RESEARCH REPORT

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Effect of Concentrated Ambient Particulate Matter on Blood Coagulation Parameters in Rats

Christine Nadziejko, Kaijie Fang, Lung Chi Chen, Beverly Cohen, Margaret Karpatkin, and Arthur Nadas

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The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI 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 130 research reports.

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HEI STATEMENT
Synopsis of Research Report III

Concentrated Ambient Particles and Blood Coagulation in Rats

INTRODUCTION

Several epidemiologic studies have shown an association between short-term increases in ambient particulate matter and short-term increases in morbidity and mortality, especially in people with cardiovascular disease. The biological mechanisms by which particulate matter may affect cardiovascular events are not well understood, however. Dr Christine Nadziejko of the New York University School of Medicine hypothesized that exposure of healthy rats to ambient particles may cause changes in blood coagulation parameters. The hypothesis that particulate matter may exert its adverse health effects by altering blood coagulation parameters is biologically relevant because clots may precipitate cardiac arrest, especially in people with cardiovascular disease. The Health Effects Institute funded the animal study described in this report to address this hypothesis.

APPROACH

The investigators measured platelet number, blood cells counts, and levels of fibrinogen, thrombin-antithrombin complex, tissue plasminogen activator, plasminogen activator inhibitor, and factor VII. Rats were exposed to concentrated New York City particles or filtered air for 6 hours; blood samples were obtained via an indwelling catheter before and after exposure. A particle concentrator was used to deliver concentrated particulate matter to the animals at a target concentration of 300 $\mu g/m^3$, which was reached on 2 of 5 experimental days. The investigators used concentrated particulate matter to increase the likelihood of finding an effect.

RESULTS AND INTERPRETATION

The investigators found no consistent differences in coagulation end points in animals exposed to concentrated particulate matter compared with animals exposed to filtered air. Due to the limited scope of the study, however, the lack of a consistent effect is difficult to interpret. Factors that may have contributed to the lack of effect in this study include the use of healthy animals, the time points evaluated, the lower than expected concentration of ambient particulate matter on exposure days, and the lack of concentration of smaller particles, where much of the particle mass from mobile sources (a major source of particulate matter) is found. In addition, animal studies generally use small numbers of individuals in comparison with epidemiology studies that investigate large populations, reducing the power to detect small changes.

The blood coagulation system is complex with many positive and negative feedback loops to maintain homeostasis. Thus the particulate matter exposure may have caused changes in parts of the coagulation system that were not investigated in the current study but might be detected with other types of measurements. For example, one could perform functional assays (such as platelet aggregation or clotting time) or investigate endothelial aspects of clot formation. The lack of a consistent effect in the current study as well as other studies does not preclude that particulate matter exposure may affect coagulation under particular circumstances (for example, in individuals with preexisting disease). Animal models that exhibit increased clot formation or vascular plaques may be more likely to show changes in blood coagulation after being challenged with exposure to particulate matter.

Some human controlled exposure and epidemiologic studies have reported changes in blood cell counts, platelet counts, fibrinogen, and factor VII in relation to air pollution, but other human studies and most animal studies have failed to find such changes. No consistent pattern has emerged so far. To complement the current study, it might be worthwhile to perform similar studies with higher concentrations of

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Dr Christine Nadziejko of New York University School of Medicine, Tuxedo, New York. The following Research Report contains the detailed Investigators' Report and a Critique on the study prepared by the Institute's Health Review Committee.

fine and ultrafine particles, particles from specific mobile and stationary sources, or other well-defined particles of known toxicity while using compromised rather than healthy animals.

In summary, this was a well-designed pilot study that will contribute to designing future experiments to address the important topic of particulate matter effects on coagulation. It found no consistent effect of moderate levels of concentrated New York City particulate matter on six blood coagulation parameters in rats. This does not preclude involvement of the coagulation pathway in adverse health effects of particulate matter on the cardiovascular system, however. In future studies, changes in coagulation may be found in compromised animals with exposure to higher levels of particulate matter or with exposure to particles of different size or composition.

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HEALTH EFFECTS INSTITUTE

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Research Report 111

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HEI STATEMENT

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

PREFACE

The Preface describes the general regulatory and scientific background for the HEI Research Program that produced this and other reports on related topics.

INVESTIGATORS' REPORT

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

CRITIQUE Health Review Committee

The Critique 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

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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 epidemiologic 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 funded research projects in 1994, 1996, and 1998. In 1994, the Particle Epidemiology Evaluation Project (Samet et al 1995, 1997) evaluated six of the time-series epidemiologic studies that had reported effects of PM on mortality; under RFA 94-2, "Particulate Air Pollution and Daily Mortality: Identification of Populations at Risk and Underlying Mechanisms," epidemiologic and toxicologic studies were funded that aimed at understanding how PM might cause toxicity and what factors might affect susceptibility. In 1996, HEI issued RFA 96-1, "Mechanisms of Particle Toxicity: Fate and Bioreactivity of Particle-Associated Compounds," which sought studies that would improve our understanding of toxicologically relevant characteristics of ambient particles. In 1998, HEI issued RFA 98-1, "Characterization of Exposure to and Health Effects of Particulate Matter," which targeted a broad and ambitious set of research goals relating to both exposure assessment and health effects. In all, HEI has issued five requests for research on PM and funded 34 studies or reanalyses over the last 5 years.

This Preface provides general regulatory and scientific background information relevant to studies funded from RFA 98-1, including the study by Dr Christine Nadziejko that is described in the accompanying Report and Critique. This is one of nine studies from RFA 98-1 that have been completed; five additional studies are in progress. The "HEI Program Summary: Research on Particulate Matter" (Health Effects Institute 1999) provides information on PM studies funded since 1996.

BACKGROUND

Particulate matter is the term used to define a complex mixture of anthropogenic and naturally occurring airborne particles. In urban environments, these particles derive mainly from combustion, including mobile sources such as motor vehicles and stationary sources such as power plants. The size, chemical composition, and other physical and biologic properties of PM depend on the sources of the particles and the changes the particles undergo in the atmosphere. The most commonly used descriptor of particles is size measured as *aerodynamic diameter*. On the basis of 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 found primarily in emissions from combustion processes, whereas 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 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 an increased number of deaths) particularly in children, the elderly, and those with cardiopulmonary conditions (Firket 1931; Logan 1953; Ciocco and Thompson 1961; 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 people thought to be sensitive to the effects of pollution.

The first NAAQSs for PM were based on controlling total suspended PM or particles up to 40 μm in diameter. In 1978, the standards were revised to regulate inhalable particles, or particles that can deposit in the respiratory tract and therefore have greater potential for causing adverse health effects. These particles measure 10 μm or smaller in aerodynamic diameter (PM_{10}). More recent epidemiologic studies, published in the early 1990s, indicated a relatively consistent association between short-term small increases in PM levels and increases in both morbidity and mortality from respiratory and cardiovascular diseases (reviewed by the

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

Preface Table 1. Current National Ambient Air Quality Standards for Particulate Matter (Set in 1997)

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

Committee of the Environmental and Occupational Health Assembly, American Thoracic Society [Bascom et al 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 also pointed to a possible role of fine particles (less than 2.5 μm in aerodynamic diameter [$PM_{2.5}$]). In 1997, the EPA decided that the evidence for the effects of fine particles was sufficient to promulgate a $PM_{2.5}$ standard while retaining the PM_{10} standard (EPA 1997) (see Preface Table 1). The next review of the PM NAAQSs is scheduled to be completed in 2003.

HEI's PARTICULATE MATTER RESEARCH PROGRAM

The wealth of epidemiologic data published in the early 1990s suggested an association between PM and health effects, but aspects of these findings 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 mixtures and levels of pollutants, they were not well supported by either human exposure chamber studies or animal inhalation studies aimed at delineating the 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 from RFA-94 was aimed at addressing these research needs. In 1994, HEI also initiated the Particle Epidemiology Evaluation Project to address the validity and replicability of key epidemiologic studies (Samet et al 1995, 1997). Out of that project evolved the National Morbidity, Mortality, and Air Pollution Study to continue the epidemiologic evaluation in a large number of cities across the US with varying levels of PM and other air pollutants (Samet et al 2000a,b). Subsequently, HEI funded studies under RFA 96-1 that would use fine and ultrafine particles to test specific hypotheses related to the role of particle constituents in PM toxicity.

With increased financial support from the EPA and industry, in January 1998 HEI requested applications targeting both exposure assessments and health effects. HEI held a workshop at the Offices of the National Research Council that brought together scientists and representatives of the EPA and the motor vehicle and oil industries to discuss research needs. Out of this discussion, RFA 98-1, "Characterization of Exposure to and Health Effects of Particulate Matter," was developed and issued. The exposure objectives included (1) characterizing personal exposure to particles in different indoor and outdoor microenvironments and in geographic locations that differ in the types and sources of particles, topography, and climate; and (2) improving particle characterization to increase the accuracy of exposure estimates in epidemiologic studies. The health effects objectives included (1) characterizing potential pathophysiologic effects caused by PM in sensitive subjects; (2) defining the relation between particle characteristics and dose, distribution, and persistence of particles in the respiratory tract; (3) identifying the kinds of particles or particle attributes that may cause toxicity; (4) investigating the diseases or conditions that affect sensitivity; and (5) delineating how copollutants affect or contribute to the physiologic response to particles. From this RFA, HEI funded a comprehensive set of exposure assessment and health effects studies.

Three exposure assessment studies are investigating personal exposure to PM in potentially sensitive population subgroups in several US and European cities with diverse climatic and geographic features. These studies are (1) characterizing indoor concentrations of and personal exposure to $PM_{2.5}$ for subjects in two European cities (Bert Brunekreef of Wageningen/Utrecht University); (2) characterizing exposure to $PM_{2.5}$, ozone, nitrogen dioxide, carbon monoxide, and sulfur dioxide in children, healthy seniors, and subjects with chronic obstructive pulmonary disease (Petros Koutrakis of Harvard School of Public Health); and (3) assessing personal exposure to $PM_{2.5}$ and

characterizing PM in terms of mass, functional groups, trace metals, polynuclear aromatic hydrocarbons, and elemental and organic carbon (Barbara Turpin of Environmental and Occupational Health Sciences Institute). A fourth study validated a newly developed method for measuring the acidic component of ultrafine particles or $PM_{0.1}$ (Beverly Cohen of New York University Medical Center).

Four human experimental and epidemiologic studies are investigating several potentially important endpoints that may help elucidate the mechanisms of particle toxicity. The epidemiologic studies are investigating the relation between PM levels and specific cardiac events: arrythmias (Douglas Dockery of Harvard School of Public Health) and nonfatal myocardial infarctions (Annette Peters of GSF-Forschungszentrum für Umwelt und Gesundheit). The experimental studies are investigating cardiovascular and pulmonary effects in healthy and asthmatic subjects exposed to ultrafine carbon particles (Mark Frampton of University of Rochester) and concentrated ambient particles (CAPs) from Los Angeles air (Henry Gong of Los Amigos Research and Education Institute).

Six animal studies are addressing a number of hypotheses about susceptibility to and toxicity of particulate matter components using different health endpoints, animal models, and types of particles. These studies were designed to investigate (1) whether coexposure to CAPs from Boston air and ozone causes a synergistic amplification of asthmatic airway inflammation and hyperresponsiveness in juvenile mice with hypersensitive airways (Lester Kobzik of Harvard School of Public Health); (2) the genetic determinants of susceptibility to morbidity and mortality from nickel particles in inbred mouse strains (George Leikauf of University of Cincinnati); (3) whether exposing healthy rats to CAPs from New York City air causes changes in blood coagulation parameters that may be involved in thrombotic effects (the study reported here; Christine Nadziejko of New York University Medical Center); (4) whether exposure to resuspended particles from Ottawa air samples causes changes in heart function and vascular parameters in adult rats (Renaud Vincent of Health Canada); (5) the effects of particle size and composition on the lung inflammatory and histopathologic responses in old rats and rats with preexisting inflammation (Fletcher Hahn of Lovelace Respiratory Research Institute); and (6) the effects of CAPs from Detroit air on the airway epithelium in rats with preexisting hypersecretory airway disease (Jack Harkema of Michigan State University).

CONTINUING RESEARCH

Many of the key questions identified in the early 1990s are still relevant and many research projects continue to address them. The research strategies have evolved, however, as results from completed 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 2000, HEI has published ten reports from its PM research program (Checkoway et al 2000; Gerde et al 2001; Godleski et al 2000; Goldberg et al 2000; Gordon et al 2000; Kobzik et al 2001; Krewski et al 2000a,b; Leikauf et al 2001; Lippmann et al 2000; Oberdörster et al 2000; Samet et al 2000a,b; Vincent et al 2001; Wichmann et al 2000). Additional PM studies that are currently under review will be published in 2002 and 2003.

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INVESTIGATORS' REPORT

Effect of Concentrated Ambient Particulate Matter on Blood Coagulation Parameters in Rats

Christine Nadziejko, Kaijie Fang, Lung Chi Chen, Beverly Cohen, Margaret Karpatkin, and Arthur Nadas

ABSTRACT

Epidemiologic studies have shown that exposure to particulate air pollution is associated with short-term increases in cardiovascular morbidity and mortality. These adverse effects of inhaled particulate matter (PM*) may be the indirect result of a PM-induced increase in blood coagulability. This explanation is biologically plausible because prospective studies have shown that increases in blood coagulation parameters are significantly associated with risk of adverse cardiovascular events.

We examined the hypothesis that acute exposure to elevated levels of PM causes prothrombotic changes in blood coagulation parameters. Rats with indwelling jugular vein catheters were exposed for 6 hours to filtered air or concentrated ambient PM in New York City air (n = 9 per group per experiment). PM less than 2.5 µm in mass median aerodynamic diameter ($PM_{2.5}$) was concentrated for animal exposures using a centrifugal concentrator. Blood samples were taken at four time points: before and immediately after exposure and at 12 and 24 hours after the start of exposure. At each time point, six coagulation parameters (platelet count, fibrinogen level, factor VII activity, thrombin-antithrombin complex [TAT] level, tissue plasminogen activator [tPA] activity, and plasminogen activator inhibitor [PAI] activity) were measured as well as all standard blood count parameters. Five concentrated-PM exposure experiments were performed over a period of 8 weeks in the summer of 1999. PM exposure concentrations ranged from 95 to 341 µg/m^3. Statistical significance was determined by two-way analysis of variance (ANOVA) on the postexposure data with time and exposure status as main effects.

There were no consistent exposure-related effects on any of the end points across the five experiments and no indication of any dose-dependent effects. Most of the statistically significant differences that were observed do not represent adverse effects. Therefore, the results of this study do not indicate that exposure to concentrated ambient PM causes adverse effects on blood coagulation in healthy rats.

INTRODUCTION

PARTICULATE AIR POLLUTION AND CARDIOVASCULAR MORBIDITY AND MORTALITY

An increasing amount of evidence suggests that the effect of PM on the cardiovascular system is a significant public health issue. Studies from recent decades show that the number of PM-associated deaths from cardiovascular disease equals or exceeds the number of such deaths from respiratory causes (Pope et al 1992; Schwartz 1994). Mortality data from Utah Valley indicate that a 100 µg/m^3 increase in PM less than 10 µm in mass median aerodynamic diameter (PM_{10}) would cause more than twice as many excess cardiovascular deaths as respiratory deaths (Pope et al 1992). Particulate air pollution has also been associated with increased cardiovascular morbidity (Poloniecki et al 1997). During an air pollution episode in Germany in 1985, hospital admissions for cardiovascular causes increased by 19% compared with a 7% increase in admissions related to respiratory disease (Peters et al 1997).

BLOOD COAGULATION AND CARDIOVASCULAR DISEASE

Some researchers have suggested that the adverse cardiovascular effects of PM may be the result of PM-induced changes in blood coagulability (Seaton et al 1995). This

* 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 111, which also includes a Critique by the Health Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr Christine Nadziejko, Department of Environmental Medicine, NYU School of Medicine, 57 Old Forge Road, Tuxedo NY 10987.

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hypothesis is based in part on the well-established finding that small prothombotic changes in coagulation parameters in a large population can have a substantial effect on the incidence and prevalence of cardiovascular disease (Di Minno and Mancini 1990; Braunwald 1997; Lowe et al 1997). Changes in coagulation play an important role in heart attack risk because heart attacks occur when a thrombus (clot) forms on an atherosclerotic plaque in a coronary artery and cuts off the blood supply to the myocardium (Braunwald 1997).

Some evidence suggests that formation of small thrombi is common in individuals with atherosclerosis (Meade et al 1993). Whether these individuals go on to have a heart attack depends in part on the balance between the thrombogenic factors involved in blood clot formation and the fibrinolytic factors that dissolve blood clots. Ischemic strokes have a similar pathogenesis except that the thrombi usually form in the carotid arteries and embolize to the cerebral arteries.

The effect of small acute changes in coagulation on heart attack risk is apparent in the association between time of day and incidence of heart attack. The risk of sudden cardiac death between 6 AM and 9 AM is 70% greater than the average risk for the rest of the day (Willich et al 1987). Evidence suggests that this association is due in part to the circadian rhythm of fibrinolytic factors, which are at their lowest levels in the early morning (Andrews et al 1996). Also, sympathetic nervous system activity is increased by standing up after lying prone (Tofler et al 1987; Andrews et al 1996). This increased sympathetic activity causes prothrombotic changes in blood coagulation parameters. These small, homeostatic effects on coagulation, which are within the normal range, translate into a significant increase in the incidence of heart attack because most of the population is exposed to the risk of getting out of bed in the morning. If inhalation of PM caused small prothrombotic changes in coagulation, increased cardiovascular morbidity and mortality would be observed due to the large number of people exposed during an air pollution episode.

EVIDENCE THAT PARTICULATE AIR POLLUTION AFFECTS BLOOD COAGULATION

The blood coagulation system is complex, consisting of cellular elements and a large number of soluble factors that are subject to extensive homeostatic regulation. The complexity implies that the coagulation system includes numerous targets for adverse effects of inhaled PM. For example, inhalation of PM could increase the risk of thrombosis by increasing platelet count, by decreasing activity of the fibrinolytic system, or by increasing the level of fibrinogen. In addition, multiple physiologic mechanisms—such as activation of the sympathoadrenal system, release of cytokines, and production of prostaglandins—can result in increased blood coagulability (Malyszko et al 1994; Kjeldsen et al 1995; Vasse et al 1996).

It is conceivable that PM and other inhaled pollutants may affect blood coagulation because almost all of the cardiac output travels through the gas exchange area of the lung, a region in which blood is separated from inhaled gas by only a few micrometers of tissue. Some researchers have suggested that inhaled PM may increase blood coagulability by causing inflammation deep in the lungs, which in turn may upregulate acute phase reactants such as fibrinogen (Seaton et al 1995). This idea gained support when Peters and colleagues (1997) reported that increased plasma viscosity was associated with an air pollution episode. Increased plasma viscosity is an indirect measure of blood coagulability because fibrinogen, which is both a blood-clotting factor and an acute phase reactant, is a major determinant of plasma viscosity.

BIOLOGICAL SIGNIFICANCE OF COAGULATION PARAMETERS

We examined whether inhalation of concentrated ambient PM has effects on coagulation parameters in healthy rats. Rats were used because this species is considered a good animal model for studying normal and pathophysiologic regulation of coagulation for extrapolation to humans (MacGregor et al 1995).

Six coagulation parameters (platelet count, fibrinogen and TAT levels, and activity of factor VII, tPA, and PAI) were selected for this study based on three criteria: relevance to cardiovascular disease, suitability for assays on rat blood, and potential applicability to human epidemiologic studies. A simplified overview of the role of the selected parameters in the coagulation system is shown in Figure 1.

Three parameters (platelet count, plasma fibrinogen level, and factor VII activity) are involved in thrombus formation (Nossel 1977). The initial step in arterial thrombosis is platelet activation. Activated platelets bind fibrinogen to form cross-linked aggregates. Activated factor VII and tissue factor initiate the blood coagulation cascade, which results in conversion of prothrombin to thrombin. Fibrin strands resulting from cleavage of fibrinogen by thrombin act to stabilize the growing platelet plug. Extensive evidence links an increase in fibrinogen content with risk for heart disease and stroke (Di Minno and Mancini 1990), and elevated activity of factor VII is strongly related to risk of death from ischemic heart disease (reviewed by Meade 1983).

Two parameters, tPA and PAI, are involved in regulating clot lysis. Since the amount of thrombin in 1 mL of blood

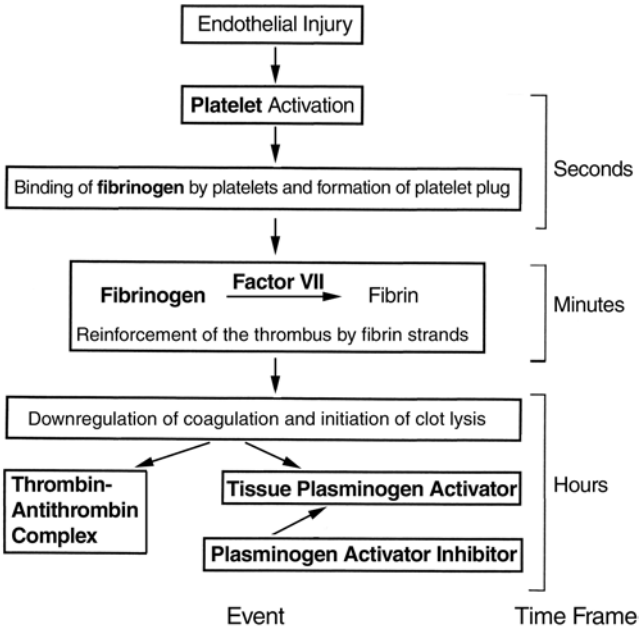

Figure 1. Simplified overview of blood coagulation system. The coagulation parameters measured in this study are indicated by bold type. The relations of these selected parameters with the rest of the coagulation system are outlined.

has the potential to clot 3 liters, inhibition of coagulation and activation of fibrinolytic factors are rapidly initiated in response to thrombosis (Nossel 1977). tPA is used clinically to treat acute myocardial infarction because it binds to thrombi and lyses them. The balance between tPA and its inhibitor, PAI, determines the rate at which thrombi are lysed. Increased levels of PAI have been linked to heart attack risk (Juhan-Vague et al 1996). Both factors are under homeostatic regulation and are relatively sensitive to outside stimuli, including emotional stress (Urano et al 1990).

We did not expect inhalation of concentrated PM to trigger thrombosis directly in exposed animals. We hypothesized that concentrated-PM exposure causes coagulation factors to change in a manner that makes thrombosis more likely (that is, PM causes prothrombotic changes). Table 1 lists the changes in coagulation parameters measured in this study that would be considered prothrombotic.

We also included in our study a sensitive in vivo indicator for activation of coagulation, TAT level (Gurfinkel et al 1994), even though activation of coagulation by inhaled PM was not expected. An indicator of in vivo intravascular coagulation was needed to interpret the biological significance of changes in the other coagulation parameters properly (see Table 1). For example, if inhalation of PM caused a decrease in plasma fibrinogen content, this finding by itself would not be an adverse effect. If fibrinogen level decreased due to intravascular clotting, however, this would suggest that PM had caused prothrombotic changes sufficient to trigger clotting—clearly an adverse effect.

SPECIFIC AIMS

Epidemiologic studies have shown that exposure to PM is associated with short-term increases in cardiovascular morbidity and mortality. The adverse cardiovascular effects of inhaled PM may be the indirect result of a PM-induced increase in blood coagulability (Seaton et al 1995; Peters et al 1997). This concept is biologically plausible because most heart attacks and strokes result from small thrombi that form on atherosclerotic plaques. Prospective studies have shown that changes in blood coagulation parameters related to thrombogenesis are significantly associated with risk of adverse cardiovascular events (Lowe et al 1997).

There is also epidemiologic evidence indicating that exposure to elevated levels of PM is associated with increases in plasma viscosity, which depends largely on fibrinogen, an important coagulation factor (Peters et al 1997). Although the study by Peters et al provides some evidence that exposure to PM is associated with increased blood coagulability, these preliminary findings need to be confirmed and expanded in controlled animal exposures.

We examined the hypothesis that acute exposure to elevated levels of PM causes prothrombotic changes in blood coagulation parameters. To test this hypothesis, rats were exposed for 6 hours to filtered air or concentrated PM. Coagulation parameters that can be related in humans to risk of heart attack or stroke were measured before exposure and at 6, 12, and 24 hours after the start of exposure.

Table 1. Biological Significance of Changes in Blood Coagulation Parameters

Parameter	Indication of Increased Risk of Thrombosis	Indication of Intravascular Coagulation
RBCs	Increased	
Platelets	Increased	Decreased
Fibrinogen	Increased	Decreased
Factor VII	Increased	Decreased
tPA	Decreased	Increased
PAI	Increased	
TAT	Not applicable	Increased

METHODS AND STUDY DESIGN

EXPERIMENTAL DESIGN

Rats (9 per group per experiment) were exposed to filtered air or concentrated PM for 6 hours by nose-only inhalation. Blood samples were drawn before exposure and at 6, 12, and 24 hours after the start of exposure. A total of 5 exposures to concentrated PM or filtered air were completed over a period of 8 weeks. Exposure days were selected based on pollution and weather forecasts from the previous day. Exposures lasted from noon to 6 PM, and both groups in each experiment were exposed at the same time in the same room.

For two control experiments, we placed a high-efficiency particulate air (HEPA) filter on the air intake of the concentrator to produce a sham exposure. In one control experiment, sham-exposed animals were compared to animals kept in their cages to identify any changes in blood coagulation parameters that might be attributable to the stress of exposure procedures. For the second control experiment, sham-exposed animals were compared to animals exposed nose only to filtered air that did not go through the concentrator. This experiment was done to determine whether there were any significant differences between the sham-exposed group and the filtered-air group.

Two pilot studies were performed to determine (1) the effect of an indwelling venous catheter on blood counts and coagulation parameters in healthy rats and (2) the effect of repeated blood sampling on peripheral blood end points.

ANIMALS AND BLOOD SAMPLING PROCEDURES

Animals

Male F344 specific pathogen–free, viral antigen–free rats (250–275 g) were purchased from the Raleigh facility of Charles River Laboratories. Rats were housed in polycarbonate cages with corncob bit bedding and were provided food and water ad libitum. Rats were maintained in rooms with controlled temperature and humidity on a 12-hour, day/night cycle.

Catheters were implanted in the jugular vein by the surgical staff at Charles River Laboratories (Raleigh NC). The catheters were made of polyurethane tubing, which is less thrombogenic than either polyethylene or silastic. The vendor filled the catheter with heparinized glycerol (40 µL of 500 IU heparin/mL final solution in glycerol) to keep the catheter patent until use. Rats were shipped to our laboratory 2 days after surgery and allowed to recover for at least 7 days. For weekly catheter maintenance, the fill solution was withdrawn, and the catheter was flushed with sterile saline and refilled with heparinized glycerol. Catheters failed in 1 of 18 rats on average.

Blood Sampling

To obtain blood samples, the rats were restrained in a thin plastic cone (DecapiCone, Braintree Scientific, Braintree MA) and a hole was cut to provide access to the catheter. Blood was sampled by first slowly withdrawing a small volume of blood (approximately 0.05 mL) to clear the catheter. A blood sample (0.8 mL) was then drawn into a syringe containing sodium citrate as an anticoagulant (1:9 vol/vol, 3.8% sodium citrate in blood). The catheter was flushed with 0.3 mL pyrogen-free sterile saline and plugged.

During preliminary studies, we noted that the first sample was often hemolyzed, but subsequent samples drawn within 24 hours were not. To minimize hemolysis, the procedure used to obtain the first sample was modified: A small volume was withdrawn to clear the catheter, the catheter was flushed with 0.5 mL saline, and a second small discard sample was removed prior to sampling.

Immediately after blood sampling, 2 aliquots were obtained from each 0.8-mL sample for total blood count (100 µL) and tPA activity (200 µL), respectively. The remaining blood was centrifuged at 2,000g (g = 980 cm/sec^2) for 10 minutes to obtain plasma. Plasma was separated from whole blood within 1.5 hours of the start of blood sampling. The plasma was divided into 4 aliquots and stored at –70°C until analysis.

EXPOSURES AND MONITORING

Filtered-Air Exposure

For the controls (filtered-air exposure), ambient air was drawn from the outside environment with a stainless-steel diaphragm pump. After passing through gas denuders (identical to those used on the concentrated PM chamber) and a HEPA filter the air was delivered to the nose-only exposure chamber.

Concentrated-PM Exposure

The concentrator system was located at the NYU School of Medicine in New York City in an eighth floor research laboratory. Air was drawn into the concentrator from a stainless steel tube that extended 1 meter from the window. Operation and validation of the centrifugal concentrator used in these experiments have been described (Gordon et al 1999). In brief, the centrifugal aerosol concentrator was a gift from the US Naval Research Laboratory (Gerber 1980).

This concentrator (diagrammed in Figure 2) used the inertial properties of particles to separate them from air. A high-volume blower was used to deliver ambient air to the inlet manifold of the centrifugal concentrator at a flow rate of approximately 100 L/min.

From the inlet manifold, the air and ambient particles traveled along a concentric annulus formed by a stationary solid outer cylinder and a porous inner cylinder rotating at 10,000 rpm. Suction applied at one end of the porous shaft drew the air through the porous cylinder and into the shaft at a rate of approximately 90 L/min. The particles moved radially outward from the inner rotor due to centrifugal force and thus were not drawn into the porous inner cylinder. The particle-enriched air exited the concentrator via the outlet manifold and was delivered to the nose-only exposure manifold at a flow rate of 7 to 10 L/min and at a positive pressure of 0.20 cm water. Calibrated orifice meters were used to monitor inlet and exhaust flow rates. An inline, stainless steel hot-wire anemometer (model 2011, TSI, St Paul MN) was used to continuously monitor the flow to the exposure chamber (CH Technology, Westwood NJ). Denuders coated with potassium iodide and lead oxide were located downstream of the anemometer to remove ozone and sulfur dioxide, respectively (Williams and Grosjean 1990). Noise was reduced by placing high-speed instruments in ventilated, soundproof enclosures and adding a laboratory-built muffler to the outlet side of the exhaust pump.

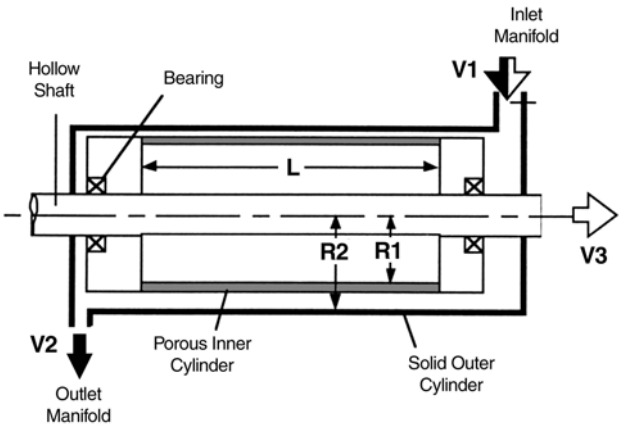

Figure 2. Gerber centrifugal concentrator (not drawn to scale). V1 (124 L/min), is the flow rate of outside air delivered to the inlet of the concentrator. V2 (10 L/min) is the flow rate of particle-enriched air from the outlet of the concentrator to the exposure chamber. V3 (114 L/min) is the flow rate of the particle-depleted air that is pulled into the porous filter and out the exhaust manifold. The length (L) of the porous filter is 30 cm; R1 and R2 are 4.445 and 5.125 cm, respectively. The inner cylinder rotation frequency is 10,000 rpm.

The aerosol concentrator was previously validated using monodisperse and polydisperse fluorescent test particles as well as ambient PM (Gordon et al 1999). These studies showed that particles ranging between 0.5 and 2.5 µm mass median aerodynamic diameter (MMAD) were concentrated most efficiently. Larger particles (> 3 µm) were removed by the inlet blower. Particles smaller than 0.5 µm were neither concentrated nor removed by the concentrator.

Concentrated PM was sampled from the stainless-steel delivery ports of the nose-only exposure manifold. Ambient PM was sampled from a tube that extended through a sampling port in the laboratory window. Data on the hourly averages for PM_{10} and $PM_{2.5}$ concentrations measured by a tapered element oscillating microbalance at the closest central monitoring station were recorded for each exposure (draft data obtained from the New York State Department of Environmental Conservation).

Filter-Sample Analyses

The PM mass concentration was determined by gravimetric filter samples (37-mm Teflon filters, Graseby/Anderson, Smyrna GA) that were weighed with an electronic microbalance (model 30, Cahn Instruments, Cerritos CA). The balance was located in a humidity-controlled chamber (30% ± 2% relative humidity), and it was calibrated daily with a class 1.1 calibration weight. Prior to weighing, filters and multiple filter blanks were conditioned for at least 48 hours in the humidity-controlled chamber. A real time aerosol monitor (RAM-1, MIE, Bedford MA) was used to monitor output of the centrifugal concentrator at ambient temperature and relative humidity during exposure experiments. Filter samples used for PM mass determination were also used for metal analysis by x-ray fluorescence spectroscopy (XRF). XRF measurements were done by Chester Labnet (Tigard OR).

Additional filter samples collected for analysis of acid and anions were sealed in the filter sample holders immediately after sample collection. Filters were removed from the sample holder in an ammonia-free hood and stored in sealed Petri dishes with citric acid–impregnated filter paper to prevent neutralization of the acid. The methods used to measure acid, sulfur species, and other anions have been previously described (Gordon et al 1999). In brief, samples were extracted from the filters with a mildly acidic solution (pH 4.0). Nitrate, sulfate, and ammonium ions were measured in the extraction medium by ion chromatography. Hydrogen ion analysis of the extraction sample was determined by measurement of pH as described by Koutrakis and colleagues (1988).

BLOOD SAMPLE ASSAYS

Peripheral Blood Cell Counts

Counts of platelets, white blood cells (WBCs), and red blood cells (RBCs) in peripheral blood were determined with a Coulter counter (model T540). Because rats have much higher platelet counts than humans, automated platelet counts on undiluted rat blood could be inaccurate. A pilot study was done to determine the effects of sample dilution on blood cell counts. Blood samples from two rats were mixed with saline at dilutions ranging from 1:0 to 1:10 vol/vol (blood to saline). Platelet, RBC counts, and WBC counts were essentially the same in diluted and undiluted blood after correction for dilution. Because sample dilution reduced the volume of blood needed, the samples used for blood cell counts were diluted 1:2 vol/vol with saline.

During experiments, it was not feasible to measure blood counts immediately after sampling. Therefore, the effect of storage time on blood cell counts was determined in blood samples from 3 rats. Samples were diluted 1:2 with saline, divided into aliquots, and analyzed at intervals ranging from 1 to 48 hours after sampling. Platelet counts declined by 3 to 7% in the first 4 hours after blood was sampled. Then, counts remained essentially the same for up to 48 hours. Other blood cell parameters were unaffected by storage time. Thus, for all experiments, blood cell counts were analyzed 4 to 24 hours after the samples were drawn.

Plasma Fibrinogen Content

Fibrinogen levels were measured by heat precipitation as described by Desvignes and Bonnet (1981). Plasma samples (15 µL) or fibrinogen reference solution (Sigma) were diluted to 600 µL with 10 mM morpholinoethane sulfonic acid buffer (pH 6.3 ± 0.05). A 255-µL portion of each diluted sample was transferred to a microtiter plate, and the optical density was determined at 405 nm. The remaining diluted samples were heated at 56°C in a water bath for 15 minutes and allowed to cool to room temperature. The optical density of the heated samples was measured at 405 nm. The difference in optical density for the heated and unheated samples was determined, and fibrinogen level (g/L) was calculated from the standard curve. As previously shown, this method agrees closely with fibrinogen measurements made by immunoassay or by thrombin clotting time (Desvignes and Bonnet 1981).

Factor VII Activity

Factor VII clotting activity was measured by adding aliquots of plasma (diluted 1/20 and 1/40) to factor VII–deficient plasma. Thromboplastin was added, and clotting time was measured using a coagulation analyzer (Diagnostica Stago, Parsippany NJ). Pooled plasma from healthy rats was used as a standard.

TAT Content

TAT was measured by an enzyme-linked immunosorbent assay (ELISA) using an ELISA kit (Enzygnost, Dade Behring, San Jose CA). As previously reported, this kit detects TAT from many species including rats (Ravanat et al 1995). We verified that this kit detects rat TAT content by infusing rats with thromboplastin (90, 450 or 2,400 µL/kg thromboplastin; Sigma) or saline over 30 minutes to activate intravascular clotting and cause TAT levels to rise (Ravanat et al 1996). Dose-dependent increases in TAT levels were measured in the thromboplastin-treated rats.

tPA and PAI Activity

tPA and PAI activities were measured using a fibrin chromogenic assay kit (Spectrolyse, Biopool International, Ventura CA). Blood sampled for tPA analysis required special preparation to prevent inactivation of tPA by endogenous inhibitors in the blood. Whole blood samples were immediately transferred to a tube containing 0.5 vol of 1.0 M acetate buffer (pH 3.9) on ice. The samples were then centrifuged, and the plasma was added to a tube containing 0.05 vol of 20% acetic acid within 30 minutes.

Thymocyte Count

After the final blood sample was drawn, each rat was killed by intraperitoneal injection of pentobarbital (65 mg/kg) and subsequent transection of the abdominal aorta. The thymus was removed and kept at 4°C until the next day. Thymocyte count was determined by a modification of the method described by Han and associates (1993). The thymus was cut into small pieces and forced through a fine nylon mesh into 10 mL of calcium-free phosphate buffered saline (PBS). The thymocytes were washed twice by centrifuging at 300*g* for 7 minutes and resuspending the cells in 10 mL PBS. The washed cells were diluted 1:9 vol/vol and counted with a model T540 Coulter counter.

STATISTICAL METHODS AND DATA ANALYSES

The statistical significance of differences between group means was tested by two-way ANOVA with exposure status (filtered air or concentrated PM) and postexposure time point (6, 12 or 24 hours) as main effects. A value of $P < 0.05$ was considered statistically significant. Data from each of the 5 experiments were analyzed separately and in combination (the latter is referred to in this report as the *combined data set*).

Because it was possible that concentrated-PM exposure might have a transient effect on some of the end points, differences in group means at each postexposure time point were tested by a two-tailed Student *t* test on the combined data set. A two-way multivariate analysis of variance was done on the combined data set with the exposure group and time as main effects and 5 coagulation parameters as dependent variables. Because blood samples were analyzed for factor VII at a later date, these data were not included in the multivariate analysis of variance. All data analysis and statistical testing was done using StatView II and Super ANOVA 1.11 (Abacus Concepts, Berkeley CA).

Baseline (preexposure) values for all end points were measured on blood sampled immediately prior to exposure. We expected that, for some blood parameters, animal-to-animal variability in baseline values might either obscure exposure-related changes or enhance them spuriously. Blood components that have long half-lives and are present in the blood at measurable levels under normal conditions may show persistent animal-to-animal variability over the course of 24 hours. For example, animal-to-animal differences in plasma fibrinogen levels could influence postexposure values because fibrinogen has a half-life of 4 days. On the other hand, because increased tPA activities are transient and normal levels are low, baseline tPA activity would not be expected to influence postexposure values.

In order to identify objectively which parameters require subtraction of baseline values to correct for persistent animal-to-animal differences, blood samples from 8 sham-exposed rats were analyzed by one-way ANOVA. Each individual animal (rat 1, rat 2, … rat 8) was the independent variable and the before and after exposure values for each end point were the dependent variables. Statistically significant animal-related differences were observed for fibrinogen level, factor VII activity, and blood platelet count. Therefore, data for these three end points were analyzed with and without subtraction of preexposure baseline values.

In order to achieve sufficient homogeneity of variances for testing by two-way ANOVA, it was necessary to remove outlier values. An extreme value was defined as an outlier if it was greater than 3 standard deviations (SDs) from the mean and occurred independently of other extreme values. The combined data set from the 5 exposure experiments containing all postexposure values for both filtered-air and concentrated-PM animals was used to calculate the mean (± 1 SD) for each end point. Values greater than 3 SDs from the mean were considered to be independent if related end points measured in that animal at that time point were not abnormal. For example, if fibrinogen content decreased markedly due to intravascular coagulation, TAT levels should increase sharply and vice versa. In addition, values measured in blood that was sampled before and after a suspected outlier value were examined to verify that the outlier value occurred sporadically in that animal. There were approximately 250 values for each of the 8 end points for a total of 2,000 data points. From this total, 20 values were identified that were greater than 3 SDs from the mean; all 20 were unrelated to temporal trends and were not associated with changes in other coagulation parameters. Removal of outlier values resulted in a gain of statistical significance for the platelet count (baseline subtracted) in experiment 4 and for TAT content in the combined data set. Removal of outliers did not result in a loss of statistical significance for any end point.

RESULTS

BLOOD SAMPLING PILOT STUDIES

Two pilot studies were performed to determine (1) the effect of an indwelling venous catheter on blood counts and coagulation parameters in healthy rats and (2) the effect of repeated blood sampling on peripheral blood end points. For comparison, Table 2 shows the range of normal values in humans for the blood coagulation parameters examined in this study. There are no published normal values in rats for these parameters. Instead, Table 2 presents the range of preexposure baseline values (mean ± 1 SD) obtained from the 84 rats used in this study as the normal range for rats.

Effect of Indwelling Catheter

The effect of an indwelling catheter was examined by assaying blood obtained from rats with jugular vein catheters and assaying blood obtained by cardiac puncture from a separate group of rats (Table 3). Blood was obtained by cardiac puncture from the group of anesthetized rats at the same time of day as blood was obtained from the catheterized rats.

Table 2. Range of Normal Values for Coagulation Parameters

Parameter	Humans	Rats[a]
Fibrinogen (g/L)	2.0–4.5	2.3–4.8
Factor VII (%)	86–124	102–166
tPA (IU/mL)	1.4–14	0.8–1.7
PAI (IU/mL)	0–15	9–18
TAT (µg/L)	1–4	0–2

[a] From baseline values of the 84 healthy male F344 rats in this study.

There was a statistically significant difference between the two groups for almost all of the parameters measured, but the differences were small in magnitude. WBC and RBC counts were significantly higher and platelet count lower in the catheterized group compared with the cardiac puncture group. These small changes in blood cell counts were probably due in part to the difference in sampling site (heart versus jugular vein) as has been previously reported (Smith et al 1986). The presence of an indwelling catheter may also have had a slight inflammatory effect as indicated by an increased WBC count. However, fibrinogen content was significantly lower in blood from catheterized rats than in blood obtained by cardiac puncture, which argues against an inflammatory effect from the catheter.

Plasma activity for tPA was the same in both groups. PAI activity was significantly higher in catheterized rats, but it was still within the normal range established by the baseline values. The value for TAT was significantly higher in the cardiac puncture group because three samples had abnormally high values. This result suggests that the tissue injury from cardiac puncture activated coagulation; it also indicates that TAT content is a sensitive end point. Overall, although values for coagulation parameters in blood sampled via catheter were statistically different from blood obtained via cardiac puncture, the indwelling catheter did not appear to cause prothrombotic changes or intravascular coagulation.

Effect of Repeated Sampling

The effect of repeated blood sampling from jugular vein catheters on blood parameters is shown in Table 4. Only two parameters showed a statistically significant change from the value at the initial time point when analyzed with one-way ANOVA and post hoc testing by a Dunnett two-tailed *t* test. As expected, RBC count decreased progressively with each successive blood sample. The decrease in WBC count at 6 and 12 hours may reflect changes related to circadian rhythm (House et al 1997). None of the parameters showed

Table 3. Blood Sampled by Cardiac Puncture Compared with Blood Sampled by Catheter[a]

Parameter	Cardiac Puncture	Jugular Vein Catheter	*P* Value[b]
WBCs ($\times 10^{-3}$/mm^3)	7.20 ± 0.26	8.55 ± 0.35	**0.008**
RBCs ($\times 10^{-5}$/mm^3)	6.99 ± 0.08	7.43 ± 0.14	**0.017**
Platelets ($\times 10^{-3}$/mm^3)	701 ± 40	592 ± 17	**0.047**
Fibrinogen (g/L)	2.86 ± 0.08	2.25 ± 0.15	**0.0023**
tPA (IU/mL)	1.3 ± 0.1	1.1 ± 0.1	0.196
PAI (IU/mL)	7.4 ± 1.3	12.4 ± 1.7	**0.034**
TAT (µg/L)	7.1 ± 2.6	1.7 ± 0.5	**0.01**

[a] All data are expressed as mean ± SE; $n = 8$ for the cardiac puncture group and 6 for the jugular vein catheter group.

[b] *P* values determined by two-tailed *t* test for all end points except TAT, which was determined by the Mann-Whitney U test. Statistically significant *P* values ($P < 0.05$) are shown in bold type.

Table 4. Blood Parameters After Repeated Sampling

	Time Blood Was Sampled (hours)[a]			
Parameter	0	6	12	24
WBCs ($\times 10^{-3}$/mm^3)	8.9 ± 0.3	**6.9 ± 0.4**	**6.8 ± 0.4**	9.6 ± 0.3
RBCs ($\times 10^{-5}$/mm^3)	7.0 ± 0.1	**6.5 ± 0.1**	**6.0 ± 0.1**	**5.7 ± 0.1**
Platelets ($\times 10^{-3}$/mm^3)	537 ± 32	562 ± 27	587 ± 33	566 ± 30
Fibrinogen (g/L)	4.0 ± 0.4	3.6 ± 0.4	3.5 ± 0.4	3.7 ± 0.4
tPA (IU/mL)	1.3 ± 0.2	1.0 ± 0.1	1.3 ± 0.3	0.9 ± 0.1
PAI (IU/mL)	4.6 ± 0.7	11.6 ± 3.1	7.3 ± 2.3	4.8 ± 0.8
TAT (µg/L)	2.5 ± 0.8	2.6 ± 0.5	1.3 ± 0.3	1.8 ± 0.4

[a] All data are mean ± SE; $n = 8$. Blood values that are significantly different from blood sampled at time point 0 (per one-way ANOVA and post hoc testing by Dunnett *t* test) are indicated by bold type.

a significant association with RBC count, which indicated that other end points should not be normalized to the RBC value.

CONTROL EXPOSURES

Cage Versus Sham Exposure

Because many of the blood parameters examined in this study can be affected by stress, the effects of restraint or noise from the concentrator could obscure subtle PM-related changes. To examine the effects of exposure conditions on blood parameters, we compared blood parameters in samples from rats sham exposed to PM with samples from rats kept in their cages under quiet conditions. Rats from both groups were killed for thymus removal 24 hours after the start of exposure. Loss of thymic lymphocytes (also termed *thymocytes*), which is an indicator of stress (Han et al 1993), was measured by counting total thymocytes.

As shown in Table 5, there were no significant differences between the groups for any parameter except fibrinogen content. In the sham-exposed group, the fibrinogen content was significantly lower due to baseline differences between the two groups. Subtraction of the baseline fibrinogen values revealed a small but significant increase in fibrinogen content in sham-exposed rats compared with cage controls. These results indicate that the stress of exposure conditions would not be expected to affect any of the blood parameters measured in this study except fibrinogen level.

Table 5. Blood Parameters of Sham-Exposed Rats Compared with Rats Maintained in Their Cages

		Hours After Start of Exposure[a]				
Parameter	Group	0	6	12	24	P[b]
WBCs ($\times 10^{-3}$/mm^3)	Cage	8.9 ± 0.3	6.9 ± 0.4	6.8 ± 0.4	9.5 ± 0.3	0.74
	Sham	7.5 ± 0.3	6.2 ± 0.3	7.9 ± 0.5	9.5 ± 0.1	
RBCs ($\times 10^{-5}$/mm^3)	Cage	7.0 ± 0.1	6.5 ± 0.1	6.0 ± 0.1	5.7 ± 0.1	0.92
	Sham	6.8 ± 0.1	6.6 ± 0.1	5.9 ± 0.1	5.6 ± 0.1	
Platelets ($\times 10^{-3}$/mm^3)						
Total	Cage	537 ± 32	562 ± 27	587 ± 33	566 ± 30	0.32
	Sham	522 ± 12	572 ± 17	544 ± 19	537 ± 17	
Baseline subtracted	Cage	0	23 ± 15	39 ± 23	25 ± 22	0.99
	Sham	0	41 ± 9	27 ± 9	20 ± 17	
Fibrinogen (g/L)						
Total	Cage	4.18 ± 0.37	**3.64 ± 0.41**	**3.48 ± 0.38**	**3.66 ± 0.38**	**0.01**
	Sham	2.71 ± 0.20	**2.82 ± 0.18**	**2.96 ± 0.14**	**3.07 ± 0.18**	
Baseline subtracted	Cage	0	**−0.54 ± 0.14**	**−0.70 ± 0.10**	**−0.52 ± 0.30**	**0.001**
	Sham	0	**0.11 ± 0.10**	**0.27 ± 0.20**	**0.37 ± 0.26**	
Factor VII (%)						
Total	Cage	134 ± 6	110 ± 11	107 ± 11	159 ± 13	0.30
	Sham	118 ± 4	100 ± 11	132 ± 13	115 ± 7	
Baseline subtracted	Cage	0	−24 ± 10	−27 ± 8	25 ± 11	0.40
	Sham	0	−18 ± 9	15 ± 11	−2 ± 6	
tPA (IU/mL)	Cage	1.5 ± 0.2	1.0 ± 0.1	1.3 ± 0.3	0.9 ± 0.1	0.20
	Sham	1.6 ± 0.4	2.2 ± 0.4	1.0 ± 0.3	1.0 ± 0.4	
PAI (IU/mL)	Cage	4.0 ± 0.7	11.6 ± 3.1	7.3 ± 2.2	4.8 ± 0.8	0.51
	Sham	7.5 ± 1.4	4.7 ± 1.1	9.3 ± 2.9	6.0 ± 2.1	
TAT (µg/L)	Cage	2.3 ± 0.6	2.6 ± 0.5	1.3 ± 0.3	1.8 ± 0.4	0.65
	Sham	1.7 ± 0.4	0.9 ± 0.4	1.7 ± 0.2	1.6 ± 0.7	
Thymocytes ($\times 10^8$ cells)	Cage				1.33 ± 0.1	0.33
	Sham				1.45 ± 0.1	

[a] Results shown are mean ± SE; n = 8 per group.

[b] P values for effect of exposure status determined by two-way ANOVA on postexposure time points (6, 12, and 24 hours). Statistically significant results ($P < 0.05$) are shown in bold type.

Filtered Air Versus Sham Exposure

The second control experiment was performed to verify that exposing rats to filtered air in the same room and at the same time as sham-exposing rats to air provided an adequate control for the effects of noise and restraint associated with concentrated-PM exposure. As shown in Table 6, the filtered-air and sham-exposed groups showed no significant differences for fibrinogen level, tPA activity, PAI activity, or peripheral blood count. However, the sham-exposed rats showed a statistically significant increase in TAT content compared with the filtered air controls. Although TAT was slightly increased in sham-exposed rats, the values were low in both groups and well within the normal range reported for humans. Also, TAT levels in sham-exposed rats tended to be lower than values obtained from rats maintained in their cages (Table 5).

Surprisingly, factor VII activities were significantly lower in sham-exposed rats than in rats exposed to filtered air. The difference between the sham-exposed and filtered-air groups was also significant after subtraction of baseline activity for factor VII. Because factor VII activities were not different in sham-exposed rats compared with the caged rats in the first control experiment, it is unlikely that the sham exposure affected factor VII activities. However, the filtered-air exposure may have caused a small but statistically significant increase in factor VII. It is also possible that the significant difference in factor VII activity between sham-exposed animals and those exposed to filtered air may have occurred by chance alone because factor VII activity was quite variable from animal to animal and over time. As described in the next section, factor VII activity did not differ significantly between concentrated-PM and

Table 6. Blood Parameters After Filtered Air or Sham Exposure

Parameter	Group	Hours After Start of Exposure[a] 0	6	12	24	P[b]
WBCs ($\times 10^{-3}$/mm^3)	Air	8.5 ± 0.4	7.0 ± 0.2	8.4 ± 0.5	8.7 ± 0.4	0.21
	Sham	8.8 ± 0.4	7.5 ± 0.3	7.8 ± 0.6	10.2 ± 0.6	
RBCs ($\times 10^{-5}$/mm^3)	Air	6.4 ± 0.2	6.4 ± 0.04	5.7 ± 0.2	5.5 ± 0.05	0.34
	Sham	6.7 ± 0.06	6.4 ± 0.1	5.3 ± 0.3	5.5 ± 0.1	
Platelets ($\times 10^{-3}$/mm^3)						
Total	Air	600 ± 26	685 ± 22	645 ± 35	658 ± 21	0.92
	Sham	588 ± 17	652 ± 12	675 ± 82	651 ± 13	
Baseline subtracted	Air	0	85 ± 26	45 ± 39	58 ± 26	0.75
	Sham	0	65 ± 15	87 ± 70	67 ± 15	
Fibrinogen (g/L)						
Total	Air	2.9 ± 0.4	3.8 ± 0.3	3.6 ± 0.4	3.8 ± 0.4	0.70
	Sham	3.0 ± 0.4	3.4 ± 0.4	3.8 ± 0.5	4.5 ± 0.8	
Baseline subtracted	Air	0	0.9 ± 0.2	0.7 ± 0.2	0.5 ± 0.5	0.34
	Sham	0	0.3 ± 0.2	0.7 ± 0.3	1.8 ± 0.6	
Factor VII (%)						
Total	Air	103 ± 5	**99 ± 10**	**124 ± 8**	**107 ± 8**	**0.02**
	Sham	105 ± 7	**81 ± 10**	**102 ± 10**	**95 ± 5**	
Baseline subtracted	Air	0	**−4.2 ± 10**	**20 ± 5**	**9 ± 7**	**0.01**
	Sham	0	**−24 ± 4**	**−3 ± 5**	**−16 ± 9**	
tPA (IU/mL)	Air	1.5 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	0.66
	Sham	1.5 ± 0.2	1.5 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	
PAI (IU/mL)	Air	14.6 ± 0.9	15.7 ± 1.0	11.7 ± 2.6	11.6 ± 1.2	0.15
	Sham	15.0 ± 1.3	15.1 ± 1.4	15.5 ± 0.7	13.9 ± 1.8	
TAT (µg/L)	Air	0.89 ± 0.13	**1.28 ± 0.12**	**0.91 ± 0.12**	**0.51 ± 0.04**	**0.01**
	Sham	0.87 ± 0.09	**1.77 ± 0.18**	**1.31 ± 0.39**	**0.94 ± 0.19**	

[a] Results shown are mean ± SE; n = 9 per group.

[b] P values determined by two-way ANOVA on postexposure time points. Statistically significant results ($P < 0.05$) are shown in bold type.

filtered-air rats except for one experiment in which the concentrated-PM rats showed a significant decrease. When baseline activity for factor VII was subtracted, however, this difference was not significant.

EFFECTS OF CONCENTRATED PM ON COAGULATION

A total of 5 concentrated-PM experiments were completed over 8 weeks in the summer of 1999. Exposure dates and concentrations of the ambient PM and concentrated PM for the 5 experiments are summarized in Table 7. The target exposure concentration, which was 300 μg/m^3, was achieved in 1 of the 5 exposures, and 1 exposure (295 μg/m^3) was close to the target concentration. PM data from the closest central monitoring station show that $PM_{2.5}$ comprised at least 80% of the PM_{10} in the air on the exposure days. There was close agreement between our gravimetric measurements of ambient PM (which was not fractionated by size) and PM_{10} measured by the central monitoring station during the hours of exposure.

Table 7 also shows the results of compositional analysis for concentrated PM and for ambient PM on each of the 5 exposure days. As expected, sulfate (SO_4^{2-}) was the predominant constituent for each of the exposures, and modest amounts of free acid (H^+) were also detected in all PM samples. Sulfate (expressed as the percentage of total sample weight) was considerably lower in the concentrated PM samples than in the ambient PM samples obtained at the same time. Because the exposures occurred during mid to late summer, much of the sulfate aerosol was presumably formed by a secondary reaction and was in a particle size range below that effectively concentrated by our centrifugal concentrator. Total elemental constituents (measured by XRF), including metals but excluding sulfur, accounted for 1 to 5% of the concentrated-PM mass and for less than 2% of the ambient PM mass. Figure 3 shows the average elemental composition of concentrated PM and ambient PM for the 5 exposures combined. Iron comprised approximately 1/8 of the total elemental mass of concentrated PM and was the second most abundant element. Ambient PM, which was sampled at the same time, contained much less iron in terms of percentage of the total elemental mass.

Table 8 summarizes the statistically significant effects of exposure to concentrated PM for each blood parameter for each of the 5 experiments and for the combined data set. Statistical significance was determined by two-way ANOVA on the postexposure data with time and exposure status as main effects. Tables containing data for each of the 5 experiments (mean ± SE for each end point for the filtered-air and concentrated-PM groups) are in Appendix A.

Exposure-related effects were not consistent for any of the end points across the 5 experiments, and none were dose dependent. tPA activity increased significantly after

Table 7. Composition of Concentrated and Ambient PM

Parameter	Experiment (Date)				
	1 (7/16/1999)	2 (7/19/1999)	3 (7/31/1999)	4 (8/24/1999)	5 (9/4/1999)
Concentrated PM (μg/m^3)[a]	341	295	205	155	95
SO_4^{2-} (% total weight)	17.41	23.33	21.72	29.63	8.23
H^+ (% total weight)	3.76	4.77	7.81	6.71	13.05
NH_4^+ (% total weight)	0.99	1.77	0.00	0.00	0.00
Elements (% total weight)[b]	1.11	2.00	1.91	2.17	5.08
Ambient PM (μg/m^3)[a]	65	88	51	36	19
SO_4^{2-} (% total weight)	57.1	48.8	62.8	48.6	37.4
H^+ (% total weight)	6.55	2.96	15.52	6.76	4.65
NH_4^+ (% total weight)	10.90	2.00	0.00	0.00	0.00
Elements (% total weight)[b]	0.65	0.88	0.72	1.46	1.01
Monitoring Station[c]					
PM_{10} (μg/m^3)	65	74	45	34	23
$PM_{2.5}$ (μg/m^3)	59	64	39	28	18

[a] Gravimetric determination.

[b] Does not include sulfur.

[c] Measured by tapered element oscillating microbalance at a central monitoring station 0.5 mile from the exposure laboratory over the same 6 hours as the concentrated ambient PM exposure.

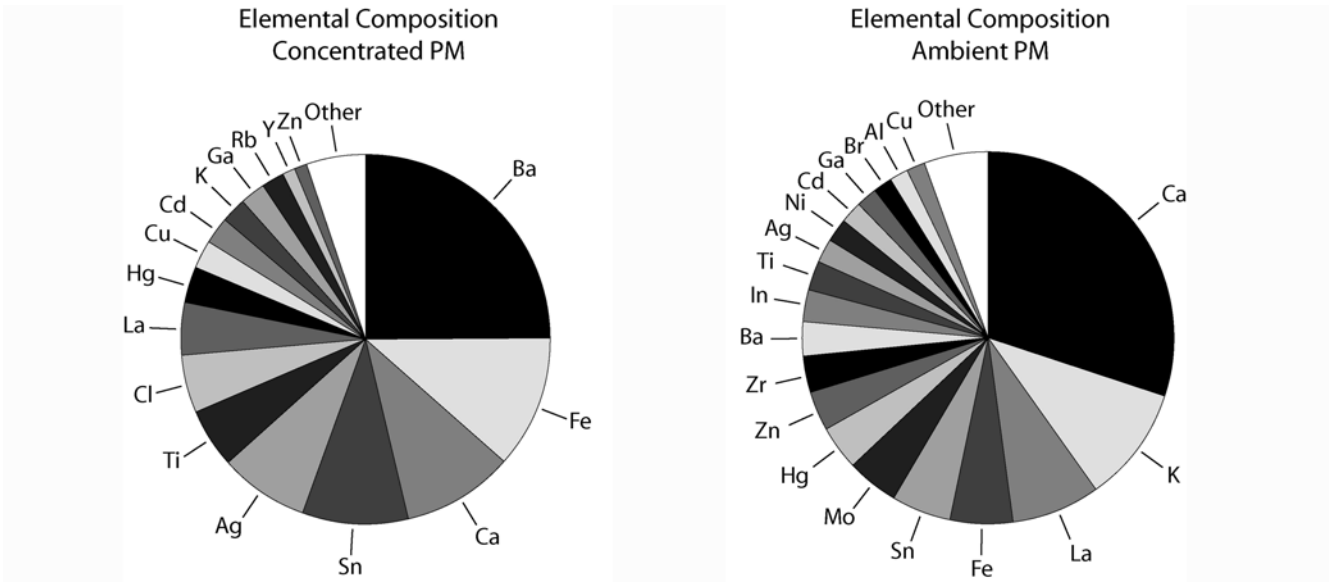

Figure 3. Elements in concentrated PM and ambient PM as percentage of total elemental mass. Samples of both concentrated and ambient PM were obtained simultaneously during the 5 exposures. Note that sulfur is excluded from the analysis. The results shown for concentrated PM and ambient PM are averages for the 5 experiments.

Table 8. Effect of Exposure to Concentrated PM on Blood Parameters (Summary)

	Experiment Number (μg PM per m^3)					
Parameter	1 (341)	2 (295)	3 (205)	4 (155)	5 (95)	Combined Data Set
WBCs	↔	↔	↔	↔	↔	↔
RBCs	↔	↔	↔	↔	↔	↔
Platelets						
Total	↔	↑	↔	↔	↔	↔
Baseline subtracted	↔	↔	↔	↓	↔	↔
Fibrinogen						
Total	↔	↔	↔	↔	↔	↔
Baseline subtracted	↔	↔	↔	↑	↓	↔
Factor VII						
Total	↔	↔	↓	↔	↔	↔
Baseline subtracted	↔	↔	↔	↔	↔	↔
tPA	↑	↔	↔	↔	↔	↔
PAI	↔	↔	↔	↔	↔	↔
TAT	↔	↔	↔	↑	↔	↑
Thymocytes	↔	↔	↑	↔	↔	↔

↔ No significant difference between exposure to filtered air and concentrated PM.

↓ Significantly decreased after exposure to concentrated PM (two-way ANOVA).

↑ Significantly increased after exposure to concentrated PM (two-way ANOVA).

concentrated-PM exposure in experiment 1, but no significant effect of concentrated-PM exposure on tPA activity was observed in experiment 2, which was performed just 3 days later during the same air pollution episode. In experiment 2, a significant PM-related increase occurred in platelet count. When the baseline levels were subtracted from the platelet data, however, the groups did not differ significantly from each other. Thymocyte count increased in experiment 3 in the concentrated-PM group, but this is not an adverse effect. Factor VII activity was significantly decreased in concentrated-PM rats in experiment 3. When the baseline activity was subtracted from the factor VII activity, however, the groups did not differ significantly from each other. In experiment 4, platelet count (baseline subtracted) decreased, fibrinogen levels (baseline subtracted) increased, and TAT levels increased significantly in the concentrated-PM group. The only change seen in experiment 5 was a significant decrease in fibrinogen levels (baseline subtracted), the opposite result from that obtained in the previous experiment.

Data from all 5 experiments were combined to increase the power to detect small but consistent changes. The mean for each end point for the filtered-air and concentrated-PM groups is shown in Table 9. TAT levels (analyzed by two-way ANOVA) increased significantly in the combined data set. In addition, differences in group means at each postexposure time point were tested by a two-tailed Student *t* test on the combined data set. The WBC count decreased significantly at 6 hours. There was no significant difference in TAT levels when each time

Table 9. Results of Experiments 1–5 Combined (the Combined Data Set)[a]

Parameter	Group	Hours After Start of Exposure 0	6	12	24	P[b]
WBCs ($\times 10^{-3}$/mm^3)	Air	8.9±0.2	6.9±0.2	8.5±0.2	9.7±0.2	0.38
	PM	8.4±0.3	6.4±0.2	8.5±0.3	9.8±0.2	
RBCs ($\times 10^{-5}$/mm^3)	Air	6.6±0.04	6.6±0.06	6.1±0.04	5.8±0.06	0.29
	PM	6.4±0.11	6.6±0.06	6.0±0.06	5.8±0.05	
Platelets ($\times 10^{-3}$/mm^3)						
Total	Air	664±12	740±13	658±12	634±13	0.48
	PM	669±11	744±12	663±11	646±10	
Baseline subtracted	Air	0	77±11	−5±12	−14±12	0.61
	PM	0	75±10	−6±11	−26±11	
Fibrinogen (g/L)						
Total	Air	3.54±0.21	3.47±0.15	3.63±0.16	3.80±0.15	0.25
	PM	3.56±0.17	3.61±0.20	3.77±0.16	3.99±0.15	
Baseline subtracted	Air	0	−0.07±0.15	0.09±0.18	0.34±0.17	0.39
	PM	0	0.05±0.12	0.21±0.13	0.43±0.17	
Factor VII (%)						
Total	Air	137±3	114±5	106±3	121±3	0.47
	PM	130±3	110±6	108±5	114±3	
Baseline subtracted	Air	0	−23±7	−31±6	−16±6	0.42
	PM	0	−20±6	−22±6	−16±5	
tPA (IU/mL)	Air	1.26±0.08	1.2±0.06	1.29±0.23	1.17±0.07	0.70
	PM	1.28±0.07	1.39±0.07	1.27±0.21	1.21±0.07	
PAI (IU/mL)	Air	12.9±0.7	15.8±0.6	12.8±0.8	11.4±0.8	0.80
	PM	14.0±0.9	15.2±0.7	13.7±0.7	10.7±0.8	
TAT (µg/L)	Air	1.00±0.07	**1.82±0.20**	**1.66±0.25**	**1.93±0.20**	**0.03**
	PM	1.18±0.23	**2.35±0.35**	**2.25±0.31**	**2.43±0.43**	
Thymocytes ($\times 10^8$ cells)	Air				2.41±0.13	0.20
	PM				2.62±0.11	

[a] Results shown are mean ± SE; 43 rats were exposed to filtered air and 41 rats were exposed to concentrated PM.

[b] P values are determined by two-way ANOVA on postexposure time points and statistically significant results ($P < 0.05$) are indicated by bold type. Underlined values indicate that there is a statistically significant difference between filtered air and concentrated PM for that time point (two-tailed t test, $P < 0.05$).

point was considered individually. A two-way multivariate ANOVA (performed on the combined data set with exposure group and time as main effects and 5 coagulation parameters as dependent variables) showed no significant effect of exposure status.

RESULTS SUMMARY

As shown in Table 8, there were 9 statistically significant results out of 72 total, more than would be expected by chance alone ($0.05 \times 72 = 3.6$). Some of the statistically significant changes of PM exposure do not represent adverse effects (namely, increased tPA activity, increased thymocyte count, decreased fibrinogen levels), or they are due to a baseline, preexposure difference in the groups (namely, increased platelet count in experiment 2, decreased factor VII activity in experiment 3). Changes after concentrated-PM exposure seen in experiment 4 (decreased platelet count and increased fibrinogen and TAT levels) could be interpreted as adverse effects, but these results were not reproducible. Data on PM composition do not suggest that PM constituents were different during that exposure. Our analysis accounted for less than one third of the total mass of PM, however, so important compositional differences may have been missed. A PM-related increase in TAT levels was statistically significant when data from all 5 exposures were combined, but the significance depended on elimination of 5 outlier values based on an a priori statistical definition of an extreme value. More importantly, a slight but statistically significant increase in TAT level was found in rats that were sham exposed compared with rats exposed to filtered air. This finding indicates that the small increase in TAT in PM-exposed rats in the combined data set could be a nonspecific effect that is unrelated to inhalation of PM.

DISCUSSION AND CONCLUSIONS

Although an increasing number of time-series studies have shown an association between elevated levels of PM and cardiovascular morbidity and mortality, establishing a biologically plausible mechanism for this association has been an elusive goal. The present study, which examined the effects of concentrated ambient PM on coagulation parameters related to cardiovascular disease risk in healthy rats, does not indicate that effects of PM on the cardiovascular system are mediated by coagulation.

Panel and epidemiologic studies that have examined the relation between PM and blood coagulation parameters have produced mixed results. Peters and colleagues (1997) reported that increased plasma viscosity was associated with an air pollution episode characterized by high levels of sulfur dioxide (SO_2) and PM. Because this pollution episode followed a sharp drop in temperature, there is concern that the effects of temperature may have confounded that study. Pekkanen and colleagues (2000) analyzed the correlation between air pollution measurements and blood levels of fibrinogen and factor VII in a large cross-sectional study done in London between 1991 and 1993. Black smoke (a measure of PM) was associated with increased fibrinogen levels only in the warm months. There were significant positive associations between fibrinogen and nitrogen dioxide and carbon monoxide independent of season. Factor VII levels were negatively associated with black smoke, carbon monoxide, and nitrogen dioxide. Seaton reported a panel study in which 112 subjects were followed for 18 months with repeated measurements of fibrinogen, factor VII, platelet, and C reactive protein (an acute phase reactant) (Seaton et al 1999). This study found a significant negative association between PM exposure and the fibrinogen level and RBC count, suggesting that PM exposure had an anticoagulant effect. Because parameters related to activation of coagulation and fibrinolysis (such as TAT content, tPA activity, or levels of fibrin degradation products) were not measured, however, one cannot rule out the possibility that PM exposure actually activated coagulation and increased the rate of consumption of coagulation factors due to increased intravascular coagulation and fibrinolysis. The fact that there was a significant positive association between PM exposure and C reactive protein provides some support for this alternative interpretation.

The current study has a number of limitations. One obvious limitation is that all the exposure studies were done in the summer, when PM was largely photochemically generated and transported from long range. Winter PM may have different effects due to different combustion sources and trapping of local pollution by temperature inversion. In addition, the centrifugal concentrator used in these studies preferentially concentrates particles in the 0.5–2.5 μm size range. It is possible that particles in the larger or smaller size fractions may affect coagulation. Another exposure-related limitation is that gaseous copollutants were removed. As described above there is some epidemiologic evidence that blood fibrinogen levels may be more strongly affected by gaseous pollutants than PM (Pekkanen et al 2000). There is increasing evidence that elevated levels of gaseous pollutants are also associated with adverse cardiovascular effects. A recent paper by Burnett and colleagues (1999) reported that most of the excess cardiovascular-related hospital admissions caused by air pollution were due to gaseous pollutants. Bouthillier and colleagues (1998) have shown that coexposure of rats to

urban air particulates and ozone had markedly greater effects on lung morphology than either pollutant alone.

Another limitation of this study is that the systemic acute phase response to mild lung inflammation may be blunted in rats compared with the response in humans. Gardiner and colleagues (2000) exposed rats to oil fly ash by tracheal instillation at concentrations of 0.3, 1.7 and 8.3 mg/kg, which was expected to cause mild to severe lung injury in a dose-related fashion. However, only the highest dose resulted in a significant increase in blood fibrinogen content. We exposed rats to 0.4 and 0.8 ppm ozone for 6 hours and found no increase in blood fibrinogen content at either concentration even though the percentage of neutrophils in the lung lavage fluid was 15% in animals exposed to 0.8 ppm ozone compared with 1% in air-exposed rats (unpublished data). In contrast, Ghio and colleagues (2000) found a small but significant increase in blood fibrinogen levels in humans exposed to concentrated ambient PM even though the percentage of neutrophils in lavage fluid was only slightly increased (8.1% in PM-exposed individuals compared with 2.7% in subjects exposed to air). More studies are needed to determine how rats and other laboratory animals compare with humans in terms of systemic responses to lung inflammation.

An animal model with cardiovascular disease might be more sensitive to hematologic effects of inhaled PM than healthy rats. Conventional laboratory animals are generally resistant to atherosclerosis, so it is difficult to produce a realistic animal model of human cardiovascular disease. However, genetically manipulated mice are showing great promise as models of human cardiovascular disease. Mice with a knockout of the apolipoprotein E gene have elevated cholesterol levels and develop blood vessel disease similar to human atherosclerosis (Dzau et al 1995). This animal model may be more sensitive to the thrombogenic effects of air pollutants because the endothelial dysfunction and disruption caused by atherosclerosis stimulates platelet activation (O'Keefe et al 1996).

On the other hand, most of the coagulation end points in our study were designed to detect procoagulant changes such as increased fibrinogen level or platelet count, which would not require activation of the coagulation cascade. It is not currently known whether animal models with cardiovascular disease are more prone to procoagulant changes in blood parameters in response to inhaled pollutants than are healthy animals. Prescott reported that individuals with elevated levels of blood fibrinogen were not more likely to have pollution-related cardiovascular events than subjects with lower baseline fibrinogen levels (Prescott et al 2000). Our previous studies of the effects of concentrated ambient PM in compromised animals utilized monocrotaline-treated rats and cardiomyopathic hamsters (Gordon et al 1998, 2000). Based on analysis of lung lavage fluid and electrocardiograph monitoring, these compromised animals did not appear to be more sensitive to effects of PM than healthy animals, and the increased animal-to-animal variability in the compromised animals tended to obscure exposure-related changes.

The sensitivity and reproducibility of measurements of hematologic and coagulation parameters in this study appear to be adequate to detect small changes. A number of statistically significant differences were detected in the various experiments even though the magnitude of the change was small. There was little animal-to-animal variability in most of the end points. Subtraction of baseline measurements was used to analyze data from the end points that showed persistent animal-related differences. Repeated sampling of blood from the same animal increased the number of observations per experiment and allowed us to test statistical significance by two-way ANOVA, which has greater statistical power than most other tests. However, the number of different coagulation parameters that were analyzed is very small. The coagulation system is as multifaceted and complex as the immune system. Even though an attempt was made in this study to examine various functional aspects of coagulation, there are many other sensitive and clinically significant parameters that could be examined. In particular, none of the end points in this study measured platelet activation, which is the first step in arterial thrombosis. It is possible that a different panel of coagulation parameters would yield different results in examining the effects of inhaled PM.

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Table A1. Results of Experiment 1[a]

Parameter	Group	Hours After Start of Exposure				P[b]
		0	6	12	24	
WBCs ($\times 10^{-3}$/mm^3)	Air	9.5 ± 0.4	6.2 ± 0.3	8.9 ± 0.5	9.8 ± 0.5	0.57
	PM	9.0 ± 0.3	6.1 ± 0.4	9.6 ± 0.2	9.8 ± 0.4	
RBCs ($\times 10^{-5}$/mm^3)	Air	6.6 ± 0.06	6.5 ± 0.09	5.9 ± 0.06	5.7 ± 0.05	0.58
	PM	6.6 ± 0.08	6.5 ± 0.11	5.8 ± 0.03	5.7 ± 0.06	
Platelets ($\times 10^{-3}$/mm^3)						
Total	Air	628 ± 32	721 ± 31	627 ± 31	622 ± 22	0.15
	PM	652 ± 20	758 ± 20	640 ± 16	658 ± 25	
Baseline subtracted	Air	0	94 ± 17	8 ± 10	−6 ± 21	0.94
	PM	0	106 ± 15	−12 ± 20	6 ± 29	
Fibrinogen						
Total	Air	3.58 ± 0.44	3.82 ± 0.46	3.66 ± 0.49	3.89 ± 0.45	0.55
	PM	3.59 ± 0.34	3.91 ± 0.46	3.78 ± 0.20	4.25 ± 0.19	
Baseline subtracted	Air	0	0.24 ± 0.09	0.09 ± 0.14	0.31 ± 0.34	0.36
	PM	0	0.31 ± 0.20	0.19 ± 0.17	0.65 ± 0.30	
Factor VII (%)						
Total	Air	138 ± 8	123 ± 8	134 ± 6	129 ± 6	0.98
	PM	126 ± 5	119 ± 6	140 ± 8	127 ± 4	
Baseline subtracted	Air	0	−15 ± 7	−3 ± 7	−8 ± 7	0.06
	PM	0	−8 ± 6	12 ± 8	1 ± 8	
tPA (IU/mL)	Air	1.14 ± 0.02	**1.19 ± 0.06**	**0.91 ± 0.07**	**0.95 ± 0.05**	**0.04**
	PM	1.07 ± 0.07	**1.39 ± 0.08**	**1.05 ± 0.06**	**1.23 ± 0.24**	
PAI (IU/mL)	Air	10.5 ± 1.2	15.3 ± 1.7	9.8 ± 1.1	10.4 ± 1.7	0.34
	PM	15.6 ± 2.7	16.5 ± 2.0	11.2 ± 1.6	11.5 ± 1.2	
TAT (μg/L)	Air	0.95 ± 0.07	1.95 ± 0.49	2.31 ± 0.21	1.15 ± 0.21	0.50
	PM	0.86 ± 0.05	1.55 ± 0.27	2.71 ± 0.95	2.70 ± 1.50	
Thymocytes ($\times 10^8$ cells)	Air				2.73 ± 0.38	0.84
	PM				2.81 ± 0.22	

[a] Results shown are mean ± SE; 8 rats were exposed to filtered air and 9 rats were exposed to 341 μg/m^3 concentrated PM.

[b] P values determined by two-way ANOVA on postexposure time points. Bold type indicates statistically significant results ($P < 0.05$).

Table A2. Results of Experiment 2[a]

Parameter	Group	Hours After Start of Exposure				P[b]
		0	6	12	24	
WBCs ($\times 10^{-3}$/mm^3)	Air	8.4 ± 0.3	7.2 ± 0.3	7.7 ± 0.4	9.1 ± 0.4	0.70
	PM	7.9 ± 0.9	6.5 ± 0.4	7.4 ± 0.4	9.8 ± 0.4	
RBCs ($\times 10^{-5}$/mm^3)	Air	6.6 ± 0.1	7.0 ± 0.1	6.2 ± 0.1	6.0 ± 0.05	0.52
	PM	6.1 ± 0.6	6.9 ± 0.1	6.1 ± 0.1	6.0 ± 0.1	
Platelets ($\times 10^{-3}$/mm^3)						
Total	Air	663 ± 18	**718 ± 23**	**604 ± 10**	**602 ± 18**	**0.001**
	PM	705 ± 18	**779 ± 20**	**672 ± 17**	**659 ± 14**	
Baseline subtracted	Air	0	54 ± 24	−57 ± 18	−61 ± 21	0.24
	PM	0	74 ± 22	−33 ± 20	−46 ± 19	
Fibrinogen (g/L)						
Total	Air	3.09 ± 0.21	3.33 ± 0.23	3.25 ± 0.20	3.32 ± 0.15	0.14
	PM	3.54 ± 0.37	3.74 ± 0.36	3.66 ± 0.31	3.42 ± 0.21	
Baseline subtracted	Air	0	0.24 ± 0.12	0.17 ± 0.20	0.23 ± 0.29	0.49
	PM	0	0.20 ± 0.07	0.13 ± 0.21	−0.11 ± 0.45	
Factor VII (%)						
Total	Air	170 ± 17	132 ± 15	98 ± 3	111 ± 4	0.36
	PM	154 ± 17	149 ± 14	98 ± 5	114 ± 6	
Baseline subtracted	Air	0	−39 ± 25	−73 ± 16	59 ± 15	0.14
	PM	0	−6 ± 22	−56 ± 15	−40 ± 17	
tPA (IU/mL)	Air	1.11 ± 0.07	1.39 ± 0.12	1.20 ± 0.09	1.12 ± 0.09	0.59
	PM	1.04 ± 0.05	1.49 ± 0.07	1.20 ± 0.07	1.15 ± 0.07	
PAI (IU/mL)	Air	16.8 ± 1.3	19.7 ± 1.0	18.6 ± 1.2	16.1 ± 1.7	0.36
	PM	17.3 ± 1.6	19.0 ± 1.1	18.4 ± 1.2	14.2 ± 1.1	
TAT (μg/L)	Air	1.04 ± 0.08	2.14 ± 0.56	2.24 ± 0.19	2.43 ± 0.30	0.98
	PM	1.12 ± 0.18	1.75 ± 0.45	2.76 ± 0.54	2.28 ± 0.31	
Thymocytes ($\times 10^8$ cells)	Air				2.63 ± 0.18	0.86
	PM				2.58 ± 0.22	

[a] Results shown are mean ± SE; 9 rats were exposed to filtered air, and 8 rats were exposed to 295 μg/m^3 concentrated PM.

[b] P values determined by two-way ANOVA on postexposure time points. Bold type indicates statistically significant results ($P < 0.05$).

Table A3. Results of Experiment 3[a]

Parameter	Group	Hours After Start of Exposure				P[b]
		0	6	12	24	
WBCs ($\times 10^{-3}$/mm^3)	Air	8.3 ± 0.3	6.7 ± 0.4	8.3 ± 0.7	10.0 ± 0.3	0.70
	PM	8.0 ± 0.4	6.0 ± 0.5	8.4 ± 0.7	10.3 ± 0.3	
RBCs ($\times 10^{-5}$/mm^3)	Air	6.6 ± 0.1	6.6 ± 0.1	6.3 ± 0.1	6.2 ± 0.1	0.70
	PM	6.6 ± 0.2	6.8 ± 0.1	6.5 ± 0.1	5.9 ± 0.1	
Platelets ($\times 10^{-3}$/mm^3)						
Total	Air	623 ± 25	751 ± 28	681 ± 26	701 ± 38	0.27
	PM	595 ± 19	727 ± 29	679 ± 32	644 ± 21	
Baseline subtracted	Air	0	128 ± 15	58 ± 28	78 ± 17	0.99
	PM	0	132 ± 26	84 ± 27	48 ± 16	
Fibrinogen (g/L)						
Total	Air	3.03 ± 0.20	3.39 ± 0.30	4.08 ± 0.35	4.08 ± 0.35	0.99
	PM	3.03 ± 0.24	3.40 ± 0.41	4.05 ± 0.39	4.12 ± 0.32	
Baseline subtracted	Air	0	0.37 ± 0.15	1.06 ± 0.18	1.06 ± 0.18	0.98
	PM	0	0.37 ± 0.22	1.03 ± 0.24	1.10 ± 0.22	
Factor VII (%)						
Total	Air	130 ± 8	**87 ± 8**	**98 ± 4**	**114 ± 9**	**0.03**
	PM	132 ± 7	**72 ± 8**	**86 ± 6**	**100 ± 9**	
Baseline subtracted	Air	0	−44 ± 12	−33 ± 7	−16 ± 11	0.24
	PM	0	−59 ± 8	−46 ± 6	−32 ± 12	
tPA (IU/mL)	Air	0.70 ± 0.07	0.80 ± 0.06	1.73 ± 1.08	1.27 ± 0.30	0.99
	PM	1.02 ± 0.18	0.87 ± 0.09	1.79 ± 1.10	1.12 ± 0.18	
PAI (IU/mL)	Air	12.1 ± 1.4	15.5 ± 1.3	8.1 ± 1.3	9.6 ± 1.9	0.66
	PM	12.9 ± 1.3	13.2 ± 1.0	11.0 ± 1.3	7.4 ± 1.5	
TAT (µg/L)	Air	0.37 ± 0.02	1.37 ± 0.42	1.07 ± 0.22	2.70 ± 0.23	0.17
	PM	0.43 ± 0.01	1.74 ± 0.34	1.44 ± 0.54	3.91 ± 1.18	
Thymocytes ($\times 10^8$ cells)	Air				**1.86 ± 0.23**	**0.02**
	PM				**2.63 ± 0.18**	

[a] Results shown are mean ± SE; 9 rats were exposed to filtered air and 8 rats were exposed to 205 µg/m^3 concentrated PM.

[b] P values determined by two-way ANOVA on postexposure time points. Bold type indicates statistically significant results ($P < 0.05$).

Table A4. Results of Experiment 4[a]

Parameter	Group	Hours After Start of Exposure				P[b]
		0	6	12	24	
WBCs ($\times 10^{-3}$/mm^3)	Air	9.1 ± 0.5	7.0 ± 0.3	8.8 ± 0.4	10.1 ± 0.3	0.59
	PM	8.6 ± 0.5	7.2 ± 0.3	9.2 ± 0.6	10.1 ± 0.7	
RBCs ($\times 10^{-5}$/mm^3)	Air	6.6 ± 0.06	6.5 ± 0.12	5.9 ± 0.07	5.8 ± 0.07	0.42
	PM	6.6 ± 0.09	6.5 ± 0.11	5.8 ± 0.13	5.7 ± 0.13	
Platelets ($\times 10^{-3}$/mm^3)						
Total	Air	692 ± 26	754 ± 24	707 ± 25	616 ± 27	0.84
	PM	711 ± 31	753 ± 32	691 ± 33	647 ± 28	
Baseline subtracted	Air	0	**61 ± 15**	**14 ± 10**	**−35 ± 20**	**0.005**
	PM	0	**42 ± 10**	**−20 ± 11**	**−74 ± 6**	
Fibrinogen (g/L)						
Total	Air	5.03 ± 0.47	3.64 ± 0.33	3.53 ± 0.28	4.09 ± 0.26	0.27
	PM	3.73 ± 0.33	2.97 ± 0.17	3.37 ± 0.24	4.18 ± 0.27	
Baseline subtracted	Air	0	**−1.39 ± 0.44**	**−1.50 ± 0.42**	**−0.71 ± 0.45**	**0.01**
	PM	0	**−0.76 ± 0.37**	**−0.36 ± 0.40**	**0.45 ± 0.52**	
Factor VII (%)						
Total	Air	121 ± 10	115 ± 11	95 ± 8	124 ± 5	0.18
	PM	121 ± 7	95 ± 9	102 ± 11	108 ± 6	
Baseline subtracted	Air	0	−6 ± 11	− 26 ± 15	1 ± 12	0.31
	PM	0	−26 ± 11	−19 ± 11	−13 ± 6	
tPA (IU/mL)	Air	1.76 ± 0.16	1.37 ± 0.10	1.12 ± 0.09	1.19 ± 0.09	0.93
	PM	1.69 ± 0.14	1.46 ± 0.15	1.06 ± 0.10	1.20 ± 0.06	
PAI (IU/mL)	Air	11.1 ± 0.8	14.9 ± 0.9	15.0 ± 1.6	12.1 ± 1.6	0.76
	PM	12.6 ± 1.5	13.0 ± 1.4	15.4 ± 1.2	12.5 ± 2.3	
TAT (µg/L)	Air	1.60 ± 0.10	**2.36 ± 0.36**	**1.55 ± 0.23**	**1.90 ± 0.76**	**0.03**
	PM	2.66 ± 1.05	**5.20 ± 1.29**	**2.70 ± 0.82**	**1.88 ± 048**	
Thymocytes ($\times 10^8$ cells)	Air				2.69 ± 0.23	0.44
	PM				2.99 ± 031	

[a] Results shown are mean ± SE; 9 rats were exposed to filtered air and 8 rats were exposed to 155 µg/m^3 concentrated PM.

[b] P values determined by two-way ANOVA on postexposure time points. Bold type indicates statistically significant results ($P < 0.05$).

Table A5. Results of Experiment 5[a]

Parameter	Group	Hours After Start of Exposure				P[b]
		0	6	12	24	
WBCs ($\times 10^{-3}$/mm^3)	Air	9.3 ± 0.9	7.6 ± 0.5	8.8 ± 0.6	9.6 ± 0.4	0.06
	PM	8.4 ± 0.7	6.6 ± 0.5	7.7 ± 0.6	9.0 ± 0.9	
RBCs ($\times 10^{-5}$/mm^3)	Air	6.3 ± 0.1	6.1 ± 0.2	6.0 ± 0.1	5.2 ± 0.3	0.69
	PM	6.3 ± 0.1	6.1 ± 0.1	5.7 ± 0.1	5.5 ± 0.1	
Platelets ($\times 10^{-3}$/mm^3)						
Total	Air	715 ± 25	757 ± 46	660 ± 25	632 ± 28	0.24
	PM	686 ± 17	704 ± 27	63 ± 28	619 ± 19	
Baseline subtracted	Air	0	42 ± 38	−70 ± 25	−68 ± 18	0.99
	PM	0	18 ± 16	−48 ± 16	−66 ± 21	
Fibrinogen (g/L)						
Total	Air	2.90 ± 0.55	3.17 ± 0.43	3.62 ± 0.42	3.67 ± 0.45	0.29
	PM	3.89 ± 0.60	4.01 ± 0.68	3.95 ± 0.60	3.92 ± 0.55	
Baseline subtracted	Air	0	**0.27 ± 0.14**	**0.73 ± 0.26**	**0.65 ± 0.38**	**0.01**
	PM	0	**0.12 ± 0.16**	**0.05 ± 0.17**	**0.03 ± 0.23**	
Factor VII (%)						
Total	Air	122 ± 10	115 ± 8	109 ± 5	127 ± 6	0.93
	PM	118 ± 6	117 ± 13	114 ± 10	122 ± 11	
Baseline subtracted	Air	0	−8 ± 3	−13 ± 7	5 ± 8	0.39
	PM	0	1 ± 11	−3 ± 8	5 ± 10	
tPA (IU/mL)	Air	1.60 ± 0.19	1.63 ± 0.14	1.44 ± 0.22	1.31 ± 0.07	0.94
	PM	1.58 ± 0.08	1.75 ± 0.16	1.26 ± 0.15	1.34 ± 0.08	
PAI (IU/mL)	Air	13.8 ± 1.6	13.5 ± 1.0	12.2 ± 1.1	8.3 ± 1.5	0.85
	PM	11.4 ± 1.5	14.0 ± 1.0	13.1 ± 0.9	7.5 ± 1.0	
TAT (µg/L)	Air	1.02 ± 0.10	1.21 ± 0.26	1.16 ± 0.20	1.31 ± 0.31	0.35
	PM	0.84 ± 0.06	1.60 ± 0.28	1.57 ± 0.30	1.18 ± 0.19	
Thymocytes ($\times 10^8$ cells)	Air				2.19 ± 0.28	0.72
	PM				2.08 ± 0.14	

[a] Results shown are mean ± SE; 8 rats were exposed to filtered air and 8 rats were exposed to 95 µg/m^3 concentrated PM.

[b] P values determined by two-way ANOVA on postexposure time points. Bold type indicates statistically significant results ($P < 0.05$).

ABOUT THE AUTHORS

Christine Nadziejko is an assistant professor in the Department of Environmental Medicine at New York University School of Medicine. She received her PhD in pathology from New York Medical College in 1985. Dr Nadziejko has specialized in cardiopulmonary effects of inhaled pollutants. Current projects in her laboratory include examination of acute and subchronic effects of PM exposure in animal models of cardiovascular disease using implanted electrocardiographic and blood pressure telemeters. She is also involved in studies examining the effects of cigarette smoke components on the activity of nitric oxide synthase in the lung. Dr Nadziejko is assistant director of the Histopathology and Experimental Animal NIEHS Core Facility in the Environmental Medicine Department. She is the pulmonary pathologist for the department and has served in that capacity in many experimental animal studies of inhaled toxicants.

Kaijie Fang is a research scientist in the Department of Environmental Medicine at New York University School of Medicine. He received his MD from Tongji Medical University in 1987. His research interests are occupational medicine and inhalation toxicology.

Lung Chi Chen is an associate professor in the Department of Environmental Medicine at New York University School of Medicine. He received his PhD in environmental medicine from New York University in 1983. Dr Chen has many years of experience in exposing animals to a wide variety of inhaled substances ranging from products of crack cocaine to kerosene heater fumes. Dr Chen is also interested in the pathogenesis of various cardiovascular diseases, and he has collaborated in studies that showed that side stream cigarette smoke promotes arteriosclerotic plaque development in a cockerel model.

Beverly Cohen is a professor in the Department of Environmental Medicine at New York University School of Medicine. She received her PhD in environmental medicine from New York University in 1979. Dr Cohen is an acknowledged expert in exposure assessment. She is coeditor of the text *Air Sampling Instruments for the Evaluation of Atmospheric Contaminants*. She is also associate editor of the journal *Aerosol Science and Technology*. Dr Cohen has been actively involved in the assessment of individual inhalation exposure to ambient and workplace aerosols. Her most recent work involves the development of a detector/monitor to measure number concentration of ultrafine ambient acid aerosols for use in assessment of human exposure to this component of $PM_{2.5}$.

Margaret Karpatkin is a professor in the Department of Pediatrics at New York University School of Medicine. She received her MD from the University of London in 1957. Dr Karpatkin is the director of Pediatric Hematology/Oncology at New York University School of Medicine. Her research examines the relation between levels of coagulant proteins and age.

Arthur Nadas is an associate professor in the Department of Environmental Medicine at the New York University School of Medicine. He received his PhD in mathematical statistics from Columbia University in 1967. His research interests focus on applications of statistics to medicine.

ABBREVIATIONS AND OTHER TERMS

ANOVA	analysis of variance
CO	carbon monoxide
H^+	free acid
HEPA	high efficiency particulate air (filter)
NH_4^+	ammonium
NO_2	nitrogen dioxide
PAI	plasminogen activator inhibitor
PBS	phosphate-buffered saline
PM	particulate matter
$PM_{2.5}$	PM less than 2.5 μm in mass median aerodynamic diameter
PM_{10}	PM less than 10 μm in mass median aerodynamic diameter (technically, this refers to the cut size of the sampler inlet or impactor stage for which there is a 50% collection efficiency)
RAM-1	real time aerosol monitor
RBC	red blood cell
SD	standard deviation
SE	standard error of the mean
SO_4^{2-}	sulfate
TAT	thrombin-antithrombin complex
tPA	tissue plasminogen activator
WBC	white blood cell
XRF	x-ray fluorescence spectroscopy

CRITIQUE
Health Review Committee

INTRODUCTION

Particulate matter (PM*) in ambient air is a complex mixture of different sizes and varying chemical composition. Epidemiologic studies have found an association between short-term increases in ambient PM levels and increases in hospital admissions and deaths from cardiovascular disease (for a review, see Environmental Protection Agency [US] 1996). The biological mechanisms by which PM may affect cardiovascular events are not well understood, however. Assessing the effects of particles in appropriate animal models is critical to understanding how PM may exert adverse health effects.

In 1998, HEI issued Request for Applications (RFA) 98-1, "Characterization of Exposure to and Health Effects of Particulate Matter," inviting proposals aimed at investigating the health effects of PM and specifically to

- characterize potential pathophysiologic effects caused by PM in sensitive subjects,
- define the relation between particle characteristics and the dose, distribution, and persistence of particles in the respiratory tract,
- identify the kinds of particles or particle attributes that may cause toxicity,
- investigate the diseases or conditions that affect sensitivity, and
- delineate how copollutants affect or contribute to the physiologic response to particles.

In response to the RFA, Christine Nadziejko and colleagues proposed to test whether acute exposure to PM alters blood coagulation (clotting) parameters. Such changes may increase the risk of heart attack. The investigators proposed to measure several blood coagulation factors in healthy rats after exposure to concentrated ambient particles alone or combined with ozone or nitrogen dioxide. In a second phase of the project, they proposed to compare the effects of exposure to concentrated ambient particles with exposure to PM from specific sources (ie, coal fly ash or diesel exhaust). The proposed studies would include dose-response and time-course experiments, and blood samples would be obtained at intervals from each rat via a jugular catheter.

The HEI Health Research Committee funded Dr Nadziejko's one-year study to investigate the effects of concentrated ambient particles on blood coagulation before performing studies with copollutants or other types of particles. In their revised plan, the investigators proposed to expose rats once for 6 hours to approximately 300 µg concentrated particles per m^3 air and to evaluate whether the catheters used for blood sampling have an effect on the blood coagulation parameters of interest. The Health Research Committee selected this study for funding because the evaluation of changes in coagulation parameters was seen as an interesting approach to understanding the possible mechanism of PM effects on cardiovascular disease.†

The intent of this Critique is to aid HEI sponsors and the public by highlighting the strengths of the study, pointing out alternative interpretations of results, and placing the report in scientific perspective.

BACKGROUND

POSSIBLE MECHANISMS UNDERLYING PM EFFECTS

The association between increased levels of PM and increased hospital admissions and death observed in epidemiologic studies is relatively consistent in different locations, but a plausible biologic mechanism that might cause such effects has not been definitively established. At the time RFA 98-1 was issued, some animal studies had suggested that exposure to particles might produce alterations in cardiac function (Costa and Dreher 1997; Godleski et al 1997; Gordon et al 1998) and pulmonary inflammation (Killingsworth et al 1997; Kodavanti et al 1997) in healthy and compromised animals.

One hypothesis, proposed by Seaton and colleagues (1995), suggested that low level inflammation caused by particles deposited deep in the lung could lead to systemic inflammation (that is, mediated by factors in the circulation) and to subsequent changes in blood coagulation, thus

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

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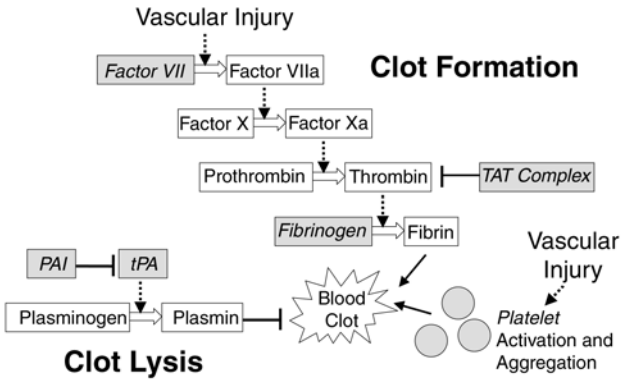
† Dr Christine Nadziejko's fifteen-month study, *Health Effects of Particles: Dosimetry and Pathophysiological Changes Related to Particle Size and Composition*, began in August 1998. Total expenditures were $241,730. The draft Investigators' Report from Dr Nadziejko and colleagues was received for review in December 1999. A revised report, received in May 2001, was accepted for publication in June 2001. During the review process, the HEI Health Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the HEI Health Review Committee's Critique.

possibly affecting cardiovascular function. Increased blood coagulation may increase risk for a heart attack by causing thrombus (clot) formation on an atherosclerotic plaque (area of blood vessel with a thickened wall in individuals with coronary artery disease). In support of this hypothesis, an epidemiologic study found an increase in blood viscosity during an air pollution episode (Peters et al 1997). Blood viscosity is largely determined by concentrations of fibrinogen, a factor involved in blood coagulation (see section on Coagulation Pathway).

Pulmonary inflammation leads to rapid increases in levels of fibrinogen, a so-called *acute phase reactant*, which is released into the circulation as part of the inflammatory response. Thus, fibrinogen plays a role in tissue inflammation and repair as well as blood coagulation. Epidemiologic evidence has found that high levels of fibrinogen and possibly other coagulation factors such as factor VII are associated with increased risk for heart attack and stroke (reviewed by Koenig 1998).

COAGULATION PATHWAY

Critique Figure 1 illustrates the main steps in the coagulation pathway and the 6 coagulation factors that Nadziejko and colleagues proposed to measure (in italics in this and the next paragraph). Coagulation, a complex process to repair injured tissue, consists of a sequence of vascular constriction, blood platelet activation and aggregation, formation of a fibrin clot, and finally clot lysis after tissue repair. The coagulation pathway involves a variety of coagulation factors that exist in inactive and active forms. As each factor is activated, it triggers activation of the next factor in the pathway. As shown in Critique Figure 1, the pathway starts with conversion of *factor VII* to the active form factor VIIa, followed by conversion of factor X to factor Xa, which in turn triggers conversion of prothrombin to thrombin. In the final step, thrombin activates *fibrinogen* conversion to fibrin, leading to a fibrin mesh around the aggregated *platelets* and resulting in a blood clot. Thrombin and fibrinogen are also involved in platelet aggregation; thrombin is involved in platelet activation as well (not illustrated).



Critique Figure 1. Coagulation cascade. Boxes: coagulation factors. Shaded boxes and italics: factors measured in this study. Open arrows indicate conversion from the inactive to the active form. Dotted arrows indicate activation of the next step in the cascade. Solid arrows indicate stimulation. Lines with a perpendicular bar indicate inhibition.

In order to prevent excessive clotting, several factors either inhibit steps in the coagulation pathway or dissolve the blood clot. Antithrombin inactivates thrombin by binding to it and forming *thrombin-antithrombin complex* (TAT). Clot lysis occurs when *tissue plasminogen activator* (tPA) converts plasminogen to plasmin. In turn, tPA is inhibited by *plasminogen activator inhibitor* (PAI). The time frame of changes in these parameters is shown in Figure 1 of the Investigators' Report. In addition, several positive and negative feedback loops exist within this pathway (not illustrated). In a healthy individual, these positive and negative feedback mechanisms slowly restore hemostatic balance.

Until recently, few animal studies had investigated fibrinogen or other blood coagulation parameters in relation to particulate air pollution. One preliminary study in rats exposed to ultrafine carbon black particles found an increase in blood coagulation factor VII, but no changes in fibrinogen (Donaldson et al 1998). By measuring changes in the levels of factors, Nadziejko and colleagues proposed to monitor changes in the coagulation process in response to exposure to PM.

TECHNICAL EVALUATION

AIM AND APPROACH

The hypothesis of this short study was that acute exposure to elevated levels of PM causes changes in blood coagulation parameters. The consequent increases in blood coagulation may lead to increased risk of heart attack or stroke, especially in people with arterial narrowing from atherosclerosis. The investigators proposed to expose healthy rats to concentrated PM from New York City air (target concentration 300 μg particles per m^3) or filtered air for 6 hours and to take blood samples from indwelling catheters to measure coagulation parameters before exposure and at 6, 12, and 24 hours after the start of exposure. Six coagulation parameters were selected (platelet number and levels of fibrinogen, TAT, tPA, PAI, and factor VII). In addition, red and white blood cells were counted to assess

the effect of repeated blood sampling; thymic lymphocytes were counted as an indicator of stress.

STUDY DESIGN AND METHODS

Exposure Procedures

Male Fischer 344 rats (250–275 g) were exposed nose only to concentrated ambient PM or filtered air for 6 hours. Five experiments were performed during 8 weeks in the summer of 1999. The investigators monitored pollution and weather forecasts to select days of potentially high PM concentrations. Each experiment involved two groups of rats, one exposed to concentrated PM and another to filtered air, both in inhalation chambers in the same room.

Two control experiments were performed to establish whether the exposure setup introduced unintended variability. First, a sham-exposed group of rats was exposed to filtered air from the concentrator by placing a high efficiency particulate air (HEPA) filter on the concentrator inlet and compared to rats that were maintained undisturbed in cages to identify any effect of stress from handling, confinement to the inhalation chamber, or noise. Second, another sham-exposed group of rats was compared to rats exposed to filtered room air that did not pass through the concentrator. The main difference between these latter two groups was therefore the noise of the concentrator; both groups were confined to nose-only inhalation chambers.

The concentrator used in this study was developed by Gordon and colleagues (1999) (Figure 2 of the Investigators' Report). The unit concentrates ambient particles between 0.5 and 2.5 μm mean aerodynamic diameter most efficiently, on average about 10-fold. Larger particles are removed, but smaller particles are neither concentrated nor removed. In an attempt to achieve the target particle concentration of 300 $\mu g/m^3$, the investigators selected days with high ambient concentrations of particles, as determined by PM_{10} and $PM_{2.5}$ (PM less than 10 μm and 2.5 μm in mass median aerodynamic diameter) data from the closest central monitoring station. Particle mass was measured by a real-time aerosol monitor and by weighing filters. Particle metal composition was determined with x-ray fluorescence spectroscopy of the gravimetric filters; separate filter samples were collected for acid and anion analysis.

Blood Sampling Procedures

To facilitate relatively stress-free blood sampling at multiple time points, animals with an implanted jugular vein catheter were purchased. Surgery took place at least 2 days before transport from the vendor to the laboratory; rats were allowed to acclimate for at least 7 days before exposure started.

The investigators performed pilot studies to establish proper blood sampling procedures and analyses and to determine whether repeated sampling or the sampling site affected the blood coagulation parameters of interest. First, they determined whether sample dilution or storage influenced platelet counts. They did this because rats have much higher platelet counts than humans; therefore, automated equipment calibrated for human blood might give false readings on undiluted rat blood. Next, they examined the possible effect of repeated sampling via a catheter on coagulation parameters. Finally, the investigators determined whether parameters differed between blood sampled by catheter or by cardiac puncture because a different location and sampling technique might affect levels of the coagulation parameters they monitored.

Blood samples (0.8 mL) were taken before exposure and at 6, 12, and 24 hours after the start of exposure. Samples were stored at –70°C until further analysis. White blood cells, red blood cells, and platelets were counted with a Coulter counter (model T540). Fibrinogen was measured by a heat precipitation assay; factor VII was measured by a clotting activity assay; TAT was measured by enzyme-linked immunosorbent assay. PAI and tPA were both measured by a fibrin chromogenic assay. To monitor stress, the investigators counted lymphocytes in the thymus of control groups at the end of the experiments because release of corticosterone during stress would decrease the number.

Statistical Analysis

The data were analyzed by analysis of variance to identify differences between rats exposed to filtered air and rats exposed to concentrated PM on each of the 5 experimental days. Additional analyses were performed on a combined data set of the 5 experiments. Because the investigators suspected that animal-to-animal variability in preexposure baseline measures might obscure treatment effects, they tested for significant differences between preexposure values of each set of filtered air and concentrated PM groups. When a significant difference was observed, additional analyses were performed on postexposure values from which the preexposure value had been subtracted.

RESULTS AND INTERPRETATION

Blood Sampling Pilot Studies

The investigators found that a 1:2 dilution of blood with saline was optimal for counting platelets and that analyses should be performed between 4 and 24 hours after sampling because counts decreased in the first 4 hours but then stabilized. They also found that coagulation parameters were not affected by repeated sampling via a jugular catheter. Only

white and red blood cell counts were decreased at 6, 12 and 24 hours after the start of exposure, which is expected due to the loss of blood plasma and cells. When they analyzed blood sampled via a catheter or via cardiac puncture, they found many small differences, suggesting that tissue injury from cardiac puncture had activated coagulation. Sampling via a catheter did not appear to cause changes indicative of increased coagulation. Thus, blood sampling via catheters seemed an appropriate procedure.

Exposure Pilot Studies

In the first pilot study, the only significant difference found between rats that were sham exposed and rats maintained in quiet cages was in fibrinogen levels, which were lower in the sham-exposed group. The authors indicated that this may have been due to baseline differences between the 2 groups. No significant difference was found in thymocyte numbers measured as an indicator of stress. Thymocyte death occurs only after sustained increases in blood corticosterone due to long-term or high intensity stress (Ashwell et al 2000), however, and thus is not a sensitive measure of stress. For this reason, this result does not necessarily indicate that the animals were not stressed during inhalation exposure.

In the second pilot study, the investigators found a significant increase in TAT levels in the sham-exposed group compared to the filtered air group. TAT levels were lower than in the first pilot experiment, however, so the significance of this finding remains uncertain. The investigators also found a significant difference in factor VII levels, but the levels were similar to the first experiment. Thus the observed difference was attributed to interindividual differences, and the investigators concluded that exposure to filtered room air was an appropriate control exposure.

Effect of Concentrated PM on Coagulation

Ambient PM levels on the 5 experimental days ranged from 19 to 88 $\mu g/m^3$, resulting in concentrated PM levels of 95 to 341 $\mu g/m^3$, lower than the target concentration of 300 $\mu g/m^3$ on 3 occasions. Some of the coagulation parameters were significantly different after exposure of rats to concentrated PM compared with measures from rats exposed to filtered air. These results are summarized in Table 8 of the Investigators' Report. Increases were observed in tPA levels, platelet counts, thymocyte counts, TAT levels, and fibrinogen levels corrected for baseline. Decreases were observed in factor VII and fibrinogen corrected for baseline (on a different experimental day). However, each of these changes was observed on one of 5 experimental days only, and no dose-related changes were observed. Examination of elemental composition of concentrated PM on each experimental day did not explain why results were different in different experiments. Analysis of a combined data set of the 5 experimental days did not provide additional insight: only one change was observed, an increase in TAT levels. Therefore, the main conclusion of this study is that moderate levels of concentrated PM did not lead to changes in coagulation in this small number of healthy rats.

DISCUSSION

The authors were among the first researchers to address the association between PM and changes in blood coagulation parameters in an experimental study. The experimental design was straightforward, and the investigators performed a range of pilot studies to optimize the experimental procedures. Due to the limited scope of the study, however, the lack of a consistent effect of concentrated PM exposure on blood coagulation is difficult to interpret.

Several reports support an association between air pollution and changes in blood coagulation. A human controlled-exposure study by Coppola and colleagues (1989) found a decrease in blood viscosity and platelet function after a 30-minute exposure to gasoline-engine exhaust (containing 206 ppm carbon monoxide; no particle measures were provided). Whether these effects were due to particles or gases remains undetermined. A preliminary report of an ongoing study, in which 4 human volunteers were exposed for 2 hours to 92 ± 25 $\mu g/m^3$ concentrated ambient PM, reported a trend for plasma fibrinogen levels to be higher compared with levels in volunteers exposed to filtered air (Petrovic et al 2000). In addition, Ghio and colleagues (2000) reported increases in fibrinogen in blood of volunteers exposed for 2 hours to 23 to 311 $\mu g/m^3$ concentrated ambient PM.

Recent epidemiology studies have reported varied results. In support of effects of PM on coagulation, Pekkanen and colleagues (2000) reported an association between daily concentrations of air pollution and plasma fibrinogen in London. In a large US population, PM_{10} was associated with increased fibrinogen levels, platelet counts, and white blood cell counts (Schwartz 2001). A study that monitored tunnel construction workers exposed to 0.3 to 1.9 mg/m^3 respirable dust in an 8-hour work shift showed increased levels of an inflammatory marker, interleukin-6, and fibrinogen levels in blood (Hilt et al 2002). In contrast, Seaton and colleagues (1999) found no changes in fibrinogen or factor VII related to air pollution in elderly individuals, but they did find increases in C-reactive protein (an inflammatory acute phase reactant) and decreases in blood platelet and red blood cell counts. Prescott and colleagues (2000) also found no evidence for interactions between PM concentrations and fibrinogen levels.

Animal studies using a variety of particle species are similarly inconclusive. A recent animal study has reported

increased plasma fibrinogen after intratracheal instillation of residual oil fly ash in healthy rats (Gardner et al 2000). Effects were seen only at the highest dose (8.3 mg/kg body weight), and no effects were observed in other blood and hemostatic parameters. The relevance of these findings to humans inhaling low concentrations of ambient particles is questionable (Gordon and Reibman 2000). Campen and colleagues (2001) investigated the effects of inhaled nickel and vanadium fine particles on cardiac and vascular parameters in rats. They found no significant changes in fibrinogen levels after rats inhaled either vanadium or nickel multiple times, but they noted a nonsignificant trend for increased fibrinogen levels after rats inhaled the highest concentrations of nickel (1.3 and 2.1 mg/m^3). The increase in fibrinogen levels 24 hours after inhaling a combination of nickel and vanadium (0.5 mg/m^3 each) was significant, however. In another study in healthy rats, exposure to 1000 µg/m^3 ultrafine carbon black particles led to increased factor VII levels but no changes in fibrinogen levels (Donaldson et al 1998, 2001). These results could not be replicated in subsequent experiments, however (K Donaldson, personal communication, April 2002). A study in dogs exposed via a tracheal tube to 203 to 360 µg/m^3 concentrated ambient PM for 6 hours on 3 consecutive days found no changes in fibrinogen levels, red or white blood cell counts, or platelet counts (Clarke et al 2000). A study in rats exposed for 1 week or 6 months to 30 to 1000 µg/m^3 diesel exhaust found no exposure-related changes in fibrinogen, TAT, factor VII, or platelet number but did find decreases in TAT and factor VII in some groups (Reed and Seagrave 2002). A study in anesthetized hamsters exposed intratracheally to 5 to 500 µg diesel exhaust particles reported a dose-dependent increase in thrombus formation (Nemmar et al 2002).

In summary, some human controlled exposure and epidemiologic studies have reported changes in blood cell counts, platelet counts, fibrinogen and factor VII levels in relation to air pollution, but other human studies and most animal studies have failed to find such changes. No consistent pattern has emerged so far.

The lack of a consistent effect of PM exposure on blood coagulation does not preclude an effect of PM exposure on coagulation under specific circumstances (for example, in individuals with preexisting disease). Some factors that may have contributed to the lack of effect in the current study include the use of healthy animals, the time points evaluated, and the lower than expected concentration of ambient PM on several exposure days. Individuals with preexisting lung or heart disease are thought to be more susceptible to the health effects of particle exposure than healthy individuals through a variety of mechanisms (eg, Burnett et al 1995; Thurston 1996; Peters et al 1997; Pope 2000). Supporting evidence has been provided by studies in which animals with preexisting pulmonary or cardiac disease appeared more vulnerable to effects of concentrated PM on lung inflammation (Costa and Dreher 1997; Gordon et al 1998; Kodavanti et al 1998). In a recent study in which dogs with an occluded coronary artery were exposed to concentrated ambient particles, no changes were observed in white blood cell or platelet counts but decreases were found in hematocrit and hemoglobin (red blood cell parameters; Savage et al 2002). The authors suggested that red blood cells may have been sequestered somewhere in the body. Another study in which hypertensive rats were used to evaluate inflammation and coagulation parameters after intratracheal instillation of resuspended oil fly ash and ambient particles reported increases in PAI-1 (Backus et al 2002).

Nadziejko and colleagues measured coagulation parameters in healthy animals. Future studies may expand the scope by testing blood coagulation parameters in compromised animals, especially animals with impaired coagulation or atherosclerosis. There are two pathways by which animals and humans may be more susceptible to effects of PM on coagulation: differences in the coagulation system (clotting mechanism) or in blood vessels (ie, increased atherosclerotic plaques, which promote clotting on their surfaces after rupture). Such clots or thombi may in turn occlude a coronary artery and thereby cause heart failure. Currently, mouse models are available in which various parts of the coagulation system have been inactivated by genetic manipulation (knockout mice). For example, mice lacking genes for the coagulation factors tPA, factor V Leiden, or thrombomodulin (a vascular surface receptor for thrombin) show increased clotting compared with wild-type mice (Carmeliet 1995). Mice lacking the gene for apolipoprotein E (apo-E, a plasma lipoprotein that degrades cholesterol) show increased atherosclerosis (Hofker et al 1998). Another animal model of interest is the hypercholesterolemic rabbit, which develops atherosclerosis due to high circulating cholesterol (Fan and Watanabe 2000). These animal models may be more likely to show changes in blood coagulation after PM exposure. In addition, using species other than rats may avoid problems arising from the high blood platelet counts in rats in comparison with humans. New studies on the effects of air pollution on cardiac and endothelial endpoints in apo-E deficient mice (Luchtel et al 2002) and hyperlipidemic rabbits (Quinlan et al 2002; Suwa et al 2002) have not evaluated coagulation parameters yet.

Experimental procedures, such as the timing of sample collection or the nature of the sampling technique, may explain the lack of effect in this study. The investigators

evaluated two blood-sampling techniques, via a catheter and via cardiac puncture, and concluded that sampling via catheter was the preferred option to avoid clotting and minimize stress during repeated sampling. Even though no obvious adverse signs (ie, clots) were observed, catheter use may still have influenced the blood coagulation parameters of interest. In addition, the animals probably were stressed during nose-only exposures, especially if they were not accustomed to the procedure. Measurement of thymocyte number may not be sensitive enough to detect whether animals were stressed or not. When animals are stressed, blood catecholamine levels increase, which in turn influence blood coagulation by increasing fibrinogen and factor VIII concentrations and increasing fibrinolysis (Jern et al 1989; Grant 1990). In this study, the small but significant differences in coagulation parameters between filtered air or sham-exposed animals and animals that were undisturbed may be manifestations of this phenomenon.

The blood coagulation system is complex with many positive and negative feedback loops to maintain homeostasis. PM exposure may have caused changes in parts of the coagulation system that went undetected in the current study but might have been detected with other types of measurements. For example, one could perform functional assays of clotting (such as platelet aggregation or clotting or prothrombin time) or investigate endothelial aspects of thrombosis. Measuring the activity of a compound may be more informative than measuring absolute concentrations (Mannucci and Giangrande 1992). For instance, a 30% change in activity of a compound may be clinically significant, regardless of the actual concentration of that compound. In addition, the impact of an increase or decrease in a single parameter on the entire system is difficult to determine. Similarly, it is unclear how big a change would be biologically significant.

Another factor that may have contributed to the lack of effect of PM on coagulation in this study was the lower than expected ambient PM concentrations and concentration factors (about 5-fold) as well as the lack of concentration of smaller particles. The Gerber concentrator was developed at New York University and has been evaluated previously (see HEI Commentary of Gordon et al 2000). The unit concentrates particles around 1.0 µm with most efficiency, while smaller particles are concentrated with much lower efficiency. Particles below 0.2 µm, where much of the mass of particles from mobile sources is found, are not concentrated. Whether smaller particles would have an effect on coagulation parameters remains to be determined because exposure to smaller particles was minimal in this study.

As with other particle concentrators, the concentration factor varied in day-to-day operation. Moreover, analysis of particle composition revealed differences between the concentrated particles and the ambient particles, illustrated by relative loss of sulfate (concentrated only about 1.5-fold) and changes in metal composition (Figure 3 of the Investigators' Report). These differences could be explained by the differential concentration of particles of different sizes or by internal components of the concentrator contributing to particle generation (discussed in Gordon et al 2000). Nevertheless, examination of elemental composition of concentrated PM on each experimental day did not explain why results were different in different experiments. To complement the current study, it might be worthwhile to perform similar studies with higher concentrations of fine and ultrafine particles, particles from specific mobile and stationary sources, or laboratory-generated particles of known toxicity (such as nickel or other transition metals).

SUMMARY AND CONCLUSIONS

This was a well-designed pilot study that will contribute to designing future experiments to address the important topic of PM effects on coagulation. It found no consistent effect of moderate levels of concentrated New York City PM on six blood coagulation parameters in healthy rats. This does not preclude involvement of the coagulation pathway in adverse health effects of PM on the cardiovascular system, however. In future studies, changes in coagulation may be found in compromised animals with exposure to higher levels of PM or with exposure to particles of different size or composition.

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