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Use of Physical Chemistry and in Vivo Exposure to Investigate the Toxicity of Formaldehyde Bound to Carbonaceous Particles in the Murine Lung

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Includes the Commentary of the Institute's Health Review Committee

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HEI HEALTH EFFECTS INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate materials), and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 120 projects at institutions in North America and Europe.

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HEI Statement

Synopsis of Research Report Number 53

Effects of Formaldehyde and Particle-Bound Formaldehyde on Lung Macrophage Functions

BACKGROUND

Exposure to formaldehyde may occur at work, at home, and outdoors. Human studies, animal inhalation studies, and cell culture assays suggest that formaldehyde potentially can cause adverse effects on human health. Therefore, the Clean Air Act Amendments of 1990 defined formaldehyde as a toxic air pollutant subject to regulatory action. Emissions from motor vehicles using gasoline and diesel fuels contribute to the outdoor levels of formaldehyde. The projected use of methanol as an alternative fuel and in fuel blends may further increase outdoor formaldehyde levels because alcohol combustion yields more aldehydes than conventional fuel combustion.

In addition to gases, the combustion of fossil fuels produces respirable carbon-based particles that can deposit in the lower respiratory tract. Because gas molecules can bind to particle surfaces, these particles may serve as carriers and transport the bound gases deep into the lungs. Formaldehyde is a water-soluble gas that normally deposits in the upper respiratory tract. Adsorption of formaldehyde to particles could carry it deeper into the lungs and alter its toxic action.

Alveolar macrophages in the lower respiratory tract protect the lungs from inhaled microorganisms. Impairment of alveolar macrophage function could compromise a person's resistance to respiratory infection. The Health Effects Institute sponsored this study to examine the effects of inhaled formaldehyde, with and without carbon particles, on alveolar macrophages.

APPROACH

Dr. George Jakab and associates exposed mice to varying levels (ranging from 0.5 to 15 parts per million [ppm]) of formaldehyde alone or to formaldehyde (5 and 2.5 ppm) mixed with carbon black particles. Carbon black particles were chosen because of their similarity to combustion-derived particles. Different alveolar macrophage functions were evaluated using two assays. First, the investigators measured the ability of alveolar macrophages to kill the bacteria *Staphylococcus aureus*. Mice were exposed to pollutants either before, after, or at both times relative to inhaling the bacteria. The number of bacteria still alive in the lungs four hours after inhaling the bacteria was counted. Second, the investigators measured the ingesting, or phagocytic, capacity of the macrophages. Mice were exposed to the pollutants, and at intervals ranging from one to 60 days later, alveolar macrophages were washed out of the lungs and their phagocytic activity was measured. Chemical analyses of the interactions between formaldehyde and carbon black particles also were conducted to determine the amount of formaldehyde potentially available for interacting with lung cells.

RESULTS AND IMPLICATIONS

An extremely high concentration (15 ppm) of formaldehyde, which is far higher than outdoor concentrations, was needed to impair the killing of *S. aureus* when mice were exposed to formaldehyde after inhaling the bacteria. However, when mice were exposed to formaldehyde both before and after inhaling the bacteria, only 1 ppm of formaldehyde was needed to impair bacterial killing. Exposing mice to formaldehyde and carbon black particles had no effect on bacterial killing but did depress phagocytosis. This depression was not apparent until five days after exposure and was maximal 25 days after exposure. The chemical analyses revealed that only 1% of the formaldehyde was bound to the carbon black particles, which may account for the absence of an effect of the combined exposure on bacterial killing. It is not known why the combined exposure depressed alveolar macrophage phagocytosis or why this effect was delayed.

These experiments demonstrated that inhalation of formaldehyde can impair the capacity of mouse alveolar macrophages to kill certain bacteria and that inhalation of formaldehyde mixed with carbon black particles can depress alveolar macrophage phagocytosis. However, the detection of these effects depended on exposure protocol and the time of assay after an exposure.

This Statement is a summary, prepared by the Health Effects Institute (HEI) and approved by the Board of Directors, of a research project sponsored by HEI from 1986 to 1990. This study was conducted by Drs. George J. Jakab and associates at The Johns Hopkins University and the University of Vermont. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee. JAKAB 53

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Use of Physical Chemistry and in Vivo Exposure to Investigate the Toxicity of Formaldehyde Bound to Carbonaceous Particles in the Murine Lung

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INVESTIGATORS' REPORT

Use of Physical Chemistry and in Vivo Exposure to Investigate the Toxicity of Formaldehyde Bound to Carbonaceous Particles in the Murine Lung

George J. Jakab, Terence H. Risby, and David R. Hemenway

ABSTRACT

Knowledge about the health effects of exposure to formaldehyde associated with automotive emissions is of pivotal importance in the risk assessment of this agent. Mobile sources emit many combustion-derived pollutants, including formaldehyde, in association with respirable carbon particles. Because it is hydrophilic, most of the inhaled formaldehyde is absorbed in the upper respiratory tract. However, if the organic vapor is adsorbed on respirable particles, formaldehyde may be deposited in the deep lung with the inhaled particles and may be available to interact adversely with cells along the lung parenchyma.

On the respiratory surface, the alveolar macrophage phagocytic system plays the pivotal role in defending the lung against infectious agents. Susceptibility to respiratory infections is a relevant and sensitive indicator of the adverse effects of air pollution because acute and chronic exposures to a variety of air pollutants have been shown to decrease pulmonary antibacterial defenses.

The goal of this research was to investigate whether exposure to formaldehyde decreases resistance to respiratory infections through dysfunctions of the alveolar macrophage phagocytic system. The study also explored whether interactions between formaldehyde and respirable carbon black particles alter susceptibility to respiratory infections and impairment of alveolar macrophage phagocytosis by delivering adsorbed formaldehyde to the deep lung with the inhaled particles.

A carbon black, Regal GR, was used in these studies as a surrogate for the carbonaceous core of Diesel particulate matter. This material was selected to represent the worst-case scenario because the carbon black was expected to adsorb formaldehyde strongly.

To accomplish this goal, mice were exposed to formaldehyde and to carbon black and formaldehyde combinations; increased susceptibility to respiratory infections was quantified by alveolar macrophage–dependent intrapulmonary killing of *Staphylococcus aureus* after an inhalation challenge with the bacterium.

The salient findings of the bactericidal studies are as follows: Fifteen parts per million (ppm)* formaldehyde impaired the intrapulmonary killing of *S. aureus* when exposure followed the bacterial challenge. One ppm formaldehyde impaired the intrapulmonary killing of *S. aureus* when exposure preceded and was continued after the bacterial challenge. Coexposures to target concentrations of 3.5 mg/m^3 carbon black and 2.5 ppm formaldehyde, or 10 mg/m^3 carbon black and 5 ppm formaldehyde after the bacterial challenge had no effect on the intrapulmonary killing of *S. aureus*. Preexposure for four hours per day for four days to target concentrations of 3.5 mg/m^3 carbon black and 2.5 ppm formaldehyde had no effect on the intrapulmonary killing of *S. aureus* when the assay was performed one day after the cessation of exposure.

To determine whether any possible effect was delayed, a surrogate assay for alveolar macrophage phagocytic function, Fc-receptor–mediated phagocytosis, was performed 1, 3, 5, 10, 14, 25, and 40 days after the cessation of exposure. After exposure to target concentrations of 10 mg/m^3 carbon black and 5 ppm formaldehyde, alveolar macrophage Fc-receptor–mediated phagocytosis was progressively suppressed through day 25; thereafter, the phagocytic potential recovered by day 40.

In order to estimate effective doses of formaldehyde bound to carbon black, which is fundamental to the biologic studies, the physical chemistry of adsorption and desorption of formaldehyde onto different carbon blacks were investigated. These studies are important because the biologic response to carbon black and formaldehyde coexposures may be governed by the physicochemical interactions between the two agents. The physicochemical experiments demonstrated that 1.0% of the formaldehyde was adsorbed on the carbon black Regal GR that was used as the surrogate for carbonaceous particles from Diesel exhaust emissions.

This Investigators' Report is one part of the Health Effects Institute Research Report Number 53, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. George J. Jakab, Department of Environmental Health Sciences, The Johns Hopkins University School of Hygiene and Public Health, 615 North Wolfe Street, Baltimore, MD 21205.

* A list of abbreviations appears at the end of this report for your reference.

The data show that only a small amount of the organic vapor was adsorbed on carbon black Regal GR; therefore, little of the formaldehyde was deposited in the deep lung with the inhaled particle. However, this small amount is sufficient to impair alveolar macrophage phagocytosis. The phagocytic dysfunction is delayed in that the impairment is progressive for 25 days after the cessation of exposure. This was followed by an incremental recovery of phagocytic potential until alveolar macrophage phagocytosis returned to normal 40 days after the end of exposure.

INTRODUCTION

Uncertainty over existing petroleum supplies has resulted in the search for alternate fuels for transportation purposes. One of the suggested fuels is methanol, because it can be produced from a variety of readily available biomasses. Methanol is a cleaner burning fuel with significantly lower nitrogen oxide emissions than either petroleum-based gasoline or Diesel fuels (Tilman et al. 1975). However, although methanol use has the potential for reducing regulated pollutants, concern exists over the environmental health impact of the increased formaldehyde emissions from methanol-supplemented fuels burned in internal combustion engines (Houser and Lestz 1980).

The character of emissions varies dramatically with the type of engine, fuel, load, and fuel-to-oxidizer ratio. For example, Diesel engines emit more nitrogen oxides and 30 to 100 times more particles than do gasoline engines (National Research Council 1982). Diesel engines also emit significantly more formaldehyde than gasoline engines. Moreover, the emission of formaldehyde from either type of engine dramatically increases when either gasoline or Diesel fuel is supplemented with methanol (Houser and Lestz 1980; Broukhiyan and Lestz 1981; Lipari and Keski-Hynnila 1986). Thus, if methanol-supplemented fuels are widely accepted, the levels of atmospheric formaldehyde probably will increase.

As would be expected by its hydrophilicity, inhaled formaldehyde is absorbed primarily in the upper respiratory tract (Egle 1972). Indeed, formaldehyde is almost entirely deposited in the anterior nasal cavity of rodents (Heck et al. 1983). Experimental studies have demonstrated that the toxic effect of formaldehyde is primarily limited to the nasal passages of rodents with little evidence of overt toxicity in the lower respiratory tract or in tissues remote from the site of deposition (National Research Council 1981a; Heck et al. 1983; Bernstein et al. 1984; Gammage and Gupta 1984; Feinman 1988). Formaldehyde is emitted with respirable carbonaceous particles by combustion sources (Amann and Siegla 1982). If the organic vapor is adsorbed on the surfaces of these respirable carbon particles, formaldehyde may be deposited in the deep lung along with the inhaled particles, as suggested by the studies of Rothenberg and colleagues (1989). Therefore, the formaldehyde may be available to interact adversely with cells along the respiratory surface.

The health effects associated with exposure to formaldehyde cover a wide range of symptoms. Most of the symptoms involve the eyes, nose, and throat and are related to the irritating properties of formaldehyde. The severity of the response is related to the exposure concentration and can vary from person to person. In general, these are the acute adverse effects from inhaling formaldehyde at various exposure levels (Bernstein et al. 1984):

1. less than 0.05 to 1.5 ppm: 50% to 70% of people exposed may report no effects;
2. 0.05 to 1 ppm: odor threshold (before accommodation occurs);
3. 0.10 to 25 ppm: upper airway and nose irritation;
4. 5 to 30 ppm: lower airway and pulmonary effects;
5. 50 to 100 ppm: pulmonary edema, inflammation, pneumonia; and
6. greater than 100 ppm: death.

Although no routine monitoring programs exist for measuring atmospheric formaldehyde concentrations, typical levels in urban areas during the summer range from 0.005 to 0.02 ppm (Cleveland et al. 1977; Hoshika 1977) and tend to fluctuate with a seasonal pattern (Tanner and Meng 1984). Ambient atmospheric levels of formaldehyde in Los Angeles range from 0.004 to 0.086 ppm, of which 55% to 75% come from mobile sources (Grosjean 1982). Estimates suggest that the percentage of formaldehyde bound to particles is approximately 1% of the amount found in the gas phase (Grosjean and Friedlander 1975; Grosjean 1982).

Particulate matter emitted by Diesel engines consists of a carbonaceous core coated with solvent-extractable, combustion-derived materials. More than 95% of the particulate matter emitted by Diesel-powered light-duty vehicles consists of submicrometer agglomerates ranging from 0.06 to 0.7 μm in diameter (National Research Council 1982). This size distribution is important because the particles fall within the respirable size range that may reach the deep lung. Carbon comprises 10% to 20% by weight of urban aerosols (Countess et al. 1980; Wolff and Klimisch 1982). Approximately half of this amount is elemental carbon, a consequence of incomplete combustion of fossil fuels (Wolff and Klimisch 1982). Limited air monitoring studies suggest that elemental carbon concentrations range from 1 to 35 $\mu g/m^3$, with averages of about 7 $\mu g/m^3$ (Countess et al. 1980; Wolff 1985). Particles emitted by Diesel engines have

high specific surface areas of 30 to 100 m^2/g. These surface areas are similar to those typical of carbon blacks, but are much larger than those of ambient noncarbonaceous aerosols (National Research Council 1982). Diesel particles are capable of adsorbing relatively large quantities of volatile organic material; the solvent-extractable fraction is typically 5% to 40%, but it may be as high as 90% (National Research Council 1982).

An amorphous aciniform carbon black, Regal GR (Cabot Corp., Boston, MA), was selected as a model particle for the carbonaceous core of Diesel particulate matter. The physical and chemical properties of this carbon black and an oxidized carbon black, Mogul L (Cabot Corp.), were determined. The physicochemistry of the intermolecular interactions between these carbon blacks and formaldehyde were quantified. These experiments included measuring intermolecular interactions between the surfaces of the carbon blacks with formaldehyde, with and without the presence of water and methanol vapors. Water and unburned methanol are major emission products from internal combustion engines operating on fuels supplemented with methanol (Lipari and Keski-Hynnila 1986) and, therefore, may affect the adsorption and desorption of formaldehyde on the carbonaceous particles.

Animal studies (LaBelle et al. 1955; Amdur 1960; Kilburn and McKenzie 1978) and human studies (Schoenberg and Mitchell 1975; Kilburn et al. 1978; Alexandersson et al. 1982; Frigas et al. 1984; Alexandersson and Henderstierna 1989; Green et al. 1989) suggest that respirable particles and formaldehyde vapors produce an interactive effect. These studies, however, used coexposures to uncharacterized particles, did not quantify the amount of particle-adsorbed formaldehyde, and used biologic endpoints that focused on the upper respiratory tract rather than events in the deep lung.

In the respiratory tract, the alveolar macrophage phagocytic system serves as the primary defense mechanism against inhaled particles that reach the distal lung (Green et al. 1977). In the alveoli, the alveolar macrophages engulf the deposited particles, thereby sequestering them from the vulnerable respiratory membrane. Resistance against staphylococcal infections assesses the in vivo integrity of the alveolar macrophage phagocytic system (Goldstein et al. 1977). The aerosol model of rodent infection (Green and Kass 1964) provides an excellent means of measuring pollutant-induced physiologic abnormalities of antibacterial activity because sufficient similarities exist between the defense mechanisms of rodents and humans to permit the use of the rodents as a surrogate (Goldstein 1984; Green 1984). Furthermore, the integrity of pulmonary antibacterial defenses is considered a relevant and sensitive indicator of the health effects of air pollution because a wide variety of air pollutants have been shown to impair the bactericidal mechanisms of the lung (Ehrlich 1980; Gardner 1984; Goldstein 1984; Pennington 1988).

In addition to in vivo bactericidal assays against *S. aureus*, ex vivo assays also can test the integrity of alveolar macrophage phagocytic function. Therefore, Fc-receptor–mediated phagocytic assays also were performed on alveolar macrophages lavaged from the lungs of mice after they were coexposed to carbon black and formaldehyde. In our hands, impairment of alveolar macrophage Fc-receptor–mediated phagocytosis correlates positively with suppression of pulmonary antistaphylococcal defenses (Gilmour et al. 1991).

The adopted threshold limit value for formaldehyde requires that a worker's eight-hour time-weighted average exposure level in their breathing zone be limited to 1 ppm. The adopted threshold limit value for carbon black is a time-weighted average of 3.5 mg/m^3 (American Conference of Governmental Industrial Hygienists 1990). In-depth investigations of the toxicology of particulate carbon black, with the emphasis on inhalation exposure, have found an absence of significant acute health effects (Nau et al. 1962; Rivin and Medalia 1983; Rivin 1986), whereas other investigators have characterized carbon black as a "nuisance dust" (Crosbie 1986; Uragoda 1989).

SPECIFIC AIMS

The goal of this study was to investigate whether exposure to formaldehyde decreases resistance to respiratory infections through dysfunctions of the alveolar macrophage phagocytic system. Additionally, the study aimed to explore whether the interactions of formaldehyde and respirable carbon black particles alter the susceptibility to infection by delivering the adsorbed formaldehyde to the deep lung with the inhaled particles. This aim was accomplished through in vivo experiments involving the alveolar macrophage–dependent intrapulmonary killing of *S. aureus*, ex vivo alveolar macrophage phagocytosis, and physicochemical experiments on the interaction of formaldehyde and carbon black. The specific objectives were:

1. to determine the concentration of formaldehyde that impairs pulmonary antibacterial defenses;
2. to determine the concentration of formaldehyde and carbon black particles that impairs pulmonary antibacterial defenses and alveolar macrophage phagocytosis; and
3. to study the physicochemical interactions between formaldehyde and respirable carbon black particles, including assessing the impact of methanol and water vapor on

formaldehyde absorption and desorption and evaluating the desorption of formaldehyde from carbon black particles into synthetic alveolar lung fluid.

METHODS

ANIMALS

White female Swiss mice weighing 20 to 23 g (Hilltop Laboratory Animals, Scottsdale, PA) and having no serologic evidence of Sendai virus infection were used. The animals were housed in filter-topped stainless-steel cages with wood shaving bedding and were provided with food and water ad libitum. The mice were acclimatized for one week prior to the beginning of each experiment. The National Institutes of Health Guidelines for care and use of laboratory animals were followed.

BACTERIAL CHALLENGE

Staphylococcus aureus (FDA strain 209P, phage type 42D) was used for bacterial challenge. Stock bacterial cultures were prepared by inoculating the organisms into a liter of brain-heart–infusion broth and incubating the suspension for 24 hours at 37°C in a rotary shaker water bath (model number G76, New Brunswick Scientific, New Brunswick, NJ). Thereafter, the bacterial preparation was concentrated 10-fold by centrifugation and resuspended in 80 mL of the brain-heart–infusion broth. After adding 20 mL of sterile glycerin, the bacterial preparation was aliquotted into 1-mL portions and frozen at −20°C.

For bacterial challenge, 1 mL of the stock bacterial preparations of *S. aureus* was inoculated into 200 mL of trypticase soy broth and incubated at 37°C in the rotary shaker water bath. After 18 hours, the culture was centrifuged (3,000 × g, 10 minutes), washed twice with 0.1 M phosphate-buffered saline (PBS, pH 7.6) and resuspended in 10 mL of trypticase soy broth.

A previously described (Ruppert et al. 1976) modification of the Henderson (1952) aerosol apparatus was used to challenge the animals via inhalation with the bacteria. The apparatus consisted, in sequence, of a Collison-type atomizer activated by compressed air, a 6- by 60-cm length of Plexiglas tubing to mix the nebulized agent with diluting air, a large Plexiglas cylindrical chamber (28- by 80-cm) containing six cylindrical wire cages, a fiber glass prefilter, two absolute type bacterial high-efficiency particulate air (HEPA) filters, and a vacuum pump. The nebulizer was activated with 15 psi of compressed air. At a rate of 6 L/min, the nebulizer produced a continuous cloud of small infectious droplets, 97% of which had an aerodynamic particle mass diameter of 3.5 μm or less (Jakab and Green 1972), as determined with an Andersen (1958) sampler.

The outlet of the nebulizer emptied into the open end of the small Plexiglas tubing, in which the bacterial cloud from the nebulizer was mixed with a larger volume of air. The secondary air entered the mixing chamber around the nozzle of the atomizer. The vacuum pump located at the downstream end of the apparatus maintained the secondary air flow of approximately 20 L/min, as measured with a hot-wire anemometer. Each end of the large cylindrical exposure chamber was removable and equipped at the center with a rectangular baffle plate suspended perpendicularly to the air stream. This baffle plate removed large droplets and provided more uniform mixing and distribution of the bacteria in the exposure chamber.

The six cylindrical cages located in the exposure chamber were made of stainless-steel woven wire. Each cage held 12 individually separated mice to prevent the huddling of animals, which would have altered the number of bacterial particles the animals inhaled. To assure more uniform distribution of the aerosol in the chamber, the cages rested on 2-cm-high offsets, which allowed a 2-cm airspace between the cages and the chamber wall. This space then was blocked by placing Plexiglas rings between the cages, thereby channeling the bacterial particles through the space holding the animals (Ruppert et al. 1976). The aerosol generation apparatus, exposure chamber, and filter system were located in a HEPA-filtered reverse laminar flow hood (Baker Co., Sanford ME). Animals were challenged for 30 minutes with the staphylococcal cloud, during which time 1×10^5 to 5×10^5 of the staphylococci were deposited in the lungs.

BACTERICIDAL ASSAY

Pulmonary bactericidal activity was assessed by previously described methods (Ruppert et al. 1976). Animals were killed by luxation of the neck either immediately (zero time) or four hours after the end of bacterial challenge. The lungs were aseptically removed, trimmed of the trachea and major bronchi, and homogenized in 3 mL of iced trypticase soy broth with an all-glass tissue homogenizer (model #K41, Tri-R Instruments, Rockville Center, NY). A 1.0-mL aliquot of the lung homogenate was diluted 10-fold in sterile PBS and a 0.1-mL aliquot of the appropriate dilution was cultured quantitatively in quadruplicate on Petri-X dishes by standard microbiologic pour-plate methods using trypticase soy agar supplemented with 5% sodium chloride. The Petri dishes were incubated for 48 hours at 37°C and thereafter visually counted with a Quebec-type bacterial colony counter. Pulmonary bactericidal activity in each animal

was calculated as the percentage of initial viable bacteria remaining four hours after the end of bacterial challenge by the following formula (Ruppert et al. 1976):

Percent viable bacteria remaining = (bacterial count [4 hours]/mean bacterial count [0 hours]) × 100.

The data are presented as the differences in the percentage of change between control and exposed values in viable bacteria remaining four hours after bacterial challenge.

ALVEOLAR MACROPHAGE FC-RECEPTOR–MEDIATED PHAGOCYTOSIS

Preparation of Antibody-Coated Erythrocytes

Sheep erythrocytes (RBCs) in Alseever's solution (Beckton and Dickinson, Hunt Valley, MD) were washed three times in RPMI tissue culture medium and resuspended in RPMI at a concentration of 5% (v:v). The RBCs were sensitized as previously described (Warr et al. 1979) by incubating them with a subhemagglutination concentration of rabbit IgG against RBCs (Cordis Laboratories, Miami, FL) for 30 minutes at 30°C. The sensitized RBCs were washed three times with RPMI and resuspended in RPMI at a concentration of 0.5% (v:v).

Collection of the Macrophages

Alveolar macrophages were obtained, and the phagocytic assay was performed as previously described (Warr et al. 1979). Briefly, mice were killed by intraperitoneal injection of sodium-pentobarbital and bled by cardiac puncture. The lungs were removed in toto and lavaged three times with 1.5 mL PBS containing 3 mM ethylenediaminetetraacetic acid. The recovered lavage fluid was centrifuged (200 × *g* for 10 minutes), and the cell pellet was resuspended at a concentration of 5×10^5 cells/mL in RPMI tissue culture medium supplemented with 10% newborn calf serum. Three to four aliquots of 200 µL of the cell suspensions were allowed to adhere to 22-mm^2 albumin coated glass coverslips in 35- × 10-mm plastic Petri dishes for 45 minutes (37°C, 5% CO_2).

Phagocytic Assay

After monolayering, the fluid was removed and immediately replaced with 200 µL of sensitized RBC medium, and the resulting suspension was incubated at 37°C for 45 minutes. After removing the RBCs by aspiration and washing the monolayers with RPMI, noningested RBCs were hypotonically lysed for 10 seconds and then rinsed several times with culture medium. The monolayers were then dried, fixed with methanol, and stained with Wright-Giemsa. The stained monolayers were read microscopically at a magnification of 1,000 × to quantify the percentage of macrophages containing RBCs and the number of RBCs ingested per phagocytic macrophage. The phagocytic index (total number of RBCs ingested by 100 macrophages) was calculated by multiplying the percentage of phagocytic macrophages by the mean number of RBCs ingested per phagocytic macrophage. One hundred macrophages were counted on each monolayer, with three to four monolayers counted per animal.

ANIMAL EXPOSURE TO FORMALDEHYDE VAPORS

Exposure Chambers

Two identical eight-cubic-foot stainless-steel and glass whole-body inhalation chambers were used to expose animals to either HEPA-filtered air (control) or to varying concentrations of formaldehyde. These chambers have been tested and described for reliable flow dynamics (Hemenway and MacAskil 1982). Airflow to the exposure chambers was maintained at 2.7 ft^3/minute, providing a theoretical rate of air turnover of 20 volume changes per hour.

Formaldehyde Generation

The method developed to generate formaldehyde vapors consisted of adding 30 g of powdered paraformaldehyde (JT Baker Co., Phillipsburg, NJ) to a 500-mL round-bottomed flask equipped with two tapered glass joints (24/40). A layer of boiling beads (glass number 3,000; 50 g) was added on top of the paraformaldehyde to minimize sporadic output of the material as air was introduced. The beads reduced pickup of sublimed paraformaldehyde and helped to stabilize the vapor evolution. Dry air was blown through the flask at a rate of 1.8 L/min. The flask was kept submerged in a constant-temperature water bath (± 0.1°C). Different concentrations of formaldehyde were generated by increasing or decreasing the temperature of the water bath. Typically, at a flow rate of 1.8 L/min through the formaldehyde generating flask, a temperature of 34°C produced a formaldehyde concentration of 0.9 ppm in the chamber (45°C produced 4.3 ppm; 54°C produced 9.2 ppm; 59°C produced 14.7 ppm). Minor adjustments in chamber concentrations were achieved by varying the air flow (± 0.2 L/min) through the formaldehyde generating flask. The formaldehyde from the flask was delivered through Teflon tubing to a small premixing chamber for final dilution with HEPA-filtered room air before entering the exposure chamber.

The formaldehyde generator had a higher mass generation rate when it was initially filled than after it had been operating awhile, as has been previously reported (Ho 1985). Therefore, after the generator was filled with paraformaldehyde, it was necessary to operate the unit overnight to provide a stabilized output for the inhalation studies. The

generator then produced stable concentrations in the range of 0.5 to 20 ppm formaldehyde with concentration variations less than 10% for one to two weeks, with the length of time dependent on the mass generation rate (higher mass flow rates depleted the paraformaldehyde at a faster rate).

Formaldehyde Monitoring

Chamber concentrations of formaldehyde were monitored continuously with an infrared vapor analyzer (Miran-103, Foxboro Co., Norwalk, CT) set at the appropriate wavelength to detect formaldehyde. The data output was monitored as a function of time by a chart recorder.

The formaldehyde monitor was calibrated at the beginning of each week with the National Institute for Occupational Safety and Health (NIOSH) chromotropic acid method (National Institute for Occupational Safety and Health 1977). Formaldehyde was generated in the exposure chamber until a steady-state concentration was reached (as determined with the Miran analyzer), at which time a 10-minute impinger (midget impinger number 7531-C, Ace Glass, Vineland, NJ) sample was obtained. The procedure was repeated at least two more times at different chamber concentrations to obtain a multipoint calibration. Triplicate samples were obtained at each concentration. After the impinger samples were assayed, a standard curve was drawn comparing the NIOSH chromotropic acid concentrations with the concentrations obtained from the Miran analyzer. The chromotropic acid analysis provided a means of calibrating the Miran analyzer with a known reference method. In this fashion, a calibration that used the Miran monitor for day-to-day real-time measurements was obtained.

Exposure Protocol

Exposure target concentrations were set before the animals were placed in the chamber. In order to place the animals in the chamber, the formaldehyde flow into the diluent air was halted, and the chamber was flushed with diluent air before the animals were placed in the chamber. Afterward, the formaldehyde flow was started again. Once the formaldehyde exposure level had been preset, evacuating the formaldehyde from the chamber, transferring the animals, and reestablishing the preset concentration took approximately five minutes. During the exposure period, the animals were housed in expanded stainless-steel wire cages and, when exposed for more than four hours (overnight), were provided food and water ad libitum.

The levels of formaldehyde were calculated using the permanent record of the formaldehyde concentrations as monitored by the chart recorder. The area under the curve was measured with an electronic graphics calculator (Numonics Corp., Lansdale, PA), and the exposure levels were reported as time-weighted averages for the exposure period. Because the real-time monitor allowed rapid adjustment for excursions from the target concentrations, the infrequent and slight drifts were immediately readjusted. The time-weighted averages were within ± 3% of the target concentrations.

CARBON BLACK AND FORMALDEHYDE COEXPOSURES

Exposure Chamber

Before the research on the coexposure of carbon black and formaldehyde could begin, it was necessary to design, construct, and test an inhalation chamber and generation system appropriate for exposing animals to the test substances. A flow-past, nose-only unit similar to a previously published design (Cannon et al. 1983) was employed. A nose-only unit was chosen because it limits the amount of fur contamination of the animals; the specific design was selected to minimize rebreathing and humidification effects on the carbon black aerosol-formaldehyde mixture test material (Stephenson et al. 1988). At the time that this research was initiated, there were no commercially available nose-only inhalation chambers using the concept of an internal distribution manifold to distribute the test material and directly transport it to the nose of each animal separately.

The inhalation chamber design used by Cannon and colleagues (1983) was not appropriate for formaldehyde because the polyvinyl chloride construction material would have adsorbed the formaldehyde or developed a surface charge that would influence wall losses of carbon black. Therefore, the system was redesigned, manufactured with 304 stainless steel, and electrically grounded. Additional modifications to the original design are detailed in our publication dealing with the design and testing of the chamber (Hemenway et al. 1990).

Figure 1 presents a schematic diagram of the chamber. The chamber has 10 levels with eight ports per level, thus accommodating 80 animals. Each level was designed to hold eight commercially available animal restrainers (Amesa Industries, Geneva, Switzerland) (Coggins et al. 1981) so that each animal's nose was located approximately 2 cm from the delivery nozzle. The 6-mm opening of the delivery nozzle reduced the carbon black aerosol vapor velocity, thereby avoiding the possible initial separation of the test aerosol or the inertial impaction on the animals' noses. Figure 2 shows the nozzle and restrainer assembly.

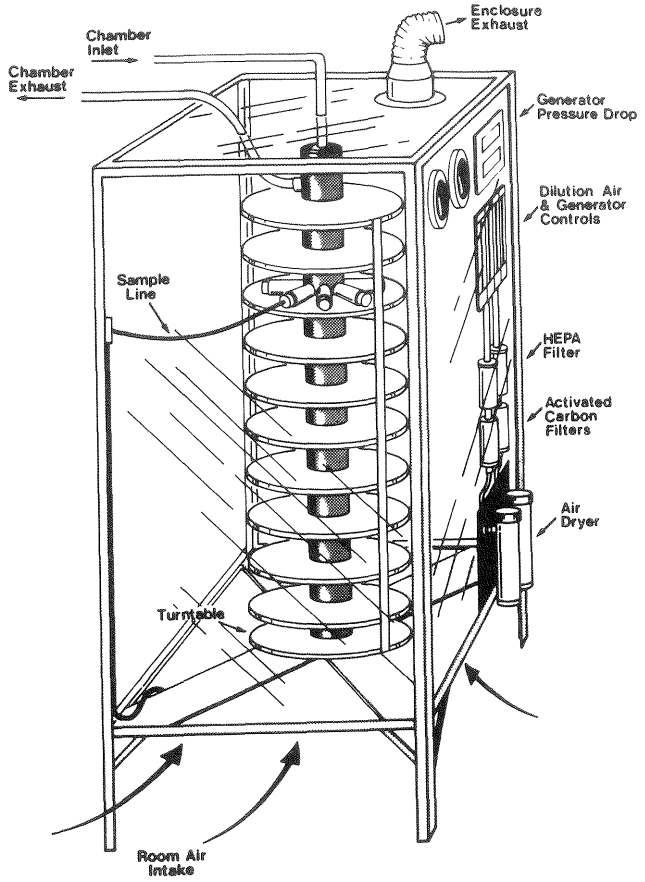

Figure 1. General layout of the nose-only inhalation chamber enclosure.

Aerosol Generation

A fluidized bed generator (Intox Products, Albuquerque, NM) (Carpenter and Yerkes 1980) was chosen to provide deagglomeration of the bulk carbon black. The generator was modified to provide for continuous generation of carbon black aerosols (Hemenway et al. 1990).

Twenty grams of carbon black Regal GR was mixed thoroughly with 1,500 g of stainless-steel shot (80 to 100 mesh powder, Hoeganes Corp., Rivertown, NJ) and placed in the bed material reservoir. The amount of stainless-steel powder and carbon black mix fed to the generator was controlled with a motorized ball valve (A22TT with EL8 actuator solenoid, Tate Engineering, Baltimore, MD) and was controlled by a timer (control timer CD4, Cole Parmer, Chicago, IL). The valve's minimum on and off cycle time was 12 seconds. Thus, the valve would rotate one complete revolution in 12 seconds, depositing a controlled amount of premixed carbon black and stainless-steel shot, with the number of rotations controlled by the timer. A 10-minute interval proved optimal to maintain stable carbon black aerosol concentrations. Variations in carbon black aerosol concentrations were achieved by premixing a larger amount of carbon black with the stainless-steel shot and by adjusting the flow rate through the fluidized bed. The dilution air then was adjusted accordingly in order to maintain a flow rate of 80 L/min through the system.

The carbon black aerosol generated by the fluidized-bed generator then was passed through a stainless-steel cyclone separator to reduce the nonrespirable fraction of the aerosol (John and Reischl 1980). The aerosol was combined with formaldehyde vapor and passed through a five-gallon ballast tank to allow time for the formaldehyde to adsorb on the carbon black and to help reduce short-term fluctuations from the generator output. As shown in Figure 3, the air flow containing the carbon black aerosol–formaldehyde mixture exited the ballast tank and entered the top of the inhalation chamber (see Figure 1, chamber inlet). Carbon black is an extremely conductive aerosol and carries a free electron. Reduction of surface charge on the particles was achieved by installing two radioactive ^{63}Ni sources. One was placed on the inside of the fluidized-bed generator approximately 5 cm from the generator exit, and the second device was placed inside the ballast tank near the tank inlet.

A 5-gallon ballast tank reservoir was used to help dampen fluctuations in the aerosol and formaldehyde generator output and to provide time for the formaldehyde to reach phase equilibrium between gas phase and adsorbed formaldehyde. Initial estimates indicated that the mean residence time of the reservoir (15 seconds) should have been more than adequate to reach steady-state conditions without significant loss of the particles to the walls or the introduction of major shifts in the particle size distribution due to time-dependent agglomeration.

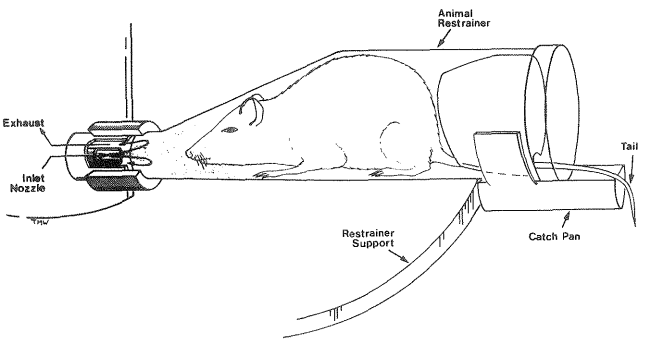

Figure 2. Typical animal restrainer and nozzle assembly, showing relationship of the aerosol entry to the animal's nose.

Aerosol Sampling

Total carbon black aerosol loadings were measured using a 25-mm, 0.45-μm-pore-size membrane filter (model DM450, Gelman Sciences Inc., Ann Arbor, MI). The filter was held

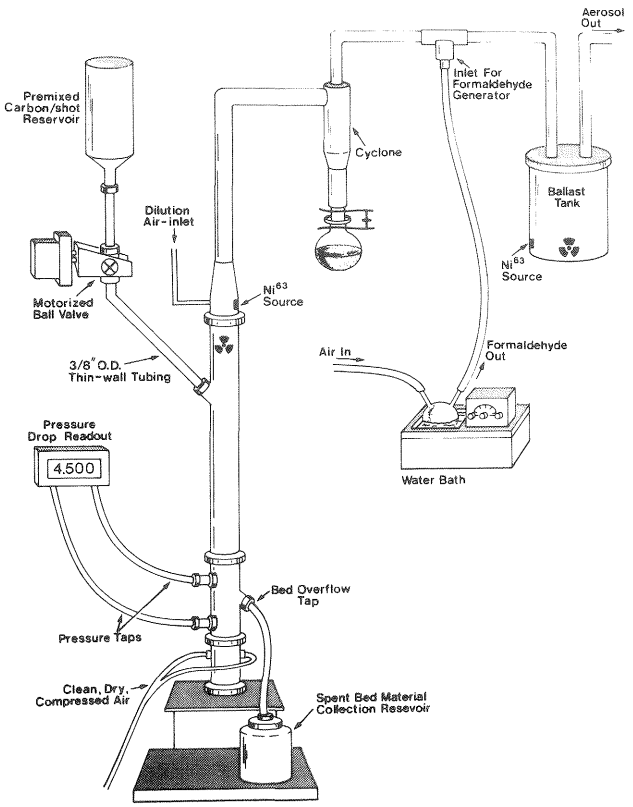

Figure 3. Carbon black aerosol and formaldehyde generation system (also referred to as the fluidized bed generator).

in an electrically conductive 25-mm open-faced filter cassette (number 300075, Nucleopore, Carmel, CA), and the flow rate through the filter was maintained with a critical orifice in the range of 200 to 300 cm^3/min. The weight of the filters before and after sampling was determined with a microbalance (model 30, Cahn, Cerritos, CA). A central sample vacuum line was placed in one corner of the chamber enclosure (Figure 1), and individual lines were attached to each level of the chamber as necessary. Therefore, if each level was sampled simultaneously, only seven animals could be accommodated per chamber level. Figure 1 shows an example of a single sample line connected to the second level. During animal exposures, four ports were sampled on the chamber levels at which animals were exposed. The ports were sampled during the entire four-hour exposure period and averaged to calculate the time-weighted exposure concentration.

Routine aerodynamic particle size measurements were made with a 10-stage Sierra impactor (model number 115, Andersen Samplers Inc., Atlanta, GA) at either the inlet to the ballast tank, the inlet to the inhalation chamber, or the exhaust from the inhalation chamber, at a flow rate of 2.75 L/min for three hours on glass fiber filters (model 220-GF, Andersen Samplers Inc.). The mass of the filters before and after sampling was recorded, and the aerodynamic particle size distribution was determined by standard methods (Lodge and Chan 1986). The impactor could not be used for the individual ports because the impactor flow rate exceeded the nozzle delivery rate. Particle size distribution was also verified with an APS-33 aerodynamic particle sizer (TSI Inc., St Paul, MN) (Hemenway et al. 1990).

For particle size distribution at the exposure ports, the samples were taken with the open-faced filter cassettes on 25-mm Gelman GN-6 filters (0.45-μm pore size) for approximately 15 minutes at a flow rate of 250 to 300 mL/min. Thereafter, the filters were clarified per the Occupational Safety and Health Administration's method V-1 with 50% dimethyl phthalate and 50% diethyl oxalate (using the pore collapse method published in the "Filter Book", Gelman Sciences, Ann Arbor, MI) and mounted on glass microscope slides. Particle size distribution was determined by standard methods (Silverman et al. 1971), using a Porton G2 graticule (Graticules Limited, Tonbridge, Kent, England) calibrated with a stage micrometer.

Real-time aerosol levels were measured at the input of the inhalation chamber using a RAM-1 aerosol monitor (GCA Technology Corp., Bedford, MA) to observe relative fluctuations in the aerosol concentration resulting from the aerosol generation system. The RAM-1 provided qualitative information about the performance of the aerosol generator. It was not used to provide reliable quantitative information because this type of instrument exhibits drifts when its optics become dirty (that is, when dirt passes through the sheath clean-up filters and deposits on the optics). In addition, such devices must be calibrated for the specific particles being used because the measurement is dependent on the aerosol's size distribution and index of refraction. This problem was encountered with the infrared spectrophotometer used for monitoring coexposure to carbon black and formaldehyde because the filters used with the instrument (available from the manufacturer with a pore size that would not significantly reduce the flow rate through the unit) were inadequate to totally protect the multiple-path optical cell from the buildup of particles on the surface. The result of this was a loss of sensitivity, precision, and accuracy. Cleaning the instrument with anhydrous ether did not rectify this problem. Therefore, the RAM-1 was used to assess the minute-to-minute operations or generator status, whereas absolute mass measurements were made using 0.45-μm-pore-size membrane filters. Numerous studies have shown that such filters have count efficiencies of greater than 99% for particles as small as 0.01 μm (Lioy and Lioy 1983). Thus, the membrane filters provided the primary standard for assessing particle mass concentration.

The performance experiments for the chamber in preparation for the biologic studies (Hemenway et al. 1990) included measurements of (1) vertical concentration profile; (2) horizontal concentration profile; (3) generator stability with time; (4) particle size distribution by Sierra cascade impactor; (5) particle size distribution by light microscopy; (6) particle size by scanning electron microscopy; (7) particle size distribution by light microscopy as a function of vertical concentration profile; and (8) an examination of the effect-of-charge ratio (carbon to stainless-steel fluidized bed materials) on vertical and horizontal concentration profile, generator stability with time, and particle size distribution.

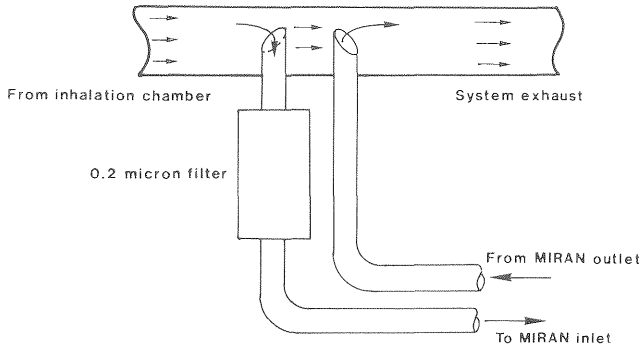

Figure 4. Sample line connection for infrared spectrometer.

Formaldehyde Generation and Monitoring

The formaldehyde generation protocol for the carbon black aerosol–formaldehyde coexposure studies was identical to that used for animal exposures to formaldehyde vapors. The formaldehyde from the generation flask was delivered through teflon tubing to an inlet port between the cyclone and the ballast tank (Figure 3).

Real-time monitoring of formaldehyde concentrations was performed using the infrared spectrophotometer at the exhaust end of the inhalation chamber. A sample point assembly (Figure 4) was built from stainless-steel and included a 0.2-μm-pore-size filter (Micronic Beta-3, Purolator Technologie, Newberry Park, CA) placed in-line to remove the carbon black particles.

The nominal sampling flow rate of the infrared spectrophotometer monitor was 25 L/min, which represents approximately one-third of the total flow rate through the chamber. It was also necessary to prevent particles from entering the unit during those periods when formaldehyde and carbon black were cogenerated. Therefore, the formaldehyde sampling point was installed on the exhaust side of the inhalation chamber to eliminate problems of the filtered exhaust reducing the mainstream aerosol concentration before inhalation by the animals. In addition, by returning the exhaust from the spectrophotometer to the main airstream (Figure 4), the overall flow through the chamber was not reduced. Preliminary studies showed identical results when the sample point assembly was placed before or after the inhalation column, indicating minimal wall loss or adsorption of the formaldehyde. Therefore, all subsequent sampling was taken on the exhaust side of the column. The 0.2-μm-pore-size filter placed before the formaldehyde monitor was periodically replaced to reduce the accumulation of carbon black. Even with the use of the filter, there was penetration of some carbon black through the filter resulting in the gradual accumulation of material on the mirrors and other components within the spectrophotometer sample compartment. As a result, the formaldehyde monitor had to be returned to the manufacturer for cleaning and replating the gold mirrors once during the course of these experiments. To check for drift or changes in sensitivity the instrument was calibrated weekly with the NIOSH chromotropic acid method (National Institute for Occupational Safety and Health 1984). Triplicate impinger samples were taken for each calibration.

ANIMAL EXPOSURES

Animal Conditioning

After the nose-only inhalation system was established, preliminary experiments indicated that immobilizing mice in restraint holders required for nose-only inhalation exposures suppressed the antibacterial defenses of the lungs. Such alterations of the biologic baseline would have confounded the experiments by making it difficult to determine whether suppression of pulmonary antibacterial defenses during carbon black aerosol–formaldehyde coexposures was due to the inhalation of the test compounds, restraint stress, or a combination of both. Therefore, before the study with carbon black aerosol–formaldehyde coexposures could be initiated, experiments were performed to determine whether the restraint-induced impairment of pulmonary antibacterial defenses could be abrogated by previous conditioning (training) experience.

The results of these experiments (Jakab and Hemenway 1989) demonstrated that (1) immobilizing mice for four hours impairs pulmonary antibacterial defenses for 20 hours after they are removed from the restraint holders; (2) training the animals in the restraint holders for four hours per day for two days abrogated the bactericidal impairment induced by restraint before the bacterial challenge; and (3) restraining the animals after the bacterial challenge also suppressed the antibacterial defenses of the lungs; this suppression was abrogated by training the animals for four

hours per day for four days. During the course of these experiments, all animals were trained in the restraining holders for four hours per day for four days before exposure in the nose-only inhalation chamber.

In addition, experiments were performed that demonstrated that conditioning of the animals for four hours per day for four days did not alter the deposition of bacteria (the number of viable organisms recovered from lungs immediately after the 30-minute bacterial challenge). Bacterial deposition was the same on day five for unrestrained animals as it was for conditioned animals.

Experimental (Restraint) Controls

Because only one nose-only inhalation exposure chamber was available for this study, experiments had to be performed for the proper surrogate controls. After completing the adaptation study, an experiment was performed to determine whether exposing trained animals to a flow of clean air at the chamber exposure ports altered pulmonary bactericidal activity, as compared with immobilized animals whose restraining holders were not inserted into the exposure ports. The restraining holders of one group of mice were inserted into the exposure ports of the nose-only inhalation chamber, and the animals were allowed to breath clean air. Under actual toxicologic inhalation exposure conditions, the air would have contained the carbon black aerosol–formaldehyde combination. Another group of restrained animals was placed on another level of the chamber and exposed only to the low-level noise generated by the air handling system. During exposure, the ports not used for animal inhalation or air sampling were plugged with rubber stoppers.

The results demonstrated that exposing the restrained mice to the clean air from the exposure ports did not alter pulmonary bactericidal activity, as compared with the restrained animals that were allowed to breath ambient room air in the chamber. All subsequent experiments with exposures to carbon black aerosol–formaldehyde mixtures were performed with similar surrogate controls.

EXPERIMENTAL DESIGN

Formaldehyde Exposure After Bacterial Challenge

The basic experimental design involved 18 animals. Six were killed immediately after the bacterial challenge to determine the number of viable bacteria deposited in the lungs. The other 12 animals were exposed for four hours and then killed. Six of these 12 animals were exposed to formaldehyde (HCHO) during the four hours, and the other six breathed ambient room air. Bacterial killing in each of these latter 12 animals was calculated by comparing the bacterial colony counts to the mean count of the six animals killed immediately (zero hour) after the bacterial challenge.

In summary,

Zero-Hour Group: bacterial challenge → assay;

Four-Hour Control Group: bacterial challenge → four hours air → assay;

Four-Hour Exposure Group: bacterial challenge → four hours HCHO → assay.

Formaldehyde Exposure Before and After Bacterial Challenge

The experimental design involved 36 animals. Eighteen animals were exposed to formaldehyde for 18 hours, and the other 18 breathed ambient room air. The following morning, all animals were challenged with bacteria and, immediately thereafter, six of the mice exposed to formaldehyde and six that had breathed ambient air were killed to determine the number of viable bacteria deposited in the lungs. Of the remaining 12 that had been exposed to formaldehyde, six were reexposed to the vapors (before and after bacterial challenge exposure), and the other six breathed air (before bacterial challenge exposure). Of the remaining 12 that were not exposed to formaldehyde before the bacterial challenge, six were exposed to the vapors (after bacterial challenge exposure), and the other six breathed air (controls). Bacterial killing in each of these latter 24 animals was calculated by comparing the bacterial colony counts to the mean count of the six respective animals killed immediately (zero hour) after the bacterial challenge.

For air exposure for 18 hours before bacterial challenge, the design is identical to that described above for "Formaldehyde Exposure After Bacterial Challenge."

In summary,

Zero-Hour Group: 18 hours air → bacterial challenge → assay;

Four-Hour Control Group: 18 hours air → bacterial challenge → four hours air → assay;

After-Exposure Group: 18 hours air → bacterial challenge → four hours HCHO → assay.

For formaldehyde exposure for 18 hours before bacterial challenge,

Zero-Hour Group: 18 hours HCHO → bacterial challenge → assay;

Before-Exposure Group: 18 hours HCHO → bacterial challenge → four hours air → assay;

Before-and-After-Exposure Group: 18 hours HCHO → bacterial challenge → four hours HCHO → assay.

During the overnight exposures, the animals were provided with food and water ad libitum. Removing the

animals from the exposure chamber, conducting the bacterial challenge, and returning the animals to the exposure chamber took less than one hour.

To assure that exposure to formaldehyde vapors before the bacterial challenge did not alter the number of staphylococci deposited in the lungs, zero-hour animals (those killed immediately after bacterial challenge) from the group exposed for 18 hours to formaldehyde, as well as mice that had breathed room air, were included in the analyses. There were no significant differences ($p>0.05$) when we compared the number of staphylococci recovered from the lungs of mice in the two groups.

Carbon Black and Formaldehyde Coexposures After Bacterial Challenge

This series of experiments was performed to mimic the experiments performed with exposure to formaldehyde vapors after bacterial challenge and used the same experimental design. All animals were conditioned for four hours per day for four days in the restraining holders before the experiment. Eighteen animals were challenged by aerosol inhalation with staphylococci and then separated into three groups. The first group was killed immediately after the bacterial challenge to determine the number of viable bacteria deposited in the lungs. The second group was exposed to the carbon black and formaldehyde combination, and the third group breathed air. Four hours after the bacterial challenge, the second and third groups were killed, and the number of viable bacteria remaining in their lungs were compared.

In summary,

Zero-Hour Group: training (4 hours/day/4 days) → bacterial challenge → assay;

Four-Hour-Exposure Group: training → bacterial challenge → four hours carbon black and HCHO → assay;

Four-Hour Control Group: training → bacterial challenge → four hours air → assay.

Carbon Black and Formaldehyde Coexposures Before Bacterial Challenge

The experimental design involved 24 animals. All mice were placed in the restraining holders. Twelve mice were exposed to the carbon black and formaldehyde combination at the chamber exposure ports; the other 12 mice were placed in the chamber, but their restraining holders were not inserted into the exposure ports. After four hours, all mice were removed from their restraining holders and returned to their normal cages for 20 hours. This procedure was repeated for four days. On the fifth day, all mice were challenged with staphylococci, and six exposed mice and six unexposed mice were killed to determine the number of bacteria deposited in the lungs. The remaining mice (exposed and unexposed) then were returned to their normal cages and assayed at four hours for comparison of intrapulmonary bacterial killing.

In summary,

Control Zero-Hour Group: (four hours air → 20 hours rest) × 4 days → bacterial challenge → assay.

Control Four-Hour Group: (four hours air → 20 hours rest) × 4 days → bacterial challenge → four hours air → assay.

Before Zero Hour Group: (four hours carbon black and HCHO → 20 hours rest) × 4 days → bacterial challenge → assay.

Before Four Hour Group: (four hours carbon black and HCHO → 20 hours rest) × four days → bacterial challenge → 4 hours air → assay.

Alveolar Macrophage Fc-Receptor–Mediated Phagocytosis

The bactericidal assays were performed a day after the cessation of carbon black and formaldehyde coexposures and showed no effect on the intrapulmonary killing of *S. aureus*. To determine whether any effect was delayed, a surrogate assay for alveolar macrophage phagocytic function, Fc-receptor–mediated phagocytosis, was performed 1, 3, 5, 10, 25, 40, and 60 days after cessation of exposure.

The experiment involved 60 to 70 animals and used a design identical to that described above for carbon black and formaldehyde coexposures before bacterial challenge, with the exception that the zero-hour animals were not required. All mice were placed in the restraining holders. One-half of the mice were exposed to the carbon black and formaldehyde combination at the chamber exposure ports; the other half of the mice were placed in the chamber, but their restraining holders were not inserted into the exposure ports. After four hours, all mice were removed from their restraining holders and returned to their normal cages for 20 hours. This procedure was repeated for four days. At the various time intervals thereafter, groups of five exposed mice and five unexposed mice were killed. Alveolar macrophage Fc-receptor–mediated phagocytosis for the two groups was compared.

Forty control mice for the coexposures were exposed for four hours per day for four days to either carbon black (20 mice) or formaldehyde (20 mice). These control experiments were performed a week (formaldehyde) and two weeks (carbon black) after the combined inhalation exposures. Mice were killed 3, 10, 25, or 40 days after the last exposure day, and the phagocytic activity of alveolar macro-

phages from groups of five exposed and five unexposed mice were compared.

CARBON BLACK PARTICLES

Amorphous carbon blacks were used in this experiment as models for the carbonaceous combustion-derived particles that are emitted from the tail-pipes of mobile sources. Both these types of carbonaceous particles have short-range graphitic order. Amorphous carbon blacks are agglomerates of aciniform aggregates with a fundamental size of 20 to 30 nm (Rivin 1986). The carbon blacks are manufactured by the furnace process, using petroleum feed stock. This process is conceptually similar to combustion processes (substoichiometric and pyrolytic reactions) occurring with heterogeneous fuels and oxidizers in Diesel engines. The advantages of using amorphous carbon blacks as opposed to particles collected from the exhaust of mobile sources are that the surface properties of carbon black particles can be well-characterized and carbon black particles are homogeneous from particle to particle. The mode of manufacture of carbon blacks makes them useful experimentally because they can be produced with defined particle sizes. Particles emitted by mobile sources are coated with sorbed materials that are deposited on their surfaces during particle formation and as the particles cool in the exhaust manifold. The adsorption of gas-phase molecules depends to a great extent upon the surface properties of the particle. The strength and selectivity of adsorption defines the identity and the amount of adsorbed molecules. Measurement of the interaction between adsorbed molecules and the particle surface provides an indication of the ease with which the adsorbed molecules are released once the particle-adsorbed molecule complex is deposited in the lung. Therefore, a thorough characterization of the physical and chemical characteristics of the carbon black particles used in this study was essential.

Four carbon blacks were selected: two pelletized carbon blacks (Regal 660 and Black Pearls L) and two carbon black powders (Regal GR and Mogul L). Regal 660 is pelletized Regal GR, and Black Pearls L is pelletized Mogul L. Pelletization is a process whereby powders are compressed into pellets without changing the surface area, surface properties, or the fundamental size of the aggregate (Rivin 1986). These carbon blacks were selected because they are commercially available, and large quantities can be obtained from a single batch and stored for future studies, thus allowing a large inhalational data base to be generated with the same particles. These carbon blacks have similar surface areas and surface properties to those found in the exhaust of mobile sources (Ross et al. 1982; Risby and Sehnert 1988). The powdered carbon blacks were used in the inhalation studies, and the pelletized carbon blacks were used to characterize the interactions between formaldehyde and carbon black surfaces by gas solid chromatography. (It is difficult to characterize these interactions by gas solid chromatography with powders that have small particle sizes because the pressure drops across the columns are very high.) The carbon blacks selected were an amorphous carbon black (Regal GR/Regal 660) and an oxidized carbon black (Mogul L/Black Pearls L).

The oxidation of carbon blacks is a process whereby the surface groups on the carbon blacks are chemically oxidized with ozone or the oxides of nitrogen. Oxidation does not significantly change the overall physical structure of the carbon black, and only the surface groups are oxidized. This process is performed in order to increase the surface polarity of the carbon black. It was expected that the adsorption of formaldehyde would be significantly different on these two carbon blacks with different polarities. The surfaces of the carbonaceous particles emitted by mobile sources are expected to have varying degrees of surface oxidation depending upon the combustion stoichiometries that existed at the time the carbon particle was produced and the age of the aerosol in the ambient atmosphere.

PHYSICAL AND CHEMICAL PROPERTIES OF PARTICLES

There are a number of physical and chemical properties of particles, environmental agents, and particle-sorbed environmental agents that must be determined in order to provide the fundamental information necessary for a comprehensive inhalation study. The particle size, density, and shape determine whether the particles are respirable and where in the respiratory tract these particles will be deposited. The deposition site, as well as the particle size and shape, define the rate and extent of clearance from the lung. The surface area of the particle determines the potential concentration burden that can be expected to be retained on the particle because the amount of material that can be associated directly with the particle surface is limited. The porosity (roughness factor) of the particle may be important to the release of the adsorbed environmental agents since if the molecules are sorbed in micro-, meso-, or macro-pores, they may not be as available as those adsorbed on the external surface. The bulk chemical properties and the surface properties of the particle are significant contributors to the particle's inherent toxicity and the identity of molecules that may be adsorbed selectively on the particle surface. This study quantified the bulk chemical properties, the surface properties, the size, shape, bulk density, true density, total and external surface areas, and roughness factor, and

characterized the endogenous molecules that are adsorbed on the carbon surfaces during the particles' manufacture.

CHEMICAL PROPERTIES OF CARBON BLACKS

Bulk Chemical Properties of the Carbon Blacks

The carbon samples were subjected to elemental analysis for carbon, hydrogen, nitrogen, and oxygen using standard methods.

Chemical Analysis of the Adsorbed Molecules Endogenous to Carbon Blacks

Known weights of the two carbon black powders (10.8038 g of Mogul L and 10.8038 g of Regal 660) were Soxhlett-extracted with 50 mL of dichloromethane for five days, and the extract was evaporated to dryness under nitrogen. The residues were redissolved in known volumes of dichloromethane and methanol (1:1) and analyzed by capillary gas chromatography electron impact mass spectrometry (4500 GC/MS system; Finnigan MAT, San Jose, CA). The separations were performed on a capillary column (30 m) coated with a substituted methyl silicone (DB-5), which was programmed to increase from 80°C to 300°C at a rate of 8°C per minute, and to remain at the final temperature for 10 minutes. The samples (2.0 μL) were injected splitless, and mass spectra were collected from 50 to 450 *m/z* in one second. The identifications and quantifications of the polycyclic aromatic hydrocarbon components of these extracts were compared to authentic standards of polycyclic aromatic hydrocarbons.

PHYSICAL PROPERTIES OF CARBON BLACKS

Specific Surface Area

The specific surface areas of the carbon blacks were determined using a surface area analyzer (Flowsorb II, Micromeritics Instrument Corp., Norcross, CA). The method used was based upon the derivation by Brunauer, Emmett, and Teller (Brunauer et al. 1938), commonly known as the B.E.T. method. Nitrogen gas was adsorbed onto the carbon surface in increasing amounts. An analysis of the quantity of nitrogen adsorbed versus the corresponding change in pressure (an adsorption isotherm) yields the quantity of nitrogen required to complete one monolayer on the surface. Nitrogen gas was used as the standard adsorbate for surface area determinations because it does not exhibit localized adsorption and can penetrate most of the pore structures and allow the morphologies of the carbon blacks to be studied.

External Surface Area

The external surface areas of the carbon blacks were calculated using the method proposed by Anderson and Emmett (1948) based on the diameters of the carbon black particles and their true densities.

Roughness Factor

If the values for the total surface area and the external surface area are compared, then it is possible to obtain a value for the roughness factor for the particle. This is a way to estimate the porosity of the carbon blacks. The actual measurement of porosity could have been performed with mercury porosimetry or from the hysteresis of the adsorption-desorption isotherms for the sorption of helium or nitrogen.

Particle Size

All carbon samples were examined by scanning electron microscopy, using a JEOL model T330 scanning electron microscope (JEOL, USA, Peabody, MA), and by photomicroscopy for determination of average particle size of the sieved pelletized carbon blacks and the count median diameter of the carbon black powders.

Apparent Density

The apparent densities of the sized carbons were determined by weighing the quantity of particles occupying a known volume in air.

True Density

The helium density is generally considered to be the true density since helium can penetrate the total pore structure of the carbon by virtue of its size, and it is assumed to have negligible adsorption at room temperature. The helium densities were determined by expanding known volumes of helium into a sample cell with or without known masses of carbon black.

Physical and Chemical Properties of Environmental Agent Complexes

The chemical and physical properties of the environmental agents that sorb onto the surfaces of particles also play a major role in the agents' resulting biological effects. The chemical and physical properties of formaldehyde are well known, and the only poorly understood parameter is the rate and extent of the polymerization of formaldehyde to paraformaldehyde. Paraformaldehyde is an undefined polymer with a low vapor pressure. Formaldehyde can be prevented from polymerization by the presence of low concen-

trations of stabilizers such as methanol. Since the rationale for this inhalation study was to determine the possible health effects associated with exposures to formaldehyde in the presence of carbon particles that may result from the use of fuels augmented by methanol for mobile source operation, it is reasonable to expect unburnt methanol as a major emission in addition to carbon particles and formaldehyde. Water is another major product of combustion and may be important since it may affect the adsorption of formaldehyde onto the surface of carbon particles.

The intermolecular forces that govern the capture and release of environmental agents by particles are determinants of the potential dose of the sorbed environmental agents on the particle and the rates and extents of their release. When a gas-phase molecule adsorbs on a surface, the partial free energy is the thermodynamic descriptor for the equilibrium capture of an agent by the particle. The relative magnitudes of the partial free energies determine which agents remain in the gas phase and which are adsorbed onto the particle. The major contributor to the partial free energy is the partial enthalpy. This is because the partial entropy, which is the result of the loss of three degrees of translational freedom, is small and not specific. The partial enthalpy results from the intermolecular forces between the adsorbate and adsorbent. The partial enthalpy varies as a function of surface coverage and this variation can be large if the surface is heterogeneous. The interactions between adsorbates and adsorbents are dependent on concentration and on the presence of other adsorbates that may be pre-adsorbed on the surface. These studies characterized the interaction of formaldehyde with the surface of an amorphous carbon black (Regal GR/Regal 660) and an oxidized carbon black (Mogul L/Black Pearls L) in order to quantify important parameters needed for selecting the carbon black for the inhalation study. These results also contributed to the interpretation of the in vivo inhalation data. The partial enthalpy that accompanied adsorption of formaldehyde onto carbon blacks in the presence and absence of water vapor, methanol vapor, and water and methanol vapors also was measured.

THE INTERACTIONS BETWEEN FORMALDEHYDE AND CARBON BLACKS

Adsorption of Formaldehyde

Preliminary experiments were performed to investigate the adsorption properties of formaldehyde by using formalin, which is a solution of formaldehyde in water stabilized with methanol. These experiments served to determine the appropriate separation conditions for the subsequent gas solid chromatographic separations using formaldehyde gas. Pelletized carbon blacks (Regal 660, Black Pearls L), sieved to obtain a fraction of uniform particle size, were packed into glass columns (1.2 m, 6 mm o.d., 2 mm i.d.) using vibration with vacuum assistance. Helium that had been dried with molecular sieve was used as the carrier gas. Considerable care was taken to assure that the chromatographic conditions corresponded to the Henry's Law region of the adsorption isotherm, and the columns were conditioned between each injection by allowing at least four hours to elapse before the next injection was made.

A specially designed apparatus was used to generate formaldehyde gas via the thermal decomposition of paraformaldehyde. The method (Vogel 1986) involves heating (180°C) paraformaldehyde that has been dried for two days over phosphorous pentoxide forming gaseous formaldehyde, which is carried by a slow stream of helium into a glass gas sampling flask. This method of formaldehyde generation, which is slightly different from the one described for animal exposures, was needed to reduce any vapor contaminants. Formaldehyde gas free of water vapor is necessary for gas chromotography. However, very dry formaldehyde gas is not a requisite for the animal inhalation studies.

A gas chromatograph with a thermal conductivity detector (Varian 920, Varian Inc., Sunnyvale, CA) was used to investigate the adsorption phenomenon of formaldehyde on the carbon black adsorbents under a variety of conditions. The pelletized carbon blacks were sieved to a mesh size of 80 to 100 and packed by vibration with vacuum assistance into glass columns (2.0 mm i.d., 1.2 m). Helium that had been passed through a molecular sieve was used as the carrier gas at a constant flow rate (1 mL/minute). Known aliquots (20 μL) of gas-phase formaldehyde in helium were introduced directly onto the column containing the carbon black using a gas-tight syringe. Isothermal retention data were recorded with a computing integrator (Spectraphysics SP4270, Spectra-Physics, San Jose, CA) at three column temperatures. The column was allowed to re-equilibrate between each run for at least four hours. The isosteric heat of adsorption was obtained from the slope of a plot of the logarithm of the specific retention volume versus the absolute column temperature. This relationship is valid providing the concentration of the solute falls within the Henry's Law region of the adsorption isotherm.

The effect of the presence of methanol and/or water on the adsorption of formaldehyde was also investigated. For these studies, the carrier gas was passed through a glass bubbler (placed in a controlled temperature bath at 41°C or 25.5°C) containing water and/or methanol with glass beads. The gas chromatographic columns were equilibrated for four hours with these mobile phases before the adsorption of formaldehyde was studied.

Desorption of Adsorbed Formaldehyde from the Surface of Carbon Blacks into Synthetic Alveolar Lung Fluid

This objective involved measuring the desorption of adsorbed formaldehyde from the surface of carbon blacks into a synthetic alveolar lung fluid using the technique of physiologically relevant pseudophase high-performance liquid chromatography (HPLC) (Risby and Jiang 1987; Sehnert and Risby 1988). This technique uses liposomal mobile phases (synthetic alveolar lung surfactant) to quantify the release of adsorbed molecules from the surfaces of environmentally relevant particles. Synthetic alveolar lung surfactant based on the composition reported by King (1982) was used since it is impossible to obtain sufficient quantities by lung lavage (Sehnert and Risby 1988). Release is quantified on the basis of retention data, which can be measured as a function of column temperature. This technique allows performance of in vitro experiments that quantify the energetics of release without the confounding variable involved by the use of in vivo systems. Since formaldehyde is difficult to detect with normal HPLC detectors, we planned to derivatize formaldehyde by post-column reaction with 2,4-dinitrophenylhydrazine and monitor with UV detection.

The Chemistry of Adsorbed Formaldehyde on the Surface of Carbon Blacks

There are a number of obvious reactions that may take place when formaldehyde is sorbed on the surface of carbon black. Carbon black–bound formaldehyde could polymerize to paraformaldehyde in the presence of water vapor and subsequently depolymerize to form formaldehyde, formic acid, and methyl formate. We considered these potential chemical reactions in detail and made some preliminary investigations.

Formaldehyde in helium, obtained by the thermal depolymerization of paraformaldehyde at 180°C, and helium saturated with water vapor (7.7%) were passed for two hours into a specially designed glass reaction vessel containing 50 g of the carbon black. After this time, the gas generation systems were disconnected, and the formaldehyde and any reaction products adsorbed on the surface of the carbon black were thermally desorbed at 180°C into an evacuated gas sampling vessel. These products were analyzed by gas chromatography with a thermal conductivity detector using a glass column (2 m, 4 mm i.d.) containing a polymeric packing material (HAYSEP Q, Alltech Associates, Deerfield, IL) (80 to 100 mesh). The isothermal column was kept at 114°C because this provided baseline separation of water, formaldehyde, methanol, methyl formate, and formic acid.

STATISTICAL ANALYSIS

Statistical analysis was performed using the differences between control groups and treated groups (excess survival of bacteria in the lungs or decrements in alveolar macrophage phagocytosis). The analysis was performed on a personal computer using a Statpak statistical program (Northwest Analytical, Inc., Portland, OR).

FORMALDEHYDE EXPOSURE

The analytical design of the formaldehyde exposure experiments was a randomized block design with treatment combinations defined by concentrations of formaldehyde and the presence or absence of exposure to formaldehyde before or after bacterial challenge. The blocks were replications of the experiments representing different days on which the experiments were performed; each concentration had six replicates.

Because of the day-to-day variability of the assay, significant differences occurred between replications. Accordingly, *F*-ratios for testing treatment effects used appropriate error variances based on variation among replications, rather than on the within-subgroup variability among the six animals in each group. The analyses were repeated using the log transformation and yielded similar results. The analysis of variance was computed for all of the data with the pertinent factors of concentration (1, 5, 10, and 15 ppm formaldehyde) and the presence or absence of formaldehyde exposure after bacterial challenge. The significant interaction between the concentration and formaldehyde exposure implies that the formaldehyde effect varied among concentrations. Duncan's multiple range test was used post-hoc to determine which groups were significantly different.

For the experiments with formaldehyde exposure before and after bacterial challenge, the analytical design was a randomized block design with the treatment combinations defined by two concentrations of formaldehyde and the absence or presence of a four-hour formaldehyde exposure after the bacterial challenge, and the absence or presence of an 18-hour formaldehyde exposure before the bacterial challenge exposure. The blocks are replications of the experiments representing different days. Six animals were used for each treatment combination. Significant variation among replications (days) was found for some concentrations, but not for others. For consistency, and erring on the conservative side, *F*-ratios for testing treatment effects utilized appropriate error variances based on variations among replications and not on the within-subgroups variability among the six animals in each subgroup. The analysis of

variance was calculated for the pertinent factors of concentration and exposure. This analysis indicated some significant differences between exposures and the interaction between exposure and concentration. Duncan's multiple range test was used post-hoc to determine which groups were significantly different.

CARBON BLACK AND FORMALDEHYDE EXPOSURE

Because the actual exposure concentrations to carbon black and formaldehyde deviated considerably from the target concentrations in the bactericidal experiments, multiple regression analysis was used to test the effects of each factor and its interaction with every other factor.

The analysis of alveolar macrophage phagocytosis experiment Number 2 was a 2-by-2 factorial design; the blocks were the presence or absence of carbon black or the presence or absence of formaldehyde.

RESULTS

FORMALDEHYDE VAPOR EXPOSURE SYSTEM

The formaldehyde generation and quantification system showed excellent stability during the 4- and 18-hour exposure periods. In general, the stability of the system during the four-hour period was better than ± 2%. During the 18-hour exposure period (overnight), the stability was ± 3%. The day-to-day variation was within ± 4%.

CARBON BLACK AND FORMALDEHYDE COEXPOSURE SYSTEM

The carbon black and formaldehyde generation and quantification system resulting from these studies has been previously described (Hemenway et al. 1990). Briefly, after conducting the performance experiments on the chamber we found that:

1. The particle size distribution was highly respirable, with at least 98% of the particle mass having an aerodynamic diameter less than 5 μm.
2. The top-to-bottom variability was less than ± 20% of the mean column concentration. Because of this, animals were always placed on adjacent levels in the middle of the exposure chamber, and aerosol samples were taken at those levels.
3. Levels 5 through 9 were used to insure the minimum exposure deviation for the laboratory animals. Characterization studies showed that these levels gave similar concentrations and exhibited the least difference from the column average concentration.

The four-hour time-weighted averages of the carbon black aerosol mass concentrations to which the animals were exposed were sampled at exposure ports adjacent to the ports where the animal restraint holders were inserted. Estimations of the target concentrations depended on the preliminary observations of the RAM-1 monitor to adjust the system as closely as possible to the target concentration. The primary measurement and verification of the exposure concentration was subsequently determined during each exposure via open-faced membrane filter samplers. The average exposure concentration typically deviated from the target concentration due, in part, to the variability of the RAM-1 as well as random wall loss factors from sampling at different points in the system. Because of this variability, and because the RAM-1 could not be used to sample at the nozzle (incompatible flow rates), we were forced to rely on the membrane filter method as the primary estimate of the average exposure concentration.

The infrared spectrophotometer also was used as a feedback control unit for setting the formaldehyde levels in a manner similar to that used with the RAM-1 for estimating the aerosol concentration. This was necessary because of drift problems that resulted in significant loss of precision and accuracy for the response of the infrared spectrophotometer used when measuring formaldehyde in the presence of the carbon black aerosol. These drift problems continued even after factory cleaning and realignment of the instrument (although the drift problems initially were reduced significantly after factory maintenance). Because of this drift, primary measurement of the formaldehyde levels was made by making daily measurements, using the chromotropic acid method.

The day-to-day variability in the exposure system was similar to the variability between experiments. For example, when mice were exposed to a target concentration of 3.5 mg/m^3 carbon black and 2.5 ppm formaldehyde, the time-weighted concentrations were 4.2 mg/m^3 and 2.5 ppm, 6.8 mg/m^3 and 3.0 ppm, 3.7 mg/m^3 and 2.7 ppm, 2.4 mg/m^3 and 3.6 ppm, and 4.4 mg/m^3 and 2.7 ppm (data from legend to Figure 8).

FORMALDEHYDE EXPOSURE AND PULMONARY ANTIBACTERIAL DEFENSES

Formaldehyde Exposure After Bacterial Challenge

The effect of exposure to increasing concentrations of formaldehyde vapors on the intrapulmonary killing of *S. aureus* is presented in Figure 5. At each concentration, six replicates of the experiment were performed with six animals each for the various groups. The results of the replicates were pooled and the data presented as the mean ± SE of the difference in the percentage of change between control and exposed values in viable bacteria remaining four hours after the end of exposure. (The bactericidal

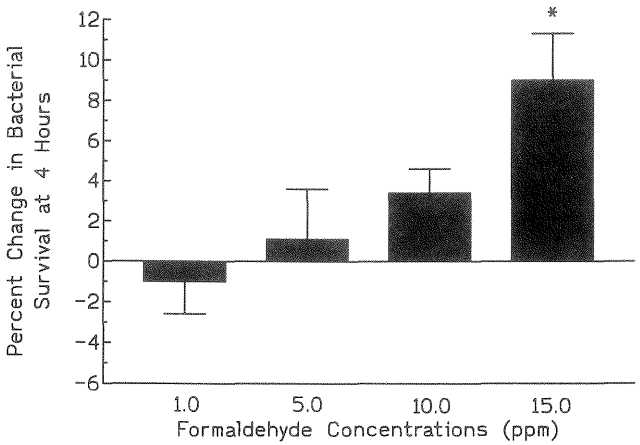

Figure 5. Comparison of intrapulmonary killing of *S. aureus* for mice exposed to various concentrations of formaldehyde for four hours after bacterial challenge. The data are presented as the difference in percentage of change between control and formaldehyde-exposed bactericidal values. Each value represents the mean ± SE of 36 determinations, * = $p < 0.05$.

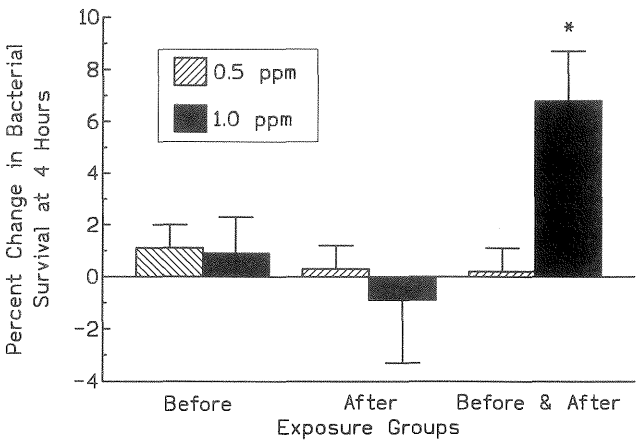

Figure 6. Comparison of intrapulmonary killing of *S. aureus* for mice exposed to either 0.5 or 1.0 ppm formaldehyde for 18 hours before bacterial challenge, four hours after bacterial challenge, or both. The data are presented as the difference in percentage of change between control and formaldehyde-exposed bactericidal values. Each value represents the mean ± SE of 36 determinations, * = $p < 0.05$.

values of each run at each concentration are detailed in Appendix Table A.1.) Statistical analysis of the bactericidal values showed that there were no significant differences ($p>0.05$) in the values between unexposed animals and those exposed to 1, 5, or 10 ppm formaldehyde. At exposure concentrations of 10 and 15 ppm, the p values were 0.1 and 0.006, respectively.

Formaldehyde Exposure Before and After Bacterial Challenge

The results of the above experiments with bacterial challenge before formaldehyde exposure showed that for the effects of formaldehyde to be toxic, in terms of suppressing the intrapulmonary killing of staphylococci, exposure to more than 10 ppm formaldehyde was needed. These experimental exposure concentrations are much higher than ambient formaldehyde levels. To determine whether the threshold for this toxic effect would be lower with a different exposure protocol, mice were exposed to formaldehyde vapors for 18 hours before bacterial challenge, and then again for four hours after bacterial challenge.

The data for these experiments at formaldehyde exposure concentrations of 0.5 and 1 ppm are presented in Figure 6. At each concentration, six replicates of the experiment were performed with six animals from each group. The results of the replicates were pooled and the data are presented as the mean ± SE of the difference in the percentage of change between control and exposed values in viable bacteria remaining four hours after the end of exposure (the bactericidal values for each run at each concentration are detailed in Appendix Tables A.2 and A.3).

Figure 6 shows that exposure to 0.5 ppm formaldehyde before, after, or before and after bacterial challenge did not affect pulmonary antibacterial defenses; nor did exposure before or after have any effect at the 1 ppm concentration. In contrast, preexposure to 1 ppm formaldehyde for 18 hours before bacterial challenge, followed by another four hours of exposure, significantly ($p<0.05$) suppressed the intrapulmonary killing of *S. aureus*. The data demonstrate that the toxic effect of formaldehyde depends on the exposure protocol. The modified exposure protocol reduced the formaldehyde threshold dose from greater than 10 ppm down to 1 ppm.

CARBON BLACK DEPOSITION IN THE LUNGS

To assure that the particles were deposited deep in the lung, a group of mice was exposed for four hours per day for four days to target concentrations of 15 mg/m^3 carbon black; 24 hours after the end of exposure, their lungs were fixed in 10% formalin, then wax embedded, sectioned, and stained with hematoxylin and eosin. Figure 7 shows that the carbon black was deposited in the lungs. Carbon black particles ingested by alveolar macrophages were also evident on the cell preparations for the Fc-receptor–mediated phagocytic assay.

CARBON BLACK AND FORMALDEHYDE COEXPOSURES AND PULMONARY ANTIBACTERIAL DEFENSES

Carbon Black and Formaldehyde Coexposures After Bacterial Challenge

Preliminary experiments indicated that exposing mice after bacterial challenge to low levels of carbon black and formaldehyde (approximately 1 mg/m^3 and 0.5 ppm, re-

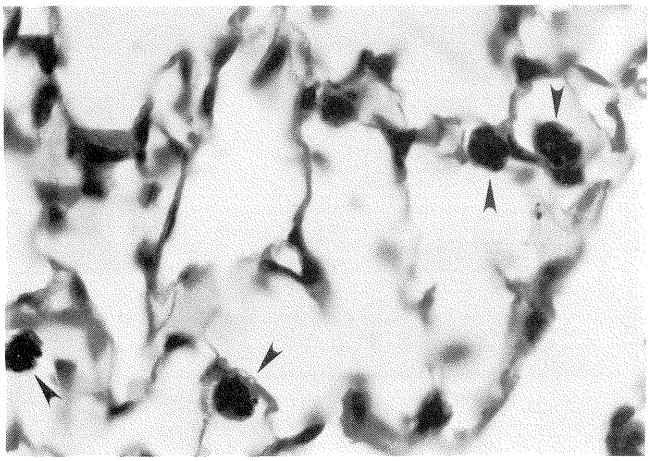

Figure 7. Photomicrograph of a lung section from a mouse exposed to 15 mg/m^3 of carbon black for four hours per day for four days showing carbon particles engulfed by alveolar macrophages. Magnification is × 1,500.

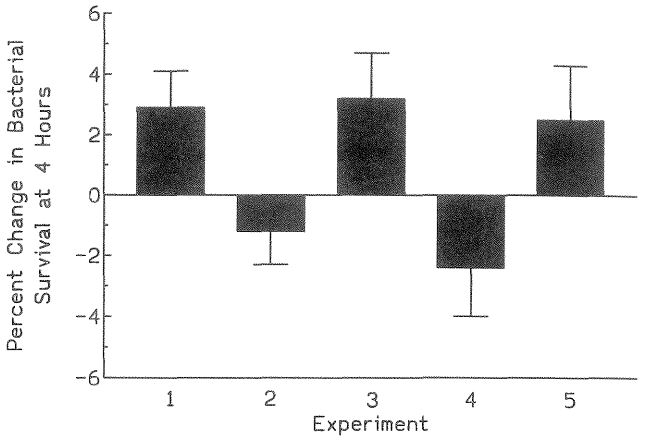

Figure 8. Comparison of intrapulmonary killing of *S. aureus* for mice exposed to target concentrations of 3.5 mg/m^3 carbon black and 2.5 ppm formaldehyde for four hours after bacterial challenge. The time-weighted concentrations were: experiment number 1, 4.2 mg/m^3 and 2.5 ppm; experiment number 2, 6.8 mg/m^3 and 3.0 ppm; experiment number 3, 3.7 mg/m^3 and 2.7 ppm; experiment number 4, 2.4 mg/m^3 and 3.6 ppm; and experiment number 5, 4.4 mg/m^3 and 2.7 ppm. The data are presented as the difference in percentage of change between control and carbon black and formaldehyde–exposed bactericidal values. Each value represents the mean ± SE of six determinations.

spectively) did not alter pulmonary antibacterial defenses. Therefore, the next series of experiments was set at target concentrations of 3.5 mg/m^3 carbon black and 2.5 ppm formaldehyde. The rationale for choosing this concentration of carbon black is that the current allowable occupational health limit for eight hours of exposure to "nuisance dusts" (which include carbon black) is a time-weighted average of 3.5 mg/m^3 (American Conference of Governmental Industrial Hygienists 1990). The rationale for using a target concentration of 2.55 ppm formaldehyde was that this concentration did not alter pulmonary bactericidal activity. The threshold limit value for formaldehyde is 1 ppm for eight hours per day, whereas the short-term exposure limit is 2 ppm for 15 minutes.

Five experiments were performed at this target concentration using 18 trained mice per experiment. Six mice were assayed immediately after bacterial challenge, and the remaining twelve were separated into either an exposed group or a control group. Because of the difficulties in achieving the precise target concentrations for carbon black and formaldehyde coexposures, the data were not pooled, and, therefore, are expressed as the mean ± SE of each individual experiment (Figure 8; bactericidal values in Appendix Table A.4). Multiple regression analysis of the data demonstrates that exposing animals to a target concentration of 3.5 mg/m^3 carbon black and 2.5 ppm formaldehyde for four hours after bacterial challenge did not alter pulmonary antibacterial defenses.

Because no effect was observed at target concentrations of 3.5 mg/m^3 carbon black and 2.5 ppm formaldehyde, experiments were initiated at target concentrations of 10 mg/m^3 and 5 ppm. Five experiments were performed at this target concentration with 18 trained mice per experiment. Multiple regression analysis of the data (Figure 9; bactericidal values in Appendix Table A.5) demonstrates that exposing animals to a target concentration of 10 mg/m^3 carbon black and 5 ppm formaldehyde for a four-hour period after bacterial challenge did not alter pulmonary antibacterial defenses.

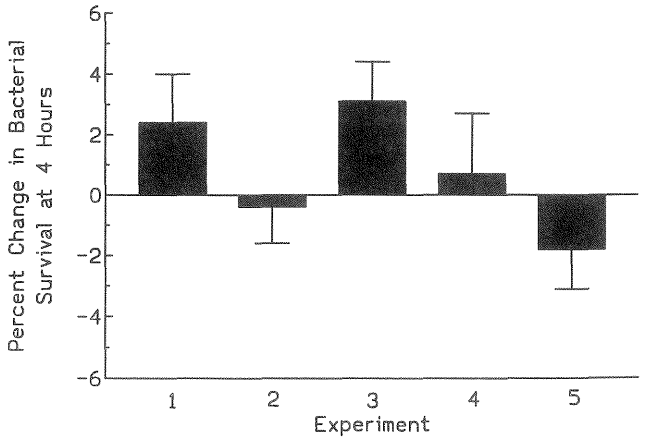

Figure 9. Comparison of intrapulmonary killing of *S. aureus* for mice exposed to target concentrations of 10.0 mg/m^3 carbon black and 5 ppm formaldehyde for four hours after bacterial challenge. The time-weighted concentrations were: experiment number 1, 4.8 mg/m^3 and 4.8 ppm; experiment number 2, 7 mg/m^3 and 4.1 ppm; experiment number 3, 13.2 mg/m^3 and 5.1 ppm; experiment number 4, 8.5 mg/m^3 and 5 ppm; and experiment number 5, 9.7 mg/m^3 and 5 ppm. The data are presented as the difference in percentage of change between control and carbon black and formaldehyde–exposed bactericidal values. Each value represents the mean ± SE of six determinations.

Carbon Black and Formaldehyde Coexposures Before Bacterial Challenge

Five experiments were performed with this protocol, using 24 trained mice per experiment. The data (Figure 10; bactericidal values in Appendix Table A.6) demonstrate that exposing animals to a target concentration of 3.5 mg/m^3 carbon black and 2.5 ppm formaldehyde for four hours per day for four days did not alter pulmonary antibacterial defenses. One experiment was performed at target concentrations of 10 mg/m^3 and 5 ppm, which also showed no effect (data not included).

ALVEOLAR MACROPHAGE FC-RECEPTOR–MEDIATED PHAGOCYTOSIS

For the first of these experiments, trained mice were exposed for four hours per day for four days to target concentrations of 10 mg/m^3 carbon black and 5 ppm formaldehyde. One, 3, 5, 10, 25, and 60 days after exposure, groups of five exposed and five control mice were killed, and their alveolar macrophage Fc-receptor–mediated phagocytic integrity was compared. For the second of these experiments, the phagocytic activity of the alveolar macrophages for the exposed and control groups was compared at intervals 1, 3, 5, 10, 14, 25, and 40 days after the end of exposure. As controls for the coexposures, mice were exposed for four hours per day for four days to either target concentrations of 10 mg/m^3 carbon black or 5 ppm formaldehyde for the second run of this experiment. These exposures were performed one week (formaldehyde) and two weeks (carbon black) after the combined inhalation exposures, and the animals were killed and assayed 3, 10, 25, and 40 days after the end of exposure.

More than 90% of the alveolar macrophages from unexposed control lungs were phagocytic, with each phagocytic macrophage ingesting approximately six erythrocytes. The phagocytic index is calculated by multiplying the percentage of phagocytic macrophages by the average number of erythrocytes ingested per phagocytic macrophage. For the above example, this was 90 × 6 = 540. Because an equal number of unexposed control mice was included for each experimental group at each time point, the data are presented as percentage of change from the phagocytic activity of control alveolar macrophages.

Initially, carbon black and formaldehyde coexposure did not alter the phagocytic activity of alveolar macrophages obtained from mice on the first and third day after the end of exposure (Figure 11; phagocytic indices in Appendix Table A.7). Phagocytic activity progressively declined through day 25 and was reestablished by day 60.

A repeat of this protocol showed the same response, with alveolar macrophage phagocytosis reestablished by day 40 (Figure 12; phagocytic indices in Appendix Table A.8). The controls demonstrated that neither the formaldehyde nor the carbon black alone had a significant effect on alveolar macrophage Fc-receptor–mediated phagocytosis (phagocytic indices in Appendix Tables A.9 and A.10).

Further analysis of the data shows that the impairment of phagocytic activity was due to the fact that fewer macrophages were phagocytic, and each phagocytic macrophage

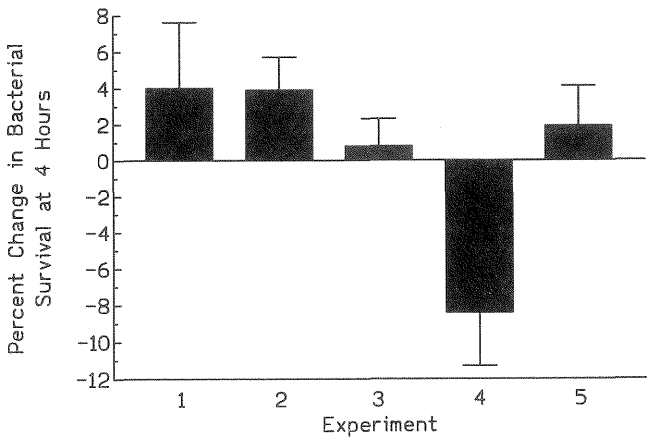

Figure 10. Comparison of intrapulmonary killing of *S. aureus* for mice exposed to target concentrations of 3.5 mg/m^3 carbon black and 2.5 ppm formaldehyde for four hours per day for four days before bacterial challenge. The time-weighted concentrations were: experiment number 1, 5.7 mg/m^3 and 2 ppm; experiment number 2, 6.1 mg/m^3 and 1.8 ppm; experiment number 3, 5.6 mg/m^3 and 1.7 ppm; experiment number 4, 6.1 mg/m^3 and 1.8 ppm; and experiment number 5, 4.7 mg/m^3 and 2.8 ppm. The data are presented as the difference in percentage of change between control and carbon black and formaldehyde–exposed bactericidal values. Each value represents the mean ± SE of six determinations.

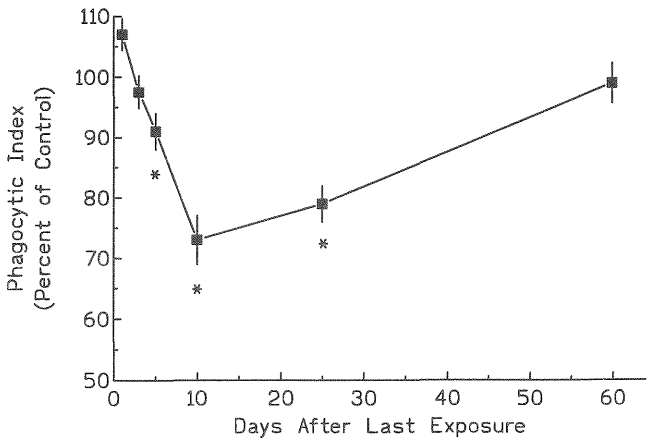

Figure 11. Comparison of alveolar macrophage Fc-receptor–mediated phagocytosis from mice exposed for four hours per day for four days to target concentrations of 10.0 mg/m^3 carbon black and 5 ppm formaldehyde (experiment number 1). Each value represents the mean ± SE of five determinations, * = $p < 0.05$.

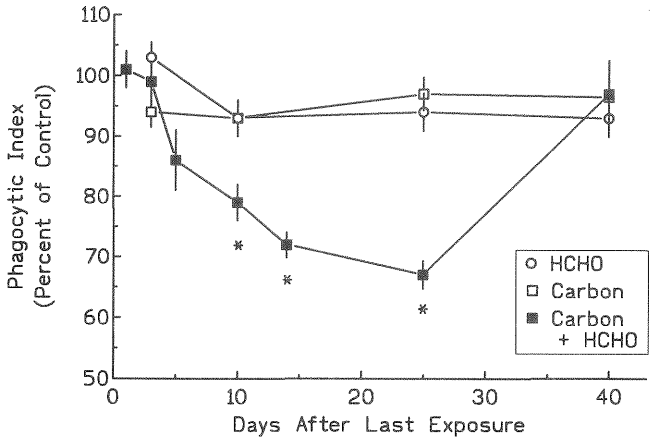

Figure 12. Comparison of alveolar macrophage Fc-receptor-mediated phagocytosis from mice exposed for four hours per day for four days to target concentrations of 10.0 mg/m^3 carbon black, 5 ppm formaldehyde, or both (experiment number 2). Each value represents the mean ± SE of five determinations, * = $p < 0.05$.

ingested fewer erythrocytes. Examining the coverslip preparations revealed no differences between the number of erythrocytes ingested by macrophages that contained carbon black and those that did not.

Finally, when the experiment was repeated, total and differential counts were performed on the cell populations lavaged from the lungs of coexposed mice. Table 1 shows that combined exposure to carbon black and formaldehyde did not induce a pulmonary inflammatory response, as quantified by the recovery of increased numbers of polymorphonuclear leukocytes and lymphocytes from lung lavages.

Table 1. Total and Differential Cell Counts of Cell Populations Lavaged from the Lungs of Mice After Coexposure to Target Concentrations of 10 mg/m^3 Carbon Black and 5 ppm Formaldehyde

Group	Total Cells ($\times 10^4$)	Alveolar Macrophages (%)	Lymphocytes (%)	Polymorphonuclear Leukocytes (%)
Day 1				
Control	13.2 ± 1.6[a]	98.4	1.5	0.1
Exposed	10.6 ± 1.3	99.1	0.3	0.3
Day 2				
Control	12.6 ± 1.2	99.3	0.6	0.1
Exposed	12.8 ± 1.4	99.2	0.1	0.7
Day 5				
Control	17.6 ± 3.2	99.2	0.7	0.1
Exposed	16.1 ± 1.7	98.9	0.7	0.4
Day 10				
Control	10.4 ± 0.8	97.9	1.7	0.2
Exposed	13.2 ± 1.2	98.8	1.1	0.1
Day 14				
Control	11.1 ± 0.6	98.0	1.8	0.4
Exposed	12.5 ± 1.9	97.3	1.5	1.2
Day 25				
Control	9.3 ± 1.4	98.9	1.0	0.1
Exposed	9.5 ± 3.3	98.9	0.9	0.2

[a] Values are expressed as means ± SE.

CHEMICAL PROPERTIES OF CARBON BLACKS

Before the particle-formaldehyde inhalation studies were initiated, the appropriate carbon black surrogate for Diesel exhaust was identified. The surfaces of the carbonaceous particles emitted by mobile sources are expected to have varying degrees of surface oxidation; thus, it was expected that the adsorption of formaldehyde would be significantly different on these two carbon blacks. The carbon blacks selected were a regular black (Regal GR/Regal 660) and an oxidized black (Mogul L/Black Pearls L). Oxidation does not significantly change the overall physical structure of the carbon black particles; however, the surface groups do contain increased oxygen.

Bulk Chemical Properties of the Carbon Blacks

The results of the elemental analysis of the carbon black samples for carbon, hydrogen, nitrogen, and oxygen are listed in Table 2. These data show that all the particles are composed mainly of carbon, quantifiable levels of hydrogen and oxygen, with minor amounts of nitrogen. These carbon blacks also contain detectable amounts of other, not quantified, elements, notably sulfur and aluminum. These elements were detected by x-ray fluorescence spectrometry during the particle sizing by scanning electron microscopy. The only carbon blacks that are different in terms of the elements quantified are Black Pearls L and Mogul L, for which the increased percentages of oxygen are balanced by reductions in the percentages of carbon. (This difference is consistent with the fact that these are oxidized carbon blacks.) Other features that may be important are the increased carbon/hydrogen ratios for Regal GR and Regal 660, perhaps signifying that the surfaces of these carbon blacks are composed of less energetic sites than the other carbon blacks.

Chemical Analysis of the Adsorbed Molecules Endogenous to Carbon Blacks

After this study was initiated, concern was expressed regarding the potential toxicities of any endogenous molecules on the surface of the carbon blacks. The identifications and quantifications of the polycyclic aromatic hydrocarbon components of these extracts were compared to authentic standards of polycyclic aromatic hydrocarbons;

Table 2. Elemental Analysis for Percent Carbon, Hydrogen, Nitrogen, and Oxygen of Carbon Black Adsorbents

Sample	% Carbon	% Hydrogen	% Nitrogen	% Oxygen	Empirical Formula
Mogul L	93.10	0.46	0.18	6.14	C_{202} H_{12} O_{10}
Black Pearls L	92.59	0.58	0.034	5.28	C_{234} H_{18} O_{10}
Regal GR	96.90	0.30	0.0043	1.42	C_{910} H_{34} O_{10}
Regal 660	97.56	0.32	0.017	1.34	C_{971} H_{38} O_{10}

Table 3. Summary of the Analyses of the Compounds Extracted from Regal 660 and Mogul L by Dichloromethane

Compound	Standard (ng/sample)	Regal 660 (ng/sample)	Mogul L (ng/sample)
Naphthalene	140	2,157	32
Acenaphthylene	280	6,071	7
Acenaphthene	140	44	2
Fluorene	28	51	3
Anthracene	14	166	11
Phenanthrene	14	ND[a]	ND
Fluoranthene	28	62	3
Pyrene	14	321	3
Chrysene	14	ND	ND
Benz[*a*]anthracene	14	ND	ND
Benzo[*b*]fluoroanthene	28	ND	ND
Benzo[*k*]fluoroanthene	14	ND	ND
Benzo[*a*]pyrene	14	ND	ND
Indene[1,2,3-*cd*]pyrene	14	ND	ND
Dibenz[*ajh*]anthracene	28	ND	ND
Benzo[*ghi*]perylene	28	ND	ND

[a] ND = not detected.

these results are contained in Table 3. Clearly, these carbon blacks contain very low levels of polycyclic aromatic hydrocarbons, and the oxidized carbon black (Mogul L) contains even less than the regular carbon black (Regal 660). Both these carbon blacks were approximately 0.01% extractable by dichloromethane.

PHYSICAL PROPERTIES OF CARBON BLACKS

The physical properties (particle size, external and specific surface areas, roughness factor, bulk and true density) that were determined for the carbon blacks are presented in Table 4. Based upon the data and consultations with the Health Effects Institute, Regal GR was selected as the model particle for all inhalation studies.

THE INTERACTIONS BETWEEN FORMALDEHYDE AND CARBON BLACKS

Adsorption of Formaldehyde

The heat of adsorption of the formaldehyde in formalin on Regal 660 was determined to be 36.5 kJ. This value is less than that previously determined for pure water on different pelletized carbon blacks (29–45 kJ) (Risby and Sehnert 1988), and lower than the heat of adsorption of water on Regal 660.

Experiments were designed to determine the appropriate separation conditions for the subsequent experiments using pure formaldehyde gas. The preliminary experiments using formalin on Black Pearls L produced far less information than did the corresponding experiments using formalin on Regal 660. The gas chromatography data obtained for Black Pearls L was uniformly irreproducible since it was impossible to observe the separation of the formalin into its components. These experiments only permitted qualitative observation that the formaldehyde eluted before the water and methanol. The data shown in Table 5 provide preliminary indications that the affinity of formaldehyde gas for Regal 660 may be low. The data for Black Pearls L was different, and these preliminary results suggest that the formaldehyde may be retained irreversibly by this oxidized carbon black, even in the presence of water and methanol. This ob-

Table 4. Physical Properties of Carbon Blacks

Carbon Black	Specific Surface Area (m^2/g)	Particle Size (μm)	External Surface Area (m^2/g)	Roughness Factor	Bulk Density (g/cm^3)	True Density (g/cm^3)
Regal GR	88	4.6	6.8×10^{-1}	1.3×10^{2}	0.52	1.95
Regal 660	94	20	1.6×10^{-2}	5.9×10^{3}	0.29	1.96
Mogul L	107	4.2	7.6×10^{-1}	1.4×10^{2}	0.28	1.92
Black Pearls L	130	22	1.4×10^{-2}	9.3×10^{3}	0.063	1.94

Table 5. Heats of Adsorption (kJ) for the Components of Formalin on Carbon Blacks Regal 660 and Black Pearls L Determined from Retention Data and Adsorption Isotherms

	ΔH (kJ)		
Carbon Black	Formaldehyde	Water	Methanol
Regal 660	36.5	68.1	84.6
Black Pearls L	Ret[a]	NS[b]	NS

[a] Ret = irreversibly retained at separation temperature.
[b] NS = not significant since the irreversible adsorption of formaldehyde changes the retention of these compounds.

servation was extremely significant because it offered the possibility of using both carbon blacks in the inhalation studies. Regal GR may rapidly release formaldehyde. Mogul L may release formaldehyde very slowly.

Studies Using Formaldehyde

The goal of this portion of the study was to determine the strength of adsorption of gaseous formaldehyde for the surface of carbonaceous particles. Understanding the selectivity of adsorption of formaldehyde under varied conditions (that is, formaldehyde alone, formaldehyde in the presence of methanol, or water, or both), was critical to the total project. Predictions made from the data by using quantitative physicochemical determinations may be directly related to the subsequent in vivo exposures.

Gas chromatographic retention data (temperatures for the separation were 80°C, 90°C, and 100°C for Regal 660; and 125°C, 140°C, and 155°C for Black Pearls L) were used to obtain values for the enthalpy of adsorption. These data are shown in Table 5. There does not appear to be a significant difference between the enthalpies of adsorption of formaldehyde on these blacks.

A number of qualitative observations can be made about the resulting gas chromatographic data obtained with the mobile phase containing water vapor and/or methanol vapor. First, the chromatographic peaks were more Gaussian in shape, which suggests that the water vapor in formalin deactivates the active sites on the carbon black surfaces which provide the heterogeneic character of the carbon black surfaces. These results also may be due to the formaldehyde interacting with sorbed water vapor via the mechanism of gas-liquid chromatography. Second, the chromatographic retention data were more consistent and less dependent upon sample volume injected, which is also indicative of gas-liquid interactions. Third, the peak areas of the eluting formaldehyde were greater than those observed with normal mobile phases, which suggests that formaldehyde is adsorbed strongly by some of the sites on the carbon surfaces and is only released slowly with dry helium. Additionally, new peaks were observed in the resulting chromatograms. These peaks are attributed to chemical reactions between water and formaldehyde at the surface of the carbon black. The results of these studies are shown in Table 6.

Prediction of the Adsorption Times for Formaldehyde on the Surface of Carbon Blacks

The adsorption times τ for formaldehyde on the surface of carbon blacks can be calculated from the heat of adsorption and other physical properties of formaldehyde, such as molecular weight (M), molar volume (V), and melting point (T_s). This calculation uses the relationship proposed by Frenkel (1924), in which τ_0 is the time of oscillation of the molecules in the adsorbed state with particular reference to vibrations perpendicular to the surface (Lindemann 1910):

$$\tau = \tau_0 \exp \Delta H^a/RT$$

$$\tau_0 = 4.75 \times 10^{-3}\{[M(V)^{0.667}]/T_s\}^{0.5}.$$

This equation and the data contained in Table 5 were used to calculate the time that formaldehyde remains associated with the surface of the carbon blacks in the presence and absence of water or methanol vapors. The results of these calculations are shown in Table 7.

Desorption of Adsorbed Formaldehyde from the Surface of Carbon Blacks into Synthetic Alveolar Lung Fluid

This objective was not achieved because formaldehyde was not retained by the carbon blacks in any aqueous mobile phase, which is consistent with the results of the gas phase adsorption studies. Therefore, an alternate aspect of the interaction of formaldehyde with carbon black was studied.

The Chemistry of Adsorbed Formaldehyde on the Surface of Carbon Blacks

Four products were observed in the reaction mixture.

Table 6. Heats of Adsorption (kJ) for Gaseous Formaldehyde on Carbon Blacks Regal 660 and Black Pearls L Determined from Retention Data Using Various Mobile Phases

	Isosteric Heat of Adsorption (kJ/mol)			
Carbon Black	Helium	Helium and Water	Helium and Methanol	Helium, Water, and Methanol
Regal 660	57.9	48.5	42.6	42.8
Black Pearls L	51.3	55.2	33.4	33.5

Table 7. Adsorption Times (seconds) at 25°C for Formaldehyde as a Function of Carbon Black and Mobile Phase Composition

	Carbon Black[a]	
Mobile Phase	Regal 660	Black Pearls L
Helium	9.0×10^{-3}	6.3×10^{-4}
Helium and Water	2.0×10^{-4}	3.0×10^{-3}
Helium and Methanol	1.9×10^{-5}	4.6×10^{-7}
Helium, Water, and Methanol	2.0×10^{-5}	4.8×10^{-7}

[a] Adsorption times are expressed in seconds.

These products were identified by the addition of known standards to the gas sampling vessel. The results of the analysis of the thermal desorption products were found to be water (3.7%), formaldehyde (94.0%), methanol (0.2%), and methyl formate (2.1%). Although no evidence of formic acid was found, it is reasonable to propose that it is formed since methyl formate was found. Clearly, the reaction products methanol and methyl formate are formed at low concentrations. Attempts were made to establish whether formic acid could be identified by washing the carbon black with water. This water also was analyzed by gas chromatography using the same conditions but no formic acid was detected. These results should be treated with caution since methanol and methyl formate could be produced by the thermal desorption of paraformaldehyde produced when formaldehyde adsorbs on the surface of the carbon black.

DISCUSSION

This study was designed to investigate whether exposure to formaldehyde decreases resistance to respiratory infections through dysfunctions of the alveolar macrophage phagocytic system. Additionally, the study explored whether the interaction of formaldehyde with respirable particles alters susceptibility to respiratory infections by delivering adsorbed formaldehyde to the deep lung with the inhaled particle. Physicochemical studies were performed to determine the equilibrium distribution of formaldehyde between the gas phase and the surface of a biologically inert particle, carbon black Regal GR. The biologic studies assessed the effect of inhalation coexposures to carbon black and formaldehyde on the antibacterial defense mechanisms of the lungs and alveolar macrophage phagocytosis.

BACKGROUND

Animal and human studies suggest that coexposure to respirable particles and formaldehyde produces increased biologic effects. Amdur (1960) showed a greater airway constrictive response in guinea pigs exposed to formaldehyde combined with sodium chloride particles than when they were exposed to formaldehyde alone. LaBelle and associates (1955) showed that the survival time of mice simultaneously exposed to formaldehyde and aerosols of diatomaceous earth, sodium chloride, and mineral oil was shorter than the survival time of controls exposed to formaldehyde alone. Frigas and colleagues (1984) described an asthmatic subject who had bronchial constriction caused by exposure to urea-formaldehyde foam, but not to gaseous formaldehyde. Other studies with humans have shown an acute bronchoconstrictive effect with low-level formaldehyde exposure that suggests concomitant particle exposure (Schoenberg and Mitchell 1975; Alexandersson et al. 1982; Kilburn et al. 1985).

Only a few studies have examined the outcome of inhaling carbonaceous particles and formaldehyde. The study conducted by Kilburn and McKenzie (1978) suggests such interactions can occur. Experimental coexposure for four hours to activated carbon and formaldehyde (700 mg/m^3 and 200 to 300 ppm; 567 mg/m^3 and 206 ppm; 805 mg/m^3 and 6 ppm, respectively) resulted in the recruitment of polymorphonuclear leukocytes to the upper airways, whereas exposure to either agent alone did not. Because particles were only rarely found in the alveoli, these researchers estimate that probably less than 10% of the particles were respirable.

In order to determine whether respirable carbonaceous aerosols have an interactive effect on the acute symptomatic and pulmonary responses to formaldehyde inhalation, Green and associates (1989) exposed human subjects for two hours each to either 3 ppm formaldehyde, 0.5 mg/m^3 respirable activated carbon, or the combination of formaldehyde and an uncharacterized carbon. Coexposure to activated carbon black and formaldehyde synergistically increased cough, but not other irritative respiratory tract symptoms. The combination produced a small (less than 5%) effect on pulmonary function, with decrements in forced vital capacity and in forced expiratory volume in three seconds.

CARBON PARTICLES

In addition to the above studies, much work has been performed on the health effects of inhalation exposure to Diesel exhaust (Strom 1984; reviewed by McClellan 1987; Marnett 1988) and other carbonaceous particles (Medalia et al. 1983; Rivin 1986). To place the experiments presented in this report in proper perspective, a description of the types of carbon particles used is warranted because carbon black is frequently confused with soot and activated carbon.

Elemental carbon can exist in an ordered crystalline structure, such as tetrahedrally hybridized face-centered cubic lattice (diamond). It also can exist as parallel layers of trigonally hybridized carbon in a hexagonal network (graphite), as a random structure with regions of ordered graphitic structure, or as a random structure (for example, amorphous). If high molecular weight organics are pyrolyzed, they produce a form of elemental carbon known as coke, char, or charcoal. These forms of carbon contain significant concentrations of heteroatoms and have relatively little graphitic structure. Activated carbon or charcoal is produced by treating these elemental forms of carbon with steam or carbon dioxide at elevated temperatures. These activation processes remove volatile impurities and increase the surface area of the resulting carbon. When gaseous or volatile lower molecular weight organics are pyrolyzed, colloidal carbon particles are produced by polymerization. Soot, Diesel particulate matter, and carbon blacks are all colloidal carbons. The only difference among these forms of carbon is the amount of partially pyrolyzed materials on the surfaces of the colloidal carbon particles. Diesel particulate matter may contain as much as 50% of the mass of solvent-extractable material. Carbon blacks are produced by well-controlled processes and can have graphitic structure, random structure with regions of graphitic structure, or a completely random structure.

Carbon black sometimes is described incorrectly as soot. This comparison is not valid for most environmental soots, which are mixtures of various forms of particulate carbon occluded with organic tars and resins (Boyland 1983). Particulate carbon exhibits variable composition and properties because it is produced by partial combustion or pyrolysis of carbonaceous matter. Some environmental soots contain more extractable organic tar than particulate carbon, with most deposited soots having very little carbon in colloidal form. For example, Diesel exhaust soot particles are chain aggregates with a primary mass median diameter of 10 to 80 nm. The specific surface area of Diesel soot is high. It depends on the outgassing temperature and is approximately 100 m^3 per g at 450°C. This high surface area adsorbs large amounts of vapor-phase organic compounds. Diesel soot consists primarily of carbon, hydrogen, oxygen, and nitrogen. The nonextractable carbonaceous core resembles carbon black. The extractable fraction (5% to 50%) is removed by solvent extraction; the relative amount of extractable material depends upon factors such as engine type and condition, fuel composition, and load. Although the health effects of Diesel exhaust soot may be of primary environmental concern, airborne soot is also generated by domestic chimneys and commercial furnace boilers. Although commercial carbon blacks differ in important respects from soots and other environmental carbonaceous particles, they are useful models for investigating the adsorption properties and health effects of these materials because they have well-defined surface properties and low quantities of endogenous adsorbed molecules. The presence of endogenous adsorbed molecules could produce bioeffects that would not have been relevant to the studies reported herein.

PHYSICOCHEMICAL STUDIES ON CARBON BLACK AND FORMALDEHYDE INTERACTIONS

Comparisons of the physical properties of the aciniform carbon blacks used in this study suggest that these blacks have extensive pore structures and do not contain long-range graphitic structure. The specific surface areas are similar for these carbon blacks and are comparable to those observed for Diesel particles (100 m^2/g) (Ross et al. 1982). Elemental analysis shows that the carbon blacks are composed mainly of carbon with quantifiable amounts of hydrogen and oxygen. Minor amounts of nitrogen were found (less than 0.1%). Mogul L and Black Pearls L contain more oxygen than Regal GR and Regal 660 because they are oxidized. Minor amounts (less than 1 μg/g carbon black) of total polycyclic aromatic hydrocarbons were found in the dichloromethane extracts by capillary gas chromatography electron impact mass spectrometric analyses. On the basis of these analyses, most of the extractable material could be accounted for by the mass of naphthalene and acenaphthalene.

The isosteric heats of adsorption of formaldehyde on the pelletized carbon blacks as a function of mobile phase were slightly larger for the nonoxidized carbon black, Regal 660. Additionally, the heats of adsorption of formaldehyde on these blacks were measured as a function of water vapor and/or methanol vapor which was added to the mobile phase. Water was added to the mobile phase since the air that is used in the nose-only exposure studies is humidified, and this water vapor could be expected to modify the adsorption of formaldehyde on the carbon blacks. The effect of methanol on the adsorption of formaldehyde on carbon black was investigated since formaldehyde, carbonaceous particles, and unburnt methanol are likely to be the major emissions from Diesel engines operated on fuels augmented with methanol. The heat of adsorption decreased with the addition of either water or methanol for the nonoxidized carbon black and decreased with the addition of methanol for the oxidized black. The heat of adsorption for formaldehyde on the oxidized carbon black in the presence of water was greater. This result can be explained by the reaction between the formaldehyde and water to form paraformaldehyde. The acidic active sites on the oxidized carbon black (Risby and Sehnert 1988) can be expected to catalyze the

polymerization of formaldehyde. This observation is supported by the appearance of additional peaks (reaction products) when the mobile phase contains water vapor. Additionally, the peak areas for formaldehyde were larger and the peak shapes were more Gaussian when the mobile phase contained water or methanol. These observations indicate that formaldehyde was interacting irreversibly with the active sites on the carbon black when the mobile phase was dry helium (that is, gas solid chromatography) and that formaldehyde was interacting with adsorbed water and/or methanol (that is, gas liquid chromatography) when mixed mobile phases were used.

A thermodynamic model has been developed (a complete description of the theory is presented in Appendix B) to predict the distribution of volatile pollutants between the gas phase and respirable particles. The resulting data may be used to estimate the dose of sorbed pollutants for inhalation studies. The utility of this model has been demonstrated by experimental confirmation of the predicted amount of adsorbed formaldehyde on the surface of a carbon black. This model may be used in a similar manner for other agents and particles of environmental origin, providing that the surface of the particle is well characterized and the chemistry of the adsorbing molecule or molecules is known, since it relies upon measurable physicochemical properties of particles, pollutant molecules, and their interactions.

The low heat of adsorption of formaldehyde on Regal GR suggests that formaldehyde does not remain adsorbed for a significant period of time. If the physical properties of the carbon blacks and the time formaldehyde remains associated with the surface of the carbon blacks are used in combination with the experimental conditions used in the nose-only exposure study, (that is, formaldehyde concentration = 6 ppm [$N_c = 7 \times 10^{-9}$] [HCHO g/cm^3]; carbon black concentration = 14 mg/m^3[$P_p = 14 \times 10^{-9}$] [Regal GR g/cm^3]), then it is possible to predict ($\sigma_a = 3\tau N_c P_p [8RT/\pi M]^{0.5}/2dp\rho_p$) the amount of formaldehyde that will be adsorbed on the surface of the carbon black (σ_a, Regal GR). The result of this calculation is that 5.0 mg of the gas-phase formaldehyde is adsorbed onto the surface of 1.0 g of Regal GR. These calculations suggest that, under the conditions used in the inhalation study, nearly all the formaldehyde remains in the gas phase (6 ppm) and a very minor amount is adsorbed on the carbon black (60 parts per billion).

This predicted conclusion was validated experimentally (Risby et al. 1990) by collecting the airborne carbon black particles in the exposure chamber on both a cascade impactor (inlet or outlet of the inhalation chamber) and an open-faced membrane filter (nose-only exposure port). Approximately 3.0 mg formaldehyde per gram of carbon black was found on the sample collected by the cascade impactor, and no formaldehyde was detected on the carbon black collected on the open-faced membrane filter. The difference between the amounts of formaldehyde found on the collected carbon black may be explained by the variations in sampling procedures. Air is drawn continuously through the carbon black collected by the membrane filter and is not drawn through the carbon black collected by the cascade impactor. There are uncertainties attached to the experimental values for the bound formaldehyde since low quantities of carbon black were collected with either sampling device. Small amounts of carbon black were collected in order to reduce any potential sampling artifacts.

Considering the small amount of gas-phase formaldehyde that remains associated with the carbon black, the solubility of formaldehyde in aqueous systems, and the significant decrease in the heat of adsorption of formaldehyde in the presence of small amounts of water vapor, it is reasonable to propose that any formaldehyde adsorbed as formaldehyde will be released immediately when the carbon black particle is deposited in the alveolar surfactant of the distal lung. This prediction was confirmed by the experimental evidence showing that an aqueous solution of formaldehyde was not retained by the carbon black.

BIOLOGIC STUDIES

In the respiratory tract, the alveolar macrophage phagocytic system serves as the primary defense mechanism against inhaled particles that reach the distal lung (Green et al. 1977). These surveillance phagocytes rapidly seek and ingest particles that reach the alveolar region, thereby sequestering them from the vulnerable respiratory membrane. If the internalized particle is nondegradable, the phagocyte serves as a storage site for the particle until it can be cleared from the lungs by alveolobronchiolar or mucociliary transport mechanisms (Green et al. 1977). If the particle is degradable, such as a bacterium, the bactericidal armamentarium of the macrophages rapidly inactivates and degrades the ingested organism (Kim et al. 1976). Studies on the defense mechanisms of the lung against inhaled *S. aureus* have shown that the intrapulmonary killing of this bacterium primarily depends on the alveolar macrophage phagocytic system (Goldstein et al. 1976). Other bacterial agents induce an inflammatory response, with the intraalveolar influx of polymorphonuclear phagocytes providing additional phagocytic defense capabilities (Pierce et al. 1977; Rehm et al. 1979, 1980). Inhalation challenges with the numbers of staphylococci used in these studies do not produce an inflammatory response (Lipscomb et al. 1983). Therefore, the bactericidal mechanisms of the lung against this organism depend primarily on the alveolar macro-

phages (Goldstein et al. 1976; Lipscomb et al 1983; Onofrio et al. 1983). The quantifications of intrapulmonary staphylococcal killing and Fc-receptor–mediated phagocytosis are recognized, respectively, as in vivo and ex vivo assays to probe the functional integrity of the alveolar macrophage phagocytic system.

Carbon black deposited in the lungs is rapidly engulfed by the alveolar macrophages; the ingestion of the particles has no effect on a subsequent phagocytic challenge. If the organic vapor is adsorbed on the carbon black, it will be deposited with the inhaled particles on the alveolar surface. The release of the organic vapor, either in the alveolar surfactant or soon afterward in the macrophages, may result in an impairment of phagocytosis.

Formaldehyde Exposure and Pulmonary Antibacterial Defenses

Formaldehyde exposure for four hours after bacterial challenge impaired the antistaphylococcal defenses of the lungs at concentrations greater than 10 ppm. Because this concentration is 10 to 20 times greater than the odor threshold and much higher than ambient levels (Bernstein et al. 1984), the exposure protocol was changed. In working with an identical antistaphylococcal model, Goldstein and colleagues (1973) found a reduced level for the toxic threshold of nitrogen dioxide when exposure to the gas preceded and was continued after bacterial challenge. Our data show that preexposure to 1 ppm formaldehyde for 18 hours before bacterial challenge, followed by another four hours of exposure after bacterial challenge, significantly suppressed the intrapulmonary killing of *S. aureus*. This change in exposure protocol reduced the threshold dose from greater than 10 ppm to 1 ppm. A concentration × time (C × T) comparison between the two exposure protocols suggests that the length of exposure is the dominant factor. However, when formaldehyde exposure was not continued after bacterial challenge, no effect was found. This, in turn, suggests that the mechanisms involved in formaldehyde-induced suppression of lung defenses against *S. aureus* may be related not only to factors that depend on C × T influences, but also to factors such as the time of bacterial challenge during the exposure protocol.

Carbon Black and Formaldehyde Coexposures and Pulmonary Antibacterial Defenses

To mimic the experiments with formaldehyde vapors, mice were exposed to target concentrations of 3.5 mg/m^3 carbon black and 2.5 ppm formaldehyde for four hours after bacterial challenge. Because this coexposure level did not alter the intrapulmonary killing of staphylococci, the concentrations were raised to 10 mg/m^3 carbon black and 5 ppm formaldehyde, which also had no effect. It should be kept in mind that the adopted threshold limit values for carbon black and formaldehyde are 3.5 mg/m^3 and 1 ppm, respectively.

An explanation for the lack of impairment of pulmonary antibacterial defenses at the high levels of carbon black and formaldehyde coexposures after bacterial challenge requires several considerations. First, intrapulmonary bacterial killing proceeds at a rapid rate after an inhalation challenge with staphylococci. It has been demonstrated that within 30 minutes of exposure, over 90% of the bacteria are ingested by the alveolar macrophages; within four hours, approximately 90% of the bacteria are killed in the lungs (Kim et al. 1976). Second, with carbon black and formaldehyde exposures after bacterial challenge, the lung burden of carbon black gradually increases over the four-hour exposure period. Third, our calculations, verified by experimental data, indicated that almost all of the formaldehyde remained in the gas phase, and a minor amount was adsorbed on the carbon black. This scenario would lead to a situation where carbon black particles are progressively delivered to the lung with very little adsorbed formaldehyde at a time when most of the staphylococci have already been internalized within the alveolar macrophages and are being killed rapidly. The intracellular killing process is already at full speed when the carbon black with nearly no adsorbate is delivered to the depth of the lung. Thus, there may not be sufficient time for the carbon black and formaldehyde combination to induce a detrimental effect on macrophage killing, and there may not be enough formaldehyde adsorbed on the carbon black to induce an effect.

Therefore, it may be that the carbon black and formaldehyde combination has to be delivered to the lung before the bacterial challenge so that it can induce an impairment in intrapulmonary bacterial killing. Preliminary experiments showed that after inhalation exposure with carbon black, most of the particles were found within alveolar macrophages. Having the macrophages already "loaded" with the carbon black and formaldehyde combination before the bacterium reaches the lungs may provide sufficient time for the particle and adsorbate complex to induce physiologic effects on the pulmonary phagocyte (that is, impairment of phagocytic uptake or dysfunctions in the intracellular killing apparatus) that would impair the intrapulmonary bactericidal activity. However, exposing animals at a target concentration of 3.5 mg/m^3 carbon black and 2.5 ppm formaldehyde for four hours per day for four days did not alter pulmonary antibacterial defenses; similarly, no effects were observed when the target concentration was increased to 10 mg/m^3 carbon black and 5 ppm formaldehyde in one experiment.

Alveolar Macrophage Fc-Receptor–Mediated Phagocytosis

To determine whether any possible adverse effect of coexposure to carbon black and formaldehyde was delayed, alveolar macrophage Fc-receptor–mediated phagocytosis was assayed at intervals up to 60 days after the end of exposure. The results showed a progressive decrement in phagocytosis through day 25; normal phagocytic potential returned by day 40. These observations were made at coexposures to a target concentration of 10 mg/m^3 of carbon black and 5 ppm of formaldehyde. Exposure to either 10 mg/m^2 of carbon black or 5 ppm of formaldehyde alone did not result in an impairment of alveolar macrophage phagocytosis.

COMMENTARY

The data raise several questions: (1) what is the relationship between Fc-receptor–mediated phagocytosis and the intrapulmonary killing of *S. aureus*? (2) would the effect have occurred at lower concentrations? and (3) what is the potential mechanism of the impaired phagocytic effect?

The in vitro phagocytic assay quantifies the ingestion of opsonized sheep erythrocytes via the alveolar macrophage membrane Fc-receptor, whereas the bactericidal assay measures not only the ingestion of staphylococci by nonspecific receptors, but also the intracellular killing of the organism. Also, in the milieu of the lung, all the ancillary support and repair mechanisms for the alveolar macrophage phagocytic system are present, whereas they are absent in vitro. This suggests that higher concentrations of inhaled toxicants may be required to suppress alveolar macrophage intrapulmonary staphylococcal killing than those needed to impair alveolar macrophage Fc-receptor–mediated phagocytic ingestion in vitro. However, comparative studies on the relative sensitivity of these in vivo and in vitro assays have not been performed. Furthermore, it may be that the various membrane receptors involved in alveolar macrophage phagocytic function (nonspecific, Fc, and complement) are affected differently by a given inhaled toxicant.

The mechanism for impairing alveolar macrophage Fc-receptor–mediated phagocytosis induced by coexposures to carbon black and formaldehyde remains obscure. The data clearly demonstrate that the functional capacity of the phagocytic cells found in the deep lung was impaired despite the adsorption of minor quantities of formaldehyde on the carbon black particles. This indicates that any amount of formaldehyde delivered to the deep lung by a particle may produce some biologic effect. The reason why the defect in alveolar macrophage phagocytosis occurred only at time intervals after the cessation of coexposure is unknown. The physicochemical studies show that an aqueous solution of formaldehyde was not retained by the carbon black, and, therefore, it is reasonable to assume that any formaldehyde adsorbed as formaldehyde was released immediately after the carbon black particle was deposited in the alveolar surfactant. However, if this were so, it would not be unreasonable to expect that the rapid desorption of formaldehyde would result in an immediate effect, rather than the observed progressive phagocytic dysfunction. Alternatively, in the milieu of the lung, the formaldehyde may not be desorbed as rapidly as the rate predicted by the physicochemical studies, because the carbon particles are in a lipophilic environment. After ingestion of the carbon black particles, desorption could occur over an extended period of time. This could result in a slow accumulation of an internal dose, which, in turn, would produce a progressive effect, as evidenced by the continuous decline in phagocytic function.

Alternatively, more formaldehyde may have reached the distal lung than was predicted by the physicochemical experiments. In these experiments, the amount of formaldehyde adsorbed on the carbon black during coexposures was quantified by the release of formaldehyde. The possibility exists that during coexposures with carbon black some formaldehyde may have been polymerized into paraformaldehyde catalyzed by the acidic active sites on the carbon black, resulting in a carbon black, formaldehyde, and paraformaldehyde complex. Then, in the alveolar macrophages, the paraformaldehyde may have decomposed slowly, producing formaldehyde and other reaction products that become increasingly available to produce the delayed and progressive alveolar macrophage phagocytic dysfunction. This mechanism appears to be possible because the gas chromatographic studies showed evidence of reaction products such as formic acid and methyl formate.

The pioneering work of Amdur (reviewed in 1985) on the enhanced biologic effect of concomitant inhalation exposures to particles and gases amply pointed out the importance of such interactions. However, such studies require a large initial investment in specialized equipment and are labor intensive. Because of this, most toxicologic studies with air pollutants involve exposure to single materials. Recently, there has been renewed interest in "real world" exposures because of concern about interactions due to inhaling pollutant mixtures (National Research Council 1988; Henderson and Schlesinger 1989). The experiments reported herein emphasize the importance of such interactions. Furthermore, they indicate that unless multiple biologic parameters are investigated at various time intervals, significant effects may be missed.

The experiments in this study demonstrated that inhala-

tion exposure to the carbon black and formaldehyde concentrations had no effect on alveolar macrophage–dependent intrapulmonary killing of *S. aureus* when the assay was performed a day after the end of exposure. In a similar manner, alveolar macrophage Fc-receptor–mediated phagocytosis also was unaffected immediately after exposure. However, from 3 through 25 days after the end of exposure, alveolar macrophage Fc-receptor–mediated phagocytosis was progressively impaired; full recovery of the phagocytic capacity occurred 40 days after the end of exposure. In contrast, exposure to either carbon black or formaldehyde alone had no significant effect on alveolar macrophage Fc-receptor–mediated phagocytosis.

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APPENDIX A. Pulmonary Antistaphylococcal Defenses and Alveolar Macrophage Fc-Receptor–Mediated Phagocytosis

Table A.1. Comparison of Intrapulmonary Killing of *Staphylococcus Aureus* in Mice Exposed to Various Concentrations of Formaldehyde for Four Hours After Bacterial Challenge

Formaldehyde Concentration (ppm)	Run Number	*n*	Percentage of Initial Viable Bacteria Remaining[a] 4 Hours		
			0 Hours	Control	Formaldehyde
1	1	6	100 ± 6.2	9.2 ± 1.3	15.5 ± 2.3
	2	6	100 ± 6.4	16.9 ± 4.1	10.9 ± 1.6
	3	6	100 ± 5.3	19.9 ± 2.9	25.3 ± 4.9
	4	6	100 ± 8.9	12.9 ± 1.8	12.1 ± 2.9
	5	6	100 ± 12.4	8.9 ± 0.8	12.6 ± 2.6
	6	6	100 ± 8.9	26.7 ± 6.1	15.1 ± 3.1
	Pool	36	100 ± 7.5	15.5 ± 2.1	14.7 ± 1.4
5	1	6	100 ± 6.4	16.9 ± 4.1	10.7 ± 2.9
	2	6	100 ± 5.3	19.9 ± 2.9	24.8 ± 12.8
	3	6	100 ± 8.9	12.9 ± 1.8	16.6 ± 5.8
	4	6	100 ± 12.2	8.9 ± 0.8	13.5 ± 2.0
	5	6	100 ± 7.4	11.0 ± 1.4	23.3 ± 3.3
	6	6	100 ± 8.7	26.7 ± 6.1	23.5 ± 3.6
	Pool	36	100 ± 7.3	16.8 ± 2.3	17.9 ± 2.3
10	1	6	100 ± 6.2	9.0 ± 1.3	13.4 ± 2.6
	2	6	100 ± 3.0	10.6 ± 2.7	10.3 ± 2.0
	3	6	100 ± 8.8	14.6 ± 2.4	12.6 ± 2.3
	4	6	100 ± 5.4	14.3 ± 4.2	23.4 ± 4.0
	5	6	100 ± 7.9	6.7 ± 0.8	13.4 ± 2.3
	6	6	100 ± 9.7	13.2 ± 3.0	15.8 ± 3.2
	Pool	36	100 ± 5.1	11.3 ± 1.1	14.7 ± 1.3
15	1	6	100 ± 3.0	10.6 ± 2.7	12.8 ± 2.5
	2	6	100 ± 8.8	14.6 ± 2.4	24.9 ± 6.7
	3	6	100 ± 5.4	14.3 ± 4.2	26.4 ± 8.3
	4	6	100 ± 7.9	6.7 ± 0.8	19.8 ± 7.1
	5	6	100 ± 9.7	13.2 ± 3.0	16.8 ± 3.1
	6	6	100 ± 7.4	11.3 ± 1.4	24.3 ± 3.4
	Pool	36	100 ± 4.5	11.9 ± 1.2[b]	20.9 ± 2.4[b]

[a] Values are expressed as means ± SE.
[b] $p < 0.05$.

Table A.2. Comparison of Intrapulmonary Killing of *Staphylococcus Aureus* in Mice Exposed to 0.5 ppm Formaldehyde for 18 Hours Before, Four Hours After, or 18 Hours Before and Four Hours After Bacterial Challenge[a]

Run Number	n	0 Hours	Percentage of Initial Viable Bacteria Remaining 4 Hours			
			Control	Formaldehyde Before Challenge	Formaldehyde After Challenge	Formaldehyde Before and After Challenge
1	6	100 ± 7.8	7.2 ± 1.7	18.4 ± 3.2	11.4 ± 2.4	7.7 ± 0.5
2	6	100 ± 4.5	14.9 ± 4.5	10.9 ± 2.5	16.4 ± 4.1	10.9 ± 1.2
3	6	100 ± 9.5	14.4 ± 2.2	12.2 ± 1.3	13.6 ± 1.6	14.8 ± 2.5
4	6	100 ± 6.4	13.7 ± 1.2	11.4 ± 2.1	16.0 ± 2.5	16.0 ± 2.2
5	6	100 ± 13.2	9.9 ± 3.1	11.3 ± 2.1	13.4 ± 1.4	12.6 ± 3.0
6	6	100 ± 7.8	15.0 ± 3.1	12.6 ± 1.1	10.9 ± 1.3	13.8 ± 1.8
Pool	36	100 ± 6.2	12.5 ± 1.2	13.6 ± 1.0	12.8 ± 1.0	12.6 ± 0.9

[a] Values are expressed as means ± SE.

Table A.3. Comparison of Intrapulmonary Killing of *Staphylococcus Aureus* in Mice Exposed to 1 ppm Formaldehyde for 18 Hours Before, Four Hours After, or 18 Hours Before and Four Hours After Bacterial Challenge[a]

Run Number	n	0 Hours	Percentage of Initial Viable Bacteria Remaining 4 Hours			
			Control	Formaldehyde Before Challenge	Formaldehyde After Challenge	Formaldehyde Before and After Challenge
1	6	100 ± 8.5	13.1 ± 2.0	11.7 ± 1.9	12.3 ± 0.6	21.7 ± 2.5
2	6	100 ± 6.8	8.6 ± 1.2	16.7 ± 7.2	8.3 ± 0.7	22.0 ± 5.4
3	6	100 ± 7.2	7.2 ± 0.7	9.7 ± 1.0	6.8 ± 0.6	16.3 ± 5.5
4	6	100 ± 10.5	9.2 ± 2.1	15.9 ± 1.8	8.4 ± 1.1	12.5 ± 3.5
5	6	100 ± 11.3	13.1 ± 2.2	14.8 ± 2.2	14.1 ± 1.1	23.5 ± 6.8
6	6	100 ± 4.7	12.4 ± 1.4	13.0 ± 1.6	9.9 ± 0.6	18.1 ± 4.3
Pool	36	100 ± 4.0	11.5 ± 0.8[b]	12.4 ± 1.2	10.6 ± 0.7	18.3 ± 2.0[b]

[a] Values are expressed as means ± SE.
[b] $p < 0.05$.

Table A.4. Comparison of Intrapulmonary Killing of *Staphylococcus Aureus* in Mice Coexposed to Target Concentrations of 3.5 mg/m^3 Carbon Black and 2.5 ppm Formaldehyde After Bacterial Challenge

Actual Concentrations			Percentage of Initial Viable Bacteria Remaining[a] 4 Hours	
Carbon Black (mg/m^3)	Formaldehyde (ppm)	0 Hours	Control	Carbon Black and Formaldehyde
4.2	2.5	100 ± 7.1	15.3 ± 1.4	18.2 ± 2.5
6.8	3.0	100 ± 8.7	17.2 ± 2.0	16.0 ± 1.4
3.7	2.7	100 ± 4.1	18.0 ± 1.8	21.2 ± 2.5
2.4	3.6	100 ± 4.2	20.3 ± 2.7	17.7 ± 1.2
4.4	2.7	100 ± 7.3	14.7 ± 1.3	17.5 ± 2.0

[a] Values are expressed as means ± SE (n = 6).

Table A.5 Comparison of Intrapulmonary Killing of *Staphylococcus Aureus* in Mice Coexposed to Target Concentrations of 3.5 mg/m^3 Carbon Black and 5 ppm Formaldehyde After Bacterial Challenge

Actual Concentrations			Percentage of Initial Viable Bacteria Remaining[a] 4 Hours	
Carbon Black (mg/m^3)	Formaldehyde (ppm)	0 Hours	Control	Carbon Black and Formaldehyde
4.8	4.8	100 ± 8.1	17.7 ± 1.7	20.3 ± 1.7
7.0	4.1	100 ± 2.9	27.5 ± 3.1	27.1 ± 1.8
13.2	5.1	100 ± 4.7	17.0 ± 0.6	20.1 ± 1.0
8.5	5.0	100 ± 17	19.5 ± 2.8	20.2 ± 2.1
9.7	5.0	100 ± 5.8	23.9 ± 1.2	22.1 ± 1.0

[a] Values are expressed as means ± SE (n = 6).

Table A.6 Comparison of Intrapulmonary Killing of *Staphylococcus Aureus* in Mice Coexposed to Target Concentrations of 3.5 mg/m^3 Carbon Black and 2.5 ppm Formaldehyde for Four Hours per Day for Four Days Before Bacterial Challenge

Actual Concentrations			Percentage of Initial Viable Bacteria Remaining[a] 4 Hours	
Carbon Black (mg/m^3)	Formaldehyde (ppm)	0 Hours	Control	Carbon Black and Formaldehyde
5.7	2.0	100 ± 9.4	18.7 ± 1.9	22.8 ± 3.6
6.1	1.8	100 ± 8.8	13.9 ± 1.9	18.0 ± 1.9
5.6	1.7	100 ± 6.8	14.7 ± 2.2	15.5 ± 1.5
6.1	1.8	100 ± 5.9	25.5 ± 2.7	17.1 ± 2.9
4.7	2.8	100 ± 7.4	20.5 ± 2.2	22.4 ± 2.2

[a] Values are expressed as means ± SE (n = 6).

Table A.7. Comparison of Alveolar Macrophage Fc-Receptor–Mediated Phagocytosis from Mice Coexposed to Target Concentrations of 10 mg/m^3 Carbon Black and 5 ppm Formaldehyde for Four Hours per Day for Four Days (Experiment Number 1)[a]

Days After Exposure	Percentage of Phagocytic Macrophages	Sheep Erythrocytes Ingested per Phagocytic Macrophage	Phagocytic Index
Day 1			
Control	92.4 ± 1.0	5.6 ± 0.1	515 ± 15
Exposed	94.4 ± 0.6	5.9 ± 0.1	556 ± 13
Day 3			
Control	96.4 ± 0.6	6.6 ± 0.2	636 ± 23
Exposed	94.0 ± 0.7	6.6 ± 0.1	620 ± 17
Day 5			
Control	92.7 ± 0.8	6.2 ± 0.1	574 ± 15[b]
Exposed	91.3 ± 0.7	5.7 ± 0.1	520 ± 12[b]
Day 10			
Control	94.0 ± 1.1	6.7 ± 0.2	630 ± 23[b]
Exposed	85.4 ± 2.2	5.4 ± 0.2	461 ± 24[b]
Day 25			
Control	90.8 ± 1.1	5.9 ± 0.1	536 ± 17[b]
Exposed	86.1 ± 1.3	5.1 ± 0.2	439 ± 19[b]
Day 60			
Control	95.2 ± 0.6	6.1 ± 0.2	581 ± 18
Exposed	93.0 ± 1.0	6.2 ± 0.2	577 ± 19

[a] Values are expressed as means ± SE (n = 5).
[b] $p < 0.05$.

Table A.8. Comparison of Alveolar Macrophage Fc-Receptor–Mediated Phagocytosis from Mice Coexposed to Target Concentrations of 10 mg/m^3 Carbon Black and 5 ppm Formaldehyde for Four Hours per Day for Four Days (Experiment Number 2)[a]

Days After Exposure	Percentage of Phagocytic Macrophages	Sheep Erythrocytes Ingested per Phagocytic Macrophage	Phagocytic Index
Day 1			
Control	95.7 ± 0.6	6.2 ± 0.2	593 ± 17
Exposed	93.5 ± 0.6	6.4 ± 0.2	598 ± 18
Day 3			
Control	93.1 ± 0.8	5.8 ± 0.1	540 ± 13
Exposed	92.4 ± 0.7	5.8 ± 0.1	536 ± 15
Day 5			
Control	95.4 ± 1.2	6.5 ± 0.3	620 ± 36
Exposed	92.3 ± 1.7	5.8 ± 0.3	535 ± 33
Day 10			
Control	95.4 ± 0.8	5.9 ± 0.2	563 ± 18[b]
Exposed	87.6 ± 0.9	5.1 ± 0.2	447 ± 17[b]
Day 14			
Control	90.4 ± 1.0	5.6 ± 0.1	506 ± 18[b]
Exposed	77.8 ± 1.0	4.7 ± 0.1	366 ± 16[b]
Day 25			
Control	95.4 ± 0.8	7.0 ± 0.1	667 ± 19[b]
Exposed	86.1 ± 1.7	5.2 ± 0.1	449 ± 15[b]
Day 40			
Control	97.9 ± 1.1	5.1 ± 0.1	499 ± 12
Exposed	98.9 ± 1.1	5.0 ± 0.1	494 ± 19

[a] Values are expressed as means ± SE (n = 5).
[b] $p < 0.05$.

Table A.9. Comparison of Alveolar Macrophage Fc-Receptor–Mediated Phagocytosis from Mice Coexposed to Target Concentrations of 5 ppm Formaldehyde for Four Hours per Day for Four Days (Experiment Number 2)[a]

Days After Exposure	Percentage of Phagocytic Macrophages	Sheep Erythrocytes Ingested per Phagocytic Macrophage	Phagocytic Index
Day 3			
Control	94.4 ± 0.6	6.7 ± 0.1	632 ± 16
Exposed	93.8 ± 1.0	7.0 ± 0.1	656 ± 16
Day 10			
Control	97.2 ± 0.5	6.2 ± 0.1	602 ± 15
Exposed	96.1 ± 0.6	5.9 ± 0.1	567 ± 17
Day 25			
Control	93.0 ± 1.0	6.8 ± 0.2	632 ± 21
Exposed	91.1 ± 0.8	6.5 ± 0.2	595 ± 20
Day 40			
Control	94.7 ± 0.3	5.9 ± 0.2	559 ± 19
Exposed	95.1 ± 0.4	5.8 ± 0.2	551 ± 18

[a] Values are expressed as means ± SE (n = 5).

Table A.10. Comparison of Alveolar Macrophage Fc-Receptor–Mediated Phagocytosis from Mice Coexposed to Target Concentrations of 10 mg/m^3 Carbon Black for Four Hours per Day for Four Days (Experiment Number 2)[a]

Days After Exposure	Percentage of Phagocytic Macrophages	Sheep Erythrocytes Ingested per Phagocytic Macrophage	Phagocytic Index
Day 3			
Control	92.6 ± 0.8	6.0 ± 0.2	556 ± 17
Exposed	90.0 ± 0.8	5.7 ± 0.1	513 ± 14
Day 10			
Control	96.1 ± 0.7	6.5 ± 0.2	624 ± 19
Exposed	94.3 ± 0.8	6.1 ± 0.2	575 ± 17
Day 25			
Control	92.3 ± 0.4	5.9 ± 0.3	545 ± 17
Exposed	93.2 ± 0.3	5.7 ± 0.3	531 ± 16
Day 40			
Control	93.7 ± 0.1	6.1 ± 0.1	571 ± 11
Exposed	93.0 ± 1.0	5.9 ± 0.1	553 ± 12

[a] Values are expressed as means ± SE (n = 5).

APPENDIX B. Phase Distribution of Volatile Pollutants: A Thermodynamic Model

Excerpted from: "A model to estimate effective doses of adsorbed pollutants on respirable particles and their subsequent release into alveolar surfactant. 1. Validation of the model for the adsorption and release of formaldehyde on a respirable carbon black" (Risby et al. 1990).

THEORY

Nonelastic Collisions Between Gas-Phase Molecules and Airborne Particles

If n gas-phase molecules collide with a unit area of a surface per unit time and remain there for an average time τ, then we will find σ molecules per unit area of surface where

$$\sigma = n\tau. \quad (1)$$

An expression has been derived for nonelastic collisions between N gas-phase molecules (molecules/cm^3) with molecular weight M moving with a velocity C (cm/sec) at a temperature T (K), and a surface of unit area (deBoer 1953). This equation is based on the assumption that the molecules are moving in all directions, that is, there is no angular dependence on the collisions with the surface (Knudsen 1934).

$$n = N\,C/4 \quad (2)$$

$$n = (8\,RT/\pi\,M)^{0.5}\,N/4 \quad (3)$$

However, if the gas-phase molecules are not colliding with a surface of unit area but are instead colliding nonelastically with spherical particles of concentration P (particle/cm^3), and mass mean particle diameter, dp (cm), we can derive a similar expression for n_p (number of gas-phase molecules colliding nonelastically with the total number of particles per unit volume-second). This derivation assumes that the gas-phase molecules are moving with an average velocity of C (cm/sec), the particles are stationary, and the mean diameters of the particles are much greater than the diameters of the gas-phase molecules.

$$n_p = N\,P\,\pi\,(dp/2)^2(8\,RT/\pi\,M)^{0.5} \quad (4)$$

The usual way to express the concentration of gas molecules, N_c, is mass/volume (g/cm^3), and the concentration of particles is mass/volume P_p (g/cm^3). Therefore:

$$N = N_c\,A/M \quad (5)$$

$$P = P_p/V_p\rho_p \quad (6)$$

$$n_p = (N_c\,A/M)(P_p/V_p\,\rho_p)\,\pi\,(dp/2)^2(8\,RT/\pi M)^{0.5} \quad (7)$$

$$n_p = N_c A P_p\,\pi\,(dp/2)^2((8\,RT/\pi\,M)^{0.5}/MV_p\,\rho_p) \quad (8)$$

where A is Avogadro's number, ρ_p is the true density of a known volume of particles (g/cm^3), and V_p is the volume of a particle (cm^3/particle).

If the particle is assumed to be spherical, then the volume of a particle is $V_p = (dp/2)^3 4\pi/3$. Therefore,

$$\pi\,(dp/2)^2/V_p = 3/2\,dp \quad (9)$$

$$n_p = 3N_c A P_p\,(8\,RT/\pi\,M)^{0.5}/2dpM\rho_p. \quad (10)$$

We can derive an equation for the number of gas-phase molecules remaining on the particles for a given period of time (σ_p = number of gas-phase molecules adsorbed on the total number of particles/unit volume), where

$$\sigma_p = n_p \tau \quad (11)$$

$$\sigma_p = 3\tau N_c A P_p (8\ RT/\pi\ M)^{0.5}/2dpM\rho_p. \quad (12)$$

This equation may be expressed in terms of σ_a (g of gas-phase molecules adsorbed on the total number of particles/unit volume).

$$\sigma_a = \sigma_p\ M/A \quad (13)$$

$$\sigma_a = 3\tau N_c P_p (8\ RT/\pi\ M)^{0.5}/2dp\rho_p. \quad (14)$$

Fractional Coverage of the Surface

The fractional coverage of the surface of an adsorbent (θ) particle by the adsorption of a gas-phase adsorbate molecule onto its surface may be described by the following equation:

$$\theta = (\Sigma n_x \tau_x S_x)/S_p \quad (15)$$

where $n_x\tau_x$ is the number of adsorbate molecules adsorbed per particle, S_x is the surface area of the adsorbate molecule (cm^2/molecule), and S_p is the surface area of the particle (cm^2/particle).

Enthalpy of Adsorption of an Adsorbate Molecule onto the Surface of an Adsorbent

The enthalpy of adsorption varies exponentially as a function of surface coverage ($\Delta H^{\theta a}{}_x$) from the enthalpy of adsorption at very low coverages ($\Delta H^{\circ a}{}_x$) to the 2/3 enthalpy of adsorption (2/3 $\Delta H^a{}_x$), when coverage approaches one monolayer, to the enthalpy of vaporization (ΔH_v) at infinite coverage. We have derived the following equation to describe this variation in enthalpy of adsorption as a function of coverage:

$$\Delta H^{\tau a}{}_x = \Delta H_v + (\Delta H^{\circ a}{}_x - \Delta H_v) \exp^{-\theta/2.46}. \quad (16)$$

Time an Adsorbate Molecule Remains on the Surface of an Adsorbent

The time τ that a molecule, with molar volume V and melting point T_s, remains associated with an adsorbent surface can be calculated from the enthalpy of adsorption (Frenkel 1924) and the vibrational frequencies of the molecules in the adsorbed state, with particular reference to vibrations perpendicular to the surface (Lindemann 1910):

$$\tau = \tau_0 \exp \Delta H^a/RT, \quad (17)$$

$$\tau_0 = 4.75 \times 10^{-3} \{(M(V)^{0.667})/T_s\}^{0.5}. \quad (18)$$

Therefore, since the enthalpy of adsorption is dependent upon surface coverage, we can calculate the time that a molecule is associated with the surface as a function of surface coverage:

$$\tau = \tau_0 \exp \Delta H^{\theta a}{}_x/RT. \quad (19)$$

Release of an Adsorbate Molecule from the Surface of Adsorbent into Lung Physiological Fluid

The process of release can be described by the following equation, in which the molecule x is displaced from the surface of the particle by molecules of physiological fluid M:

$$X^a + (V_x/V_m)\ M^s \leftrightarrow X^s + (V_x/V_m)\ M^a, \quad (20)$$

where V_x and V_m are the molar volumes of the adsorbate x and physiological fluid M, and superscripts a and s refer to adsorbed or to solution phases.

It is possible to describe this process in terms of changes in enthalpy by the following equation (Snyder 1968; Karger et al. 1978):

$$\Delta H_{release} = (V_x/V_m)\ \Delta H^a{}_m + \Delta H^s{}_{x,m} - \Delta H^a{}_{x,m} - \Delta H^a{}_x. \quad (21)$$

$\Delta H^{\Theta a}{}_x$ is dependent upon surface coverage. It has been established that under normal circumstances any adsorbed molecule that is adsorbed onto the surface at greater than one monolayer in coverage is released immediately from the surface because the enthalpy of adsorption between the supramonolayer adsorbed molecule and the molecules of the first layer of adsorbate are weak. The driving force for this release is increased entropy. Therefore, the variation in the enthalpy of adsorption of the adsorbate molecules in the first layer can be described in the following manner:

$$\Delta H^{\theta a}{}_x = \Delta H^{\circ a}{}_x\ e^{-\theta/2.46}. \quad (22)$$

Additionally, since the physiological fluid is interacting with the surface of the particle, which is covered by one monolayer of a combination of the adsorbate and solvent molecules, then $\Delta H^a{}_m = (2/3)\ \Delta H^{\circ a}{}_m$.

Therefore, $\Delta H^a{}_{x,m} = (2/3)\ \Delta H^{\circ a}{}_x - (2/3)\ \Delta H^{\circ a}{}_m$, and $\Delta H^s{}_{x,m}$ is the enthalpy of solution of molecule in the physiological fluid. Therefore, the final form of the equation is as follows:

$$\Delta H_{release} = (2/3)\ (V_x/V_m)\ \Delta H^{\circ a}{}_m + \Delta H^s{}_{x,m} - (2/3)\Delta H^{\circ a}{}_x + (2/3)\ \Delta H^{\circ a}{}_m - \Delta H^{\circ a}{}_x\ e^{-\theta/2.46}, \quad (23)$$

$$\Delta H_{release} = (2/3)\ [(V_x + V_m)/V_m]\ \Delta H^{\circ a}{}_m + \Delta H^s{}_{x,m} - (2/3)\ \Delta H^{\circ a}{}_x - \Delta H^{\circ a}{}_x\ e^{-\theta/2.46}. \quad (24)$$

Time Before an Adsorbate Molecule is Released from the Surface of an Adsorbent into Physiological Fluid

The time that a molecule remains associated with an adsorbent surface can be calculated from the enthalpy of release and the vibrational frequencies of the molecules in the adsorbed state, with particular reference to vibrations perpendicular to the surface:

$$\tau = \tau_0 \exp \Delta H_{release}/RT, \quad (25)$$

$$\tau_0 = 4.75 \times 10^{-3} \{(M(V)^{0.667})/T_s\}^{0.5}. \quad (26)$$

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Terence H. Risby received his Ph.D. in Chemistry from London University in 1970. He was Assistant Professor in the Department of Chemistry at Pennsylvania State University from 1972 to 1978. He joined the faculty at The Johns Hopkins University School of Hygiene and Public Health in 1978 and was promoted to the rank of Professor in 1987. Since 1989 he has been Director of the Division of Environmental Chemistry and Biology in the Department of Environmental Health Sciences. An analytical chemist, Dr. Risby's research focuses on the application of physicoanalytical techniques to the elucidation of fundamental questions in atmospheric pollution from mobile sources.

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ABBREVIATIONS

HCHO	formaldehyde
HPLC	high-performance liquid chromatography
NIOSH	National Institute of Occupational Safety and Health
PBS	phosphate-buffered saline
ppm	parts per million
RBCs	sheep erythrocytes

INTRODUCTION

A Request for Applications (RFA 85–1) that solicited proposals for "Health Effects of Aldehydes" was issued by the Health Effects Institute (HEI) in the summer of 1985. In response to the RFA, Dr. George Jakab, from the Johns Hopkins University School of Hygiene and Public Health in Baltimore, MD, submitted a proposal entitled, "The Relationship Between Particulate-Bound Acrolein and Respiratory Infections." The four-year project began in October 1986, and total expenditures were $530,774. The Investigators' Report was received at the HEI for review in March 1991. The revised report was received in October 1991 and was accepted for publication by the Health Review Committee in December 1991. During the review of the Investigators' Report, the Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary. The Health Review Committee's Commentary is intended to place the Investigators' Report in perspective as an aid to the sponsors of the HEI and to the public.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1990. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Sections 202(a), (b)(1), (g), and (h) and Sections 207(c)(4), (5), and (6) impose specific requirements for reducing motor vehicle emissions of certain oxidants (and other pollutants) and, in some cases, provide the EPA with limited discretion to modify those requirements.

Formaldehyde has been regulated by the EPA under emission standards for organic material hydrocarbon equivalents produced in connection with the operation of various classes of vehicles using methanol fuel. Several changes in the Clean Air Act instituted by the 1990 Amendments to the Act deal with formaldehyde. Section 211(k) of the Act, as added by Section 219 of the 1990 Amendments, establishes a program for the use of reformulated gasoline. The program is designed, at least in part, to reduce the "emissions of toxic air pollutants" such as formaldehyde. Similarly, the "clean-fuel vehicle" program emission standards set out in Section 243, as added by the 1990 Amendments, require that certain formaldehyde emission targets be met.

Section 202(l) of the Clean Air Act, as added by Section 206 of the 1990 Amendments, requires the EPA to "complete a study of the need for, and feasibility of, controlling emissions of [certain] toxic air pollutants." Section 202(l) states that the study "shall focus on those categories of emissions that pose the greatest risk to human health or about which significant uncertainties remain." Defined as a "hazardous air pollutant" under Section 301 of the 1990 Amendments, formaldehyde is one of the three emissions specifically mentioned in Section 202(l)(1).

SCIENTIFIC BACKGROUND

Aldehydes are normal constituents of emissions from internal combustion engines that use diesel and gasoline fuels (Marnett 1988). The concentrations of aldehydes that are emitted from motor vehicles vary with the mode of operation and the fuel. In general, diesel-fueled engines emit more aldehydes than gasoline-fueled engines (Swarin and Lipari 1983). Recent amendments to the Clean Air Act of 1970 (U.S. Congress 1991) address the production and use of vehicles that use alternative fuels, such as methanol and ethanol. Although the combustion of alcohol fuels should produce only carbon dioxide and water, actual combustion is an incomplete process that produces aldehydes as by-products. As a result, outdoor air concentrations of some aldehydes, particularly formaldehyde from methanol fuels and acetaldehyde from ethanol fuels, may increase in the near future. In addition to their direct release from combustion sources, aldehydes are formed from hydrocarbons during photochemical reactions in the atmosphere.

A National Ambient Air Quality Standard does not exist for formaldehyde; therefore, outdoor concentrations are not regularly monitored. However, measurements made in urban areas during the summer indicate that outdoor levels can range from 0.005 parts per million (ppm)* to 0.020 ppm (Graedel 1988). In Los Angeles, outdoor concentrations ranging from 0.004 to 0.086 ppm have been reported (Grosjean 1982). These concentrations are considerably lower than those found in occupational settings (upper concentrations ranging from 0.5 ppm to greater than 1.0 ppm) (Occupational Safety and Health Administration 1985) or indoor air (0.03 ppm in older homes to 4.2 ppm in mobile homes that use urea-formaldehyde foam insulation) (National Research Council 1981).

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

TOXICITY OF FORMALDEHYDE

Formaldehyde is a very water-soluble compound, and when inhaled, more than 98% of the gas is absorbed by the upper respiratory tract; thus, little of the gas penetrates to the lower respiratory tract (Egle 1972). Formaldehyde also is very reactive chemically and forms cross-links with reactive groups on proteins, DNA, and RNA. These properties of formaldehyde contribute to its toxicity and site of toxic action.

Experimental and epidemiological studies indicate that exposure to formaldehyde causes a wide range of toxic effects (reviewed by Bernstein et al. 1984, by Heck et al. 1990, by Leikauf 1991, and by Marbury and Krieger 1991). Formaldehyde is a carcinogen in laboratory animals. Chronic exposure to formaldehyde concentrations greater than 5 ppm has produced nasal carcinomas in rats (Swenberg et al. 1980; Albert et al. 1982; Kerns et al. 1983). Tumor incidence rates indicate a nonlinear dose-response. Mutations produced by formaldehyde may be related to cross-links formed between DNA and proteins that lead to subsequent errors in DNA replication (reviewed by Heck et al. 1990). These errors present a plausible explanation for the observed carcinogenic effects. Whether long-term exposure to formaldehyde is associated with an increased risk of cancer for humans is not yet known. The EPA (1987) and the International Agency for Research on Cancer (1987) have categorized formaldehyde as a probable human carcinogen, indicating that although there is limited evidence of carcinogenicity in humans, there is sufficient evidence of carcinogenicity in laboratory animals.

Formaldehyde irritates the eyes and upper respiratory tract. The concentration at which humans experience such sensory irritation varies considerably, but the majority of persons report some symptoms when exposed to levels ranging from 0.5 to 1.5 ppm (reviewed by Bernstein et al. 1984, and by Leikauf 1991). The effects of sensory irritation on the respiratory tract can be measured with tests of pulmonary function. Changes in pulmonary function have been documented in rodents but are less demonstrable in humans. In laboratory animals, exposure to 0.3 to 0.95 ppm formaldehyde decreased breathing frequency and increased pulmonary resistance (Amdur 1960; Kane and Alarie 1977; Chang et al. 1981; Barrow et al. 1983). An increase in resistance to airflow in and out of the lung indicates a narrowing, or constriction, of the airway's diameter. When healthy humans and persons with asthma were experimentally exposed to 0.3 to 3.0 ppm formaldehyde, changes in respiratory function did not occur (Anderson and Molhave 1983; Sheppard et al. 1984; Sauder et al. 1986; Schachter et al. 1986; Green et al. 1987; Kulle et al. 1987; Witek et al. 1987). At levels greater than 10 ppm, formaldehyde can cause transient bronchoconstriction in humans (Leikauf 1991). Formaldehyde also may induce bronchial hyperreactivity. Bronchial hyperreactivity is defined as an increased responsiveness of the airways to bronchoconstrictive agents. Although healthy persons appear unaffected by experimental exposure to formaldehyde (Sauder et al. 1986; Kulle et al. 1987; Green et al. 1987), Witek and associates (1987) reported an effect on hyperreactivity in some persons with asthma.

In addition to its nonspecific irritative properties, formaldehyde can adversely affect individuals through immunologically mediated mechanisms. Direct contact of formaldehyde solutions with the skin can cause allergic reactions in sensitive individuals (National Research Council 1981). In contrast with findings from clinical studies noted above, it has been possible to detect airway hyperreactivity in workers chronically exposed to formaldehyde gas in occupational settings (Burge et al. 1985; Nordman 1985).

Formaldehyde exposure also can depress mucociliary clearance of the upper respiratory tract in a dose-dependent fashion. Inhibition of mucociliary clearance following exposure to formaldehyde has been reported in animals and humans (reviewed by Leikauf 1991). Little is known about what impact formaldehyde has on the host defense systems of the lower respiratory tract.

FORMALDEHYDE AND RESPIRABLE-SIZED PARTICLES

In addition to aldehydes and numerous other organic gases, the combustion of fossil fuels produces respirable carbon-based particles. Diesel engines release approximately 10 times more particles than uncontrolled gasoline engines and approximately 100 times more particles than gasoline engines equipped with catalytic converters (Zweidinger 1982). During combustion, gaseous molecules can bind, or adsorb, to the surface of these carbon-based particles. When these particles are inhaled, they are deposited throughout the respiratory tract. Between 7% and 13% of the particles can be expected to deposit in the lower or alveolar region of the lung (Yu and Xu 1987). Thus, these carbon-based particles can serve as carriers of the adsorbed organic gases and affect their deposition site in the lung. In the case of water-soluble organic compounds, such as formaldehyde, which normally deposit in the upper airways, adsorption to particles may carry them deeper into the lung where they normally would not deposit. A question of concern is whether changes in deposition site of organic gases from the upper respiratory tract to the lower respiratory tract would affect the toxic action of the gases.

In experimental studies, carbon black particles can be used as a surrogate for diesel particles. Although the two

types of particles differ in the amount of organic material that can be extracted from them by solvents, carbon black is similar in structure to the carbon core of diesel particles and can be produced to have physical properties similar to those of diesel particles. The Investigators' Report contains a thorough description of the various types of carbon black particles and their properties.

Also reviewed in the Investigators' Report are studies that evaluated the effects of coexposure of formaldehyde and various aerosols. The findings indicate that the combination of formaldehyde with aerosols may enhance the toxicity of the gas. To summarize briefly, airway constriction was greater in guinea pigs when they were exposed to a combination of formaldehyde and noncarbonaceous particles than when they were exposed to the formaldehyde alone (Amdur 1960). When humans were exposed to formaldehyde and activated carbon particles, cough increased (Green et al. 1989). In hamsters exposed to formaldehyde and activated carbon, polymorphonuclear leukocytes entered the upper airways (Kilburn and McKenzie 1978). The effects of formaldehyde and particles in the lower respiratory tract are unknown. The integrity of the alveolar macrophage system could serve as an indicator as to whether formaldehyde bound to particles has an effect in this region of the lung.

DEFENSE MECHANISMS IN THE LOWER RESPIRATORY TRACT

Located in the lower respiratory tract, alveolar macrophages have the major responsibility of protecting the lungs from inhaled toxins and microorganisms (reviewed by Green et al. 1977, and by Brain 1985). When the lung is exposed to bacteria or viruses, macrophages release factors that stimulate an influx of other defense cells, such as polymorphonuclear leukocytes. These defense cells first bind microorganisms to their surfaces and then ingest, or phagocytize, them. Then, macrophages and other phagocytes kill the microorganisms with a variety of degradative enzymes and highly reactive species of oxygen. The impairment of alveolar macrophage functions would decrease the host's resistance to respiratory tract infection.

Green and Kass (1964) developed a model system to assay the intrapulmonary microbicidal capacity of animals in vivo. In this model, mice are challenged with an aerosol of bacteria in conjunction with exposure to a toxic agent. The lungs are removed, and the number of live bacteria remaining in the lungs is counted. A high bacterial count indicates that the lung's antibacterial defenses have been adversely affected by exposure to the toxic agent. Several other assays are available to assess alveolar macrophage function. For example, animals can be exposed to a pollutant, after which the alveolar macrophages are washed out of the lungs by bronchoalveolar lavage, and various functions of the cells are measured in culture.

Several air pollutants have been evaluated for their impact on the susceptibility of laboratory animals to pulmonary infection. Inhalation of oxidants, such as nitrogen dioxide and ozone, has been shown to increase mortality resulting from respiratory infection, to decrease intrapulmonary killing capacity, and to decrease alveolar macrophage phagocytic and killing activities in culture (reviewed by McGrath 1985, and by Pennington 1988). Because of their chemical properties, a significant fraction of inhaled oxidants reaches the distal regions of the lung where macrophages reside. In contrast, the majority of inhaled aldehydes deposit in the upper airways. Although the effect of formaldehyde on susceptibility to respiratory tract infection is not known, Jakab (1977) reported that acrolein adversely affected pulmonary defense mechanisms.

JUSTIFICATION FOR THE STUDY

The HEI was interested in supporting studies that would evaluate the health effects of three aldehydes (acrolein, acetaldehyde, and formaldehyde) at concentrations relevant to those present in automotive emissions. The Institute also was interested in determining the individual and combined effects of these aldehydes and their interactions with carbon particles. Three specific areas of interest outlined in the RFA were: (1) the effects of aldehydes on susceptibility to respiratory infections; (2) the interactive effects of aldehydes to produce cancer; and (3) the clinical human studies and new animal models to evaluate hypersensitivity to formaldehyde.

Dr. Jakab proposed to determine whether exposure to low levels of acrolein increased the susceptibility or severity of upper and lower respiratory infections. Because acrolein from combustion sources is often associated with respirable-sized particles, its site of toxic action could be shifted from the upper respiratory tract, where acrolein vapor is normally absorbed, to the lower respiratory tract, where particles deposit.

At the time of the proposal, Dr. Jakab had spent over 10 years evaluating pulmonary defense mechanisms against infections. He and his colleagues have focused their research on the mechanisms by which biological and chemical agents suppress host defenses and increase susceptibility to respiratory tract infections, both bacterial and viral. Previous studies included the effects of acrolein on the intrapulmonary killing of *Staphylococcus aureus* in mice

(Jakab 1977). Dr. Terence Risby, a coinvestigator, has expertise in characterizing the physical and chemical properties of particle surfaces and in how these properties influence the adsorption and desorption of chemical agents.

The HEI Research Committee reviewed the proposal favorably but requested that formaldehyde, instead of acrolein, be used in the studies. Formaldehyde was considered to be of greater regulatory concern than acrolein. The Research Committee also recognized the importance of including a physical chemist, Dr. Risby, to evaluate the bioavailability of the adsorbed formaldehyde in the proposed studies.

SPECIFIC AIMS AND STUDY DESIGN

The major goal of Dr. Jakab's study was to assess whether inhalation of formaldehyde, alone or in combination with carbon black particles, would interfere with alveolar macrophage function in mice and, thus, potentially decrease resistance to pulmonary infection. As part of this goal, the investigators evaluated the physicochemical interactions between formaldehyde and carbon black particles, with particular attention to those factors related to the adsorption and desorption of formaldehyde from the particles.

The specific aims of the study were: (1) to determine the concentration of formaldehyde that impairs pulmonary antibacterial defenses; (2) to determine the concentration of formaldehyde and carbon black particle complexes that impairs pulmonary antibacterial defenses; and (3) to study the physicochemical interactions between formaldehyde and carbon black particles. This aim included assessing the impact of methanol and water vapor on formaldehyde adsorption and evaluating the desorption of formaldehyde from carbon black particles into synthetic alveolar lung fluid.

For the first two specific aims, female Swiss mice were exposed to formaldehyde (0.5, 1, 5, 10, or 15 ppm) or to mixtures of formaldehyde and carbon black particles (2.5 ppm formaldehyde plus 3.5 mg/m^3 carbon black or 5 ppm formaldehyde plus 10 mg/m^3 carbon black). Two parameters of alveolar macrophage function, bacterial killing and phagocytosis, were used to evaluate the effects of exposure to the pollutants. For the first parameter, the investigators measured the intrapulmonary killing of *S. aureus*. Immediately after a 30-minute exposure to an aerosol of bacteria, the number of bacteria deposited in the lungs was determined. Animals then were exposed to the pollutant or to ambient air for four hours, and the number of viable bacteria remaining in the lungs was measured. Additional experiments were performed in which the animals were exposed to the formaldehyde both before (for 18 hours) and after (for four hours) the challenge with the bacterial aerosol. For the second parameter, the investigators measured alveolar macrophage Fc-receptor–mediated phagocytosis. Animals were exposed to 5 ppm formaldehyde, 10 mg/m^3 carbon black, or both for four hours a day for four days. Alveolar macrophages were removed from the lungs by bronchoalveolar lavage at intervals up to 60 days after pollutant exposure and their phagocytic activity was assayed in vitro. The isolated macrophages were incubated with sheep red blood cells that had been coated with specific antibody molecules. With Fc-receptor–mediated phagocytosis, the alveolar macrophages recognize one domain, the Fc end, of the antibody molecule. The alveolar macrophages attach to the Fc end of the molecule and then proceed to ingest the whole red blood cell. In this study, the number of macrophages containing red blood cells and the number of red blood cells per macrophage were used as an index of phagocytic activity.

For the third specific aim, several physical and chemical properties of carbon black particles and particle-bound formaldehyde were evaluated. The investigators conducted elemental analysis and chemical analysis of endogenous adsorbed molecules of the carbon black. They also measured specific surface area, external surface area, particle size, apparent density, and true density, and calculated the porosity of the particle samples. To evaluate the interactions between the formaldehyde and the carbon black particles, the authors investigated the adsorption and desorption properties of the aldehyde. Information on these properties can be used to predict the bioavailability of the formaldehyde in the lung.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The investigators met most of the objectives of their study. They demonstrated that at sufficiently high concentrations, formaldehyde impaired the intrapulmonary killing of *S. aureus*, and they observed an interesting, but as yet unexplained, delayed effect of the combined exposure of carbon black and formaldehyde on the phagocytic activity of isolated alveolar macrophages. They contributed valuable information to the understanding of the physicochemical interaction between formaldehyde and carbon black particles. The investigators also developed a thermodynamic model to predict the distribution of pollutants between the gas phase and adsorption onto solid particles.

ASSESSMENT OF METHODS AND STUDY DESIGN

Some compromise in experimental design occurred to accommodate two different aims. The first question of

whether formaldehyde will adsorb onto a respirable carbon black particle and produce effects deeper in the lung than formaldehyde alone is an important one. To answer it, the optimal study design would have included endpoints that reflected the known irritant effects of formaldehyde on airways. For example, small airway bronchoconstriction, which reflects irritant effects on lower respiratory tract airway function, would have been an appropriate endpoint. The second question addressed by the study is whether formaldehyde interferes with pulmonary defenses; additional endpoints reflecting alveolar macrophage function could have been used to evaluate this issue. By attempting to address both questions, some diffusion of the specific aims inevitably occurred.

In the formaldehyde and carbon black experiments that used intrapulmonary bacterial killing as an endpoint, the investigators did not use the same concentration of formaldehyde (15 ppm) or the same exposure protocol (1 ppm for 18 hours before and four hours after the bacterial challenge) that had produced a positive effect in the formaldehyde alone experiments. Therefore, they could not test for an interactive effect.

The investigators performed careful and appropriate preliminary exposure experiments and determined that immobilization of mice in a nose-only exposure apparatus impaired pulmonary antibacterial defenses; this factor could potentially have confounded their results. The investigators demonstrated that a conditioning period for the mice prior to the exposure would abrogate the effect of restraint. In addition, they carefully evaluated appropriate conditions for surrogate controls (restrained animals exposed to clean air but not inserted into the nose-only chamber ports) for the nose-only inhalation studies.

STATISTICAL ANALYSIS

The statistical analysis plan described in the Statistical Methods section of the report did not always correspond with the statistical procedures used in the Results section. Better documentation of the application of the statistical analysis to the data is needed. For example, a randomized block analysis was described, with formaldehyde presence or absence and formaldehyde concentration as the main treatment variables and their interaction as the featured hypothesis. Calculation of an *F*-ratio was described, appropriately constructed from between-run variance rather than from within-run variance. Finally, a Duncan procedure was mentioned for the post-hoc analysis of significant treatment effects. In the Results section, however, neither *F*-ratios, tests for interaction of treatment effects, nor Duncan procedures were reported. It is not clear whether the analysis, which was detailed in the Methods section, was either performed or used to interpret the data. The statistical inferences depicted in bar graphs in Figure 5 and reported in the text appear to be drawn from some other, unspecified analysis. Formaldehyde concentrations were analyzed separately, rather than as a treatment factor. Control and exposed animals were pooled across runs, thus abolishing the distinction between sources of variance. The *p* values were provided without any indication of what statistic they represented. If a different analytical method was used, then that approach should have been described. This is not a trivial concern. For example, in some cases an inappropriate pooling of between-run and within-run variation could lead to a severe underestimation of variance and, thus, to spurious statistical significance (Type I error).

RESULTS AND INTERPRETATION

A statistically significant decrease in the intrapulmonary killing of *S. aureus* was observed in mice exposed to 15 ppm formaldehyde for four hours immediately after bacterial challenge; experiments with 10 ppm were negative. Exposures to carbon black with 2.5 or 5 ppm formaldehyde also showed no effect. An experiment with carbon black plus 15 ppm formaldehyde was not conducted. Experiments were performed in which mice were exposed to lower levels of formaldehyde (0.5 to 1 ppm) for 18 hours before bacterial challenge, for four hours after bacterial challenge, and for both time periods. Bacterial survival significantly increased 7% in animals exposed to 1 ppm formaldehyde both before and after bacterial challenge. No follow-up of this protocol with a carbon black coexposure was attempted. Experiments with 3.5 mg/m^3 carbon black and 2.5 ppm formaldehyde for four hours per day for four days before bacterial challenge showed no effect.

Because of the similarities between human and rodent lung defense systems, the monitoring of bacterial infection used by the investigators was a valid means for evaluating the potential impact of an inhaled pollutant on a person's resistance to infection (Green 1984). However, *S. aureus* is not a representative human lung pathogen, and other bacterial as well as viral microorganisms would have been more relevant (Pennington 1988).

An interactive effect of formaldehyde and carbon black was observed when alveolar macrophage Fc–receptor-mediated phagocytosis was assayed. Neither 10 mg/m^3 carbon black nor 5 ppm formaldehyde alone produced any effect up to 40 days after the exposure ended. However, when the animals were exposed to both pollutants, a delayed effect of combined exposure was observed. The observed impairment of alveolar macrophage Fc-receptor–mediated phagocytosis is potentially of great interest, but it is diffi-

cult to explain why this effect was delayed. The biological mechanism by which formaldehyde, which is rapidly metabolized, can produce such a delayed effect is not clearly identified. The half-life of alveolar macrophages in rodents, which is 27 days, could only explain the duration of an effect, not the time at which the effect was maximal. Because the majority of alveolar macrophage precursors are recruited from a bone marrow pool rather from the lungs, it is unlikely that the replacement macrophages would be exposed to the formaldehyde as late as 25 days after the animals were exposed.

The physicochemical studies showed that little of the gas-phase formaldehyde was bound to the carbon black and that the adsorbed aldehyde was highly soluble in an aqueous medium. The addition of water or methanol vapor decreased the heat of adsorption of the aldehyde to carbon black. These results are in agreement with findings from the recent study by Rothenberg and coworkers (1990), who analyzed the interactions between formaldehyde and indoor dust.

IMPLICATIONS FOR FUTURE RESEARCH

This study raises a number of issues deserving careful follow-up. Most importantly, these investigations suggest the need for careful assessment of the possible role of formaldehyde exposure in potentiating pulmonary infection, with or without associated particles. The concentration of formaldehyde needed to impair intrapulmonary bacterial killing of *S. aureus* (15 ppm) was orders of magnitude higher than outdoor concentrations; however, when the protocol was changed to include exposure of the mice to the formaldehyde before, as well as after, the bacterial challenge, the concentration needed decreased 15-fold to 1 ppm. It is not known why changing the pollutant exposure to both before and after bacterial challenge had such an effect on the threshold of response. Other potential studies include looking at different microorganisms. *Staphylococcus aureus* is a gram positive bacterium, and the effects of exposure of formaldehyde on gram negative bacteria or viruses may be different and worthy of investigation.

Carbon black particles were used as a surrogate for diesel particles. However, the presence of extractable organic compounds on diesel particles may influence the adsorptive and desorptive properties of the particles for formaldehyde. Furthermore, diesel particles are not the only particles that could conceivably adsorb formaldehyde. Other particles found in outdoor and indoor environments may be more effective carriers of formaldehyde to the lower respiratory tract. The applicability of the physicochemical findings and the model developed by the investigators to other particle types and pollutants may be of interest. Some analyses of the adsorption and desorption of formaldehyde on indoor dust samples have been conducted recently by Rothenberg and associates (1990).

The observation of a delayed interactive effect of the combined exposures on alveolar macrophage phagocytosis needs confirmation and explanation. Given the findings from the physicochemical studies that little of the formaldehyde was bound to the particles and that the formaldehyde desorbed rapidly from the particles in aqueous solutions, it is difficult to conceive of a biologic mechanism by which impairment of phagocytosis was delayed. In their report, the investigators provided some interesting speculations that relate primarily to issues of dose. However, because of the heterogeneity of alveolar macrophages, selective sampling of macrophages by bronchoalveolar lavage, and kinetics of cell turnover, it is also possible that those macrophages most affected by the formaldehyde were not sampled until 25 days after exposure. Thus, although the depression of phagocytosis was real, the time of maximal depression may have been artifactual because of sampling technicalities. These speculations could be explored in future studies.

CONCLUSIONS

The investigators evaluated the effect of formaldehyde, a highly reactive water-soluble gas, on the integrity of the alveolar macrophage bactericidal and phagocytic functions. The investigators also determined whether coexposure of the formaldehyde and carbon black particles, which might increase the penetration of the formaldehyde to the lower respiratory tract, would potentiate any toxicity of formaldehyde to alveolar macrophages. Finally, they studied the physicochemical interactions between the formaldehyde and carbon particles and developed a thermodynamic model to predict some of these interactions.

In this series of well conducted and controlled experiments, the investigators detected few effects on alveolar macrophage functions. When mice were exposed to formaldehyde after inhaling an aerosol of *S. aureus*, very high concentrations (15 ppm) of formaldehyde, which are orders of magnitude higher than outdoor concentrations, were needed to impair intrapulmonary killing of the bacteria. When exposure to the gas preceded, as well as followed, the bacterial challenge, a slight impairment of bacterial killing was observed with lower concentrations (1 ppm) of formaldehyde. Coexposure of mice to formaldehyde and carbon black particles (2.5 ppm formaldehyde plus 3.5 mg/m^3 car-

bon black or 5 ppm formaldehyde plus 10 mg/m^3 carbon black) had no effect. The absence of an effect of combined exposure on intrapulmonary bacterial killing was attributed to the finding that only 1% of the formaldehyde bound to the carbon particle. The investigators, however, did not duplicate the exposure concentration and exposure protocol that had produced a positive effect with the gas alone.

When a different assay of alveolar macrophage function was used, the investigators observed an interactive effect of formaldehyde and carbon black. Alveolar macrophage Fc-receptor–mediated phagocytosis was impaired after exposure to 5 ppm formaldehyde and 10 mg/m^3 carbon black; neither of these agents alone had an effect on phagocytosis. This impairment was not apparent until five days after exposure, and maximal impairment occurred 25 days after exposure. The biological mechanism responsible for this delayed impairment is not known.

In summary, these experiments demonstrated that only high concentrations of formaldehyde impaired intrapulmonary killing of *S. aureus*. Exposure protocol was an important variable when assessing intrapulmonary bactercidal activity, and changes in protocol affected the sensitivity of the assay. Carbon black particles were not effective carriers of formaldehyde to the lower respiratory tract, and no effect on intrapulmonary killing was observed with combined exposures of formaldehyde and carbon black particles. However, alveolar macrophage phagocytosis was depressed several days after coexposures to formaldehyde and carbon black, a finding difficult to explain mechanistically. As noted by the investigators, a valuable conclusion drawn from this study is the importance of using different exposure protocols and evaluating multiple endpoints at different time intervals after exposure. Differences in experimental results may help to formulate hypotheses on mechanisms of action for further investigation.

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