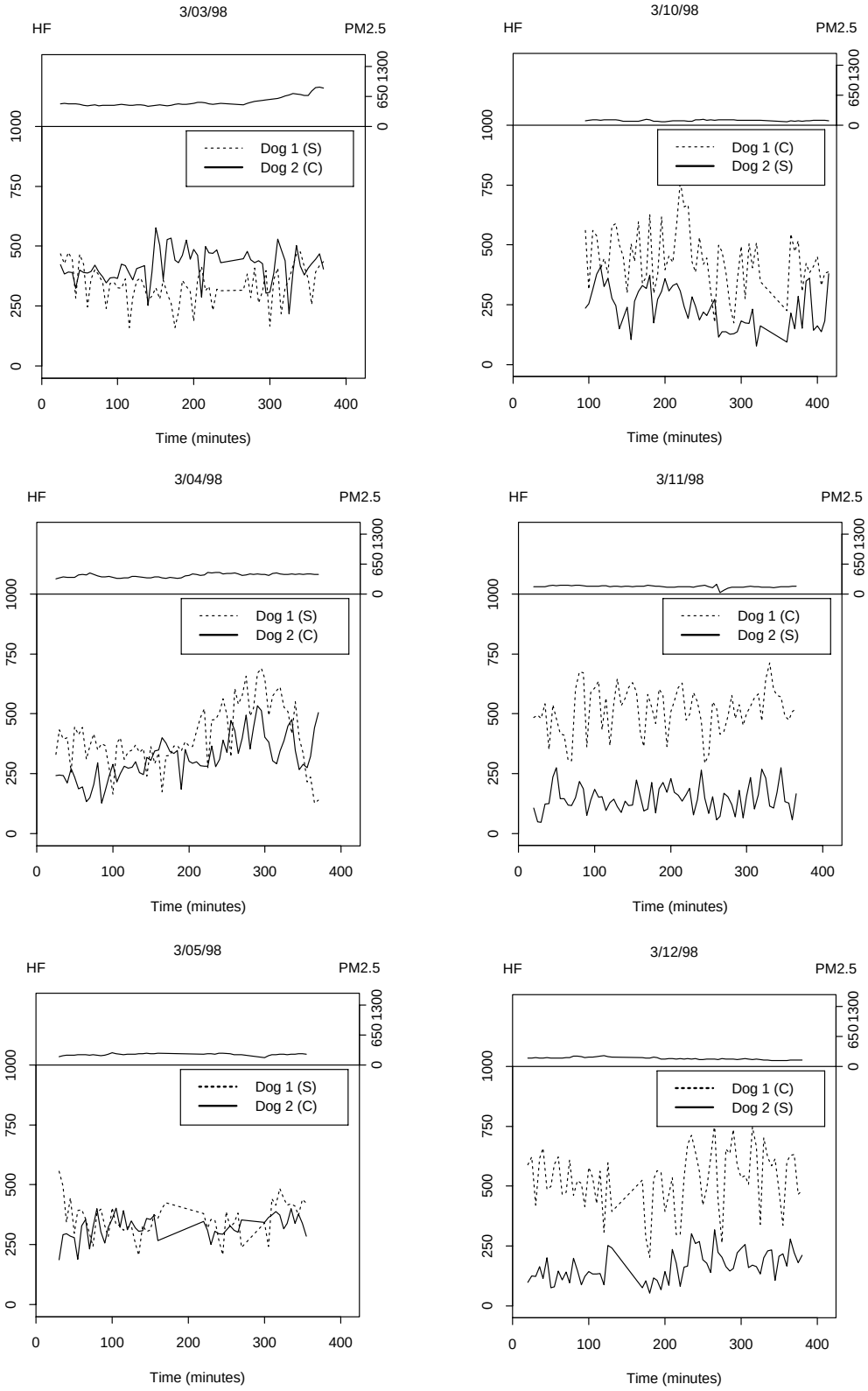


Figure 33. HF patterns of CAPs (C) and sham (S) exposure on crossover days in March 1998 to dogs 1 and 2 with coronary occlusion. The CAPs mass concentration is shown in the upper panel for each day. These patterns can be compared with the pair shown in Figure 34 and with their PEF pattern in Figure 35.

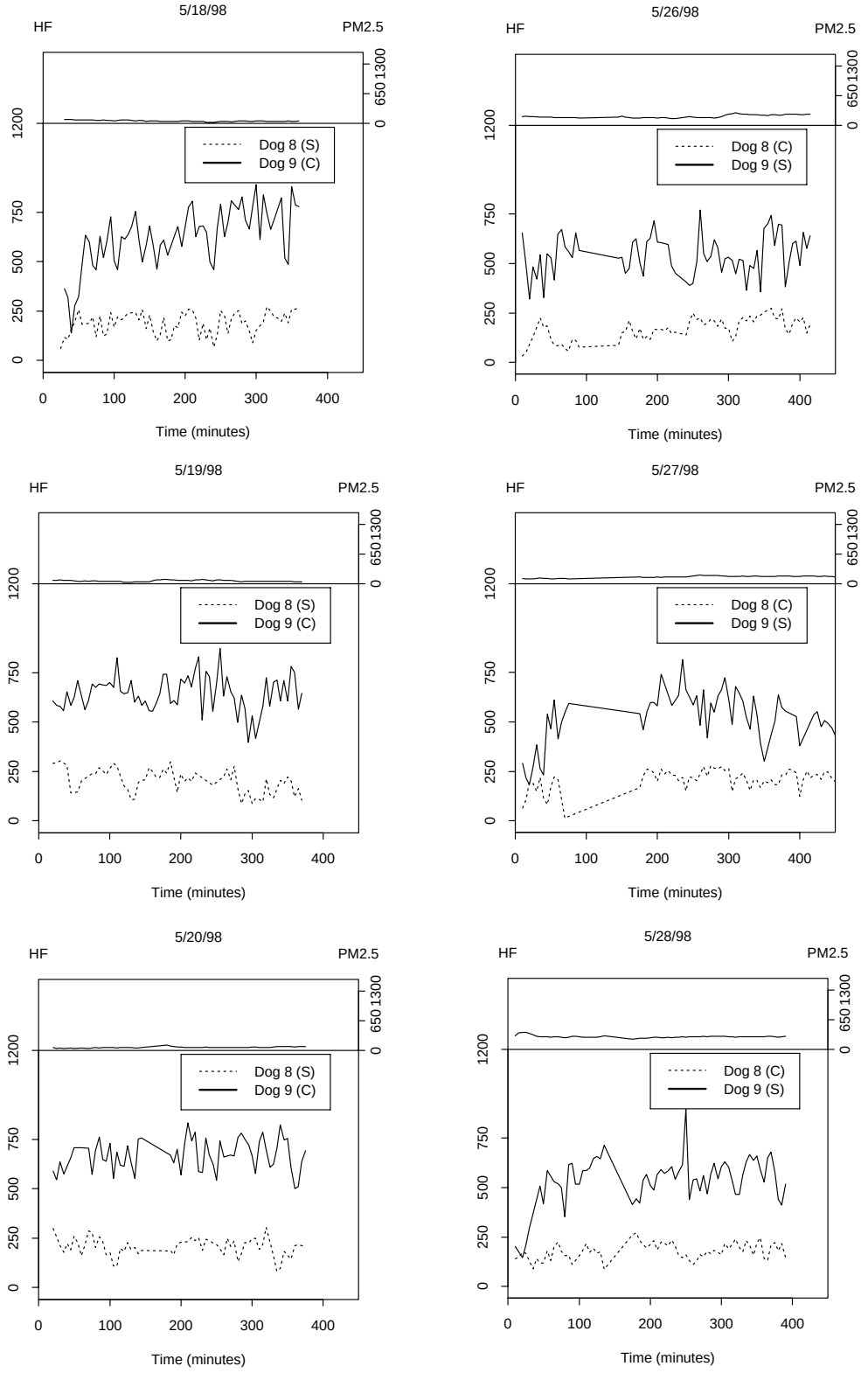


Figure 34. HF patterns of CAPs (C) and sham (S) exposures on crossover days in May 1998 to dogs 8 and 9 with coronary occlusion. The CAPs mass concentration is shown in the upper panel for each day. These patterns can be compared with the pair shown in Figure 33 and with their PEF pattern in Figure 36.

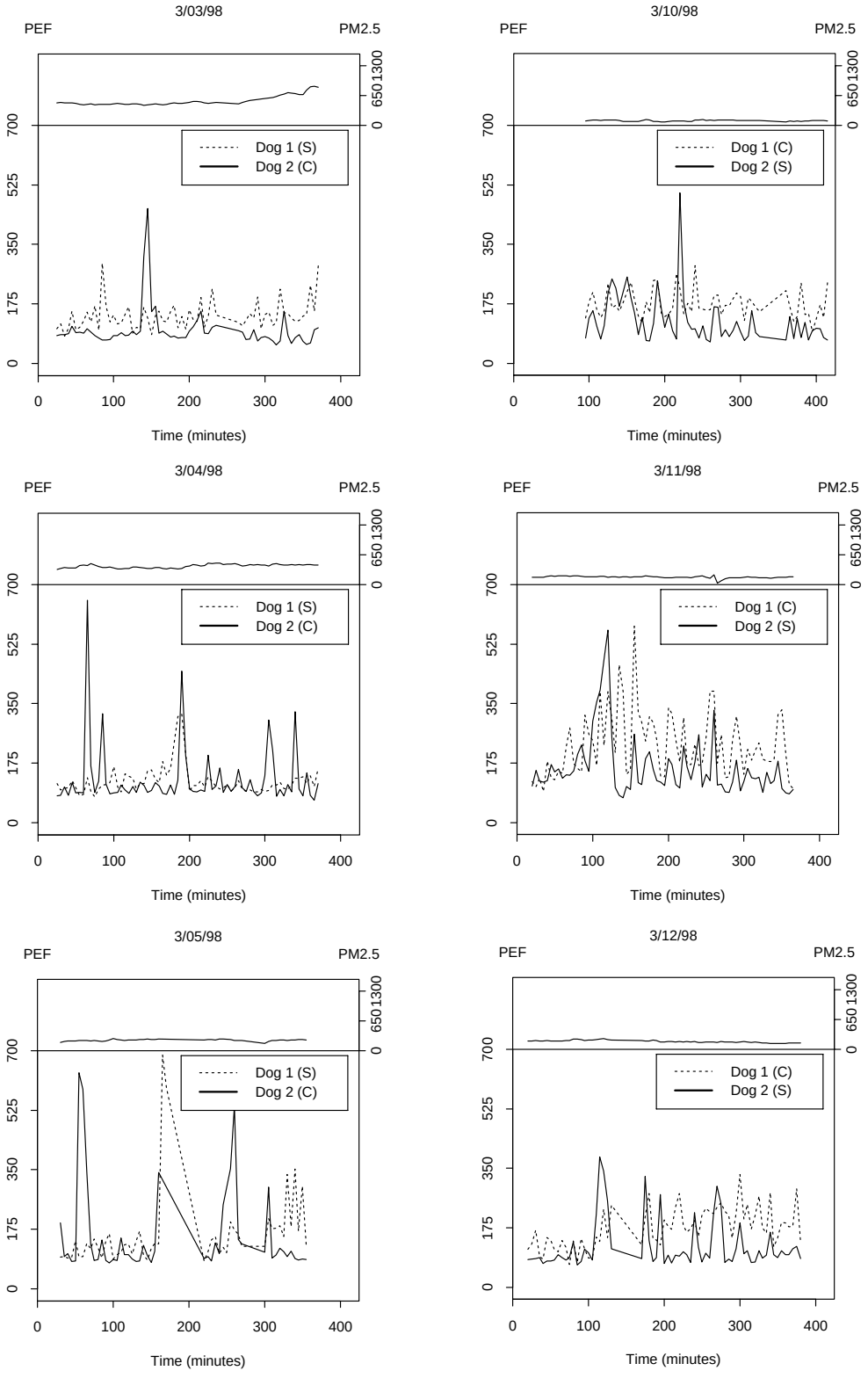


Figure 35. PEF patterns of CAPs (C) and sham (S) exposures on crossover days in March 1998 to dogs 1 and 2 with coronary occlusion. The CAPs mass concentration is shown in the upper panel for each day. These patterns can be compared with the pair shown in Figure 36 and with their HF pattern in Figure 33.

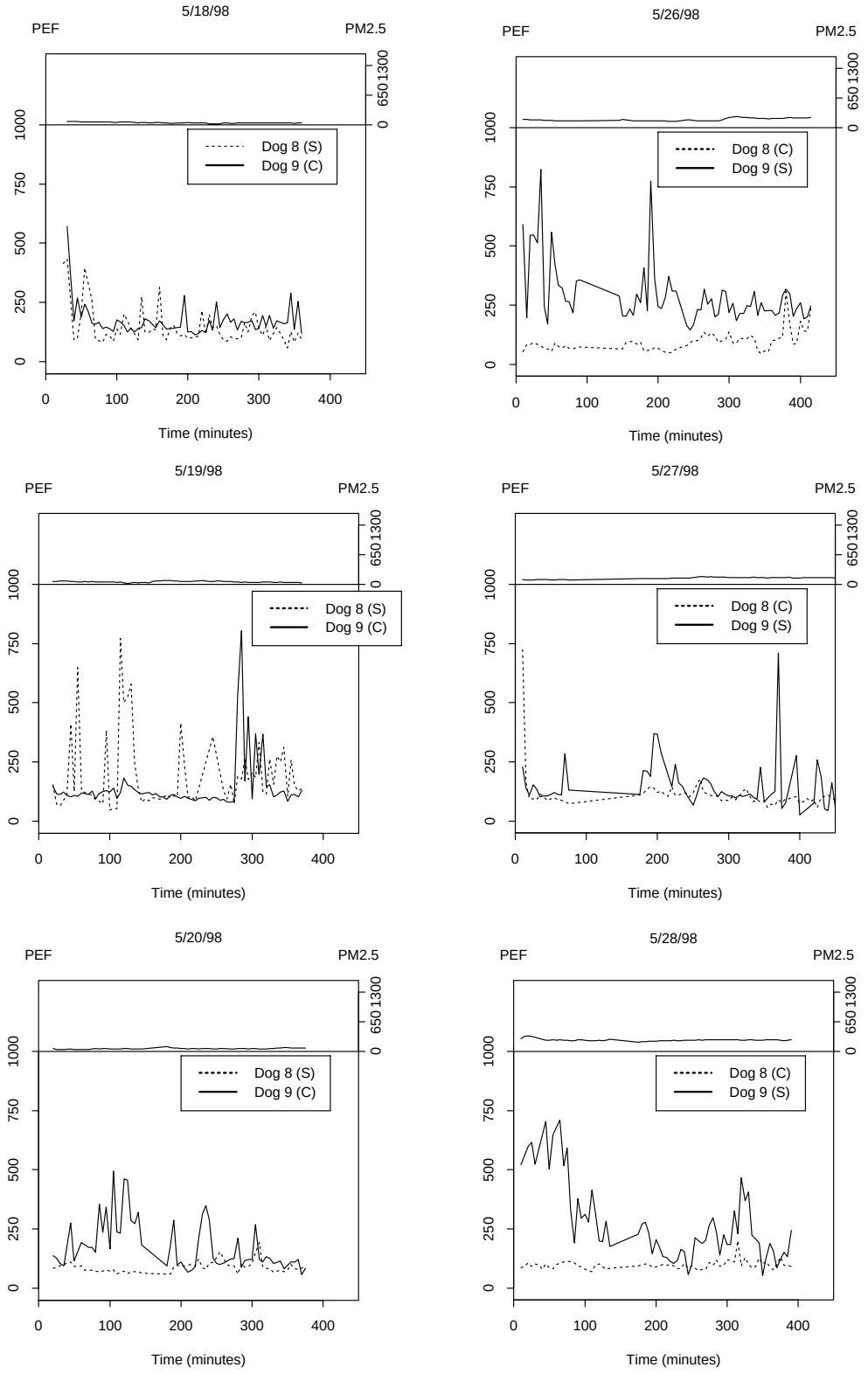


Figure 36. PEF patterns of CAPs (C) and sham (S) exposures on crossover days in May 1998 to dogs 8 and 9 with coronary occlusion. The CAPs mass concentration is shown in the upper panel for each day. These patterns can be compared with the pair shown in Figure 35 and with the HF pattern in Figure 34.

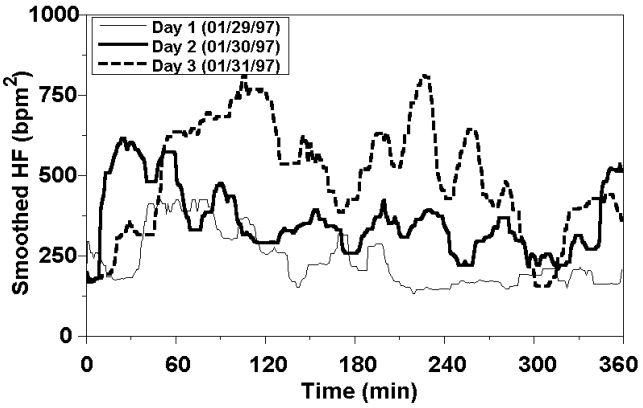


Figure 37. HF pattern over three days of CAPs exposure in a dog in the coronary artery occlusion study. Note the high levels of HF and presence of surges in this parameter.

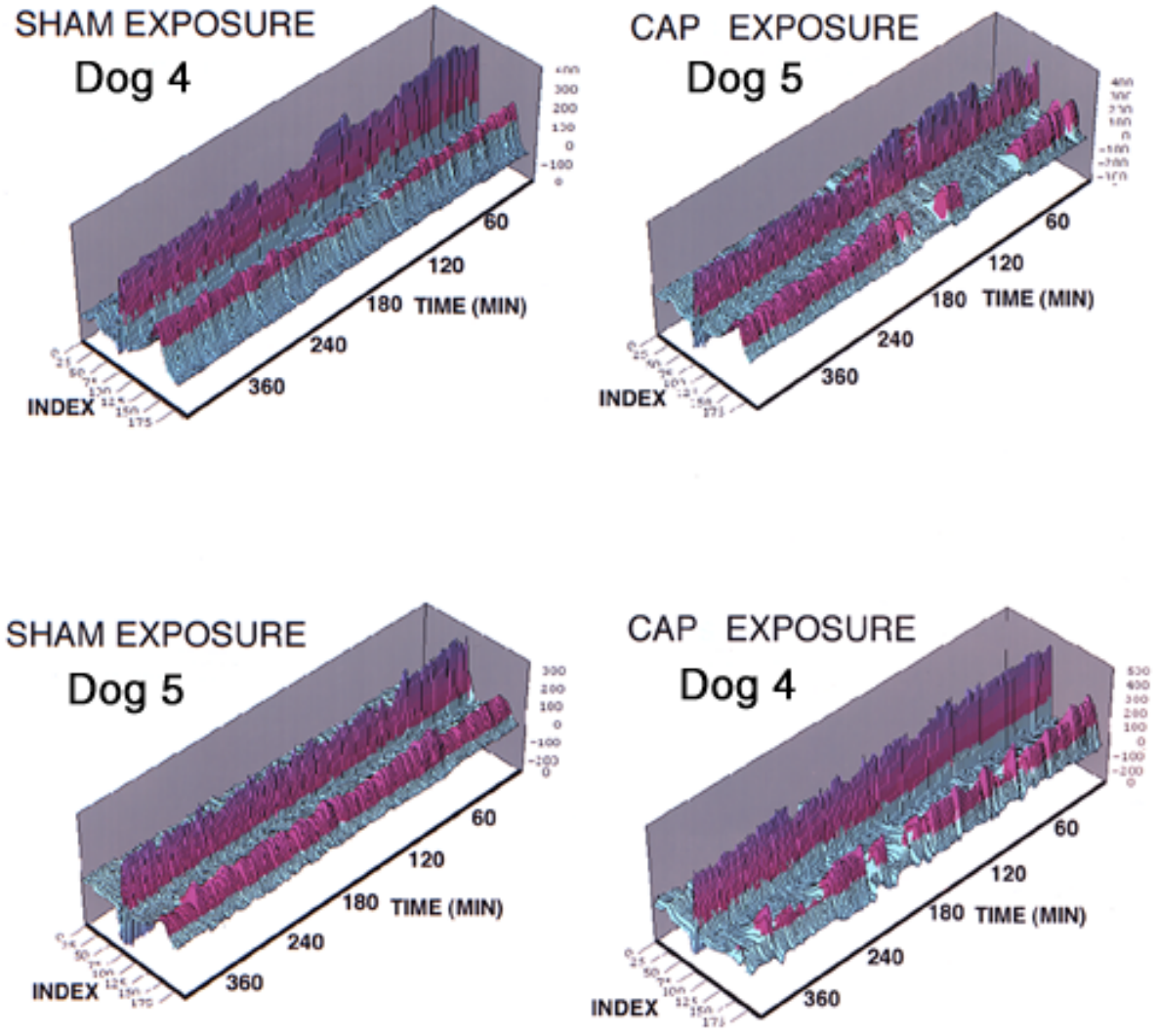


Figure 38. Three-dimensional patterns of response of dogs in the coronary occlusion protocol on the first day of exposure in each crossover. Note again the regular patterns in the sham-exposed dogs and the T wave inversion and changes in the CAPs-exposed dog. Note especially dog 5 at 60 minutes into the exposure. This point compares to the highest HF measurement on that day (1-29-97) in Figure 37. The ECG at this time is shown in Figure 39.

Table 21. Cardiac Measurements for Dogs with Coronary Occlusion Comparing Each Dog's Response to CAPs or Sham Exposure

	Number of Days		LF Power of HRV (beats/min^2/Hz)		HF Power of HRV (beats/min^2/Hz)		LF/HF Ratio		Mean HR (beats/min)		HR SD (beats/min)	
Animal	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs
1	3	3	65.82	103.60	371.78	489.19	0.20	0.26	70.70	70.47	23.60	27.16
2	3	3	135.05	71.86	171.56	344.35	1.08	0.25	89.39	87.95	23.84	22.85
4	3	3	32.16	44.39	452.88	537.28	0.48	0.18	91.67	90.61	21.43	24.79
5	3	3	26.75	28.29	127.09	363.73	0.34	0.09	103.63	75.69	13.13	21.27
8	3	3	39.88	36.41	192.51	166.42	0.36	0.37	63.98	63.47	17.39	16.15
9	3	3	74.30	84.58	501.33	623.69	0.18	0.16	85.43	83.15	26.86	29.08
Mean			62.33	61.52	302.86	420.78	0.44	0.22	84.13	78.56	21.04	23.55
Paired *t* test			$p = 0.52$		$p = 0.05$		$p = 0.12$		$p = 0.17$		$p = 0.11$	

Table 22. Respiratory Measurements for Dogs with Coronary Occlusion Comparing Each Dog's Response to CAPs or Sham Exposure

	f (breaths/min)		V_T (mL)		$\dot{V}$ (mL/min)		PIF (L/min)		PEF (L/min)	
Animal	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs
1	29.60	19.05	51.89	79.01	1568.22	1494.83	112.07	146.37	141.55	194.20
2	33.57	25.62	63.32	78.55	1797.32	1629.21	141.73	136.20	133.01	126.31
4[a]	62.10	40.62								
5[a]	47.22	45.92								
8	22.78	22.85	60.02	59.14	1546.88	1533.41	132.98	133.05	136.24	122.99
9	33.55	27.58	95.03	84.25	2986.29	2159.60	252.75	189.14	241.27	170.13
Mean	38.14	30.27	67.56	75.24	1974.68	1704.26	159.88	151.19	163.02	153.41
Paired *t* test	$p = 0.03$		$p = 0.21$		$p = 0.12$		$p = 0.35$		$p = 0.36$	

[a] Respitrace system used for respiratory measurements.

Table 23. Respiratory Measurements for Dogs with Coronary Occlusion Comparing Each Dog's Response to CAPs or Sham Exposure

	EIP (msec)		T_I (sec)		EEP (msec)		T_E (sec)		Pau_{enh}		*T*rel (sec)		Pau	
Animal	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs
1	78.79	90.75	1.11	1.24	941.74	2108.62	1.54	2.89	5.44	10.51	0.49	0.54	3.03	5.63
2	59.26	66.86	0.75	0.98	1068.04	1497.73	1.84	2.42	3.56	3.40	0.51	0.75	3.20	3.03
4[a]														
5[a]														
8	67.89	59.58	1.07	0.94	1539.42	1419.82	2.29	2.34	7.39	4.17	0.55	0.61	4.90	3.81
9	59.97	69.88	0.83	1.10	974.92	1262.47	1.64	1.94	3.84	1.87	0.56	1.06	2.92	1.57
Mean	66.48	71.77	0.94	1.07	1131.03	1572.16	1.83	2.40	5.06	4.99	0.53	0.74	3.51	3.51
Paired *t* test	$p = 0.17$		$p = 0.13$		$p = 0.10$		$p = 0.07$		$p = 0.49$		$p = 0.07$		$p = 0.50$	

[a] Respitrace system used for respiratory measurements.

Table 24. Cumulative Effects of $PM_{2.5}$ on Cardiac Parameters by Day for Studies of Dogs with Coronary Occlusion[a]

LF Power of HRV		HF Power of HRV		LF/HF Ratio		Mean HR		HR SD	
Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$
5/18/98	**−0.1139**	5/26/98	−0.0121	5/18/98	**−0.1571**	5/26/98	**−0.9345**	5/18/98	−0.0092
3/11/98	−0.0188	3/10/98	−0.0091	3/11/98	**−0.0726**	5/27/98	**−0.5963**	3/4/98	−0.0008
3/4/98	**−0.0157**	5/28/98	0.0018	3/12/98	**−0.0320**	3/5/98	**−0.4612**	3/11/98	0.0006
3/3/98	0.0053	5/27/98	0.0063	3/4/98	**−0.0286**	5/28/98	**−0.4447**	5/19/98	0.0015
5/20/98	0.0061	5/20/98	0.0097	3/3/98	**−0.0259**	3/4/98	**0.0519**	5/20/98	0.0020
3/5/98	0.0069	3/4/98	**0.0133**	3/5/98	−0.0160	3/3/98	**0.3439**	5/28/98	0.0027
3/12/98	0.0089	3/5/98	**0.0243**	5/19/98	−0.0052	5/20/98	0.5000	3/5/98	0.0034
5/28/98	0.0101	3/3/98	**0.0333**	5/28/98	0.0068	3/11/98	**0.5678**	5/27/98	0.0056
5/19/98	0.0140	5/19/98	**0.0334**	5/20/98	0.0075	5/19/98	0.7087	3/3/98	**0.0091**
5/27/98	0.0270	5/18/98	**0.0375**	5/27/98	0.0167	3/12/98	**1.0354**	3/12/98	**0.0096**
5/26/98	**0.1324**	3/12/98	**0.0380**	5/26/98	**0.1373**	5/18/98	**1.3216**	5/26/98	**0.0128**
3/10/98	**0.1364**	3/11/98	**0.0506**	3/10/98	**0.1541**	3/10/98	**2.3859**	3/10/98	**0.0177**

[a] All measurements were controlled for f and $\dot{V}$, and all except mean HR were controlled for heart rate. All data except mean HR are presented as log values. Boldface type indicates significance at $p < 0.05$. Data are presented from the lowest negative to the highest positive value for $\hat{\beta}_j^C$, which is the estimated CAPs effect on a particular day based on comparison to chambermate sham corrected for CAPs dog as sham.

Table 25. Cumulative Effects of $PM_{2.5}$ on Pulmonary Parameters by Day for Studies of Dogs with Coronary Occlusion[a]

f		V_T		$\dot{V}$		PIF		PEF	
Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$
5/27/98	**−0.0700**	5/19/98	**− 0.1574**	5/19/98	**−0.2130**	5/19/98	**−0.1967**	5/19/98	**−0.2203**
3/10/98	**−0.0568**	5/18/98	**−0.0752**	5/18/98	**−0.0563**	5/18/98	**−0.0794**	5/18/98	**−0.1055**
3/5/98	**−0.0533**	5/28/98	−0.0014	3/5/98	**−0.0527**	5/20/98	**−0.0407**	3/3/98	**−0.0614**
5/19/98	**−0.0512**	5/20/98	−0.0009	3/3/98	**−0.0303**	3/5/98	**−0.0179**	5/20/98	**−0.0193**
5/26/98	**−0.0510**	3/3/98	0.0019	5/20/98	−0.0296	3/3/98	**−0.0133**	3/5/98	**−0.0168**
3/3/98	**−0.0346**	3/4/98	0.0034	3/4/98	**−0.0158**	3/4/98	**− 0.0093**	5/26/98	−0.0133
5/20/98	**−0.0228**	3/5/98	0.0052	5/26/98	0.0040	5/28/98	0.0093	3/4/98	−0.0007
3/4/98	**−0.0214**	3/11/98	**0.0283**	5/28/98	0.0077	5/26/98	**0.0301**	5/28/98	0.0064
3/12/98	−0.0056	3/12/98	**0.0513**	5/27/98	0.0143	3/11/98	**0.0350**	3/11/98	**0.0307**
5/28/98	0.0079	5/26/98	**0.0575**	3/11/98	**0.0424**	5/27/98	**0.0528**	3/10/98	**0.0435**
3/11/98	**0.0111**	5/27/98	**0.0789**	3/12/98	**0.0462**	3/12/98	**0.0546**	3/12/98	**0.0781**
5/18/98	0.0247	3/10/98	**0.1042**	3/10/98	0.0472	3/10/98	**0.0832**	5/27/98	**0.1030**

[a] All data are presented as log values. Boldface type indicates significance at $p < 0.05$. Data are presented from the lowest negative to the highest positive value for $\hat{\beta}_j^C$, which is the estimated CAPs effect on a particular day based on comparison to chambermate sham corrected for CAPs dog as sham.

Table 26. Cumulative Effects of $PM_{2.5}$ on Pulmonary Parameters by Day for Studies of Dogs with Coronary Occlusion[a]

EIP		T_I		EEP		T_E		Pau_{enh}	
Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$	Date	$\hat{\beta}_j^C$
5/19/98	−0.0240	5/28/98	**−0.011**	5/18/98	**−0.1327**	5/18/98	**−0.0806**	5/18/98	**−0.3774**
5/18/98	−0.0220	3/11/98	−0.0014	3/11/98	−0.0172	3/11/98	−0.0106	5/19/98	**−0.1843**
5/28/98	**−0.0218**	5/18/98	0.0006	5/20/98	−0.0168	5/28/98	−0.0065	5/20/98	**−0.1659**
3/11/98	−0.0107	3/12/98	0.0033	5/28/98	0.0038	5/20/98	0.0043	5/26/98	**−0.1020**
3/12/98	0.0033	3/4/98	**0.0107**	3/12/98	0.0225	3/12/98	0.0123	3/11/98	−0.0186
3/4/98	**0.0056**	3/3/98	**0.0126**	3/4/98	**0.0280**	5/19/98	0.0180	3/12/98	−0.0103
5/27/98	0.0069	3/5/98	**0.0189**	5/19/98	0.0397	3/4/98	**0.0216**	5/28/98	0.0045
3/5/98	0.0072	5/26/98	**0.0272**	3/3/98	**0.0530**	3/3/98	**0.0400**	3/3/98	**0.0137**
3/3/98	**0.0117**	5/27/98	**0.0317**	3/10/98	0.0589	3/10/98	**0.0521**	3/4/98	**0.0339**
5/26/98	**0.0225**	5/20/98	**0.0416**	5/26/98	**0.0701**	3/5/98	**0.0652**	3/10/98	0.0620
3/10/98	0.0245	3/10/98	**0.0636**	3/5/98	**0.0876**	5/26/98	**0.0654**	3/5/98	**0.0628**
5/20/98	**0.0813**	5/19/98	**0.0780**	5/27/98	**0.1939**	5/27/98	**0.0990**	5/27/98	**0.1721**

[a] All data are presented as log values. Boldface type indicates significance at $p < 0.05$. Data are presented from the lowest negative to the highest positive value for $\hat{\beta}_j^C$, which is the estimated CAPs effect on a particular day based on comparison to chambermate sham corrected for CAPs dog as sham.

exhibited surges of increasing HF. This profile differs from HF observed in normal dogs exposed to CAPs, which tended to have an increasing temporal trend. On one occasion, Dog 5 showed a surge in HF accompanied by a number of changes in ECG morphology (Figure 38), including inverted T waves corresponding to the HF surges at about 60 minutes on the first day. The ECG during this period (Figure 39) exhibits clearly visible T wave alternans (arrows) that is characterized by alternating larger and smaller T waves. The sham-exposed dog (Dog 4) in Figure 38 had no consistent morphologic change in ECG, no visible T wave alternans, and an HF pattern typical of sham exposure. When the dogs crossed over the following week, the CAPs-exposed animal (Dog 4) showed more variation in the T wave than did the sham dog. Despite finding examples of T wave alternans in ECG data, assessment of T wave alternans in all dogs of the coronary occlusion studies, performed the same way as in normal animals, revealed no significant increase or decrease in this phenomenon during the exposure.

During the coronary occlusion procedure, the CAPs-exposed dogs ($n = 4$) had more rapid development of ST elevation typical of ischemia compared to sham-exposed dogs. Time from occlusion to the onset of ST-segment elevation was defined as the time of the first beat in which subsequently sustained or increased ST elevation (> 3 mm) was noted. This determination was made by a cardiologist unaffiliated with this project who assessed the data blinded to treatment groups. A significant decrease in the time to ST elevation in the occlusion after exposure was observed in the CAPs-exposed group versus the sham-exposed dogs. In comparisons using a paired t test, ST elevation occurred over 40 seconds faster with CAPs as opposed to sham exposure ($p < 0.0005$) (Figure 40) during the occlusion at the end of exposure.

When the heights of ST segment elevation before and after exposure to CAPs were compared for 6 animals, the peak ST-segment elevation was greater after exposure (post = 5.7 ± 0.3 mm; pre = 2.2 ± 0.6 mm; $p < 0.05$ by paired Student t test). Sham animals showed no differences between the before and after exposure measurements of this parameter. Peak heart rate did not differ significantly between the two occlusions in either treatment group. Thus, another measurement supports the assertion that exposure to CAPs potentiates ischemic ECG changes in this model.

Serum samples were collected to measure changes in the enzymes LDH and CPK in relation to the occlusions and exposures. These enzymes can increase after episodes of myocardial ischemia, and marked increases indicate infarction. Initially, in the first coronary occlusion experiment, both LDH and CPK were in the normal range (mean ± SE = 186 ± 34.0 U/L and 148.0 ± 22 U/L, respectively, compared with a normal LDH range of 30 to190 U/L and normal CPK of 20 to 200 U/L). At 24 hours after the first series of LAD coronary artery occlusions, LDH increased to 397.5 ± 95.5 U/L and CPK increased to 286.5 ± 40.5 U/L. Similar increases in cardiac enzymes were measured after

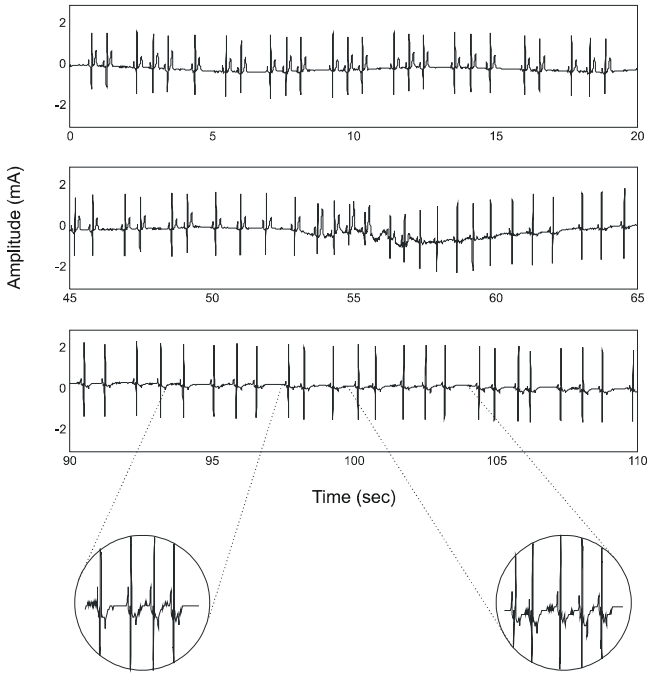


Figure 39. Continuous ECG pattern after approximately 60 minutes of exposure in an animal with coronary artery occlusion. This figure is a beat by beat display of the changes identified at 60 minutes in Figure 37 (1/29/97) and Figure 38. Note T wave inversion in the second panel and then visible T wave alternans in the third panel. That is, the T wave alternates between a larger and smaller size as illustrated in the magnified inserts. This ECG segment corresponds to the highest HF measurement of that day as shown in Figure 37.

a second episode of coronary artery occlusions on the third day of exposure. In subsequent occlusions, changes in CPK varied from baseline. With all data included, a significant ($p = 0.05$) increase in CPK occurred (SD baseline ± mean, 156.25 ± 97.53; postocclusion, 3,340 ± 3,807). The postocclusion CPK has a very large standard deviation, however, because only two dogs showed a change. For all data, LDH also increased significantly ($p = 0.05$) from baseline (mean 100 ± 38 compared to 172 ± 80). Both of these values are within the normal range, however. The enzyme data are interpreted to indicate that ischemia, but not myocardial infarction, had been produced.

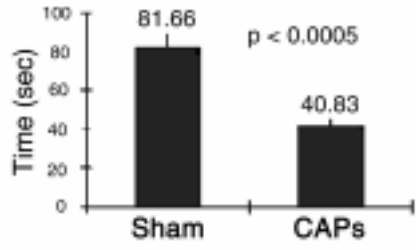


Figure 40. Latency of ST segment elevation with coronary occlusion after CAPs or sham exposure. Time to development of ST changes was significantly shorter with CAPs exposure ($p < 0.0005$).

DISCUSSION AND CONCLUSIONS

The studies reported here use concentrated ambient air particles to assess biologic responses associated with inhalation of particulate ambient air pollution. These studies are very complex in certain aspects of design, specific methods, results, and interpretations. A primary consideration is whether the air particles studied in these experiments represent particulate air pollution in the northeastern United States. We chose to conduct our studies in Boston, a reasonable choice, we believe, because most of the epidemiologic studies linking ambient particles to adverse health outcomes have been conducted in similar environments. Moreover, two major air pollution health effects studies, the original six cities study (Ferris et al 1979) and the current EPA Metropolitan Ambient Air Quality Study (MAAQS) being conducted in five cities, are associated with the Boston metropolitan area (Watertown, one of the original six cities, is a suburb of Boston). In addition, several particle exposure studies for the MAAQS study conducted by our Environmental Chemistry Laboratory (Harvard School of Public Health) in northeastern cities suggest that fine particle exposures do not vary spatially within a given metropolitan area (Philadelphia, Burton et al 1996; Washington DC, Suh et al 1997; and Boston preliminary results). The data for these studies all were collected at sites chosen according to EPA site selection criteria for particle mass concentration measurements.

Our intake at Harvard School of Public Health meets these same EPA criteria and is placed approximately 30 feet above ground level and 100 meters from the nearest street. Traffic on this street is composed primarily of automobiles with a small number of trucks and buses. A small parking lot is located on the opposite side of the building. There are no local point sources of airborne particulates. No large industrial sources are known in our vicinity, which is approximately one mile from downtown Boston. A well-controlled, small oil-burning power plant with a tall stack and a modern pollution control system is located within 0.2 mile. Outdoor air with a usual fine mass concentration of 5 to 15 $\mu g/m^3$ enters the laboratory via a plenum at the second-floor level connected to the inlet of the three-stage concentrator. The mass and chemical composition of fine particles in the metropolitan Boston area have been extensively studied as part of the six cities pollution study (Ferris et al 1979). Spengler and Thurston (1983) had characterized ambient particles in Boston and compared the chemical composition to that of other US cities. On the basis of these considerations, we felt confident that the ambient particles collected at the intake of our exposure facility would represent the Boston area and

our findings represent the northeastern United States. Airborne fine mass in the northeastern US is dominated by regional transport of secondary particles composed largely of sulfates and OC, especially during the summer (Burton et al 1996; Suh et al 1997). In addition to secondary sulfate and organic particles, ambient fine particle mass concentration in Boston is likely to be influenced by marine aerosol and local source emissions, including automobile exhaust and residual oil combustion.

Only a few studies have examined the urban Boston ambient particle mass concentrations and composition specifically, and most of these studies are quite dated. In a very early study of the composition and sources of Boston ambient particulate matter, Hopke et al (1976) performed a source apportionment analysis on total suspended particulate mass samples collected at several sites throughout metropolitan Boston during spring of 1970. The major sources of ambient total suspended particles that he identified, using factor analysis and hierarchical cluster analysis, included crustal material (mostly coarse particles, larger than 2.5 μm), marine aerosol, oil burning residue, motor vehicle exhaust, and refuse incineration. Most of the particle mass was attributed to local sources, which is not surprising as the analysis was based on total suspended particulate mass rather than fine mass.

The mass and chemical composition of fine particles in the metropolitan Boston area have been studied in the past as part of the six cities pollution study (Ferris et al 1979). As part of this study, Thurston and Spengler (1985) examined ambient fine and coarse particle mass concentrations and composition at a single site in the metropolitan Boston area from June 1979 to June 1981. Using principal components analysis, they identified six major source classes, including coal combustion emissions, crustal material, motor vehicle exhaust, refuse incineration, residual oil combustion, and marine aerosol. Using back-wind trajectory analysis to assess the relative contributions of regional transport and local sources, they found regional transport contributed more to sulfate mass than local emissions did although a relatively large fraction of particulate sulfate remained unapportioned.

Olmez and Hermann (1990) measured particulate fine mass concentrations and composition at one site in the metropolitan Boston area and a second site in a rural area 100 km to the west. Composition patterns at the urban and rural sites, subjected to factor analysis, were used to identify regional and local sources, including regional coal combustion emissions, crustal material, local oil combustion emissions, marine aerosol, and other sources of anthropogenic particulates.

Determining the relative contributions of local and distant sources is a challenging task. To date, no dispersion or receptor model is accurate enough to identify and quantify sources of fine particulate matter. For this reason, the EPA is initiating multimillion-dollar programs for particle characterization and model development initiatives that will enable scientists to develop and validate accurate source apportionment methods.

Recently an air pollution monitoring study was conducted in South Boston to investigate the relative contributions of local and regional sources to ambient fine mass concentrations (Koutrakis et al 1999). The data, collected as part of this South Boston Air Quality Study (SBAQS), can be used to formulate contributions of local and regional sources. On average, at least 50% to 70% of Boston ambient fine mass is associated with sources located outside the metropolitan area. This is supported by the observation that particle concentrations vary little within the greater Boston area. On a yearly basis, less than half of the particle mass originates from local sources, but the relative importance of distant and local sources has significant diurnal and seasonal variability. For instance, during a cold winter the majority of ambient fine particle exposures might be associated with local sources such as vehicles and domestic heating. Apportioning of CAPs exposures requires a large number of measurements and application of sophisticated statistical models. These efforts will improve with more data than is available with the number of measurements in this research project.

The more recent information on the observed particulate mass concentrations and composition of Boston ambient fine mass is summarized in Table 28. This table also includes data (Spengler and Thurston 1983) characterizing ambient particles in Boston and comparing the chemical composition to other US cities as part of the Harvard Six Cities study. The annual means and standard errors of mass and elemental ambient concentrations are shown in Table 28 along with similar measures from the SBAQS study (2-year mean, January 1995 through March 1997). For comparison, the mean and standard errors for the same elements for all CAPs exposures analyzed to date are shown as well as those for the set of 24 dog crossover exposures. It is interesting to note the reduction of Pb and Br in Boston ambient particles from the time of the Spengler and Thurston study (1979 to 1981) to the present day due to elimination of lead from gasoline. The difference in iron concentrations is also noteworthy but not as easily explained; however, our concentrator studies appear to have higher iron and silicon levels than would be found if one were to multiply the mean current ambient levels for these elements times the mean mass concentration factor.

Table 27. Effects of $PM_{2.5}$ on Cardiac and Respiratory Parameters in Dogs with Coronary Occlusion[a]

	Using Cumulative Exposure Dose			Using Actual Exposure Dose		
Response	$\hat{\beta}^C$	SE	p Value[b]	$\hat{\beta}^C$	SE	p Value
log LF	-3.4×10^{-3}	2.7×10^{-3}	NS	-5.0×10^{-6}	1.0×10^{-4}	NS
log HF	2.2×10^{-2}	1.9×10^{-3}	< 0.0001	1.3×10^{-3}	1.0×10^{-4}	< 0.0001
log LF/HF ratio	-2.5×10^{-2}	3.7×10^{-3}	< 0.0001	-1.3×10^{-3}	2.0×10^{-4}	< 0.0001
Mean HR	2.1×10^{-1}	1.6×10^{-2}	< 0.0001	1.6×10^{-2}	2.8×10^{-3}	< 0.0001
log HR SD	2.8×10^{-3}	6.0×10^{-4}	< 0.0001	1.0×10^{-4}	3.4×10^{-5}	< 0.0001
log V_T	1.0×10^{-2}	2.4×10^{-3}	< 0.0001	6.0×10^{-4}	1.0×10^{-4}	< 0.0001
log $\dot{V}$	-9.0×10^{-3}	3.0×10^{-3}	< 0.004	-6.0×10^{-4}	2.0×10^{-4}	< 0.0006
log f	-2.1×10^{-2}	2.2×10^{-3}	< 0.0001	-1.2×10^{-3}	1.0×10^{-4}	< 0.0001
log EEP	3.1×10^{-2}	4.5×10^{-3}	< 0.0001	2.1×10^{-3}	2.0×10^{-4}	< 0.0001
log EIP	4.4×10^{-3}	1.9×10^{-3}	< 0.02	2.0×10^{-4}	9.5×10^{-5}	NS
log T_E	2.2×10^{-2}	2.5×10^{-3}	< 0.0001	1.4×10^{-3}	1.0×10^{-4}	< 0.0001
log T_I	1.1×10^{-2}	1.5×10^{-3}	< 0.0001	6.0×10^{-4}	7.7×10^{-5}	< 0.0001
log PEF	-4.0×10^{-4}	2.0×10^{-3}	NS	-2.0×10^{-4}	1.0×10^{-4}	NS
log PIF	3.0×10^{-4}	2.0×10^{-3}	NS	4.8×10^{-5}	1.0×10^{-4}	NS
log Pau_{enh}	6.7×10^{-3}	5.1×10^{-3}	NS	-7.8×10^{-5}	2.0×10^{-4}	NS

[a] Each response was calculated from 12 days.

[b] NS = Not significant.

Table 28. Annual Concentrations of Mass and Elemental Ambient Fine Mass Measured in Previous Boston Air Pollution Studies[a]

Element	Spengler and Thurston (1983)[b]	Oh et al (1998)[c,d]	104 (Total) CAPs Exposures[c]	24 Normal Dog Crossover Exposures[c]
Mass ($\mu g/m^3$)	17.3 ± 0.5	13.5 ± 0.6	254.9 ± 20.3	372.0 ± 57.6
Si (ng/m^3)	100 ± 1	74 ± 7	4699 ± 467	5478 ± 625
S ($\mu g/m^3$)	1.8 ± 0.1	1.5 ± 0.1	20.8 ± 1.8	28.9 ± 4.9
Cl (ng/m^3)	84.0 ± 10.0	25.8 ± 9.5	2054 ± 727	941 ± 497
Ca (ng/m^3)	41 ± 2	37 ± 3	2064 ± 213	2483 ± 370
V (ng/m^3)	22.0 ± 0.1	10.2 ± 0.7	99 ± 12	143 ± 30
Mn (ng/m^3)	3.6 ± 0.16	1.2 ± 0.09	92 ± 7	132 ± 11
Fe (ng/m^3)	350 ± 20	64 ± 3	3499 ± 288	5046 ± 518
Ni (ng/m^3)	8.5 ± 0.4	8.7 ± 0.8	71 ± 8	116 ± 24
Se (ng/m^3)	0.59 ± 0.04	0.99 ± 0.08	17 ± 2	31 ± 8
Br (ng/m^3)	88.0 ± 4.0	2.9 ± 0.2	72 ± 7	104 ± 22
Pb (ng/m^3)	329.0 ± 13.0	8.1 ± 0.4	139 ± 9	201 ± 22

[a] Values are expressed as means ± SE.

[b] Mass concentrations were determined using β-gauge attenuation and the elemental composition of fine mass was determined using x-ray fluorescence.

[c] Mass concentrations were determined using gravimetric analysis and the elemental composition of fine mass was determined using x-ray fluorescence.

[d] Unpublished data from South Boston Air Quality Study.

Overall, this table suggests that our concentrator studies are representative of Boston air which, as noted above, is similar to that of other eastern US cities.

Also of interest is the diurnal variability in pollutant concentrations. Figure 41 shows typical daily variation for concentrations of PM_{10}, $PM_{2.5}$, BC (a surrogate for EC), and CO measured in South Boston as part of the SBAQS (Koutrakis et al 1999). $PM_{2.5}$ and PM_{10} were measured continuously using TEOM monitors; all concentrations represent hourly averages. The BC concentrations measured in Boston typically peak during the morning hours (6 AM to 9 AM), most likely due to the contribution of vehicular emissions during morning rush hour, which is also the time when local emissions are often trapped beneath the inversion layer. It is important to note that experimental exposures can be timed to maximize or minimize the contribution of local CAPs sources. The data in Figure 41 can be compared to the continuous measures of CAPs shown in Figures 12 through 15 and Figures 33 and 34. During some exposures, higher levels early in the day correspond to the locally generated morning peak. Because of the preparation time required for dog exposures, however, it is difficult to capture the peak contribution of local sources during the early morning hours, and our studies are more representative of the levels from 9 AM to 3 PM.

The seasonal variation in concentrations of particulate fine mass and sulfate in Boston has also been investigated (Koutrakis et al 1999; Spengler and Thurston 1983). Both studies found that a notable increase in the sulfate (and fine mass) concentration occurred during the summer months, which is similar to seasonal variations in other eastern US locations (Altshuller 1980). The study by Spengler and Thurston (1983) showed that particulate sulfate concentration accounts for as much as 60% of the total fine mass concentration during the summer months and for as little as 40% during the winter months. Comparisons among the 1983 data of Spengler and Thurston, the recent results of SBAQS, and our data based on concentrated particles suggest that, with the exception of lead concentrations, only modest changes in the composition of Boston air have occurred over this time period. In our CAPs studies, a number of days had relatively high lead levels for the era of low-lead fuel. The transition metal concentrations were also relatively high compared to percentages found in other monitoring studies.

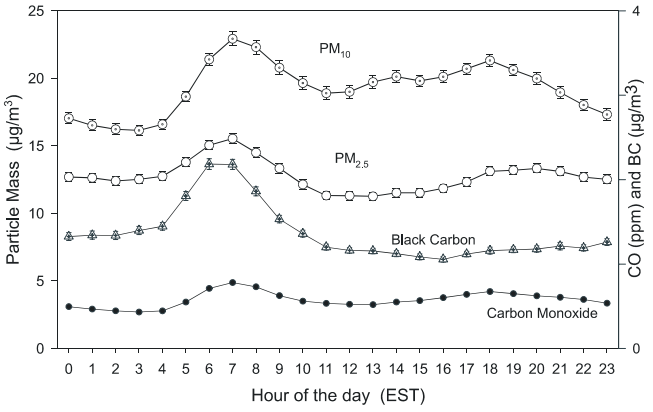


Figure 41. Average diurnal variation in particulate concentration. Continuous mass measurements over 24 hours between January 1995 and March 1997 in Boston, Massachusetts, illustrate hourly changes in mass.

The northwest trajectory pattern was associated with significant changes in canine cardiac response, but the relation between daily levels of metals and the northwest and north-northwest trajectory patterns was not statistically significant. Nickel, however, was quite close to being significant in this small sample, and additional studies could support such a relation; this would have important implications, especially because these trajectory patterns extend outside the United States. In a recent study, Gao and coworkers (1996) found substantial accumulations of vanadium, nickel, manganese, arsenic, copper, and zinc in central and eastern Canada. In a study of back-trajectories for Dorset, Ontario, a substantial proportion of the transport of metal particles to Dorset clearly emanated from the same Canadian regions noted in our trajectory patterns. Finally, although our studies reported here include all four seasons, we do not have enough repetitions of crossover experiments in the summer to make summer versus winter comparisons. Additional summer studies added to this data base could provide useful insights and answer questions about summer and winter differences.

Figure 1, viewed in relation to the biologic findings of these studies, appears to reveal an important response via the pulmonary chemoreflex pathways with considerable vagally mediated effects. Our studies have shown statistically significant biologic effects in dogs exposed to CAPs. These effects are strongest in HRV parameters. The pattern of HRV provides a measure of cardiac response to the autonomic control system, based on the fact that the two divisions of the autonomic nervous system exert distinct influences on the pattern of beat-to-beat fluctuations. The parasympathetic nervous system exerts very rapid control and influences HF fluctuations in the range of 0.15 to 1.00 Hz, which is close to the respiratory frequency (corresponding to rates of 9 to 50 breaths per minute). The sympathetic nervous system exerts more sluggish control, and its frequency control range is 0.05 to 0.15 Hz (Levy 1995). Spectral analysis was performed by decomposing the HRV signals into a sum of sine waves of different amplitudes and frequencies.

By calculating the power spectral density, we can infer effects of inhaled particles on both sympathetic and parasympathetic control of the heart. Essentially, a change in HF power implies an effect on parasympathetic or vagal control of the heart. A change that affects primarily the LF component of HRV implies a sympathetic nervous system contribution. These influences are generally characterized by the ratio LF/HF power because the LF component represents some mixture of effects that can not be ascribed to the sympathetic nervous system. If an exposure directly affected the heart such that its response to vagal or sympathetic stimulation changed, these measurements would also detect such an effect.

In our studies exposure of normal dogs to CAPs affected both LF and HF power. The mean data, as well as the propensity for these changes on individual days, show these effects. In the overall mean data, no significant changes occurred in the LF/HF ratio; however, in individual dogs in crossover studies, and in the overall analysis of the crossover studies, significant increases did occur in the LF/HF ratio. In Table 13, 10 days show significant increases and 5 days show significant decreases. The magnitude of the increases tends to be two to five times the magnitude of the decreases. This, as well as the overall crossover analyses, suggests considerable sympathetic influence during many exposures, as well as parasympathetic effects, as shown by the decrease in heart rate (13 days show significant decrease, as well as a significant overall decrease), increased standard deviation (11 days show significant increase), and HF (8 days show significant increase).

The findings in Table 16, in which data from all crossover studies are used in the statistical model, show clearly that regardless of how these data are assessed in relation to the exposure, the LF/HF ratio indicates significant sympathetic influence. Further, the overall decrease in heart rate with exposure indicates an increase in parasympathetic influence. Thus, our data suggest a perturbation in sympathetic as well as parasympathetic control of the heart mediated via the vagus nerve during particle exposure on some days. These effects may be important as they suggests a disruption of beat-to-beat control of heart rhythm.

Recent studies by Pope and colleagues (1999) show a similar influence on sympathetic and parasympathetic control of the heart associated with particulate air pollution increases in an elderly human cohort. In those studies, using the time domain of HRV, reductions in the standard deviation of normal-to-normal heart beat (NN) intervals, and the standard deviation of the averages of NN intervals, indicated the presence of sympathetic effects correlated to particulate levels in all five-minute segments of the recording. Increases in the square root of the mean of squared differences between adjacent NN intervals indicated a parasympathetic effect. Thus, both canine and human studies show the same direction of changes in HRV with exposure to particulate air pollution.

Perhaps the most significant supporting data for the importance of particulate effects on cardiac mechanisms comes from a new collaborative pilot study with Dockery and colleagues (Peters et al 1999). The relation between air particulate exposure and spontaneous discharge of the automatic implantable cardioverter/defibrillator (AICD) was examined in a pilot study of 99 patients attending the Beth Israel Deaconess Medical Center AICD clinic. The results demonstrate a highly significant increase in the firing rate of AICDs during the first two days following elevated $PM_{2.5}$ (relative risk = 1.33, 95% CI:1.09–1.63, $p < 0.01$). This human finding carries important implications because it suggests that air particles can perturb cardiac electrophysiologic function to the extent that life-threatening disturbances in heart rhythm can result. The relation of these human findings to decreases in the time to ST elevation after CAPs exposure and coronary occlusion remains speculative, but both the human and canine findings warrant continued investigation.

Increases in HF and HRSD along with decreases in heart rate classically are thought to predict a good outcome in patients with coronary artery disease, but as outlined in Figure 2, this may not be the case in all instances. Kasanuki and coworkers (1997) have reported idiopathic ventricular fibrillation accompanied by vagal activation in patients without obvious heart disease. The patients exhibited impressive increases in HF before ventricular fibrillation as well as changes very similar to the vagally induced trends seen in our normal dogs and particularly similar to the HF surges associated with T wave alternans in the coronary occlusion animals. Thus, although a vagally mediated mechanism of effect was not our primary hypothesis, this mechanism appears to have a strong influence in our data and is coming to be recognized in the literature as important in normal individuals as well as those with underlying heart disease. Excessive vagal discharge can lead to potentially fatal arrhythmias, such as atrial fibrillation and idiopathic ventricular fibrillation (Kanasuki et al 1997); however, our results in normal animals do not suggest a level of vagal activity that would be expected to cause such arrhythmias.

The consistency of HRV effects was evident in our mean data analysis, in the analyses of individual dogs and days in the crossover design, and in the overall data analysis of the crossover studies. Our choice of statistical analyses for the crossover studies demonstrated these HRV effects on a day-to-day basis. Chambermates often tended to behave

similarly over a six-hour exposure. For instance, a steady decrease in a particular parameter for both the sham and CAPs dogs across the six-hour exposure could have reflected a chamber effect for this parameter. Likewise, common nonlinear trends across the six-hour exposure could have represented similar responses to surrounding conditions or feedback between chambermates. If parameters exhibited the same trend for both control and CAPs dogs, we recognized that CAPs effects might be obscured. We controlled for these trends by fitting semiparametric models that assumed an arbitrary, nonlinear function common to sham and CAPs dogs particular to that day. We allowed for dog heterogeneity by including a separate curve intercept in the model for each dog. Deviations from parallelism of the overall trends in the curves were interpreted to represent a cumulative CAPs effect. We accounted for the repeated measures form of the data over each six-hour exposure by assuming that the residual error followed a first-order autoregressive process. This statistical approach proved to be particularly powerful for these analyses. Because of the complexity of the study and the variation in CAPs toxicity from day to day, using such an approach confirmed distinct differences that were obvious in individual data but were obscured in data sets grouped by simple comparisons of CAPs or sham results.

Fitting the semiparametric model defined by equation (1) to the crossover data gained several advantages over simpler approaches. First, the sample size reduction that resulted when the responses for a given dog and day were summarized by a single measure was avoided. Thus, the resulting analysis of continuous data provided increased power to detect exposure effects. In addition, this approach recognized that concentration level and other intrinsic properties of CAPs varied over each six-hour exposure. This finding allowed for the possibility that these variations might induce within-day changes in the biologic parameters, whereas an analysis at the day level using integrated mass measurements would detect only between-day effects.

The semiparametric analyses also avoided the potential for bias in several ways. The nonparametric estimation of a smooth baseline trend (using LOESS) for each day avoided the strict assumption of linearity for each response over each six-hour exposure. In addition, the model estimated CAPs effects while it simultaneously controlled for dog-to-dog differences and day-to-day variability. Applied to the cardiac parameters, the model also controlled for respiratory performance and heart rate, which is important to assure that the findings are not due simply to known interrelations among these measurements.

The relation of CAPs exposure to changes in ST segment in the coronary occlusion protocols is important. Several factors may account for the earlier onset of ST segment elevation in CAPs versus sham exposure. Plausible mechanisms, which may act independently or synergistically, include exposure-related endothelial dysfunction, altered cardiac metabolism, and responses to organic aerosol components. Endothelium maintains intrinsic control of vascular tone in normal coronary arteries and affects phasic vasoaction through a variety of mechanisms (Chiodo 1993; Ghaleh et al 1995). Under certain conditions, such as inflammation or damage resulting from disease, age, or exposure to free radicals, endothelial dysfunction occurs (van Hinsbergh 1992) and substances can evoke paradoxical effects. Bradykinin normally acts as a vasodilator, but vasoconstriction can result after removal of endothelium or presumed endothelial damage from advanced age (Mantelli et al 1995). Intracoronary acetylcholine (important in the chemoreflex) also ordinarily promotes vasorelaxation, but in cases of even minimal atherosclerosis, a vasoconstrictor effect has been observed (Ludmer et al 1986). This effect may explain the finding of Kasanuki and colleagues (1997) that transient elevations in vagally mediated HRV are observed immediately before episodes of idiopathic ventricular fibrillation.

Recent work also has demonstrated that a byproduct of cardiac inflammation causes the migration of myocyte intracellular calcium stores to the myoplasm (Eley et al 1991). This disequilibrium impairs muscle function and viability and may increase sensitivity to ischemia. Semivolatile organic molecules from a variety of sources are present in CAPs. Certain molecules, either at the lung surface or within the circulation via the lung epithelium, may mimic intrinsic neurotransmitters, hormones, and cytokines to trigger systemic effects. At the present time, endothelial dysfunction has to be considered a potential cause of reducing ST segment elevation latency. Exposure to CAPs results in modest pulmonary inflammation, as these studies show. Volatile compounds in the CAPs aerosol may generate free radicals, on interaction with cells, in either soluble or particulate forms of metals; these substances may temporarily damage endothelium or impair function.

Modest increases in neutrophils after exposure to CAPs were detected in assessed populations even though other inflammatory parameters were less clear. This evidence suggests inflammation in the lung, therefore, as a mechanism of response to CAPs. Previous studies from our laboratory (Pierce et al 1996; Killingsworth et al 1997; Tsai 1997) and others (Costa et al 1994; Ghio et al 1992), using fly ash or its metal components as surrogates for particulate air pollution, have shown metals to be an important factor in lung inflammation. Although these studies have

provided support for the role of metals in the air as a causative agent, this role has not been widely accepted because metals usually constitute a small fraction (< 10%) of the composition of ambient particles.

Evidence is accumulating that metals may have a role in any toxic response to complex air particles. Our studies in rats (Killingsworth et al 1997) showed changes in cardiac macrophages associated with fly ash exposure. Neutrophils in BAL fluid were significantly elevated in monocrotaline-treated rats and were further increased by fly ash. Fly ash alone induced MIP-2 mRNA expression in BAL cells from normal animals; MIP-2 immunostaining was positive in the heart and lungs of monocrotaline-treated rats exposed to either air or particles; and fly ash enhanced MIP-2 immunostaining in the lung and heart of monocrotaline-treated animals. Less MIP-2 immunostaining was detected in the cardiac and alveolar macrophages of normal rats exposed to fly ash alone. Such immunostaining in the heart was concentrated in cardiac macrophages, as determined by concomitant staining with the common leukocyte marker OX-1. These data suggest that exposure to fly ash results in lung inflammation and that proinflammatory signals are evident in the heart. Further studies on that heart tissue, using analytical electron microscopy with electron energy loss analysis, have revealed particles in cardiac macrophages that have the same elemental signature as the particles used for exposure (unpublished observations). These findings suggest that particulates containing metal may migrate to the heart to contribute to the effects observed. In addition, Watkinson and associates (1998) have reported deaths in rats exposed to intratracheally instilled residual oil fly ash. Cardiac effects, including bradyarrhythmia, were observed in the ECGs of these animals immediately before death. The findings of the study reported here have implications for defining mechanisms of mortality because we have used concentrated air particles rather than a surrogate and have observed similar inflammatory and cardiac-related effects.

Because people receive multiple exposures to increased ambient particulates throughout their lives, it is reasonable to record multiple exposures with the same dog. Presumably, people return to their individual baselines between exposures to such increased particulate levels. There does not appear to be an increased effect from multiple exposures, and no evidence in our data suggests additive or synergistic effects from multiple exposures. Perhaps the most important reason to perform multiple exposures of the same dog is to identify any day-to-day variation in the inherent toxicity of ambient particulate. Our examination of Boston air particulate in vitro (Goldsmith et al 1998) shows that on an equal-mass basis, the ability of ambient air particles to induce active oxygen radicals and proinflammatory cytokines varies from none to marked. In that study, roughly a quarter of the particulate samples were nonreactive; each sample represented one day's collection. In our studies reported here, essentially negative cardiac responses occurred in about the same number of experimental days. That is, on about 25% of the exposure days in normal animals, there was no response whatsoever to the particulate; these days, regardless of the mass level, were indistinguishable from sham exposure days. The failure to respond resulted from a lack of toxicity in the exposure rather than a nonresponsive animal. Our multiple-exposure design has shown that we have not had any dogs that never respond. Thus, from the data reported here, it appears likely that the biologic responses were related to the composition of the exposure.

IMPLICATIONS OF FINDINGS

1. Disturbances in HRV have been associated clinically with risks of fatal arrhythmia. With exposure to CAPs, we observed an increase in both LF power and LF/HF ratio measurements of HRV as well as increases in HF power and decreases in heart rate. These changes appear to be due to disturbance of autonomic balance either directly or through the respiratory system. Further elucidation of this underlying mechanism could provide important insights into the pathophysiologic consequences of exposure to air particulates.
2. In our study, responses to particulate exposure varied from animal to animal and from day to day in the same animal. This finding implies the capability to identify relations in the continuous display of cardiac dynamics with the spontaneously varying concentration of the particles during the exposure period. The intrinsic lability and sensitivity of HRV are useful tools for defining relations to potential cardiotoxic constituents of particles.
3. The studies reported here are the first to carry out a large-scale, essentially random selection of dates for exposure with concentrated ambient particles. The random dates were both a strength and a weakness of these studies. The weakness stems from the fact that compositional variability was such that insufficient power was available to define significant differences for some comparisons. However, the strength lies in the robust predictors of response that emerged from these data. For example, the relation of air mass trajectory patterns to cardiac responses affords the possibility of planning and executing exposures on the

basis of predicted trajectory patterns. Thus, it may be possible to plan for the variation in the exposure composition to better test compositional hypotheses.

4. After coronary artery occlusion, the heart exhibits increased proclivity for abnormalities in the ECG, particularly in the T wave. The observed decrease in the time from occlusion to ST-segment elevation in the case of CAPs exposure compared to sham exposure has considerable implication for the development of potentially fatal pathology. Elucidation of mechanisms contributing to this change is an important goal for continued research in this area.
5. Methodologic advances from this study are numerous. These include
   - the overall design of these experiments;
   - successful demonstration of the use of an ambient particle concentrator to expose animals to "real-world" particles;
   - development of methods to collect continuous respiratory and cardiac data, with a minimum of artifact, over a six-hour exposure in awake, unrestrained dogs;
   - successful use of acute coronary occlusion in dogs as a mechanism to increase sensitivity to inhaled ambient particles, thus modeling a known high risk factor in people;
   - successful demonstration of the utility of HRV analysis to assess autonomic nervous system balance in health effects studies of particulate air pollution;
   - development of innovative data analysis methods including our three-dimensional ECG plot analysis approach, which provides a sensitive means for detecting subtle alterations in ECG morphology for all phases of the ECG (including P waves, PQ interval, R wave, P and Q wave morphology, and T wave); and
   - application of statistical methods, including transitional and semiparametric regression models, in the crossover design and thus creation of increased statistical power to detect exposure effects by using all individual responses, and reducing bias through general assumptions on the baseline trend of each response over time.

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APPENDIX A. Studies of Dogs With Chronic Bronchitis

The second specific aim in our original proposal to HEI included studies on dogs with chronic bronchitis. The rationale for such studies was based on the concept that underlying pulmonary inflammation would potentiate the effects of inhaled ambient particles. Indeed, we had observed such potentiation in studies of rodents with chronic bronchitis (Godleski et al 1996) and with other models of pulmonary inflammation and fly ash (Killingsworth et al 1997). The chronic bronchitis model produced in dogs by chronic exposure to sulfur dioxide (SO_2) had been used in our laboratory and was well characterized (Drazen et al 1982; Shore et al 1987). Protocols used by various investigators have included a gradual increase in concentration, ultimate levels in the 200 ppm range, a minimum of 2 hours per day of exposure, and at least 4 weeks of exposure. In our studies, the animals underwent BAL before any exposure and then were exposed for one week for 2 hours each day to 49.9 ± 2.27 ppm SO_2 (Figure A.1). The following two weeks, the concentration was increased to 183.6 ± 7.94 ppm SO_2 for 2 hours each day; for the next two weeks, for 3 hours each day. BAL was then done to determine the level of pulmonary inflammation and mucus production, and the dogs had ECG leads implanted as described in the Methods section of the full report. After recovery from BAL and the implantation procedure (2 weeks), the dogs again were exposed to SO_2

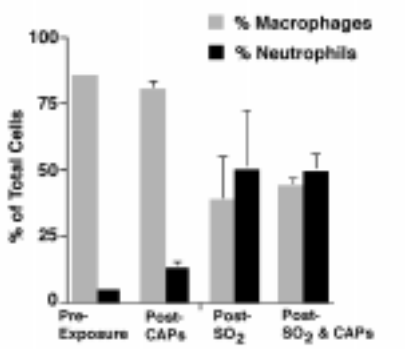

Figure A.1. BAL cells obtained from dogs in chronic bronchitis studies at baseline, after development of model, and after CAPs exposures.

Table A.1. Exposure Parameters for CAPs and Sham Exposure of Dogs with Chronic Bronchitis

Date	Animal (n = 2)	MMAD ± GSD (μm)	Mass CAPs (μg/m^3)	SO_4 CAPs (μg/m^3)	Mass Ambient (μg/m^3)	SO_4 Ambient (μg/m^3)	Concentration Factor	
							Mass	SO_4
12/9/96	10	0.22 ± 2.9	210.0	41.1	10.7	3.0	19.6	13.7
	11	0.22 ± 2.9	210.0	41.1	10.7	3.0	19.6	13.7
12/10/96	10	0.22 ± 2.9	119.0	38.2	10.7	2.9	11.1	13.2
	11	0.22 ± 2.9	119.0	38.2	10.7	2.9	11.1	13.2
12/11/96	10	0.22 ± 2.9	195.0	62.6	14.0	3.8	13.9	16.5
	11	0.22 ± 2.9	195.0	62.6	14.0	3.8	13.9	16.5
5/12/97	10	0.23 ± 2.4	242.1	50.7	11.5	3.5	21.1	14.5
	11		Sham					
5/13/97	10	0.23 ± 2.4	475.4	106.4	18.9	2.9	25.2	36.7
	11		Sham					
5/14/97	10	0.23 ± 2.4	310.0	71.9	9.2	2.4	33.7	30.0
	11		Sham					
5/20/97	10		Sham					
	11	0.18 ± 3.4	265.0	87.3	7.4	2.4	35.8	36.4
5/21/97	10		Sham					
	11	0.18 ± 3.4	177.0	61.2	5.5	1.7	32.2	36.0
5/22/97	10		Sham					
	11	0.18 ± 3.4	173.3	12.5	5.5	0.6	31.5	20.8

Table A.2. Cardiac Measurements for Two Dogs with Bronchitis Comparing Each Dog's Response to CAPs or Sham Exposure

	Number of Days		LF Power of HRV (beats/min^2/Hz)		HF Power of HRV (beats/min^2/Hz)		LF/HF Ratio		Mean HR (beats/min)		HR SD (beats/min)	
Animal	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs
10	3	6	64.82	54.81	496.50	375.82	0.21	0.18	95.51	110.00	24.34	20.76
11	3	6	48.48	35.21	229.24	194.06	0.25	0.22	80.82	72.33	18.52	16.46
Mean			56.65	45.01	362.87	284.94	0.23	0.20	88.17	91.16	21.43	18.61
Paired t test			p = 0.04		p = 0.19		p = 0.04		p = 0.41		p = 0.08	

Table A.3. Respiratory Measurements for Two Dogs with Chronic Bronchitis Comparing Each Dog's Response to CAPs or Sham Exposure

	f (breaths/min)		V_T (mL)		$\dot{V}$ (mL/min)		PIF (L/min)		PEF (L/min)	
Animal	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs
10	24.13	72.11	116.97	122.50	2128.38	4623.81	155.05	294.65	170.83	262.73
11	125.72	61.44	59.84	130.33	6794.98	2471.58	374.52	281.14	311.67	147.39
Mean	74.93	66.77	88.41	126.41	4461.68	3547.69	264.78	287.89	241.25	205.06
Paired t test	p = 0.45		p = 0.22		p = 0.42		p = 0.44		p = 0.41	

Table A.4. Respiratory Measurements for Two Dogs with Chronic Bronchitis Comparing Each Dog's Response to CAPs or Sham Exposure

	EIP (msec)		T_I (sec)		EEP (msec)		T_E (sec)		Pau_{enh}		Trel (sec)		Pau	
Animal	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs
10	45.20	32.49	1.42	0.89	797.57	719.54	1.81	1.56	2.54	1.41	0.72	0.54	1.85	1.50
11	30.48	50.41	0.33	0.88	133.34	1063.06	0.45	2.41	1.02	0.80	0.22	1.02	0.95	1.43
Mean	37.84	41.45	0.88	0.88	465.46	891.30	1.13	1.98	1.78	1.11	0.47	0.78	1.40	1.46
Paired *t* test	$p = 0.43$		$p = 0.50$		$p = 0.28$		$p = 0.29$		$p = 0.19$		$p = 0.32$		$p = 0.45$	

for 3 weeks at 190.2 ± 8.43 ppm for 3 hours per day. CAPs exposures were then carried out on three consecutive days.

Double CAPs, double sham, and crossover design studies were done with this disease model. The exposure parameters of the CAPs used in these studies are described in Table A.1. Concentrations achieved in these studies were in the same range as used in the other experiments of the main report, and air mass trajectories were often from the northwest direction, which was found to be a significant factor in our other studies.

During CAPs exposures, dogs with chronic bronchitis did not exhibit consistent changes in ECG morphology or in cardiovascular parameters such as median heart rate and HF (Table A.2). Continuous data for HF throughout exposures are illustrated for the double CAPs and double sham. In this data set, only one sham exposure day showed the pattern of increasing HF throughout the exposure, and no effect of CAPs on HF was observed. In crossover studies with dogs with chronic bronchitis, again no consistent effect was seen in either respiratory or cardiac parameters with CAPs exposure (Tables A.2, A.3, and A.4). Indeed, the more obvious changes in both cardiac and respiratory measurements tended to be an increase or decrease in parameter response in the sham-exposed chambermate and no change in the CAPs animal. Such effects could be statistically and biologically significant, but the lack of any pathophysiologic consistency in responses as compared to the normal dogs or dogs with coronary occlusion suggested that this particular model was unlikely to be a productive line of investigation.

We did not expect the dogs with chronic bronchitis to show fewer cardiac and respiratory parameter changes than normal dogs. Since this was a limited study, it is unclear at this time whether the lack of effect is related to the model or some other factor.

These dogs had significant pulmonary inflammation associated with the SO_2 exposure, but this level of inflammation did not increase with this CAPs exposure.

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APPENDIX B. CO Concentrations

Table B.1. Ambient Carbon Monoxide Average Hourly Concentrations Measured During Crossover Design Studies with Normal Dogs[a]

Date	Hour 1	Hour 2	Hour 3	Hour 4	Hour 5	Hour 6
6/16/97	0.2	0.2	0.2	0.3	0.2	0.1
6/17/97	0.5	0.4	0.4	0.5	0.6	0.5
6/18/97	0.5	0.8	NA	NA	0.7	0.9
6/23/97	0.3	0.3	0.3	0.3	0.3	0.3
6/24/97	0.4	0.4	0.4	0.4	0.4	0.4
6/25/97	0.5	0.5	0.5	0.5	0.6	0.7
11/4/97	0.7	0.6	0.6	0.6	0.7	0.8
11/5/97	0.5	0.4	0.5	0.5	0.6	0.6
11/6/97	0.5	0.5	0.5	0.6	0.7	0.8
11/12/97	0.4	0.5	0.6	0.7	0.8	0.8
11/13/97	0.2	0.1	0.1	0.1	0.1	0.2
11/14/97	0.2	0.2	0.1	0.2	0.2	0.3
12/3/97	0.6	0.7	0.9	1.0	1.2	1.3
12/4/97	1.0	1.0	1.1	1.2	1.3	1.2
12/5/97	1.6	1.5	1.1	1.2	1.3	1.3
12/9/97	1.6	1.3	1 .0	1.1	1.5	2.2
12/10/97	1.4	1.1	1.1	1.0	1.0	1.0
12/11/97	0.7	0.7	0.7	0.6	0.7	0.6
12/16/97	1.5	1.0	0.7	0.5	0.5	0.7
12/17/97	0.9	0.9	0.6	0.6	0.3	0.4
12/18/97	0.9	0.2	0.1	0	0.1	0.1
1/27/98	1.3	1.0	0.4	0.3	0.3	0.4
1/28/98	0.3	0.3	0.3	0.3	0.4	0.4
1/29/98	0.4	0.4	0.4	0.4	0.4	0.4

[a] Values are given in ppm. Ambient CO was measured outside Room 102 near the concentrator inlet. NA = Not available

APPENDIX C. HEI Quality Assurance Report

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

Quality Assurance Audits

Date	**Phase of Study Audited**
January 14, 1998	Facility audit included animal care areas, inhalation exposure chamber, filter weighing room and offices for computer evaluation/classification of EKG data in Deaconess Hospital. Procedures for data collection, uploading, classification, editing and archiving of cardiac data were audited. Data printouts for one animal were selected at random and the data trail was audited.
October 7–9, 1998	Follow-up on previous audit findings and draft final report audit.

Written reports of each inspection were provided to the Director of Research of the Health Effects Institute who transmitted these findings to the Principal Investigator. These quality assurance audits demonstrated that the study was conducted by a well-coordinated, experienced team of professionals according to the study protocol and standard operating procedures. The report appears to be an accurate representation of the study.

B. Kristin Hoover, Audit Coordinator

ABOUT THE PRIMARY AUTHORS

John J Godleski is a clinical and experimental pathologist with expertise in inhalation toxicology and physiology. He received his MD from the University of Pittsburgh School of Medicine. His postgraduate training in pathology was at Massachusetts General Hospital, and he received additional research training in physiology at Harvard School of Public Health. He served as a research scientist at the EPA laboratories at Research Triangle Park NC and as assistant professor of pathology at Medical College of Pennsylvania in Philadelphia. He has been on the faculty of Harvard Medical School and Harvard School of Public Health since 1978. He has produced more than 100 publications including studies in pulmonary pathology, deposition and clearance of inhaled particles, respiratory physiology, and inhalation toxicology.

Richard L Verrier is a cardiovascular physiologist. He received his PhD from University of Virginia. He is associate professor of medicine at Harvard Medical School and director of the Institute for Prevention of Cardiovascular Disease at Beth Israel Deaconess Medical Center. His research focuses on neural, behavioral, and environmental triggers of sudden cardiac death and arrhythmias. He specializes in computerized analysis of electrocardiographic markers including heart rate variability and T wave alternans. Dr Verrier is the author of more than 130 publications.

Petros Koutrakis is an environmental scientist. He received his PhD from University of Paris. His research activities focus on the development of human exposure measurement techniques and the investigation of sources, transport, and fate of air pollutants. He has developed and patented novel techniques, including the HAPC, that have been used extensively by air pollution scientists. Dr Koutrakis is technical editor-in-chief for the *Journal of Air & Waste Management Association*, a broad environmental journal that concentrates on science and engineering in the areas of air pollution and hazardous waste. He has written over 100 publications.

Paul Catalano is a biostatistician. He received his ScD from Harvard School of Public Health and is currently associate professor of biostatistics there. Dr Catalano's major interests involve research in methods for the analysis of multiple outcomes and repeated measures and their application to environmental dose-response modeling and quantitative risk assessment. He has written publications on statistical methods and has collaborated on various studies.

OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH

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ABBREVIATIONS AND OTHER TERMS

AICD	automatic implantable cardioverter/ defibrillator
ANOVA	analysis of variance
AR1	first-order auto regressive process
β-*N*-AG	β-*N*-acetyl glucosaminidase
BAL	bronchoalveolar lavage
BC	black carbon
CAPs	concentrated ambient particles
CBC	complete blood count
CO	coronary occlusion (used in equations)
CO	carbon monoxide
CPK	creatinine phosphokinase
EC	elemental carbon
ECG	electrocardiogram
EEP	end expiratory pause
EIP	end inspiratory pause
EPA	US Environmental Protection Agency
FID	flame ionization detector
GSD	geometric standard deviation
HAPC	Harvard ambient particle concentrator
HF	high frequency heart rate variability
HRSD	heart rate standard deviation
HRV	heart rate variability
IC	ion chromatography
IL-1	interleukin-1
IL-8	interleukin-8
LAD	left anterior descending coronary artery
LDH	lactate dehydrogenase
LF	low frequency heart rate variability
LOD	limit of detection
LOESS	locally weighted regression
MI	myocardial infarction
MMAD	mass median aerodynamic diameter
NN	normal-to-normal heart beat intervals
OC	organic carbon
Pau	pause
Pau_{enh}	enhanced pause
PEF	peak expiratory flow
PIF	peak inspiratory flow
PM	particulate matter
$PM_{2.5}$	particulate matter less than 2.5 microns in aerodynamic diameter
PM_{10}	particulate matter less than 10 microns in aerodynamic diameter
TB	transbronchial
TC	total carbon
*T*E	expiratory time
TEOM	tapered element oscillating microbalance
*T*I	inspiratory time
TNF	tumor necrosis factor
$\dot{V}$	minute ventilation
*V*I	virtual impactor
*V*T	tidal volume
WBC	white blood cell
XRF	x-ray fluorescence

INTRODUCTION

Epidemiologic studies have indicated that short-term exposure to low-level increases in particulate matter (PM)* is associated with an increase in morbidity and daily mortality, particularly in individuals with cardiopulmonary conditions (reviewed in US Environmental Protection Agency 1996). A plausible biological mechanism linking low-level particle exposure and pathophysiologic effects has not been established, however. Assessing the effects of PM in appropriate animal models is critical to learning how PM may cause adverse health effects.

In 1994, HEI issued RFA 94-2 to address these and other outstanding issues in PM research. John Godleski and colleagues at Harvard School of Public Health responded with a proposal to study the effects of exposure to concentrated ambient particles (CAPs) in dogs. In his application, Godleski hypothesized that normal dogs exposed to PM would show inflammatory responses in the airways and changes in pulmonary mechanical measurements. In dogs with cardiopulmonary conditions, Godleski proposed that exposure to PM might lead to changes in cardiac function and potentially fatal arrhythmia. To maximize the possible effects, he planned to use a piece of equipment, the Harvard ambient particle concentrator, developed by his collaborators to concentrate particles up to 30 times their level in ambient air. Because the changes induced might be quite subtle, he proposed to use sophisticated cardiology techniques to monitor any CAPs-induced changes. As epidemiology studies suggested that individuals with cardiac or pulmonary diseases were particularly sensitive to PM effects, Godleski proposed to study the effects of concentrated particles in dogs that modeled these conditions. The HEI Research Committee funded the proposal because it thought the study was an innovative exploratory study designed to identify possible adverse effects of PM in a relevant animal model.†

Godleski submitted a draft report in July 1998. As with all HEI-funded studies, Godleski's report was reviewed by the HEI Health Review Committee and external peer reviewers who, for this report, were experts in human and veterinary cardiology as well as the generation and characterization CAPs. Following the recommendations of the reviewers, Godleski submitted a revised report in January 1999. Because of the complexity of the revised report, Dr Godleski and his collaborators met with a panel comprising the HEI Health Review Committee and consultants to discuss the study in detail. Following these discussions, the Review Committee accepted the revised report for publication in February 1999 and suggested a small number of further revisions. In May 1999, Dr Godleski submitted a final revision to the report along with a new statistical analysis performed after the panel had reviewed the previous draft report.

JUSTIFICATION FOR THE STUDY

POLLUTANT EFFECTS ON RESPONSES IN THE AIRWAYS

Although epidemiology studies have described an association between exposure to short-term increases in PM and short-term increases in morbidity and mortality due to cardiopulmonary causes, a plausible pathophysiologic mechanism for such an effect has not been definitively demonstrated to date. Prior to the application for the current study, a number of studies had tried to establish such a link by investigating the potentially toxicologic effects, particularly to the airways, of different components of PM in controlled animal exposures. For example, Gearhart and Schlesinger (1989) showed that long-term inhalation of sulfuric acid decreased mucociliary clearance of particles from the bronchial area in rabbits. Amdur and Chen (1989) indicated that a 3-hour aerosol exposure of sulfuric acid adsorbed onto zinc oxide impaired oxygenation of blood across the guinea pig alveolar capillary membrane and increased indicators of an inflammatory response in bronchoalveolar lavage (BAL). In a long-term study of a respirable sulfite aerosol in beagles, Heyder and colleagues (1992) reported few changes in respiratory lung function, a small decrease in alveolar macrophage phagocytic capacity, and a small increase in BAL inflammatory parameters. Unless the exposure to pollutants was at very high levels, however, responses in animal studies did not lead to acute mortality (Mauderly 1994).

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

† Dr John J Godleski's 3-year study, *Mechanisms of Morbidity and Mortality from Exposure to Ambient Air Particles*, began in July 1995 with total expenditures of $609,494. The Investigators' Report from Dr Godleski and colleagues was received for review in July 1998. A revised report, received in February 1999, was accepted for publication in May 1999. During the review process, the HEI Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary.

Because some epidemiology studies had suggested that individuals with preexisting inflammatory lung diseases might be particularly sensitive to PM, and thus that particles might exacerbate ongoing inflammatory responses, attention focused on pollutant effects in animal models that modeled these conditions. In 1994, Costa and colleagues showed that intratracheal instillation of residual oil fly ash, a highly toxic combustion-generated urban particulate, increased inflammation in the airways and led to the death of rats which had previously been injected with monocrotaline to induce pulmonary hypertension and right-heart hypertrophy (Costa et al 1994). The study did not, however, suggest a mechanism that linked the inflammatory response to pulmonary and cardiac effects of PM.

To explain the possible link of PM and cardiovascular effects, Godleski and colleagues proposed that effects of PM on the normal heart might be mediated by the release from lung cells of proinflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNFα) and chemokines such as interleukin-8 (IL-8). Levels of these mediators were known to increase in human cardiac ischemia and myocardial infarction (MI) (Abe et al 1993; Basaran et al 1993; Blum et al 1994; Strieter et al 1993; Vaddi et al 1994). In addition, Seaton and colleagues (1995) hypothesized that the deposition of particles in airways might result in the release of acute inflammatory mediators, which could in turn induce or increase levels of circulating hematologic factors, such as fibrinogen. As fibrinogen could increase plasma viscosity, the coagulability of blood would be affected, increasing the likelihood of thrombus formation. Thus, these groups proposed that effects of PM on the cardiovascular system might be a consequence of inducing an acute inflammatory response in the airways.

USE OF CARDIAC PARAMETERS TO MEASURE PM EFFECTS

As a number of agents were known to affect neural control of the heart, and the hearts of animals with cardiopulmonary diseases were known to be less stable electrically than those of normal animals, Godleski and colleagues proposed an alternative hypothesis to explain how PM might induce mortality in animals with impaired cardiopulmonary systems. They suggested that PM might induce potentially fatal arrhythmias, possibly via a mechanism directly affecting neural control of the heart and in the absence of inflammatory responses. To monitor cardiac responses, Godleski and colleagues measured changes in the canine electrocardiogram (ECG) pattern, which shares many features of the human ECG. Their assessment included changes in the ST segment, the ECG interval that starts at the end of the QRS complex and ends at the end of the T wave.

The investigators also used two noninvasive cardiologic techniques that have been used experimentally to identify patients at risk for potentially fatal arrhythmias. The first was heart rate variability (HRV), the fluctuations in heart rate which occur in normal individuals. A substantial body of evidence indicates that reduced HRV is associated with cardiac mortality after MI in humans and dog models (Kleiger et al 1987; Bigger et al 1992; Odemuyiwa et al 1991). Depressed HRV has also been specifically associated with arrhythmic complications following acute MI (eg, sudden cardiac death and ventricular tachycardia) (Odemuyiwa et al 1991) and with increased risk for the development of ischemia-induced ventricular arrhythmias following balloon angioplasty (Airaksinen et al 1999).

Normal variability in heart rate is under the influence of the autonomic nervous system, that portion of the nervous system which controls the viscera (for example, glandular secretions and movements of the gastrointestinal tract). The two branches of the autonomic nervous system, the sympathetic and the parasympathetic (or vagal) nerves, exert opposing effects on heart rate: the sympathetic arm to increase it and the parasympathetic to slow it down. By mathematical transformation of the heart rate data, HRV can be broken down into components of differing frequencies that reflect differential control of heart rate by the two arms of the autonomic nervous system. This dynamic is depicted in Figure 9 of the Investigators' Report. High frequency (HF) components (0.15 to 1.00 Hz) reflect the modulatory influence of the parasympathetic nervous system on the heart, and low frequency (LF) components (0.05 to 0.15 Hz) reflect control by both sympathetic and parasympathetic mechanisms. (Very low frequency [VLF] components of HRV [0.05 Hz] are not shown in the figure.) The LF/HF ratio has been proposed as an index of the balance between the sympathetic and parasympathetic nervous systems (Malliani et al 1991).

The influence of the autonomic nervous system on the susceptibility to ischemia-induced ventricular arrhythmias has been extensively studied in humans and animal models (Lombardi et al 1987; Hull et al 1990). These studies show that stimulation of the sympathetic system evokes fibrillation whereas surgical or pharmacologic blocking of the sympathetic nervous system is anti-fibrillatory. Manipulations of the parasympathetic nervous system show effects opposite of manipulation of the sympathetic system. Thus, increases in the LF/HF ratio are thought to represent a condition of sympathetic activation and parasympathetic (vagal) withdrawal and thus more likely to result in arrhythmias.

The second noninvasive electrophysiologic parameter Godleski and colleagues studied was T wave alternans. The T wave phase of the ECG measures repolarization of the heart's electric potential; T wave alternans is a beat-to-beat change in the amplitude of the T wave that repeats once every other beat (ie, it has an ABABAB… pattern, depicted in Figure 39 of the Investigators' Report). The presence of either T wave alternans that is visible on the surface ECG or microscopic T wave alternans that is invisible to the naked eye but can be seen by using sophisticated signal processing of high-fidelity ECG tracings has been associated with an increased risk for subsequent ventricular arrhythmias (Schwartz and Malliani 1975; Rozanski and Kleinfeld 1982; Puletti et al 1980; Rosenbaum et al 1994; Nearing et al 1991; Estes et al 1997). In a recent report, a causal link was found between T wave alternans and ventricular fibrillation (Pastore et al 1999).

Godleski was the first to attempt to assess the relationships among cardiac, inflammatory and pulmonary parameters in the response to inhaled concentrated particles in normal dogs and in dogs with cardiopulmonary conditions that model relevant human diseases.

TECHNICAL EVALUATION

AIMS AND OBJECTIVES

The overall goal of the study was to determine whether inhalation of urban air particles produces systemic effects that become manifested in subtle ECG changes associated with fatal arrhythmia. The investigators considered two pathways by which PM might exert its effects: (1) a pathway mediated via the autonomic nervous system, perhaps in the absence of significant pulmonary inflammation, and (2) a mechanism operating as a result of pulmonary inflammation.

The specific aims of the study were as follows.

Aim 1: To assess mortality and morbidity from exposure to CAPs using normal adult dogs. The investigators tested the hypothesis that exposure to CAPs might induce cardiac rhythm changes, inflammatory responses, and changes in pulmonary mechanical measurements. (In their original application, the investigators theorized that CAPs would not induce changes in cardiac parameters in normal dogs; after finding positive preliminary data, however, the investigators evaluated cardiac changes in the normal dog response to CAPs.)

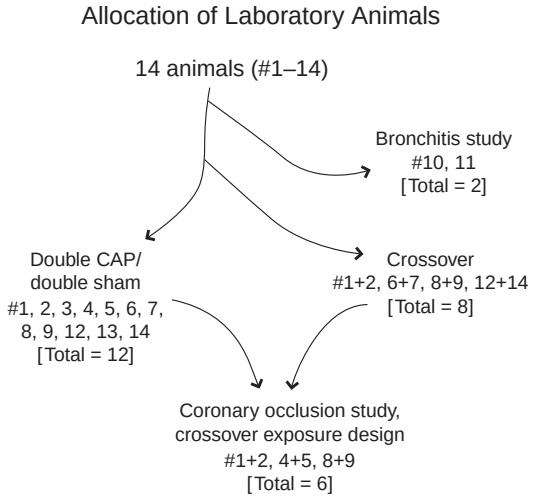
Aim 2: To study dogs with chronic bronchitis, induced by increasing concentrations of sulfur dioxide, in order to determine whether preexisting pulmonary inflammation potentiated CAPs-induced changes in cardiac rhythm, resulting in increased morbidity or mortality. Because of limited time and the need to concentrate his resources, Dr Godleski did not pursue this aim beyond a limited number of experiments, which are summarized in the Results section.

Aim 3: To study dogs with a coronary occlusion, induced by mechanical occlusion of the left anterior descending coronary artery, in order to determine whether the presence of coronary artery disease potentiated PM effects on the heart (in particular, whether CAPs would induce changes in cardiac parameters associated with fatal arrhythmias that may lead to morbidity or mortality).

STUDY DESIGN

The investigators exposed 14 dogs to CAPs or filtered air for six hours a day on three consecutive days (see figure below). Two of these animals were used exclusively for the bronchitis study (Aim 2), and 12 were used in studies of normal dog responses (Aim 1). Six of these 12 dogs were used in the coronary occlusion study (Aim 3).

Godleski and colleagues used two study designs to assess the effects of exposure. The first was a *crossover design*, in which one of a pair of dogs was initially exposed to CAPs and the other dog in the pair was simultaneously exposed to filtered air (sham or control exposure). In the following week, the dogs crossed over to receive the other exposure (ie, sham received CAPs or CAPs received sham). Exposure of eight normal dogs (4 pairs) in the crossover study resulted in 24 days of exposure information. On one of the days, however, a poor connection resulted in loss of cardiac information so the investigators presented 23 days of crossover exposure data. The six dogs (3 pairs) with a coronary occlusion were exposed in the crossover study and produced 18 days of exposure information.



The second design was a *double CAPs/double sham design*: two dogs were exposed at the same time either to CAPs or to filtered air, and at a later date two dogs were exposed to filtered air or to CAPs. Twelve dogs were exposed using the double CAPs/double sham design, but not all 12 received both CAPs and sham exposure under this protocol.

METHODS

Harvard Ambient Particle Concentrator

Under the leadership of Dr Petros Koutrakis and his research group, the investigators designed and developed one of the first instruments for concentrating ambient PM, the Harvard ambient particle concentrator (HAPC).

The HAPC comprises the following major components:

- *Particle Pre-size Selective Inlet*, which collects ambient air particles from an intake outside the building, and removes particles of aerodynamic diameter larger than 2.5 µm.
- *Virtual Impactors*, which separate particles based on their aerodynamic size. Input air flows through a series of slit-nozzle virtual impactors. Based on the geometry and flow dynamics of the system, the majority of the particles between 0.15 and 2.5µm are collected in a reduced volume. This increases the concentration of particles in this size range by a factor of approximately 3.5 (one-stage concentrator). Further concentration can be achieved by passing the concentrated particles through additional stages. In the current study, the HAPC used a three-stage concentrator, able to concentrate particles approximately 30-fold. (Recent modifications using four stages can concentrate particles up to 80-fold.)
- *High-Volume Pumping Unit* In the HAPC, the particle-laden air is pulled through the system by a pump placed downstream of the exposure chamber. This design ensures that if particles are generated within the pump they do not contaminate the atmosphere to which the animals are exposed. Dogs are thus exposed to particles at a negative pressure, similar to a modest elevation in altitude.

The goal of the HAPC was to concentrate the PM components of ambient air pollution in unaltered form. One advantage of the concentrator approach is that it affords exposure to PM at higher than ambient levels, and so it can be used on days on which ambient pollution is low. Animals were exposed to ambient levels of pollutant gases such as nitrogen oxides and carbon monoxide (CO) in the exposure chamber, but the impact of these gases on the study endpoints was not assessed. Ozone present in the pollutant mix was likely removed by the aluminum housing of the HAPC. The investigators did not measure levels of ambient gases other than CO (see below, Methods Section 3), which is known to affect the cardiovascular system. In future studies, controlled levels of these gases may be added to the chamber to assess their impact on CAPs exposure.

One issue with the HAPC was that its performance varied during the study, as demonstrated by the large variability in particle concentration factor (7- to 45-fold) reported on different days. The investigators noted that the concentration factor was most strongly influenced by the negative operating pressure of the HAPC, which was optimal at a 2.5-inch pressure drop per stage (7.5 inch total), but varied from 7.5 to 25 inches in the study. They found that alignment of the slits of the virtual impactor was critical for optimal operation; misalignment increased the negative operating pressure and resulted in particle loss and thus a decreased concentration factor. They also found that high ambient concentration and high relative humidity resulted in particle loss. Many of these problems of concentrator operation were found and solved in the course of the study: most of the lower concentration factors were obtained in the earliest experiments. Nonetheless, it will be critical for future studies to ensure that more consistent levels of CAPs can be administered daily over the course of a study.

Animals

All experiments were carried out with careful attention to the animals' health and with the approval of the Harvard School of Public Health's Animal Welfare Committee. Godleski and colleagues exposed 14 female mongrel dogs (retired breeders) less than 5 years of age and weighing 14 to 17 kg to CAPs. A permanent tracheostomy performed on each dog required minimal maintenance after healing. Dogs were exposed to CAPs or filtered air via a tube inserted at the tracheostomy site, a method which the investigators had previously shown to be the most efficient for exposing dogs to both gaseous and particulate pollutants. In addition, measurements of respiratory rate, air flow and BAL measurements were made through this site. Dogs were trained so that they would be comfortable and without anxiety in the chamber during the six-hour exposure. The investigators exposed the dogs to CAPs or filtered air for six hours a day on three consecutive days.

For the coronary occlusion studies, the investigators implanted a balloon around the dog's left anterior descending coronary artery. To induce occlusion, they inflated the balloon with saline and held it for 5 minutes

before releasing it. A second 5-minute occlusion conducted 20 minutes later was followed by a third 5-minute occlusion at the end of the 6-hour exposure to CAPs or air.

In the initial part of this study (using dogs 4 and 5), a coronary flow probe, an epicardial thermistor for measuring temperature, a pericardial catheter for pericardial fluid sampling and drug delivery, and bipolar electrodes for ECG recording over the myocardium supplied by the left anterior descending coronary artery were used to monitor effects of the occlusion. As these additional instruments tended to induce inflammation, the investigators implanted only the balloon occluder in studies of the four other dogs (1, 2, 8, and 9).

Physical and Chemical Characterization of Ambient Particle Exposures

The investigators characterized both ambient and concentrated particles using several methods employing Teflon, Nucleopore, and quartz filters. They determined ambient fine particle ($PM_{2.5}$) mass and ion concentrations from samples collected during each 6-hour exposure. Separate samples were collected to determine strong acidity (H^+). They collected size-fractionated samples of ambient $PM_{2.5}$ over the entire 18-hour (3 × 6 hours) exposure. They then determined the integrated particle mass for each sized fraction. The investigators collected separate samples during each exposure to characterize the concentrated particles for mass, ions, elemental and organic carbon, trace metals and endotoxins.

Data on the mass and sulfate concentrations of ambient and concentrated particles were used to determine the concentration efficiency of the concentrator. Data on sulfate, trace metal, and elemental and organic carbon composition of the CAPs were used for the component analysis; these components accounted for an average of 82% of the total particle mass. Data on metal content primarily were included in the statistical analysis of response parameters.

During all exposure periods, the investigators made continuous measurements (integrated over 5-minute intervals throughout the exposure period) of the levels of nonvolatile mass components and black carbon in the concentrated particles. Weather data, including wind speed and direction, barometric pressure, outside temperature and relative humidity from Boston's Logan International Airport were obtained from the National Weather Service. The continuous measurements of mass and black carbon were used in the statistical model.

PM composition varies with season, however, and it is possible that all dogs were not subject to the full heterogeneity of particles during the course of the study. For example, most of the crossover experiments were performed over a short period in winter (November to January), and it is known that sulfate levels are higher in summer than in fall or winter. This may also account for the high number of exposure days in the crossover experiments in which the trajectory of the particles was from the northwest. Future studies should include observations over a wider annual exposure range.

Endpoints

Cardiac Function ECGs were recorded using the Marquette 8500 Holter monitor, which allows capture of the ECG signal for almost every heartbeat throughout a six-hour exposure. ECG electrodes were placed subcutaneously in six locations.

The investigators measured LF and HF components of HRV as the primary measurement of cardiac autonomic activity and determined the LF/HF ratio. Complex demodulation of the T wave provided a quantitative measure of vulnerability to ventricular fibrillation, thereby quantifying cardiac vulnerability under diverse conditions. T wave alternans was assessed by demodulation of the ECG data. ECG morphology was also assessed using a three-dimensional display that provided a topographic image of the ECG across the entire duration of the experiment. These images were used as a pattern-recognition tool to detect subtle changes in ECG morphology.

For the coronary occlusion study, the investigators measured elevation of the ST segment, the cardinal ECG manifestation of ischemia resulting from abrupt, complete coronary artery occlusion (for example, blockage by a thrombus). In this event blood flow ceases and acute MI, commonly referred to as a *heart attack*, ensues. Elevation of the ST segment occurs early after MI and resolves after 1 to 2 days. The investigators also measured plasma levels of lactate dehydrogenase (LDH) and of creatinine phosphokinase (CPK), which has been used previously to assess ischemic damage. The investigators did not measure plasma levels of cardiac troponin I or T, more sensitive and specific markers of ischemic damage (Adams et al 1993; Bodor et al 1995; McLaurin et al 1997; Falahati et al 1999) because they reported that the standard radioimmunoassay for human troponin was unable to detect the canine protein.

Respiratory Monitoring and Analysis of Rate, Breathing Volumes, and Airflow Airflow was monitored through the tracheotomy tube allowing breath by breath analysis for the entire six-hour exposure. The breathing parameters measured were: time for inspiration, time for expiration, peak inspiratory and expiratory flows, tidal volume, relaxation

time, (time of volume decay of the expiration volume to 30%), minute ventilation, breathing frequency and inspiratory and expiratory pauses, pause and enhanced pause (Pau_{enh}).

Inflammatory Responses To determine the composition of cells in the lungs, the investigators performed a differential count on cells obtained from BAL via transbronchial biopsies on the third day of exposure. In pilot studies, the investigators also measured the cytokines IL-8, IL-1, and TNFα in cells obtained from the BAL and from lung tissue obtained by lung biopsy. They also measured plasma fibrinogen, a marker previously associated with coagulation events, and total and differential peripheral white blood cell (WBC) counts.

Statistical Analysis

The investigators began their statistical analyses of CAPs effects by comparing the mean response to CAPs against the mean response as a sham for each dog and for each cardiac and respiratory parameter, using a set of simple paired *t* tests. Results of this analysis for normal dogs are shown in Tables 10 through 12 of the Investigators' Report. These tables incorporate data from both crossover and double CAPs/double sham experiments. Tables 21 through 23 of the Investigators' Report show data from this analysis derived from dogs with a coronary occlusion.

During the study, the investigators determined that they needed to account for the variability they observed in CAPs concentrations and in the dogs' cardiac and respiratory parameters during the exposure period. To take this variability into account, the investigators performed a second analysis, fitting a semiparametric model to the cardiac and pulmonary responses of dogs involved only in the crossover experiments. The model included a term for exposure dose that was calculated from the $PM_{2.5}$ mass concentration in the air and the animal's minute volume. Because this model was based on controlling simultaneously for both the response of the CAPs dog and of the chambermate sham dog, only data from the crossover studies were used in this analysis. Results from double CAPs/double sham exposures could not be analyzed in the same way because they lacked this simultaneous control.

This analysis was performed independently for each cardiac and pulmonary parameter. The model fitting involved three major components. First, it removed so-called nuisance day effects, possibly related to chamber effects and environmental stimuli or feedback between chambermates. Next, it estimated for each day of the crossover (23 in the normal dogs, 18 in the coronary occlusion study) a separate linear regression coefficient that quantified the difference between responses to the CAPs and the sham exposures. Third, it produced a coefficient that measured the average difference between the CAPs response and the sham response. The analysis took into account correlations across time for a given subject by modeling the model errors as an autoregressive process (AR1 model). For each parameter, they calculated an estimated regression coefficient $\{\hat{\beta}_j^C\}$ to define the difference in response between a CAPs-exposed and a sham-exposed animal on each crossover exposure day.

This analysis provided a means for the investigators to illustrate that on some days the response of the CAPs-exposed animal was greater than that of the sham during the exposure period (positive $\{\hat{\beta}_j^C\}$) or lower than that of the sham (negative $\{\hat{\beta}_j^C\}$). Results of this analysis on individual days for normal dogs are shown in Tables 13 through 15, and for dogs with a coronary occlusion, in Tables 24 through 26. The investigators performed an additional analysis that combined data from every crossover exposure day for each parameter. Results of this analysis are shown in Tables 16 and 27 for normal and coronary occlusion dogs, respectively. These tables show the overall magnitude of CAPs effects on each cardiac and pulmonary parameter, a negative number indicating that the response to CAPs was lower than in the control.

Several concerns about the semiparametric analysis make interpretation of the results derived by this approach difficult, however; in particular, as discussed in the following paragraphs, the appropriateness and correctness of this model cannot be judged. As a result, the investigators' conclusions about changes in cardiopulmonary responses, and especially those involving changes in HRV, which are derived from this analysis, are somewhat weakened.

The investigators have not adequately validated the statistical model. They assume implicitly that the error terms in their semiparametric model are independent, normally distributed, and have mean zero and constant variance, after allowing for an autoregressive time dependence. The investigators appear to have examined residuals to assess whether the data were normally distributed, but the small number of dogs used in the analysis precludes the possibility of evaluating the validity of the model in a general population of dogs. In addition, the authors did not have much statistical power to look for problems. The use of AR1 seems to have substantially reduced serial correlations, but the authors did not tell us about the degree of independence of the residuals. Departures from these and other assumptions might have affected the results.

Another issue is that the investigators propose that the error terms of the variables in the model they use are independent after allowing for autoregressive processes. Dog-day

and dog-treatment interactions as well as temporal trends within week and across weeks may have occurred, suggesting that errors might not be independent. The investigators examined carryover effects, but their statistical power to detect such effects was likely to have been low. They acknowledge many of these problems but had no effective way to deal with them without more data.

Other concerns relate to the secondary analytic procedure employed. This procedure is valid only for large samples (that is, for a large number of dogs and a large number of observations per dog). In the crossover study, only a small number of dogs were analyzed (8 normal dogs and 6 coronary occlusion dogs), and the investigators did not examine the properties of the model for small samples by either theoretical or empirical approaches. Thus, the properties of the procedure for such a small number of dogs are not known, and future studies should include extensive simulations. Although the investigators analyzed 3,038 observations to obtain results in the normal dogs, these observations were obtained from only 8 dogs tested multiple times. Similarly, the small numbers of dogs inhibits analysis of dog-specific effects (including interactions with other variables) and thus the significance of the very small *p* values associated with CAPs effects in Tables 16 and 27 is difficult to evaluate. These *p* values result from multiple measurements on a small number of dogs rather than comparisons of a large number of dogs. With only a small number of dogs available, the contribution of each dog to the results is important because only a limited number of crossover experiments were performed; the normal dog study had only four different pairings (Dogs 1 and 2, 6 and 7, 8 and 9, and 12 and 14).

Although the coefficients given in Table 16 provide a means to summarize the magnitude and direction of CAPs effect on each cardiac or pulmonary parameter, these numbers tend to obscure the substantial variability of each parameter on different exposure days. For example, Table 16 indicates that combining the data for all crossover exposure days produces an overall significantly greater LF response in the CAPs dogs than in the sham animals. Table 13 shows 13 out of 23 exposure days on which the LF power of HRV was significantly greater in the CAPs-exposed dogs than in the sham animals, 4 days on which the sham dogs showed greater LF response than the CAPs dogs, and 6 days on which the CAPs and sham dogs showed no significant differences in LF.

In summary, interpretation of data derived by this approach may well be correct, but the evidence Godleski and colleagues present is not conclusive. Although their models and analyses are impressive, they have not resolved questions associated with the small number of dogs involved nor the unestablished sensitivity and validity of the methods. Firm resolution of the issues will require a study design and statistical methods that enhances the strength of conclusions, validates whatever model is selected, and provides substantially more data across dogs, days, and times.

RESULTS

Normal Animals

Cardiopulmonary response to CAPs was variable.

In their first analytic approach to determine the effects of CAPs in normal dogs, the investigators used a paired *t* test to compare the means of responses to CAPs and to sham exposure for every normal dog, including all crossover and double CAPs/double sham experiments. The results were not corrected for heart rate or respiratory rate, which can influence HRV. The investigators noted small CAPs-induced increases in both LF and HF components of HRV but no change in the LF/HF ratio (Table 10 of the Investigators' Report). A similar analysis of respiratory parameters showed small but not significant changes (Tables 11 and 12).

The investigators next analyzed day-to-day responses in the crossover experiments using a semiparametric model. Cardiac parameters (apart from heart rate average) were controlled for heart rate, respiratory frequency and minute volume. Three distinct patterns of response were found (Tables 13 through 15):

- No response. The investigators state that this was observed on about 25% of the exposure days in normal animals. As every dog showed some change in cardiac parameters at least once over the course of the study in response to CAPs exposure, the investigators concluded that no dog was a nonresponder to CAPs.
- An increased LF/HF ratio (resulting from increased LF and a smaller increase in HF) and a decreased heart rate on 10 of the 23 exposure days. These cardiac responses were associated with changes in pulmonary parameters: decreased respiratory frequency, tidal volume, minute volume, and peak flows with corresponding increases in respiratory cycle times and enhanced pause.
- A decreased LF/HF ratio (resulting predominantly from decreased LF) and decreased breathing cycle times accompanied by increased heart rate, respiratory flow, and volumes on five of the exposure days (ie, a pattern of response opposite to that described above).

Because CAPs either increased or decreased the LF/HF ratio on most exposure days, the investigators interpreted

these changes to indicate that exposure to CAPs affected both the sympathetic and parasympathetic arms of the autonomic nervous system. Because the increase in LF/HF ratio occurred on twice as many days as did a decrease, and because the magnitude of ratio increases was 2 to 5 times higher than the magnitude of ratio decreases, the investigators interpreted their findings as indicating a predominance of sympathetic nervous system effects. The investigators' interpretation that CAPs affected control of the autonomic nervous system may not be unreasonable. These changes might have been due to random fluctuations, however, resulting from repeated statistical testing of data from a small set of animals. Further, while responses may be qualitatively similar in humans, important quantitative differences may include a different balance between LF and HF or between sympathetic and parasympathetic effects.

To address the criticism that the HRV changes they reported were random, the investigators performed an additional semiparametric statistical analysis combining all exposure data from the crossover experiments with normal dogs to estimate an overall CAPs effect (Table 16 of the Investigators' Report). This analysis indicated that CAPs exposure resulted in a significant increase in LF and the LF/HF ratio but a decrease in heart rate; these effects appeared to depend on CAPs dose. As a consequence, the investigators argued that this further analysis bolstered their interpretation: exposure of normal dogs to CAPs results in changes in both sympathetic and parasympathetic arms of the autonomic nervous system. This analysis may be sound, but as described in the Statistical Analysis section, the correctness of the model and its applicability to a small sample size needs to be validated. In the absence of a validation study, the levels of significance of the data reported in Table 16 should be interpreted cautiously. Validation of the significance of these findings may be achieved in future studies with larger numbers of animals and with dogs of different ages, sexes and sizes.

Variability in day-to-day aerosol composition and exposure concentrations may account for the response variation. To explain the large variations in the responses they observed, the investigators attempted to characterize the components of the ambient or concentrated PM on different exposure days. They noted a large variation in ambient PM and CAPs composition on different exposure days (Tables 3 and 8). The investigators performed a principal components analysis to try to determine whether some component of PM, such as a metal, might be responsible for these cardiac effects. Using this approach, however, they did not identify a unique component of the particles which could be associated with cardiopulmonary response, such as particle mass concentration, although they did find a trend associating metal components of the PM and cardiopulmonary responses. As described in the preceding paragraph, when the investigators analyzed their combined data for all crossover exposure days with a semiparametric approach (Tables 16 and 27), they showed a relationship between CAPs dose and cardiac and respiratory responses, but the validity of this relationship needs further evaluation. More sophisticated analyses of the composition data may be helpful in trying to identify whether cardiopulmonary responses are associated with specific PM components.

Their analysis of weather conditions on different exposure days suggested that the direction from which the particles came affected the cardiopulmonary response of the exposed animals (Figure 21). This trajectory analysis indicated that PM which derived from exposure from the north or northwesterly directions was associated with a significant increase in HF and decrease in heart rate. Increase in LF was associated with all trajectories. Table 7 of the Report indicates, however, that the northwest trajectory prevailed on over half the exposure days (13/23), making comparisons difficult with other specific trajectories. Further work is needed to characterize and compare the components of PM from different directions.

CAPs exposure had little or no effect on markers of inflammation. The investigators found an increase in BAL neutrophils of CAPs-exposed animals compared to controls ($p = 0.05$), but no differences in any other BAL parameter measured (Figures 27 through 30). Pilot studies of bronchial biopsies showed minimal inflammation, no neutrophil infiltration, and no evidence of positive staining of IL-8, IL-1, or TNFα. Since the results from a small number of biopsies were essentially negative, the investigators did not fully assess all biopsy tissue. They also found no changes in peripheral total WBC count, differential count or fibrinogen level. These findings indicate that if changes in cardiopulmonary parameters occur in response to CAPs, they take place in the absence of an obvious inflammatory response.

CAPs decreased levels of T wave alternans. The investigators reported a 25% decrease in T wave alternans in the CAPs-exposed animals compared to sham in a simple *t* test, which was significant at the $p < 0.05$ level. This was based on the analysis of three 5-minute periods of 23 days of exposure which corresponded to peaks in the LF/HF ratio.

Bronchitis Model

Cardiopulmonary responses to CAPs were inconsistent in a limited number of animals. Two animals with chronic bronchitis were exposed to CAPs on six days and to sham on three days. CAPs exposure resulted in a statistically significant decrease in LF and LF/HF ratio, but no change in HF (Appendix A, Table A.2) or any respiratory parameter (Appendix A, Tables A.3 and A.4). As noted in Appendix A, CAPs exposure did not cause consistent changes in ECG morphology or cardiovascular parameters such as HF or median heart rate. The investigators also noted that when they compared responses in sham and CAPs-exposed animals, changes in cardiac or respiratory parameters were larger in the former than the latter. The investigators recognized that these results might be statistically or biologically relevant but did not pursue them and devoted their resources to the coronary occlusion model.

Coronary Occlusion Model

Exposure to CAPs resulted in significant cardiac effects. Possibly the most biologically significant finding of the study was that during coronary occlusion at the end of the 6-hour exposure, CAPs-exposed dogs showed a more rapid development of ST elevation, an indicator of cardiac ischemia, than sham-exposed dogs (exactly one half the time) (Figure 40). (The time to ST-segment elevation during preexposure occlusion was similar in CAPs-exposed and sham-exposed animals.) In addition, the magnitude of ST-segment elevation in CAPs exposed (but not sham-exposed) animals was larger during the occlusion after exposure compared to the occlusion before exposure. These results were based on results from the four animals in which a coronary occlusion was induced in the latter part of the study; response times were compared using a paired t test. The investigators reasonably concluded that PM lowered the ischemic threshold in animals predisposed to ishemia. They did not provide data on the CAPs-induced extent of elevation of the ST segment (over how much of the heart the ischemia was occurring), which would have strenghthened the conclusion that the effects of CAPs were associated with ischemic coronary disease.

The investigators did document increased plasma levels of the ischemia markers LDH and CPK in the CAPs-exposed animals after occlusion. Using all the data, the investigators noted an increase in CPK after occlusion, but this finding had a large standard deviation because only two dogs showed a change. Similarly, LDH increased when all data were used, but even the increased values were within the normal range.

The mean of paired *t* test data from all dogs with coronary occlusion indicated that CAPs exposure increased HF and decreased respiratory frequency (Tables 21 and 23), but these differences were driven by large changes predominantly in one dog for each parameter. CAPs exposure did not lead to statistically significant changes in the LF/HF ratio (Table 21) or to changes in other pulmonary parameters measured (Tables 22 and 23). When data were analyzed in the semiparametric model, CAPs-exposed dogs with coronary occlusion showed increases in HF on 7 of the 12 exposure days and changes in LF/HF ratio on 7 of the 12 exposure days (Table 24). Respiratory parameters showed decreases in frequency associated with decreased minute volume and peak flows along with increases in inspiration time, expiration time, and Pau_{enh} (Tables 25 and 26). Effects on T wave alternans were noted in a small number of animals (Figure 39), some showing visible fluctuation (Figure 38). Overall, however, changes in T wave alternans were not different between CAPs and sham animals. No ventricular arrhythmia was observed during coronary occlusions.

DISCUSSION

DOGS AS MODELS FOR HUMAN CARDIOVASCULAR RESPONSES

One of the strengths of Godleski and colleagues' study was its attempt to measure responses to PM in an animal which shares many features of the human cardiovascular system, particularly in the activation sequence of the heart, average heart rate, and heart size. Because of these similarities, dogs have been used routinely for testing agents which might be cardiotoxic or therapeutic in humans. For these reasons, results obtained with PM effects on measures such as ST segment elevation in the dog may be more effectively extrapolated to humans than results obtained in species such as rodents.

Human and dog cardiovascular systems differ, however, in important ways. First, although the average heart rate is similar in the two species, the heart rate of the healthy dog is highly variable—over a five- to sevenfold range from a sleeping to an excited state—and HRV in normal dogs varies over a much greater range than any other tested species. Thus, the biological or clinical significance of changes in normal canine HRV, and the potential impact of such changes on arrhythmia induction in normal animals is uncertain. One consequence of the normal dog's variable heart rate is that beat-to-beat variations referred to as *sinus arrhythmias* are extremely common but occur much less frequently in humans. Canine sinus arrhythmias are clinically

inconsequential and are related for the most part to the mechanics and to the nervous control of breathing. Thus, even if an agent were to induce this type of arrhythmia in a normal dog, it is unlikely that the arrhythmia would have clinical significance. Second, the nerve centers which control cardiac and ventilatory function are closer together in the dog than in other species examined. Because of this unique proximity, impulses to the ventilatory center spill over into the nerves controlling cardiac function. Thus, in the dog, an agent that affects breathing rate can change the heart rate by a mechanism that is unlikely to occur in humans. Thus, care must be taken in extrapolating changes in canine HRV to humans.

FINDINGS IN NORMAL DOGS

In this study, Godleski and colleagues generated a number of interesting findings in normal dogs. Their introduction of sophisticated measures of cardiac function such as HRV and T wave alternans into particle toxicology studies is an important contribution to the field, and these parameters may become useful features of future human and animal studies.

They highly variable cardiopulmonary responses to CAPs ranged from approximately 100 to 1,000 $\mu g/m^3$ (140 to 1,400 μg/animal) and were likely due to variability in particle composition. Although the study did not demonstrate convincingly a significant association between any PM component (including mass) and the resulting pattern of cardiopulmonary responses, their suggestion that metals may be associated with particle effects in the dogs merits further study. This conclusion is consistent with previous findings from these investigators and others who have shown that metals, and transition series metals in particular, are important components of particle exposure effects in rodents (Dreher et al 1997; Killingsworth et al 1997; Kodavanti et al 1997; Watkinson et al 1998). In addition, the investigators' suggestion that metals associated with particles arriving in Boston from a north or northwesterly direction may be associated with a particular pattern of cardiopulmonary responses (increases in HF and heart rate standard deviation) is intriguing. Further studies performed in different seasons when particle compositions are known to differ, and more information about how local and distant sources of particles influence the trajectory are needed to assess how these issues may affect health endpoints.

The investigators' analysis of all exposure experiments (Tables 10 through 12) suggests that PM exposure does not have large effects on cardiopulmonary parameters including HRV in the normal dog. The investigators' interpretation of day-to-day differences in crossover experiments (Tables 13 through 16) as suggesting that CAPs exposure resulted in changes in HRV, and in particular to increased LF components, is not unreasonable. Godleski and colleagues did not directly examine the mechanism of CAPs effects. Possibly the changes in neural control of cardiac response could have resulted from either direct impaction of particles on the airway epithelium and sensory nerve endings, or alternatively, by an indirect mechanism, possibly mediated by cytokines released by cells recruited to the epithelium.

At the same time, interpretation of the investigators' findings in normal dogs and of changes in HRV in particular is currently not clear. First, the results are based on multiple testing of a limited number of animals, and the investigators' statistical analysis of these results should be interpreted with caution because their model requires validation. Second, the clinical significance of changes in HRV in normal humans has not been established (aside from the fact that it is a measure of autonomic nervous system balance) and even less is known about the clinical significance of HRV changes in normal dogs. Similar considerations apply to the investigators' finding of decreased T wave alternans. In human clinical studies, decreased T wave alternans is an independent marker of *good* outcome in patients with heart disease. Thus, in the normal dog response to CAPs, the investigators report a finding that would be interpreted as proarrhythmic (increased LF) and one considered antiarrhythmic (decreased T wave alternans). The value of these parameters as predictive markers in normal populations of humans and other species still needs to be established.

Other recent toxicologic and epidemiology studies have examined the effects of PM on cardiac parameters. Gordon and coworkers tested CAPs in a range from 30 to 600 $\mu g/m^3$ (corresponding to approximately 2 to 40 μg/animal) derived from New York City air using a different concentrator air (Gordon et al 1998; Gordon et al 2000). They found that normal rats and rats with pulmonary hypertension induced by injecting monocrotaline showed small CAPs-induced increases in heart rate but no effect on ECG patterns or on the induction of arrhythmias. Normal hamsters and hamsters with cardiomyopathy showed no changes in response to CAPs. These investigators did not measure HRV in the CAPs-exposed rats or hamsters. Differences in results between Godleski and colleagues and those of Gordon may be due to a number of issues including differences in the sensitivity of the respective animal models to PM effects, the concentrations of critical PM components inhaled, or differences between components of the ambient PM mixtures in New York and Boston.

In contrast to the findings of Gordon and colleagues, Watkinson and coworkers (1998) showed that normal rats exposed to varying concentrations of the highly toxic combustion product residual oil fly ash particles exhibited multiple ECG changes characteristic of arrhythmia. Differences in the results may be the consequence of intratracheal administration of highly toxic residual oil fly ash in contrast to the aerosol of concentrated ambient air.

Godleski and colleagues' interpretation of their findings, namely, that the cardiopulmonary responses associated with CAPs exposure were the result of perturbations of the autonomic nervous system, is consistent with the results of recent epidemiology studies of particulate exposure and cardiovascular parameters (Liao et al 1999; Pope et al 2000). Liao and colleagues (1999) reported that in an elderly population exposure to elevated levels of ambient PM was associated with decreased HRV and that the association was strongest in individuals with cardiac disease. In a different elderly population, Pope found that increased levels of PM exposure were associated with increased HF and heart rate and decreased overall HRV (Pope et al 2000).

Godleski and colleagues' finding that CAPs exposure resulted in only modest changes in airway inflammation (an increase in neutrophil number in BAL) may be important because one theory of how particles or CAPs may act is via the induction of an inflammatory response (Seaton et al 1995). Studies of concentrated particle effects on inflammatory responses in normal rats have been conflicting: Li and coworkers (1997) reported increased levels of some mediators, while Gordon and associates (1998) did not. Differences in response in these studies might have been due to the levels, composition, or method of exposure to the pollutants, which were distinct in each study. Thus, the role of inflammatory response in the induction of cardiac events in both normal animals and those with cardiopulmonary disease remains unresolved.

CORONARY OCCLUSION MODEL

The study has produced intriguing data on the effects of CAPs in animals with a coronary occlusion. The investigators's finding that CAPs exposure in dogs with a coronary occlusion resulted in a more rapid development of ST segment elevation than in control animals may be highly important: ST segment elevation may indicate or even contribute to a greater susceptibility to ventricular fibrillation because it is associated with an increased heterogeneity in the time required for electrical inactivation of the ventricles. The decreased time to ST segment elevation suggests that exposure to CAPs rendered the myocardium of these dogs more susceptible to ischemia following cessation of blood flow during the coronary occlusion. Although this model does not mimic an MI, the findings are particularly relevant to humans who suffer an acute MI (heart attack); these individuals show elevation of the ST segment rapidly after MI onset and are at risk for malignant ventricular arrhythmias during the first 24 to 48 hours after the onset of coronary occlusion.

A number of mechanisms may explain CAPs effects on the ST segment elevation. Components of the CAPs may interact with a specific epicardial cell ion channel, I_{to}, which is believed to generate the ST segment elevation (Lukas and Antzelevitch 1993). Alternatively, CAPs components may affect the cardiac endothelium, alter cardiac metabolism, or cause damage as a consequence of free radical generation, and one of these events then leads to electrophysiologic changes.

Whatever the mechanism, the decrease in the time to ST elevation suggests that CAPs exposure may increase the prevalence of arrhythmias, which can be fatal among individuals who happen to suffer an ischemic event during an air pollution episode. This sequence of events would be consistent with the current information that individuals most at risk from sudden death are those with ischemia; the risk of someone without heart disease dying suddenly with or without heavy pollution is very small. Thus, the findings of Godleski and colleagues extrapolated to humans support an ischemic etiology for the increased mortality associated with increased air pollution. Godleski and colleagues did not study whether CAPs exposure raised the incidence of cardiac ischemia, but this would also be worth investigating in future studies.

Godleski and colleagues' findings that animals with an induced coronary occlusion may be susceptible to serious arrhythmias are consistent with results from another study of PM effects on animals with a compromised cardiopulmonary systems: namely, residual oil fly ash effects on monocrotaline-treated rats with pulmonary hypertension (Watkinson et al 1998). Similarly, the induction of arrhythmias may account for the previously described deaths of monocrotaline-treated rats following exposure to residual oil fly ash (Costa et al 1994; Killingsworth et al 1997). Godleski and colleagues' findings in the coronary occlusion model are also consistent with a recent epidemiology pilot study investigating the effects of particulate pollution in a susceptible human population, patients with a history of cardiovascular disease who have an implanted defibrillator (Peters et al 2000). The study indicated that during the first two days after elevated $PM_{2.5}$ exposure, the incidence of fibrillator discharge increased significantly to prevent the induction of arrhythmia. Expansion of this pilot study to include measurements of the cardiac parameters evaluated

by Godleski and colleagues, in particular HRV, ST segment analysis, and T wave alternans, would be important in attempting to further define how air pollution may induce arrhythmia.

The investigators' reported findings that CAPs exposure on a number of days increased HF components of HRV and decreased the LF/HF ratio in animals with coronary occlusion are intriguing. As discussed previously, the body of literature in humans with cardiac problems supports the notion that increases in HF are associated with good outcome (eg, Kleiger et al 1987; Odemuyiwa et al 1991; Bigger et al 1992); in addition, studies in dogs with coronary occlusion indicate that either decreasing sympathetic input or stimulating the parasympathetic arm of the autonomic nervous system is antifibrillatory (eg, Myers et al 1974; Janse et al 1985; Puddu et al 1988; Collins and Billman 1989; Vanoli et al 1991). Seen in this light, Godleski and colleagues's finding could be interpreted as *decreasing* rather than increasing the risk of arrhythmias and sudden death. Godleski and colleagues cite some situations in which increases in HF may be proarrhythmic: a study of six patients with a rare condition known as *idiopathic ventricular fibrillation* (Kasanuki et al 1997) and other conditions in which parasympathetic stimulation may result in fatal bradyarrhythmias (Blattberg and Levy 1969; Deutschmann et al 1994; Viskin et al 1996). Although in these cases the increased HF and stimulation of the parasympathetic arm of the autonomic nervous system may be proarrhythmic, the majority of the literature suggests the opposite conclusion. Thus, it is difficult to conclude that the observed HRV changes in CAPs-exposed animals during coronary occlusion are cardiotoxic. It is also worth noting that Godleski and colleagues reported HRV changes only during exposure, rather than after the occlusion. This may make comparison of the results with published information on post MI follow-up studies more difficult.

In the current study no significant differences in T wave alternans were observed between the CAPs-exposed dogs and controls during coronary occlusion. One possible interpretation of these data is that the dogs with occlusion were simply not vulnerable to ischemia-induced ventricular fibrillation. Alternatively, CAPs exposure may have had some influence on T wave alternans but was not detectable in these experiments. This is possible as elevation of the heart rate using either exercise (Estes et al 1997) or invasive atrial pacing (Rosenbaum et al 1994) is generally required for optimal ECG differentiation of normal individuals from patients who are at risk for ventricular arrhythmias, and the investigators did not attempt to artificially manipulate the heart rate in these experiments. The finding that some CAPs-exposed dogs with coronary occlusion showed visible fluctuations in beat-to-beat T wave morphology, even in the absence of overall changes in T wave alternans, may have electrophysiologic significance and merits further study.

It is important to note, however, that although the dog has been the model of choice for many studies of cardiac ischemia, the canine occlusion model used by Godleski and colleagues is not entirely analogous to the most common form of human cardiovascular disease. In the current study, the acute ischemic event was superimposed on a normal dog's cardiovascular system. In contrast, the acute event in humans generally takes place in the context of a long-term atherosclerotic cardiovascular condition which develops over many years. As dogs do not suffer from atherosclerosis, the effects of PM in dogs cannot be tested in a model similar to the human disease. Another canine model which may be applied in future studies of CAPs effects would involve investigating the effects of CAPs in animals which have recovered from an MI. Evaluating the effects of CAPs exposure on cardiac electrophysiologic parameters such as HRV and T wave alternans in this model would also be valuable. Future studies of the effects of CAPs on cardiac responses in models of chronic pulmonary infection, asthma or chronic obstructive pulmonary disease would also help to define the link between air pollutants and subpopulations who are considered at highest risk from their effects.

SUMMARY AND CONCLUSIONS

This was a complex, technically challenging study with potentially important implications for future research on particulate pollution. In this innovative exploratory study, Godleski and colleagues addressed new and plausible hypotheses for linking the findings from epidemiology studies with potential mechanisms of particle effects. They used the HAPC to expose dogs to a real-life pollutant, ambient particles in a concentrated form. This methodology is now in use in several laboratories and is likely to yield important information about the effects of concentrated particles in humans and other species. Further studies will need to address issues such as ensuring reproducible delivery of concentrated particles on a daily basis, and assessing the impact of pollutant gases on CAPs exposure effects.

The investigators' most important finding with potential significance for humans was that during the coronary occlusion dogs exposed to CAPs showed more rapid elevation of the ST segment of their ECG traces than controls. The result suggests that in susceptible individuals (that is,

those with ischemic heart disease) particle pollution may lead to increased prevalence of arrhythmias which are fatal. This is a plausible and important mechanism to explain the association of increased cardiopulmonary mortality and exposure to particle pollution. This finding was derived, however, from observations made in only four animals—a total of eight measurements in the crossover study—and the investigators did not provide information about the CAPs-induced extent of ST elevation. If the finding can be confirmed in additional animals and consolidated by further information, the conclusion that CAPs have a major effect on electrophysiologic responses associated with ischemic events would be strengthened.

In normal dogs, the investigators reported CAPs-induced changes in cardiac parameters and HRV in particular, as well as changes in respiratory performance parameters, and interpreted these findings to indicate that CAPs affected the balance between the sympathetic and parasympathetic arms of the autonomic (involuntary) nervous system. The investigators attributed the observed changes to a component or components of PM, which varied with the direction that PM was transported to Boston. They did not find an obvious link to the concentration of any measured PM component. Definitive interpretation of the investigators' findings in normal dogs, however, is not possible. The changes in respiratory and cardiac parameters such as HRV were highly variable, and further studies are needed to determine the source of this variability. It is important to note that the clinical significance of changes in HRV and in T wave alternans in normal dogs, and in humans who do not have preexisting heart disease, is currently undetermined. More extensive research is needed to determine the biological and clinical significance of such changes and establish that they are plausibly linked to an increased risk of fatal arrhythmia in humans. In the absence of such information, it is difficult to conclude that changes in control of the autonomic nervous system are the underlying cause for the increased mortality associated with exposure to particulate pollution.

Finally, it is worth noting that the major part of the study rests on the observation of 12 dogs, of which 6 were used in the coronary occlusion part of the study. Most of the analysis of normal dog responses is based on four pairs of animals exposed on 23 days of the crossover experiments, during which the composition of the CAPs and the cardiopulmonary responses were highly variable. The interesting results generated in this study need to be expanded in further studies involving more exposure days and more animals and at different PM exposure sites.

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