TOXICOLOGICAL SCIENCES **93(2)**, 390–399 (2006)
doi:10.1093/toxsci/kfl062
Advance Access publication July 13, 2006

Acute Pulmonary and Systemic Effects of Inhaled Coal Fly Ash in Rats: Comparison to Ambient Environmental Particles

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Received April 17, 2006; accepted July 6, 2006

Although primary particle emissions of ash from coal-fired power plants are well controlled, coal fly ash (CFA) can still remain a significant fraction of the overall particle exposure for some plant workers and highly impacted communities. The effect of CFA on pulmonary and systemic inflammation and injury was measured in male Sprague-Dawley rats exposed to filtered air or CFA for 4 h/day for 3 days. The average concentration of CFA particulate matter less than 2.5 μm ($PM_{2.5}$) was 1400 μg/m^3, of which 600 μg/m^3 was PM_1. Animals were examined 18 and 36 h postexposure. Chemical analysis of CFA detected silicon, calcium, aluminum, and iron as major components. Total number of neutrophils in bronchoalveolar lavage fluid (BALF) following exposure to CFA was significantly increased along with significantly elevated blood neutrophils. Exposure to CFA caused slight increases in macrophage inflammatory protein-2, and marked increases in transferrin in BALF. Interleukin-1β and total antioxidant potential in lung tissues were also increased in rats exposed to CFA. Histological examination of lung tissue demonstrated focal alveolar septal thickening and increased cellularity in select alveoli immediately beyond terminal bronchioles. These responses are consistent with the ability of CFA to induce mild neutrophilic inflammation in the lung and blood following short-term exposure at levels that could be occupationally relevant. However, when comparing the effects of CFA with those of concentrated ambient particles, CFA does not appear to have greater potency to cause pulmonary alterations. This study furthers our understanding of possible mechanisms by which specific sources of particulate air pollution affect human health.

Key Words: **coal fly ash; BALF; IL-1β; TNF-α; MIP-2; $PM_{2.5}$; inhalation; pulmonary toxicology; concentrated ambient particles; iron; inflammation.**

Coal fly ash (CFA) consists of anthropogenic particles formed by mineral transformation in high-temperature combustion processes. CFA is a primary particle emitted, along with soot as a solid from the power plant stack. Exposure to CFA is a health concern because coal is a major power source used world wide, especially in developing countries such as China and India, and human exposure to particulate matter is associated with increased respiratory and cardiac disease.

Primary emissions from modern power plants are controlled by either an electrostatic precipitator or a fabric filter, but the collection efficiency of both technologies is lowest for particles with an aerodynamic diameter in the range 0.1–1 μm (Helble, 2000). As a result, CFA emissions are dominated by particles that can travel long distances in the atmosphere and that can be inhaled into the lung. Power plant workers are exposed to CFA, especially during boiler maintenance, and construction workers are exposed to collected CFA that is recycled into cement and construction fill material. In the United States, there is no specific workplace limit for CFA, so occupational exposures to CFA is regulated as a nuisance dust under ''Particulates (Insoluble) Not Otherwise Classified'' (Meij and te Winkel, 2001). The occupational limit for these types of particles is an 8-h limit of 10 mg/m^3 for the inhalable size range and 3 mg/m^3 for respirable particles (ACGIH, 2001), which is two to three orders of magnitude higher than for typical ambient particulate matter (PM) concentration.

Besides in the workplace, exposure to CFA may occur by living near clusters of power plants. Time-average ambient concentrations of CFA in the United States are low because pollution controls and tall stacks have eliminated extreme local concentrations of CFA. However, CFA emissions remain a regional-scale source of PM. For example, primary PM from the seven existing coal-fired power plants in Utah represents 30% of the primary PM_{10} emitted by all regulated stationary sources statewide. In northwest Colorado, the Craig and Hayden coal-fired power plants were found to contribute about 15% of the time-averaged $PM_{2.5}$ mass close to the Hayden plant but less than 5% at more remote sites (Chow and Watson, 2002). Coal combustion along the Ohio River Valley may be an

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important contributor to $PM_{2.5}$ mass in Pittsburgh, PA, when wind is from the southwest (Anderson *et al.*, 2002). Model predictions for China show the maximum annual averaged PM_{10} and SO_2 concentrations caused by existing power plants are 52.6 and 33.6 $\mu g/m^3$, respectively (Xue *et al.*, 2005).

Due to health concerns with CFA exposure, inhalation and instillation studies with CFA have been conducted in a number of laboratory rodents. The following observations have been made from these studies: (1) CFA particles are retained in the lung (Raabe *et al.*, 1982); (2) airway resistance is affected following exposure to CFA (Chen *et al.*, 1990); and (3) CFA causes increased immune cell activity (Dormans *et al.*, 1999) and inflammation in the lung (Gilmour *et al.*, 2004). Reviews and research reports discuss the formation of particles during coal combustion and the implications of these particles for human health (Lighty *et al.*, 2000), the mechanisms and biological effects of iron mobilization from CFA (Ball *et al.*, 2000), CFA particle characteristics responsible for effects on lung epithelial cells (Aust *et al.*, 2002), the effects of nanoparticles from coal combustion (Donaldson *et al.*, 2005), and occupational effects of chronic exposures to CFA (Borm, 1997).

The purpose of this study was to use controlled exposure to a well-characterized particle type to gain insights into the induction of pulmonary inflammation by exposure to particulate air pollution. The approach was to physicochemically characterize CFA and then to examine the acute effects of inhaled resuspended CFA aerosol in rats at concentrations that are relevant to occupational limits. CFA is both an emission of current concern (Fields, 2004), and an excellent prototype particle due to the extensive literature on CFA formation mechanisms (Lighty *et al.*, 2000). In contrast to prior inhalation studies with CFA, this study examined a wide variety of highly sensitive pulmonary and systemic indicators of inflammation and oxidative stress that are relevant to current mechanistic toxicological hypotheses. Although some of the measured biochemical parameters may indicate small changes that are not biologically significant in healthy animals, these changes may be biomarkers that give insights into underlying mechanisms leading to increased susceptibility. Responses induced by a specific pollutant are best placed in context by comparison to studies with real-world ambient particles. Therefore, another objective of this study design was to compare the effect of CFA, a defined-source particle, to responses reported for the mixture of emission sources contributing to the concentrated ambient particles (CAPs) used by recent studies in the United States and Europe.

MATERIALS AND METHODS

Animals. Forty-eight male Sprague-Dawley rats, 8 weeks of age (260–270 g) and free of respiratory pathogens or disease, were purchased from Harlan (San Diego, CA). Prior to CFA exposure, animals were acclimated to nose-only exposure tubes for 5 days (2, 3, 4, 4, 4 h, on days 1–5, respectively). Animals were handled in accordance with standards established by the U.S. Animal Welfare Acts as set forth in the National Institutes of Health guidelines (Institute of Laboratory Animal Resources (1996) as well as the Animal Care and Use committee of the University of California, Davis. Rats were housed in plastic cages with TEK-Chip pelleted paper bedding (Harlan Teklad, Madison, WI) and maintained on a 12-h light/12-h dark cycle. All animals had access to water and Laboratory Rodent Diet 5001 (LabDiet, Brentwood, MO) *ad libitum* except during each daily acclimation or exposure period.

Experimental design. Rats were exposed to filtered air or aerosolized CFA in a nose-only exposure system for 4 h/day for 3 days and examined 18 or 36 h after the last exposure to CFA. These necropsy times were selected to measure acute responses and to approximate the 1- to 3-day lag time between increases in PM concentration and human effects commonly observed in epidemiology studies.

Coal fly ash. The particulate material used for inhalation was derived from a bulk sample of fly ash collected by air pollution control equipment on a 400-MW power plant burning bituminous coal from multiple mines in the Wasatch Plateau, UT, coal field. Size-fractionated material, enriched in 0.4- to 2-μm-sized particles, was extracted from the bulk fly ash by mechanical resuspension and aerodynamic separation using a previously described method (Veranth *et al.*, 2000). The capture efficiency of typical coal power plant air pollution control equipment is at a minimum for the size range of CFA particles used in the current study, but is still > 90% (Lighty *et al.*, 2000). Size-dependent differences in CFA composition have been reviewed (Lighty *et al.*, 2000). Extracting particles in the minimum efficiency size range from captured power plant fly ash produces an aerosol similar to the emitted particles.

CFA particle generator. CFA was aerosolized using a belt feeder and fluidized bed system shown in Figure 1 and previously described by Teague *et al.* (2005). In brief, $PM_{2.5}$ size-fractionated particles were premixed with 100- to 200-μm-diameter glass beads and loaded into a dust feed for delivery to a vibrating fluidized bed system for aerosolization of both CFA and glass beads. The aerosol subsequently passed through a cyclone separator to remove the glass beads (Fig. 2), while the CFA remained aerosolized (Fig. 3) passing through a krypton-85 source (to reduce particle agglomeration) to the nose-only inhalation system. Particle concentration during exposure was monitored by a continuous-reading, light-scattering, dust concentration monitor and filter connected to the exposure chamber. A Grimm Series 1.108 Aerosol Spectrometer (GRIMM Aerosol Technik GmbH, Douglasville, GA) extracted a 1.2 Lpm flow, and a proprietary algorithm converted light scattering into particle mass concentration using the default calibration for occupational monitoring (PM_{10}, $PM_{2.5}$, and PM_1). The particle feeder settings of the aerosolization system were adjusted as needed to achieve the target time-averaged concentration for exposure. The aerosol spectrometer was operated in the 16-channel mode to measure particle size distribution. Mass of CFA collected on the filter was used to correct the aerosol spectrometer output to obtain the actual mass concentration during exposures. The filter sample was collected at 3 l/min on a 25-mm Pallflex EMFAB TX40HI20-WW filter using an InTox filter housing connected to a diaphragm pump and a bellows-type dry gas meter. Control animals were exposed to air passed through a high efficiency particulate air (HEPA) filter (Airguard, Louisville, KY).

Bronchoalveolar lavage and biochemical assay of bronchoalveolar lavage fluid. Eighteen or 36 h after the last exposure to CFA, rats were anesthetized by ip injection of pentobarbital (100–150 mg/kg body weight). Blood was collected from the caudal vena cava and placed in a collection tube for serum and in a tube containing ethylenediaminetetraacetic acid for determination of hematology parameters including complete blood counts. Animals were exsanguinated via the abdominal aorta. The trachea was cannulated, and the lungs were lavaged with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS; pH 7.4) at a volume equal to 35 ml/kg body weight. Three in-and-out lavages were performed using the same aliquot to maximize recovery of cells and biochemical markers from the lungs. Bronchoalveolar lavage fluid (BALF) was

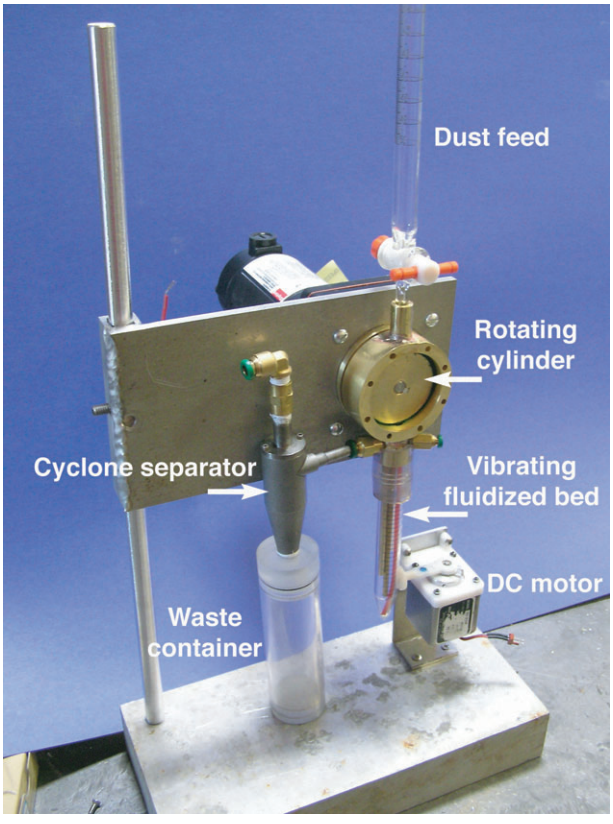

FIG. 1. CFA aerosol generator system consisting of (1) rotary feed, (2) vibrating fluidized bed, (3) cyclone separator, and (4) dilution control system. The fluidized bed consists of a 15-ml conical test tube into which the test material is delivered. Filtered air is added to the conical test tube at the bottom of the cone. Particle aerosolization is accomplished by mechanical agitation and turbulent airflow, passing the stream of particle-laden air through a cyclone that readily separates suspended CFA particles from the glass beads by means of the large difference in aerodynamic diameter. The CFA is further diluted with air as it passes through a krypton-85 charge neutralizer, prior to introduction into the nose-only exposure unit (Teague *et al.*, 2005).

centrifuged at 2000 rpm for 10 min at 4°C. The supernatant was removed and stored at − 80°C for biochemical analyses. The cell pellet was resuspended in Ca^{2+}/Mg^{2+}-free PBS, and 100 µl of this suspension was used to determine total cell count and viability. Cell viability was measured by exclusion of 0.4% trypan blue (Sigma, St Louis, MO), an indicator of irreversible loss of plasma membrane integrity. A second aliquot of cells was centrifuged using a Shandon Cytospin (Thermo Shandon, Inc., Pittsburgh, PA) to prepare cell differential slides. The slides were dried at room temperature and stained with HEMA 3 (Fisher Scientific International, Inc.) (Biochemical Sciences, Inc., Swedesboro, NJ). Macrophages, neutrophils, and lymphocytes were counted using light microscopy (1000 cells per sample).

BALF supernatant was analyzed for protein content using a Coomassie Plus Protein Assay Kit (Pierce, Rockford, IL) and bovine serum albumin (BSA) standards from Sigma/Aldrich Chemicals. BALF supernatant was analyzed for albumin content using a commercially available kit (Diasorin, Inc., Stillwater, MN). N-acetyl-β-D-glucosaminidase (NAG) activity was measured in BALF supernatant using a commercially available kit and standards from Roche Diagnostics (Indianapolis, IN). γ-Glutamyltransferase (GGT) activity was measured in the BALF supernatant using a commercially prepared kit from Thermo Trace, Ltd (Melbourne, Australia). Transferrin concentration in BALF supernatant was measured using a Transferrin SPQ II kit from Diasorin, Inc. Total antioxidant status of BALF supernatant was determined using a kit from Randox Laboratories, Inc. (Ardmore, U.K.). These assays were modified and adapted for use on a KONLAB clinical chemistry analyzer (Thermo Clinical Labsystems, Espoo, Finland).

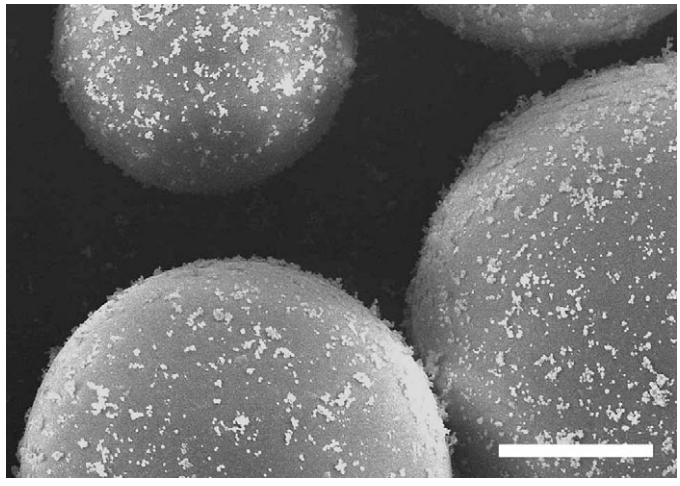

FIG. 2. Scanning electron micrograph of glass beads (100–200 µm diameter) collected from the waste receptacle, with CFA particles still present on the surfaces of the beads. Through mechanical agitation and airflow turbulence in the fluidized bed system, the bulk of the CFA particles become aerosolized as single particles or simple particle clusters not attached to these glass beads. Scale bar is 50 µm.

Levels of Interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), and macrophage inflammatory protein-2 (MIP-2) in the BALF in BALF supernatant were determined using rat enzyme-linked immunosorbent assay (ELISA) kits obtained from Biosource International (Camarillo, CA) and used according to the manufacturer's instructions. Concentrations are reported as pg/ml.

BALF supernatant was analyzed for total glutathione (GSH) and glutathione disulfide (GSSG) content using a Bioxytech reduced to oxidized glutathione-412 (GSH/GSSG-412) kit from Oxis International, Inc. (Portland, OR) according to manufacturer's instructions. Concentrations are reported as µM and as the GSH/GSSG ratio, defined as (GSH-2GSSG)/GSSG.

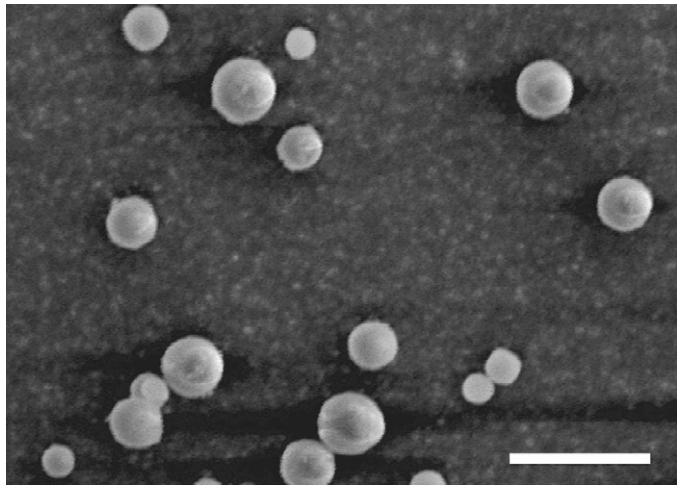

FIG. 3. Scanning electron micrograph of CFA particles collected from a port in the nose-only exposure system. Virtually all particles fall within the $PM_{2.5}$ size range. Scale bar is 5 µm. No fragments or glass shards from the beads are present, suggesting that the fluidized bed system does not cause destruction of the glass beads or the CFA particles.

Hematology parameters. Whole blood was collected as described above and analyzed using a Mascot Hemavet 850 analyzer (CDC Technologies, Oxford, CT). This system determines a variety of hematologic parameters, including red blood cell count, white blood cell count, and percentages of neutrophils, lymphocytes, monocytes, eosinophils, and basophils.

Lung tissues for biochemistry and histopathology. Six animals per group (separate from those used for collection of BALF) were anesthetized by ip injection of pentobarbital (100–150 mg/kg body weight). Blood was collected from the caudal vena cava and placed in a tube containing citrate for collection of plasma. Animals were exsanguinated via the abdominal aorta. The trachea was cannulated, the chest cavity was opened by a midline incision, and the right mainstem bronchus was ligated. Right lung lobes were snap frozen individually in liquid nitrogen and stored at − 80°C until analysis of cytokines, chemokines, glutathione, and total antioxidant potential. The left lung was inflation fixed by intratracheal instillation of 4% paraformaldehyde at 30 cm of water pressure for 1 h. The lung was sliced into pieces, dehydrated in a series of graded ethanol and embedded in paraffin for use in histochemical studies. Sections of paraffin-embedded lung tissue were cut at a thickness of 5 μm and stained with hematoxylin and eosin (H&E) or Prussian blue iron stain. Airways and the lung parenchyma were examined for the presence of cellular changes and inflammation in H&E-stained sections, while the presence of CFA particles in tissues was detected using Prussian blue iron stain.

Cytokines and chemokines in lung tissue. The right-middle lung lobe was homogenized in T-PER Reagent (Pierce, Rockford, IL) at a ratio of 0.1 g tissue to 1 ml T-PER Reagent. The homogenate was centrifuged, and the supernatant was collected according to the manufacturer's instructions. Protein concentration in the supernatant was determined using the BCA Protein Assay from Pierce with BSA as the standard. Levels of IL-1β, TNF-α, and MIP-2 in the supernatant were determined using rat ELISA kits obtained from Biosource International and used according to the manufacturer's instructions. Concentrations are reported as pg/μg protein.

Glutathione in lung tissue. The right caudal lung lobe was divided in half. One half of the lobe used to measure total glutathione was homogenized in ice-cold 5% metaphosphoric acid at a ratio of 0.1 g tissue to 0.5 ml 5% metaphosphoric acid. The homogenate was placed on ice for 15 min and then centrifuged at 12,000 × g for 15 min at 4°C. The supernatant was aliquoted and stored at − 80°C. The other half of the lobe used to measure oxidized glutathione was homogenized as described, but with the addition of 1-methyl-2-vinylpyridinium trifluoromethanesulfonate. Lung tissue supernatant was analyzed for total glutathione (GSH) and glutathione disulfide (GSSG) content using a Bioxytech GSH/GSSG-412 kit from Oxis International, Inc. according to manufacturer's instructions. Concentrations are reported as nmol/mg tissue and as the GSH/GSSG ratio, defined as (GSH-2GSSG)/GSSG.

Total antioxidant potential in lung tissue. The accessory lobe of the lung was homogenized in ice-cold PBS at a ratio of 0.1 g tissue to 1 ml PBS. The homogenate was centrifuged at 3000 × g for 15 min at 4°C. The supernatant was removed and stored at − 80°C. A colorimetric, quantitative assay from OxisResearch (Portland, OR) was used according to the manufacturer's instructions to determine total antioxidant potential. Results are presented as nmol uric acid equivalents/μg protein.

Statistics. All numerical data were calculated as the mean and standard deviation. Comparisons between animals exposed to CFA and filtered air were made by Student's *t*-test or, where appropriate, by analysis of variance followed by Fisher's protected least significant difference posttest. Comparisons were considered significant if a value of $p < 0.05$ was observed. Statistical analysis was performed with StatView 5.0.1 (SAS Institute, Inc., Cary, NC).

RESULTS

CFA Aerosol Characteristics

The chemical composition of the aerodynamically size-fractionated CFA powder is shown in Table 1. Average concentration of CFA in the $PM_{2.5}$ range was 1400 ± 150 μg/m^3, of which 600 ± 70 μg/m^3 was in the PM_1 range. The composition was analyzed by ion chromatography for soluble ions, by x-ray fluorescence (XRF) for elements, and by thermal/optical reflectance for carbon fractions. These methods are the same as used by ambient air monitoring networks; therefore, the data are useful for comparing to ambient PM samples. The cumulative chemical composition data percentages do not equal 100% because oxygen and hydrogen from mineral oxides, chemically bound water, and organic molecules are not measured. XRF analysis is also subject to artifacts from x-ray adsorption within the sample.

TABLE 1
Size-fractionated CFA Composition (All Data in %)[a]

Soluble ions by atomic absorption or ion chromatography	
Chloride	0.003
Nitrate	ND[b]
Phosphate	ND
Sulfate	3.97
Ammonium	0.03
Sodium ion	0.37
Potassium ion	0.06
Calcium ion	3.67
Carbon fractions by thermal/optical reflectance	
Organic carbon 1	0.05
Organic carbon 2	0.29
Organic carbon 3	0.34
Organic carbon 4	0.46
Pyrolyzed carbon	0.09
Elemental carbon 1	0.50
Elemental carbon 2	1.40
Elemental carbon 3	1.28
Carbonate carbon	0.35
Elements by X-ray fluorescence	
Sodium	1.00
Magnesium	0.14
Aluminum	4.92
Silicon	12.43
Phosphorus	0.22
Sulfur	1.26
Potassium	1.23
Calcium	5.64
Titanium	0.65
Vanadium	0.06
Chromium	0.02
Manganese	0.03
Iron	4.16
Cobalt	ND
Nickel	0.004
Copper	0.02
Zinc	0.04
Selenium	0.009

[a]Analysis performed on size-fractionated samples enriched for $PM_{2.5}$.
[b]Not detected.

Particle Deposition

The particle size distribution of the resuspended CFA is shown in Figure 4. A lognormal size distribution equation (Hinds, 1982)

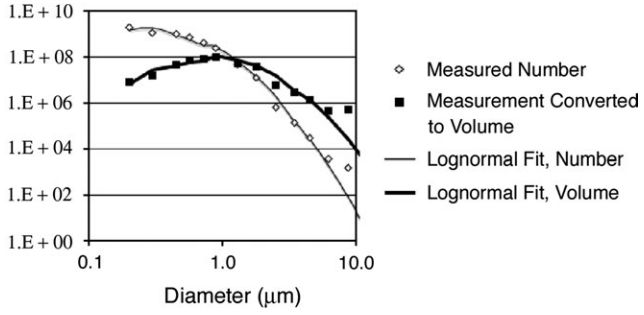

FIG. 4. Size distribution of the resuspended CFA aerosol. Particle number (#/m^3) was measured by the laser light scattering instrument operating in 16-channel mode, and volume (μm^3 of particles/m^3 of air) was calculated assuming spherical particles. The lognormal size distribution equation was fit to the measured data.

was fit to the measured aerosolized particle number distribution. The actual aerosol particle size generated can be approximated by a count median diameter of 0.3 μm and a geometric standard deviation of 1.4, which corresponds to a mass mode near 1 μm. Using this information, deposition of the CFA aerosol generated was modeled using MPPD V1.0 software developed by Chemical Industry Institute of Toxicology (CIIT Center for Health Research) and Rijksinstituut voor volksgezondheid en milieu (RIVM National Institute for Public Health and the Environment) (Asgharian *et al.*, 2002). The model inputs include measured particle size distribution, time-averaged aerosol concentration during exposure, and observed breathing rate. Based on the MPPD model, the total CFA mass deposited over the 3-day exposure was 32 μg per rat, and the regional deposition was head, 25%; tracheobronchial, 20%; and pulmonary, 55%.

Animal Responses (BALF, Blood, Lung Tissue)

All BALF, blood, and lung tissue parameters showing statistically significant changes at either postexposure time are summarized in Table 2. These changes included a significant increase in neutrophils, both in the lung BALF and in the blood (Fig. 5), and increases in cytokines such as MIP-2 in BALF and IL-1β in lung tissue (Fig. 6). Other significant changes included increased levels of transferrin, lung tissue total antioxidant potential, plasma protein, and blood complement 4. Biological endpoints showing no statistically significant changes at either time point are shown in Table 3.

Lung Histopathology

Following CFA exposure, a moderate elevation in the number of alveolar macrophages was noted within the bronchiole-alveolar duct regions of the lungs both 18- and 36-h postexposure (Fig. 7). This region of the lungs was associated with a subtle, but obvious increase in the cellularity of alveolar septal tips immediately beyond terminal bronchioles (Fig. 7B). Although not every bronchiole-alveolar duct region was involved, there was a clear distinction between the sham control animals and those exposed to CFA both 18- and 36-h postexposure. Staining with Prussian blue iron stain demonstrated in a small fraction of alveolar macrophages the presence of iron-positive cytoplasmic inclusions (inset, Fig. 7B), suggestive of phagocytosized CFA particles.

TABLE 2
Parameters Showing Statistically Significant Difference from Control at the Same Postexposure Time ($p < 0.05$). $N = 5$ or 6

	Filtered air	CFA
18-h postexposure		
BALF % macrophages	99.3 ± 0.3	97.9 ± 1.1
BALF % neutrophils	0.3 ± 0.2	1.5 ± 1.0
BALF neutrophils, cells/ml	260 ± 160	1400 ± 860
BALF MIP-2, pg/ml	156 ± 13	181 ± 12
BALF transferrin, mg/dl	5.6 ± 1.6	9.7 ± 3.6
Plasma protein, g/dl	6.4 ± 0.1	6.9 ± 0.4
Lung tissue total antioxidant potential (nmol uric acid equivalent/μg protein)	0.091 ± 0.005	0.097 ± 0.005
36-h postexposure		
BALF % macrophages	99.2 ± 0.3	98.7 ± 0.4
BALF % neutrophils	0.4 ± 0.2	0.9 ± 0.4
BALF neutrophils, cells/ml	250 ± 100	825 ± 480
BALF gamma glutamyl transferase activity, U/l	4.9 ± 0.2	4.4 ± 0.4
Hematocrit %	40.5 ± 6.0	48.5 ± 4.8
Mean corpuscular volume fl	53.7 ± 1.8	56.3 ± 1.4
Blood neutrophil %	16.0 ± 2.4	23.8 ± 5.6
Blood lymphocyte %	77.2 ± 3.7	68.8 ± 6.4
Blood complement 4, mg/dl	70.9 ± 29	105 ± 21
Lung tissue IL-1β, pg/μg protein	0.060 ± 0.002	0.066 ± 0.004

DISCUSSION

The purpose of this study was to examine pulmonary and systemic effects of CFA at occupationally relevant exposure concentrations on a variety of biological indicators/markers. Sprague-Dawley rats exposed to resuspended CFA rich in silica, aluminum, iron, and calcium demonstrated a mild pulmonary inflammatory response and associated cytokine increase. CFA caused subtle thickening and increased cellularity of the centriacinar (bronchiole-alveolar duct) region. Positive staining of alveolar macrophages for iron and increased transferrin, an iron-binding protein, in BALF was likely to be due to the iron associated with CFA. These pulmonary effects (maximum at 18-h postexposure) of CFA were associated with moderate, but significant systemic neutrophilic inflammation, decreased lymphocytes, increased complement 4, and increased hematocrit at 36 h. These later systemic changes could be critical in potential cardiovascular effects of CFA. Some of these statistically significant changes may be too small to indicate biologically significant tissue damage, but the results are valuable both as biomarkers of exposure and as indicators of underlying mechanisms.

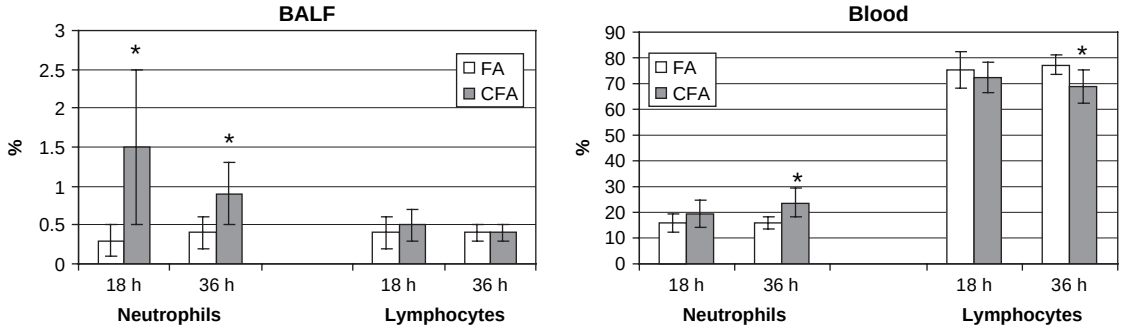

FIG. 5. Cell differential for the BALF and blood at 18- and 36-h postexposure. FA, filtered air. *Denotes statistically different than filtered air control ($p < 0.05$).

Few systemic effects have been reported in animals exposed to CFA. No changes in hematological parameters were observed in rabbits intratracheally instilled with a Nigeria bituminous CFA with a size range of 4–5 μm mean particle diameter (Ogugbuaja *et al.*, 2001). Pulmonary toxicology studies using CFA particles have generally shown few effects following instillation of 2–10 mg/kg in rats (Borm, 1997). In contrast, our study demonstrates small, but significant systemic effects. These differences may be a reflection of the importance of particle size, composition, and the exposure methods used in this study.

Although the increase in neutrophils due to inhaled CFA was significant compared with that observed in studies using CAPs at similar mass concentrations (Cassee *et al.*, 2005; Kodavanti *et al.*, 2005), the proportion of polymorphonuclear leukocytes (PMNs) measured in BALF was only 1.5% of the total cell number, which can represent baseline neutrophil levels in some rat strains. Therefore, it is possible that the detection power of increased neutrophilic inflammation was much greater in our study using Sprague-Dawley rats compared with Wistar Kyoto and spontaneously hypertensive rats.

Two time points were used in our study in an attempt to more efficiently capture possible pulmonary and systemic biological responses of CFA following a 3-day exposure. Most pulmonary biomarkers such as neutrophilic inflammation and proinflammatory cytokines were found to be increased at 18-h postexposure. However, systemic neutrophilic inflammation was most evident at 36 h associated with a decrease in circulating lymphocytes and a concomitant increase in complement 4 and hematocrit. Our results indicate by 36-h postexposure that there is a general trend of recovery in many of the pulmonary parameters, including cytokine levels, while systemic changes become most prominent at this postexposure time. The data imply that systemic inflammation peaked after pulmonary inflammation had already subsided, but confirming this observation and developing a plausible mechanistic explanation would require data at additional time points.

Time-related changes in inflammation and injury markers are likely to be affected by specific PM components, which might act via different mechanisms and temporal patterns. The time course of effect and recovery are also likely to be influenced by the extent of lung injury caused by particles. Pulmonary messenger RNA expression for IL-1β and MIP-2 increased 1 day following intratracheal instillation of diesel in rats and returned to control levels 7 days postexposure, with a second increase again at 30 days postexposure (Rao *et al.*, 2005).

Few systemic responses have been reported in animals following exposure to combustion source or ambient particles. A small, but significant increase in circulating neutrophils may

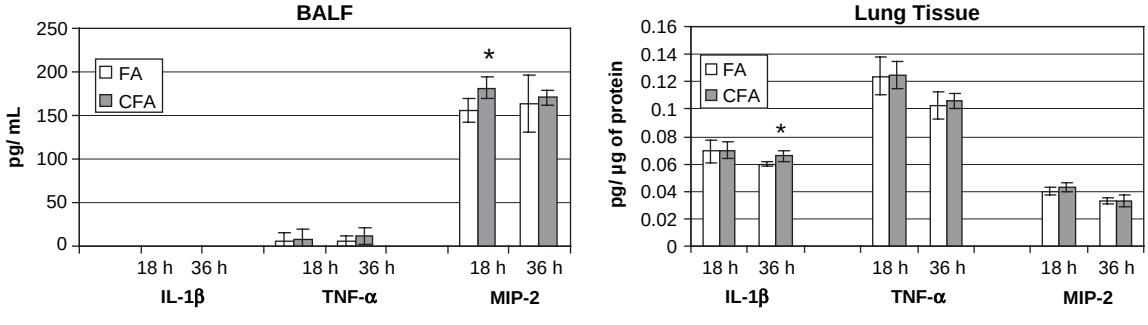

FIG. 6. Cytokines IL-1β, TNF-α, and MIP-2 in the BALF and lung tissue at 18- and 36-h postexposure. FA, filtered air. *Denotes statistically different than filtered air control ($p < 0.05$).

TABLE 3
Measured Parameters Showing no Statistically Significant Change at either Time Point after Exposure. Mean and Standard Deviation are Shown for 18-h Postexposure for the Filtered Air and CFA Groups. $N = 5$ or 6

	Filtered air	CFA
BALF		
Total cell # ($\times 10^4$)/ml	8.2 ± 2.6	9.7 ± 1.7
Macrophage # ($\times 10^4$)/ml	8.2 ± 2.5	9.5 ± 1.7
Lymphocyte %	0.4 ± 0.2	0.5 ± 0.2
Lymphocytes # ($\times 10^2$)/ml	3.4 ± 1.8	5.0 ± 1.8
Eosinophil %	0.03 ± 0.08	0.03 ± 0.05
Eosinophils, #/ml	43 ± 105	30 ± 47
Nonviable cells %	7.0 ± 1.8	7.8 ± 2.0
IL-1β, pg/ml	BLD	BLD
TNF-α, pg/ml	5.2 ± 11	7.7 ± 11
Total glutathione, μM	BLD	BLD
Glutathione disulfide, μM	BLD	BLD
GSH/GSSG ratio	BLD	BLD
Albumin, μg/ml	10.3 ± 2.9	12.8 ± 3.8
NAG, U/l	3.6 ± 1.5	3.3 ± 0.95
Protein, μg/ml	73 ± 21	92 ± 14
Ferritin, ng/ml	37 ± 6.6	42 ± 9.7
Iron, μg/dl	3.4 ± 1.9	4.6 ± 2.1
Unsaturated iron binding capacity, μg/dl	BLD	BLD
Total antioxidant status, mmol/l	0.16 ± 0.04	0.19 ± 0.04
Lung tissue		
TNF-α, pg/μg protein	0.124 ± 0.014	0.125 ± 0.010
MIP-2, pg/μg protein	0.040 ± 0.003	0.043 ± 0.003
Total glutathione, nmol/mg tissue	1.6 ± 0.23	1.6 ± 0.16
Glutathione disulfide, nmol/mg tissue	0.022 ± 0.005	0.019 ± 0.005
GSH/GSSG ratio	76 ± 24	88 ± 22
Hematology		
Red blood cell count, 10^6/mm^3	9.7 ± 0.3	9.1 ± 2.2
Hemoglobin concentration, g/dl	17 ± 2.5	17 ± 3.3
Mean corpuscular hemoglobin, pg	17.2 ± 2.4	18.7 ± 2.4
Mean corpuscular hemoglobin concentration, g/dl	29.2 ± 4.3	33.2 ± 4.7
Platelets, 10^3/mm^3	428 ± 121	561 ± 133
White blood cell count, 10^3/mm^3	10.7 ± 1.6	10.3 ± 3.6
Monocyte %	7.8 ± 3.2	7.6 ± 2.1
Eosinophil %	0.6 ± 1.3	0.4 ± 0.5
Basophil %	0.2 ± 0.4	0.2 ± 0.4
Serum		
Alpha 2 macroglobulin, mg/dl	25 ± 13	23 ± 10
Complement, 3 mg/dl	863 ± 83	968 ± 108
c-Reactive protein, μg/ml	156 ± 14	137 ± 17
Heptoglobin (HPT), mg/dl	386 ± 95	391 ± 129

BLD, below limit of detection for assay.

suggest a release of these cells in the circulation from the bone marrow. Such changes in bone marrow stimulation and the appearance of band cells in the circulation have been reported in animals exposed to particles (Mukae *et al.*, 2001). The significance of increased circulating levels of complement 4 is not clear and will require further study to elucidate its source and biological significance.

The observation of positive iron staining in alveolar macrophages and the elevation of transferrin, an iron-binding protein, in BALF following exposure to CFA may indicate increased availability of iron from inhaled CFA particles. Similar results have been reported in the lungs of rats exposed to iron-containing particles (Ghio *et al.*, 1998), where transferrin levels were elevated 24 h after instillation, but were decreased to control levels by 96-h postinstillation. These data suggest that iron may modulate some of the responses caused by particles. Ball *et al.* (2000) reviewed generation of reactive oxygen species by CFA-associated iron via the Fenton reaction.

A unique feature of this study was the ability to aerosolize over a cumulative period of 12 h a limited amount (1 g) of size-fractionated CFA sample from a specific power plant burning a specific coal. The composition of CFA depends on the mineralogy of the coal, combustion conditions, and types of equipment used to control NO_x and SO_2 (Lighty *et al.*, 2000). *In vitro* results have shown that both the bioavailability of transition metals and the cytokine signaling responses vary with the size of particles and the coal source (Smith *et al.*, 1998, 2000). The aerosol generation methods used in this study provide data on biological responses to a controlled exposure with particles of known and consistent chemical composition.

The same aerodynamically separated CFA sample that was used in this study was also used in an *in vitro* toxicology study in which IL-6 and IL-8 were measured in BEAS-2B cells. CFA was less potent for cytokine induction *in vitro* than the soil-derived $PM_{2.5}$ particles (Veranth *et al.*, 2006). The degree of neutrophilic inflammation induced *in vivo* by CFA in our study was mild, which is similar to what has been reported with other CFA studies. It is also possible that the different chemical components in the CFA interact to modify the inflammatory response. For example, neutrophilic inflammation has been negatively correlated with the level of soluble iron in oil combustion particles (Kodavanti *et al.*, 1998).

The effects of a more widely studied, but less well-characterized pollutant, CAPs, can be compared to the effects of CFA. Table 4 lists the fold increases over control for the rat responses measured in this study and CAPs studies conducted by Cassee *et al.* (2005) and Kodavanti *et al.* (2005). Although rat strains and procedures differed between studies, the particle concentrations and exposure durations were similar: 1400 μg/m^3, 4 h × 3 days (this CFA study); 144–2758 μg/m^3 CAPs, 4 h × 2 days (Kodavanti *et al.*, 2005); and 270–3720 μg/m^3 CAPs, 6 h × 1 day (Cassee *et al.*, 2005). Both CAPs studies used $PM_{2.5}$ aerosol, but detailed particle size distribution data were not reported for the CAPs. Sulfate was present at 3.97% and nitrate was not detected in the CFA, while average composition was 26.3% sulfate and 1.6% nitrate for the eastern U.S. CAPs (Kodavanti *et al.*, 2005) and 17.8% sulfate and 18.3% nitrate for the Netherlands CAPs (Cassee *et al.*, 2005).

Small changes in parameters associated with inflammation and oxidative stress were observed in all three studies, suggesting some commonalities in the effect of ambient particles

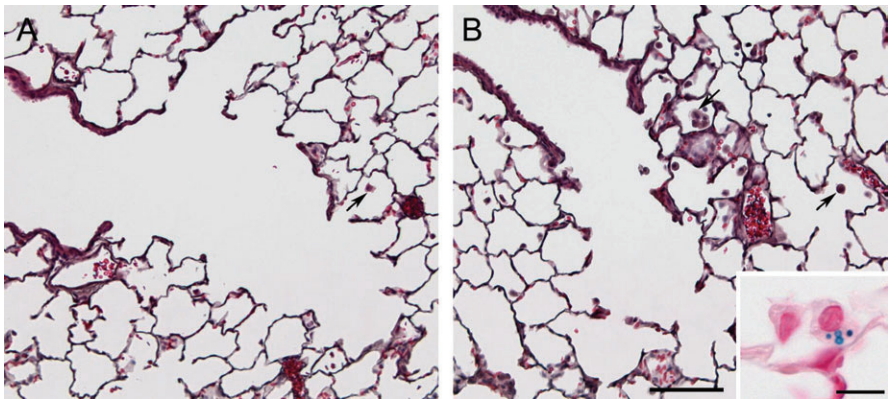

FIG. 7. Light micrograph of the terminal bronchiole-alveolar duct region from the lungs of an animal exposed to filtered air (nose only) (A) or to CFA particles (B). An increase in the number of alveolar macrophages (arrows) is evident in the alveolar airspaces following exposure to CFA. The inset in panel B shows two alveolar macrophages with one cell containing four distinct iron-positive cytoplasmic inclusions. The magnification for panels A and B is identical (scale bar is 100 μm). The scale bar in the inset of panel B is 10 μm.

and CFA. Although the reported CAPs studies represent a mean of multiple repeat studies done with chemically heterogeneous particles relative to CFA used in this study, these similarities in inflammatory response may indicate common mechanistic pathways that are likely to be induced regardless of varied composition and quantities of different PM samples.

In conclusion, this study shows that primary mineral ash particles in the $PM_{2.5}$ size range from coal-fired power plants can induce mild, but statistically significant time-dependent alterations in pulmonary and systemic parameters that are associated with inflammation. Acute inhalation exposure of healthy adult rats to CFA resulted in responses at particle

TABLE 4
Comparison of CFA and CAPs for BALF and Blood Parameters[a]

	This study $PM_{2.5}$ CFA		Kodavanti $PM_{2.5}$ CAPs		Cassee $PM_{2.5}$ CAPs	
Rat strain	Sprague-Dawley	Sprague-Dawley	Wistar Kyoto	SH	Wistar	SH
Experiment condition	18 h	36 h			Ozone pretreated	
BALF parameters						
Total protein	1.26	0.94	0.80	1.02	**1.23**	0.94
GGT activity	0.96	**0.90**	1.09	**1.34**		
NAG activity	0.92	1.04	0.97	1.13	**1.14**	0.97
Glutathione	BLD	BLD	1.39	1.10	1.18	1.00
Total antioxidant potential (tissue)	**1.07**	1.03				
Uric acid (BALF)			1.29	1.14	1.16	1.04
Total cells	1.18	1.26	**0.63**	1.03	**0.89**	1.04
Macrophages	1.16	1.25	**0.58**	1.02	**0.97**	0.99
Neutrophils	**5.38**	**3.30**	**1.38**	1.13	1.16	**1.45**
TNF-α	1.48	1.88	1.25	ND	0.96	0.98
MIP-2	**1.16**	1.05	1.05	ND	1.02	1.00
Blood parameters						
Red blood cells	0.94	1.13	1.00	1.01	**1.02**	1.00
Hemoglobin	1.00	1.20	1.00	1.01	**1.02**	1.00
Hematocrit	0.90	**1.20**	1.00	1.01	**1.01**	0.99
Mean corpuscular volume	0.96	**1.05**			1.00	1.00
Mean corpuscular hemoglobin	1.09	1.00			1.01	1.00
Mean corpuscular hemoglobin concentration	1.14	0.95			1.00	**0.996**
White blood cell count	0.96	1.12	0.94	1.02	0.99	0.98

[a]Expressed as the ratio of the exposure value to the corresponding control value.

Rats exposed to CFA (1400 μg/m^3, 4 h × 3 days), CAPs (144–2758 μg/m^3, 4 h × 2 days) (Kodavanti *et al.*, 2005), and CAPs (270–3720 μg/m^3, 6 h × 1 day) (Cassee *et al.*, 2005). Bold denotes statistically different from filtered air control as reported by the original publication. BLD, below limit of detection; ND, not determined; SH, spontaneously hypertensive rat.

concentrations relevant to current occupational exposure limits for nuisance dusts. CFA particles are not biologically inert; however, for the BALF, lung tissue, and blood endpoints measured CFA particles are also not highly potent compared to an equal mass of $PM_{2.5}$ CAPs. Air quality management will benefit if toxicology research can identify the specific emissions that are most important for causing the morbidity and mortality associated with increased air pollution. Since CFA is typically less than 1–5% of the ambient PM mass, the comparison of CFA and CAPs study results suggests that CFA alone is a minor, but incremental, contributor to the potency of the ambient air PM mixture.

ACKNOWLEDGMENTS

This research was funded by the Health Effects Institute contracts 97-8 and 02-3, U.S. Environmental Protection Agency (USEPA) STAR grants 829215 and 831714, USEPA Center grant R832414, National Institute for Occupational Safety and Health (NIOSH) grant OH07550 and National Institute of Environmental Health Sciences (NIEHS) grants K25011281, and ES05707. We would like to thank Imelda Espiritu, Julian J. Recendez, Judy H. Richards, Mette C. Schladweiler, and Stephen V. Teague for their technical assistance and Dr Judith Chow at the Desert Research Institute, who provided the elemental composition data. We would also like to thank Dr Suzette Smiley-Jewell for editorial assistance in the preparation of this manuscript.

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