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Pulmonary Toxicity of Inhaled Diesel Exhaust and Carbon Black in Chronically Exposed Rats

Part I: Neoplastic and Nonneoplastic Lung Lesions

Joe L. Mauderly, M. Burton Snipes (Study Director), Edward B. Barr, Steven A. Belinsky, James A. Bond, Antone L. Brooks, I-Yiin Chang, Yung S. Cheng, Nancy A. Gillett, William C. Griffith, Rogene F. Henderson, Charles E. Mitchell, Kristen J. Nikula, and David G. Thomassen

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Includes the Commentary of the Institute's Health Review Committee

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HEI HEALTH EFFECTS INSTITUTE

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HEI Statement

Synopsis of Research Report Number 68

COMPARISON OF THE CARCINOGENICITY OF DIESEL EXHAUST AND CARBON BLACK IN RAT LUNGS

BACKGROUND

Emissions from diesel engines are a complex mixture of gaseous vapors and soot particles. The soot particles are a public health concern because they are of a respirable size and contain organic compounds adsorbed onto their surfaces. Many of these compounds can damage the cellular genetic material (DNA), and have been shown to cause cancer in laboratory animals. A number of laboratories have demonstrated that inhaling high concentrations of diesel engine exhaust for a prolonged period of time causes lung tumors in rats. However, in the 1980s, questions were raised about which constituents of diesel engine exhaust (the soot particles, or their adsorbed organic compounds, or both) were responsible for this tumorigenic effect. The Health Effects Institute and the Department of Energy sponsored Dr. Joe L. Mauderly and coworkers to help resolve this question. This is an important issue because the first steps in assessing risk are to identify the hazardous agent(s) and to determine the mechanisms through which they act before extrapolating to humans the results from animal studies. Such risk assessments are being currently undertaken by the U.S. Environmental Protection Agency, the California Environmental Protection Agency, and the World Health Organization.

APPROACH

Dr. Mauderly and coworkers exposed F344/N rats to clean air or to one of two levels (2.5 or 6.5 mg of particles/m^3 of diesel exhaust or air) of either emissions from a light-duty diesel engine or carbon black particles. The exposures lasted for 16 hours/day, 5 days/week, for 24 months. The carbon black particles were similar to the soot particles in the diesel engine exhaust; however, they contained markedly lower amounts of adsorbed organic compounds. Approximately 100 times less organic material could be extracted from carbon black than from diesel exhaust particles. Also, in contrast to extracts of diesel exhaust soot, carbon black extracts produced little or no response in bacterial mutagenicity assays. Thus, they served as a surrogate for diesel exhaust particles that are relatively free of mutagenic organic compounds. The investigators determined the number and types of tumors that formed in the rats, and assessed the possible contributions of a number of factors (such as tissue injury, or clearance or translocation of inhaled particles) known to be linked with the development and progression of lung cancer. They also ascertained whether the exposures resulted in the formation of DNA adducts (the products of chemicals or their metabolites reacting with the DNA) in lung tissue or in cells isolated from lungs. The results of the DNA adduct research will be discussed at a later date, with the results of a companion study, in Part II of this Research Report.

RESULTS AND INTERPRETATION

The results of this carefully conducted study demonstrate that prolonged exposure to diesel engine exhaust and carbon black particles produces nearly identical carcinogenic and noncarcinogenic effects in this strain of rats. No significant differences were noted between the two exposure materials in the resulting incidence, number, or types of lung tumors. These results may be considered surprising because, compared with diesel soot, the carbon black particles were relatively free of mutagenic organic compounds. Both exposures caused injury to lung tissue, including inflammation, cell proliferation, and fibrosis. These lesions progressed in number and size as the dose of particles increased. At both exposure concentrations, diesel soot and carbon black accumulated in the rat lungs and, after three months of exposure, normal particle clearance mechanisms were impaired.

Dr. Mauderly's results, and recent findings from other laboratories, suggest that (1) the small respirable soot particles in diesel exhaust are primarily responsible for lung cancer developing in rats exposed to high concentrations of diesel emissions, and (2) at high particle concentrations, the mutagenic compounds adsorbed onto the soot play a lesser role, if any, in tumor development in this species. The mechanism by which insoluble particles cause lung tumors in heavily exposed rats is not known. However, it is clear that the tumors are associated with an impairment of the process by which inhaled material is cleared from the lungs; the impaired clearance

process leads to a progressive accumulation of particles and damage to the surrounding tissues. This response appears to be dependent upon the species; for example, results from other studies have shown that hamsters do not develop lung tumors after exposure to high concentrations of particles, and the limited data available for mice are equivocal.

More information is needed about the mechanisms by which inhaled diesel exhaust and other particles cause lung tumors in rats before the results of the rat bioassay are used to predict lung cancer risk in humans. For example, we need to know if the same mechanism that produces lung cancer in rats also operates in humans, and if the sequence of events that leads to pulmonary tumors developing in rats after exposure to high concentrations of particles also occurs in humans exposed to much lower concentrations of particles, as in ambient settings. Dr. Mauderly's findings do not support extrapolating the rat carcinogenicity data to humans on the basis of the amount of organic material deposited in the lungs. However, uncertainties are associated also with extrapolating the data from rats to humans on the basis of the particle concentration or the lung burden of particles.Furthermore, the outcome of prolonged exposure to low levels of particles or particle-bound carcinogens, under conditions in which the lungs do not have a proliferative response to particles is unknown.

This Statement, prepared by the Health Effects Institute (HEI) and approved by the Board of Directors, is a summary of a research project sponsored by HEI from 1988 to 1992. This study was conducted by Dr. Joe L. Mauderly of the Inhalation Toxicology Research Institute, Lovelace Biomedical and Environmental Research Institute, Albuquerque, NM. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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ABSTRACT

This study compared the pulmonary carcinogenicities and selected noncancer effects produced by chronic exposure of rats at high rates to diesel exhaust and carbon black. The comparison was intended to provide insight into the likely importance of the mutagenic organic compounds associated with the soot portion of diesel exhaust in inducing pulmonary carcinogenicity in diesel exhaust–exposed rats. The role of the organic fraction has become important in judging the usefulness of the substantial data base on carcinogenicity in rats for predicting lung cancer risk for humans, and for determining the most appropriate method of extrapolating results across species and exposure concentrations.

Rats were exposed chronically to either diesel exhaust or carbon black, which served as a surrogate for diesel exhaust soot with much reduced mutagenic activity associated with its organic fraction. The sequestration of particles in the lung and the induction of pulmonary neoplasia and nonneoplastic changes in the lung were compared in detail. Samples also were provided to collaborators to examine adduct formation in lung DNA and hemoglobin.

Approximately 140 female and 140 male F344/N rats were exposed for 16 hours per day, 5 days per week for up to 24 months, beginning at eight weeks of age, to diesel exhaust or carbon black at 2.5 mg or at 6.5 mg particles/m^3 of air, or to clean air as controls. The diesel exhaust was generated by light-duty engines burning certification fuel and operating on an urban-duty cycle. The carbon black was selected because it had particle size and surface area characteristics similar to those of diesel exhaust soot, but markedly less mutagenic activity associated with its organic fraction when analyzed using procedures typically used in studies of diesel soot. Rats were killed after 3, 6, 12, 18, or 23 months of exposure to measure lung and lung-associated lymph node burdens of particles, lung weight, bronchoalveolar lavage indicators of inflammation, DNA adducts in whole lung and alveolar type II cells, and chromosome injury in circulating lymphocytes, and to perform histopathologic assessment. In addition, after 3 and 18 months of chronic exposure, one group of rats was acutely exposed to radiolabeled carbon black particles or to fluorescent microspheres. These exposures were conducted to examine the clearance of radiolabeled particles and the sequestration of the fluorescent microspheres in the lungs. These experiments provided information on clearance overload and particle dosimetry. The growth characteristics of lung neoplasms also were examined by transplanting neoplastic cells into athymic mice.

Exposures to diesel exhaust and carbon black caused nearly identical effects, in similar relation to exposure, in all parameters measured. Both diesel exhaust and carbon black exposures caused marked, dose-related slowing of particle clearance by three months, and progressive accumulation of particles in lungs and lymph nodes. Lavage demonstrated persistent inflammation and cytotoxicity; and nonneoplastic changes consisting of inflammation, epithelial proliferation, and fibrosis were progressive and related to dose. Lung DNA adducts were slightly increased by diesel exhaust exposure, and DNA adducts in type II cells were increased by both diesel exhaust and carbon black exposure. No exposure-related chromosome damage was found in circulating lymphocytes. The incidence of primary lung neoplasms was increased significantly and related to dose in all exposed groups. The types of neo-

This Investigators' Report is Part I of Health Effects Institute Research Report Number 68, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Part II: Diesel Exhaust and DNA Damage, by Randerath and associates, will be published in 1995. Correspondence concerning this Investigators' Report may be addressed to Dr. Joe Mauderly, Lovelace Inhalation Toxicology Research Institute, P.O. Box 5890, Albuquerque, NM 87185.

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plasms resulting from diesel exhaust and carbon black exposure were identical, and there was no significant difference between the carcinogenicities of the two materials. The same types of neoplasms were induced by both diesel exhaust and carbon black and these neoplasms also had similar growth characteristics when transplanted. The sequestration and translocation of tracer particles were similarly affected by diesel exhaust and carbon black exposure.

The finding that nearly identical carcinogenicity and noncancer effects resulted from diesel exhaust and carbon black exposure suggested that the organic compounds associated with diesel exhaust soot may not have played an important role in the pulmonary carcinogenicity of diesel exhaust in rats exposed chronically to high concentrations. In the present study, very low levels of mutagenic organic residues were associated with carbon black when compared with diesel exhaust. It is possible that these organic residues might have been extracted from the carbon black by more exhaustive procedures than the ones used in this study. However, our findings suggest that pulmonary carcinogenicity induced by diesel exhaust in rats exposed to high concentrations of particles probably does not occur by the chemical mechanisms that have been hypothesized based on the mutagenicity of diesel exhaust soot extracts studied in the past. These findings do not prove that mutagenic activity associated with diesel exhaust does not contribute to lung cancer risk in humans exposed to low concentrations for longer times. However, they suggest that lung cancer risk assessments that use the amount of deposited organic material as the comparative dose term should not be used to extrapolate cancer risks for humans from rat carcinogenicity data. Because the mechanisms by which inhaled diesel exhaust and carbon black cause lung neoplasms in rats remain undefined, it is not known if lung cancer might be produced in humans by similar mechanisms.

INTRODUCTION

The background of concern for the potential pulmonary carcinogenicity of diesel engine exhaust has been reviewed in detail (Mauderly 1992). Diesel exhaust is a complex mixture of gases, vapors, and soot particles. The soot consists of respirable, chain-aggregate, elemental carbon particles having a high specific surface area onto which a mixture of organic compounds is adsorbed. The organic fraction typically constitutes from 5% to 30% of the total soot mass when it is extracted using modestly rigorous solvent procedures. More than 450 compounds have been identified in the organic fraction, including several known mutagens and carcinogens (Opresko et al. 1984). The potential for diesel exhaust–induced chemical carcinogenesis was demonstrated in the 1950s when the organic fraction extracted with solvents was shown to cause mouse skin tumors (Kotin et al. 1955). Concern for diesel exhaust–induced lung cancer in humans intensified in the late 1970s with the prediction of increased diesel use in the U.S. light-duty vehicle fleet and the report by the U.S. Environmental Protection Agency (EPA)* that diesel exhaust soot extract was mutagenic in the Ames *Salmonella* assay (Huisingh et al. 1978).

Bioassays aimed at carcinogenic hazard assessment demonstrated diesel exhaust to be a pulmonary carcinogen in rats. During the 1980s, chronic inhalation bioassays of diesel exhaust–exposed rats, mice, and Syrian hamsters, conducted in the U.S., Germany, Japan, and Switzerland, produced generally consistent results (Mauderly 1992). The lung tumor incidence was increased in a dose-related manner in rats exposed repeatedly for 24 months or longer to diesel exhaust at weekly soot exposure rates above approximately 120 $mg/m^3 \cdot hr$ (concentration × time). Studies that included groups exposed to filtered (gas and vapor phases) diesel exhaust demonstrated that the response required the presence of soot. The lung tumor response of mice to whole diesel exhaust was questionable, with significant increases evident only in females of cancer-sensitive strains. The bioassays of hamsters were uniformly negative.

By the late 1980s, it was considered plausible that the lung tumor response of rats to chronic diesel exhaust inhalation might be attributable to the initiating action of genotoxic metabolites of the soot-associated organic compounds, coupled with the promoting action of the inflammatory and epithelial proliferative responses also caused by exposures at levels that are known to induce cancer. Several studies demonstrated that diesel exhaust soot–associated organic compounds, including the carcinogens benzo[*a*]pyrene and 1-nitropyrene, could be eluted from soot after deposition in the rat lung (Sun et al. 1988). These studies also showed that association with particles caused inhaled organic compounds to be retained longer in the lung than when inhaled in pure form, thus prolonging the availability of the compounds for metabolism by lung cells. A preliminary study of diesel exhaust–exposed rats from a group having an increased tumor incidence showed that the group mean total lung DNA adduct level was higher than that of controls (Wong et al. 1986). Although the ranges of DNA adduct levels in the lungs of exposed and control rats overlapped,

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

these data suggested that the diesel exhaust exposure was genotoxic and bolstered the chemical carcinogenesis theory. The attention of several investigators shifted toward identifying the key genotoxic compounds in the organic fraction, and biodirected chemical fractionation suggested that a major portion of the bacterial mutagenicity was attributable to nitroaromatic species (Howard et al. 1990).

Other evidence, however, suggested that the carcinogenicity of diesel exhaust in rats might reflect a nonspecific response of the species to the accumulation of the carbonaceous particles in the lung, and might have little to do with the organic fraction. Soon after the rat bioassay results became known, it was noted (Vostal 1986) that lung cancer was significantly increased only in groups exposed to diesel exhaust sufficient to cause a progressive lung accumulation of soot and an accompanying complex of inflammation, epithelial hyperplasia and metaplasia, and focal fibrosis, and that exposures of humans were unlikely to produce these effects. Moreover, it was noted that other particles without a bioavailable organic fraction, such as titanium dioxide and oil shale, caused similar effects in rats (Vostal 1986). At the same time, it was shown that intratracheally instilled activated carbon particles could cause lung cancer in rats (Kawabata et al. 1986). These observations led to speculation that the mechanism by which diesel exhaust caused lung tumors in rats might not be expected to occur in humans, and that the rat bioassay results might not, therefore, be appropriate for predicting cancer risk for humans inhaling diesel exhaust.

An expanding bioassay data base brought a broadening awareness that chronic inhalation exposures to a wide range of poorly soluble respirable particles without a bioavailable mutagenic organic fraction produced lung cancer in rats if exposures were sufficiently high and observation times sufficiently long. Responses common to all such carcinogenic exposures were particle sequestration in the deep lung, inflammation, epithelial hyperplasia and metaplasia, and fibrosis. Measurements of the clearance of tracer particles from the lung during continued chronic exposure demonstrated that the progressive accumulation of lung burdens of particles was attributable to an overloading of macrophage-mediated clearance, and the term "lung overload" was coined for this phenomenon (Morrow 1988). The mechanism of clearance overload of rats by repeated dust exposure and the determinants of dust sequestration in the lung were the subjects of considerable study and debate at the time the present research began. It now appears that volumetric loading is critical to macrophage mobility, and the adequacy of macrophage-mediated clearance to prevent build-up of particle burdens in the lung represents a balance between macrophage recruitment and particle deposition rates (Morrow 1992). Two particular areas in which information continues to be lacking are the rapidity of movement of newly deposited particles into sequestered compartments, and the amount of dust collected in lung-associated lymph nodes during chronic exposure.

Concurrent with the bioassay and chemical fractionation efforts were attempts to determine human cancer risk from diesel exhaust exposures by epidemiology. Early attempts yielded equivocal results; however, considerable attention was drawn to the publication by the Harvard University group of case-control (Garshick et al. 1987) and retrospective cohort (Garshick et al. 1988) studies indicating relative risks on the order of 1.4 for lung cancer among railroad workers with long-term exposures to diesel exhaust. At the present time, the weight of the epidemiological evidence from approximately 30 studies (Mauderly 1992) suggests that heavy occupational exposures to diesel exhaust, such as those in the mining and transportation industries, are probably associated with an increase in lung cancer risk. However, epidemiology has not resolved the risk issue with certainty. The modest magnitude of the apparent increase (relative risks generally in the range of 1.2 to 1.5), coupled with a lack of exposure data and uncertainties about the effectiveness of controls for confounding factors, have precluded the confident assignment of unit cancer risks solely from epidemiological data. After reviewing the bioassay and epidemiological data available in 1988, the International Agency for Research on Cancer judged that there was sufficient evidence for the carcinogenicity of whole diesel exhaust and soot extracts in animals, but only limited evidence for carcinogenicity in humans; thus, diesel exhaust was classified as "probably carcinogenic" to humans (International Agency for Research on Cancer 1989).

At the time the present research was begun, it had clearly become critical to determine whether or not the soot-associated organic compounds were important in the lung cancer response of rats. Most estimates of unit lung cancer risk for humans have been developed from extrapolation of the rat bioassay data or from the comparative mutagenic potencies of diesel exhaust soot extract and known human chemical carcinogens (Mauderly 1992). The former requires confidence that the mechanism by which diesel exhaust induces lung tumors in rats would also occur in humans, and the latter assumes that the genotoxicity of the soot-associated organic fraction is responsible for carcinogenesis in rats. The importance of knowing whether or not the rat

tumors were caused primarily by the soot-associated organic fraction was heightened by the suggestion in the EPA's draft diesel exhaust health assessment document (U.S. Environmental Protection Agency 1990) that unit risks for diesel exhaust–induced lung cancer in humans might be extrapolated from rat bioassay data using the amount of organic material deposited in the lung as the equivalent dose term.

Determining the specificity of the rat lung tumor response to diesel exhaust (i.e., its relation to the organic fraction) also lay at the center of a broader debate on the design and usefulness of rodent cancer bioassays per se. Research on diesel exhaust helped focus debate on whether or not rat pulmonary carcinogenicity data collected under dust "overloading" conditions are useful for hazard assessment and risk extrapolation. It has been proposed that much of the excess tumor incidence observed in bioassays results from increased cell proliferation (mitogenesis) which, in turn, results from dosing at rates higher than expected for human exposures, and that this carcinogenicity is not predictive for human cancer risk (Ames and Gold 1990). It is still apparent, however, that even under overloading conditions, bioassay data reveal marked differences in carcinogenic potential among dusts and that not all dust exposures causing overload cause increased lung tumor incidences in rats (Mauderly et al. 1990). Because of the large national investment in the rat bioassay for assessing the carcinogenic hazards of inhaled materials, and the tendency, lacking good epidemiological data, to extrapolate the rat cancer data to estimate unit risks for humans, it is very important to improve our ability to interpret the rat data and place it in its proper context.

The research described below was designed to address certain key elements of the information needs described above. The principal issue was the importance of the genotoxicity of the soot-associated organic compounds in the lung tumor response of rats to chronically inhaled diesel exhaust. It would have been desirable to address this issue directly by exposing rats to whole diesel exhaust with and without the soot-associated organic fraction, but extracting this fraction, resuspending the soot, and reconstituting the remaining elements of whole diesel exhaust for chronic, high-level exposures were deemed impractical. Alternatively, it was decided to expose rats to one type of carbon black, selected from among the many types available, that would simulate the elemental carbon matrix of organic-free diesel exhaust soot. We felt it was not necessary to add the gas and vapor fractions to the carbon black exposure atmosphere because it had been shown previously that filtered whole diesel exhaust was not carcinogenic to rats (Heinrich et al. 1986; Brightwell et al. 1989). Thus, we decided to expose rats to whole diesel exhaust, aerosolized carbon black, or clean air as controls. Rats were exposed to the same air concentrations of diesel exhaust soot and carbon black particles, although we predicted that the difference between the particle size distributions of the two materials would cause differences in the rate of pulmonary deposition of the two particles. Because of the general similarity of median aerodynamic particle size between the two materials and the lack of data for lung accumulation of carbon black in chronically-exposed rats, we deemed it impractical to attempt to achieve exactly identical cumulative lung burdens of diesel exhaust soot and carbon black by using different exposure concentrations.

The carbon black selected (Elftex-12 furnace black, Cabot, Boston, MA) was chosen from among the many types available, based on previous experience at this Institute (Wolff et al. 1990). It is known to have particle size characteristics and a specific surface area (43 m^2/g, per Cabot) generally similar to those of diesel soot, but very little solvent-extractable organic material and bacterial mutagenic activity when assayed using procedures applied to diesel exhaust soot. It was also found to cause particle sequestration and inflammatory responses similar to those caused by diesel exhaust in rats exposed by inhalation for several weeks.

Although it was recognized that the carbon black was not devoid of organic matter, it was considered a useful approximation of diesel exhaust soot with markedly reduced organic mutagenic activity. Knowledge of the magnitude and mutagenicity of the organic fraction of diesel exhaust soot, including the data used in comparative potency estimates of cancer risk, has been based on short-term Soxhlet or ultrasonic extractions using single or blended solvents, and mutagenicity testing in *Salmonella* strains TA98 and TA100. Based on our previous studies using Soxhlet or ultrasonic extraction that showed little difference in total bacterial mutagenic activity (Brooks et al. 1979; Royer et al. 1979), dual ultrasonic extraction was routinely used at this Institute for diesel exhaust soot (see Appendix A). Using this approach, a sample of Cabot Elftex-12 carbon black had been shown to have only 0.04% extractable organic compounds by mass, and to have an extractable fraction and specific mutagenic activity that were 825 and 200 times less, respectively, than diesel exhaust soot (Wolff et al. 1990). Organic residues adhere tenaciously to carbon black, and exhaustive lengthy or high-temperature extractions are required to fully remove them (International Agency for Research on Cancer 1983). For example, Locati and associates (1979) extracted five furnace blacks for 350 hours with benzene using the Soxhlet method and found that at least 150 hours was required to extract 95% of the

organic fraction, which ranged from 0.025% to 0.142% of the carbon black mass (252 to 1417 μg per gram). It was considered very unlikely that organic residues would dissociate from the carbon black under physiological conditions in the lung. Neal and colleagues (1962) found no elution of polycyclic aromatic hydrocarbons from furnace black or channel black after 180 hours of incubation with agitation at body temperature in gastric and intestinal fluid simulants, citric acid mixtures, or other body fluid and foodstuff media. Therefore, the carbon black used in the present study was considered to be a reasonable model for diesel exhaust soot having little or no bioavailable mutagenic organic activity.

Because the occurrence and mechanism of carcinogenicity in rats were the focal concerns, rather than detailing exposure-response relations, exposure levels were selected for which it was certain that diesel exhaust would induce significant pulmonary carcinogenicity. Because the carcinogenicity of carbon black was unknown, the study was designed with two exposure levels to increase the probability that an adequate comparison between the carcinogenicities of diesel exhaust and carbon black could be made. Measures of the accumulation of soot and carbon black particles in lung and lung-associated lymph nodes, lung inflammation, the efficiency of tracer particle clearance, and the movement of tracer particles into sequestered compartments were included to address lung overload issues and to allow the amount of accumulated material to be used as one of the comparative dose terms for relative carcinogenicity. Radiolabeled carbon black was used as the tracer particle for clearance studies because the use in all previous studies of tracers with physicochemical properties quite different from those of diesel exhaust soot had left the applicability of the clearance data to soot clearance in question.

This research also was designed to examine the chemical genotoxicity of diesel exhaust directly, largely through collaborations between this Institute (Inhalation Toxicology Research Institute [ITRI]) and other laboratories. Whole-lung DNA adducts in samples collected serially throughout the exposure were measured by Dr. Kurt Randerath, Baylor College School of Medicine, using DNA extracted at ITRI. Whole-lung DNA adducts after three months of exposure also were measured at ITRI and by Dr. Alan Jeffrey, Columbia University, as an interlaboratory comparison of analytical techniques and results. Dr. Jeffrey also measured hemoglobin adducts in erythrocytes collected at selected times.

Studies at ITRI had demonstrated differences among the levels of DNA adducts induced in different regions of the respiratory tracts of rats exposed to diesel exhaust (Bond et al. 1988). To further refine our understanding of the localization of diesel exhaust–induced DNA adducts, this study included analysis of DNA adducts in alveolar type II epithelial cells. Both type II cells and Clara cells were known to be able to metabolize mutagenic organic compounds such as those associated with diesel exhaust soot. For example, type II cells can metabolize benzo[*a*]pyrene to reactive metabolites, and this metabolism is enhanced by exposure to xenobiotics (Bond et al. 1983). Moreover, type II cells are thought to be the progenitor cells for replacing damaged type I epithelium (Adamson and Bowden 1974; Evans et al. 1973), and are undoubtedly important in alveolar epithelial proliferative responses. Finally, recent studies had provided evidence that type II cells are the progenitors of at least some types of peripheral lung tumors in rats (Hahn et al. 1989; Belinsky et al. 1990).

Because markers of exposure to diesel exhaust and resulting genotoxicity would be useful in epidemiological studies, this study also included an exploratory evaluation of whether or not chromosome damage in circulating lymphocytes might reflect diesel exhaust exposure.

SPECIFIC AIMS

This research was conducted in response to the Health Effects Institute's Request for Applications No. 86-2, "Health Effects of Diesel Emissions," issued in October, 1986. The overall goal of this project was to improve our understanding of the usefulness of rat lung cancer bioassay data for assessing the carcinogenic hazard of inhaled diesel exhaust and for estimating human lung cancer risk from inhaled diesel exhaust. The research strategy was to compare key health effects resulting from chronic inhalation exposure of rats to diesel exhaust and carbon black. The three specific aims of this project were as follows.

1. To compare the pulmonary carcinogenicities of diesel exhaust and carbon black in rats exposed chronically by inhalation at two exposure rates at which diesel exhaust would predictably cause lung cancer. The carcinogenicities were compared on the basis of the occurrence of lung neoplasms, the prevalence of neoplasms over time, the types of neoplasms, and the growth characteristics of neoplasm tissue transplanted subcutaneously into nude mice. Because the carcinogenicity of diesel exhaust in rats was thought to be linked to the development and progression of noncancer lesions, this study also included comparisons of noncancer histopathology between diesel exhaust– and carbon black–exposed rats.

2. To determine the genotoxicity of inhaled diesel exhaust and compare it to that of carbon black. The genotoxicities were compared by evaluating DNA adducts in lung tissue and alveolar type II cells, and chromosome damage in circulating lymphocytes.
3. To improve the understanding of the kinetics of clearance overload with inhaled particles in the rat by comparing particle clearance, translocation, and sequestration phenomena caused by exposure to diesel exhaust and carbon black. Particle clearance was evaluated during continuous chronic exposure, using carbonaceous tracer particles that simulated diesel exhaust soot. Particle translocation and sequestration were evaluated by measuring the portions of inhaled tracer particles in different anatomic locations in the lung at sequential times after inhalation. Particle accumulation was evaluated by measuring amounts of diesel exhaust soot and carbon black in the lung and lung-associated lymph nodes serially during exposure.

METHODS

EXPERIMENTAL DESIGN

Female and male F344/N rats were exposed by inhalation for up to 24 months to diluted whole diesel exhaust, carbon black aerosolized in air, or filtered air as sham-exposed controls, beginning at 8 (7-9) weeks of age. The rats were exposed 16 hours per day, 5 days per week to diesel exhaust and carbon black at two identical target particle concentrations, a low concentration of 2.5 mg/m^3 and a high concentration of 6.5 mg/m^3. The rats were entered into the study in two approximately equally sized blocks (A and B) separated by three weeks to accommodate animal availability and entry activity schedules. Rats in both blocks were divided into the five experimental groups. The chronic exposures of blocks A and B began on February 8, 1989 and March 1, 1989, respectively. The experimental groups and numbers of rats entered into the chronic exposure chambers are outlined in Table 1.

The majority of the rats, approximately 200 per experimental group, were observed for life span to evaluate body weight, survival, and carcinogenicity. Necropsies were performed when these rats died or were euthanized when moribund, and tissues were collected from major organ systems. The exposures were terminated at 24 months, and the remaining rats were transferred to an animal housing room where they were maintained in shoebox cages with hardwood-chip bedding (Murphy Forest Products, Montville, NJ) until mortality reached approximately 90%. All remaining rats in blocks A and B were killed and necropsies were performed between March 21 and 25, and between April 10 and 12, 1991, or 41 to 45 and 40 to 42 days after the end of the 24-month exposures, respectively. When a lung mass was observed that was of sufficient size that the entire mass was not required for histopathology (larger than 3 to 4 mm in diameter), the excess portion was transplanted subcutaneously into immunodeficient nude mice to determine the growth characteristics and to propagate tissue for future use.

As summarized in Table 2, some of the rats from each experimental group were used for evaluations requiring special procedures or that they be killed at times different than those specified above. Three female and three male rats selected randomly from rats in block A were killed after 3, 6, 12, 18, or 23 months of exposure for multiple evaluations. The left lung was used for bronchoalveolar lavage, measurement of particle burden, and extraction of DNA for

Table 1. Exposure Groups of F344/N Rats Used to Compare Pulmonary Carcinogenicities of Diesel Exhaust and Carbon Black

Exposure Group	Target Particle Concentration (mg/m^3)	Number of Rats Entered into Exposure Chambers						
		Female			Male			
		Block A	Block B	Total	Block A	Block B	Total	Total Rats
Low carbon black	2.5	70	65	135	70	68	138	273
High carbon black	6.5	70	66	136	70	69	139	275
Low diesel exhaust	2.5	70	65	135	70	68	138	273
High diesel exhaust	6.5	70	66	136	70	69	139	275
Sham-exposed control	0	70	65	135	70	69	139	274
Total		350	327	677	350	343	693	1,370

adduct analysis. Because of a technical difficulty with the original DNA extraction, DNA was later extracted from the frozen right intermediate lobes for reanalysis of the 3- and 6-month responses. The right diaphragmatic lobe was fixed for histopathology, and the remaining lobes were frozen as archival samples. Lung-associated lymph nodes were collected to measure particle burdens. Blood was collected for serological detection of microbial pathogens, and for analyses of hemoglobin adducts in erythrocytes and chromosome injury in circulating lymphocytes.

Serial analyses of lung DNA adducts and blood hemoglobin adducts were performed by Dr. Kurt Randerath, Baylor College of Medicine, and Dr. Alan Jeffrey, Columbia University, respectively, using samples collected at ITRI, coded, and shipped frozen to the collaborators. The animal identification code was not broken until the collaborators submitted results of their initial analyses to the Principal Investigator. Coded lung DNA from the rats killed at three months was analyzed for adducts at all three laboratories for a comparison of analytical results. Samples of the solvent-extracted organic fraction of diesel exhaust soot collected at the six month point also were provided to the collaborators for assays of composition and genotoxicity.

Five females and five males from the high diesel exhaust, high carbon black, and control groups of block A were killed after three months of exposure for analysis of DNA adducts in alveolar type II epithelial cells. Eight females and eight males from all groups of block B were exposed once by inhalation to fluorescent latex microspheres after 3 or 18 months of chronic exposure; they were killed serially during continued chronic exposure to determine the movement of tracer particles into sequestered anatomic locations, such as the lung interstitium and alveolar macrophage aggregates. Eight males and eight females from all groups of block B were exposed once to radiolabeled carbon black after three months of chronic exposure, and the clearance of the tracer particles from the lung during continued chronic exposure was determined by serially counting thoracic radioactivity. The chronic exposures of these rats

Table 2. Special Evaluations of Female and Male F344/N Rats

Evaluations	Exposure Block	Months of Exposure				
		3	6	12	18	23
Regularly scheduled intervals (3 females, 3 males)[a]	A					
Lung and lymph node particle burdens		X	X	X	X	X
Histopathology		X	X	X	X	X
Serology for pathogens		X	X	X	X	X
Collection of archival tissue		X	X	X	X	X
Bronchoalveolar lavage				X	X	X
Chromosome injury in lymphocytes		X				
Lung DNA for adducts		X	X	X	X	X[b]
Erythrocytes for hemoglobin adducts		X				
DNA adducts in type II alveolar cells (5 females, 5 males)[c]	A	X				
Sequestration of fluorescent particles (8 females, 8 males)	B	X[d]			X[d]	
Clearance of radiolabeled carbon black (8 females, 8 males)	B	X[e]			X[e]	

[a] All *n* values indicate number of animals from each of the five exposure groups. Therefore, a total of 15 males and 15 females were killed at each regularly scheduled interval.

[b] DNA for the 23-month adduct analysis was taken from the lungs of 9 to 10 rats from both blocks A and B of the groups exposed to high diesel exhaust and high carbon black, and the control group only. These rats died or were killed after a mean of 696 (638 to 772) days of exposure.

[c] For this evaluation, rats from only three exposure groups were used: high diesel exhaust, high carbon black, and control.

[d] Two males and two females were killed at 1, 4, 28, and 90 days after inhaling tracer particles.

continued, and clearance was reevaluated by a second inhalation of radiolabeled carbon black after 18 months of chronic exposure.

PROCEDURES

Animals, Assignment, and Maintenance

Female and male F344/N rats were obtained from the barrier-maintained, specific pathogen–free, ITRI animal colony at five to seven weeks of age and placed in inhalation exposure chambers ventilated with filtered air for a two-week preexposure acclimation period. Five acclimation chambers, identical to the experimental exposure chambers, were used for both entry blocks. Midway through the acclimation of each block, one female and one male rat were selected randomly from each acclimation chamber and killed; necropsies to detect subclinical disease were performed, and serum was collected for serological surveillance for pathogenic microorganisms (described below). The remaining rats were assigned to the five different exposure groups using a software program (Path-Tox, Xybion Medical Systems, Cedar Knolls, NJ) that randomized each gender by body weight measured nine days before the start of exposure. Upon assignment, each rat received a unique alphanumeric tail tattoo. The rats were then moved to their respective experimental exposure chambers and were seven to nine weeks old (mean = 57 days) when the chronic toxicant or sham exposures began.

The rats were housed for 24 months, or until they died or were killed, in wire cages within glass and stainless-steel inhalation exposure chambers having a volume of 2 m^3 (H-2000, Hazleton Systems, Aberdeen, MD). As they grew, the male rats were moved to progressively larger individual cages in accordance with National Institutes of Health guidelines (National Institutes of Health 1985). All rats were housed initially in cages having a floor area of 271 cm^2, and males were housed after nine months of age in cages having a floor area of 387 cm^2. At the final cage sizes, each chamber housed 58 females and 58 males. Cage maps showing the position of each rat were maintained throughout the study and used as checklists during twice–daily animal observations. The checklists were verified against tattoo identification when rats were moved to clean cages, and the six cage units within each chamber were rotated clockwise weekly. Animal status was also verified at weighing and whenever rats were removed from their chambers.

The exposure chambers were maintained at an airflow of 425 ± 57 L/min, providing 15 air changes per hour, a relative humidity between 40% and 70%, and a temperature of 24°C ± 1°C. Standard fluorescent lighting was on a 12-hour cycle (on 0600 to 1800). The exposure chambers were washed weekly. Bacteriostatic liners in excreta trays below each cage unit were changed twice daily, and trays were washed daily. A pelleted ration (Certified Wayne Lab Blox, Allied Mills, Chicago, IL) and tap water were available at all times.

After 24 months, surviving rats were transferred from the exposure chambers to an animal housing room where they were maintained in plastic cages with hardwood-chip bedding (P.J. Forest Products Corp., Montville, NJ) and filter tops. The rats were provided with pelleted ration and tap water ad libitum, and the cages were cleaned weekly. Relative humidity was 14% to 36%, and room temperature was 23°C ± 2°C. Standard fluorescent lighting was on a 12-hour cycle (on 0600 to 1800).

Exposures and Exposure Materials

The rats were exposed 16 hours per day (4:00 p.m. to 8:00 a.m.), 5 days per week (Monday through Friday) for up to 24 months to diesel exhaust, carbon black, or filtered air. Methods for generating and analyzing exposure atmospheres and conducting exposures are described in greater detail in Appendix A. Exposures were omitted on certain holidays in addition to weekends, but lapses never exceeded three consecutive days. During the 24 months, both blocks were exposed for a total of 508 exposure days averaging 15.85 hours each.

Diesel exhaust exposures were conducted using the facility described previously (Mokler et al. 1984) and used in previous research sponsored by the Health Effects Institute (Mauderly et al. 1987a; Mauderly et al. 1989). Diesel exhaust was generated using two 1988 model LH6 General Motors 6.2-L V-8 engines burning D-2 control fuel meeting EPA certification standards. The engines were fitted with four-speed automatic transmissions, mounted on test stands, and operated continuously by computer on the Federal Test Procedure urban certification cycle during exposures. The system was calibrated to simulate operation of a Chevrolet C1500 pickup having a gross weight of 2,773 kg. Exhaust passed through a standard exhaust system, including muffler, and was then diluted serially with filtered air to the desired soot concentration.

The particle concentration in each diesel exhaust chamber was measured gravimetrically by daily filter samples. The particle size distribution was measured at the beginning, midpoint, and end of exposures using a cascade impactor (Multijet Cascade Impactor, In-Tox Products, Albuquerque, NM) and a parallel-flow diffusion battery, as previously described (Cheng et al. 1984). Previous studies of diesel exhaust soot had shown it to have a bimodal size distribution in exposure chambers when evaluated in this way, rather than by a single instrument (Cheng et al. 1984);

thus, the same approach was applied to compare in detail the size distributions of diesel exhaust soot and carbon black in chambers in the present study. Filter samples of soot were extracted twice using dichloromethane and sonication (Royer et al. 1979) to determine the fraction of particle mass constituted by organic material and to obtain extract for assaying mutagenicity. The bacterial mutagenicity of the solvent extract was measured using a modification of the Ames *Salmonella* assay (Ames et al. 1975) and tester strains TA98 (revealing frameshift mutations) and TA100 (revealing both frameshift and base pair substitution mutations). Bag samples were taken weekly, alternately from one of the two chambers at each exposure level, for analysis of gases and vapors.

The carbon black (Elftex-12, Cabot, Boston, MA) was aerosolized using air jet dust generators (Jet-O-Mizer Model 0101, Fluid Energy, Hatfield, PA) and diluted with filtered air to the desired chamber concentrations. Measurements of particle concentration, gas and vapor concentrations, particle size distribution, mass fraction of solvent-extractable organic material, and mutagenicity of the extract were conducted as described above for diesel exhaust exposures.

The chambers housing sham-exposed controls were ventilated continuously with ambient air passed through high-efficiency particulate air filters. Concentrations of particles generated from the animals and feed and the background concentrations of vapors and gases were measured as described above for diesel exhaust exposures. The same air supply was used to ventilate diesel exhaust and carbon black exposure chambers during nonexposure hours.

The mean concentrations of the exposure atmospheres over the entire exposure period are presented in Table 3. Exposure concentrations were very close to the target values and varied little during the study. Cumulative mean particle concentrations ranged from −2.6% (high diesel exhaust) to +0.8% (high carbon black) of target values. Gas and vapor concentrations measured in the carbon black exposure chambers were similar to those in the sham exposure atmosphere, indicating that little, if any, of the background vapor-phase hydrocarbon fraction adsorbed to the carbon black. The ratios of mean net (with background subtracted) particle concentrations for the high- versus low-exposure levels were 2.63:1 for diesel exhaust and 2.70:1 for carbon black. The mean particle concentration was 3.5% higher for carbon black than for diesel exhaust at the high level, and 0.8% higher for carbon black than for diesel exhaust at the low level.

The particle size distributions of diesel exhaust soot and carbon black are summarized in Table 4. Particle size was similarly bimodal for both diesel exhaust and carbon black, with large-size modes of 2.0 μm mass median aerodynamic diameter (MMAD), and small-size modes of 0.1 μm mass median diffusion diameter (MMDD). The portions of particles in the larger and smaller modes differed between the two exposure materials, however, with approximately 23% in the larger mode for diesel exhaust and 67% in the larger mode for carbon black. Because of this difference in size distribution, we estimated that the pulmonary deposition of carbon black in rat lungs would be approximately 69% of that of diesel exhaust soot, or that the pulmonary deposition of diesel exhaust soot would be approximately 45% greater than that of carbon black.

The mass fraction of particle-associated, solvent-extractable organic matter was determined by ultrasonic extraction with dichloromethane. The weighted mean mass

Table 3. Concentrations[a] of Key Constituents of Exposure Atmospheres Collected at Chamber Midpoint During 24 Months of Exposure

Constituent[b]	Carbon Black		Diesel Exhaust		Control Exposure
	Low	High	Low	High	
Total particles	2.46 ± 0.03	6.55 ± 0.06	2.44 ± 0.02	6.33 ± 0.04	0.05 ± 0.02
Carbon monoxide	0.70 ± 0.06	0.69 ± 0.06	10.30 ± 0.23	26.85 ± 0.52	0.78 ± 0.06
Carbon dioxide	2,010 ± 64	1,820 ± 65	4,470 ± 77	7,390 ± 87	2,210 ± 58
Hydrocarbon vapors	4.37 ± 0.09	4.21 ± 0.08	6.47 ± 0.15	8.13 ± 0.20	4.53 ± 0.08
Total oxides of nitrogen	0.030 ± 0.004	0.033 ± 0.004	8.79 ± 0.32	23.45 ± 0.69	0.033 ± 0.003
Nitrogen dioxide	0.027 ± 0.004	0.029 ± 0.004	0.73 ± 0.05	3.78 ± 0.18	0.023 ± 0.002

[a] Values are expressed as means ± SE of weekly mean values for particles and weekly values for vapors and gases. Values for diesel exhaust and carbon black groups presumably include the background concentrations of particles, gases, and vapors listed for sham exposures.

[b] All values are given in ppm except total particles, which is given in mg/m^3.

fraction of organic material associated with diesel exhaust soot for the two engines during the course of the exposures was 8.22% and varied little during the study. However, the extremely low mass fraction of extractable organic material on carbon black made precise quantification difficult. Values ranging from 0.04% to 0.29% were obtained in preexposure trials and during the course of exposures, resulting in a mean value of 0.12%. The diesel exhaust soot, therefore, contained approximately 68 times more extractable organic matter than carbon black.

The mutagenicity of the dichloromethane extracts of diesel exhaust soot and carbon black was assessed using the Ames *Salmonella typhimurium* assay (Figure 1). As observed previously (Clark and Vigil 1980; Bechtold et al. 1984), the mutagenicity of diesel exhaust soot extract was higher in tester strain TA100 than in strain TA98, and slightly higher without metabolic activation by rat liver S-9 microsomal fraction than with activation. Very little mutagenic activity was associated with the carbon black extracts. The specific mutagenic activities of the extracts in strain TA98, calculated from the mean responses after subtracting background response to the vehicle control, were 0.25 revertants/μg for diesel exhaust and 0.003 for carbon black, an 83-fold difference. The specific activities in strain TA100 were 0.68 revertants/μg for diesel exhaust and 0.017 for carbon black, a 40-fold difference. Combining the differences in extractable fraction and specific mutagenicity yielded a mutagenicity in revertants per unit of particle mass that was approximately 5,700 times higher for diesel exhaust than for carbon black in strain TA98, and 2,700 times higher in TA100.

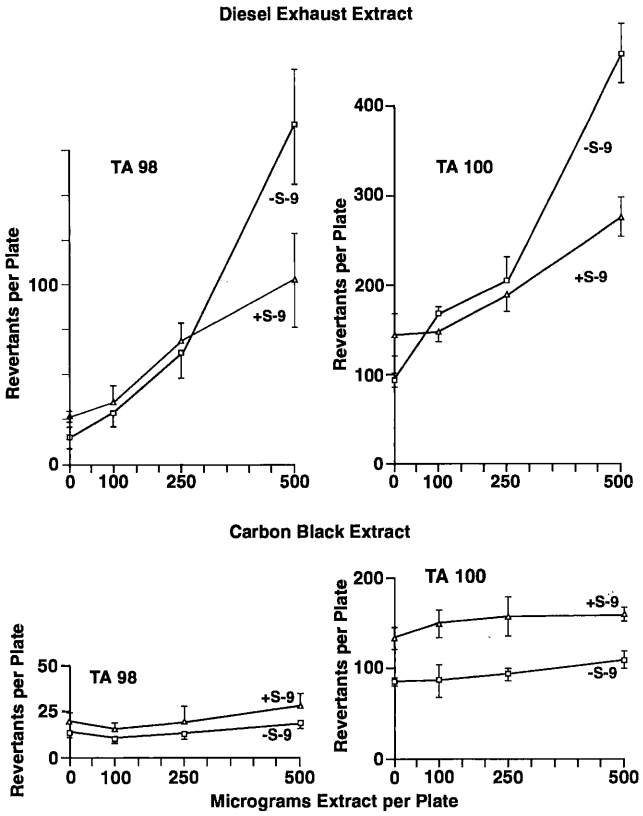

Figure 1. Mutagenicity of dichloromethane extracts of diesel exhaust and carbon black in tester strain TA98 and TA100 *S. typhimurium* in the Ames assay (Ames et al. 1975). Mutagenicity is expressed as the mean ± SD revertants per plate from triplicate plates seeded with 1.9×10^8 organisms each and treated with 0 (vehicle control), 100, 250, or 500 μg of extract with and without metabolic activation by rat liver S-9 microsomal fraction. Additional details are given in Appendix A.

Table 4. Particle Size Distributions of Diesel Exhaust Soot and Carbon Black Measured by the Cascade Impactor and Parallel-Flow Diffusion Battery During the First Quarter, at the Midpoint, and During the Final Quarter of the Exposures

			Large-Fraction, Cascade Impactor			Small-Fraction, Parallel-Flow Diffusion Battery		
Particles	Sample Time	No. of Samples	Percent of Total	Mean MMAD (μm)	GSD	Percent of Total	Mean MMDD (μm)	GSD
Diesel exhaust soot	First quarter	9	18.7	2.24	2.16	81.3	0.11	2.28
	Midpoint	12	26.0	1.84	1.97	74.0	0.09	2.16
	Third quarter	6	25.4	1.92	2.15	74.6	0.09	2.02
	Mean		23.4	2.00	2.09	76.6	0.10	2.15
Carbon black	First quarter	4	58.7	1.93	1.84	41.3	0.10	2.40
	Midpoint	14	64.4	1.87	1.86	35.6	0.09	2.14
	Third quarter	6	78.0	2.04	1.83	22.0	0.10	1.94
	Mean		67.0	1.95	1.84	33.0	0.10	2.16

Measurements of Effects

General Toxicity. All rats were observed twice daily, before and after exposures, for morbidity and mortality. The clinical condition of the rats was assessed more carefully during weekly transfers to clean cages and during weighing, which occurred monthly until 22 months and biweekly thereafter. The rats were weighed using a computer-interactive system for logging data, and the data were analyzed for individual monthly weight gains and weight differences among experimental groups using a toxicology data base system (Pathtox, Xybion Medical Systems, Cedar Knolls, NJ). Animal status logs and survival data were maintained using the same data base.

Necropsy, Histopathology, and Transplantation of Neoplasms. Procedures for histopathological evaluations are described in greater detail in Appendix B. Necropsy personnel were on duty at the time of preexposure and postexposure observations, including weekends; thus, rats found moribund or dead were usually necropsied immediately. Dead rats were held under refrigeration if necropsy was not started immediately. Moribund rats were euthanized by intraperitoneal injection of sodium pentobarbital. Lungs and all major organ systems were observed for gross abnormalities, and approximately 40 tissues from major organs (see Appendix B) were preserved in 10% neutral buffered formalin. The following tissues were routinely processed for histological evaluation by paraffin embedding, sectioning at 5 μm, and staining with hematoxylin and eosin: lung (right apical, right cardiac, right diaphragmatic lobes and left lung, cut sagittally along the axial airway); bronchial lymph node, liver, kidney, and thoracic lesions or masses, if found. Only the lungs and suspected lung neoplasms or tumors were examined microscopically because the focus of the study was on lung pathology; previous studies had revealed little nonpulmonary disease in diesel exhaust–exposed rats, and funding was not sufficient for examining all tissues.

Beginning at approximately 15 months of exposure, necropsy procedures were altered to facilitate special collections of the anticipated lung neoplasms. Lung neoplastic tissue was allocated for three uses in the following order of priority: (1) fixation for microscopic evaluation; (2) transplantation into athymic (nude) mice; and (3) preservation by freezing as an archival sample. Instrument kits and materials for aseptic tissue collection and transplantation were prepared and placed on standby, personnel responsibilities were defined, and notification procedures were activated for the remainder of the study. After euthanasia, the pelt was wiped with 70% alcohol and reflected from the incision, sterile drapes were applied, and a sterile technique was used for dissection until it was determined whether or not a gross lesion suspected of being a lung neoplasm was present.

Lungs containing suspected neoplasms less than 5 mm in diameter were fixed with 4% buffered paraformaldehyde. For larger lesions, a portion approximately 2 mm by 3 mm was dissected using a sterile blade and placed in cold phosphate-buffered saline containing antibiotics for transplantation. A sample approximately 3 to 4 mm thick of the suspected neoplasm and adjacent lung was fixed in paraformaldehyde for histopathology. If a portion of the lesion remained, it was placed in a cryovial, frozen immediately in liquid nitrogen, and stored at –80°C as an archival sample. A portion of apparently nonneoplastic lung tissue from each tumor-bearing rat was similarly frozen. Nonneoplastic lung tissue consisted of the right apical and intermediate lobes if they were considered free of neoplasms, or portions of other lobes as necessary. The remaining lung was fixed with 4% buffered paraformaldehyde. A portion of lung tissue from the next group- and gender-matched rat to die that did not have a suspected lung neoplasm was similarly frozen as an approximately matched specimen for potential analysis of adducts or molecular changes in rats with and without lung neoplasms. Neoplasm tissue for transplantation was placed in sterile Dulbecco's phosphate-buffered saline containing 100 units/mL penicillin and 100 mg/mL streptomycin. The tissue was cut into approximately 2-mm cubes, placed on ice, and transported to the room housing the athymic mice.

Male NCr-nu mice were obtained at 5 to 6 weeks of age from stock originating from the National Cancer Institute (Simonsen Laboratories, Gilroy, CA). The mice were housed in sterile isolator cages (Micro-Isolator, Lab Products, Maywood, NJ) on autoclaved hardwood-chip bedding in a barrier-maintained room using special procedures to reduce potential for infection. The mice were fed a pelleted, autoclaved diet (NIH-31, Autoclavable Rat and Mouse Diet, Zeigler Bros., Gardners, PA) and sterile water ad libitum. All manipulations, including weekly cage changes, were done within a laminar-flow hood.

Six- to twelve-week-old mice were anesthetized lightly with carbon dioxide, and neoplasm tissue was injected subcutaneously at multiple dorsolateral sites using a sterile 11-gauge trocar. In addition to daily health checks, the mice were observed twice weekly for growth of masses at the injection sites. When a mass reached approximately 1 cm in diameter, the mouse was killed using carbon dioxide, the mass was excised using a sterile technique, tissue from the mass was divided, and a portion was reimplanted into another mouse using the procedure described above. The remainder of the mass was frozen rapidly in liquid nitrogen

as an archival sample. After the third such transplantation, a sample was fixed for histological evaluation, and the majority of the mass was cut, using a sterile scalpel, into 2-mm cubes and cryopreserved in 7.5% dimethylsulfoxide in Dulbecco's Modified Eagle's medium.

Procedures for Animals Killed at Scheduled Intervals. Three male and three female rats from each exposure group were selected randomly from those living at the time; they were anesthetized by halothane, weighed, and killed by exsanguination. Blood was collected in evacuated tubes containing anticoagulant. Erythrocytes for analysis of hemoglobin adducts were separated, washed in phosphate-buffered saline, coded, and shipped frozen to Dr. Jeffrey. Lungs were removed, trimmed, and weighed in aggregate and as individual lobes. The right diaphragmatic lobe was cannulated and fixed for histopathology with 10% neutral buffered formalin at a constant airway pressure of 25 cm of fixative for 4 to 6 hours and submerged in the same fixative for 24 hours. The liquid displacement (external) volume of the lobe was then measured. When rats were killed at 23 months, the right diaphragmatic lobe was fixed with 4% buffered paraformaldehyde, instead of formalin, to facilitate molecular analyses. The right apical, cardiac, and intermediate lobes were frozen in liquid nitrogen and stored at –80°C as archival samples. Tissues from several major organs were fixed in 10% neutral buffered formalin for histopathology (see Appendix B), and selected tissues were processed for evaluation. Only lungs and suspected lung tumors were examined microscopically. Morphometric analysis of parenchymal versus nonparenchymal components and of selected parenchymal subcomponents was performed using sections collected at 3, 6, and 12 months (Appendix B).

The lung-associated (posterior parathymic and paratracheal) lymph nodes were removed, weighed, frozen, and retained for measurement of particle burdens.

Serum from one female and one male rat from each experimental group was collected at each time point when a group of rats were killed and was submitted for independent serological surveillance for pathogens (Standard Level II Antibody Profile, Microbiological Associates, Rockville, MD). The surveillance included pneumonia virus of mice, reovirus Type 3, Sendai, lymphocytic choriomeningitis virus, Kilham rat virus, Toolan H-1, rat coronavirus sialodocryoadenitis, *Mycoplasma pulmonis*, and cilia-associated respiratory bacillus.

Bronchoalveolar lavage of the left lung was performed, and the lavagate was analyzed as described previously (Henderson et al. 1988). The bronchus of the left lung was cannulated, and the lung was lavaged using two washes of 2.0 mL each for females and 2.5 mL each for males. The recovered fluid was pooled, cells were removed by centrifugation, and both total and differential cell counts were performed. The supernatant was analyzed for cytoplasmic and lysosomal enzymes and for total protein.

The amount of diesel exhaust soot or carbon black in the left lung was estimated using a light absorption method as described previously (Henderson et al. 1987). The tissues were homogenized in saline, and the extinction of light at 620-nm wavelength by homogenates was compared with standard curves constructed from tissue samples spiked with particles. Total lung particle burdens were estimated using weights of the whole organs and of the portions analyzed.

The analyses of particle lung burdens provided estimates based on light absorption of homogenates, rather than absolute values based on chemical analysis. The results are known to be influenced by the degree of dispersion of the particles, as well as their concentration, and may also be influenced by the absorbance characteristics of diseased lung tissue (Henderson et al. 1987). Although the results are approximations of the lung burdens, the method had provided values for rats chronically exposed to diesel exhaust that were consistent with predicted accumulations, and had coefficients of variation (standard deviations divided by means) of 13% to 17% for lung burdens over a 30-fold range (Wolff et al. 1987). This degree of intersubject variability is similar to those of other measures of particle lung burdens. Analysis of selected samples demonstrated that lavage before lung burden analysis removed less than 5% of diesel exhaust soot or carbon black from lungs of chronically exposed rats.

The amounts of diesel exhaust soot or carbon black in the lung-associated lymph nodes were measured by analysis of inorganic carbon. The nodes were digested with acid, the washed residue of inorganic carbon was converted to carbon dioxide, and the carbon dioxide was quantified using an infrared spectrometer. The spectrometer was calibrated by constructing standard curves using lymph node tissue spiked with known amounts of diesel exhaust soot or carbon black.

The left lung homogenates were also used for DNA extraction for adduct analysis. The DNA was extracted using the Gupta modification (Gupta 1984) of the Marmur procedure (Marmur 1961). The DNA was coded and shipped frozen to collaborators. Collaborators were given the codes indicating the animal, gender, and experimental group after their initial results for the coded samples were received by the Principal Investigator.

Chromosome Damage in Circulating Lymphocytes. The preliminary evaluation of chromosome alterations in circulating lymphocytes of exposed rats was conducted using

blood from rats killed after three months of exposure. The procedure was exploratory, and was to have been repeated at later times if results suggestive of a significant treatment effect were observed at three months. Because results at three months did not suggest that the approach was useful, the assay was not repeated.

Blood was obtained from rats from the high diesel exhaust, high carbon black, and control groups by cardiac puncture after they had been killed. White blood cells (primarily lymphocytes) were isolated by centrifugation and cultured as described by Klingerman and associates (1981). Three cultures were used to measure the frequency of sister chromatid exchanges. The blood was placed in RPMI-1640 medium (Gibco, Gaithersburg, MD) containing 25 nM *N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid (HEPES) buffer, L-glutamine, and 10% heat-inactivated fetal bovine serum, and incubated at 37°C in 5% carbon dioxide. Phytohemagglutinin was added to stimulate cell division. After 24 hours, 10 μM bromodeoxyuridine was added to label the dividing cells. Colchicine was added three hours before harvest to arrest the cells in metaphase. Cells were harvested at three six-hour intervals, beginning at 48 hours after the addition of phytohemagglutinin, and examined by microscope to determine when they had undergone the second mitotic division. The methods for harvest and preparation of cells have been reported elsewhere (Wolff and Perry 1974). The frequency of sister chromatid exchanges was determined microscopically.

The frequency of micronuclei was also measured to determine if the exposures had clastogenic effects. Blood was placed in two cultures and incubated as described above, but no bromodeoxyuridine or colchicine was added. Cytochalasin-B was added at 3 μg/mL to block cytokinesis and enable scoring of micronuclei. Cells were harvested after 72 hours and 94 hours of incubation, and the frequency of micronuclei in binucleated cells was determined microscopically.

DNA Adducts in Alveolar Type II Cells. The methods and results for this portion of the study were published in detail (Bond et al. 1990). Briefly, separate groups of five female and five male rats from each of the high diesel exhaust, high carbon black, and control groups of block A were killed after three months of exposure by intraperitoneal injection of sodium pentobarbital, the thorax was opened, and the lung vasculature was perfused with saline until white. The lung was removed, lavaged with saline, and incubated with protease. Lungs from one female and one male were pooled, resulting in five samples per group, and further incubated with enzymes in a shaking bath.

Tissue and media were filtered through a 70-μm nylon mesh, the filtrate was centrifuged, and the resulting cell pellet was suspended in medium and layered onto a discontinuous Percoll gradient (Sigma, St. Louis, MO) for further centrifugation. The cell fraction at the interface between the 35% and 42.5% Percoll layers was used for flow cytometric isolation of type II cells (FACSTAR Plus, Becton-Dickinson, Mountain View, CA). Type II cells were separated at a purity greater than 80% by autofluorescence characteristics, as confirmed by microscopic evaluation. The type II cell population isolated from rats in this manner has been shown to include hyperplastic type II cells, but it is possible that a portion of the size distribution including extremely large type II cells might have been excluded. Type II cell DNA was isolated by the Gupta modification (Gupta 1984) of the Marmur procedure (Marmur 1961), and adduct levels were measured by ^{32}P-postlabeling (Bond et al. 1988) using the nuclease P_1 procedure (Reddy and Randerath 1986).

Clearance of Radiolabeled Carbon Black. A group of eight female and eight male rats from block B of each experimental group was exposed once by inhalation to carbon black radiolabeled with the gamma emitter ^{7}Be after 3 months and again after 18 months of chronic exposure to evaluate particle clearance. The [^{7}Be]CB was prepared by Dr. Leonard Mausner, Brookhaven National Laboratory, using an accelerator-induced, proton spallation reaction (Mausner et al. 1984) to induce a tracer level of ^{7}Be in carbon black; the carbon black used for this procedure came from the same stock used for chronic exposures. Different batches of [^{7}Be]CB were prepared for the two exposures because ^{7}Be has a physical half-life of 53 days. The [^{7}Be]CB was aerosolized as an aqueous suspension using a Lovelace nebulizer, and the rats were exposed through the nose only (Raabe et al. 1973) for four hours to achieve approximately 8 nCi of initial lung activity (30 μg of mass). The rats were held in plastic cages with hardwood-chip bedding and feed and water provided ad libitum for three days after [^{7}Be]CB exposure to allow for early excretion of radioactivity, and were then returned to their chronic exposure chambers.

The rats underwent whole-body counts of radioactivity to determine the amounts of ^{7}Be present at 0, 4, 7, 13, 28, 35, 42, 56, 73, 84, 98, 112, and 126 days after exposure to [^{7}Be]CB, and the counting data were used to evaluate particle clearance from the lungs. The counts on day 7 were used to represent the "initial lung burden" of [^{7}Be]CB. By day 7, radioactivity in the head, large airways, and gastrointestinal tract was negligible due to early mucociliary clearance from the lung. The counting data were corrected for physical decay of radioactivity, and curves for each group were plotted as percentages of the day-7 counts. A

nonlinear regression analysis was used to fit two-component negative exponential functions to the clearance of radioactivity.

Particle Sequestration. The translocation and sequestration of tracer particles were evaluated by histopathology and morphometry after 3 and 18 months of chronic exposure. At these two times, eight female and eight male rats selected randomly from block B of each of the five experimental groups were exposed once for four hours by nose-only inhalation to an aerosol containing a mixture of 1-μm-diameter fluorescent latex microspheres (Polysciences, Warrington, PA) and [^{7}Be]CB. The aerosol or latex particles were generated by nebulizing an aqueous suspension of 1.7×10^{9} particles/mL, which was found in preliminary trials to produce an aerosol containing over 95% single particles. Exposure conditions were adjusted to deposit a predicted 10^{8} latex particles in the lungs of each rat. The rats were simultaneously exposed to [^{7}Be]CB generated as described above. The ratio of latex particles to [^{7}Be]CB was used to estimate the initial lung burden of latex particles by counting the radioactivity. The rats were held for three days after exposure in cages with plastic bedding and feed and water provided ad libitum to allow for early excretion of radioactivity, and then were returned to their chronic exposure chambers.

Two females and two males from each group of 8 described above were anesthetized with sodium pentobarbital and killed by exsanguination at 1 (24 hours), 4, 28, and 90 (87 to 91) days after inhalation of the tracer particles. Rats killed at 28 and 90 days were withheld from chronic exposure during the night before they were killed to preclude the presence of recently deposited particles in the lung. These rats were injected with heparin, anesthetized with sodium pentobarbital, and killed by exsanguination. Their lungs were fixed in situ using an intravascular perfusion procedure adapted from Brain and coworkers (1984). Lungs and lung-associated lymph nodes were collected, the liquid displacement volume of the lung was measured after an additional 24 hours of fixation, and the tissues were stored in cold 10% neutral buffered formalin until further processing.

The right diaphragmatic and left lung lobes were divided sagittally along the main axial airway, and the distal portion of the lobe was cut from each face of the divided lobe, resulting in two tissue blocks from each of two lobes per rat. These blocks were embedded in glycol methacrylate, and the temperature was kept at –10°C throughout processing to maintain polymerization and microsphere fluorescence. Sections 2 μm thick were cut from the blocks, stained with toluidine blue, coded, and randomized for evaluation. Two sections per rat, one from each lobe, were examined using a 10× eyepiece and 40× objective on an epi-fluorescence microscope (Carl Zeiss, Oberkochen, Germany) with a blue excitation filter.

Each of the two sections per rat were scanned, each fluorescent microsphere was counted, and its location was scored using the anatomical identifiers listed in detail in Appendix G. The groups were compared using data summarized into percentages of microspheres in three locations: single alveolar macrophages, aggregated macrophages, and other locations, because the primary interest was in examining movement of particles into macrophage aggregates. All macrophage aggregates in the alveolar region were considered a single category, whether lumenal or interstitial in location.

Statistical Evaluations

The criterion for statistical significance was set at $p < 0.05$ for all analyses.

Survival data were analyzed using the life table method, and the differences in survival patterns were tested using log rank tests (Kalbfleisch and Prentice 1980).

The significances of group differences in body weight were analyzed using procedures standard in the toxicology data base software (Path-Tox, Xybion Medical Systems, Cedar Knolls, NJ), which included one-way analysis of variance and Dunnett's test of significance by group and gender at each measurement time.

The significances of differences in lung weight and bronchoalveolar lavage parameters were analyzed by multiple pairwise comparisons done using BMDP software (BMDP7D; BMDP Statistical Software 1993). Because the Levene test for equality of variances (Levene 1960) demonstrated that group variances were often unequal, t values used to estimate significances of all group differences were derived using separate (rather than pooled) variances. The Bonferroni method was used to adjust t values for multiple contrasts.

The influences of exposure material, exposure level, and gender on the development of nonneoplastic lung lesions were examined using a prevalence model. The severity score for each lesion except squamous cysts was converted to a binary variable indicating whether or not a selected score was achieved. The observation of a squamous cyst was already a binary variable. The binary variable was taken to represent the prevalence of the lesion at death. Because most of the lesions were not observed in control rats and the prevalences at the low exposure levels were always much higher than in control rats, differences from control prevalences were not tested. Rather, comparisons focused on differences due to exposure level, type of exposure, and gender. The model of prevalence was a logistic

regression model with the logit of the lesion prevalence being a linear function of time. The significances of the factors tested were judged by the reduction of the deviance caused by adding the factor as a function of time to the model. The p value was calculated for each factor based on the likelihood ratio test, which was the reduction of model deviance and the reduction of degrees of freedom compared with a chi-squared distribution (McCullagh and Nelder 1983).

The significances of group differences in the lung neoplastic response to exposure were also examined using a logistic regression model of neoplastic prevalence at the times of death of the rats (McKnight and Crowley 1984). This model, which had been used previously at ITRI for evaluating carcinogenic responses to diesel exhaust (Mauderly et al. 1987b), was very simple, having a common intercept term for neoplastic prevalence at the start of exposure and terms for the increasing neoplastic prevalences with time for each exposure group. Prevalence was the probability that a rat living at a given time had a lung neoplasm. Because the neoplasms did not appear to alter the risk of death, the times when the rats died or were killed or euthanized were regarded as providing random samples of times at which rats were examined for neoplasms (Dinse and Lagakos 1983). The significances of terms in the model were tested using likelihood ratio tests. These tests were similar to Hoel-Walburg tests (Hoel and Walburg 1972), except that prevalence was modeled as a continuous, rather than a discrete, function of time (Dinse and Lagakos 1983; Dinse 1985).

The data for clearance of [^{7}Be]CB were plotted as percentages of the radioactivity measured at day 7 after inhalation. The significances of differences between curves for exposed and control groups were determined using repeated measures analysis of variance (Jennrich and Schluchter 1986) and pairwise comparisons using the Bonferroni adjustment for multiple contrasts. Two-component negative exponential functions were fit to counting data for each group using SAS software (SAS Institute, Cary, NC). The two components were forced to sum to 100% of the day-7 lung burdens, and slopes were constrained to being zero or negative. Values for the slope (half-time of clearance) and portion of the total curve represented by each of the two components resulted from the curve fits.

The data for the anatomic locations of fluorescent microspheres were expressed as percentages of the total microspheres counted for each rat. Because of variability in the numbers of microspheres observed, no attempt was made to quantify the absolute numbers of microspheres by time and location. A few rats with less than five microspheres were omitted from the analyses. Student t tests were used to compare percentages among groups. A method for adjusting for multiple contrasts by Bootstrap resampling (Westfall and Young 1989) and SAS PROC MULTITEST (SAS 1992) was used rather than the Bonferroni adjustment, because of the large number of contrasts among highly correlated data.

Quality Assurance

This study was conducted under the spirit of Good Laboratory Practices, a designation indicating that quality assurance procedures designed to meet EPA and Federal Drug Administration Good Laboratory Practices standards were employed, but certain record-keeping procedures deviated slightly from strict compliance with the Good Laboratory Practices. This study was conducted under an approved protocol referencing approved standard operating procedures for the repetitive laboratory procedures. Changes in the protocol were allowed only by means of approved protocol amendments. A formal Quality Assurance Project Plan (Appendix C) was developed and approved before the study began, both by the ITRI approval process and by Health Effects Institute. The plan specified quality control checks of all data at generation, study inspections at approximately three-month intervals by ITRI's Quality Assurance Unit for conformance to protocol and standard operating procedures, a 10% audit of all types of data at the end of the study, and an audit of the final report for conformance to the protocol.

The data audits performed in preparation for the final report exceeded those prescribed in the Quality Assurance Project Plan. One hundred percent quality control checks were performed for lung and lymph node burdens of particles, lung weights, survival, histopathology (including lung neoplasms), lung morphometry, and tumor transplant data. These audits consisted of an independent verification that all data contained in files used for summary computations and analyses were consistent with original entries, that the neoplasms and other lesions ascribed to exposure groups could be tracked to the correct individual rat, and that the sources of transplanted neoplasms were properly ascribed to individual rats and individual lesions within rats.

The protocol was formally reviewed and approved by ITRI's Animal Research Committee, which included a nonemployee community member. Animals were maintained and used according to the recommendations in the NIH publication, "Guide for the Care and Use of Laboratory Animals" (1985). Furthermore, ITRI has an approved Public Health Service Animal Welfare Assurance (NIH Assurance No. 3083-01). The study was conducted in facilities fully accredited by the American Association for the Accredita-

tion of Laboratory Animal Care, registered under the Animal Welfare Act (Reg. No. 85-R003), and in full compliance with the Act's provisions.

RESULTS

GENERAL HEALTH OBSERVATIONS

Survival

The survival of the rats differed slightly, but significantly, among the exposure groups. Estimates of the survival curves of each gender and exposure group after 400 days of exposure are shown in Figure 2. The curves were generated using the Kaplan-Meier method (Kalbfleisch and Prentice 1980). The significances of differences in survival patterns were tested using the log-rank test (Harrington and Flemming 1982) and were found to be $p < 0.026$ for females and $p < 0.001$ for males. There was a consistent pattern of shorter survival time among males than among females, but the relationships between survival and exposure were not consistent between females and males. The fractions of surviving female rats exposed to low carbon black, high carbon black, and high diesel exhaust were lower than those of the females in the control group after approximately 600 days of exposure, but the difference was small. The fractions of surviving male rats exposed to low carbon black and high carbon black were consistently lower than those of the males in the control group after approximately 500 days, and the survival of the male rats exposed to high diesel exhaust was reduced after approximately 600 days.

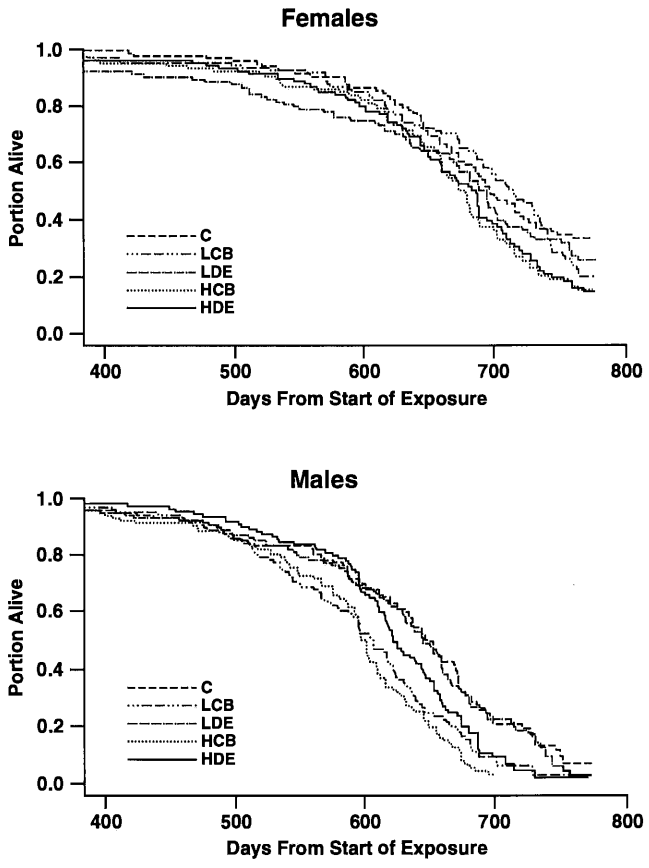

Figure 2. Survival of female and male rats after 400 days of exposure. Curves were generated using the Kaplan-Meier method (Kalbfleisch and Prentice 1980).

Survival of the rats in the five exposure groups is expressed quantitatively in Table 5 as the median survival time in days after initiation of exposure, with the first day of exposure counted as day 1. These median survival data can be transformed to days of age by adding 57 days, the mean age of the rats when exposures began. The median life span of the sham-exposed rats was 753 days of age (24.8 months) for females and 696 days of age (22.9 months) for males. The percentages of rats alive at 23 months of exposure are also listed in Table 5. These values further illustrate differences in survival late in the exposure period, and the reason we conducted the final interim killing at 23 instead of 24 months.

The high carbon black exposure significantly reduced the median life span of both female and male rats, although only by 3.0% and 6.3%, respectively. The survival of males was also significantly shortened (by 5.3%) by the low carbon black exposure. The high diesel exhaust exposure caused a small (2.6%), but significant, shortening of the median survival of females, and a slightly less (2.4%), and insignificant, shortening in males. At the median survival of 23 months, only 6% and 2% of the male rats exposed to low carbon black and high carbon black, respectively, were alive.

Body Weight

The body weights of both females and males were significantly affected by all treatments except the low diesel exhaust exposures. The body weights of females and males in all exposure groups are presented in Figure 3 as the mean weights of all living rats at each weighing time. The mean weights of both female and male rats exposed to high diesel exhaust were significantly lower than the respective mean values for control rats from exposure day 29 onward. Significant reductions in mean weights of the female and male rats exposed to high carbon black first occurred on days 309 and 449, respectively. Significant reductions in mean weights of both the female and male rats exposed to low carbon black occurred on day 509. Although mean weights

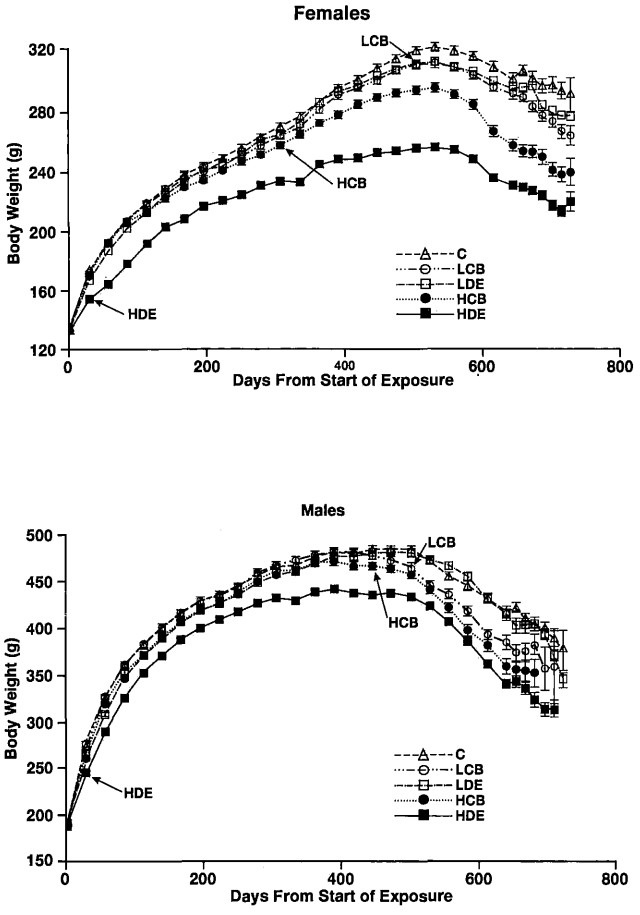

Figure 3. Body weights of female and male rats in the five exposure groups. Values represent means ± SE for gender-specific groups that varied from 139 rats at the first weighing to 2 rats at the last. Arrows indicate the time at which the respective group mean value became significantly different from the control mean at $p < 0.05$.

of female and male rats exposed to low diesel exhaust were significantly lower than those of the control group at one or more weighing times during the study, these differences were not persistent. Additional data on body weight are contained in Appendix D, which lists the mean weights of rats killed at scheduled intervals.

The influence of exposure on body weight late in the study is more easily seen in Figure 4, in which the mean weights of the exposed groups at 673 days (approximately 22 months) are expressed as percentages of the gender-specific control mean values. This time was selected because of the diminished group sizes at later times (for example, fewer than 10 males were alive in the group exposed to high carbon black at later weighing times). Mean weight reductions of 25% in females and 18% in males exposed to high diesel exhaust, occurred at that time, and reductions of 16% in females and 14% in males exposed to high carbon black. Mean reductions of 6% in females and 9% in males exposed to low carbon black, occurred, but the weights of both female and male rats exposed to low diesel exhaust were only 2% lower than those of the control group.

ACCUMULATION OF PARTICLES IN LUNGS AND LYMPH NODES

Lung Burdens of Particles

The exposures caused progressive, dose-related accumulations of diesel exhaust soot and carbon black particles in the lungs, although the two materials accumulated at different rates. The accumulation of particle lung burdens is illustrated in Figure 5, which shows the mean lung burdens of rats killed at intervals from 3 to 23 months. The figure shows that, as observed in a previous study (Mauderly et al. 1987b), the accumulation of particles tended to accelerate

Table 5. Effects of Exposure on Survival

	Females			Males		
Exposure Group	Median Survival in Days[a]	Percent Difference from Control Group	Number Alive at 23 Months	Median Survival in Days[a]	Percent Difference from Control Group	Number Alive at 23 Months
Control group	696	–	48	639	–	19
Low carbon black	707	+1.6	55	605[b]	–5.3	6
High carbon black	675[b]	–3.0	35	599[b]	– 6.3	1
Low diesel exhaust	687	–1.3	42	649	+1.6	20
High diesel exhaust	678[b]	–2.6	36	624	–2.4	8

[a] Exposures began at a mean of 57 (46 to 66) days of age; therefore, 57 days should be added to the values in these columns to obtain median life span values for the groups.

[b] The survival curve is significantly ($p < 0.05$) different from the curve for rats in the control group.

in all groups after 12 months of exposure. After 23 months, mean lung burdens of the groups exposed to carbon black had reached 21.0 mg (low exposure) and 38.5 mg (high exposure), and the mean lung burdens of the groups exposed to diesel exhaust had reached 40.7 mg (low) and 85.4 mg (high). Diesel exhaust soot accumulated more rapidly than carbon black, to a degree exceeding that predicted from differences in the size distributions of the two particles. The lung burdens of the groups exposed to low diesel exhaust and high carbon black were similar from 12 to 23 months, although there was a 2.7-fold difference between the mean particle exposure concentrations (see Table 3). The lung burden data are presented in greater detail in Appendix D.

The accumulation of lung burdens of particles per unit of lung weight is presented in Figure 6, in which the time course of particle accumulation is plotted per unit of lung weight. These data are presented in greater detail in Appendix D. Figure 6 illustrates that the relationship between increasing lung burden and increasing lung weight was similar after 12 months for the two groups exposed to diesel exhaust and for the two groups exposed to carbon black, but the relationship differed between diesel exhaust and carbon black. After 23 months of exposure, the combined mean weight-normalized lung burden was 13.8 mg/g lung for the groups exposed to diesel exhaust, and 8.1 mg/g lung for the groups exposed to carbon black, a 1.7-fold difference. This finding reflected the greater increase in lung weight per unit of particle dose, expressed as lung burden, for rats exposed to carbon black than for rats exposed to diesel exhaust (see section on lung weight below). The lung burdens are also presented in milligrams per gram of mean lung weight of the control group in Appendix D. When plotted against exposure time (not shown), the curves for these data appeared identical to those of the lung burdens in Figure 5. After 23 months of exposure, lung burdens normalized in this manner ranged from 13.2 mg/g of control lung weight for the group exposed to low carbon black, to 55.1 mg/g of control lung weight for the group exposed to high diesel exhaust.

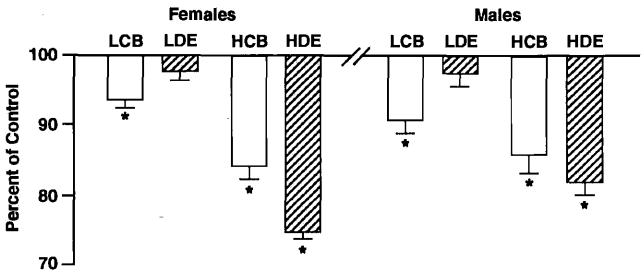

Figure 4. Impact of treatment on body weight late in the exposure period. The mean ± SE weights of the exposed rats are shown as percentages of the control mean values at 673 days after the first exposure. Asterisks (*) indicate significant ($p < 0.05$) differences from the control means.

Lung-Associated Lymph Node Burdens of Particles

The accumulation of particles in the lung-associated lymph nodes with time is illustrated in Figure 7, which shows the mean lymph node particle burdens when the rats were killed. More detailed data are contained in Appendix D. Particles accumulated progressively in the lymph nodes during exposure; however, the rate of accumulation was slowed after 18 months at the higher exposure levels of both diesel exhaust and carbon black. Unlike the lung burdens, the lymph node burdens of diesel exhaust soot and carbon black were similar at both exposure levels throughout the exposure. The combined (diesel exhaust and carbon black)

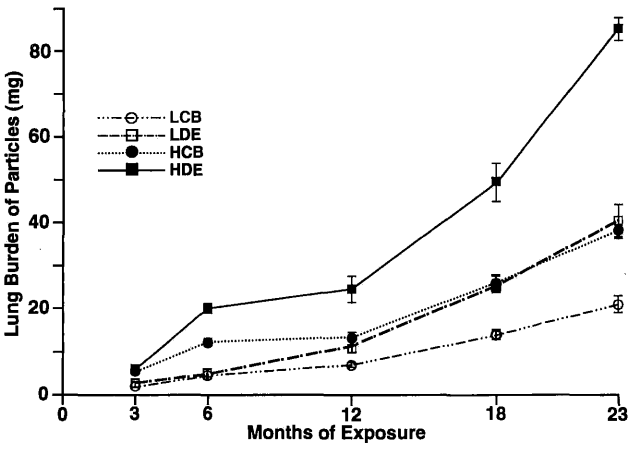

Figure 5. The accumulation of lung burdens of particles is shown as the mean ± SE of the combined lung burdens of females and males killed after 3, 6, 12, 18, or 23 months of exposure.

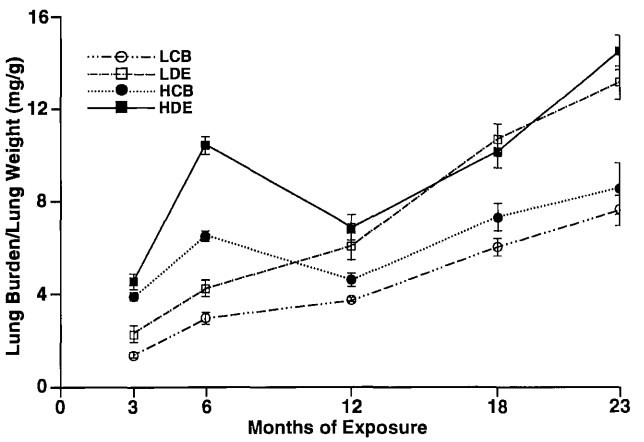

Figure 6. The relationships between lung burdens of particles and lung weights at each time when rats were killed are shown as the combined means ± SE for females and males.

mean lymph node burdens at 23 months were 2.4 mg for the low-level exposures, and 3.2 mg for the high-level exposures, a 1.3-fold difference, compared with a 2.6-fold difference in particle exposure concentrations. The accumulation of lung-associated lymph node burdens, therefore, was not closely proportional to the exposure concentrations.

LUNG WEIGHT

The lung weights of rats killed during the exposure are illustrated in Figure 8 as the mean weights of females and males combined. Lung-weight data are presented in greater detail in Appendix D. The lung weights of all groups increased in a nearly linear manner throughout the exposure, and lung weights of all exposed groups were significantly increased. Significant increases in lung weight above the control group mean value occurred at 6 months for both high exposure groups, at 18 months for the group exposed to low diesel exhaust, and at 23 months for the group exposed to low carbon black (only the variability prevented the mean for the group exposed to low carbon black from reaching significance at 18 months).

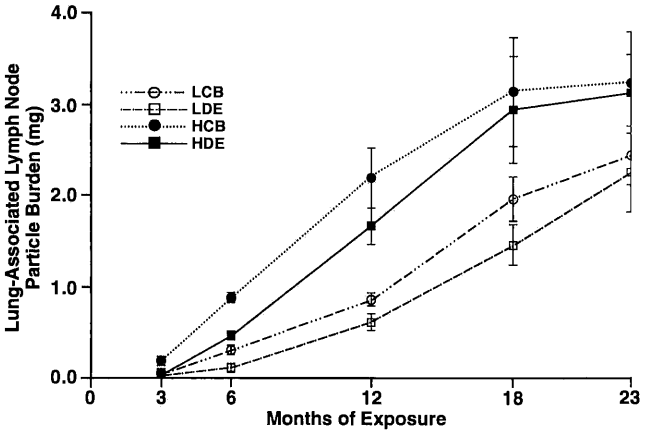

Figure 7. The combined mean ± SE particle burdens in lung-associated lymph nodes of females and males at each time when rats were killed.

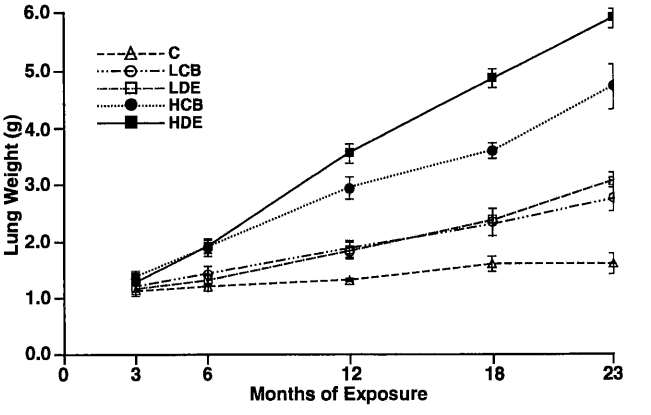

Figure 8. The combined mean ± SE lung weights for females and males at each time rats were killed.

The increase in lung weight above the growth-related increase of the control group was presumed to reflect the inflammatory, proliferative, and fibrotic lesions resulting from the exposures (described below) and the accumulation of particles in the lung. The accumulated mass of particles contributed a negligible fraction of the increase in weight; for example, the 85-mg lung burden of the group exposed to high diesel exhaust at 23 months was only 2% of the 4.3-g increase in lung weight above that of the control group.

INFLAMMATORY RESPONSE

Bronchoalveolar lavage was not planned in the original study design, but was added later. The inflammatory and cytotoxic responses in bronchoalveolar lavage fluid were first evaluated in rats killed after 12 months of exposure, and were also evaluated at 18 and 23 months. Data for the concentrations of white blood cells, selected cytoplasmic and lysosomal enzymes, and total protein in recovered lavage fluid at each measurement time are presented in detail in Appendix E.

Inflammation and cytotoxicity were clearly evident at 12 months (Figure 9). The concentration of white blood cells was elevated approximately three- to four-fold above the control value at 12 months in all exposed groups, but the increases were not closely correlated with either the exposure concentrations or lung burdens. Indeed, the per-unit value of either particle exposure concentration or lung burden was greater in both low-exposure groups than in the high-exposure groups (data not shown). Increases in both macrophages and neutrophils contributed to the increase in white cells. Although the higher exposure levels were associated with slightly higher increases in neutrophils in both diesel exhaust and carbon black groups, values for the groups exposed to high carbon black and low diesel exhaust were similar; this finding is parallel to the lung burdens at 12 months (see Figure 5).

The increases in the concentrations of the cytoplasmic enzyme lactate dehydrogenase, the lysosomal enzyme beta glucuronidase, and total protein were all roughly proportional to exposure concentration, and did not differ greatly between diesel exhaust and carbon black exposures. The interpretations of these changes in rats after chronic exposure to diesel exhaust have been discussed elsewhere (Henderson et al. 1985, 1988). Release of lactate dehydrogenase is associated with cytotoxicity, but the source cell type (or types) is not distinguished by this assay. Release of beta glucuronidase is associated with increased macrophage

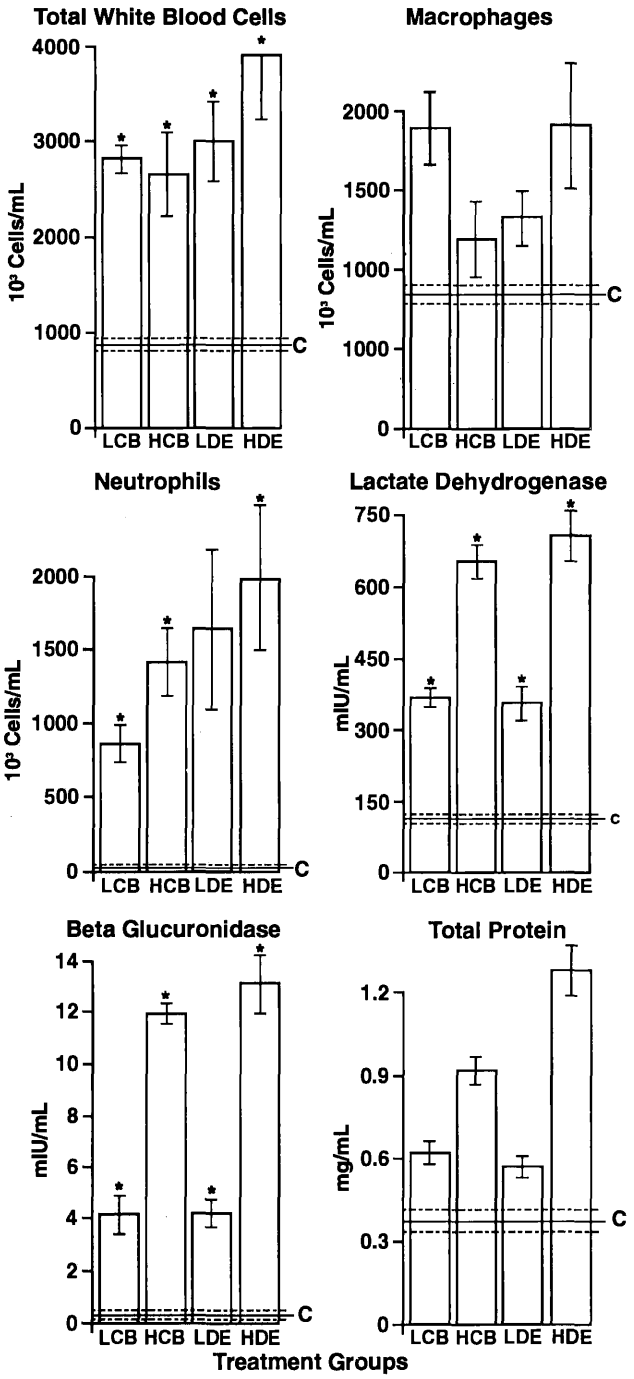

Figure 9. The inflammatory response is illustrated by expressing the combined female and male means ± SE of concentrations of several constituents in fluid recovered by bronchoalveolar lavage of lungs removed after twelve months of exposure. Concentrations are expressed per milliliter of recovered fluid, and values for controls are shown as horizontal lines crossing the bars. The variances of the control group for neutrophils, lactate dehydrogenase, and beta glucuronidase are shown as standard deviations, because the standard error lines would be indistinguishable from the mean lines; other variances for controls are standard errors. Asterisks indicate statistically significant differences from the control values.

activity, and perhaps macrophage death. Increases in total protein are commonly associated with inflammation and are indicative of increased transudation of serum proteins from capillaries to alveolar spaces.

MORPHOLOGICAL RESPONSES

The nonneoplastic and neoplastic morphological responses to exposure are described and summarized in this section and in Appendix F. A complete listing of all rats examined for histopathology and the abnormalities observed for each rat is presented in Appendix H, and is available upon request from the Health Effects Institute.

Nonneoplastic Lung Lesions

The nonneoplastic lung lesions are described below first for each group of rats killed at scheduled intervals, and then for rats that died spontaneously or were euthanized in moribund condition. The incidence and severity of nine classifications of nonneoplastic lesions were scored using a semiquantitative scale, and the lungs of rats killed at 3, 6, and 12 months were also evaluated by morphometry, as described in Appendix B. The data resulting from these evaluations are presented in detail in Appendix F.

Rats Killed at Three Months. Lesions related to exposures to carbon black or diesel exhaust consisted of alveolar macrophage hyperplasia, alveolar epithelial hyperplasia, and chronic-active inflammation (Appendix Table F.1). These lesions were absent in the control group. Alveolar macrophage hyperplasia was present in the lungs of all exposed rats. Increased numbers of enlarged macrophages containing particles were found scattered throughout the lungs. In the groups exposed to low carbon black (Figure 10A) and low diesel exhaust (Figure 10B), there was a minimal tendency toward localization of the macrophages in the centriacinar region. The centriacinus is located at the junction of the last conducting airway (the terminal bronchiole) and the alveolar region, where gas exchange occurs. This localization was more pronounced in both high exposure groups (Figures 10C and 10D), and particularly in the group exposed to high carbon black. The macrophages were predominantly located within the lumens of alveolar ducts and alveoli. A few particle-containing macrophages were located in the interstitium of the centriacinus, particularly in the group exposed to high carbon black. The severity of the macrophage hyperplasia increased with exposure concentration. The severity was similar for each exposure concentration of carbon black and diesel exhaust.

Alveolar epithelial hyperplasia consisted of an increased number of hypertrophic alveolar epithelial cells lining al-

veolar septa. This lesion was most often located with the alveolar macrophage hyperplasia. There was a slight increase in the incidence and severity of this lesion with exposure concentration, and the response to carbon black was slightly greater than that to diesel exhaust.

Chronic-active inflammation was seen in one rat exposed to high diesel exhaust. This was characterized by the presence of focal aggregates of lumenal or interstitial neutrophils in addition to alveolar macrophage hyperplasia, and by evidence of cell injury in the form of degenerate inflammatory cells or cell debris. A mononuclear or mixed inflammatory cell infiltrate sometimes thickened the interstitium in foci of chronic-active inflammation.

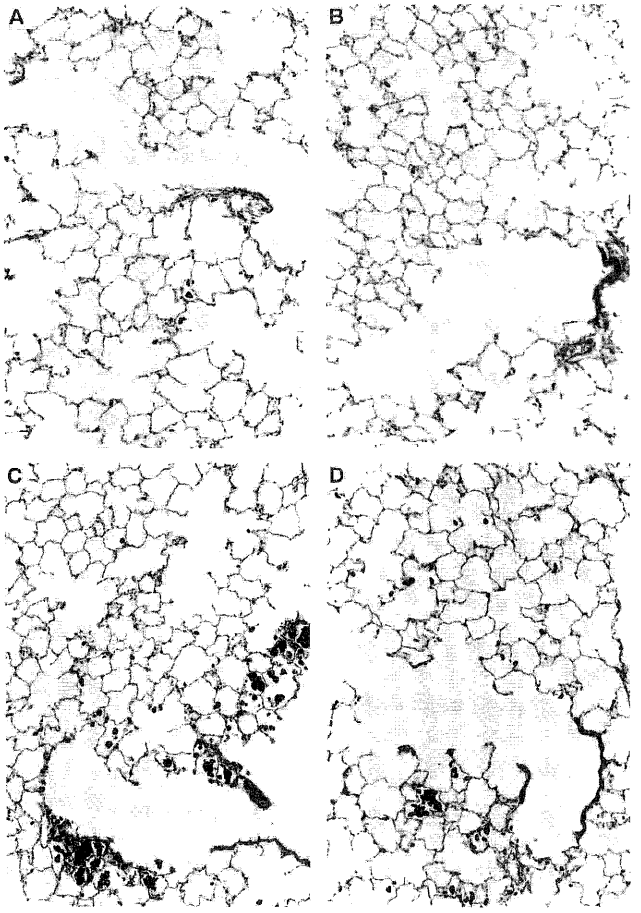

Figure 10. Photomicrographs illustrating the distribution of macrophage hyperplasia in the lungs of rats exposed to carbon black or diesel exhaust for three months. Each panel shows the distal portion of a terminal bronchiole, the first alveolar duct, and peripheral alveoli. In rats exposed (A) to low carbon black and (B) to low diesel exhaust, macrophages were scattered and were minimally localized in the alveoli that are immediately adjacent to alveolar ducts. In rats exposed to (C) high carbon black and (D) to high diesel exhaust, the macrophages were predominately localized in the alveolar out-pocketings of alveolar ducts and in the immediately adjacent alveolar ducts. Magnification = × 121.

Morphometry at three months demonstrated that the portion of the lung volume occupied by single macrophages in the alveolar lumen was increased in a dose-related manner in all exposed groups (Appendix Table F.2). These increases paralleled the histopathologic observation of alveolar macrophage hyperplasia. However, the histopathologic diagnosis of hyperplasia referred to an increased number of single or aggregated macrophages, while the increased volume fraction could have been due to an increase in either the number or volume (size) of macrophages. Morphometry confirmed that most of the increase in macrophage volume was in the form of single macrophages at three months.

There were slight increases in the volume fractions of the lung occupied by aggregated macrophages and macrophage-associated fibrosis in the group exposed to high carbon black. These increases correlated with the histopathologic observations of macrophage localization and particle-containing macrophages in the interstitium in the group exposed to high carbon black. Macrophage localization is not synonymous with macrophage aggregation, but as macrophages become localized they are more apt to be aggregated. Similarly, interstitialization of macrophages is not synonymous with macrophage-associated fibrosis, but the interstitialization can cause a thickening of the alveolar septum, which could be counted as macrophage-associated fibrosis during the point-counting procedure used for morphometry.

Rats Killed at Six Months. Histopathologic lesions related to exposure consisted of alveolar macrophage hyperplasia, alveolar epithelial hyperplasia, chronic-active inflammation, septal fibrosis, and alveolar proteinosis (Appendix Table F.3). None of these lesions were observed in control rats. The alveolar macrophage hyperplasia and the alveolar epithelial hyperplasia occurred in all exposed rats. The severities of these lesions were similar in rats exposed to diesel exhaust and carbon black, and increased similarly with concentration. In both low-exposure groups, macrophages were restricted to the immediate centriacinar region, and only a few, scattered macrophages were located in more peripheral alveoli. In both high-exposure groups, the macrophages were predominantly located in the centriacinus, but the zone of involvement was enlarged and included more of the alveoli adjacent to the centriacinus. In all groups, the macrophages were predominantly intraluminal. Interstitial macrophages containing particles were present but uncommon. The alveolar epithelial hyperplasia (characterized by foci of hypertrophic, hyperplastic, alveolar epithelial cells forming a cuboidal lining of the septa) was predominantly found in alveoli containing numerous particle-containing macrophages (Figure 11).

Chronic-active inflammation was found in one rat exposed to low carbon black and in one rat exposed to high diesel exhaust. Septal fibrosis consisted of interstitial fibrosis that predominately affected alveolar septa in foci where macrophages were aggregated in the lumen or interstitium and where there was concomitant alveolar epithelial hyperplasia. This lesion was present in two rats each from the high-exposure groups. Alveolar proteinosis was characterized by an accumulation of eosinophilic, granular or refractile, homogeneous, acellular material in the alveoli. Soot particles were present within this material. This lesion was present in one rat exposed to high diesel exhaust.

Morphometric data for rats killed at six months are summarized in Appendix Table F.4. The volume fraction of the lung occupied by single macrophages in the alveolar lumen was increased in a dose-related manner in all exposed groups, paralleling the histological macrophage hyperplasia. The volume fractions of the lung occupied by aggregated macrophages and macrophage-associated fibrosis were increased in the rats exposed to carbon black, but little increase was seen in rats exposed to diesel exhaust. In contrast, the histopathological observations discussed above suggested that the aggregation of macrophages and macrophage-associated fibrosis would be more similar in rats exposed to carbon black and diesel exhaust, but this may have reflected the difficulty in measuring a change in such a small compartment of the total lung volume by morphometric sampling techniques. The volume fraction of macrophages in terminal bronchioles was also increased in the group exposed to high carbon black, with only a suggestion of an increase in the group exposed to high diesel exhaust.

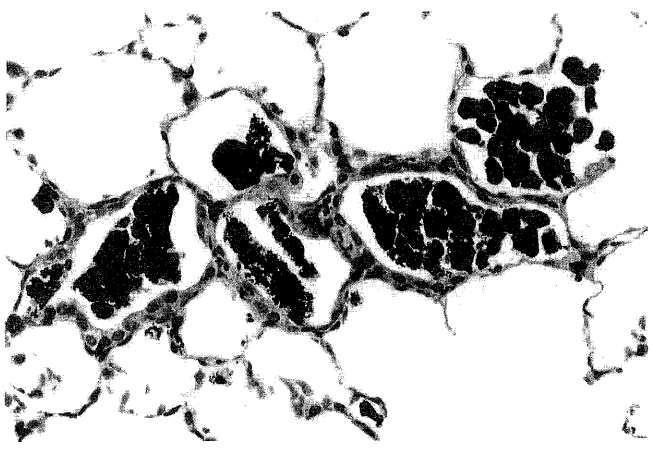

Figure 11. Photomicrograph illustrating alveolar epithelial hyperplasia in a rat exposed to low carbon black for six months. Hypertrophic, hyperplastic epithelial cells line alveoli that contain numerous macrophages that contain particles. Note that the alveoli that do not contain macrophages do not contain hypertrophic and hyperplastic epithelium. Magnification = × 368.

Rats Killed at Twelve Months. Exposure-related histopathological lesions consisted of alveolar macrophage hyperplasia, alveolar epithelial hyperplasia, chronic-active inflammation, septal fibrosis, alveolar proteinosis, and bronchiolar-alveolar metaplasia (Appendix Table F.5). No lesions were present in the control group. Alveolar macrophage hyperplasia and alveolar epithelial hyperplasia were present in all exposed rats. The severity of these lesions increased with exposure concentration, and were similar for rats exposed to carbon black and diesel exhaust. However, the severity of alveolar macrophage hyperplasia was slightly greater in the group exposed to high diesel exhaust than in the group exposed to high carbon black.

Chronic-active inflammation was present in one rat each from both groups exposed to carbon black, in two rats from the group exposed to low diesel exhaust, and in all rats from the group exposed to high diesel exhaust (Figure 12A). The severity of the chronic-active inflammation was higher for the group exposed to high diesel exhaust than the other groups. Cholesterol clefts, by-products of necrosis, were components of the lesion scored as chronic-active inflammation. Septal fibrosis was present in two rats each from the low-exposure groups, in four rats from the group exposed to high carbon black, and in three rats from the group exposed to high diesel exhaust. Alveolar proteinosis (Figure 12B) was present in all rats from high-exposure groups, in one rat exposed to low carbon black, and in two rats exposed to low diesel exhaust. The severity of the proteinosis was greater in the group exposed to high diesel exhaust than in the group exposed to high carbon black. The term bronchiolar-alveolar metaplasia was used to describe the presence of columnar, ciliated cells lining the alveolar ducts and adjacent alveoli; this lesion is sometimes called alveolar bronchiolization. This lesion was present in two rats exposed to high carbon black and three rats exposed to high diesel exhaust.

The histological characteristics of the macrophage hyperplasia, alveolar epithelial hyperplasia, and septal fibrosis were generally similar to those described for rats killed at six months, except that the extent of the lesions was increased for each group so that a larger zone around the centriacinus was involved, and the lesions were focally more severe in the centriacinus. The number and size of interstitial macrophages and macrophage aggregates had markedly increased compared with earlier times. For each exposure concentration, the zone of macrophage hyperplasia with aggregation of macrophages was greater for the groups exposed to diesel exhaust than for the groups ex-

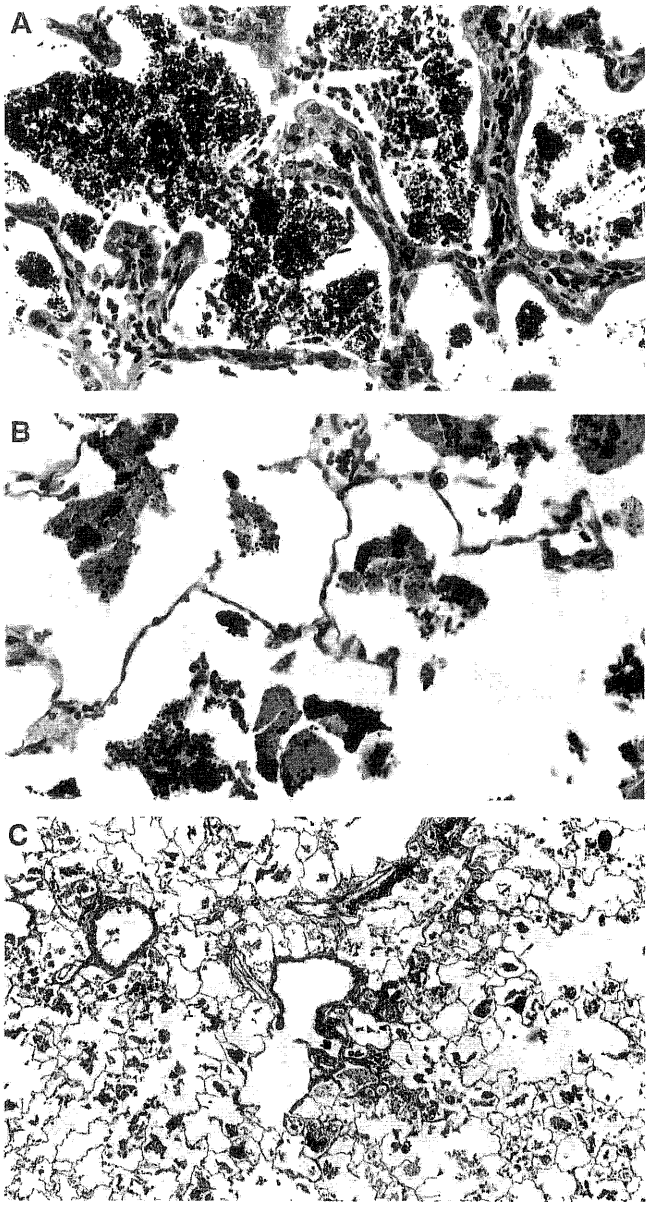

Figure 12. Photomicrographs illustrating exposure-related lesions in rats exposed to high diesel exhaust for twelve months. A: Chronic-active inflammation with neutrophils and cholesterol clefts (cleft-like empty spaces with pointed ends). Magnification = × 368. This photomicrograph also shows numerous soot-containing macrophages, alveolar epithelial hyperplasia, and a minimal increase in collagen in the alveolar septa. B: Alveolar proteinosis. Magnification = × 368. The homogeneous, acellular material (proteinosis) is located in the alveolar lumens. Diesel exhaust soot is found within this material. Note the typical lack of inflammation, alveolar macrophage hyperplasia, and septal fibrosis in association with this lesion. C: Distribution of the exposure-related lesions. Magnification = × 59. Although the lesions are more concentrated in the centriacinar region (center of the photomicrograph), alveoli peripheral to this region are also affected. Soot-containing macrophages, soot mixed with proteinosis, and debris are found in the peripheral alveoli. Note the progression from earlier changes in rats exposed to high diesel exhaust as illustrated in Figure 10D.

posed to carbon black. The increased zone of involvement was particularly evident in the group exposed to high diesel exhaust (Figure 12C).

Morphometric data obtained at 12 months are summarized in Appendix Table F.6. The volume fractions of the lung occupied by single macrophages in the alveolar lumens were increased in a dose-related manner in all exposed groups, and a significantly greater increase was found in the group exposed to high diesel exhaust than in the group exposed to high carbon black (Figure 13). The volume fractions of the lung occupied by aggregated macrophages and by macrophage-associated fibrosis were increased in a dose-related manner in all exposed groups, but values for the groups exposed to carbon black and diesel exhaust were similar at each exposure level.

Rats Killed at Eighteen Months. The same categories of histopathological lesions seen after 12 months of exposure were present at 18 months of exposure (Appendix Table F.7), and none of these lesions were seen in the control group. Alveolar macrophage hyperplasia, alveolar epithelial hyperplasia, and septal fibrosis were present in all exposed rats. The severity of alveolar macrophage hyperplasia was the same for both high-exposure groups and the group exposed to low diesel exhaust, but was slightly less

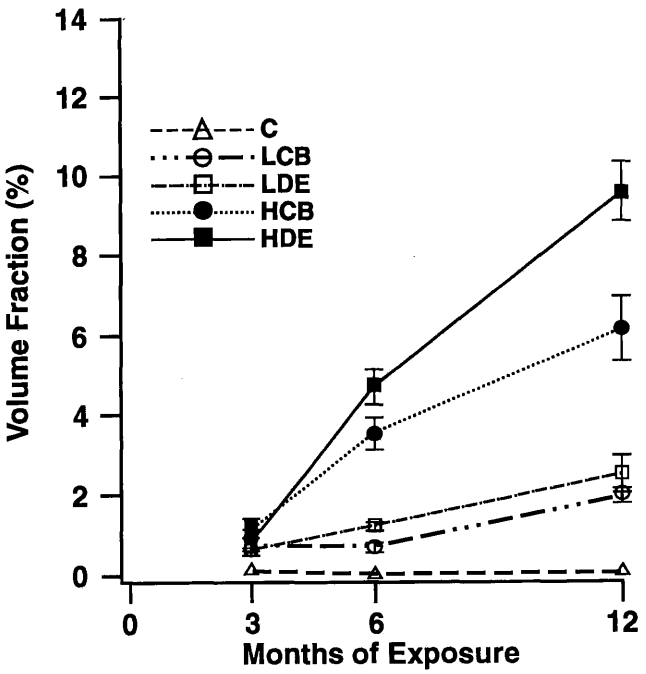

Figure 13. The fractions of the lungs occupied by single alveolar macrophages in lungs of rats examined morphometrically are expressed as mean ± SE percentages of lung volume. Values for the groups exposed to high diesel exhaust and high carbon black were significantly different from those of the control group at twelve months. Methods are given in Appendix B.

for the group exposed to low carbon black. There was a slight trend for the severity of alveolar epithelial hyperplasia to increase with dose and for diesel exhaust exposure to cause a greater increase than carbon black exposure. Overall, there was little difference among groups. The severity of septal fibrosis was mild in all rats from low-exposure groups, and it was moderate in all rats exposed to high diesel exhaust. It varied between mild and moderate for rats exposed to high carbon black.

Chronic-active inflammation and bronchiolar-alveolar metaplasia (Figure 14) were present in most exposed rats, and the severity increased slightly with exposure concentration. Alveolar proteinosis was present in the lungs of rats in most high-exposure groups, but not the low-exposure groups. The severity of alveolar proteinosis increased with exposure concentration and was greater for the groups exposed to high diesel exhaust than the group exposed to high carbon black.

The main progression from the lesions at 12 months were increased incidences and severities of septal fibrosis and bronchiolar-alveolar metaplasia, and, in the low-exposure groups, chronic-active inflammation. Concomitant with the increase in septal fibrosis was an increase in the interstitial aggregation of macrophages containing particles (Figure 15). There was also an increase in the amount of carbon black or diesel exhaust soot that was associated with alveolar proteinosis or debris. Most of the debris appeared to be the remains of macrophages. The proportion of alveolar space that contained carbon black or diesel exhaust soot that was not within viable macrophages was greater for the group exposed to high diesel exhaust than for the group exposed to high carbon black.

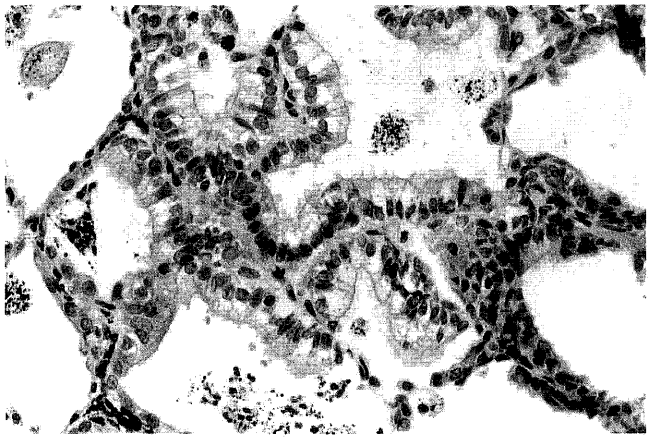

Figure 14. Photomicrograph illustrating bronchiolar-alveolar metaplasia in a rat exposed to high carbon black for eighteen months. Tall, columnar, ciliated cells line the alveoli. Magnification = × 368.

In some animals, particularly in the high-exposure groups, there were foci where the epithelial hyperplasia differed in character from that seen earlier. In some foci, the amount of alveolar epithelial hyperplasia was greater than expected relative to the amount of macrophage hyperplasia and septal fibrosis. In these foci, the alveolar epithelial hyperplasia seemed to occur almost independently from these lesions. Also, in some foci, whether or not macrophage hyperplasia and fibrosis occurred, there were papillary projections of hyperplastic epithelium, or there was a multilayering of hyperplastic epithelial cells.

Rats Killed at Twenty-Three Months. Histopathological lung lesions associated with carbon black or diesel exhaust exposure in rats killed at 23 months included those seen previously, and included three additional lesions: focal fibrosis, squamous metaplasia, and squamous cysts (Appendix Table F.8). None of these lesions were present in the control group. Alveolar macrophage hyperplasia, al-

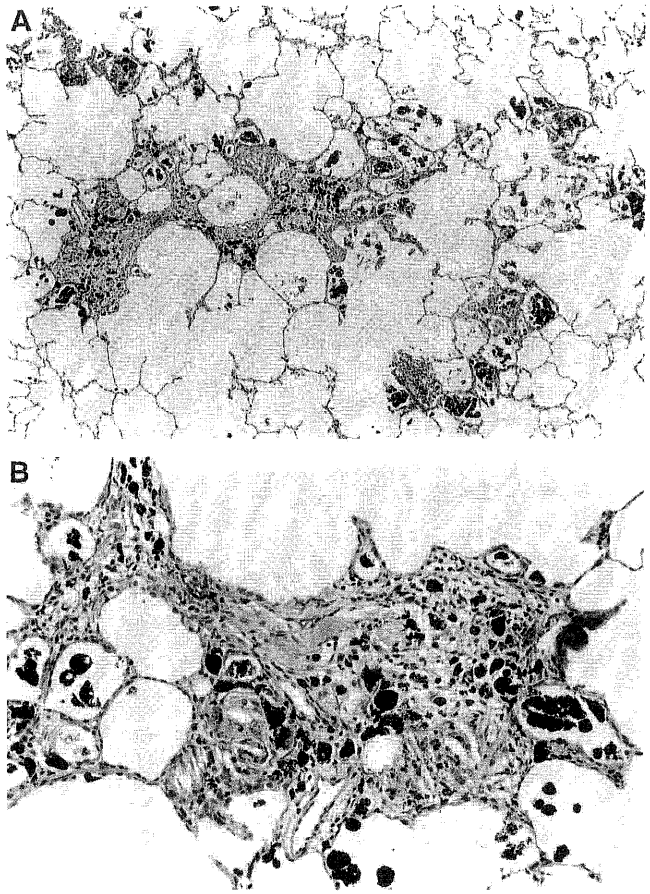

Figure 15. Photomicrographs illustrating septal fibrosis and interstitial aggregation of macrophages in a rat exposed to low carbon black for eighteen months. A: The extent of the septal fibrosis. Note that although the septa are thickened due to the fibrosis, the parenchymal architecture is not obliterated. Magnification = × 59. B: The lesion shown in panel A magnified to show the incorporation of particle-containing macrophages. Magnification = × 184.

veolar epithelial hyperplasia, and septal fibrosis were present in all exposed rats. Although the severity of the lesions increased slightly with dose, the severity scores were similar for all exposed groups. Chronic-active inflammation, alveolar proteinosis, and bronchiolar-alveolar metaplasia were present in the majority of exposed rats, and the severity of the lesions increased with exposure concentration. The severity of the alveolar proteinosis was greater in the group exposed to high diesel exhaust than in the group exposed to high carbon black; and the severities were similar in the groups exposed to high carbon black and low diesel exhaust.

Focal fibrosis was a well-circumscribed, nodular focus of fibrosis that obliterated the pulmonary architecture (Figure 16A). The center of the lesion was usually composed of dense collagen bundles and a few, small alveolar structures lined by cuboidal epithelium (Figure 16B). A thick rim of hyperplastic, sometimes papillary, epithelium often surrounded the periphery of the scirrhous nodule. This lesion was present in four rats from both high-exposure groups and in one rat exposed to low diesel exhaust. Squamous metaplasia of the alveolar epithelium was seen in one rat exposed to high diesel exhaust.

Squamous cysts were seen in two rats exposed to high carbon black and in one rat from each of the diesel exhaust groups. The term squamous cyst was used to denote a cyst lined by well-differentiated, stratified squamous epithelium with a central keratin accumulation (Figure 17A). The lesion was sharply demarcated (Figure 17B), except in those areas in which there was extension into adjacent alveoli (Figure 17C). The squamous epithelium had few mitotic figures, and dysplasia was absent. Growth of the lesion appeared to occur by keratin accumulation and by peripheral extension into the alveolar spaces.

For both exposure concentrations, the carbon black particles, whether in macrophages or outside cells, tended to be localized more to the centriacinar region than diesel exhaust soot, leaving correspondingly more particle-free alveoli in rats exposed to carbon black than in rats exposed to diesel exhaust.

Rats Killed Six Weeks After the End of Exposure. The same constellation of histopathological lesions observed at 23 months was present in exposed rats that were killed after the end of exposure (Appendix Table F.9). A few control rats also had lung lesions at that time, the most common of which was alveolar epithelial hyperplasia, which occurred in 4 of 34 animals.

Alveolar macrophage hyperplasia, alveolar epithelial hyperplasia, chronic-active inflammation, septal fibrosis, alveolar proteinosis, and bronchiolar-alveolar metaplasia occurred in all rats from high-exposure groups, and in most rats from low-exposure groups. Chronic-active inflammation occurred in 72% of the rats exposed to low carbon black, and 76% of the rats exposed to low diesel exhaust. Alveolar proteinosis occurred in 56% of the rats exposed to low carbon black, and 80% of the rats exposed to low diesel exhaust. Bronchiolar-alveolar metaplasia occurred in 94% of the rats exposed to low carbon black, and 92% of the rats exposed to low diesel exhaust. These lesions were similar in groups exposed to carbon black and diesel exhaust and were increased only slightly at the higher exposure levels. The exceptions were alveolar proteinosis and bronchiolar-alveolar metaplasia, which differed clearly between the low and high exposures. Focal fibrosis, alveolar squamous metaplasia, and squamous cysts occurred in a minority of the rats exposed to low carbon black, high carbon black, and low diesel exhaust, but focal fibrosis occurred in 54% of the rats exposed to high diesel exhaust. The incidences of squamous metaplasia were dose-related, and the inci-

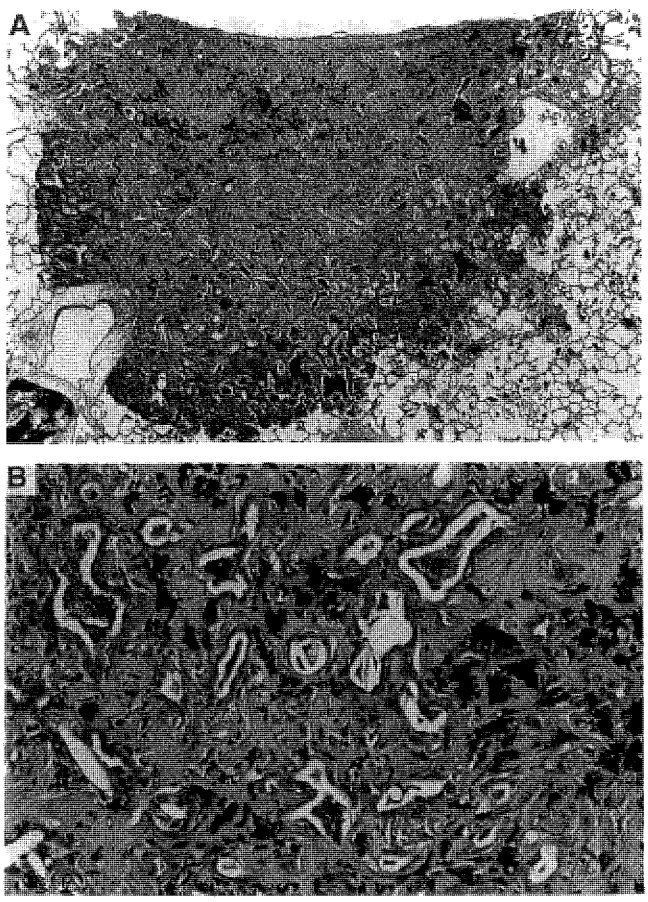

Figure 16. Photomicrographs illustrating focal fibrosis in a rat exposed to high carbon black for twenty-three months. A: The obliteration of the pulmonary architecture. Magnification = × 29. B: The collagen surrounding small spaces, which are the remnants of alveoli. Numerous particle-containing macrophages are found within this lesion. Magnification = × 184.

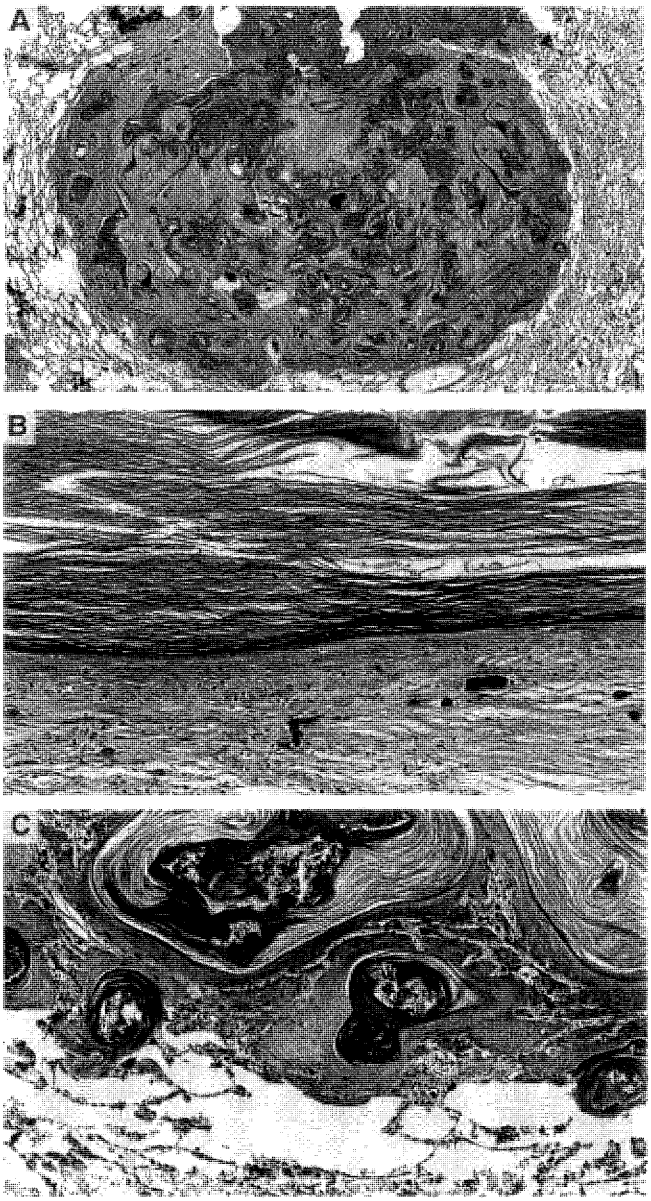

Figure 17. Photomicrographs illustrating a squamous cyst in a rat exposed to high carbon black when rats were killed at the end of exposure. A: This space-occupying lesion can be seen to compress the surrounding pulmonary parenchyma. The center of the squamous cyst is filled with keratin, a small amount of necrotic debris, and carbon black. Magnification = × 24. B: The typical structure of the edge of a keratin cyst where it borders the pleura or an area of fibrosis. In these areas, the cyst is sharply demarcated from the underlying fibrous tissue. The stratified squamous epithelium shows an orderly differentiation and keratinization. Magnification = × 184. C: An area where the cyst wall is complex and not as flattened as the wall in panel B. Squamous metaplasia extends into the adjacent alveoli. There is still an orderly differentiation and keratinization of the epithelium. Magnification = × 147.

dences of squamous cysts were higher in the groups exposed to carbon black than in the groups exposed to diesel exhaust.

The morphologic characteristics of most exposure-related lesions did not differ between the rats killed at 23 months and those killed after the end of exposure, and the incidences and severities of alveolar macrophage hyperplasia, alveolar epithelial hyperplasia, chronic-active inflammation, and septal fibrosis were similar to those in rats killed at 23 months. A progression noted in several lung sections from the rats killed after the end of exposure was alveoli in the ventral (dependent) portions of the lung that were filled with carbon black particles or diesel exhaust soot, debris, proteinosis, and lesser numbers of macrophages and inflammatory cells (Figure 18). This filling of alveoli had been present in small foci earlier, but was much more severe at this time. The severity of alveolar proteinosis and bronchiolar-alveolar metaplasia, and the incidence of squamous metaplasia, had increased in all exposure groups compared with those at 23 months. The incidence of focal fibrosis had also increased in the low-exposure groups, and the incidence of squamous cysts had increased in the group exposed to low carbon black.

Rats That Died or Were Euthanized. Lung lesions in rats that died or were euthanized in moribund condition had the same characteristics, and their incidences and severities bore the same relations to exposure and exposure time as lesions in rats that were killed. In Appendix Tables F.10

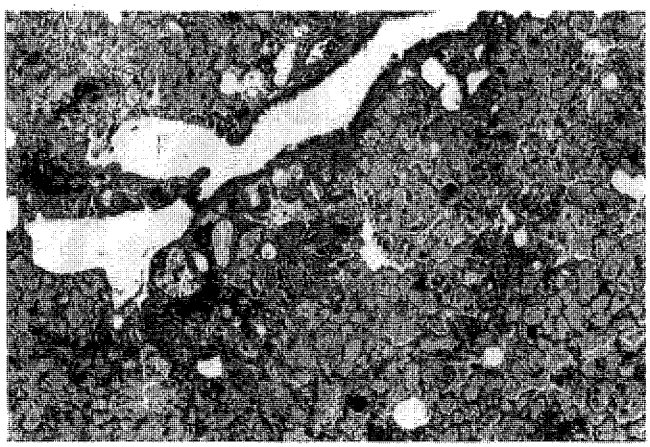

Figure 18. Photomicrograph illustrating exposure-related lesions in the ventral portion of a lung lobe from a rat exposed to high carbon black when rats were killed at the end of exposure. A terminal bronchiole and the first alveolar ducts are evident as spaces. Most of the parenchymal architecture is obscured due to the filling of alveoli with particles, debris, proteinosis, macrophages, and inflammatory cells. In the center of the micrograph, there is an area of bronchiolar-alveolar metaplasia and marked alveolar epithelial hyperplasia, but these alterations are difficult to discern at this low magnification and with the filling of the airspaces. Magnification = × 59.

through F.15, the lesions are tabulated for three time intervals: less than 12 months, 12 to 18 months, and from 18 months to the final killing.

Statistical Significance of Trends in Nonneoplastic Lesions. The statistical significances of trends in the prevalence of nonneoplastic lesions were examined by gender and exposure. The data from the rats that were killed were combined with those from rats that died or were euthanized. The prevalence of each lesion was defined at a particular severity score, which was selected to show best the progression from a low prevalence at early death times to a higher prevalence at later death times. It was assumed that the prevalences of lesions changed as linear functions of exposure time on a logistic scale, and that the factors used in the regression model altered the slopes of the functions. The progression in prevalence of three nonneoplastic lesions, alveolar proteinosis, alveolar epithelial hyperplasia, and squamous cysts, is illustrated in Figure 19.

The prevalence of macrophage hyperplasia was examined at a severity score of three or higher because most of the animals had severity scores of one or two at the early times. We found that the prevalence was higher for rats exposed to diesel exhaust than for those exposed to carbon black ($p < 0.001$), and higher for females than for males ($p < 0.001$). The gender difference was smaller than the exposure difference, and only became significant for severity scores of three or larger.

The prevalence of alveolar epithelial hyperplasia was also examined at a severity score of three or higher. A higher prevalence ($p < 0.001$) was found for rats exposed to diesel exhaust than for rats exposed to carbon black (Figure 19). The prevalence was higher for females than for males, but the difference was small and of marginal statistical significance ($p = 0.035$).

A severity score of one or higher was used to examine the prevalence of chronic-active inflammation because this lesion occurred gradually throughout the exposure. The prevalence was higher for rats exposed to diesel exhaust than for rats exposed to carbon black ($p < 0.001$) and higher for females than for males ($p < 0.001$).

A severity score of two or higher was used to examine the prevalence of septal fibrosis. The rats exposed to diesel exhaust had a significantly higher prevalence than rats exposed to carbon black ($p < 0.001$). There was no significant gender difference in the prevalence of septal fibrosis ($p = 0.15$).

A severity score of one or higher was used to examine the prevalence of alveolar proteinosis. Prevalences were

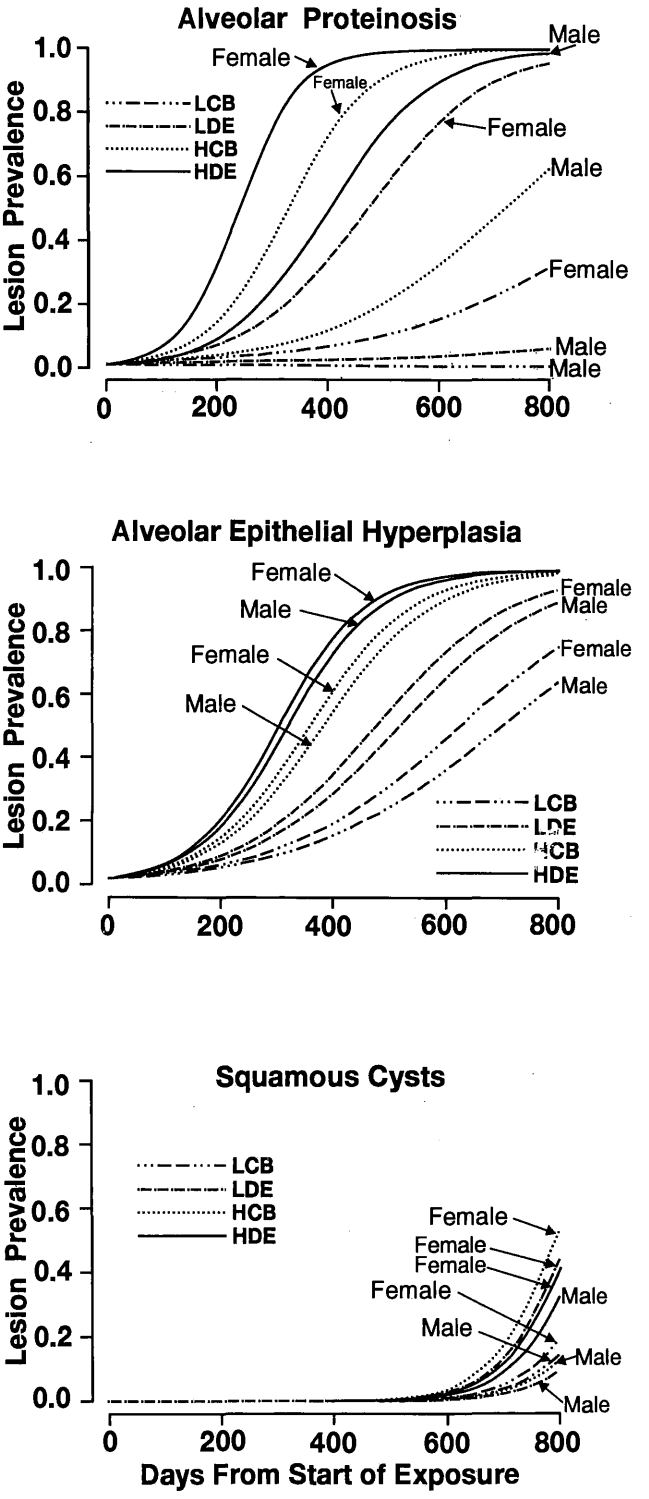

Figure 19. The prevalences of three nonneoplastic lesions observed in rats that died, or were killed or euthanized. The data plotted are derived from incidence and severity data summarized in Appendix Tables F.10 through F.15. The prevalences shown are for severity scores of one or higher for alveolar proteinosis, and three or higher for alveolar epithelial hyperplasia and for all squamous cysts. F20= female, M20= male.

higher for rats exposed to diesel exhaust than for rats exposed to carbon black, and the prevalence was higher for females than for males (both $p < 0.001$) (Figure 19).

A severity score of one or higher was used to examine the prevalence of bronchiolar alveolar metaplasia. The prevalence was higher for rats exposed to diesel exhaust than for rats exposed to carbon black, and higher for females than for males (both $p < 0.001$).

A severity score of one or higher was used to examine the prevalence of focal fibrosis. Neither the difference due to exposure ($p = 0.45$) nor that due to gender ($p = 0.08$) was significant.

A severity score of one or higher was used to examine the prevalence of alveolar squamous metaplasia. There was no significant difference due to exposure ($p = 0.49$), but females had a marginally significantly higher prevalence than males ($p = 0.012$).

The prevalence of squamous cysts was evaluated separately because these were discrete lesions that were not given a severity score; however, the same methods were used for statistical evaluation of the prevalence of squamous cysts that were used for the other nonneoplastic lesions. No squamous cysts were observed in any group before 18 months (Figure 19). There were no significant differences in the prevalence of squamous cysts due to either exposure ($p = 0.14$) or gender ($p = 0.47$). The prevalence of squamous cysts was significantly higher at the high levels of exposure than at the low levels of exposure ($p < 0.001$).

Neoplastic Lung Lesions

In considering the lung neoplasm results, one must recognize that the data resulted from a sampling procedure that almost certainly underestimated the actual number of neoplasms present. Although all gross lesions suspected of being neoplasms were examined, many of the neoplasms were not visible on gross examination and were discovered only during histopathological evaluations. Because the lungs were not serially step-sectioned and examined in their entirety, the results presented below can only be considered a statistical sampling of the occurrence of neoplasms. The comparisons among exposure groups were considered valid on the basis that the same sampling procedure was used for all groups, but the comparisons are relative, rather than absolute.

The primary lung neoplasms were classified as adenomas, adenocarcinomas, squamous cell carcinomas (Figure 20A), adenosquamous carcinomas, and one malignant neoplasm not otherwise specified. The adenomas were subclassified into two morphologic patterns: alveolar and papillary. The adenocarcinomas were subclassified into four morphologic patterns: alveolar, papillary, tubular, and solid. Adenomas or adenocarcinomas that exhibited more than one morphologic pattern were categorized according to the predominant pattern. Two of the squamous cell carcinomas arose from the epithelium of squamous cysts,

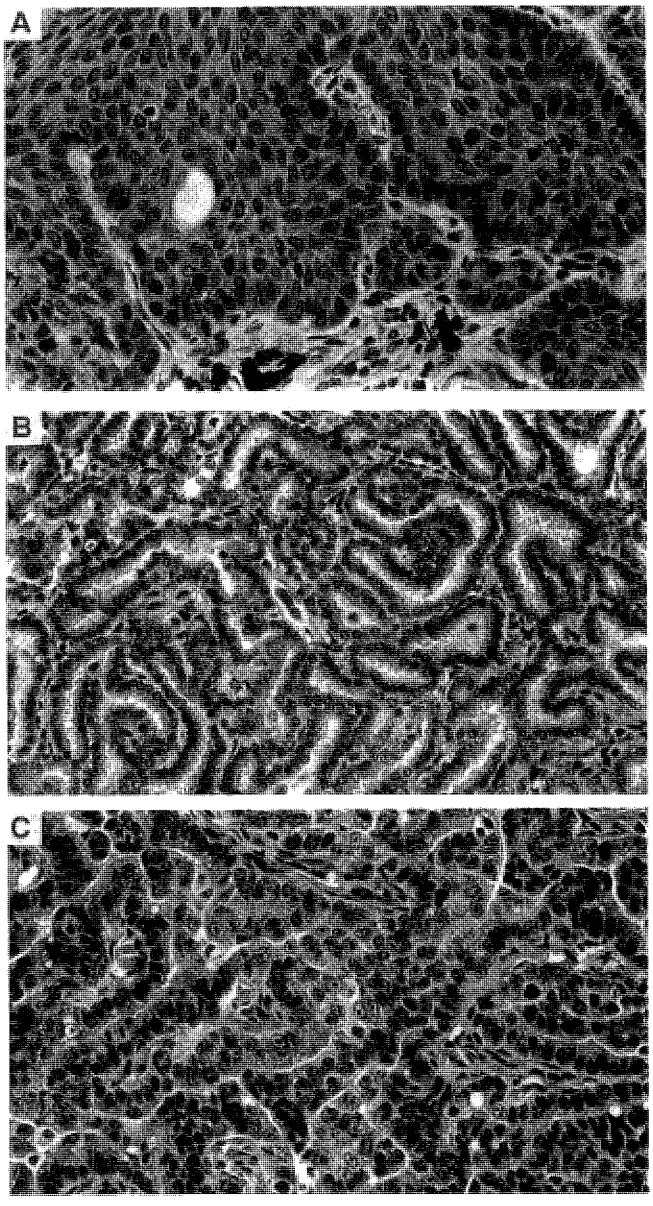

Figure 20. Photomicrographs illustrating neoplasms in rats exposed to high diesel exhaust when rats were killed at the end of exposure. A: A nonkeratinizing squamous cell carcinoma. Note that the polyhedral squamous cells have anaplastic nuclei and that they do not show an orderly differentiation, in contrast to the squamous cyst shown in Figure 18. B: A papillary adenoma with a single layer of uniform, small, epithelial cells lining the septa. C: A papillary adenocarcinoma with multiple, disorderly layers of epithelial cells lining the septa. Features of anaplasia include the large size and hyperchromasia of the nuclei, the variable size and shape of the nuclei, and the frequent mitotic figures. All magnifications = × 368.

and these were subclassified as squamous cell carcinomas arising from squamous cysts. Adenosquamous carcinomas were neoplasms that showed morphologic patterns of both squamous cell carcinoma and adenocarcinoma. The primary lung neoplasm classified as a "malignant neoplasm not otherwise specified" had both a mesenchymal (spindle cell and stromal) component and an epithelial component.

All of the primary lung neoplasms appeared to arise from the parenchyma; none appeared to arise from the conducting airways. Some of the smaller adenomas could be seen to arise from the parenchyma adjacent to the alveolar ducts, the area where alveolar epithelial hyperplasia was most commonly located. The key features differentiating the adenomas from foci of hyperplasia were that adenomas exhibited uniform, regular cells causing distortion of the alveolar architecture, a unitized structure forming a discrete mass, and an expansive growth that sometimes caused compression of the surrounding lung. The adenocarcinomas were differentiated from adenomas by their cellular anaplasia (Figure 20B and 20C), their invasion, in some cases, of lymphatics, blood vessels, or bronchial walls, and their metastasis to regional lymph nodes.

There was evidence that the pathogenesis of adenomas and adenocarcinomas formed a continuum that began with alveolar epithelial hyperplasia. In some cases, the adenomas and adenocarcinomas were surrounded by epithelial hyperplasia. Several adenocarcinomas appeared to arise within adenomas, and the morphological features of the neoplasms suggested that they arose from the alveolar epithelium. However, a progression from squamous metaplasia to a benign squamous neoplasm to a squamous cell carcinoma was not clearly evident. The location of the squamous cell carcinomas and the observation of squamous metaplasia in the alveolar region suggested that the squamous cell carcinomas arose from alveolar epithelium, but a benign squamous neoplasia linking metaplasia and carcinoma was not evident.

The occurrence of the different types of lung neoplasms is summarized in Table 6, which lists the total number of rats examined for neoplasms, the number of each type of neoplasm observed, and the number of rats having each type of neoplasm by gender and exposure group. A total of 114 to 118 rats of each gender from each exposure group was examined by gross necropsy and microscopic evaluation of the lungs. No neoplasm type or morphology was distinctive for carbon black or diesel exhaust exposure. Adenomas, adenocarcinomas, and squamous cell carcinomas occurred in all exposure groups, except that there were no squamous cell carcinomas in rats exposed to low carbon black. Of the three adenosquamous carcinomas, two occurred in rats exposed to high carbon black and one occurred in a rat exposed to high diesel exhaust. The preponderance of neoplasms were adenomas and adenocarcinomas, which occurred more often in females than in males. This gender difference was most striking at the higher levels of exposure to diesel exhaust and carbon black. In contrast, more squamous cell carcinomas occurred in males than in females.

Three rats from the control group had primary lung neoplasms, with each rat having an adenoma, an adenocarcinoma, or a squamous cell carcinoma. Both the alveolar and papillary patterns of adenomas and adenocarcinomas were seen in rats exposed to both levels of carbon black and diesel exhaust. The two less common patterns of adenocarcinomas, tubular and solid, were observed in rats exposed to both levels of carbon black and diesel exhaust, except there were no tubular adenocarcinomas in rats exposed to low carbon black and no solid adenocarcinomas in rats exposed to low diesel exhaust.

Only single lung neoplasms were observed in rats in the control group, but the data in Table 6 demonstrate that multiple neoplasms were observed in numerous rats in the exposed groups. A total of 141 neoplasms, 56 benign and 85 malignant, were observed in a total of 96 rats bearing neoplasms. The multiplicity of neoplasms is summarized more succinctly in Table 7, which lists the numbers of neoplasm-bearing rats having one, two, or three or more neoplasms, and the percentages of neoplasm-bearing rats having different numbers of neoplasms. Multiplicity of neoplasms was related to dose, and slightly more prevalent among the groups exposed to diesel exhaust than among the groups exposed to carbon black. One neoplasm-bearing rat exposed to low carbon black had both a malignant and a benign neoplasm; the rest had single neoplasms. One neoplasm-bearing rat exposed to low diesel exhaust had two benign and one malignant neoplasms. Only approximately 69% of neoplasm-bearing rats exposed to high carbon black and 55% of the neoplasm-bearing rats exposed to high diesel exhaust had single lung neoplasms; approximately 13% and 18%, respectively, had three or more neoplasms. The percentage of neoplasm-bearing rats exposed to high carbon black with multiple benign neoplasms (approximately 15%) was slightly larger than the percentage with multiple malignant neoplasms (approximately 12%); however, the percentage of neoplasm-bearing rats exposed to high diesel exhaust with multiple benign neoplasms (approximately 17%) was less than that with multiple malignant neoplasms (approximately 31%).

The appearance of lung neoplasms in rats exposed to diesel exhaust occurred late during the exposure, as observed previously (Mauderly et al. 1987b), and this was also true for the groups exposed to carbon black. The observation of neoplasms with time is illustrated in Figure 21, in which the cumulative number of rats with one or more benign or malignant neoplasms is plotted over time after initiating the exposures. For all exposure groups (exclud-

Table 6. Numbers of Different Types of Lung Neoplasms Observed, and Numbers of Rats with Each Type of Neoplasm[a, b]

	Control			Low Carbon Black			High Carbon Black			Low Diesel Exhaust			High Diesel Exhaust		
Tumor Type	Female 114	Male 118	Total 232	Female 116	Male 115	Total 231	Female 114	Male 115	Total 229	Female 114	Male 114	Total 228	Female 115	Male 115	Total 230
Adenoma															
Number of neoplasms	0	1	1	2	1	3	17	0	17	6	2	8	22	5	27
Rats with neoplasms	0	1	1	2	1	3	13	0	13	5	2	7	19	4	23
Adenocarcinoma															
Number of neoplasms	0	1	1	6	1	7	23	1	24	3	1	4	32	3	15
Rats with neoplasms	0	1	1	6	1	7	20	1	21	3	1	4	19	3	22
Squamous cell carcinoma															
Number of neoplasms	0	1	1	0	0	0	1	2	3	1	2	3	1	2	3
Rats with neoplasms	0	1	1	0	0	0	1	2	3	1	2	3	1	2	3
Adenosquamous carcinoma															
Number of neoplasms	0	0	0	0	0	0	1	1	2	0	0	0	1	0	1
Rats with neoplasms	0	0	0	0	0	0	1	1	2	0	0	0	1	0	1
Malignant tumor not otherwise specified[c]															
Number of neoplasms	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
Rats with neoplasms	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0

[a] Several individual rats had multiple types of tumors, or multiple tumors of a single type, or both; thus, these rats are counted more than once in this table.

[b] The number at the top of each column is the number of animals examined, including all rats that underwent gross necropsy and microscopic examinations of the lung whether the rats died spontaneously, or were euthanized or killed.

[c] This tumor was of a mixed mesenchymal and epithelial type.

ing controls), the first neoplasms observed were malignant, and the number of neoplasm-bearing rats increased slightly more rapidly for malignant than for benign neoplasms. The first neoplasm observed was a squamous cell carcinoma in a male rat exposed to high diesel exhaust for 448 days (approximately 15 months). The first neoplasms in other groups were an adenosquamous carcinoma in a male rat exposed to high carbon black for 497 days, a malignant neoplasm not otherwise specified in a female exposed to low carbon black for 526 days, a squamous cell carcinoma in a male rat exposed to low diesel exhaust for 568 days, and an adenoma in a male rat from the control group at 632 days. At both exposure levels, lung neoplasms tended to increase earlier in groups exposed to carbon black than in groups exposed to diesel exhaust. Substantial numbers of rats with neoplasms did not begin to be counted in any group until after approximately 600 to 650 days (approximately 20 months). The finding of a substantial percentage of the neoplasms in all groups when rats were killed after the end of exposure suggested that the prevalence of neoplasms was probably still increasing at the termination of the study.

It could not be determined conclusively if the lung neoplasms were the primary cause of death, but neoplasms that had metastases, occupied 33% or more of the lung volume, invaded vessels, or involved substantial necrosis, hemorrhage, infarction, or secondary infection were considered fatal. A few other neoplasms having similar, but less extensive features, were considered contributory to death. Using

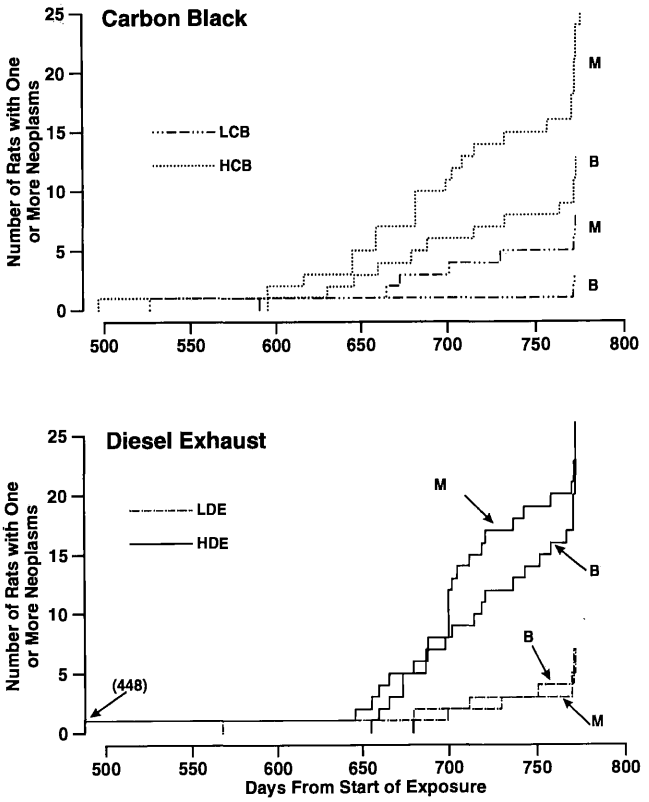

Figure 21. The cumulative number of rats in each exposure group observed to have one or more malignant (M) or benign (B) lung neoplasms. Females and males are combined.

Table 7. Percentages of Rats Bearing Neoplasms That Had One, Two, or Three or More Lung Neoplasms[a]

	Control Group		Low Carbon Black		High Carbon Black		Low Diesel Exhaust		High Diesel Exhaust	
Number of Neoplasms	Number of Rats	%	Number of Rats	%	Number of Rats	%	Number of Rats	%	Number of Rats	%
Benign										
1	1	100	3	100	11	84.6	6	85.7	19	82.6
2	0	0	0	0	0	0	1	14.3	4	17.4
3 or more	0	0	0	0	2	15.4	0	0	0	0
Malignant										
1	2	100	8	100	22	88.0	7	100	18	69.2
2	0	0	0	0	2	8.0	0	0	5	19.2
3 or more	0	0	0	0	1	4.0	0	0	3	11.6
Benign or malignant										
1	3	100	9	90	22	68.8	12	92.3	21	55.3
2	0	0	1	10	6	18.8	0	0	10	26.3
3 or more	0	0	0	0	4	12.5	1	7.7	7	18.4
Total rats with neoplasms	3		10		32		13		38	

[a] The numbers of rats having either benign or malignant neoplasms are counted separately; thus, individual rats may be counted in both categories. The total number of rats with neopolasms is the sum of those listed in the "Benign or malignant" category.

these criteria and considering accompanying histopathological findings, only eleven neoplasms were considered to have been fatal or contributory to death. Among females, no neoplasms occurred in the control group and only one neoplasm, the malignant neoplasm not otherwise specified in a female rat exposed to low carbon black, was considered to be fatal at the lower levels of exposure. Among females at the higher levels of exposure, two papillary adenocarcinomas, one tubular adenocarcinoma, and one solid adenocarcinoma were considered fatal or contributory to death in the group exposed to high carbon black, and one alveolar adenocarcinoma, one papillary adenocarcinoma, and one squamous cell carcinoma were considered fatal or contributory to death in the group exposed to high diesel exhaust. No neoplasms were considered fatal among males in the low-exposure or control groups. Among males at the higher levels of exposure, one squamous cell carcinoma was considered fatal in the group exposed to high carbon black, and two squamous cell carcinomas were considered fatal or contributory to death in the group exposed to high diesel exhaust.

Although not a primary lung neoplasm, mononuclear cell leukemia, which often affected the lungs, was among the major causes of death. The pulmonary manifestations of leukemia were considered fatal if malignant cells packed the vessels or if extravascular leukemic infiltrates occupied more than a third of the lung volume and were associated with hemorrhage and necrosis. Among females, pulmonary leukemia was considered the primary cause of death in 22 control rats, 28 rats exposed to low carbon black, 28 rats exposed to high carbon black, 29 rats exposed to low diesel exhaust, and 25 rats exposed to high diesel exhaust. Among males, pulmonary leukemia was considered the primary cause of death in 31 control rats, 31 rats exposed to low carbon black, 26 rats exposed to high carbon black, 36 rats exposed to low diesel exhaust, and 27 rats exposed to high diesel exhaust. These findings indicate that the incidence of fatal pulmonary leukemia was not related to exposure. Other rats with little or no expression of leukemia in the lung died from the manifestations of leukemia in other organs, particularly the spleen.

Because of the sampling strategy for microscopic evaluation of the lungs and the fact that neoplasms too small to be observed grossly were identified by microscopy, the absolute incidence of lung neoplasms could not be determined. The data most closely representing conventional measures of incidence are listed in Table 8. In this table,

Table 8. Summary of Numbers and Percentages of Rats Examined for Lung Neoplasms That Had One or More Neoplasms[a]

Group	Gender	Number of Susceptible[b] Rats Examined for Neoplasms	Rats with Malignant Neoplasms		Rats with Malignant or Benign Neoplasms	
			Number	Percent of Susceptible Rats	Number	Percent of Susceptible Rats
Control	Female	105	0	0	0	0
	Male	109	2	1.8	3	2.8
	Both	214	2	0.9	3	1.4
Low carbon black	Female	107	7	6.5	8	7.5
	Male	106	1	0.9	2	1.9
	Both	213	8	3.8	10	4.7
High carbon black	Female	105	21	20.0	28	26.7
	Male	106	4	3.8	4	3.8
	Both	211	25	11.8	32	15.2
Low diesel exhaust	Female	105	4	3.8	8	7.6
	Male	105	3	2.9	5	4.8
	Both	210	7	3.3	13	6.2
High diesel exhaust	Female	106	21	19.8	29	27.4
	Male	106	5	4.7	9	8.5
	Both	212	26	12.3	38	7.9

[a] Each rat with one or more neoplasms was counted only once in each neoplasm category.

[b] Values include all rats examined by gross necropsy and microscopy except rats killed at 3, 6, and 12 months. The first lung neoplasm was observed between 12 and 18 months of exposure; thus, all rats that died spontaneously, or were euthanized in moribund condition plus those killed at 18 months or later were considered to be at risk of lung neoplasms. The total number of rats examined, including those killed at 3, 6, and 12 months, is listed in Table 6.

the numbers of rats that had one or more lung neoplasms are given by gender and exposure group. In addition, the numbers of rats with neoplasms are shown as the percentages of all rats that were at risk of and examined for lung neoplasms. The number of rats considered to have been at risk of developing observable lung neoplasms included all rats that died or were euthanized or killed, the lungs of which were examined microscopically, but excluded those rats killed at 3, 6, and 12 months. These rats killed early in the exposure period were excluded because the first lung neoplasm was observed at approximately 15 months. Therefore, the numbers of rats listed in Table 8 for each exposure group are 18 (nine females and nine males) fewer than the total numbers whose lung were examined histologically (listed in Table 6).

The percentage of rats with one or more benign or malignant lung neoplasms was 1.4% for the control group, and ranged from 4.7% for the group exposed to low carbon black to 17.9% for the group exposed to high diesel exhaust. The percentages of rats with neoplasms were not strikingly different between the two low-exposure groups or between the two high-exposure groups, regardless of whether only malignant or both malignant and benign neoplasms were considered. Among all exposed groups, a several-fold greater percentage of females than males had neoplasms. At least in part, this difference may have been related to the longer survival time of the females, which allowed greater expression of the late-occurring neoplasms.

The prevalence of lung neoplasms was considered a better measure of carcinogenicity than the crude incidence described above, both because of the sampling limitations and because of the exposure-related shortening of survival. Although the life-span shortening was not striking, the groups with shortened survival had fewer rats at risk of developing neoplasms late in the study. Because the prevalence of neoplasms was increasing in all groups at the end of the study, the expression of carcinogenicity in groups with shortened survival was probably suppressed. Prevalences of neoplasms were calculated using actual data, and were modeled using a logistic regression approach.

The prevalences of rats with one or more lung neoplasms among rats examined for neoplasms were calculated using data for rats that died or were euthanized or killed within selected time intervals during the study. Figure 22 illustrates the prevalences of rats with one or more malignant or benign lung neoplasms. The figure illustrates clearly that the prevalences of lung neoplasms were related to exposure concentration, were higher in females than in males, and were very similar for the two low-exposure groups and for the two high-exposure groups. The prevalence of lung neoplasms was highest for females that died during the last interval, after exposures were terminated. The appearance

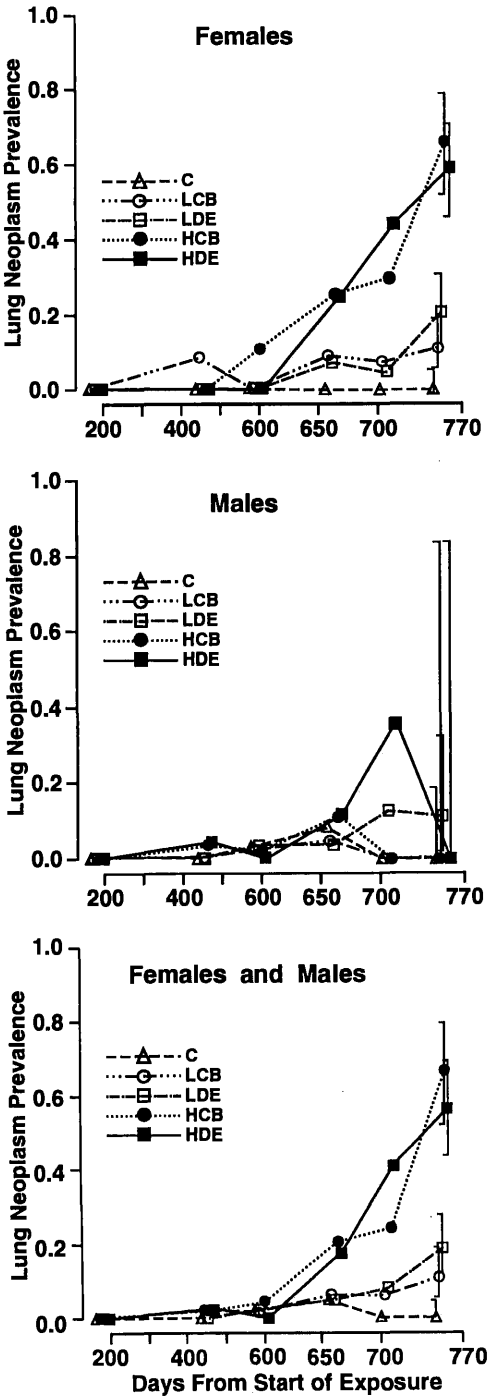

Figure 22. The prevalence of rats observed to have malignant or benign lung neoplasms during various intervals after 200 days of exposures. Prevalences are shown for females and males separately and for the combined genders. The data points shown are centered on the intervals representing < 365 days, 365 to 548 days, 548 to 639 days, 639 to 684 days, 684 to 730 days, and > 730 days (after end of exposure). The prevalences represent the fractions of rats that died, or were euthanized or killed during each interval that had one or more malignant or benign lung neoplasms. The error bars represent the SE of the estimates of prevalence, based on the numbers of rats observed during the last interval. Error bars for other intervals are omitted for clarity.

of the prevalence curves for males during the last two intervals reflects the small numbers of rats still living at that time. No males exposed to high carbon black lived to the last interval. Only two males exposed to high diesel exhaust lived to the last interval, and neither had lung neoplasms. The combined female and male prevalences of benign and malignant neoplasms during the final interval, after exposures ended, were 11% for the group exposed to low carbon black, 18% for the group exposed to low diesel exhaust, 67% for the group exposed to high carbon black, and 57% for the group exposed to high diesel exhaust.

Statistical comparisons of the lung neoplastic responses between genders and among exposure groups were done using logistic regression modeling, as described in the Methods section, and this procedure also gave estimates of the lifetime incidences of lung neoplasms. The appropriateness of the neoplasm prevalence model was predicated on the assumption that the prevalences of neoplasms were the same among rats that died, or were euthanized in moribund condition, or killed. Upon testing this assumption using a likelihood ratio test, it was found that there was no significant difference ($p = 0.13$) in neoplasm prevalence between the group of rats that died or were euthanized and the group of rats that were killed, indicating that the assumption was reasonable. The judgment that few lung tumors caused death, and the assumption of similar neoplasm prevalences in rats that died, or were euthanized or killed were based on the histopathology described above, rather than on statistical power. The statistical power for determining the significant difference in neoplasm responses among the three groups (based on manner of death) was tested using Kaplan-Meier estimates of survival, the scheduled times at which rats were killed, the percentage of rats that were killed but not evaluated for histopathology, and the estimates of neoplasm prevalence in rats that died, or were euthanized, or killed. The power estimated by 518 repeated simulations was 49% at $\alpha = 0.05$ and 74% at $\alpha = 0.20$; thus, the power for determining statistically that the neoplasm prevalence did not differ among the groups of rats that were killed, or died, or were euthanized was low. The following results were obtained using data for all rats examined for neoplasms, including those killed at all times.

The following model provided the best description of the data:

$$\text{logit}[p(t)] = -13.8(\pm 1.4) + 0.0136(\pm 0.0021)t + 0.0023(\pm 0.0009)t\, I_{low} + 0.0049(\pm 0.0009)t\, I_{high},$$

where $p(t)$ is the prevalence of primary malignant and benign neoplasms at time t in days after the start of exposure; $\text{logit}[p(t)] = \log(p(t)/[1 - p(t)])$ is the logit function of the prevalence; I_{low} is an indicator function that is equal to one for animals exposed to the low concentration of either carbon black or diesel exhaust, and is equal to zero for all other animals; and I_{high} is similarly defined for animals exposed to the high concentrations of either pollutant.

The statistical power for detecting differences between neoplasm responses to diesel exhaust and carbon black was estimated by calculating the power for determining a 25% difference at both exposure levels (that is, response to carbon black was equal to 75% of the response to diesel exhaust, or, based on the above model, $0.0049 \times 0.75 = 0.0037$/day (neoplasm prevalence/day) at the high exposure and $0.0023 \times 0.75 = 0.0017$/day at the low exposure). These coefficients were used to simulate the tumor response in the study along with the Kaplan-Meier estimates of survival, the scheduled times when rats were killed, and the number of rats that were killed but were not studied histopathologically. Based on 250 simulations, the power was 72% at $\alpha = 0.05$ and 92% at $\alpha = 0.20$, which indicated good power for a small change in the slope of neoplastic prevalence.

Using a likelihood ratio test, we found that the coefficient for the low-level exposure was statistically significant ($p < 0.01$), indicating that the prevalences of lung neoplasms were significantly elevated in both low-exposure groups compared with the prevalence in the control group. We also found that neither the terms for the exposure material (diesel exhaust or carbon black) nor for gender were statistically significant. The lack of significance ($p = 0.86$) of the exposure material was shown by adding two additional terms to the above model, one for the change over time due to exposure to the low concentration of diesel exhaust or carbon black and one for the change over time due to exposure to the high concentration of diesel exhaust or carbon black. The lack of significance ($p = 0.12$) of gender was shown by adding an additional term to the above model for the change over time for female rats only. Models for the prevalence of malignant lung neoplasms, excluding the benign neoplasms, yielded similar results. These results suggested that there was no significant difference in carcinogenicity between genders or between carbon black and diesel exhaust at the same exposure levels. It should be noted that, although the model demonstrated no significant difference between genders, this was due in part to the large estimated error of the neoplastic prevalence in males late in the study. Figure 22 illustrates that the prevalences were clearly higher for females than for males during the later study intervals, but that the error bars for the male prevalences overlapped with the prevalences for females.

The lifetime incidence of lung neoplasms can also be modeled by logistic regression; for this, we assume that survival times were identical among exposure groups.

When the neoplasms have no effect on the time of death, the incidence of lung neoplasms, $\lambda(t)$, can be estimated from the prevalence by

$$\lambda(t) = -\frac{1}{1-p(t)} \times \frac{d}{dt} p(t),$$

where $(d/dt)p(t)$ is the derivative of neoplasm prevalence. Also, the lifetime incidence of neoplasms for a group can be estimated by

$$\int_0^T S(t)\lambda(t)dt,$$

where $S(t)$ is the survival of a group at time t, and t is when the rats were killed after the end of exposure. This incidence was estimated for each exposure group and for each gender. Separate estimates were made for each gender because of differences in survival between the genders; that is, male rats had lower estimated incidences because of their shorter survival time. The estimated lifetime neoplasm incidences calculated in this manner were 1.0% for males in the control group, 1.9% for females in the control group, 2.6% for males exposed to low carbon black, 9.0% for females exposed to low carbon black, 4.7% for males exposed to low diesel exhaust, 8.2% for females exposed to low diesel exhaust, 8.3% for males exposed to high carbon black, 25.9% for females exposed to high carbon black, 13.0% for males exposed to high diesel exhaust, and 26.5% for females exposed to high diesel exhaust. These estimated lifetime incidences were all clearly greater for females than for males.

The above lifetime lung neoplasm incidences modeled by logistic regression are compared in Table 9. The incidences for females and males are compared separately using two particle dose terms: the exposure concentration, and the weight-normalized lung burden. Neither dose term fully normalized the estimated incidences of carcinogenicity among groups. In females, the estimated incidence per unit of exposure concentration was higher for carbon black than for diesel exhaust for the low-level exposure, but the normalized incidences were similar for the high-level exposure. In males, the estimated incidence per unit of exposure concentration was higher for diesel exhaust than for carbon black at both levels. The estimated incidence for females per unit of weight-normalized lung burden was higher for carbon black than for diesel exhaust at both exposure levels, and incidences for males normalized in this manner were slightly higher for diesel exhaust at both levels.

Table 9. Comparison of Estimated Lifetime Incidence of Lung Neoplasms per Unit of Two Particle Dose Terms at 23 Months

		Particle Dose		Estimated Incidence		Net Incidence/Particle Dose	
Treatment Group	Gender	Exposure Concentration (mg/m^3)	Weight-Normalized Lung Burden[a] (mg/g)	Incidence[b] (%)	Net Incidence (% – C)[c]	Net Incidence/ Exposure Concentration ([% – C]/[mg/m^3])	Net Incidence/ Weight-Normalized Lung Burden ([% – C]/[mg/g])
Control	Female	0	0	1.9	0	–	–
	Male	0	0	1.0	0	–	–
Low carbon black	Female	2.46	13.9	9.0	7.1	2.9	0.51
	Male	2.46	12.4	2.6	1.6	0.7	0.13
High carbon black	Female	6.55	30.0	25.9	24.0	3.7	0.80
	Male	6.55	20.2	8.3	7.3	1.1	0.36
Low diesel exhaust	Female	2.44	29.2	8.2	4.3	1.8	0.15
	Male	2.44	22.7	4.7	3.7	1.5	0.16
High diesel exhaust	Female	6.33	64.9	26.5	24.6	3.9	0.38
	Male	6.33	45.4	13.0	12.0	1.9	0.26

[a] Lung burden was divided by the mean lung weight of the females or males in the control group to produce a normalized index of the concentration of particles in the lung (data in Appendix D.5.).

[b] Lifetime incidence of lung neoplasms estimated by logistic regression modeling.

[c] C = value for control group.

In summary, the above results indicate that the occurrence of lung neoplasms was significantly elevated at both the low and high exposures of both carbon black and diesel exhaust. The overall expressions of pulmonary carcinogenicity were similar in rats exposed to carbon black and diesel exhaust, both in the types of neoplasms observed and in the portions of rats with neoplasms. A larger portion of females than males had neoplasms regardless of exposure material. Although multiple neoplasms were induced in some rats by both carbon black and diesel exhaust, the multiplicity was slightly greater among rats exposed to diesel exhaust. There was little difference between the carcinogenicities of carbon black and diesel exhaust per unit of exposure concentration, but carbon black was more carcinogenic than diesel exhaust per unit of lung burden.

Growth of Transplanted Neoplasms

Most lung masses suspected of being neoplasms were too small to accommodate both histopathology and transplantation. A total of 49 suspect lung masses were transplanted into nude (athymic) mice, 29 from rats exposed to carbon black and 20 from rats exposed to diesel exhaust (Table 10). After the primary masses were classified by microscopy, we found that a total of 18 adenocarcinomas, 5 squamous cell carcinomas, and 26 squamous cysts had been transplanted. Neither the histologic appearance of the primary masses, nor the growth characteristics of the small number of transplanted portions of the primary mass, differed between carbon black and diesel exhaust exposure. Overall, 33% of the adenocarcinomas and 60% of the squamous cell carcinomas grew after transplantation. From this small sample, therefore, it appeared that squamous cell carcinomas had more aggressive growth characteristics than adenocarcinomas. None of the transplanted squamous cysts grew, further supporting the view that these lesions should not be classified as "neoplasms."

EFFECTS ON DNA

DNA Adducts in Alveolar Type II Cells

The effects of three-month exposures to diesel exhaust and carbon black on DNA adduct levels in lung type II alveolar epithelial cells of control rats, and rats exposed to both high diesel exhaust and high carbon black have been published in greater detail than presented here (Bond et al. 1990). Autoradiograms demonstrated the presence of several distinct adducts in DNA from both control and exposed rats. None of the adducts have been identified; however, none of the adduct spots comigrated with an authentic benzo[*a*]pyrene diolepoxide adduct standard. The chromatographic patterns were similar among the three groups, but there were fewer individual adduct spots (eight distinct spots) on the autoradiograms than observed in whole-lung DNA from rats exposed to diesel exhaust or carbon black in this (Randerath et al. 1994) and previous (Wolff et al. 1990) studies. The intensity of individual adduct spots varied among the groups, and some were not present in all groups.

Both diesel exhaust and carbon black exposures increased the levels of most individual adduct spots above those observed in the control group (Figure 23). Because a blurred zone of radioactivity near the chromatographic origin of the autoradiograms was not counted as an "adduct," the adduct levels may have represented underestimates of the true values. The total adduct levels were similar for the groups exposed to high carbon black and high diesel exhaust (20.1 and 23.2 per 10^9 bases, respectively), and both were significantly elevated above the adduct level in the control group (6.1 per 10^9 bases).

DNA Adducts in Lung Cells at Three Months

The results of the analyses of lung DNA adducts in three-month samples analyzed at ITRI are reported as part of the interlaboratory comparison described in the final report of the accompanying project, "Diesel Exhaust and

Table 10. Growth of Lung Neoplasms and Squamous Cysts in Athymic Mice

	Carbon Black			Diesel Exhaust			Total	
Primary Neoplasm	Number Implanted	Number Growing	Percent Growing	Number Implanted	Number Growing	Percent Growing	Number Implanted	Number Growing
Adenocarcinoma	8	2	25	10	4	40	18	6
Squamous cell carcinoma	2	1	50	3	2	67	5	3
Squamous cyst	19	0	0	7	0	0	26	0
Total	29	3	10	20	6	30	49	9

DNA Damage," by Dr. Randerath. In brief, the total adduct levels and 5 of the 16 individual adducts were increased in a dose-related manner at three months when compared with the control group. The mean ± SE total adduct level for the control group was 38 ± 2.4, for the group exposed to low diesel exhaust was 53 ± 3.9, and for the group exposed to high diesel exhaust was 83 ± 8.5. There was no dose-related increase in adduct levels in DNA from rats exposed to carbon black.

Chromosome Damage in Circulating Lymphocytes

No treatment-related differences in chromosome damage were found in blood lymphocytes from rats in the control group, or from rats exposed to high diesel exhaust or high carbon black and killed after three months of exposure. The assay was hampered by a small mitotic response of the cultured lymphocytes, which resulted in a small number of opportunities to observe damage. Only one chromosome aberration, a chromatid deletion, was found in the control group, and none were observed in either of the high-exposure groups. A preliminary assay of the frequency of micronuclei in binucleated lymphocytes found no difference between the control group and groups exposed to high diesel exhaust. Therefore, despite the low mitotic response, these results suggested that the high exposures did not cause observable chromosome damage. For this reason, the other three-month samples were not analyzed, and the assay was not repeated at later times when animals were killed.

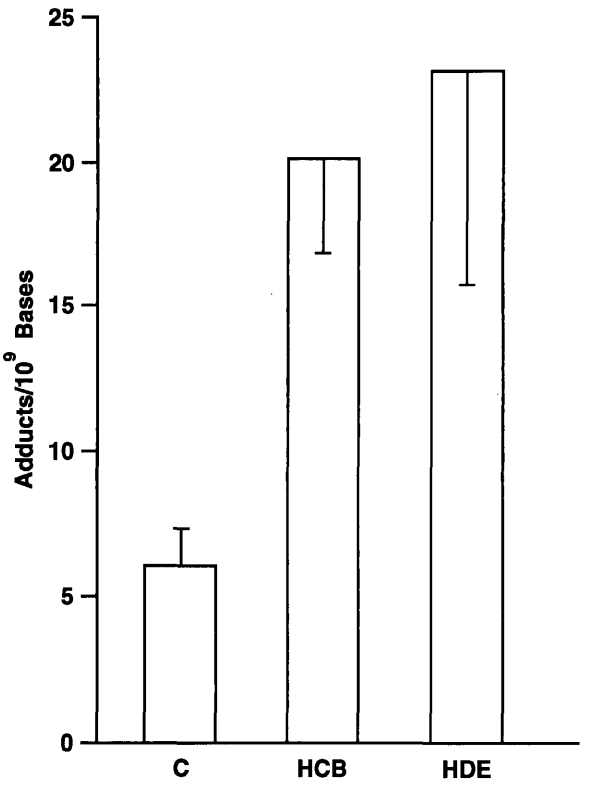

Figure 23. The total levels of DNA adducts in Type II alveolar epithelial cells from control rats and rats exposed to high carbon black and high diesel exhaust for three months. The bars represent the mean ± SE of four to five rats per group. The adduct levels of both exposed groups were significantly higher than that of the control group.

PARTICLE CLEARANCE AND TRANSLOCATION

Clearance of Radiolabeled Carbon Black

The diesel exhaust and carbon black exposures both caused dose- and time-related delays in clearing the [^{7}Be]CB tracer particles, as illustrated in Figure 24. Because the radiotracer particles were from the same lot of carbon black used for exposures, we assumed that their behavior would mirror the clearance behavior of the carbon black and diesel exhaust soot particles inhaled daily the same time periods during the exposure.

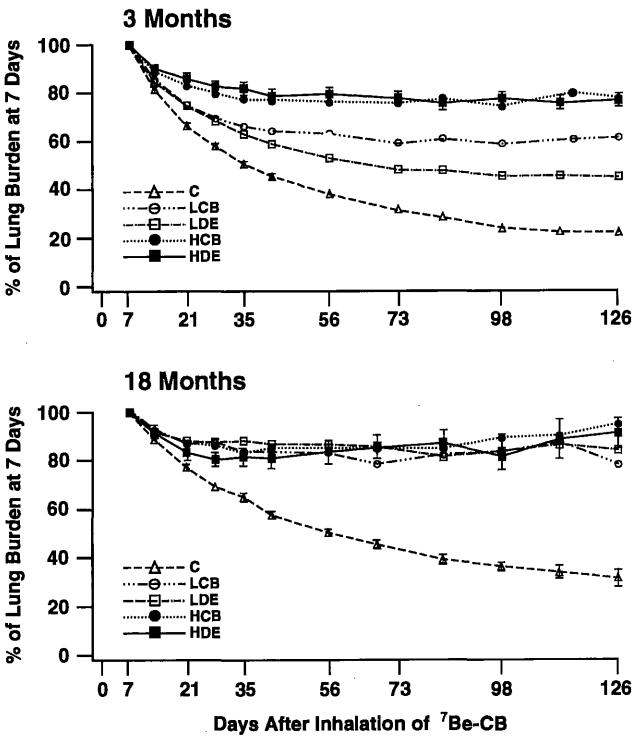

Figure 24. The clearance of radiolabeled carbon black particles from the lungs of rats exposed once by inhalation after three or eighteen months of chronic exposure. The whole-body radioactivity measured at seven days after inhalation of the tracer particles was assumed to be 100%. Data represent the mean ± SE group percentages corrected for the decay of the radiolabeled particles. For clarity, and because the degree of variability was similar among the groups, error bars are presented for only the control group and the group exposed to high diesel exhaust. The rats continued to be exposed during the 126-day counting period.

The patterns of clearance from lungs of rats in the control group were similar to those observed previously in healthy rats (Snipes 1989). Analysis of variance demonstrated a small, but significant, difference in clearance between females and males, with curves of data from females in all exposure groups showing slower clearance than data from males at three months, but faster clearance than data from males at 18 months. Because exposure effects were similar for females and males, the analysis of differences among exposure groups was performed using combined data. The curves of all exposed groups were significantly different from those of controls at both 3 and 18 months. At three months, however, the curves of the low-exposure groups were clearly separated from those of the high-exposure groups, but at 18 months, curves of all exposed groups were nearly identical.

Data for the magnitudes and slopes of the faster and slower components of the pulmonary retention parameters that result from fitting two-component negative exponential curves to the counting data are presented in Table 11. The slight increase from 43% to 55% between 3 and 18 months in the portion of clearance in the slower component of the control group was interpreted as an aging effect. At three months, there was a dose-related increase of the portion of clearance of the slower component in rats exposed to carbon black or diesel exhaust. The magnitude of this component was increased from 43% in controls to 59% and 76% in the groups exposed to low carbon black and high carbon black, respectively, and was increased to 47% and 79% in the groups exposed to low diesel exhaust and high diesel exhaust, respectively. There were proportionate decreases in the magnitudes of the faster components of the exposed groups. A striking finding was that the slope of the slower component was reduced to zero or near zero in all exposure groups. This suggested that long-term clearance was essentially stopped by exposure to carbon black and diesel exhaust at both exposure levels by three months of exposure. Despite the retarded long-term clearance in exposed rats, there were differences in overall clearance between the exposure levels of rats exposed to carbon black or diesel exhaust. Of the seven-day lung burden of tracer particles, at 126 days, 59% remained in the lungs of rats exposed to low carbon black, 43% in the lungs of rats exposed to low diesel exhaust, 76% in the lungs of rats exposed to high carbon black, and 75% in the lungs of rats exposed to high diesel exhaust.

The magnitudes and slopes of the clearance components of all exposed groups were similar at 18 months; no differences related to dose or exposure material were apparent. The faster clearance component represented only 12% to 19% of the total for the exposed groups, which was less than one-half the value for the control group. The magnitudes and slopes of the slower clearance components were also similar for the exposed groups. In contrast to the finding that only 31% of the seven-day lung burden of [^{7}Be]CB remained in the lungs of the control group at 126 days after inhalation, 82% to 86% remained in the lungs of the exposed groups. These findings suggest that the clearance of carbon black tracer particles was probably near maximally

Table 11. Pulmonary Clearance of [^{7}Be]CB Inhaled by Rats After 3 and 18 Months of Chronic Exposure

		Pulmonary Retention Parameters[a]				
		Faster Component		Slower Component		Percent of ^{7}Be
Month	Exposure Group	A_1	A_2	B_1	B_2	Retained on Day 126
3	Control	57.4	−0.049	42.6	−0.0061	21
	Low carbon black	40.8	−0.068	59.2	0	59
	High carbon black	24.0	−0.100	76.0	0	76
	Low diesel exhaust	53.0	−0.043	47.0	−0.00074	43
	High diesel exhaust	20.9	−0.083	79.1	−0.00040	75
18	Control	44.8	−0.037	55.2	−0.0051	31
	Low carbon black	18.5	−0.077	81.5	0	82
	High carbon black	13.9	−0.159	86.1	0	86
	Low diesel exhaust	11.7	−0.196	88.3	−0.00058	82
	High diesel exhaust	17.4	−0.171	82.6	0	83

[a] $A_1^{(A2t)} + B_1^{(B2t)}$, where t is time in days from day 7 after inhalation of [^{7}Be]CB. Two-component exponential equations forced A_1 plus B_1 to equal 100, and the slopes A_2 and B_2 were constrained from exceeding zero.

retarded in both high-exposure groups at three months, because there was little further change in these groups at 18 months, and that the clearance in both low-exposure groups was similarly retarded by 18 months.

Sequestration of Tracer Particles

The sites where fluorescent microspheres were located at different times after inhalation were summarized into four primary regions: alveolar, peribronchiolar, bronchiolar, and "other." The categories of microsphere localization within each of these main regions are described in detail in Appendix Table G.1. Data for the distribution of microspheres retained at 1, 4, 28, and 90 days after inhalation of microspheres after 3 and 18 months of chronic exposure are presented in detail in Appendix Tables G.2 and G.3, respectively. These data represent the percentages of microspheres observed at each time. The variability in the total number of microspheres observed and the lack of precise data for initial microsphere lung burdens precluded meaningful quantification of total microsphere retention.

Figures 25 and 26 illustrate the percentages of microspheres in three sites, single alveolar macrophages, aggregated macrophages in alveoli or in the alveolar interstitium, and "other," for the low and high exposure groups at three months, and Figures 27 and 28 present similar information obtained at 18 months. These figures do not include the 90-day sampling time because particle locations at that time were not different from those at 28 days after inhalation. Only small percentages of the particles were found in bronchiolar or peribronchiolar locations in any group at any time; most were in some compartment in the alveolar region. The majority of particles retained within the alveolar region were retained in either single alveolar macrophages or aggregated alveolar macrophages in alveolar lumens or in the interstitium; a small percentage was located outside of macrophages.

Figure 25 shows the distribution of the microspheres in the control group, and in both low-exposure groups at 1, 4, and 28 days after inhalation of the microspheres after three months of chronic exposure. The majority of microspheres were retained within single alveolar macrophages at all times in all three groups. However, by 28 days, 26% were in aggregated macrophages in the alveolar region in the group exposed to low carbon black. The difference between the low-exposure groups in the percentage of microspheres in aggregated versus single macrophages was significant.

At one day after inhalation, the majority of microspheres in the rats from low-exposure groups were in heavily-pigmented macrophages, and fewer were within macrophages with little carbon black or diesel exhaust soot. The distribution of location of the macrophages with microspheres

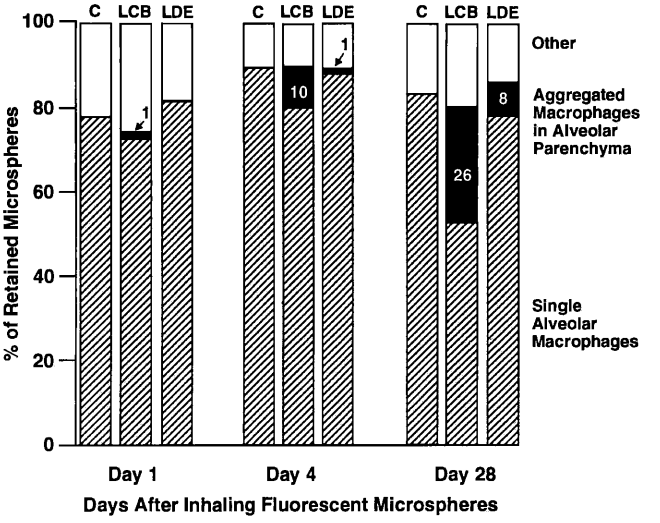

Figure 25. Percentages of retained microspheres in single alveolar macrophages, aggregated macrophages in the alveolar parenchyma, or in other sites for the control group and the groups exposed to low carbon black and low diesel exhaust that inhaled the microspheres after three months of chronic exposure. See Appendix G.1 for a listing of the categories of anatomic sites where microspheres were scored.

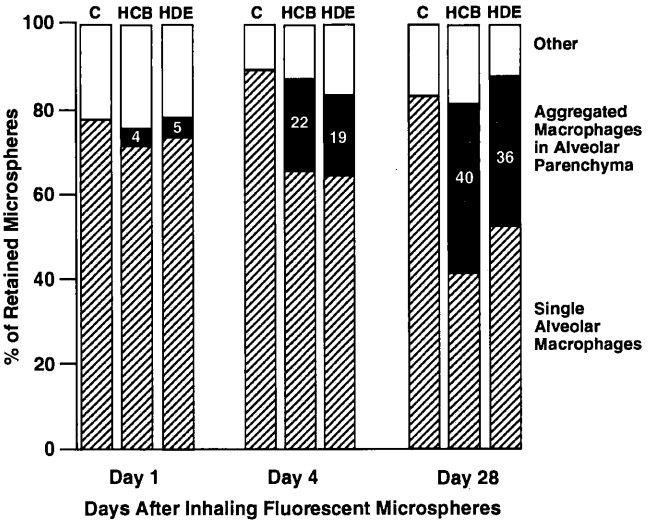

Figure 26. Percentages of retained microspheres in single alveolar macrophages, aggregated macrophages in the alveolar parenchyma, or in other sites for the control group and the groups exposed to high carbon black and high diesel exhaust that inhaled the microspheres after three months of chronic exposure. See Appendix G.1 for a listing of the categories of anatomic sites where microspheres were scored.

was similar to the distribution of all macrophages, and there was minimal tendency toward localization in the centriacinar region. In the control group, and in rats exposed to low carbon black and low diesel exhaust, 7%, 7%, and 4%, respectively, of the microspheres were located in the alveolar interstitium, but not in macrophages, and this was reduced to 2% to 4% at 28 days (data not shown).

These results suggest that, at the low exposure levels, most of the retained, newly inhaled particles would be retained in single alveolar macrophages for up to 90 days. These results coincided with the histopathologic and morphometric observations that most of the macrophages (or the volume of macrophages) were single alveolar macrophages at this time during the exposure. These results also suggest, however, that during the 28 days after inhalation, there was a tendency for greater retention of microspheres in the aggregated macrophages in the group exposed to low carbon black than in the group exposed to low diesel exhaust.

Figure 26 shows the distribution of the microspheres in the control group, and both high-exposure groups at 1, 4, and 28 days after inhalation of the microspheres after three months of chronic exposure. At one day, the majority of the microspheres retained in the rats in high-exposure groups were within heavily pigmented, single alveolar macrophages in the centriacinar region. Fewer microspheres were in less heavily pigmented macrophages or in macrophages outside the centriacinar region. In the rats exposed to high carbon black and high diesel exhaust, 8% and 6%, respectively, of the retained microspheres were in the alveolar interstitium outside of macrophages at one day after inhalation.

By 28 days after inhalation, 40% and 36% of the retained microspheres were in aggregated macrophages in the alveolar region in the groups exposed to high carbon black and high diesel exhaust, respectively, and these percentages were not significantly different. The histopathology showed that the macrophages tended to be localized, and therefore were apt to be aggregated, at the high exposure levels; however, the morphometry showed an approximately 10-fold greater volume fraction of the lung occupied by single than by aggregated macrophages. Taken together, these results suggest a preferential retention of the inhaled microspheres, along with carbon black or diesel exhaust soot, in aggregated macrophages. The portions of microspheres in alveolar interstitium outside of macrophages were reduced to 3% to 4% at 28 days.

Figure 27 shows the distribution of the microspheres in the control group, and both low-exposure groups at 1, 4, and 28 days after inhalation of the microspheres after 18 months

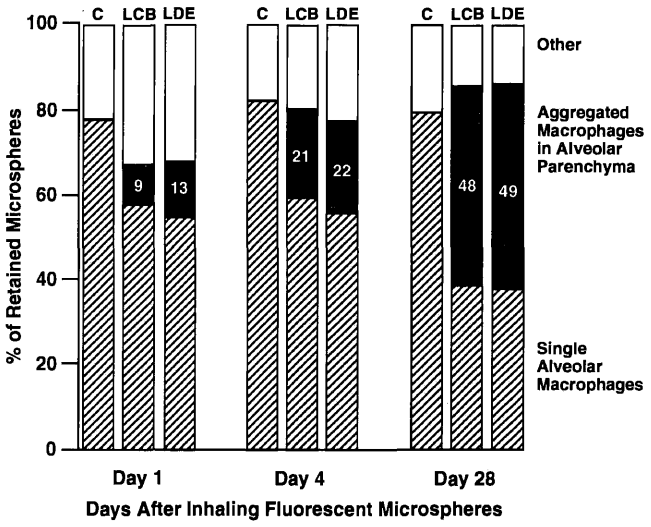

Figure 27. Percentages of retained microspheres in single alveolar macrophages, aggregated macrophages in the alveolar parenchyma, or in other sites for the control group and the groups exposed to low carbon black and low diesel exhaust that inhaled the microspheres after eighteen months of chronic exposure. See Appendix G.1 for a listing of the categories of anatomic sites where microspheres were scored.

of chronic exposure. At one day, slightly more than one-half of the retained microspheres were in single alveolar macrophages, a lower fraction than observed at one day after 3 months of chronic exposure. Of the inhaled microspheres, 9% and 13% were in aggregated macrophages in the alveolar region in the groups exposed to low carbon black and low diesel exhaust, respectively, and approximately 33% were in other sites. For the control group, and the groups exposed to low carbon black and low diesel exhaust, 10%, 20%, and 22%, respectively, of the retained microspheres were located outside of macrophages in the alveolar interstitium at one day after inhalation. This was reduced to 6% to 10% at 28 days. By 28 days after the inhalation of microspheres, 48% and 49% of the retained microspheres were in aggregated macrophages in the alveolar region in the groups exposed to low carbon black and low diesel exhaust, respectively, and less than 40% were in single alveolar macrophages in both of these groups. There were no significant differences between the low-exposure groups in the locations of microspheres.

Histopathology demonstrated a greater aggregation of macrophages at 18 months than at 3 months of chronic exposure, so the fact that more microspheres were in aggregated macrophages at day one after inhalation at 18 months than at 3 months reflected the differences in overall macrophage distribution. However, the increasing percentage of microspheres in aggregated macrophages during the sub-

sequent 28 days after inhalation at 18 months was probably not due solely to an increase in aggregated macrophages. Because the volume of aggregated macrophages would not have changed greatly during that time, the microsphere data suggest a preferential retention (less clearance) of microspheres in aggregated, than in single, macrophages.

Figure 28 shows the distribution of the microspheres in the control group, and both high-exposure groups at 1, 4, and 28 days after inhalation of microspheres after 18 months of chronic exposure. There were no significant differences between the fractional location of microspheres in the high-exposure groups of rats. At day one after inhalation, 22% and 23% of the microspheres were in aggregated macrophages in the alveolar region in the groups exposed to high carbon black and high diesel exhaust, respectively, and only approximately 40% were in single alveolar macrophages. The majority of microspheres were in heavily pigmented macrophages in the centriacinar region. Also, at day one, nearly 40% of the microspheres were in sites other than aggregated or single macrophages in the alveolar region. In groups exposed to high carbon black and high diesel exhaust, 25% and 22%, respectively, of the retained microspheres were located outside macrophages in the alveolar interstitium at one day after inhalation. This was reduced to 7% to 9% at 28 days.

By 28 days after inhalation, 52% and 48% of the retained microspheres were in aggregated macrophages in the alveolar region in the groups exposed to high carbon black and high diesel exhaust, respectively, and the fractions in single macrophages and other locations were reduced. As described above for the low-exposure groups, these results probably reflected less efficient clearance of microspheres from aggregated macrophages than from other locations.

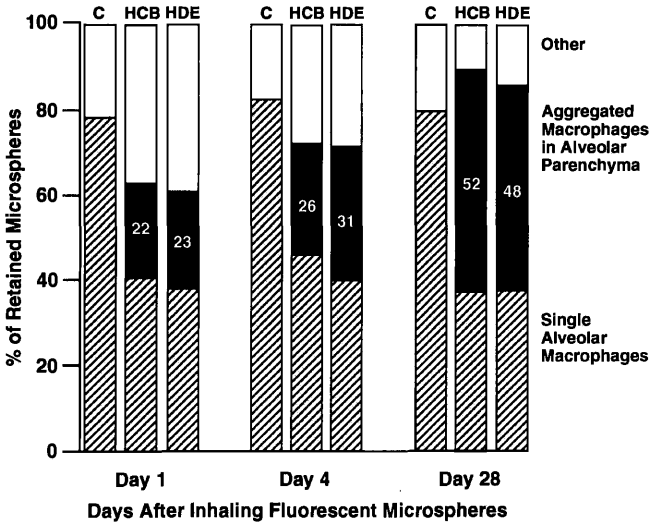

Figure 28. Percentages of retained microspheres in single alveolar macrophages, aggregated macrophages in the alveolar parenchyma, or in other sites for the control group and the groups exposed to high carbon black and high diesel exhaust that inhaled the microspheres after eighteen months of chronic exposure. See Appendix G.1 for a listing of the categories of anatomic sites where microspheres were scored.

In summary, for all groups and at all times, the majority of retained microspheres were found in macrophages in the alveolar region. The distribution of microspheres appeared to be similar to the distribution of carbon black or diesel exhaust soot. After 18 months of chronic exposure, more of the newly inhaled particles that were retained for 28 days were retained in aggregated macrophages in the alveolar parenchyma than were retained in single alveolar macrophages or in other sites in the lung. At one day after inhalation, 4% to 8% of retained microspheres were in the interstitium outside of macrophages in exposed rats at 3 months of chronic exposure, and 20% to 25% were in the same location in exposed rats at 18 months of chronic exposure. Other than the difference noted between the two low-exposure groups at three months, these results show that the location of retained microspheres, and thus, the pattern and kinetics of particle sequestration, were the same for rats exposed to carbon black and those exposed to diesel exhaust.

DISCUSSION AND CONCLUSIONS

ADEQUACY OF STUDY AND ITS RELATION TO PREVIOUS FINDINGS

The experimental design provided a good comparison of the pulmonary carcinogenicities of diesel exhaust and carbon black in rats exposed chronically to a high concentration of either material. The study was not designed to examine the full spectrum of dose-response relationships for lung disease induced by exposure to carbon black or diesel exhaust; both of the exposure levels used were very high. Even the low exposures, particularly to carbon black, met the common definition of "maximum tolerated dose" because of the effects on body weight and survival. The group sizes and the exposure time allowed for an adequate evaluation of carcinogenesis. The two particle exposure concentrations produced dose-related significant increases in lung neoplasms in both groups exposed to carbon black or diesel exhaust at incidences low enough to allay concern that the responses might have been saturated.

The study did not directly test the importance of soot-associated mutagenic organic compounds in the lung cancer response of rats to inhaled diesel exhaust. However, to the extent that the carbon black used is accepted as a surrogate for organic-free diesel exhaust soot, the importance of the organic fraction of diesel exhaust soot was tested indirectly.

The carbon black had a small amount of organic material when extracted using procedures commonly applied to diesel soot. No attempt was made to exhaustively extract the carbon black using the high temperatures and extraction times of over 100 hours that are required to remove tenacious organic residues (Locati et al. 1979; International Agency for Research on Cancer 1983). It is believed, although certainly not proven in this study, that the small amount of tightly bound organic residue that could be removed in this manner would have little, if any, bioavailability. Considering both the mass fraction of solvent-extractable matter and its mutagenicity, the mutagenic organic content of the carbon black was 2.6 or 5.5×10^{-3} (depending on bacterial strain) times greater than that of the diesel exhaust soot, when measured using methods commonly applied to diesel exhaust soot. If the organic residues on carbon black are presumed to have played a role in its carcinogenicity, it must also be presumed possible that similar organic residues on diesel exhaust soot might have played a role in its carcinogenicity. Overall, this study provided a reasonable indirect evaluation of the likely importance of the more easily extracted organic fraction of diesel exhaust soot in its pulmonary toxicity in rats exposed to high concentrations of soot.

This study reproduced both the neoplastic and nonneoplastic pulmonary effects of diesel exhaust that have been observed repeatedly in chronically exposed rats (Mauderly 1992). No adverse effect of diesel exhaust exposure was observed that could not have been predicted. The key finding of this study was that the effects of carbon black were nearly the same as those of diesel exhaust under the same exposure conditions. These findings demonstrated clearly that chronic inhalation exposure to organic-poor, fine carbon particles having a size and specific surface area generally similar to those of diesel exhaust soot can cause lung cancer in rats under conditions of heavy lung loading. This concurs with previous reports that activated carbon (Kawabata et al. 1986) and carbon black (Heinrich et al. 1994; Pott et al. 1994) that have higher specific surface areas than the carbon black in this study are carcinogenic in rat lungs when instilled or given by inhalation.

COMPARATIVE NONNEOPLASTIC TOXICITIES OF DIESEL EXHAUST AND CARBON BLACK

The nonneoplastic responses to carbon black and diesel exhaust exposure were qualitatively and quantitatively similar and were also similar to those reported previously for rats chronically exposed to diesel exhaust (Mauderly 1992). The magnitudes of most responses to both materials were generally related to particle exposure concentrations, with some parameters reflecting the greater lung burdens of diesel exhaust soot than of carbon black. Logistic regression modeling demonstrated several significantly greater nonneoplastic responses in rats exposed to diesel exhaust than in rats exposed to carbon black, and in females than in males.

The small, but significant, effects on survival appeared primarily in males, and the survival of males was shortened slightly more by carbon black than by diesel exhaust. The life-span shortening of both females and males was greater in the group exposed to high carbon black than in the group exposed to low diesel exhaust. Body weight was significantly reduced relative to exposure concentration, with the group exposed to high diesel exhaust most affected. The reductions in body weight for both females and males were greater in the groups exposed to high carbon black than in the groups exposed to low diesel exhaust. Increases in lung weight were related to exposure concentrations of both carbon black and diesel exhaust, but the increase was greater in the group exposed to high carbon black than in the group exposed to low diesel exhaust. Bronchoalveolar lavage indicators of inflammation and cytotoxicity were elevated in all groups relative to exposure concentration and lung burden of particles. Elevations of cell counts were similar for the groups exposed to high carbon black and low diesel exhaust, but enzymes and protein were elevated more in the group exposed to low diesel exhaust. The incidences and severities of histopathological scores tended to be related to the lung burden of particles, and responses of the group exposed to high diesel exhaust were greater than those of the group exposed to high carbon black. However, both the incidences and severities tended to be slightly higher for the group exposed to high carbon black than for the group exposed to low diesel exhaust, which had a similar lung burden. Clearance of radiolabeled carbon black was delayed relative to dose at three months, but the clearance of particles in all exposed groups was similarly impaired at 18 months. At three months, the tracer particle clearance of the group exposed to high carbon black was more severely impaired than that of the group exposed to low diesel exhaust, but all exposed groups were similarly impaired at 18 months. At three months, greater percentages of retained microspheres were found in aggregated macrophages in the group exposed to high carbon black than in the group exposed to low diesel exhaust, but there was little difference between the retention patterns of these two groups at 18 months.

The above findings indicate that overall, the nature and magnitude of nonneoplastic responses to diesel exhaust and carbon black were very similar at similar exposure

concentrations. However, carbon black generally produced greater effects than diesel exhaust per unit of particle lung burden.

COMPARATIVE CARCINOGENICITIES OF DIESEL EXHAUST AND CARBON BLACK

The findings clearly demonstrated that the carcinogenic responses to diesel exhaust and carbon black exposure were very similar. Exposures to carbon black and diesel exhaust caused dose-related increases, of similar magnitudes, in the incidences and prevalences of the same types of malignant and benign lung neoplasms. Neoplasms of a given type produced by carbon black and diesel exhaust exposure had similar growth characteristics when transplanted. Logistic regression modeling did not demonstrate significant differences between the neoplastic responses to carbon black and diesel exhaust at either exposure level. The numbers of rats exposed to carbon black that had neoplasms tended to increase slightly earlier than the numbers of tumor-bearing rats exposed to diesel exhaust. The crude incidences, the lifetime incidences estimated by logistic regression, and the multiplicity of neoplasms tended to be slightly higher among rats exposed to diesel exhaust than among rats exposed to carbon black, but the differences were small. Lung neoplasms were more prevalent among females than among males exposed to both materials, although the error of the estimates based on smaller numbers of males at later times prevented the gender difference from being statistically significant by logistic regression modeling. Carcinogenicity appeared more closely related to exposure concentration than to lung burden of particles, as reflected by the occurrence of more lung neoplasms in the group exposed to high carbon black than in the group exposed to low diesel exhaust.

IMPORTANCE OF THE ORGANIC FRACTION OF SOOT IN THE PULMONARY CARCINOGENICITY FOUND IN RATS EXPOSED TO HIGH CONCENTRATIONS OF DIESEL EXHAUST

The usefulness of the results of this study for estimating the importance of the organic fraction of diesel exhaust soot relies upon the assumption that carbon black served as a surrogate for diesel exhaust soot with no, or at least a markedly reduced, content of genotoxic organic compounds. This assumption, in turn, implies that organic residues that were not removed by the repeated soxhlet extraction procedure used in this study would not have played a significant role in carcinogenicity. Although these assumptions appear reasonable, they were not proven by this study.

Overall, carbon black was not significantly less carcinogenic than diesel exhaust in F344/N rats exposed chronically at the high rates used in this study. Based only on this comparison, the organic fraction of diesel exhaust soot appeared to have had little importance in the expression of neoplasia. If the organic fraction of diesel exhaust soot played any role at all in the subtle differences between the expressions of lung neoplasia in the groups exposed to diesel exhaust and carbon black, the degree of its influence was at least very small in comparison to the three orders of magnitude difference between the mutagenic organic contents of carbon black and diesel exhaust soot measured using procedures commonly used in studies of diesel exhaust. Alternate hypotheses may include one or more of the following.

1. Neoplasms caused by diesel exhaust and carbon black have different pathogenesis. The soot-associated organic compounds play an important role for diesel exhaust, but the pathogenesis of neoplasms induced by carbon black exposure does not involve organic mutagens.
2. The tightly bound organic residues on carbon black play an important role in the pathogenesis of neoplasms induced by carbon black exposure, and produce the same outcome as the more readily extracted organic compounds in diesel exhaust soot.
3. Neoplasia induced by diesel exhaust is caused by tightly bound organic compounds that parallel the effect of the tightly bound organic residues on carbon black, but the more readily extracted organic material in diesel exhaust soot plays a minor role in carcinogenesis.

This study did not disprove any of the above alternate hypotheses. However, the hypothesis indicated as most likely by the present findings is that the organic fraction of diesel exhaust soot, extracted and studied in the manner giving rise to the hypothesis that organic mutagens initiate carcinogenesis induced by diesel exhaust exposure in rats, did not play an important role in the carcinogenesis observed in this study.

PATHOGENESIS OF LUNG NEOPLASMS IN RATS EXPOSED CHRONICALLY TO DIESEL EXHAUST AND CARBON BLACK

The pathogenesis of the pulmonary neoplasms observed in rats exposed to high concentrations of particles in this and other studies of diesel exhaust and carbon black is unknown. The theory that initiation by particle-associated organic mutagens is not necessarily involved in the rat's response to diesel exhaust has been evolving during the

past few years based, in large part, on the growing data base showing similar responses induced by chronic exposure to high concentrations of a wide range of organic-poor particles (Mauderly 1993a). A prevalent view at present is that a range of respirable particles having different physical and chemical characteristics, but generally low cytotoxicity, may cause lung neoplasms by similar mechanisms in rats exposed to high concentrations of particles. The mechanisms by which a variety of dusts might induce similar types of pulmonary neoplasms in rats with accompanying chronic inflammatory, proliferative, and fibrotic responses are unknown, but the range of possible pathogenetic mechanisms that have been identified was recently reviewed (Lechner and Mauderly 1993). Several potential pathways involve increased cell division caused by the release of cytokines and growth factors from activated macrophages, the presence of which is universal in rat lungs during heavy, repeated exposure to dusts (Ames and Gold 1990; Driscoll et al. 1990; Morrow 1992). Other potential pathways involve genotoxicity and other forms of cell injury caused by active oxygen species released by phagocytes and other cells (Cerutti 1985). Still other potential pathways involve perturbations of cell division caused by the physical presence of particles in cells.

The possibility that exposure-induced alterations of DNA adduct levels played a role in the carcinogenicity in this study cannot be completely dismissed. Both diesel exhaust and carbon black altered levels of at least some whole lung and type II cell DNA adducts at some time during the exposure, although there was no evidence that new adducts were formed. Changes in whole-lung DNA adduct levels are described in detail in Part II of this Research Report, "Diesel Exhaust and DNA Damage," by Randerath and coworkers. The more limited results from analysis of the three-month, whole-lung DNA samples at ITRI were generally consistent with the detailed results of Randerath and associates. It is possible that the effects of DNA alterations induced in rats by endogenous, or ubiquitous compounds might be amplified by the proliferative influence of heavy particle exposures.

These findings, in concert with the carcinogenicity results, suggest some interim conclusions regarding the possible role of organic compounds and lung DNA adducts in lung neoplasms induced by carbon black and diesel exhaust exposure in rats. First, the repeated deposition of fine, high-surface-area, carbonaceous particles in the lungs of rats alters DNA in lung cells, perhaps regardless of the organic content of the particle. The adducts changed have not been identified, but appear to be present in unexposed rats.

Second, the possibility that the adduct changes caused by diesel exhaust and carbon black did, in fact, play a role in carcinogenesis cannot be completely dismissed at this time. It is not known which, if any, cell characteristics or functions are affected by changes in levels of these adducts; neither is the sequence of events leading to formation of the neoplasms fully understood. The failure to observe novel exposure-related adducts strongly suggests that the adduct changes played little or no role in carcinogenesis. The possibility exists, however, that one or more of the DNA alterations observed in rats exposed to diesel exhaust or carbon black comprised a common step in the pathogenesis of neoplasms. If this is true, the possibility also exists that the same mechanism might play a role in the carcinogenicity of other inhaled materials.

Third, because the changes in DNA adduct levels differed somewhat between rats exposed to diesel exhaust and those exposed to carbon black, the possibility that diesel exhaust soot–associated organic compounds might have played a role in the adduct changes cannot be entirely dismissed at this time.

In summary, the results of this study strongly suggest, but certainly do not prove, that the soot-associated organic compounds did not play an important role in the carcinogenicity of diesel exhaust. Although this study does not resolve the mechanisms by which the lung neoplasms induced by diesel exhaust or carbon black were produced in the rats, the findings support the notion that similar responses can be caused by different particles under similar conditions that include exposure to high concentrations of particles.

IMPLICATIONS OF FINDINGS FOR UNDERSTANDING POTENTIAL HUMAN LUNG CANCER RISK FROM DIESEL EXHAUST

This study was designed to improve our understanding of the pulmonary neoplastic response of rats to heavy, chronic exposures to diesel exhaust. It added little new information to our understanding of the relationships among exposure, dose, and response for lung cancer induced by diesel exhaust exposure in rats, except to reinforce previous findings of carcinogenicity at high levels of exposure. In this view, the present study added little to our ability to predict unit risks for lung cancer in humans from rat bioassay data, and does not suggest changes from previous estimates of risk derived from rodent data.

This study addressed, indirectly, the likely role of diesel exhaust soot–associated organic compounds in the pathogenesis of the rat lung neoplasms, but it is not clear that the conclusions suggested by the results can be transferred to

human risk estimates. As described above, the present epidemiological data base suggests a small increase in risk of lung cancer among humans with occupational exposure to high concentrations of diesel exhaust (Mauderly 1992). However, it is not likely that the estimated exposure levels for the individuals in the populations studied caused the degree of lung loading with soot and the accompanying nonneoplastic lung disease that occurred in groups of rats with significantly increased lung cancer incidences. Humans may be exposed for many years longer than is possible for rats; and it is not certain if the mechanisms by which the shorter-term, higher-level exposures of rats induce lung neoplasms might parallel the mechanisms in humans that might cause lung cancer from diesel exhaust exposure. Indeed, confirmation that soot-associated genotoxic organic compounds do not play an important role in the response of rats would not ensure that these compounds play no role in potential human carcinogenesis.

Present data suggest that both the nonneoplastic and neoplastic effects of heavy, chronic exposures to diesel exhaust soot and other particles differ between rats and other animals (Mauderly 1994a,b). The available data suggest that mice and Syrian hamsters are less susceptible than rats to the development of particle-induced lung neoplasms. Moreover, the limited comparisons possible between the nonneoplastic responses of rat and human lungs to heavy dust loading suggest that the alveolar epithelial proliferative and metaplastic responses prevalent in dust-exposed rats are not typical of human responses (Mauderly 1994a). The greatest body of information on human lungs exposed to high concentrations of dust comes from coal miners, in whose lungs dust has accumulated in amounts similar to the loading of lungs of rats exposed to high concentrations of particles, but in whom epithelial hyperplasia, metaplasia, and neoplasia have not been characteristic (Mauderly 1994a). In summary, at this time, it is not known which, if any, animal species serves as a useful model for the responses of human lungs to heavy, chronic dust exposure.

ADVANCEMENTS IN UNDERSTANDING THE SEQUESTRATION OF INHALED PARTICLES

The information provided by this study on the anatomic location of tracer particles with time after inhalation, during continued chronic exposure to diesel exhaust and carbon black, is unique and has provided several useful insights. First, inhaled particles are incorporated into macrophage aggregates quite rapidly in heavily-loaded lungs. At 1 day after the microspheres were inhaled after 18 months of chronic exposure, 9% to 13% and 23% were found in aggregated macrophages in the low and high exposure groups, respectively. These results suggest that either (1) up to approximately 20% of macrophages that ingest particles move rapidly into aggregates, or (2) up to approximately 20% of the particles are ingested by macrophages already in aggregates, or (3) both factors played a role. It must be considered that these findings are also undoubtedly influenced by the greater clearance of single than of aggregated macrophages during the first day after particle deposition.

On the other hand, the results show that no more than approximately one-half of the particles inhaled during chronic exposure and retained 28 days later are retained in aggregated macrophages. There was a dose-related retention of microspheres in aggregated macrophages at 28 days after inhalation of microspheres at 3 months, ranging from 8% to 26% for the low exposure group and 36% to 40% at the high exposure group. At 18 months, the percentages of microspheres retained at 28 days after inhalation were similar for all exposed groups, ranging from 48% to 52%. These results suggest that only one-half of the 28-day-old particles in the lungs of rats chronically exposed to high concentrations of particles are in macrophage aggregates. The other one-half is in other locations; of this half, approximately two-thirds (one third of the total) are still in single alveolar macrophages.

Finally, the results indicate that 4% to 8% (after 3 months of chronic exposure) and 20% to 25% (after 18 months of chronic exposure) of newly inhaled particles retained at 1 day after inhalation of particles were located in the alveolar interstitium outside of macrophages. Considering that overall particle clearance was slowed markedly, this represents an interestingly large fraction of deposited material located in the interstitium as apparently free particles soon after exposure. It is not known if these particles were phagocytized by mobile cells, transported into the interstitium and released, or if they moved through the epithelium as free particles. Regardless, this finding suggests that, in rats exposed to high concentrations of particles for long times, the alveolar interstitium receives a considerable exposure to fresh particles that are not enclosed in macrophages.

CURRENT INFORMATION NEEDS

This study filled an important information need by suggesting that the organic material associated with diesel exhaust soot may be of little importance in the carcinogenicity of diesel exhaust in rats. It also emphasized the importance of a better understanding of the mechanisms by which diesel exhaust, carbon black, and other poorly soluble particles cause lung cancer in rats. This information

is of more than academic interest; it is critical to our judgment of the utility of such bioassay data for estimating lung cancer risks to humans. The finding that the mutagenic organic fraction of diesel exhaust is not important in the response of rats does not necessarily mean that the response of rats has no relation to carcinogenic responses of humans.

One important route of experimentation to resolve this issue is to determine the similarity between molecular changes in lung neoplasms and proliferative lesions in rats that develop neoplasms in response to diesel exhaust, carbon black, and other particles, and the molecular changes in human lung cancers and proliferative lesions. This can begin at the level of screening for expression of oncogenes and tumor suppressor genes thought important in human lung cancer. The full resolution of the issue, however, will only come as our understanding of the carcinogenic process evolves. Regardless of our incomplete understanding of carcinogenesis, the demonstration of similar molecular changes in rat and human neoplasia would support the relevance of the rat responses to human cancer risk.

As described recently (Mauderly 1994a), another route of inquiry that would help define the usefulness of rat inhalation bioassays of respirable particles would be to compare rat and human responses to dusts for which a substantive data base exists for humans exposed to high particle concentrations. Perhaps the best opportunity is coal dust, for which a large epidemiological data base for workers with known, heavy exposures exists. It is generally thought that coal dust exposures, even those producing obvious pneumoconiosis, are not associated with a significant increase in risk of lung cancer. If this is true, then it would be useful to know the dose-response relation for lung cancer in rats exposed to coal dust. Surprisingly, there is not an adequate data base for rats exposed to coal dust. The most extensive and recent investigation conducted by the National Institute for Occupational Safety and Health (Lewis et al. 1989) involved only a single exposure concentration, 2 mg/m^3 of respirable dust, which resulted in a small particle lung burden. The crude incidence of malignant and benign lung neoplasms was not significantly elevated in the rats exposed to coal dust, or in rats exposed in the same study to diesel exhaust at the same particle concentration. A good examination of the relevance of the rat bioassay to human lung cancer risk from inhaled particles would be to compare the carcinogenicity and lung burden data from coal miners with the carcinogenicity results from a modern bioassay of rats exposed to coal dust, including multiple exposure concentrations and measurements of particle lung burdens.

A third investigative path that would help our understanding of the relevance of data from rat inhalation bioassays of respirable particles is the study of particle retention and clearance in humans exposed to dust. Not only are there few data for the quantities of particles retained in the lungs of workers exposed to high concentrations of dust, there also is little information on the degree to which particle clearance is overloaded in these populations. It would be of great interest to know if particle clearance is impaired, and to have data for particle retention kinetics in workers with ongoing dust exposures. This would require that populations be identified and tracer techniques be employed.

Finally, the most important information needed to resolve the health risks of human exposures to diesel exhaust is better epidemiological information on health effects in populations with known exposures. The opportunities for obtaining this information are limited, and the low level of effects predicted at current exposure levels may preclude clear definition even if populations with defined exposures were identified. Regardless, any such opportunity should be pursued.

IMPLICATIONS OF FINDINGS

The key implication of the results of this study is the strong suggestion that the organic compounds associated with soot might play little or no role in the pulmonary carcinogenicity of diesel exhaust in rats exposed to high concentrations of particles. While not confirmed by this study, the lack of role of the organic fraction implied by the results has several interrelated implications.

The question about the role of soot-associated organic mutagens in the rat lung neoplastic response to diesel exhaust raised by this study suggests that caution should be used in extrapolating from the carcinogenicity in rats exposed to high concentrations of diesel exhaust to predict on the basis of chemical carcinogenesis the possible carcinogenicity in humans exposed to diesel exhaust at lower rates. In particular, it does not appear reasonable to use the delivered dose of soot-associated organic compounds as the comparative dose term for extrapolation. Although it is possible that soot-associated organic compounds may contribute to lung cancer in humans exposed to diesel exhaust, they may not contribute to the response in rats.

The present results contribute to the growing doubts about the validity of extrapolating from the carcinogenicity in rats exposed chronically to particles at high exposure rates to estimates of risk of carcinogenicity in humans exposed at low rates or for short times using any comparative dose term. A logical comparative dose term would seem to be the lung burden of particles. It appears likely, but is not certain, however, that there is a threshold related

to clearance overload for the relationship between lung burden and carcinogenesis in rats, but it is very uncertain whether or not a similar threshold might exist for humans. Unit risks of similar magnitude have been calculated for lifetime exposures of rats and humans using exposure concentration as the comparative dose term for linking bioassay and epidemiological data (Mauderly 1992). The similarity of these estimates lends the greatest confidence to the usefulness of extrapolating the rat results to humans, but this confidence is eroded by the weakness of the exposure data for humans and the uncertainty about the similarity of the pathogenesis of the cancers in rats and humans.

Another implication of the present results is the concern they raise for the potential human lung cancer risk from inhaled fine carbon particles. It would be useful to have dose-response information from rats exposed at lower rates, and to examine closely the data from workers exposed chronically to carbon black or similar particles to evaluate the potential for carcinogenicity among workers. Published data (Smith and Musch 1982) suggest that the higher time-weighted average exposures of workers in the carbon black industry are under 0.5 mg/m^3, and that elevated rates of lung cancer have not been identified in association with occupational exposures to carbon black. Close evaluation of data from carbon black workers might help place the results from rats in a clearer context, particularly if additional human exposure data have been collected. Concerning environmental exposures, the present results raise the possibility that fine airborne carbon should be a special subclass of concern among airborne particulate matter.

Finally, the results of this study, in concert with the growing data base from other studies, suggest the need to search for opportunities to more directly compare responses of rat and human lungs to heavy particle exposures. The most extensively documented responses of human lungs to heavy dust loading are those that have occurred in coal miners. There appears to be an opportunity to fill an information gap and "calibrate" the general responses of animals to those of humans by conducting exposure-dose-response studies of multiple species exposed to coal dust.

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REFERENCES

Adamson IY, Bowden DH. 1974. The type 2 cell as progenitor of alveolar epithelial regeneration. Lab Invest 30:35–42.

Ames BN, Gold LS. 1990. Too many rodent carcinogens: Mitogenesis increases mutagenesis. Science 249:970–971.

Ames BN, McCann J, Yamasaki E. 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test. Mutat Res 31:347–364.

Bechtold WE, Dutcher JS, Mokler BV, Lopez JA, Wolf I, Li AP, Henderson TR, McClellan RO. 1984. Chemical and biological properties of diesel exhaust particles collected during selected segments of a simulated driving cycle. Fundam Appl Toxicol 4:370–377.

Belinsky SA, Foley JF, White CM, Anderson MW, Maronpot RR. 1990. Dose-response relationship between O^6-methylguanine formation in clara cells and induction of pulmonary neoplasia in the rat by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Cancer Res 50:3772–3780.

BMDP Statistical Software. 1983. Description of Groups [strata] with Histograms and Analysis of Variance, Version 1987. BMDP Statistical Software, Los Angeles, CA.

Bond JA, Johnson NF, Snipes MB, Mauderly JL. 1990. DNA adduct formation in rat alveolar type II cells: Cells potentially at risk for inhaled diesel exhaust. Environ Mol Mutagen 16:64–69.

Bond JA, Wolff RK, Harkema JR, Mauderly JL, Henderson RF, Griffith WC, McClellan RO. 1988. Distribution of DNA adducts in the respiratory tract of rats exposed to diesel exhaust. Toxicol Appl Pharmacol 96:336–346.

Bond JA, Mitchell CE, Li AP. 1983. Metabolism and macromolecular covalent binding of benzo[*a*]pyrene in cultured Fischer-344 rat lung type II epithelial cells. Biochem Pharmacol 32:3771–3776.

Brain JD, Gehr P, Kavet RI. 1984. Airway macrophages. Am Rev Respir Dis 129:823–826.

Brightwell J, Fouillet X, Cassano-Zoppi AL, Bernstein D, Crawley F, Duchosal F, Gatz R, Perczel S, Pfeifer H. 1989.

Tumours of the respiratory tract in rats and hamsters following chronic inhalation of engine exhaust emissions. J Appl Toxicol 9:23–31.

Brooks AL, Li AP, Dutcher JS, Clark CR, Rothenberg SJ, Kiyoura R, Bechtold WE, McClellan RO. 1984. A comparison of genotoxicity of automotive exhaust particles from laboratory and environmental sources. Environ Mutagen 6:651–668.

Brooks AL, Clark CR, Royer RE. 1979. Mutagenic characterization of particulate materials in diesel exhaust. In: Inhalation Toxicology Research Institute Annual Report, 1978-1979, LF-69, pp 237–238. Inhalation Toxicology Research Institute, Albuquerque, NM.

Cheng YS, Yeh HC, Mauderly JL, Mokler BV. 1984. Characterization of diesel exhaust in a chronic inhalation study. Am Ind Hyg Assoc J 45:547–555.

Cerutti PA. 1985. Prooxidant states and tumor promotion. Science 227:375–381.

Clark CR, Vigil CL. 1980. Influence of rat lung and liver homogenates on the mutagenicity of diesel exhaust particulate extracts. Toxicol Appl Pharmacol 56:110–115.

Dinse GE. 1985. Testing for a trend in tumor prevalence rates: I. Nonlethal tumors. Biometrics 41:751–770.

Dinse GE, Lagakos SW. 1983. Regression analysis of tumor prevalence data. Appl Stat 32:236–248.

Driscoll KE, Maurer JK, Crosby LL. 1990. Overload on lung clearance is associated with activation of alveolar macrophage tumor necrosis factor and fibronectin release. J Aerosol Med 3(Suppl. 1):83–92.

Evans MJ, Cabral LJ, Stephens RJ, Freeman A. 1973. Renewal of alveolar epithelium in the rat following exposure to NO_2. Am J Pathol 70:175–182.

Garshick E, Schenker MB, Munoz A, Segal M, Smith TJ, Woskie SR, Hammond KS, Speizer FE. 1987. A case-control study of lung cancer and diesel exhaust exposure in railroad workers. Am Rev Respir Dis 135:1242–1248.

Garshick E, Schenker MB, Munoz A, Segal M, Smith TJ, Woskie SR, Hammond KS, Speizer FE. 1988. A retrospective cohort study of lung cancer and diesel exhaust exposure in railroad workers. Am Rev Respir Dis 137:820–825.

Gupta RC. 1984. Nonrandom binding of the carcinogen *N*-hydroxy-2-acetylaminofluorene to repetitive sequences of rat liver DNA in vivo. Proc Natl Acad Sci USA 81:6943–6947.

Hahn FF, Herbert RA, Gillett NA, Guilmette RA, Rebar AH, Singh G. 1989. Pathogenesis of alpha radiation–induced lung cancer in the rat: Results to 450 days after inhalation exposure. In: Inhalation Toxicology Research Institute Annual Report, LMF-126, pp. 121–122. Lovelace Biomedical and Environmental Research Institute, Albuquerque, NM.

Harrington DP, Flemming TR. 1982. A class of rank test procedures for censored survival data. Biometrika 69:553–566.

Heinrich U, Dungworth D, Pott F, Schulte A, Peters L, Dasenbrock C, Levsen K, Koch W, Creutzenberg O. 1994. The carcinogenic effects of carbon black particles and tar/pitch condensation aerosol after inhalation exposure of rats. Ann Occup Hyg (in press).

Heinrich U, Muhle H, Takenaka S, Ernst E, Fuhst R, Mohr U, Pott F, Stöber W. 1986. Chronic effects on the respiratory tract of hamsters, mice and rats after long-term inhalation of high concentrations of filtered and unfiltered diesel engine emissions. J Appl Toxicol 6:383–395.

Henderson RF, Benson JM, Hahn FF, Hobbs CH, Jones RK, Mauderly JL, McClellan RO, Pickrell JA. 1985. New approaches for the evaluation of pulmonary toxicity: Bronchoalveolar lavage fluid analysis. Fundam Appl Toxicol 5:451–458.

Henderson RF, Pickrell JA, Jones RK, Sun JD, Benson JM, Mauderly JL, McClellan RO. 1988. Response of rodents to inhaled diluted diesel exhaust: Biochemical and cytological changes in bronchoalveolar lavage fluid and in lung tissue. Fundam Appl Toxicol 11:546–567.

Henderson RF, Waide JJ, Mauderly JL, McClellan RO. 1987. A rapid method for determining soot content of lungs in diesel-exposed rats. J Appl Toxicol 7:357–360.

Hoel DG, Walburg HE. 1972. Statistical analysis of survival experiments. JNCI 49:361–372.

Howard PC, Hecht SS, Beland FA, eds. 1990. Nitroarenes: Occurrence, Metabolism, and Biological Impact. Plenum Press, New York, NY.

Huisingh J, Bradow R, Jungers R, Claxton L, Zweidinger R, Tejada S, Bumgarner J, Duffield F, Waters M, Simmon VF, Hare C, Rodriquiz C, Snow L. 1978. Application of bioassay to the characterization of diesel particle emissions. In: Application of Short-term Bioassay in the Fractionation and Analysis of Complex Environmental Mixtures (Waters MD, Nesnow S, Huisingh JL, Sandhu SS, Claxton L, eds.) pp. 381–418. Plenum Press, New York, NY.

International Agency for Research on Cancer. 1989. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol 46, Diesel and Gasoline Engine Exhausts and Some Nitroarenes. International Agency for Research on Cancer, Lyons, France.

International Agency for Research on Cancer. 1983. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol 33, Polynuclear Aromatic Hydrocarbons, Part 2. International Agency for Research on Cancer, Lyons, France.

Jennrich RI, Schluchter MD. 1986. Unbalanced repeated-measures models with structured covariance matrices. Biometrics 42:805–820.

Kalbfleisch JD, Prentice RL. 1980. The Statistical Analysis of Failure Time Data. Wiley & Sons, New York, NY.

Kawabata Y, Iwai K, Udagawa T, Tukagoshi K, Kazue H. 1986. Effects of diesel soot on unscheduled DNA synthesis of tracheal epithelium and lung tumor formation. In: Carcinogenic and Mutagenic Effects of Diesel Engine Exhaust (Ishinishi N, Koizumi A, McClellan R, Stöber W, eds.) pp. 213–222. Elsevier Science Publishing Co., New York, NY.

Klingerman AD, Wilmer JL, Erexson GL. 1981. Characterization of a rat lymphocyte culture system for assessing sister chromatid exchange after in vivo exposure to genotoxic agents. Environ Mutagen 3:531–543.

Kotin P, Falk HL, Thomas M. 1955. Aromatic hydrocarbons III: Presence in the particulate phase of diesel engine exhausts and the carcinogenicity of exhaust extracts. Arch Ind Health 11:113.

Lechner JF, Mauderly JL. 1994. Sequence of events in carcinogenesis: Initiation and promotion, protooncogenes and tumor suppressor genes. In: Toxic and Carcinogenic Effects of Solid Particles in the Respiratory Tract, pp. 235–252. International Life Sciences Institute Press, Washington, DC.

Levene H. 1960. Robust tests for equality of variance. In: Contributions to Probability and Statistics (Olkin I, ed.) pp. 272–292. Stanford University Press, Palo Alto, CA.

Lewis TR, Green FHY, Moorman WJ, Burg JR, Lynch DW. 1989. A chronic inhalation toxicity study of diesel engine emissions and coal dust, alone and combined. J Am Coll Toxicol 8:345–375.

Locati G, Fantuzzi A, Consonni G, Gotti IL, Bonomi G. 1979. Identification of polycyclic aromatic hydrocarbons in carbon black with reference to cancerogenic risk in tire production. Am Ind Hyg Assoc J 40:644–652.

Marmur JA. 1961. Procedure for the isolation of deoxyribonucleic acid from micro-organisms. J Mol Biol 3:208–218.

Mauderly JL. 1992. Diesel exhaust. In: Environmental Toxicants: Human Exposures and Their Health Effects, Chapter 5 (Lippmann M, ed.) pp. 119–162. Van Nostrand Reinhold, New York, NY.

Mauderly JL. 1994a. Contribution of inhalation bioassays to the asessment of human health risks from solid airborne particles. In: Toxic and Carcinogenic Effects of Solid Particles in the Respiratory Tract, pp. 335–366. International Life Sciences Institute Press, Washington, DC.

Mauderly JL. 1994b. Non-cancer pulmonary effects of chronic inhalation exposure of animals to solid particles. In: Toxic and Carcinogenic Effects of Solid Particles in the Respiratory Tract, pp. 43–56. International Life Sciences Institute Press, Washington, DC.

Mauderly JL, Bice DE, Carpenter RL, Gillett NA, Henderson RF, Pickrell JA, Wolff RK. 1987a. Effects of Inhaled Nitrogen Dioxide and Diesel Exhaust on Developing Lung. Research Report Number 8. Health Effects Institute, Cambridge, MA.

Mauderly JL, Bice DE, Cheng YS, Gillett NA, Henderson RF, Pickrell JA, Wolff RK. 1989. Influence of Experimental Pulmonary Emphysema on Toxicological Effects from Inhaled Nitrogen Dioxide and Diesel Exhaust. Research Report Number 30. Health Effects Institute, Cambridge, MA.

Mauderly JL, Cheng YS, Snipes MB. 1990. Particle overload in toxicological studies: Friend or foe? J Aerosol Med 3:S169–S187.

Mauderly JL, Griffith WC, Henderson RF, Jones RK, McClellan RO. 1987b. Diesel exhaust is a pulmonary carcinogen in rats exposed chronically. Fundam Appl Toxicol 9:208–221.

Mauderly JL, Griffith WC, Henderson RF, Jones RK, McClellan RO. 1990. Evidence from animal studies for the carcinogenicity of inhaled diesel exhaust. In: Nitroarenes (Howard PC, Hecht SS, and Beland FA, eds.) pp. 1–13. Plenum Press, New York, NY.

Mausner LF, Prach T, Richards P. 1984. Activation of carbon particles with Be-7 by proton irradiation (abstract). J Labelled Comp Radiopharm 21:1268.

McCullagh P, Nelder JA. 1983. Generalized Linear Models. Chapman & Hall, London, England.

McKnight B, Crowley J. 1984. Test for differences in tumor incidence based on animal carcinogenesis experiments. J Am Stat Assoc 79:639–648.

Mokler BV, Archibeque FA, Beethe RL, Kelly CPJ, Lopez JA, Mauderly JL, Stafford DL. 1984. Diesel exhaust exposure system for animal studies. Fundam Appl Toxicol 4:270–277.

Morrow PE. 1988. Possible mechanisms to explain dust overloading of the lungs. Fundam Appl Toxicol 10:369–384.

Morrow PE. 1992. Dust overloading of the lungs: Update and appraisal. Toxicol Appl Pharmacol 113:1–12.

National Institutes of Health. 1985. Guide for the Care and Use of Laboratory Animals. NIH Publication No. 85-23. U.S. Department of Health and Human Services, National Institutes of Health, Bethesda, MD.

Neal J, Thornton M, Nau CA. 1962. Polycyclic hydrocarbon elution from carbon black or rubber products. Arch Environ Health 4:598–606.

Opresko DM, Holleman JW, Ross RH, Carroll JW. 1984. Problem Definition Study on Emission By-Product Hazards from Diesel Engines for Confined Space Army Workplaces. Oak Ridge National Laboratory Report No. 6017. Oak Ridge National Laboratory, Oak Ridge, TN.

Pott F, Dungworth DL, Heinrich U, Muhle H, Kamino K, Germann P-G, Roller M, Rippe RM. 1994. Lung tumours in rats after intratracheal instillation of dusts. Ann Occup Hyg (in press).

Raabe OG, Bennick JE, Light ME, Hobbs CH, Thomas RL, Tillery MI. 1973. An improved apparatus for acute inhalation exposure of rodents to radioactive aerosols. Toxicol Appl Pharmacol 26:264–273.

Randerath K. 1994. Diesel Exhaust and DNA Damage. Research Report Number 68 Part II. Health Effects Institute, Cambridge, MA. In press.

Reddy MV, Randerath K. 1986. Nuclease P_1–mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. Carcinogenesis 7:1543–1551.

Royer RE, Sturm JC, Walter RA. 1979. Extraction and fractionation of organics from diesel exhaust particles. In: Inhalation Toxicology Research Institute Annual Report, LMF-69, pp. 207–210. Lovelace Biomedical and Environmental Research Institute, Albuquerque, NM.

SAS Institute. 1992. SAS/STAT software: Changes and enhancements, pp. 371–405. SAS Technical Report P-229. SAS Institute, Cary, NC.

Smith RG, Musch DC. 1982. Occupational exposure to carbon black: A particulate sampling study. Am Ind Hyg Assoc J 43:925–930.

Snipes MB. 1989. Long-term retention and clearance of particles inhaled by mammalian species. Crit Rev Toxicol 20:175–211.

Sun JD, Bond JA, Dahl AR. 1988. Biological disposition of vehicular airborne emissions: Particle-associated organic constituents. In: Air Pollution, the Automobile, and Public Health (Watson AY, Bates RR, Kennedy D, eds.) pp. 299–322. National Academy Press, Washington, DC.

U.S. Environmental Protection Agency. 1990. Health Assessment Document for Diesel Emissions. Office of Health and Environmental Assessment, Environmental Criteria and Assessment Office, Research Triangle Park, NC.

U.S. Environmental Protection Agency. 1977. Protection of the environment. In: U.S. Code of Federal Regulations, Title 40, Part 8B, Subpart B, Section 86, pp. 113–178. U.S. Government Printing Office, Bethesda, MD.

Vostal JJ. 1986. Factors limiting the evidence for chemical carcinogenicity of diesel emissions in long-term inhalation studies. In: Carcinogenic and Mutagenic Effects of Diesel Engine Exhaust (Ishinishi N, Koizumi A, McClellan R, Stöber W, eds.) pp. 381–396. Elsevier Science Publishing Co., New York, NY.

Westfall PH, Young SS. 1989. *p*-Value adjustments for multiple tests in multivariate binomial models. J Am Stat Assoc 84:780–786.

Wolff RK, Bond JA, Henderson RF, Harkema JR, Mauderly JL. 1990. Pulmonary inflammation and DNA adducts in rats inhaling diesel exhaust or carbon black. Inhalation Toxicol 2:241–254.

Wolff RK, Henderson RF, Snipes MB, Griffith WC, Mauderly JL, Cuddihy RG, McClellan RO. 1987. Alterations in particle accumulation and clearance in lungs of rats chronically exposed to diesel exhaust. Fundam Appl Toxicol 9:154–166.

Wolff S, Perry P. 1974. Differential Giemsa staining of sister chromatids and the study of chromatid exchanges without autoradiography. Chromosoma 48:341–353.

Wong D, Mitchell CE, Wolff RK, Mauderly JL, Jeffrey AM. 1986. Identification of DNA damage as a result of exposure of rats to diesel engine exhaust. Carcinogenesis 7:1595–1597.

APPENDIX A. Generation and Characterization of Exposure Atmospheres

GENERATION OF DIESEL EXHAUST

Diesel exhaust was generated and characterized in a manner similar to previous long-term exposures conducted for the Health Effects Institute at ITRI (Cheng et al. 1984; Mokler et al. 1984; Mauderly et al. 1989), except that a more recent engine type was used, and the engine operating system was calibrated to simulate a different vehicle. The 1988 General Motors Model LH6 6.2-L engine was chosen as the current model of the most common light-duty engine manufactured in the United States. A light-duty engine was desired because the engine operating system could not accommodate a heavy-duty type without substantial modification, and because the vast majority of the previous rat carcinogenicity data were produced using light-duty engines of various types. Automatic transmissions were required by the engine operating system, and Type 09PB Hydramatic four-speed transmissions were used as appropriate for the vehicle application. Two identical engine-transmission sets were mounted in the exhaust generation test cell and used alternately for exposures at approximately one-month intervals throughout the study. Both engine-transmission sets and accessory parts were purchased by competitive bidding solicited from seven regional General Motors dealerships without knowledge of the specific purpose for which they were to be used.

The engines were operated on test stands with eddy current dynamometers (A-300, Zollner, Kiel, Federal Republic of Germany) and 561-kg inertial flywheels (Pohl Associates, Hatfield, PA) using electronic throttle actuators (DYNC-14000 Plus 4, Barber-Coleman, Rockford, IL). The system was calibrated and operated by computer (LSI-11/2, Digital Equipment, Maynard, MA) to simulate a 1988 Model C1500 Chevrolet one-half ton pickup with a weight of 2,773 kg, a frontal area of 2.58 m^2, a differential ratio of 3.42, a tire rolling radius of 32.5 cm, and a driveshaft:ground speed ratio of 27.8 rpm/kmph. The frontal area was calculated from a photograph, and the rolling radius was measured directly, using a representative vehicle on a dealership lot. Engine and driveshaft speeds were measured and compared with computer tables of desired speeds at one-second intervals using an equation that computed the required throttle position. Voltage was applied to the dynamometer as required for braking to maintain desired speeds.

Exhaust passed through a standard exhaust system of headers, collectors, muffler, and tailpipe before it was injected into a dilution tunnel with an internal diameter of 35.6 cm. Approximately 5.5 m downstream, a portion of the exhaust was diverted into a serial dilution-distribution system aerodynamically designed to minimize the deposition of soot in the tubing. Excess exhaust was vented to the atmosphere via a roof stack. Excess exhaust could also be used for high-volume collections of soot. The residence time of the exhaust in the dilution system was approximately three seconds.

For exposures, the engines were operated to simulate vehicles continuously repeating the 23-minute EPA Federal Test Procedure (also known as US-72) urban fuel economy certification cycles (U.S. Environmental Protection Agency 1977) with one exception. The drive shaft was not braked to a complete stop, as specified by the cycle, because of the complexity of the programming that would have been required. At idle "stop," the drive shaft turned at a simulated ground speed of 4.8 to 8.0 kmph. The cycle simulates operation for approximately 12 km at an average speed of 31 kmph, using a combination of idle, acceleration, deceleration, and cruise modes.

The engine intake air was from the same filtered source used in the exposure chambers. The engines were cooled by a normally pressurized closed-loop coolant system using a 50% mixture of ethylene glycol antifreeze in water. A single-pass flow of tap water was used for the other side of the heat-exchange system. The engines were maintained on the schedule for normal use described in the 1988 Chevrolet pickup owner's manual. The crankcase oil (SAE 30, SG-SF/CC-CD, Pennzoil Products, Oil City, PA), and oil filter were changed at recommended simulated mileage intervals.

Fuel was purchased in bulk, maintained in underground tanks, piped to daily supply tanks in the engine room, and filtered before routing to the standard engine-fuel systems. D-2 Diesel control fuel (Lot K-001, August 8, 1988, Phillips Chemical Company, Borger, TX) was purchased to meet EPA fuel economy certification specifications. The specifications provided by the supplier were confirmed by independent blind analysis (National Institute for Petroleum and Energy Research, Bartlesville, OK), which included a sample of ordinary diesel fuel obtained locally. The independent and supplier's specifications matched closely and were within EPA tolerances. In brief, the fuel had the following characteristics: specific gravity was 0.8501; sulfur was 0.39 wt %; aromatics were 33.9 wt %; carbon was 86.8 wt %; hydrogen was 13.01 wt %; cetane index was 46.9; 50% distillation point was 267°C. The fuel contained 30 ppb of DuPont FOA No. 11 antioxidant to enhance stability.

Before generating exhaust for exposures, the engines were operated on the EPA Highway Fuel Economy Test cycle (U.S. Environmental Protection Agency 1977) for the

equivalent of 500 miles as a break-in procedure. For daily exposures, the engines were warmed up by idling for one minute, operating through a single Highway Fuel Economy Test cycle, and then returning to idle before switching to the Federal Test Procedure cycle for exposures. The engines operated without any problem for the duration of the study, but intermittent difficulties with the control systems resulted in different cumulative usage of the two engines. One engine was operated for a total of 3,824 hours, equivalent to approximately 78,000 miles, and its average fuel was 14.0 simulated mpg. The other engine was operated for 5,284 hours, equivalent to approximately 112,000 miles, and its average fuel use was 13.5 mpg.

GENERATION OF CARBON BLACK

Carbon black was aerosolized using air jet dust generators (Jet-O-Mizer Model 0101, Fluid Energy, Hatfield, PA) supplied from reservoirs by screw feeders (Model 102, Accurate, Whitewater, WI) and diluted with filtered air to the desired chamber concentrations. Bulk Elftex-12 carbon black (Cabot, Boston, MA) was transferred upon receipt to sealed one-gallon metal containers and stored under air at room temperature until transfer to the generator reservoirs without further preparation.

PARTICLE CHARACTERIZATION

Concentrations of diesel exhaust soot and carbon black particles were measured by identical methods using two filter samples taken sequentially from each exposure chamber throughout each daily exposure. Two filter holders containing 47-mm glass fiber filters (Type AE, Gelman, Ann Arbor, MI) were attached to sampling probes placed at the chamber midpoints and connected to vacuum lines fitted with critical orifices to control flow. Sampling was controlled by electronic timers. At the end of each exposure, the filters were placed in Petri dishes and weighed to the nearest 10 g using an electronic balance. The collected mass and sampling flow rate were used to calculate air concentrations of particles.

The particle size distributions of diesel exhaust soot and carbon black were measured by identical methods using a combination of a Lovelace multijet cascade impactor and a parallel-flow diffusion battery, as described previously (Cheng et al. 1984).

The organic fraction of the mass that was associated with particles and extractable with solvents was determined by ultrasonic extraction with dichloromethane (Royer et al. 1979). Extractions of diesel exhaust soot were done using filters collected from the exposure chambers. Because of the small extractable fraction of carbon black, extractions were done using bulk carbon black rather than filters. Amber glass jars were used for extractions to minimize photolysis.

Four 47-mm filters collected from the diesel exhaust chambers as described above contained up to approximately 5 mg soot each; the soot was extracted twice by sonication in an ultrasonic bath (Model MF4.6, Mettler Electronics, full power at 200 W) for one hour with 25 mL of glass-distilled dichloromethane. Particles were removed from the extract by vacuum filtration using Millipore Teflon filters with a 0.2-μm pore size. The extract was reduced to approximately 2 to 3 mL by evaporation, taken to dryness under a flow of nitrogen, weighed, and stored at –80°C. For carbon black extraction, 10 g of bulk carbon black and 500 mL of dichloromethane were placed in an amber glass jar, extracted by sonication for one hour, and then filtered, concentrated, dried, weighed, and stored at –80°C, as described for diesel exhaust extract.

The bacterial mutagenicities of the extracts of diesel exhaust soot and carbon black particles were measured at ITRI early in the study using a modification of the Ames *Salmonella* assay (Ames et al. 1975) as previously described (Brooks et al. 1984). A series of trials using the TA98 tester strain with and without metabolic activation with liver S-9 microsomal fraction demonstrated a mutagenic response of diesel exhaust soot extract qualitatively similar to that obtained from diesel exhaust soot samples collected from previous ITRI exposures. The same series of trials demonstrated negligible mutagenicity of carbon black extract. However, the response of the ITRI TA98 strain to the positive control compounds 2-nitrofluorene, 2-aminoanthracene, and benzo[*a*]pyrene was variable and less than historic values from the Institute. Persistent uncertainties about the quantitative response led to the decision to re-evaluate the mutagenicity of the exposure materials by independent analysis later during the exposures.

The mutagenicity of dichloromethane extracts of diesel exhaust soot and carbon black particles collected during the second year of the chronic exposure was determined under quality control procedures meeting EPA and Food and Drug Administration Good Laboratory Practices by an independent laboratory (Microbiological Associates, Rockville, MD). Samples of bulk extract, shipped and stored frozen, were dissolved in dimethylsulfoxide, diluted to 100, 250, or 500 μg/0.1 μL, and added as 0.1 mL of solution to 100 mL assay plates containing 1.9×10^8 of *S. typhimurium* from

cultures obtained directly from Dr. Bruce Ames, University of California at Berkeley. Both strain TA98, which reverts to histidine independence by frameshift mutations, and TA100, which reverts to histidine independence by frameshift and base pair substitution mutations, were used. Mutagenicity was determined both with and without metabolic activation by adding Aroclor 1254–induced rat liver S-9 microsomal fraction. All assays were done in triplicate. Positive control compounds evaluated concurrently in the same assay included 2-aminoanthracene and 2-aminofluorene for strain TA98, and 2-aminoanthracene and sodium azide for strain TA100.

MEASUREMENT OF GAS AND VAPOR CONCENTRATIONS

Samples for gas and vapor analysis were collected weekly from chambers housing each of the five exposure groups, alternating between the two chambers housing each group. Samples were collected in Tedlar bags from the chamber midpoint. The samples were analyzed for carbon monoxide and carbon dioxide by infrared absorption (Model 865, Beckman Instruments, Fullerton, CA), oxides of nitrogen by chemiluminescence (Model 8840 Nitrogen Oxides Analyzer, Monitor Labs, San Diego, CA), and hydrocarbon vapors by flame ionization (Model 400, Beckman). The instruments were calibrated every six months using a multipoint calibration curve by single-point checks before every measurement. Cylinders of calibration gases were acquired for carbon monoxide, carbon dioxide, nitric oxide, and methane, and were prepared gravimetrically to within 2% of the desired concentration. Methane was used as the calibration gas for the hydrocarbon analyzer. The nitrogen dioxide analyzer was calibrated using a permeation device (VICI Metronics, Santa Clara, CA), which was accurate to within 2%.

APPENDIX B. Methods for Histopathology and Morphometry

TISSUES COLLECTED

A listing of tissues collected from rats at routine necropsies of rats that died or were euthanized, and at necropsies performed on rats that were killed at scheduled intervals is given in Table B.1.

Table B.1. Tissues Collected at Necropsy

Rats That Died or Were Euthanized	Rats Necropsied at Scheduled Intervals
Adrenal glands	Adrenal glands
Brain	
Clitoral glands	
Esophagus	
Eyes	
Femur	
Gross lesions and regional lymph nodes	Gross lesions
Harderian gland (with skull)	
Heart and aorta	
Intestine, large (cecum, colon, rectum)	
Intestine, small (duodenum, jejunum, ilium)	
Kidneys	Kidneys
Larynx	Larynx
Liver	Liver
Lungs and bronchi	Lung, right diaphragm lobe
Lymph nodes (mandibular, mesenteric, bronchial, mediastinal, lumbar)	
Mammary gland and adjacent skin	
Muscle, thigh	
Nasal cavity	Nasal cavity
Nerve, sciatic	
Oral cavity (larynx and pharynx)	
Ovaries	
Pancreas	
Parathyroid glands	
Pituitary glands	
Preputial glands	
Prostate	
Seminal vesicles	
Skin	
Spleen	Spleen
Stomach (forestomach and glandular	
Testes, epididymis, and vaginal tunic of testes	
Thymus	
Thyrod glands	Thyroid glands
Tissue masses and regional lymph nodes	
Tongue	
Trachea	Trachea
Urinary bladder	
Uterus	
Zymbal's glands (with skull)	

HISTOPATHOLOGICAL EVALUATION OF TISSUES

Histopathological evaluations focused on the lungs because previous inhalation bioassays had not revealed significant nonlung pathology, and because the goal of this project was to compare the effects of diesel exhaust and carbon black on the lungs. Lung sections were stained with hematoxylin and eosin and examined by light microscopy. For most rats that died or were euthanized, four sections of lung were examined: one each from the right apical, right cardiac, and right diaphragmatic lobes, and one from the left lung. Additional lung sections were cut for some rats to evaluate specific lesions noted on gross examination. The lung histopathology of rats killed at scheduled intervals was evaluated by examining five or six sections of the right diaphragmatic lobes because other lobes were used for various purposes. In some rats, additional sections were taken to evaluate lesions in other lobes.

All sections examined were scored using standard lesion terminology, and results were entered into a computer data base (Path-TOX, Xybion Medical Systems, Cedar Knolls, NJ). Nonneoplastic lesions were scored as present or absent using the following classification list. Squamous cysts were considered nonneoplastic, by current interpretation at this Institute, and by consensus of a panel of experts reviewing our lesion terminology and tumor classification scheme at the pathology workshop described below. The number of individual cysts was noted. The term "multiple" was used as a qualifying term to describe structures consisting of either multilocular cysts or several adjacent cysts that could not be distinguished from each other.

NONNEOPLASTIC LESIONS

Alveolar macrophage hyperplasia
Alveolar proteinosis
Alveolar epithelial hyperplasia
Squamous metaplasia of alveolar epithelium
Bronchiolar-alveolar metaplasia (bronchiolization)
Bronchiolar epithelial hyperplasia
Septal fibrosis
Focal fibrosis
Chronic-active inflammation
Uremic pneumonia
Alveolar histiocytosis
Squamous cyst

If present, the severity of the lesion was scored on a scale of one to four indicating the percentage of the lung or structure involved as described in the list below. The severity ranking could be raised or lowered one grade due to the intensity of the reaction to staining (or lack thereof).

GRADING SCALE

1. < 10% of the lung affected.
2. 10 to 25% of the lung affected.
3. 25 to 50% of the lung affected.
4. > 50% of the lung affected.

Primary lung neoplasms were categorized and noted as present or absent. If more than one neoplasm was present and there was no evidence of metastasis from a single primary neoplasm, then each neoplasm was considered individually. Because of the multiplicity of neoplasms, the microscopic size of some of the neoplasms, and the sampling method used, it is certain that not all neoplasms that occurred in the rats were observed. The description of the tumor incidence and prevalence among the experimental groups, therefore, must be taken as the results of a statistical sampling of the lungs. To avoid a bias from over-sampling, neoplasms observed by microscopy were counted only if (1) they were observed on a standard cut; (2) they were observed on one of the sections from the right diaphragmatic lobe of a rat killed at a scheduled interval; or (3) they were observed grossly as masses at necropsy. Additional microscopic neoplasms observed by chance on a section taken to categorize a lesion noted at necropsy were not counted.

PRIMARY LUNG NEOPLASMS

Adenoma
  Alveolar pattern
  Papillary pattern
  Mixed pattern
Adenocarcinoma
  Alveolar pattern
  Papillary pattern
  Tubular pattern
  Solid pattern
  Mixed pattern
Carcinoma, squamous cell
Carcinoma, squamous cell, arising from a squamous cyst
Carcinoma, adenosquamous
Neoplasm, malignant, not otherwise specified

The subclassification by pattern of tumors with complex morphologies was based on the predominant pattern ob-

served. Neoplasms that had metastasized from other organs to the lung were entered as metastatic neoplasms. Pulmonary large granular cell leukemia (mononuclear cell leukemia) and histiocytic sarcoma were classified as multicentric neoplasms.

CLASSIFICATION WORKSHOP

A pathology workshop was held at this Institute on March 12 and 13, 1992, to review the lesions observed in the lungs of rats in this study, to evaluate the extent to which a consensus existed regarding the classification of problematic lesions, and to ensure that the classification scheme used in this study was consistent with current majority opinion. Three internationally recognized, U.S. expert experimental pulmonary pathologists were selected for their experience and because they were known to represent a range of views on tumor classification. These individuals included Dr. Donald Dungworth from the University of California at Davis, Dr. Ernest McConnell (consultant) of Raleigh, NC, and Dr. Roger Renne from the Battelle Pacific Northwest Laboratories, Richland, WA. These pathologists met with Dr. Nikula, the ITRI pathologist for this study, and Dr. Hahn, supervisor of ITRI's Pathology Group and also internationally recognized in the field. All five pathologists are board certified and have extensive experience in examining rat lung tumors resulting from chronic inhalation exposures to particles.

These five individuals reviewed and discussed a spectrum of lesions, and also classified selected lesions independently before discussion. Dr. Renne served as chairman of the workshop, and summarized the workshop proceedings and conclusions in the attached report. The workshop was also attended by observers who included Dr. Joe Mauderly, Principal Investigator of this study; Dr. Maria Costantini, Health Effects Institute project officer for the study; and Dr. Jack Harkema, a third ITRI board-certified, expert experimental pulmonary pathologist.

As noted in the summary report that follows, there was good general agreement among the five pathologists regarding the classification of most lesions. As expected, there was not always unanimous agreement. There was a clear disagreement on the classification of squamous cysts, with one pathologist consistently classifying these lesions as benign neoplasms, while the other four considered them to be nonneoplastic. The classification of these lesions used in the present report, therefore, can be taken to represent a majority, but not unanimous, view of the current thinking of expert pathologists.

SUMMARY REPORT, REVIEW OF LUNG LESIONS FROM DIESEL EXHAUST AND CARBON BLACK CHRONIC INHALATION STUDY IN RATS

At the request of Health Effects Institute Research Committee, selected lung lesions observed in F344/N rats exposed chronically to diesel exhaust or carbon black were reviewed and discussed by a group of pathologists at the Inhalation Toxicology Research Institute on March 12 and 13, 1992.

Participants

Visiting Consultants

- Dr. Donald Dungworth, University of California at Davis
- Dr. Ernest McConnell, Consultant, Raleigh, NC
- Dr. Roger Renne, Battelle Pacific Northwest Laboratories, Richland, WA

ITRI Staff

- Dr. Kristen Nikula
- Dr. Fletcher Hahn

Nonvoting Observers

- Dr. Jack Harkema, ITRI
- Dr. Joe Mauderly, ITRI
- Dr. Maria Costantini, HEI

Agenda

Welcome and review of study *Dr. Mauderly*

Format of review *Dr. Hahn*

Presentation of examples of lesions induced by diesel exhaust and carbon black *Dr. Nikula*

Individual review of representative lesions and polling of diagnoses *Dr. Renne*

Discussion of terminology and criteria for classifying lesions observed *Dr. Renne*

Summary Report Written by Dr. Renne: Terminology

The following terminology scheme and proposed criteria for classification of lesions observed inthis study, proposed by Dr. Nikula (with minor modifications), was considered acceptable by the voting participants for classification of the lesions observed in this study.

Hyperplasia, Bronchiolar Epithelium. Increased numbers of Clara cells and/or ciliated epithelial cells lining lumens of terminal bronchioles; cellular atypia not present.

Hyperplasia, Alveolar Epithelium. Increased numbers of type II epithelial cells lining alveoli and alveolar ducts. Normal alveolar architecture is not altered by this prolifera-

tive change, although proliferating epithelial cells may fill the alveolar lumens, and concomitant inflammation and/or fibrosis may result in loss of normal architecture. Cellular atypia is not present.

Squamous Metaplasia, Alveolar or Bronchiolar Epithelium. Replacement of normal alveolar or bronchiolar mucosal epithelium with stratified squamous epithelium. Accumulation of keratinized or nonkeratinized metaplastic epithelium within the confined space of the lung parenchyma may distort the normal architecture, but no evidence of cellular atypia, loss of polarization with the basement membrane, or abnormal differentiation is present.

Metaplasia, Bronchiolar-Alveolar. Replacement of normal epithelium lining the alveoli and alveolar ducts with Clara cells and/or ciliated epithelial cells typical of bronchiolar epithelium.

Squamous Cyst. Discrete cyst in the lung parenchyma, filled with keratinaceous debris and lined by keratinizing stratified squamous epithelium. Keratinization of the lining epithelium is orderly, with no evidence of dysplasia. The lining epithelium appears to extend into the surrounding alveoli and may encroach upon adjacent bronchioles, but this is not interpreted as invasion. Rupture of a cyst with release of keratinaceous debris may induce a marked inflammatory response in the surrounding lung parenchyma.

Note: There were differences of opinion among the participants in the interpretation of the microscopic features of the lesions classified as squamous cysts in this study. Following prolonged examination and discussion of these lesions, a majority (four of five participants) felt that there was insufficient morphologic evidence to classify these lesions as neoplasms, and that they should be classified as squamous cysts, with no connotation of neoplasia. A minority opinion was that these lesions should be classified as benign squamous epithelial neoplasms. There was a consensus that these lesions arose from foci of squamous metaplasia of the alveolar or bronchiolar epithelium, and that they could progress to squamous cell carcinoma. The crux of the debate was whether or not the morphologic features of the epithelium lining the cysts were indicative of neoplasia. Four of the five participating pathologists felt that more evidence of the neoplastic nature of these cysts was required before they could be classified as neoplasms.

Adenoma (alveolar, papillary, or mixed pattern). A discrete mass of proliferating alveolar or bronchiolar epithelium that distorts the normal alveolar architecture. Cells resembling cuboidal alveolar or bronchiolar epithelium fill the alveoli, forming papillae, pseudoalveoli, or a mixture of these patterns, but neither atypia of individual cells nor invasion of adjacent tissue is present.

Adenocarcinoma (alveolar, papillary, tubular, solid, or mixed pattern). A mass of proliferating bronchiolar or alveolar epithelium forming papillae, pseudoalveoli, solid sheets of cells, or a mixture of these patterns, and that contains cells with characteristics of cellular atypia (enlarged, hyperchromatic nuclei, abnormal cell size and shape, or abnormal mitotic figures), and/or invasion of adjacent tissue.

Carcinoma, Squamous Cell. A discrete mass of proliferating squamous epithelium originating from metaplastic bronchial, bronchiolar, or alveolar epithelium and containing areas of cellular atypia, loss of polarization of epithelial maturation with basement membrane, and/or invasion of adjacent tissue.

Carcinoma, Squamous Cell, Arising from Squamous Cyst. A squamous cell carcinoma as defined above, present in continuity with or immediately adjacent to a squamous cyst as defined above.

Carcinoma, Adenosquamous. A primary lung neoplasm having the morphologic characteristics and microscopic features of malignancy defined above for both an adenocarcinoma and a squamous cell carcinoma.

Neoplasm, Malignant, Not Otherwise Specified. A proliferative lesion apparently originating in the lung, that has cytologic or histologic characteristics of malignancy (cellular atypia, disorganization, invasion), but with insufficient cytologic or histologic structure to identify its tissue of origin. This diagnostic term should be used to classify only tumors that are insufficiently differentiated to recognize either an epithelial (carcinoma, not otherwise specified) or connective tissue (sarcoma, not otherwise specified) origin.

Summary of Results

Despite the general agreement among the members of the group on criteria for classification of the proliferative lung lesions examined from this study, differences of opinion were present on the classification of some individually examined borderline lesions, as noted in the results tabulated in Table B.2. The proliferative lung lesions induced in this study represent a continuum from hyperplasia to malignancy, and the potential biologic behavior of some of

these lesions was not definable from histologic sections. This task was made more difficult by the background of intense inflammation and fibrosis also induced by exposure to the test materials.

Table B.2. Results of Polling Diagnoses on Individually Examined Representative Lesions

Slide Number	Animal Number	Diagnosis (Number of Votes)
T706	4914	Two adenomas and multifocal hyperplasia (5)
T826	4915	Primary lesion: adenoma (4) hyperplasia (1)Second lesion: squamous cell carcinoma (3)benign squamous tumor (2)Third lesion: hyperplasia (5)
M824	4915	Adenoma and multifocal hyperplasia (5)
T738	4914	Primary lesion: adenoma (5)
T729	4914	Primary lesion: multifocal hyperplasia (5)
G757	4914	Primary lesion: squamous cyst (4)

METHODS USED FOR MORPHOMETRY OF LUNGS AFTER THREE, SIX, AND TWELVE MONTHS OF EXPOSURE

Special effort was expended to describe lung lesions after three, six, and twelve months of exposure in quantitative terms using morphometric techniques. These time points were selected because, at the high rates of exposures used, macrophage-based particle clearance was already overloaded. A detailed description of the condition of the lung at these times was desired to aid in interpreting the particle clearance data.

The right diaphragmatic lung lobe was removed, weighed, and perfused with 10% neutral buffered formalin at a constant pressure of 25 cm fixative for 4 to 6 hours. The bronchus was then ligated, the lobe was immersed in fixative for 24 hours, and the external lobe volume was measured by water displacement. The lobe was sectioned serially at 4-mm intervals, after a 1- to 4-mm random start. The tissue blocks were embedded in paraffin, cranial face down. A 5-μm section was then cut from each block and stained with hematoxylin and eosin.

Morphometry was performed in two steps, using an inverted microscope equipped with a projection arm. In the first step, the volume fraction of parenchymal versus nonparenchymal structures was determined using the point-counting method of planimetry via a magnification objective of 10×. Nonparenchymal structures are the structural elements of the lung that do not function directly in gas exchange. The nonparenchymal structures consist of arteries, veins, lymphatics, bronchial-associated lymphoid tissue, the conducting airways and their walls proximal to the terminal bronchiole, and the pleura. A six-point grid was used to count every fourth field in every section. A random number table was used to select the field in which the counting began. An average of 172 total points were counted for each rat.

In the second step, the volume fractions of various parenchymal components were determined. A magnification objective of 40 × and a 20-point grid were used, and every 14th field on every other section was counted. Points landing on nonparenchymal components were not counted. An average of 918 points were counted for each rat. The volume fractions of eight parenchymal components were determined: alveolar interstitium; alveolar lumenal airspace; alveolar lumenal single macrophage; terminal bronchiolar wall; terminal bronchiolar airspace; terminal bronchiolar macrophage; aggregated macrophages in the alveolar parenchyma; and macrophage-associated fibrosis.

The value representing the volume fraction of each component within the parenchyma was multiplied by the volume fraction of parenchyma obtained in step one to estimate the percentage of total lung for each parenchymal component. This value could then be multiplied by the total volume of the lobe (measured by volume displacement) to estimate the absolute volume of each component within the lobe. Although the morphometric techniques were applied to the right diaphragmatic lobe, it is thought that the volume fraction data expressed as a percentage of the lung volume can be applied to the entire lung. There is no evidence to suggest that the right diaphragmatic lobe is not representative of the remainder of the lung.

APPENDIX C. Quality Assurance Project Plan

QUALITY ASSURANCE PROJECT PLAN

Date:

August 10, 1988

Title:

INFLUENCE OF PARTICLE-ASSOCIATED ORGANIC COMPOUNDS ON CARCINOGENICITY OF INHALED DIESEL EXHAUST

Investigators:

Joe L. Mauderly, Principal Investigator

Co-Investigators: E. E. Barr, W. E. Bechtold, J. A. Bond, D. G. Burt, A. L. Brooks, Y. S. Cheng, N. A. Gillett, J. R. Harkema, R. F. Henderson, M. B. Snipes, D. G. Thomassen

Sponsors:

Health Effects Institute
Office of Health and Environmental Research, U.S. Department of Energy

Project Officer (HEI):

Dr. Maria Constantini

Laboratory:

Lovelace Inhalation Toxicology Research Institute
Lovelace Biomedical and Environmental Research Institute
Albuquerque, New Mexico

PREPARED BY: Dr. J. L. Mauderly, PI 8/10/88

REVIEWED BY: Ms. D. L. Harris, ITRI QA Officer 8/15/88

APPROVED BY: Dr. R. K. Jones, Associate Director 8/15/88

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A. PROJECT DESCRIPTION

The purpose of this project is to provide an improved estimate of the importance of the role of particle-associated organic compound in the pulmonary carcinogenicity of chronically inhaled diesel exhaust in the rat. The goal is to place in clearer context results of previous studies demonstrating that diesel exhaust can be a pulmonary carcinogen in the rat. The focal issue is whether or not (or to what extent) the same carcinogenic response would result from exposures to carbonaceous particles similar to diesel soot, but devoid of solvent-extractable, mutagenic organic compounds. The project combines elements of a chronic carcinogenicity bioassay, and ancillary mechanistic studies of a more exploratory nature.

Rats will be exposed repeatedly for 24 months to whole, diluted diesel exhaust or to carbon black at the same particle concentrations, or to clean air as controls. Two concentrations of diesel exhaust and carbon black will be used. The primary endpoint will be lung tumor incidence. Secondary issues will be addressed by other endpoints, including lung burdens of particles, levels of lung DNA adducts, adduct levels in different respiratory tract tissues and cells, histopathology, chromosomal injury in lymphocytes, hemoglobin adducts, and the movement and clearance of radiolabeled and fluorescent tracer particles.

This project will be funded jointly by the Health Effects Institute (HEI) and the Department of Energy (DOE). The project was originally proposed in response to HEI Request For Application No. 86-2, "Health Effects of Diesel Emissions", and

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included exposures to single levels of each material. The HEI-funded portion will be conducted under U.S. DOE Funds In Agreement No. DE-FI04-88AL52257, Research Agreement No. 88-3 between HEI and DOE (hereafter called "Research Agreement"). The DOE-funded portion extends the project to include second exposure levels of diesel exhaust and carbon black. This effort will be included under the DOE Office of Health and Environmental Research-funded project, "Carcinogenicity of Mixtures of Inhaled Organics".

B. PROJECT ORGANIZATION AND RESPONSIBILITIES

All of the activities encompassed by this quality assurance (QA) project plan will be conducted at the Inhalation Toxicology Research Institute (ITRI) by ITRI staff. Certain activities are related to collecting and preparing biological samples to be analyzed by two collaborators, Dr. Alan Jeffrey at Columbia University and Dr. Kurt Randerath at Baylor College of Medicine. This QA project plan encompasses only the activities related to those samples which are conducted at ITRI.

The primary responsibility for both the conduct and the QA aspects of this project lies with the Principal Investigator (PI), Dr. Joe Mauderly, who is both PI of the HEI-funded project and coordinator of the DOE project. This project will be conducted under the oversight of the ITRI QA unit and the ITRI QA Officer, Ms. Dorothy Harris. The project will be conducted within the guidelines of Article IX (Quality Assurance and Reports) of the Research Agreement and the ITRI QA Program Plan, as revised on 1/4/88, and according to revisions which might

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occur during the course of the project. Within HEI, the project will be administered by Dr. Jane Warren, Director of Research, through Dr. Maria Constantini, Project Officer.

Day-to-day responsibilities for the conduct of this research will be shared by the PI and scientific co-investigators as outlined in Table 1.

TABLE 1

Scientific Responsibilities

Individual	Responsibilities
Joe L. Mauderly, D.V.M.	Project coordination, In-Life and carcinogenicity data, Interactions with collaborators
Edward E. Barr, M.S.	Exposure system assembly and daily exposure operations
William E. Bechtold, Ph.D.	Extraction of organics from particles, gas and vapor analyses
David G. Burt, D.V.M.	Animal production, maintenance, and daily observations
James A. Bond, Ph.D.	Extraction of DNA, distribution of DNA adducts
Antone L. Brooks, Ph.D.	Mutagenicity of particle extracts, chromosomal damage in lymphocytes
Yung S. Cheng, Ph.D.	Supervisor of chronic exposure section and measurement of particle size distribution
Nancy A. Gillett, D.V.M., Ph.D.	Movement of fluorescent tracer particles
William C. Griffith, B.S.	Statistical design and evaluation
Jack R. Harkema, D.V.M., Ph.D.	Necropsy, histopathology
Rogene F. Henderson, Ph.D.	Measurements of lung burdens of particles

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M. Burton Snipes, Ph.D.	Tracer particle exposures, clearance of radiolabeled carbon black
David G. Thomassen, Ph.D.	Transplantation of tumors into nude mice, preservation of tumor tissue

C. QUALITY ASSURANCE OBJECTIVES

1. General Objectives

The overall QA objective is to obtain results useful as input into consideration of standards for airborne automotive emissions. To this end, the project will be conducted in a manner consistent with Institute standards and such that the results can withstand external scrutiny by HEI, the Environmental Protection Agency (EPA), the contributing automotive manufacturers, and the general scientific community. The aim is to produce results suitable for publication as an HEI Research Report and in the peer-reviewed scientific literature.

Some of the procedures to be performed are exploratory in nature, and detailed Standard Operating Procedures (SOPs) and specific quantitative objectives for the resulting data cannot be specified at study start. Other procedures, such as the exposures, animal maintenance and observations, routine analyses, and necropsy/pathology can be conducted according to well-defined SOPs and often within specific standards of accuracy. Most of the parameters to be measured constitute results, rather than control points, and acceptable limits for the resulting values cannot be specified. Among the parameters characterizing the exposure atmospheres, only the mass concentration of particles serves as a controlling parameter.

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2. Specific Quality Assurance Objectives

ANIMALS

Species/Strain: Rat/F344/N

Source: ITRI colony derived from NIH stock

Identification: Unique individual alphanumeric tail tattoo

Health Surveillance: Daily observation. Serology on selected rats at each major sacrifice for pathogenic viruses and mycoplasma.

Animal Care Program. ITRI is accredited by the American Association for Laboratory Animal Care.

TEST ARTICLES

Name/Source a. Diesel exhaust (see section on generation)

b. Carbon black, CAS No. 1333-86-4, Elftex-12, Cabot Corp, Boston, MA, Lot No. 2868

Characterization: Measurement of particle size distribution, gravimetric analysis of fraction extractable by methylene chloride, and mutagenicity of extract in Salmonella strain TA-98 (Ames assay)

Reanalysis: Characterization at study start, at 12 months of exposure, and at study end. Reanalysis for new batches of diesel fuel or carbon black.

EXPOSURE ATMOSPHERE GENERATION AND MONITORING

Generation: a. Diesel exhaust

Two 1988 Model LH6 General Motors 6.2 L V-8 diesel engines with automatic transmissions, on test stands and operated by computer on Federal Test Procedure urban cycle. D-2 diesel control fuel (Phillips Chemical Co.) meeting EPA certification standards in CFR 40, Chapter 1 (86.113-78). Fuel analyzed by independent laboratory to meet specifications after delivery to ITRI, and stored in underground tanks containing only fuel for this study.

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b. Carbon black

Jet-O-Mizer dust generator (Model 0101, Fluid Energy Corp., Hatfield, PA)

Exposure Atmosphere Calibration: Daily quantitative gravimetric analysis of filters from each exposure chamber. The daily concentrations will be maintained at +/- 20% of the mean target concentration stated in the study protocol.

Particle Sizing: The particle size distribution will be measured at study start, mid-study, and study end by cascade impactor and parallel-flow diffusion battery. The particles of both test atmospheres are known to be polydisperse and to have a bimodal distribution.

Gas/Vapor Measurements: a. Monitoring

Gas/vapor concentrations in each exposure and control atmosphere will be measured only once weekly, since concentrations in exhaust are known to closely parallel particle concentrations. Samples will be collected in Tedlar bags.

b. Instruments

Beckman Model 865 IR Analyzer for CO and CO_2
Beckman Model 400 IR Analyzer for vapor-phase hydrocarbons
Monitor Labs Model 8840 Analyzer for NO and NO_2

c. Calibration

Calibration with NBS-traceable gas standards each day of use

PROCEDURES AND MANAGEMENT OF DATA AND SPECIMENS

Data: All data will be maintained in hardbound ITRI laboratory notebooks, in looseleaf ring binders, or in electronic media separate from data from other projects. All data will be maintained in ITRI archives for a minimum of three years after acceptance of the final report. Data will be made available to HEI or its representatives for examination by arrangement with ITRI.

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Procedures:	All routine procedures will be performed according to current, approved ITRI SOPs. These procedures include animal maintenance, exposures, necropsy/pathology, DNA extraction and adduct analysis, extraction of organics from particles, bacterial mutagenicity assays, assays of chromosome damage and whole-body counting of radioactivity. The approaches to be taken for non-routine procedures will be described to the degree possible in the approved study protocol. This includes exposures to radiolabeled carbon black and fluorescent particles, evaluation of fluorescent particle movement, and transplantation of tumors. Methods development and the procedures used to collect the study data by these procedures will be documented in ITRI laboratory notebooks.
Animal Records:	Each rat will be uniquely identified and traceable to litter of origin. Exposure chamber maps and necropsy records will document the disposition of each rat. The Path-Tox toxicology data base software (Xybion Medical Systems, Cedar Knolls, NJ) will be used in this project. This system will be used to randomize rats by weight class into experimental groups, to record and summarize body weight data, and as a necropsy/pathology data base.
Exposure Records:	Raw and summary data from each measurement of the exposure atmospheres and all exposure logs will be retained.
Sample Custody:	Certain biological samples are to be preserved for potential study beyond the scope of the present protocol. These samples include DNA extracted from lung, frozen lung tissue, frozen tumor tissue, fixed tissues, and tissue sections. All of these samples will be uniquely identified by the animal ID number and date of origin. A log of samples retained, and their location, will be maintained by the PI. The disposition of these samples at the end of the project will be determined by agreement between ITRI and HEI, and will be based on evaluation techniques available at that time.
Samples For Collaborators:	Certain biological samples will be collected and provided to collaborators for analysis. This will include lung DNA at one time for inter-laboratory comparison, and lung DNA and blood erythrocytes at multiple times for analysis by

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single collaborators. These samples will be prepared and coded by ITRI technicians under the supervision of the PI, and provided to collaborators identified only by unique alpha-numeric code and collection date. The key to sample codes will be provided to collaborators after they provide the PI with initial results from each set of samples.

QUALITY CONTROL AND QUALITY ASSURANCE PROCEDURES

Data Quality Control:	A 100% QC check will be done by study personnel soon after data collection. The individual checking the data will initial and date the data document.
QA Unit Performance Auditing:	The QA Unit will perform inspections at approximately 3-month intervals to assure conformance to the approved study protocol and SOPs. The phases of work to audit will include the study protocol (protocol amendments and log of deviations), study start, inhalation exposure system, test article preparation and administration, animal necropsy, and endpoint measurements.
QA Unit Systems Auditing:	Raw and compiled data will be reviewed at approximately 3-month intervals during the study duration for good record-keeping practices. In addition, a random sample of approximately 10% of the data will be audited for completeness and accuracy.
QA Unit Final Report Validation:	All (100%) of the report text will be audited for conformance to the approved protocol. A random 10% of report data will be audited for accuracy of reporting.

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APPENDIX D. LUNG AND LYMPH NODE PARTICLE BURDENS

Table D.1 Body Weights, Lung Weights, and Lung Burdens of Particles of Rats Killed After Three Months of Exposure

		Body Weight (g)		Lung Weight (g)		Lung Weight/Body Weight (g/kg)		Lung Burden (mg)		Lung Burden/ Lung Weight (mg/g)		Lung Burden/ Control Group Lung Weight[a] (mg/g)		Lymph Node Burden (mg)		Lymph Node Burden/Lung Burden (%)	
Group	Gender	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	Female	210	10	0.94	0.08	4.47	0.20										
group	Male	376	10	1.32	0.02	3.52	0.06										
	Both	293	92	1.13	0.22	4.00	0.53										
Low	Female	221	10	1.06	0.01	4.81	0.23	1.71	0.17	1.61	0.18	1.82	0.18	0.004	0.008	0.23	0.53
carbon	Male	348	41	1.35	0.14	3.90	0.05	1.65	0.23	1.22	0.05	1.25	0.18	0.026	0.023	1.60	1.54
black	Both	284	74	1.21	0.19	4.35	0.52	1.68	0.19	1.41	0.25	1.53	0.35	0.015	0.020	0.91	1.28
High	Female	208	5	1.21	0.03	5.82	0.26	4.94	0.25	4.08	0.12	5.26	0.27	0.173	0.049	3.52	1.13
carbon	Male	360	24	1.56	0.16	4.33	0.17	5.85	0.22	3.77	0.31	4.42	0.17	0.188	0.074	3.23	1.28
black	Both	284	85	1.38	0.22	5.07	0.84	5.39	0.54	3.93	0 27	4.84	0.51	0.180	0.057	3.38	1.09
Low	Female	207	13	1.02	0.02	4.93	0.23	2.80	0.84	2.76	0.89	2.98	0.90	0.010	0.016	0.39	0.74
diesel	Male	341	27	1.31	0.14	3.85	0.12	2.46	0.65	1.85	0.33	1.85	0.49	0.022	0.008	0.89	0.14
exhaust	Both	274	76	1.16	0.19	4.39	0.62	2.63	0.70	2.31	0.78	2.42	0.90	0.016	0.013	0.64	0.55
High	Female	176	16	1.03	0.08	5.87	0.13	4.72	1.53	4.59	1.52	5.03	1.63	0.023	0.014	0.52	0.31
diesel	Male	342	15	1.54	0.12	4.51	0.13	6.98	0.33	4.53	0.21	5.27	0.25	0.004[b]	0.006	0.060	0.080
exhaust	Both	259	92	1.29	0.29	5.19	0.75	5.85	1.58	4.56	0.97	5.15	1.05	0.016	0.015	0.340	0.340

[a] Lung burden divided by mean lung weight of control group females or males, as appropriate.

[b] Values for two male rats were both near zero. This finding is unexplained.

Table D.2 Body Weights, Lung Weights, and Lung Burdens of Particles of Rats Killed After Six Months of Exposure

		Body Weight (g)		Lung Weight (g)		Lung Weight/Body Weight (g/kg)		Lung Burden (mg)		Lung Burden/ Lung Weight (mg/g)		Lung Burden/ Control Group Lung Weight[a] (mg/g)		Lymph Node Burden (mg)		Lymph Node Burden/Lung Burden (%)	
Group	Gender	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	Female	247	20	1.03	0.04	4.17	0.16										
group	Male	411	13	1.40	0.07	3.40	0.14										
	Both	329	91	1.21	0.21	3.78	0.44										
Low	Female	240	8	1.17	0.06	4.88	0.11	3.26	0.52	2.78	0.32	3.18	0.51	0.233	0.092	7.03	1.94
carbon	Male	432	23	1.71	0.15	3.95	0.18	5.55	1.60	3.22	0.70	3.98	1.15	0.370	0.033	6.97	1.59
black	Both	336	106	1.44	0.31	4.42	0.53	4.41	1.65	3.00	0.54	3.58	0.90	0.302	0.097	7.00	1.59
High	Female	229	6	1.68	0.11	7.32	0.30	11.00	0.37	6.57	0.58	10.72	0.36	0.766	0.045	6.98	0.59
carbon	Male	422	59	2.12	0.36	5.00	0.20	13.71	1.72	6.53	0.55	9.81	1.23	0.978	0.117	7.14	0.22
black	Both	326	112	1.90[b]	0.34	6.16	1.29	12.35	1.85	6.55	0.51	10.27	0.95	0.872	0.140	7.06	0.41
Low	Female	229	21	1.15	0.08	5.02	0.28	4.88	1.83	4.18	1.26	4.75	1.78	0.133	0.032	3.03	1.54
diesel	Male	411	27	1.49	0.13	3.62	0.07	6.46	0.40	4.34	0.22	4.62	0.28	0.069	0.042	1.09	0.71
exhaust	Both	320	102	1.32	0.21	4.32	0.79	5.67	1.47	4.26	0.81	4.69	1.14	0.101	0.048	2.06	1.51
High	Female	204	27	1.74	0.19	8.56	0.39	18.93	1.77	10.92	0.95	18.45	1.72	0.471	0.022	2.49	0.13
diesel	Male	369	36	2.10	0.19	5.70	0.46	20.81	1.48	9.96	0.96	14.90	1.06	0.445	0.014	2.15	0.20
exhaust	Both	287	95	1.92[b]	0.26	7.13	1.61	19.87	1.79	10.44	1.00	16.68	2.33	0.458	0.022	2.32	0.24

[a] Lung burden divided by mean lung weight of control group females or males, as appropriate.

[b] Significantly different from control group value for both genders at $p < 0.05$ by multiple comparison.

Table D.3 Body Weights, Lung Weights, and Lung Burdens of Particles of Rats Killed After 12 Months of Exposure

		Body Weight (g)		Lung Weight (g)		Lung Weight/Body Weight (g/kg)		Lung Burden (mg)		Lung Burden/ Lung Weight (mg/g)		Lung Burden/ Control Group Lung Weight[a] (mg/g)		Lymph Node Burden (mg)		Lymph Node Burden/Lung Burden (%)	
Group	Gender	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control group	Female	279	14	1.09	0.05	3.92	0.21										
	Male	463	41	1.58	0.13	3.42	0.22										
	Both	370	104	1.33	0.28	3.67	0.34										
Low carbon black	Female	253	21	1.59	0.06	6.28	0.31	6.16	0.54	3.85	0.25	5.64	0.50	0.763	0.156	12.30	1.57
	Male	493	10	2.17	0.22	4.41	0.54	7.94	1.05	3.65	0.12	5.03	0.66	0.949	0.114	12.13	2.45
	Both	397	132	1.94	0.36	5.16	1.10	7.05	1.23	3.75	0.21	5.34	0.62	0.856	0.159	12.22	1.84
High carbon black	Female	272	21	2.56	0.07	9.48	0.96	12.19	2.51	4.74	0.86	11.16	2.30	1.471	0.090	12.54	3.44
	Male	458	24	3.31	0.34	7.23	0.65	15.13	0.47	4.48	0.44	9.58	0.30	2.934	0.134	19.89	0.29
	Both	365	104	2.94[b]	0.47	8.36	1.43	13.36	2.41	4.64	0.66	10.53	1.84	2.202	0.808	15.48	4.71
Low diesel exhaust	Female	295	30	1.67	0.20	5.65	0.11	9.84	3.15	5.85	1.39	9.01	2.89	0.746	0.256	7.26	0.73
	Male	422	43	2.03	0.39	4.92	1.50	12.44	1.60	6.30	1.55	7.89	1.01	0.467	0[c]	4.05	0.14
	Female + Male	359	77	1.85	0.34	5.29	1.03	11.15	2.65	6.07	1.34	6.45	2.03	0.606	0.207	5.65	1.90
High diesel exhaust	Female	254	22	3.18	0.08	12.57	0.90	20.77	8.69	6.56	2.76	19.02	7.96	1.227	0.191	6.54	2.39
	Male	431	21	3.95	0.21	9.15	0.17	28.33	3.97	7.18	0.96	17.95	2.52	2.099	0.113	7.47	0.66
	Both	343	99	3.56[b]	0.43	10.86	1.96	24.55	7.33	6.87	1.88	18.49	5.31	1.663	0.498	7.01	1.65

[a] Lung burden divided by mean lung weight of control group females or males, as appropriate.

[b] Significantly different from control group value for both genders at $p < 0.05$ by multiple comparison.

[c] The mean vlue for this group represents two male rats with identical lymph node burdens.

Table D.4 Body Weights, Lung Weights, and Lung Burdens of Particles of Rats Killed After 18 Months of Exposure

		Body Weight (g)		Lung Weight (g)		Lung Weight/Body Weight (g/kg)		Lung Burden (mg)		Lung Burden/ Lung Weight (mg/g)		Lung Burden/ Control Group Lung Weight[a] (mg/g)		Lymph Node Burden (mg)		Lymph Node Burden/Lung Burden (%)	
Group	Gender	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control group	Female	330	31	1.45	0.44	4.47	1.65										
	Male	471	17	1.77	0.03	3.76	0.09										
	Both	401	81	1.61	0.33	4.12	1.12										
Low carbon black	Female	308	19	2.01	0.08	6.57	0.64	12.06	1.18	5.98	0.36	10.04	0.98	1.478	0.152	12.30	1.32
	Male	396	22	2.69	0.69	6.79	1.74	15.96	1.12	6.15	1.33	9.00	0.63	2.461	0.362	15.50	2.75
	Both	352	52	2.35	0.57	6.68	1.18	14.01	2.37	6.06	0.88	9.52	0.93	1.969	0.593	13.90	2.61
High carbon black	Female	296	7	3.71	0.44	12.50	1.22	22.64	1.68	6.15	0.75	18.85	1.40	1.997	0.211	8.85	1.14
	Male	416	33	3.50	0.11	8.43	0.53	29.93	1.31	8.56	0.51	16.88	0.74	4.318	1.150	14.42	3.66
	Both	356	69	3.60[b]	0.31	10.47	2.38	26.29	4.21	7.36	1.44	17.87	1.47	3.158	1.471	11.63	3.90
Low diesel exhaust	Female	314	11	2.24	0.12	7.14	0.32	21.68	1.58	9.71	1.15	18.05	1.31	1.258	0.085	5.96	0.96
	Male	456	38	2.52	0.12	5.54	0.36	29.32	2.55	11.68	1.29	16.54	1.44	1.659	0.822	5.66	2.23
	Both	385	82	2.38[b]	0.19	6.34	0.93	25.50	4.60	10.70	1.54	17.29	1.48	1.458	0.530	5.81	1.41
High diesel exhaust	Female	247	8	4.59	0.14	18.63	0.60	41.18	4.98	8.96	1.01	34.29	4.14	3.491	2.034	9.01	4.98
	Male	458	11	5.17	0.37	11.29	0.63	58.03	6.85	11.31	1.96	32.73	3.87	2.428	1.024	4.02	1.10
	Both	352	116	4.88[b]	0.40	14.96	4.06	49.61	10.67	10.14	1.90	33.51	3.68	2.959	1.451	6.52	4.12

[a] Lung burden divided by mean lung weight of control group females or males, as appropriate.

[b] Significantly different from control group value for both genders at $p < 0.05$ my multiple comparison.

Table D.5 Body Weights, Lung Weights, and Lung Burdens of Particles of Rats Killed After 23 Months of Exposure

		Body Weight (g)		Lung Weight (g)		Lung Weight/Body Weight (g/kg)		Lung Burden (mg)		Lung Burden/ Lung Weight (mg/g)		Lung Burden/ Control Group Lung Weight[a] (mg/g)		Lymph Node Burden (mg)		Lymph Node Burden/Lung Burden (%)	
Group	Gender	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	Female	266	44	1.25	0.09	4.74	0.68										
group	Male	392	29	1.99	0.19	5.06	0.39										
	Both	329	77	1.62	0.43	4.90	0.53										
Low	Female	282	18	2.42	0.31	8.55	0.58	17.29	3.64	7.16	1.25	13.89	2.92	1.782	0.449	10.40	2.21
carbon	Male	377	17	3.12	0.58	8.31	1.82	24.67	2.35	8.11	1.93	12.43	1.18	3.124	0.155	12.77	1.74
black	Both	330	54	2.77[b]	0.57	8.43	1.21	20.98	4.88	7.64	1.56	13.16	2.15	2.453	0.795	11.59	2.20
High	Female	268	19	4.95	0.13	18.50	0.82	36.91	6.79	7.46	1.40	29.65	5.46	2.076	0.549	5.88	2.41
carbon	Male	329	39	4.48	1.48	13.45	3.46	40.13	1.78	9.70	3.46	20.22	0.89	4.448	0.584	11.06	1.08
black	Both	299	43	4.72[b]	0.97	15.98	3.56	38.52	4.78	8.58	2.66	24.93	6.24	3.262	1.395	8.47	3.29
Low	Female	305	12	2.91	0.07	9.53	0.15	36.30	7.47	12.45	2.36	29.15	6.00	1.458	0.916	3.52	1.93
diesel	Male	426	31	3.26	0.38	7.65	0.88	45.14	7.42	13.83	1.08	22.74	3.74	3.062	0.142	6.44	0.75
exhaust	Both	366	69	3.08[b]	0.31	8.59	1.18	40.72	8.24	13.14	1.81	25.95	5.69	2.260	1.070	4.98	2.06
High	Female	235	4	5.86	0.56	25.00	2.56	80.73	4.47	13.85	1.48	64.85	3.59	2.251	0.323	2.75	0.20
diesel	Male	327	38	5.98	0.37	18.54	2.97	90.13	4.15	15.11	1.41	45.41	2.09	4.029	0.323	4.47	0.64
exhaust	Both	281	56	5.92[b]	0.43	21.77	4.32	85.43	6.43	14.48	1.46	55.13	10.97	3.140	1.060	3.61	1.07

[a] Lung burden divided by mean lung weight of control group females or males, as appropriate.

[b] Significantly different from control group value for both genders at $p < 0.05$ by multiple comparison.

APPENDIX E. Bronchoalveolar Lavage Fluid Results

Table E.1. Concentrations of Constituents in Bronchoalveolar Lavage Fluids from Rats Killed After 12 Months of Exposure

		Control Group (n = 2F, 3M)		Low Carbon Black (n = 3F, 3M)		High Carbon Black (n = 3F, 3M)		Low Diesel Exhaust (n = 3F, 3M)		High Diesel Exhaust (n = 3F, 2M)	
Parameter	Units[a]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total white blood cells	10^3/mL	874	133	2,820[b]	357	2,659[b]	1,075	3,004[b]	1,024	3,915[b]	1,636
Macrophages	10^3/mL	850	148	1,889[b]	556	1,192	575	1,328	417	1,911	957
Neutrophils	10^3/mL	14	22	864[b]	306	1,417[b]	559	1,641	1,330	1,979[b]	1,205
Lactate dehydrogenase	mIU/mL	112	9	368[b]	48	653[b]	83	356[b]	87	705[b]	124
Beta glucuronidase	mIU/mL	0.26	0.15	4.13[b]	1.82	11.94[b]	0.92	4.20[b]	1.28	13.10[b]	2.77
Glutathione reductase	mIU/mL	34	19	33	3	48	5	32	5	57	8
Protein	mg/mL	0.37	0.10	0.62	0.10	0.92	0.12	0.57	0.09	1.28	0.22

[a] Units are given per milliliter of lavage fluid.

[b] Significantly different from control group value at $p < 0.05$ by multiple comparison.

Table E.2. Concentrations of Constituents in Bronchoalveolar Lavage Fluids from Rats Killed After 18 Months of Exposure

		Control Group (n = 3F, 3M)		Low Carbon Black (n = 3F, 3M)		High Carbon Black (n = 3F, 3M)		Low Diesel Exhaust (n = 3F, 3M)		High Diesel Exhaust (n = 3F, 3M)	
Parameter	Units[a]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total white blood cells	10^3/mL	591	112	2,149	1,264	3,899[b]	2,095	3,454[b]	1,198	4,889[b]	1,290
Macrophages	10^3/mL	575	109	1,259[b]	376	1,877	1,576	1,227	466	1,765[b]	411
Neutrophils	10^3/mL	9	14	865	997	1,976[b]	1,188	2,188[b]	943	3,033[b]	930
Lactate dehydrogenase	mIU/mL	86	20	280[b]	90	553[b]	51	376[b]	99	832[b]	171
Beta glucuronidase	mIU/mL	0.25	0.17	5.25[b]	2.97	11.13[b]	2.65	7.00[b]	2.46	19.62[b]	5.97
Glutathione reductase	mIU/mL	20	2	32	7	52	5	40	10	72	13
Protein	mg/mL	0.33	0.05	0.69	0.25	0.98	0.16	0.78	0.21	1.57	0.39

[a] Units are given per milliliter of lavage fluid.

[b] Significantly different from control group value at $p < 0.05$ by multiple comparison.

Table E.3. Concentrations of Constituents in Bronchoalveolar Lavage Fluids from Rats Killed After 23 Months of Exposure

		Control Group (n = 3F, 3M)		Low Carbon Black (n = 2F, 3M)		High Carbon Black (n = 3F, 1M)		Low Diesel Exhaust (n = 2F, 3M)		High Diesel Exhaust (n = 1F, 3M)	
Parameter	Units[a]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total white blood cells	10^3/mL	664	176	1,583[b]	484	1,806[b]	367	2,892[b]	539	–[c]	–
Macrophages	10^3/mL	660	178	726	153	855	207	1,482	472	–	–
Neutrophils	10^3/mL	4	5	854	506	943[b]	268	1,410[b]	157	–	–
Lactate dehydrogenase	mIU/mL	76	16	335[b]	96	535[b]	177	456[b]	96	740[b]	148
Beta glucuronidase	mIU/mL	0.09	0.06	4.07[b]	1.38	9.63	4.56	6.06[b]	1.43	18.01[b]	5.88
Glutathione reductase	mIU/mL	19	2	40[b]	6	51	12	49[b]	9	70[b]	15
Protein	mg/mL	0.26	0.09	0.71	0.11	1.04	0.24	0.93	0.24	1.66	0.22

[a] Units are given per milliliter of lavage fluid.

[b] Significantly different from control group value at $p < 0.05$ by multiple comparison.

[c] Values were not measured due to cell lysis from inadvertent contamination.

APPENDIX F. Nonneoplastic Histopathology Results

Table F.1. Summary of Incidence[a] and Severity[b] of Major Nonneoplastic Lesions in Lungs of Rats Killed After Three Months of Exposure

	Control Group		Low Carbon Black		High Carbon Black		Low Diesel Exhaust		High Diesel Exhaust	
Lesion	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)
Chronic-active inflammation	0		0		0		0		1	
Alveolar macrophage hyperplasia	0		6	1.00 ± 0	6	2.00 ± 0	6	1.00 ± 0	6	1.50 ± 0.55
Alveolar epithelial hyperplasia	0		4		6	2.00 ± 0	2	0.33 ± 0.52	5	0.83 ± 0.41
Alveolar septal fibrosis	0		0		0		0		0	
Focal fibrosis	0		0		0		0		0	
Alveolar proteinosis	0		0		0		0		0	
Bronchiolar alveolar metaplasia	0		0		0		0		0	
Alveolar squamous metaplasia	0		0		0		0		0	
Squamous cyst	0		0		0		0		0	

[a] Incidence = number affected/number examined, as indicated in column heading. In each group, 3 females and 3 males were examined.

[b] Severity = mean ± SD of all rats examined (for a discussion of the severity scale 1 to 4, see Appendix B). Severity was not scored for squamous cysts.

Table F.2. Summary of Morphometric Data for the Right Diaphragmatic Lung Lobes of Rats Killed After Three Months of Exposure[a]

Component	Control Group (Mean ± SD)	Low Carbon Black (Mean ± SD)	High Carbon Black (Mean ± SD)	Low Diesel Exhaust (Mean ± SD)	High Diesel Exhaust (Mean ± SD)
Lobe weight (g)	0.35 ± 0.07	0.36 ± 0.05	0.41 ± 0.06	0.41 ± 0.20	0.41 ± 0.10
Fixed lobe external volume (cc)	2.55 ± 0.49	2.21 ± 0.42	2.61 ± 0.68	2.10 ± 0.44	2.64 ± 0.64
Alveolar lumenal airspace (%)[b]	71.26 ± 2.75	68.27 ± 4.79	69.65 ± 3.25	70.69 ± 2.16	69.23 ± 2.07
Alveolar lumenal single macrophage (%)	0.07 ± 0.10	0.72 ± 0.29	1.16 ± 0.70	0.61 ± 0.24	0.83 ± 0.34
Aggregated macrophages (%)	0 ± 0	0.03 ± 0.08	0.10 ± 0.10	0 ± 0	0 ± 0
Alveolar interstitium(%)	19.62 ± 3.59	20.43 ± 4.45	20.40 ± 1.37	21.05 ± 1.72	20.86 ± 1.99
Macrophage associated fibrosis (%)	0 ± 0	0.03 ± 0.08	0.14 ± 0.13	0 ± 0	0 ± 0
Terminal bronchiolar airspace (%)	1.94 ± 1.80	2.23 ± 1.39	1.51 ± 0.95	1.52 ± 1.36	2.18 ± 0.99
Terminal bronchiolar macrophages (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Terminal bronchiolar wall (%)	0.12 ± 0.16	0.17 ± 0.20	0.18 ± 0.19	0.13 ± 0.12	0.12 ± 0.19
Nonparenchymal structures (%)	7.0 ± 2.28	8.11 ± 2.53	6.84 ± 2.40	5.99 ± 1.52	6.78 ± 2.88

[a] Values are expressed as means ± SD; $n = 6$.

[b] Volume fractions of parenchymal and nonparenchymal components expressed as percentage of lobe volume. See Appendix B for definitions of components and explanations of calculations used to derive volume fractions.

Table F.3. Summary of Incidence[a] and Severity[b] of Major Nonneoplastic Lesions in Lungs of Rats Killed After 6 Months of Exposure

	Control Group		Low Carbon Black		High Carbon Black		Low Diesel Exhaust		High Diesel Exhaust	
Lesion	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)
Chronic-active inflammation	0		1	0.17 ± 0.41	0		0		1	0.17 ± 0.41
Alveolar macrophage hyperplasia	0		6	1.33 ± 0.52	6	2.17 ± 0.41	6	1.33 ± 0.52	6	2.83 ± 0.41
Alveolar epithelial hyperplasia	0		6	1.33 ± 0.52	6	2.00 ± 0	6	1.17 ± 0.41	6	2.33 ± 0.52
Alveolar septal fibrosis	0		0		2	0.33 ± 0.52	0		2	0.67 ± 1.03
Focal fibrosis	0		0		0		0		0	
Alveolar proteinosis	0		0		0		0		1	0.17 ± 0.41
Bronchiolar-alveolar metaplasia	0		0		0		0		0	
Alveolar squamous metaplasia	0		0		0		0		0	
Squamous cyst	0		0		0		0		0	

[a] Incidence = number affected/number examined, as indicated in column heading. In each group, 3 females and 3 males were examined.

[b] Severity = mean ± SD of all rats examined (for a discussion of the severity scale 1 to 4, see Appendix B). Severity was not scored for squamous cysts.

Table F.4. Summary of Morphometric Data for the Right Diaphragmatic Lung Lobes of Rats Killed After Six Months of Exposure[a]

Component	Control Group (Mean ± SD)	Low Carbon Black (Mean ± SD)	High Carbon Black (Mean ± SD)	Low Diesel Exhaust (Mean ± SD)	High Diesel Exhaust (Mean ± SD)
Lobe weight (g)	0.35 ± 0.07	0.43 ± 0.12	0.54 ± 0.09	0.38 ± 0.08	0.55 ± 0.07
Fixed lobe external volume (cc)	2.81 ± 0.52	2.91 ± 0.96	3.18 ± 0.62	2.64 ± 0.68	3.19 ± 0.43
Alveolar lumenal airspace (%)[b]	70.24 ± 3.08	67.75 ± 2.61	69.43 ± 3.46	68.85 ± 2.03	67.63 ± 2.83
Alveolar lumenal single macrophage (%)	0.02 ± 0.04	0.71 ± 0.32	3.58 ± 1.0	1.24 ± 0.27	4.74 ± 1.09
Aggregated macrophages (%)	0 ± 0	0.46 ± 0.35	0.56 ± 0.52	0.04 ± 0.11	0.01 ± 0.03
Alveolar interstitium (%)	20.37 ± 2.27	20.01 ± 1.56	18.26 ± 2.24	19.56 ± 2.05	19.30 ± 2.07
Macrophage-associated fibrosis (%)	0 ± 0	0.28 ± 0.23	0.49 ± 0.41	0.02 ± 0.06	0.04 ± 0.09
Terminal bronchiolar airspace (%)	0.96 ± 0.81	1.78 ± 0.64	1.49 ± 1.06	2.49 ± 1.64	1.33 ± 1.20
Terminal bronchiolar macrophages (%)	0 ± 0	0 ± 0	0.20 ± 0.05	0 ± 0	0.03 ± 0.04
Terminal bronchiolar wall (%)	0.10 ± 0.16	0.07 ± 0.09	0.05 ± 0.06	0.18 ± 0.20	0.20 ± 0.17
Nonparenchymal structures (%)	8.31 ± 2.44	8.94 ± 2.47	6.11 ± 0.97	7.62 ± 2.75	6.72 ± 2.96

[a] Mean ± SD, $n = 6$.

[b] Volume fractions of parenchymal and nonparenchymal components expressed as percentage of lobe volume. See Appendix B for definitions of components and explanations of calculations used to derive volume fractions.

Table F.5. Summary of Incidence[a] and Severity[b] of Major Nonneoplastic Lesions in Lungs of Rats Killed After 12 Months of Exposure

	Control Group		Low Carbon Black		High Carbon Black		Low Diesel Exhaust		High Diesel Exhaust	
Lesion	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)
Chronic-active inflammation	0		1	0.17 ± 0.41	1	0.17 ± 0.41	2	0.33 ± 0.52	6	1.50 ± 0.55
Alveolar macrophage hyperplasia	0		6	2.00 ± 0	6	3.00 ± 0	6	2.00 ± 0	6	3.67 ± 0.52
Alveolar epithelial hyperplasia	0		6	2.00 ± 0	6	2.83 ± 0.41	6	1.83 ± 0.41	6	3.00 ± 0
Alveolar septal fibrosis	0		2	0.33 ± 0.52	4	1.17 ± 0.98	2	0.33 ± 0.52	3	1.50 ± 1.64
Focal fibrosis	0		0		0		0		0	
Alveolar proteinosis	0		1	0.17 ± 0.41	6	1.33 ± 0.52	2	0.50 ± 0.84	6	2.33 ± 0.52
Bronchiolar-alveolar metaplasia	0		0		2	0.50 ± 0.84	0		3	0.67 ± 0.82
Alveolar squamous metaplasia	0		0		0		0		0	
Squamous cyst	0		0		0		0		0	

[a] Incidence = number affected/number examined, as indicated in column heading. In each group, 3 females and 3 males were examined.

[b] Severity = mean ± SD of all rats examined (for a discussion of the severity scale 1 to 4, see Appendix B). Severity was not scored for squamous cysts.

Table F.6. Summary of Morphometric Data for the Right Diaphragmatic Lung Lobes of Rats Killed After 12 Months of Exposure[a]

Component	Control Group (Mean ± SD)	Low Carbon Black (Mean ± SD)	High Carbon Black (Mean ± SD)	Low Diesel Exhaust (Mean ± SD)	High Diesel Exhaust (Mean ± SD)
Lobe weight (g)	0.40 ± 0.12	0.54 ± 0.14	0.73 ± 0.26	0.51 ± 0.10	1.00 ± 0.12
Fixed lobe external volume (cc)	3.46 ± 0.82	3.81 ± 0.90	4.13 ± 0.69	3.41 ± 0.84	4.39 ± 0.66
Alveolar lumenal airspace (%)[b]	70.41 ± 5.14	67.87 ± 4.09	67.36 ± 3.00	70.24 ± 3.28	63.50 ± 3.40
Alveolar lumenal single macrophage (%)	0.05 ± 0.07	1.99 ± 0.46	6.17 ± 2.00	2.55 ± 1.16	9.63 ± 1.85
Aggregated macrophages (%)	0 ± 0	0.70 ± 0.59	0.84 ± 0.37	0.42 ± 0.41	0.99 ± 0.50
Alveolar interstitium (%)	18.20 ± 1.53	18.21 ± 1.05	16.42 ± 1.63	19.11 ± 1.76	17.74 ± 0.62
Macrophage-associated fibrosis (%)	0 ± 0	0.41 ± 0.35	1.15 ± 0.35	0.29 ± 0.25	1.20 ± 0.55
Terminal bronchiolar airspace (%)	0.84 ± 0.58	1.33 ± 1.29	1.18 ± 0.51	1.08 ± 0.82	0.98 ± 0.60
Terminal bronchiolar macrophages (%)	0 ± 0	0 ± 0	0.09 ± 0.09	0 ± 0	0.02 ± 0.04
Terminal bronchiolar wall (%)	0.13 ± 0.17	0.20 ± 0.21	0.10 ± 0.18	0.07 ± 0.09	0.08 ± 0.09
Nonparenchymal structures (%)	10.37 ± 4.02	9.29 ± 2.40	6.67 ± 2.62	6.24 ± 1.50	5.87 ± 2.65

[a] Mean ± SD, $n = 6$.

[b] Volume fractions of parenchymal and nonparenchymal components expressed as percentage of lobe volume. See Appendix B for definitions of components and explanations of calculations used to derive volume fractions.

Table F.7. Summary of Incidence[a] and Severity[b] of Major Nonneoplastic Lesions in Lungs of Rats Killed After 18 Months of Exposure

	Control Group		Low Carbon Black		High Carbon Black		Low Diesel Exhaust		High Diesel Exhaust	
Lesion	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)
Chronic-active inflammation	0		6	1.17 ± 0.41	6	2.00 ± 0	5	0.83 ± 0.41	6	1.50 ± 0.55
Alveolar macrophage hyperplasia	0		6	2.33 ± 0.52	6	3.00 ± 0	6	3.00 ± 0	6	3.00 ± 0
Alveolar epithelial hyperplasia	0		6	2.17 ± 0.41	6	2.83 ± 0.41	6	2.33 ± 0.52	6	3.17 ± 0.41
Alveolar septal fibrosis	0		6	2.00 ± 0	6	2.50 ± 0.55	6	2.00 ± 0	6	3.00 ± 0
Focal fibrosis	0		0		0		0		0	
Alveolar proteinosis	0		2	0.33 ± 0.52	4	1.33 ± 1.21	1	0.17 ± 0.41	5	3.00 ± 1.55
Bronchiolar-alveolar metaplasia	0		3	1.00 ± 1.10	6	1.83 ± 0.75	5	1.33 ± 0.82	6	2.67 ± 1.51
Alveolar squamous metaplasia	0		0		0		0		0	
Squamous cyst	0		0		0		0		0	

[a] Incidence = number affected/number examined, as indicated in column heading. In each group, 3 females and 3 males were examined.

[b] Severity = mean ± SD of all rats examined (for a discussion of the severity scale 1 to 4, see Appendix B). Severity was not scored for squamous cysts.

Table F.8. Summary of Incidence[a] and Severity[b] of Major Nonneoplastic Lesions in Lungs of Rats Killed After 23 Months of Exposure

	Control Group		Low Carbon Black		High Carbon Black		Low Diesel Exhaust		High Diesel Exhaust	
Lesion	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)
Chronic-active inflammation	0		4	0.67 ± 0.52	6	1.83 ± 0.75	5	1.17 ± 0.75	5	1.67 ± 0.82
Alveolar macrophage hyperplasia	0		6	3.00 ± 0	6	3.50 ± 0.55	6	3.17 ± 0.41	6	3.83 ± 0.41
Alveolar epithelial hyperplasia	0		6	3.00 ± 0	6	3.17 ± 0.41	6	2.83 ± 0.41	6	3.67 ± 0.52
Alveolar septal fibrosis	0		6	2.83 ± 0.41	6	3.00 ± 0	6	2.67 ± 0.52	6	3.17 ± 0.75
Focal fibrosis	0		0		4	1.33 ± 1.21	1	0.33 ± 0.82	4	1.33 ± 1.21
Alveolar proteinosis	0		2	0.33 ± 0.52	4	1.83 ± 1.72	4	1.50 ± 1.38	6	3.83 ± 0.41
Bronchiolar-alveolar metaplasia	0		5	1.33 ± 0.82	6	2.50 ± 0.84	6	1.83 ± 0.41	6	2.67 ± 1.21
Alveolar squamous metaplasia	0		0		0		0		1	0.17 ± 0.41
Squamous cyst	0		0		2		1		1	

[a] Incidence = number affected/number examined, as indicated in column heading. In each group, 3 females and 3 males were examined.

[b] Severity = mean ± SD of all rats examined (for a discussion of the severity scale 1 to 4, see Appendix B). Severity was not scored for squamous cysts.

Table F.9. Summary of Incidence[a] and Severity[b] of Major Nonneoplastic Lesions in Lungs of Rats Killed 6 Weeks After Exposures Ended

	Control Group		Low Carbon Black		High Carbon Black		Low Diesel Exhaust		High Diesel Exhaust	
Lesion	Incidence (Affected/34)	Severity (Mean ± SD)	Incidence (Affected/18)	Severity (Mean ± SD)	Incidence (Affected/12)	Severity (Mean ± SD)	Incidence (Affected/25)	Severity (Mean ± SD)	Incidence (Affected/13)	Severity (Mean ± SD)
Chronic-active inflammation	3	0.09 ± 0.29	13	1.00 ± 0.77	12	1.75 ± 0.45	19	1.28 ± 0.84	13	1.77 ± 0.60
Alveolar macrophage hyperplasia	2	0.06 ± 0.24	18	2.89 ± 0.47	12	3.42 ± 0.52	25	3.20 ± 0.41	13	3.62 ± 0.51
Alveolar epithelial hyperplasia	4	0.18 ± 0.52	18	3.06 ± 0.64	12	3.67 ± 0.49	25	3.16 ± 0.47	13	3.92 ± 0.27
Alveolar septal fibrosis	1	0.03 ± 0.17	18	2.67 ± 0.49	12	3.00 ± 0.43	25	2.80 ± 0.41	13	3.38 ± 0.65
Focal fibrosis	0		5	0.50 ± 0.86	4	0.75 ± 1.14	7	0.68 ± 1.14	7	1.31 ± 1.38
Alveolar proteinosis	0		10	0.78 ± 0.81	12	3.50 ± 0.67	20	2.16 ± 1.21	13	4.00 ± 0
Bronchiolar-alveolar metaplasia	1	0.06 ± 0.34	17	2.00 ± 0.91	12	3.08 ± 0.67	23	2.40 ± 1.00	13	3.54 ± 0.66
Alveolar squamous metaplasia	0		1	0.11 ± 0.47	4	0.75 ± 1.14	1	0.04 ± 0.20	4	0.54 ± 0.88
Squamous cyst	0		5		5		3		2	

[a] Incidence = number affected/number examined, as indicated in column heading. In each group, 3 females and 3 males were examined.

[b] Severity = mean ± SD of all rats examined (for a discussion of the severity scale 1 to 4, see Appendix B). Severity was not scored for squamous cysts.

Table F.10. Summary of Incidence[a] and Severity[b] of Major Nonneoplastic Lesions in Lungs of Female Rats That Died or Were Euthanized During the First 12 Months of Exposure

	Control Group		Low Carbon Black		High Carbon Black		Low Diesel Exhaust		High Diesel Exhaust	
Lesion	Incidence (Affected/1)	Severity (Mean ± SD)	Incidence (Affected/3)	Severity (Mean ± SD)	Incidence (Affected/3)	Severity (Mean ± SD)	Incidence (Affected/7)	Severity (Mean ± SD)	Incidence (Affected/5)	Severity (Mean ± SD)
Chronic-active inflammation	0		0		0		0		1	0.20 ± 0.45
Alveolar macrophage hyperplasia	0		3	1.67 ± 0.58	2	2.00 ± 1.73	7	1.86 ± 0.90	4	2.00 ± 1.23
Alveolar epithelial hyperplasia	0		2	1.00 ± 1.00	1	1.00 ± 1.73	5	1.29 ± 1.25	4	1.80 ± 1.30
Alveolar septal fibrosis	0		0		1	0.33 ± 0.58	2	0.86 ± 1.46	2	0.80 ± 1.10
Focal fibrosis	0		0		0		0		0	
Alveolar proteinosis	0		0		0		0		2	1.40 ± 1.95
Bronchiolar-alveolar metaplasia	0		0		0		0		2	0.60 ± 0.89
Alveolar squamous metaplasia	0		0		0		0		0	
Squamous cyst	0		0		0		0		0	

[a] Incidence = number affected/number examined, as indicated in column heading. In each group, 3 females and 3 males were examined.

[b] Severity = mean ± SD of all rats examined (for a discussion of the severity scale 1 to 4, see Appendix B). Severity was not scored for squamous cysts.

Table F.11. Summary of Incidence[a] and Severity[b] of Major Nonneoplastic Lesions in Lungs of Male Rats That Died or Were Euthanized During the First 12 Months of Exposure

	Control Group		Low Carbon Black[c]		High Carbon Black		Low Diesel Exhaust		High Diesel Exhaust	
Lesion	Incidence (Affected/6)	Severity Mean ± SD	Incidence (Affected/–)	Severity Mean ± SD	Incidence (Affected/4)	Severity Mean ± SD	Incidence (Affected/2)	Severity Mean ± SD	Incidence (Affected/1)	Severity Mean±SD
Chronic-active inflammation	0		–		0		0		1	2.00 ± –
Alveolar macrophage hyperplasia	0		–		4	2.75 ± 0.96	2	3.00 ± 1.41	1	3.00 ± –
Alveolar epithelial hyperplasia	0		–		4	1.75 ± 0.98	2	3.00 ± 1.41	1	2.00 ± –
Alveolar septal fibrosis	0		–		1	0.25 ± 0.50	2	2.00 ± 1.41	0	
Focal fibrosis	0		–		0		0		0	
Alveolar proteinosis	0		–		0		0		1	3.00 ± –
Bronchiolar-alveolar metaplasia	0		–		0		0		0	
Alveolar squamous metaplasia	0		–		0		0		0	
Squamous cyst	0		–		0		0		0	

[a] Incidence = number affected/number examined, as indicated in column heading. In each group, 3 females and 3 males were examined.

[b] Severity = mean ± SD of all rats examined (for a discussion of the severity scale 1 to 4, see Appendix B). Severity was not scored for squamous cysts.

[c] No male rats exposed to low carbon black died during this interval.

[d] No standard deviations are given because only one rat was euthanized.

Table F.12. Summary of Incidence[a] and Severity[b] of Major Nonneoplastic Lesions in Lungs of Female Rats That Died or Were Euthanized Between 12 and 18 Months of Exposure

	Control Group		Low Carbon Black		High Carbon Black		Low Diesel Exhaust		High Diesel Exhaust	
Lesion	Incidence (Affected/10)	Severity (Mean ± SD)	Incidence (Affected/6)	Severity (Mean ± SD)	Incidence (Affected/12)	Severity (Mean ± SD)	Incidence (Affected/14)	Severity (Mean ± SD)	Incidence (Affected/9)	Severity (Mean ± SD)
Chronic-active inflammation	0		0		7	0.83 ± 0.94	5	0.57 ± 0.85	3	0.56 ± 0.88
Alveolar macrophage hyperplasia	0		6	2.50 ± 0.55	12	3.42 ± 0.52	14	2.64 ± 0.84	9	3.67 ± 0.50
Alveolar epithelia hyperplasia	0		6	2.33 ± 0.52	12	3.08 ± 0.52	14	2.57 ± 0.85	9	3.56 ± 0.53
Alveolar septal fibrosis	0		6	2.00 ± 0.63	12	2.83 ± 0.39	13	2.21 ± 0.98	9	3.22 ± 0.83
Focal fibrosis	0		0		2	0.33 ± 0.78	2	0.21 ± 0.58	0	
Alveolar proteinosis	0		0		9	1.42 ± 1.08	7	1.00 ± 1.18	9	3.67 ± 0.50
Bronchiolar-alveolar metaplasia	0		3	0.67 ± 0.82	9	1.33 ± 1.07	12	1.71 ± 0.91	9	2.89 ± 0.93
Alveolar squamous metaplasia	0		0		0		0		0	
Squamous cyst	0		0		0		0		0	

[a] Incidence = number affected/number examined, as indicated in column heading. In each group, 3 females and 3 males were examined.

[b] Severity = mean ± SD of all rats examined (for a discussion of the severity scale 1 to 4, see Appendix B). Severity was not scored for squamous cysts.

Table F.13. Summary of Incidence[a] and Severity[b] of Major Nonneoplastic Lesions in Lungs of Male Rats That Died or Were Euthanized Between 12 and 18 Months of Exposure

	Control Group		Low Carbon Black		High Carbon Black		Low Diesel Exhaust		High Diesel Exhaust	
Lesion	Incidence (Affected/14)	Severity (Mean ± SD)	Incidence (Affected/32)	Severity (Mean ± SD)	Incidence (Affected/28)	Severity (Mean ± SD)	Incidence (Affected/19)	Severity (Mean ± SD)	Incidence (Affected/17)	Severity (Mean ± SD)
Chronic-active inflammation	0		1	0.63 ± 0.35	5	0.25 ± 0.59	2	0.11 ± 0.32	7	0.59 ± 0.80
Alveolar macrophage hyperplasia	1	0.14 ± 0.54	32	2.00 ± 0.51	28	3.25 ± 0.58	19	2.53 ± 0.61	17	3.65 ± 0.49
Alveolar epithelial hyperplasia	0		32	1.97 ± 0.82	28	3.11 ± 0.79	19	2.47 ± 0.70	17	3.24 ± 0.75
Alveolar septal fibrosis	1	0.07 ± 0.27	24	1.16 ± 0.88	28	2.68 ± 0.86	15	1.63 ± 1.01	17	3.00 ± 0.71
Focal fibrosis	0		0		1	0.07 ± 0.38	0		0	
Alveolar proteinosis	0		0		4	0.21 ± 0.57	0		15	2.24 ± 1.30
Bronchiolar-alveolar metaplasia	0		1	0.03 ± 0.18	8	0.43 ± 0.74	0		8	0.65 ± 0.79
Alveolar squamous metaplasia	0		1	0.03 ± 0.18	1	0.11 ± 0.57	0		0	
Squamous cyst	0		0		0		0		0	

[a] Incidence = number affected/number examined, as indicated in column heading. In each group, 3 females and 3 males were examined.

[b] Severity = mean ± SD of all rats examined (for a discussion of the severity scale 1 to 4, see Appendix B). Severity was not scored for squamous cysts.

Table F.14 Summary of Incidence[a] and Severity[b] of Major Nonneoplastic Lesions in Lungs of Female Rats That Died or Were Euthanized After 18 Months of Exposure

Lesion	Control Group		Low Carbon Black		High Carbon Black		Low Diesel Exhaust		High Diesel Exhaust	
	Incidence (Affected/59)	Severity (Mean ± SD)	Incidence (Affected/75)	Severity (Mean ± SD)	Incidence (Affected/72)	Severity (Mean ± SD)	Incidence (Affected/55)	Severity (Mean ± SD)	Incidence Affected/74)	Severity (Mean ± SD)
Chronic-active inflammation	2	0.03 ± 0.18	18	0.32 ± 0.62	40	0.92 ± 0.90	20	0.49 ± 0.72	49	1.11 ± 0.90
Alveolar macrophage hyperplasia	2	0.03 ± 0.18	75	2.69 ± 0.54	72	3.35 ± 0.48	55	2.93 ± 0.47	74	3.85 ± 0.36
Alveolar epithelial hyperplasia	4	0.09 ± 0.34	75	2.73 ± 0.62	72	3.43 ± 0.58	53	2.62 ± 0.81	74	3.62 ± 0.54
Alveolar septal fibrosis	1	0.02 ± 0.13	71	2.29 ± 0.84	72	2.96 ± 0.46	49	2.14 ± 1.03	72	3.12 ± 0.79
Focal fibrosis	0		12	0.33 ± 0.81	22	0.58 ± 0.95	6	0.16 ± 0.50	36	0.97 ± 1.09
Alveolar proteinosis	0		14	0.23 ± 0.51	71	2.75 ± 0.87	44	1.69 ± 1.10	74	3.82 ± 0.53
Bronchiolar-alveolar metaplasia	0		44	0.96 ± 0.91	69	2.74 ± 1.01	47	1.91 ± 1.09	73	3.38 ± 0.79
Alveolar squamous metaplasia	0		5	0.11 ± 0.42	17	0.36 ± 0.72	4	0.13 ± 0.47	13	0.34 ± 0.78
Squamous cyst	0		4		8		0		10	

[a] Incidence = number affected/number examined, as indicated in column heading. In each group, 3 females and 3 males were examined.

[b] Severity = mean ± SD of all rats examined (for a discussion of the severity scale 1 to 4, see Appendix B). Severity was not scored for squamous cysts.

Table F.15. Summary of Incidence[a] and Severity[b] of Major Nonneoplastic Lesions in Lungs of Male Rats That Died or Were Euthanized After 18 Months of Exposure

Lesion	Control Group		Low Carbon Black		High Carbon Black		Low Diesel Exhaust		High Diesel Exhaust	
	Incidence (Affected/78)	Severity (Mean ± SD)	Incidence (Affected/67)	Severity (Mean ± SD)	Incidence (Affected/68)	Severity (Mean ± SD)	Incidence (Affected/76)	Severity (Mean ± SD)	Incidence (Affected/81)	Severity (Mean ± SD)
Chronic-active inflammation	1	0.04 ± 0.34	7	0.13 ± 0.42	21	0.53 ± 0.86	15	0.24 ± 0.51	51	1.05 ± 0.92
Alveolar macrophage hyperplasia	0		67	2.28 ± 0.49	68	3.29 ± 0.52	76	2.96 ± 0.60	81	3.69 ± 0.46
Alveolar epithelial hyperplasia	2	0.04 ± 0.25	67	2.16 ± 0.62	68	3.09 ± 0.66	76	2.99 ± 0.58	80	3.58 ± 0.63
Alveolar septal fibrosis	1	0.01 ± 0.11	61	1.82 ± 0.85	67	2.74 ± 0.76	74	2.65 ± 0.69	79	3.21 ± 0.72
Focal fibrosis	0		3	0.06 ± 0.30	15	0.44 ± 0.89	4	0.11 ± 0.48	16	0.44 ± 0.94
Alveolar proteinosis	0		0		17	0.38 ± 0.73	2	0.04 ± 0.26	78	3.05 ± 1.08
Bronchiolar-alveolar metaplasia	0		8	0.12 ± 0.33	44	1.27 ± 1.12	22	0.38 ± 0.71	67	1.89 ± 1.16
Alveolar squamous metaplasia	0		1	0.02 ± 0.12	2	0.06 ± 0.34	1	0.01 ± 0.12	7	0.19 ± 0.65
Squamous cyst	0		0		2		0		4	

[a] Incidence = number affected/number examined, as indicated in column heading. In each group, 3 females and 3 males were examined.

[b] Severity = mean ± SD of all rats examined (for a discussion of the severity scale 1 to 4, see Appendix B). Severity was not scored for squamous cysts.

APPENDIX G. Sequestration of Fluorescent Particles

Table G.1 Categories of Anatomic Locations Used to Evaluate the Sequestration of Fluorescent Particles

Anatomic Location	Description
Other[a]	
Undetermined	Unable to identify location of bead
Extracellular	Bead that is not within any cell or within the interstitium (with the exception of an extracellular bead that is on the cilia of the bronchiolar epithelium, or in association with debris)
Bronchus-associated lymphoid tissue	Bead within lymphoid tissue associated with a major airway (either in a macrophage or not)
Bronchiolar Region	
Bronchiolar Epithelium	Bead (either within a macrophage or not) that is on the cilia of the bronchiolar epithelium
Alveolar Region	
Interstitium	Bead that lies within the septum of an alveolus but is not contained in a macrophage aggregate
Thickened interstitium (rats killed at 18 months only)	Bead that lies within the thickened tissue surrounding a macrophage aggregate, but not in a macrophage aggregate (also a bead within thickened areas due to type II cell hyperplasia, large granular cell leukemia, or in lymphoid tissue not associated with a bronchus)
Single alveolar macrophage	Bead contained by a macrophage that is not associated with more than one other macrophage
Aggregated macrophages in the alveolar parenchyma	Bead contained by a macrophage that is clumped within a group of three or more macrophages together
Single alveolar macrophage in debris (rats killed at 18 months only)	Bead contained within a single macrophage that is in association with debris comprised of soot[b], proteinosis, and the remains of effete, soot-packed macrophages that are no longer intact (viable) cells
Debris (rats killed at 18 months only)	Bead that is not within a cell but is associated with soot, proteinosis, and what looks like the remains of soot-packed macrophages that are no longer intact (viable) cells
Peribronchiolar Region	
Interstitium	Bead that lies within the bronchiolar epithelium, basement membrane, or muscle of a terminal bronchiole (not on the cilia, but within the wall)
Single peribronchiolar macrophage	Bead contained by a macrophage that is not associated with more than one other macrophage and is located immediately adjacent to a terminal bronchiole
Aggregated macrophages in the peribronchiolar region	Bead contained by a macrophage that is clumped within a group of three or more macrophages and is located immediately adjacent to a terminal bronchiole
Debris	Bead that is not within a cell but is associated with soot, proteinosis, and the remains of soot-packed macrophages that are no longer intact, and the head is in an area just adjacent to a terminal bronchiole

[a] For the purpose of graphical representation of the data by regions, the categories of undetermined, extracellular, and bronchus-associated lymphoid tissue were combined and labeled as "Other." The three remaining regions were the bronchiolar region, the alveolar region, and the peribronchiolar region.

[b] The term "soot" is used here to indicate either diesel exhaust soot or carbon black particles.

Table G.2 Summary of Locations of Retained Fluorescent MicrospHeres in the Lungs of Rats That Inhaled Microspheres After 3 Months of Exposure, Expressed as Percentages of Microsperes Observed

		Control Group		Low Carbon Black		High Carbon Black		Low Diesel Exhaust		High Diesel Exhaust	
Location	Days After Inhalation	Mean[a]	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Single alveolar macrophages	1	77.77	4.50	72.72	11.64	71.45	4.86	81.43[b]	4.40	73.49	12.90
	4	89.43	3.15	79.99	1.41	65.13	5.45	87.91	6.12	64.40	10.75
	28	83.34	8.80	53.55	9.58	41.06	3.91	78.16	3.89	52.15	2.63
	90	75.68[c]	7.20	49.75c	12.13	42.24	4.54	52.31	8.98	56.68	3.51
Aggregated macrophages in alveolar parenchyma	1	0.00	0.00	1.17	0.62	4.24	2.54	0.25[b]	0.42	4.61	3.38
	4	0.00	0.00	9.76	5.35	22.46	6.46	1.17	1.04	19.10	4.89
	28	0.00	0.00	26.53	10.87	40.03	7.80	7.82	6.21	35.65	5.69
	90	0.00[c]	0.00	38.73c	7.63	44.02	6.05	33.64	6.44	29.72	4.24
Other[d]	1	22.23	4.50	26.12	11.63	24.31	6.41	18.33b	4.67	21.90	10.18
	4	10.57	3.15	10.26	5.32	12.41	1.53	10.92	6.47	16.50	6.17
	28	16.66	8.80	19.92	6.63	18.91	11.51	14.01	8.97	12.20	4.59
	90	24.32[c]	7.20	11.52c	4.51	13.74	7.77	14.05	7.30	13.60	0.87

[a] Mean ± SD; $n = 4$ per exposure group each time, except where noted by footnote b or c.

[b] $n = 3$.

[c] $n = 2$.

[d] All locations in the lung other than in single alveolar macrophages or in aggregated macrophages in the alveolar parenchyma.

Table G.3. Summary of Locations of Retained Fluorescent Microspheres in the Lungs of Rats That Inhaled Microspheres After 18 Months of Exposure, Expressed as Percentages of Microspheres Observed

		Control Group		Low Carbon Black		High Carbon Black		Low Diesel Exhaust		High Diesel Exhaust	
Location	Days After Inhalation	Mean[a]	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Single alveolar macrophages	1	78.01	7.65	57.87	5.25	40.21	4.50	54.63	6.95	37.99	4.58
	4	82.12	2.81	59.64	8.88	45.93	4.73	55.83	5.69	39.88	9.40
	28	79.35[b]	8.70	38.31	12.38	36.91	8.77	37.47	11.41	37.19c	15.16
	90	71.26[c]	9.64	48.71c	3.80	38.43[c]	14.64	39.80[b]	10.01	35.09[b]	6.65
Aggregated macrophages in alveolar parenchyma	1	0.00	0.00	9.25	3.26	22.50	4.12	13.27	2.37	22.81	3.36
	4	0.11	0.21	20.85	5.30	25.86	4.44	21.82	1.69	31.15	3.13
	28	0.00[b]	0.00	47.55	15.45	52.09	13.26	48.86	15.08	48.04c	9.01
	90	0.68[c]	0.97	38.06c	8.75	45.14[c]	15.55	53.78[b]	8.77	46.58[b]	4.95
Other[d]	1	21.99	7.65	32.88	7.97	37.29	2.92	32.09	7.97	39.20	1.78
	4	17.78	2.88	19.51	10.40	28.21	6.92	22.35	6.55	28.97	6.93
	28	20.65[b]	8.70	14.15	5.03	10.99	7.25	13.67	4.34	14.77c	6.15
	90	28.05[c]	10.61	13.23c	4.95	16.43[c]	0.91	6.43[b]	3.49	18.32	7.94

[a] Mean ± SD, $n = 4$ per exposure group each time, except where noted by footnote b or c.

[b] $n = 3$.

[c] $n = 2$.

[d] All locations in the lung other than in single alveolar macrophages or in aggregated macrophages in the alveolar parenchyma.

APPENDIX H. Histopathological Findings Listed for Individual Rats

This appendix contains a listing of pulmonary histopathological findings for individual rat lungs that were examined microscopically. It is available on request from the Health Effects Institute, 141 Portland Street, Suite 7300, Cambridge, MA 02139.

ABOUT THE AUTHORS

Joe L. Mauderly, the principal investigator of this project, received a doctorate in veterinary medicine from Kansas State University in 1967, and served as a laboratory animal veterinarian and physiologist for the U.S. Air Force at the U.S. Army Natick Laboratories, Natick, MA. He joined the Inhalation Toxicology Research Institute in 1969 and held a succession of research and management positions before becoming Director in 1989. He is currently president of the Lovelace Biomedical and Environmental Research Institute and Director of the Inhalation Toxicology Institute. Dr. Mauderly's primary research interests are the effects of inhaled toxic materials on the structure and function of the lung, relationships between lung lesions and respiratory functional changes, pulmonary carcinogenesis from inhaled particles, and the extrapolation of toxicological findings in laboratory animals to estimates of health risk for humans.

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James A. Bond received his Ph.D. degree in pharmacology in 1979 from the University of Washington. He was a toxicologist in the Chemical/Toxicology Group at the Inhalation Toxicology Research Institute from 1981-1989. His primary research interests are studying the biological fate of xenobiotics in various biological systems focusing on the role that metabolism (bioactivation and inactivation) play in the development of xenobiotic-induced toxicity and carcinogenicity. He is currently head of the Biochemical Toxicology Department at the Chemical Industry Institute of Toxicology in Research Triangle Park, NC.

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Morris Burton Snipes received his Ph.D. in physical biology in 1971 from Cornell University. He is currently a toxicokineticist at the Inhalation Toxicology Research Institute. His research interests relate primarily to studies on the biokinetics of inhaled materials, with emphasis on the respiratory tract and its associated lymphatic system. His work includes evaluation of similarities and differences among laboratory animal species with regard to respiratory tract retention and clearance of inhaled materials.

David G. Thomassen received his Ph.D. in genetics in 1980 from the University of Wisconsin at Madison and performed postdoctoral research at the National Institutes of Environmental Health Sciences and at the National Cancer Institute. He was a cell biologist at the Inhalation Toxicology Research Institute from 1986 through 1992, where his research interests were on the mechanisms of radiation and chemically induced multistage progression to respiratory neoplasia. He is currently a program manager for health effects research in the Office of Energy Research, U.S. Department of Energy, Germantown, MD.

PUBLICATIONS RESULTING FROM THIS RESEARCH

Bond JA, Johnson NF, Snipes MB, Mauderly JL. 1990. DNA adduct formation in rat alveolar type II cells: Cells potentially at risk for inhaled diesel exhaust. Environ Mol Mutagen 16:64–69.

Nikula KJ, Snipes MB, Barr EB, Griffith WC, Henderson RF, Mauderly JL. 1994. Influence of particle-associated organic compounds on the carcinogenicity of diesel exhaust. In: Toxic and Carcinogenic Effects of Solid Particles in the Respiratory Tract, pp. 464–468. International Life Sciences Institute Press, Washington, DC.

Nikula KJ, Snipes MB, Barr EB, Griffith WC, Henderson RF, Mauderly JL. 1994. Comparative pulmonary toxicities and carcinogenicities of chronically inhaled diesel exhaust and carbon black in F344 rats. Fundam Appl Toxicol (in press).

ABBREVIATIONS

[^{7}Be]CB	carbon black labeled with beryllium-7
C	controls (in figures)
EPA	Environmental Protection Agency
GSD	geometric standard deviation
HCB	high carbon black (in figures)
HDE	high diesel exhaust (in figures)
HEPES	*N*-2-hydroxyethyl-piperazine-*N*-2-ethane sulfonic acid
ITRI	Inhalation Toxicology Research Institute
LCB	low carbon black (in figures)
LDE	low diesel exhaust (in figures)
MMAD	mass median aerodynamic diameter
MMDD	mass median diffusion diameter

INTRODUCTION

Diesel engines were developed at the end of the last century with the objective of improving the low thermal efficiency of the early spark-ignited gasoline engines. Because of their efficiency and durability, diesel engines are predominantly used in heavy-duty trucks, buses, construction equipment, locomotives, and ships. Despite their advantages, potential health concerns are associated with the use of diesel engines because of their emissions. Diesel engine exhaust consists of a complex mixture of gases, liquids, and particulate aerosols (soot) (International Agency for Research on Cancer 1983, 1989). The soot particles are of particular concern because they are of a respirable size and have mutagenic and carcinogenic compounds, such as polynuclear aromatic hydrocarbons (PAHs)* and nitro-polynuclear aromatic hydrocarbons adsorbed to their surfaces. Diesel exhaust has been shown to induce lung cancer in rats when inhaled at high concentrations for a prolonged period of time. The International Agency for Research on Cancer (1989) has classified diesel exhaust as a potential human carcinogen; however, the extent of toxicity of the many exhaust components, alone or in combination with the other components, has not been fully evaluated.

Since its inception in 1983, the Health Effects Institute (HEI) has funded research to improve our understanding of the potential health effects of diesel engine exhaust and its various components. Our objective is to provide the necessary scientific information to regulatory agencies so that they can conduct sound risk assessments. The first phase of HEI's diesel program began in the early 1980s when the Institute initiated research on the mutagenic constituents of diesel engine exhaust, particularly the nitropyrenes. In 1986, in response to emerging information that exposure to high concentrations of diesel exhaust caused lung tumors in rats, HEI issued a Request for Applications (RFA 86-2) that solicited research proposals to determine the role of particulate matter in eliciting biologic responses, and to assess levels of exposure and dose. In response to this RFA, Dr. Joe Mauderly of the Inhalation Toxicology Research Institute in Albuquerque, NM, submitted a proposal entitled "Influence of Particle-Associated Organic Compounds on the Carcinogenicity of Inhaled Diesel Exhaust." The proposed work was linked to that of two other investigators, Dr. Kurt Randerath (Baylor College of Medicine, Houston, TX), and Dr. Alan Jeffrey (Columbia University, New York, NY), who submitted separate proposals. After Dr. Mauderly's proposal had undergone internal and external review, HEI approved the project, which began in March 1988. Total HEI expenditures for this project were $918,415. Additional financial support from the Department of Energy was made available to the investigators to fund a portion of this project. The Investigators' Report was received at HEI for review in December 1992, and a revised report was accepted for publication by the Health Review Committee in November 1993. During the review of Dr. Mauderly's Report, the Review Committee and Dr. Mauderly 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 Health Effects Institute and to the public.

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

REGULATORY BACKGROUND

Diesel engines are not only more fuel-efficient than spark-ignited gasoline engines, but they also are inherently low emitters of carbon monoxide and hydrocarbons. Diesel engines also offer a 10% to 25% reduction in carbon dioxide emissions over gasoline engines (Deluchi 1992). Because of these advantages and issues related to fuel availability, balance of trade, and global warming, interest has been renewed in the use of diesel engines in this country.

Despite these advantages, however, increased use of diesel engines raises public health concerns because they emit much higher levels of particles and oxides of nitrogen than gasoline engines. The particles in diesel exhaust are of concern because they are of a respirable size and contain hundreds of organic compounds, some of which are mutagenic and carcinogenic, adsorbed to their surfaces. These organic compounds arise from the incomplete combustion of diesel fuel and engine lubricants. Because of concerns regarding the potential adverse health consequences of inhaling diesel exhaust, the U.S. Environmental Protection Agency (EPA) sets emission standards for diesel engines and vehicles under Section 202 of the Clean Air Act, as amended in 1990.

Section 202(a)(1) of the Act directs the Administrator of the EPA to "prescribe (and from time to time revise) . . . standards applicable to the emissions of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgement cause, or

contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Section 202(a)(3)(i) of the Act, as amended by Section 201 of the 1990 Amendments, specifically directs the Administrator to set standards for the "emissions of hydrocarbons, carbon monoxide, oxides of nitrogen, and particulate matter from classes . . . of heavy-duty vehicles or engines . . . ."

The EPA has taken a variety of regulatory actions with respect to diesel engines and vehicles under the authority given it by Sections 202(a)(1) and 202(a)(1)(i). The EPA has set emissions standards that are initially applicable to all engines and vehicles produced in a given model year. Engines and vehicles of the same class that are produced in the succeeding years also must comply with these existing standards, unless the EPA establishes a new set of standards.

With respect to heavy-duty diesel engines and vehicles, the EPA issued emissions standards in 1980 that specified limits for hydrocarbons, carbon monoxide, and oxides of nitrogen that were applicable to engines and vehicles produced in the 1985 model year. New standards were later promulgated for the 1988 and 1989 model years; the 1988 model-year standards, initially promulgated in 1985, added limits on particulate matter emissions. Emissions standards for the 1991 and 1994 model years also have been set by the EPA. Those standards were most recently revised by the EPA in 1989 and 1990.

The 1990 Amendments of the Clean Air Act include several provisions that deal with diesel engines, vehicles, and fuels. Beginning in 1994, light-duty trucks must meet a particle emissions standard of 0.10 or 0.12 g/mile, depending on the size of the truck. For diesel engines in heavy-duty applications, 1994 emissions standards for hydrocarbons, oxides of nitrogen, carbon monoxide, and particulate matter are 1.3, 5.0, 15.5, and 0.10 grams/brake horsepower•hour (g/bhp•hr), respectively. Several provisions in the 1990 Amendments focus on emissions for buses. Section 202(f) of the Act, as added by Section 207(b) of the 1990 Amendments, puts forth a more stringent standard for particulate matter emissions of 0.05 g/bhp•hr for buses. In 1993, in order to achieve the more stringent particulate emissions standard, the EPA implemented regulations for diesel fuel quality. The new limits on sulphur content in diesel fuels represent a five-fold reduction over previously uncontrolled levels.

At the time RFA 86-2 was issued, it was known that exposure to high concentrations of diesel exhaust caused lung tumors in rats; however, it was uncertain which constituents of diesel exhaust (soot particles, adsorbed organic compounds, or both) were responsible for the tumorigenic effect. Assessing the potential health risk for human populations exposed to diesel engine exhaust relies heavily on extrapolating data obtained from animal studies. Because animal studies usually require exposures to concentrations of diesel exhaust much higher than those encountered in ambient environments, many uncertainties are involved in extrapolating data from animals to humans, or from high-dose exposures to low-dose exposures. Thus, identifying the key causative agents and knowing the mechanisms by which diesel exhaust induces carcinogenesis in rats would allow better estimates to be made for humans. Such information also would direct the development of emissions-control technology and reformulated fuels. Dr. Mauderly and coworkers were funded by HEI to help determine the relative contribution of soot particles and particle-associated organic compounds to carcinogenesis induced by diesel exhaust in rats. Such research is essential to the informed regulatory decision-making required by the Clean Air Act.

SCIENTIFIC BACKGROUND

Diesel engine exhaust consists of gases, vapors, and soot. The soot consists of carbon particles with adsorbed organic compounds, inorganic salts, and metals. It is both physically and chemically complex, and contains over 450 organic compounds (International Agency for Research on Cancer 1989). These organic compounds arise from incomplete combustion of fuel and engine lubricants. Due to their submicron aerodynamic diameter, diesel particles are readily respirable and deposited in the lungs; 7% to 13% of the particulate mass inhaled by a human is estimated to be deposited in the lungs and airways (Yu et al. 1986). The organic compounds adsorbed to the carbon particles consist of aliphatic and heterocyclic compounds and their derivatives, and PAHs and their derivatives. Some of the PAHs present in diesel soot, such as benzo[*a*]pyrene and the nitropyrenes, are genotoxic and have been found to cause cancer in laboratory animals. The gases and vapors include oxides of carbon, nitrogen, and sulphur; nitric and nitrous acids; aliphatic compounds; heterocyclic compounds; and water. In the vapor phase, toxic organic species include acrolein, ammonia, benzene, 1,3-butadiene, formaldehyde, formic acid, hydrogen cyanide, hydrogen sulfide, methane, methanol, and toluene (International Agency for Research on Cancer 1989).

A number of factors cause concern about the potential carcinogenicity of diesel exhaust; the topic has been reviewed extensively by the International Agency for Research on Cancer (1989) and Mauderly (1992). One of the first indications of the potential carcinogenicity of diesel exhaust was the work of Kotin and coworkers (1955), who used acetone to extract diesel exhaust particles from filters

and painted the extracts on the skin of C57 black mice. In this experiment, skin tumors were produced, the first one appearing after 13 months. Of the mice thus exposed, 85% grew tumors, and approximately 65% of the tumors were carcinomas. In other early studies, benzo[*a*]pyrene, a known constituent of diesel exhaust, was shown to induce tumors in skin painting tests with mice and other animals (reviewed by International Agency for Research on Cancer 1983). These early findings supported the hypothesis that carcinogenic PAHs were the tumorigenic agents in this simple animal model. Skin painting studies in SENCAR mice have been used to assess tumor initiation, promotion, and complete carcinogenicity for several exhaust extracts of diesel engines, spark-ignition gasoline engines, and coke oven emissions (Nesnow et al. 1982, 1983). Overall, a rough correlation has been shown between the potency of an emission extract in the skin tumorigenesis assay and its benzo[*a*]pyrene concentration.

In the late 1970s, EPA researchers conducted bacterial mutagenicity bioassays with diesel soot extracts (Huisingh et al. 1978). They used methylene chloride to extract organic matter from soot particles that were collected from two light-duty diesel engines, and found that the organic matter was mutagenic in the *Salmonella typhimurium* assay. Although this assay (Ames et al. 1975) was intended to be a screening assay and not a proof of carcinogenicity, the findings were consistent with the hypothesis that the mutagenic and carcinogenic organic compounds adsorbed to the carbon core were tumorigenic, and that the carbon core acted as a vehicle for these compounds to come in contact with the respiratory tract. In view of the anticipated increase in the use of diesel engines in the U.S. light-duty truck fleet at that time, considerable effort was made in bioassay-directed fractionation protocols to identify mutagens and potential carcinogens. Some 450 chemicals were identified in diesel exhaust and many have tested positive in one or more mutagenicity assays (International Agency for Research on Cancer 1989). The organic matter extractable from diesel exhaust particles has been shown to cause gene mutations, DNA damage, and chromosomal effects in mammalian systems (International Agency for Research on Cancer 1989). Efforts directed at identifying key mutagens have revealed that, in the *Salmonella* mutagenicity assay, 55% of the mutagenic activity is contained in 19% of the organic mass, and that this active mass fraction contains PAHs (Bechtold et al. 1984). In 1986, Wong and associates (1989) demonstrated that, in the lungs of rats exposed chronically to the same concentration of diesel exhaust that caused an increased incidence of lung cancer, the level of DNA adducts increased as well. Biodirected fractionation suggested that nitro-PAHs, some of which are potent mutagens, contributed the majority of the bacterial mutagenicity (Howard et al. 1990).

The importance of organic compounds in the carcinogenic response to diesel exhaust exposure also was suggested by experiments conducted by Grimmer and colleagues (1987) who reported that, when the hydrophobic fraction of an extract of diesel exhaust was implanted in the tracheas of rats (20 mg of fraction/rat), it produced a lung tumor incidence comparable to that found in inhalation experiments (Heinrich et al. 1986). When the hydrophobic fraction was separated into its component parts, it yielded four subfractions representing different classes of PAHs and their derivatives. The subfractions that contained nonaromatic compounds, polynuclear aromatic compounds with 2 or 3 rings, and the polar-polynuclear aromatic compounds were devoid of carcinogenic activity even though they comprised more than 95% of the weight of the original carcinogenic fraction. Therefore, these investigators concluded that the majority of the carcinogenic activity resided in the subfraction that contained PAHs with 4 to 7 rings, which include most of the well-known PAH carcinogens such as benzo[*a*]pyrene, benz[*a*]anthracene, and the dibenzanthracene and dibenzpyrene series of isomers. Furthermore, when the hydrophobic subfractions were reconstituted and tested again, they produced the same carcinogenic response as the original unfractionated hydrophobic fraction, which suggests that nearly all of the carcinogenic activity in this assay of diesel exhaust extract originated from the subfraction of PAHs with 4 to 7 rings.

The question of whether the contents of the organic compounds of diesel engine exhaust are actually available for biologic reactions when diesel soot is deposited in the respiratory tract has been addressed by several investigators. Early investigations (Brooks et al. 1980; Siak et al. 1981) indicated that the mutagens in the organic surface layer of diesel exhaust particles may not be bioavailable. Sun and coworkers (1984) showed that, when F344 rats inhaled radiolabeled benzo[*a*]pyrene coated onto diesel exhaust particles, the majority of the particle-associated radioactivity that was retained in their lungs was from benzo[*a*]pyrene (65% to 70%); 13% to 17% was metabolized to benzo[*a*]pyrene-phenols, and 5% to 18% was metabolized to benzo[*a*]pyrene-quinones. A similar experiment by Bond and coworkers (1986), in which rats inhalated either pure areosol of radiolabeled 1-nitropyrene or radiolabeled 1-nitropyrene coated onto diesel exhaust particles, demonstrated that within one hour after the exposure, more than 90% of the radiolabeled 1-nitropy-

rene was metabolized; furthermore, the lungs of the rats exposed to 1-nitropyrene coated onto diesel exhaust particles contained nearly five times more radioactivity than those exposed to a pure aerosol of radiolabeled 1-nitropyrene. These results suggest that some of the benzo[*a*]pyrene and 1-nitropyrene bound to diesel exhaust particles is bioavailable, and that these PAHs may be retained in the lungs longer when they are associated with diesel exhaust particles than when they are not. However, it is not clear whether the way these radiolabeled compounds were bound to the surface of diesel exhaust particles in these experiments is comparable to the way organic compounds are adsorbed onto the carbon matrix of diesel exhaust particles during the combustion process.

In the 1980s, several long-term inhalation bioassays using rats, mice, and Syrian hamsters exposed to diesel engine exhaust were conducted by different investigator groups. These studies have been reviewed by the International Agency for Research on Cancer (1989). In general, the results of these studies were negative for hamsters (Brightwell et al. 1986; Heinrich et al. 1986) and equivocal for mice (Pepelko and Peirano 1983; Heinrich et al. 1986; Takemoto et al. 1986). However, lifetime exposures of rats to sufficiently high concentrations of airborne diesel soot caused an increase in lung tumor frequency (Heinrich et al. 1986; Ishinishi et al. 1986; Iwai et al. 1986; Mauderly et al. 1987; Brightwell et al. 1989). All of the lung tumors were of epithelial origin, and included benign and malignant forms of adenomatous and squamous tumors. The tumor types appeared to arise from areas of the lung in which a proliferation of the bronchiolar epithelium and swelling of type II cells of the alveolar epithelium was apparent (Ishinishi et al. 1986). Soot particle accumulation, cell damage, inflammation, and fibrosis seemed to be associated with tumor formation (Ishinishi et al. 1986; Mauderly et al. 1987). A notable feature of these long-term exposure regimens was the progressive accumulation of particles in the lungs and impairment of the clearance functions.

The animal bioassays discussed above also provided some evidence that particles play a role in lung tumors induced by diesel exhaust. When groups of rats were exposed to whole diesel exhaust and compared with other groups exposed to filtered exhaust from which the particulate phase had been removed, no tumors were observed in the rats exposed to filtered diesel exhaust; however, tumor incidence was significantly increased in the rats exposed to the unfiltered diesel exhaust (Heinrich et al. 1986; Iwai et al. 1986; Brightwell et al. 1989). This indicated that the particulate phase was important, but did not prove whether the organics bound to the particles, or the particles themselves, or both had a role in tumor induction. However, in one study with mice, filtration of diesel exhaust did not affect tumor incidence (Heinrich et al. 1986).

Theoretical caluclations also point to the importance of the particulate matter itself, rather than the adsorbed organic compounds, in the results of the rat bioassay. Using data from a study in which rats were exposed by inhalation for 2.5 years to diesel exhaust at 4 mg/m^3, Heinrich and associates (1986) estimated that approximately 6 to 16 mg of total organic matter was bioavailable in the rat lung. They then calculated that the total deposition of benzo[*a*]pyrene would be approximately 0.1 to 0.5 μg over the life time of the rat. Based on the known tumorigenic potency of benzo[*a*]pyrene, Heinrich and coworkers concluded that this amount of benzo[*a*]pyrene was not sufficient to induce the numbers of tumors observed in the rats exposed to diesel exhaust. Another study that pointed to the importance of particles in the rat tumorigenic response was that of Kawabata and associates (1986), who found that activated carbon particles instilled into the tracheas of rats caused lung tumors.

Vostal (1986) noted three factors that limited the use of the animal bioassay data. First, the tumors were produced only when animals were exposed to high concentrations of inhaled particles. Second, the inhalation experiments did not show a linear, dose-dependant relation between the delivered dose and the production of tumors; tumors were produced only after a definite amount of particles had accumulated in the lung. Vostal argued that the existence of a no-effect level (below the threshold) was not typical for chemical carcinogens. Third, exposure to high concentrations of other insoluble particles, such as silica, titanium dioxide, and shale and coal dust, also caused lung tumors in rats, which suggested a possible role for diesel exhaust particles in the carcinogenic process.

Concurrent with the rat bioassays and bioassay-directed fractionation experiments, epidemiologic studies were conducted to evaluate the potential carcinogenic hazard of exposure to diesel exhaust (reviewed by the International Agency for Research on Cancer 1989 and Mauderly 1992). The epidemiologic evidence indicates a modest increase (relative risk of approximately 1.2 to 1.5) in lung cancer incidence associated with occupational exposure to diesel exhaust. In most studies, confounding factors such as cigarette smoking were not taken into account, and no data are available on the airborne concentration of diesel exhaust particles at the actual time of exposure.

Hence by 1986, evidence had accumulated that diesel exhaust is carcinogenic in rats under conditions of high particle lung burdens; however, it remained to be determined whether the observed lung tumors were induced by (1) a genetic mechanism mediated by the mutagenic hydrocarbons bound to the diesel particles, (2) an epigenetic (nonmutagenic) mechanism associated with high particle lung burdens resulting in cell proliferation, hyperplasia, and cancer, or (3) a combination of genetic and epigenetic effects. Determining which of these mechanisms applied to carcinogenesis induced by diesel exhaust in the rat model could be resolved only experimentally. Dr. Mauderly and coworkers set out to address this question by comparing the relative carcinogenicities of diesel exhaust and carbon black in F344/N rats. Carbon black is similar to the elemental carbon core of diesel exhaust particles; however, the carbon black selected for this study (Elftex-12) contains significantly less organic material bound to its surface than diesel exhaust particles. Furthermore, although extracts of diesel exhaust particles are mutagenic in bacterial assays, similar extracts of carbon black show little or no response. At about the same time, researchers at the Fraunhoffer Institute in Germany also initiated similar studies in which female Wistar rats were exposed to diesel exhaust and a different type of carbon black (Printex 90). A summary of the results of the German study has been published recently (Heinrich et al. 1994) and is discussed later in this Commentary.

RATIONALE FOR THE STUDY

Studies addressing the potential health effects of diesel engine exhaust have been part of the Health Effects Institute's research program since its inception. In 1986, when animal bioassays indicated that long-term exposure to whole diesel exhaust caused lung tumors in rats, the HEI Research Committee identified two major areas in which further research was needed. Applications for research proposals to address these issues were therefore solicited under RFA 86-2.

First, the HEI Research Committee determined that research was needed to clarify the role of diesel engine exhaust particles in the induction of lung tumors in rats, particularly with respect to the heavy burdens of particles found in the lungs of exposed animals where tumors developed. Two sets of proposals were sought: (1) to evaluate the influence of the physical and chemical characteristics of diesel exhaust particles on various in vivo or in vitro toxicological endpoints relevant to the carcinogenic process; and (2) to assess the impact of emerging technologies that would control emissions on the reduction of exhaust particles and their associated biological activity.

Second, the Research Committee noted that techniques to estimate effective doses of diesel engine emissions needed to be developed and applied to laboratory animals and exposed human populations. The Committee was specifically interested in improving the methods used to detect and quantify DNA and protein adducts. Methods for determining the actual exposure of selected human populations to diesel engine emissions also were solicited.

In their original application, Dr. Mauderly and coworkers indicated that their main goal was to compare the tumorigenic effect of chronic exposure of F344/N rats to high concentrations of diesel engine exhaust with those produced by carbon black. This part of the study would provide information on the relative contributions of carbon particles and particle-associated organic compounds in causing lung tumors in laboratory rats. They also proposed to examine particle clearance, and the formation of DNA adducts induced by diesel exhaust and carbon black in the lungs and other regions of the respiratory tract, and chromosomal aberrations induced in the circulating blood cells.

Some modifications were made to the original design as a result of discussions between the HEI Research Committee and Dr. Mauderly. The major modification to Dr. Mauderly's proposal was that the studies on genetic changes were to be conducted in collaboration with two other applicants. One of the applicants, Dr. Kurt Randerath of Baylor College of Medicine, proposed to measure adducts in DNA extracted from the lungs of rats exposed to diesel exhaust, carbon black, or clean air throughout the course of the study. He also proposed to compare the pattern of adducts found on the skin of mice painted with diesel exhaust extract to the pattern reported for adducts found from cigarette smoke exposure. The other applicant, Dr. Alan Jeffrey of Columbia University, proposed to examine hemoglobin adducts, and to identify the major genotoxic components in diesel particulate extract. The Research Committee decided that the exposures and tumorigenesis studies would be conducted by the investigators in Dr. Mauderly's laboratory, and the genotoxicity issues were to be primarily addressed by Drs. Randerath and Jeffrey. An interlaboratory comparison of the DNA adduct studies was to be conducted after three and six months of exposure to ensure that, if an exposure-related difference in the nature and level of adducts was observed, the results were common and consistent among the three laboratories. The Research Committee thought these three projects represented a comprehensive set of studies that would address questions about the relative contribution of the diesel exhaust particles and the associated organic

constituents to carcinogenesis induced by diesel exhaust. Furthermore, they also would allow the genetic changes induced by exposure to diesel engine exhaust and carbon black to be correlated with lung tumor formation. (Dr. Randerath's report will be published in 1995 as Part II of this Research Report. Because of technical problems, Dr. Jeffrey's study was not completed; the draft final report of his project is available upon request from HEI.)

TECHNICAL EVALUATION

OBJECTIVES AND STUDY DESIGN

The principal objective of Dr. Mauderly's study was to determine whether prolonged exposure of rats to either carbon black or diesel exhaust produced comparable lung tumor response. The effects of other factors linked to the development and progression of carcinogenesis, such as tissue injury, clearance and translocation of inhaled particles, genetic changes in target tissues, and chromosomal aberrations in circulating blood cells, also were assessed. The specific aims of the study follow.

1. **To compare the pulmonary carcinogenicity of diesel exhaust soot and carbon black in rats exposed by inhalation for 24 months to two concentrations of particles (2.5 or 6.5 mg/m^3).*** In his original application, Dr. Mauderly proposed evaluating one concentration of each test material (6.5 mg/m^3), which, on the basis of other experiments, was expected to result in a lung tumor incidence of approximately 30% in the animals exposed to diesel exhaust. Financial support for the second set of exposure groups at 2.5 mg/m^3 was provided by the Department of Energy. The second exposure concentration was expected to produce a lung tumor incidence of 10% in the animals exposed to diesel exhaust, and would provide important dose-response information.

   Carcinogenicity among the groups was to be compared on the basis of (1) the occurrence of lung neoplasms, (2) the prevalence of neoplasms over time, (3) neoplasm type, and (4) growth of neoplastic tissue transplanted subcutaneously into nude mice. Because the carcinogenicity of diesel exhaust in rats was thought to be linked to the development and progression of noncancerous lesions, this study also compared nonneoplastic responses of rats to diesel exhaust and carbon black.

   Beginning at 7 to 9 weeks of age, male and female F344/N rats were exposed by inhalation for 16 hours per day, 5 days per week, for 24 months to concentrations of diluted whole light-duty diesel engine exhaust or carbon black at 2.5 mg or 6.5 mg/m^3. The control group was exposed to filtered air. The majority of the rats were observed throughout their life span to evaluate carcinogenicity. In addition, information was obtained on body weight, survival time, markers of inflammation in lung lavage fluid, and nonneoplastic lung lesions. Exposures were terminated at 24 months, and the remaining rats were held in the exposure chambers ventilated with filtered air until overall mortality reached approximately 90% to allow the tumors to develop. Suspected neoplasms in the lungs were examined microscopically. A portion of tissues from the lung neoplasms discovered at necropsy were used for histopathological measurements; a portion of the remaining tissues were transplanted subcutaneously into immunodeficient nude mice to observe the growth characteristics of the neoplasms and to propagate tissue for future use.

2. **To determine the genotoxicity of inhaled diesel exhaust soot and compare it with that of carbon black.** Dr. Mauderly evaluated DNA adduct levels in lung tissues and alveolar type II cells. They analyzed DNA adducts in type II cells because of evidence that these cells can metabolize mutagenic organic compounds, such as those associated with diesel soot (Bond et al. 1988). Furthermore, they are thought to be progenitor cells for replacing damaged type I epithelium, and therefore are important in assessing responses to exposure in the epithelial proliferative process (Adamson et al. 1974). Evidence also indicates that type II cells are progenitors of at least some types of peripheral lung tumors in rats (Belinsky et al. 1990). Because markers of exposure to diesel exhaust and the resulting genotoxicity would be useful in epidemiologic studies, Dr. Mauderly conducted an exploratory evaluation of whether or not chromosomal damage in circulating lymphocytes might reflect diesel exhaust exposure. Because their preliminary findings at three months did not indicate chromosomal damage, they did not repeat the assays at later time points.

3. **To evaluate the role of particle overload in tumors induced by diesel exhaust in rats by comparing clearance kinetics, and the translocation and sequestration of diesel exhaust soot and carbon black.**

* Dr. Mauderly and associates exposed rats to two levels of diesel exhaust and carbon black on the basis of the particle concentration. Therefore, the exposure levels of 2.5 mg and 6.5 mg of diesel exhaust are given in milligrams of particles per cubic meter of exhaust, which contained other vapors and gases. The exposure levels of 2.5 mg and 6.5 mg of carbon black are given in milligrams of particles per cubic meter of air. Throughout the Investigators' Report and Commentary, both exposure concentrations are referred to as diesel exhaust or carbon black at 2.5 mg or 6.5 mg/m^3.

Dr. Mauderly and his coworkers conducted a very well-designed and thorough study. Two strengths of this investigation were that a large number of rats were studied, and statistical methods were applied that enabled the investigators to draw quantitative inferences. Pathologic examination of the neoplastic and nonneoplastic lesions was conducted in an exemplary manner. The study was conducted according to Good Laboratory Practices, and employed quality assurance procedures designed to meet U.S. EPA and U.S. Food and Drug Administration standards. The Quality Assurance Project Plan developed and approved by both the Inhalation Toxicology Research Institute and HEI specified quality control checks of all data, study inspections at approximately three-month intervals for conformance to protocol and standard operating procedures, an audit of all types of data at the end of the study, and an audit of the final report for conformance to the protocol. The data audits performed for this study exceeded those prescribed in the Quality Assurance Plan.

In summary, the overall goals of the study were achieved. The methodology was carefully conceived and rigorously validated. The experimental data are consistent and the interpretations and conclusions drawn by the investigators for the most part are appropriate.

ASSESSMENT OF METHODS

Test Atmospheres

Diesel exhaust was generated by two 1988-model LH6 General Motors light-duty engines. The carbon black (Elftex-12, Cabot, Boston, MA) was aerosolized using an air jet dust generator and diluted with filtered air to the appropriate concentration before exposure. Both particles were generally similar in their mean aerodynamic particle size; however, compared with diesel soot particles, the carbon black had approximately 100 times less organic material associated with its carbon core, and, compared with extracts of diesel soot particles, similar extracts of carbon black showed little or no response when tested in a bacterial mutagenicity assay.

The investigators measured particle concentration gravimetrically after collecting the samples on a filter. They carefully examined the size distribution of the carbon black and diesel exhaust particles using two different instruments, a cascade impactor and a flow diffusion battery. These data indicate that although the mean aerodynamic size of the two types of particles were similar, important differences were apparent in the size distributions. Carbon black and diesel exhaust aerosol particles had a bimodal (large and small) size distribution, with carbon black containing a greater proportion (approximately 67%) of particles in the large-size mode, and diesel exhaust containing a greater proportion (approximately 77%) of particles in the small-size mode. These bimodal distributions are useful for estimating inhaled particle deposition, which is determined by inserting the data from the smaller-mode (diffusion-dominated) and the larger-mode (inertia-dominated) particles into two separate equations, the sum of which can predict the initially deposited dose. If a single instrument, such as a multistage low-pressure impactor had been used, it is likely that a single-modal distribution would have been observed and this information would have been lost.

The fraction of particle mass constituted by the organic material adsorbed on diesel exhaust and carbon black particles was determined by extracting filter samples of diesel exhaust or raw samples of carbon black (because of the very low mass associated with carbon black) by ultrasonication in methylene chloride. The results suggest that the diesel exhaust soot used in this study contained approximately 100 times more extractable organic matter than the carbon black. Using a modified Ames *Salmonella* assay, the investigators determined that the mutagenic activity of a methylene chloride extract of diesel exhaust was higher in strain TA100 than strain TA98. In strain TA100, the specific mutagenic activity of diesel exhaust extract was 83-fold higher than the extract from carbon black; in strain TA98 the difference was 40-fold.

It is possibile that ultrasonication with the solvent methylene chloride might not have been sufficiently rigorous to extract all bound organic constituents from the particles. Therefore, the HEI Review Committee investigated whether a greater amount of organic material bound to the carbon core could have been extracted, and whether the measured mutagenic activity would have increased, if more rigorous extraction procedures had been used. An independent consulting firm first extracted the carbon black particles using methylene chloride and ultrasonication. The methylene chloride extract was separated by filtration, and the carbon black residue then was extracted further with toluene under heat using a Soxhlet apparatus. It was determined that the total mass of the organic material associated with carbon black that was extracted by methylene chloride and ultrasonication and then by toluene using a Soxhlet was in the same range reported by the investigators when they used only methylene chloride and ultrasonication. The mutagenicity results with TA98 were similar to those reported by Dr. Mauderly and his coworkers; with TA100 there appeared to be a small increase in mutagenic activity, suggesting that some additional PAHs

might have been extracted with the Soxhlet. These results confirm Dr. Mauderly's observation that the carbon black they used contained significantly less (approximately 100 times) organic material associated with its carbon core than diesel exhaust, and that carbon black extracts showed little or no response in bacterial mutation assays.

Histopathology

Dr. Mauderly and coworkers focused their histopathological evaluations on the lungs. Lung neoplasms were scored as present or absent, and nonneoplastic lesions were scored using a severity grading scale. The primary lung neoplasms were categorized as adenomas, adenocarcinomas, squamous cell carcinomas, squamous cell carcinomas arising from squamous cysts, adenosquamous carcinomas, malignant neoplasms, or neoplasms not otherwise specified. The diagnosis of cancer was based on the presence of any one of the following criteria: cellular (largely nuclear) atypia, including frequency of mitosis; vascular invasion; pleural invasion; and transplantability. The nonneoplastic lesion types were categorized as alveolar-macrophage hyperplasia, alveolar epithelial hyperplasia, alveolar proteinosis, squamous metaplasia of alveolar epithelium, bronchiolar-alveolar metaplasia, bronchiolar epithelial hyperplasia, septal and focal fibrosis, chronic active inflammation, uremic pneumonia, alveolar histiocytosis, or squamous cyst.

At the request of the HEI Research Committee, a panel of three pathologists met with two pathologists from Dr. Mauderly's team during the course of the study to discuss the results of the pathologic evaluation, and to perform an independent evaluation of selected lung lesions. These pathologists, who had extensive experience with cancers of the respiratory tract in animals and humans, reviewed and discussed a spectrum of lesions, and also classified selected lesions independently. In general, the pathologists agreed on the classification of most lesions. There was, however, disagreement on the classification of the neoplastic lesions classified as squamous cysts. The crux of the disagreement was whether or not the morphologic features of the epithelium that lined the cysts were indicative of neoplasia. Four of the five pathologists considered that more evidence of the neoplastic nature of these cysts was required, and therefore classified the squamous cysts as nonneoplastic; one pathologist consistently classified these lesions as benign neoplasms. The classification of the lesion by the investigators in this report, therefore, can be taken to represent a majority, but not a unanimous, view of the current thinking of expert pathologists. A list of panel members and the details of the panels' findings can be found in Appendix B of the Investigators' Report.

Upon completion of the study, the HEI Review Committee requested that an independent pathologist (Dr. Marvin Kushner, Huntington, NY) review representative lesions from animals killed at different intervals during the study. These included seventeen neoplasms, fourteen squamous metaplasias or squamous cysts, and four typical lesions (two lesions each from animals exposed to carbon black or diesel exhaust) from each of the 3-, 6-, 12-, 18-, 24-month, and final necropsies. Dr. Kushner was essentially in agreement with diagnoses of Dr. Mauderly's team of pathologists. He also concurred with the diagnosis of the squamous cysts as nonneoplastic lesions. He noted that the serial changes of these lesions emphasized their origins in areas of squamous metaplasia that became confluent and then extended by recruiting bordering alveoli. Centers of alveolar metaplasia were observed in ghost outline in the keratinized centers of the lesions, as were foci of incorporated pigment.

Genotoxicity

A subset of rats was killed at intervals during the exposure, and lung tissue was obtained for DNA adduct analysis of lung cells and lung alveolar type II cells. The results of DNA adduct analysis in the alveolar type II cells have been published (Bond et al. 1990). A discussion of Dr. Mauderly's analysis of DNA adducts in lung tissue from the three-month exposure samples will be included in Part II of this Research Report, which will be published in 1995.

Chromosomal damage was determined by measuring sister chromatid exchanges and micronuclei in circulating white blood cells. As mentioned earlier, because the three-month samples did not show any differences related to treatment, the investigators did not conduct further analyses of chromosomal damage.

Particle Lung Burden

Particle lung burden was evaluated by measuring the concentration of diesel exhaust and carbon black particles in the lungs and lung-associated lymph nodes at regular intervals during exposure. For their lung measurements, the investigators used a light absorption method, which compares curves describing the extinction of light at a 620-nm wavelength by homogenates of the lung tissue with standard curves prepared from tissue samples spiked with known amounts of diesel soot or carbon black particles. Because particles can aggregate, and because factors such as size, structure, number, and impurities of these aggregates can influence the measurements of light scattering and absorption, it is possible that this method produced artifacts and, thus, did not produce a precise measure of

particle lung burden. It is also important to note that total particle lung burdens were estimated by analyzing portions of lung tissue, assuming that carbon presence was uniform throughout the lung; this assumption was not verified by histologic evaluation.

Measurements of particle burden in lung-associated lymph nodes were made using a different procedure. The nodes were digested and the inorganic carbon was converted into carbon dioxide that was measured spectrophotometrically. The spectrometer was calibrated by constructing standard curves generated by spiking lymph node tissue with known amounts of diesel soot or carbon black particles. This digestion and oxidation method avoids some of the potential artifacts of the light scattering and absorption methods used for particle lung burden, and thus it yields more reliable data. Because of these uncertainties in particle burden measurements, the investigators appropriately have not placed undue emphasis on the quantitative estimates of particle lung burden.

Particle Clearance

Particle clearance was evaluated in a subset of animals by using ^{7}Be-labeled carbonaceous ([^{7}Be]CB) tracer particles that were prepared from the same lot of carbon black used for the chronic exposure. After three months of chronic exposure to either diesel exhaust or carbon black, the animals were exposed to a single dose of radiolabeled [^{7}Be]CB; thoracic radioactivity was measured at intervals from 0 to 126 days after exposure to the radiolabeled tracer particles to determine the effects of lung particle burden on clearance mechanisms. The chronic exposure to diesel exhaust or carbon black continued, and clearance was evaluated again after 18 months of exposure by a second inhalation of radiolabeled [^{7}Be]CB. At both clearance evaluation times (3 and 18 months), the measurements on days 0 through 7 were variable; therefore, the investigators reported clearance measurements starting seven days after exposure to the tracer particles. Thus, it is not known what fraction of the initially deposited tracer load the day-seven "baseline values" represent.

Particle Translocation

In order to determine the movement and location of the inhaled particles, a different subset of rats received a single exposure by inhalation to an aerosol mixture of fluorescent latex microspheres (1 μm in diameter) and [^{7}Be]CB once after 3 months and once after 18 months of chronic exposure to diesel exhaust or carbon black. The initial microsphere lung burden was estimated by measuring radioactivity and determining the ratio of the latex microspheres to the [^{7}Be]CB particles. To determine the concentrations and sites where the fluorescent tracer microspheres deposited at different times, selected rats from each exposure group (diesel exhaust, carbon black, and control) were examined at 1, 4, 28, and 90 days after they inhaled the microspheres. Histopathologic analysis of sections of fixed lung and lung-associated lymph node tissues was conducted to determine the anatomic locations of the fluorescent microspheres. Because the primary interest was in examining the movement of particles into macrophage aggregates, the data were categorized into percentages of microspheres in either single alveolar macrophages, aggregated macrophages, or other locations. Liquid displacement volume of the lung was determined, and morphometric measurements of the volume fraction of the lungs occupied by aggregated or single macrophages were used to determine the concentration of the microspheres. Because of the large variation in the microsphere data, and the fact that the measurements of initial microsphere lung burdens were estimates, the reported percentages of the fluorescent microspheres indicate relative distributions of particles in single or aggregate alveolar macrophages, and not quantitative amounts distributed in these compartments.

STATISTICAL METHODS

Dr. Mauderly and coworkers analyzed changes in a number of endpoints measured over time. These analyses were thorough and appropriate, and are described clearly in the Investigators' Report. Statistical analyses were conducted on measurements of body weight, lung weight, bronchioalveolar lavage parameters, DNA adducts, survival, nonneoplastic lesions, lung neoplastic responses, and particle clearance and translocation. In general, the methods used by the investigators are reasonable and the inferences reported are appropriate and valid. As discussed below, some uncertainties exist in the analyses of the effects of exposure and gender on the nonneoplastic lesions and particle clearance.

Neoplastic lesions

The investigators examined the significance of group differences in the lung neoplastic response to exposure using a logistic regression model of neoplasm prevalence at the times the rats died or were euthanized or killed. (Neoplasm prevalence is defined as the probability that a rat living at a given time had a lung neoplasm). The investigators determined crude neoplasm incidence (percentage of rats with neoplasms), the lifetime incidence estimated by logistic regression, and the multiplicity of neoplasms (neoplasm-bearing rats having more than one neoplasm). The measurements of lifetime incidence were conducted rigor-

ously. However, in the analyses of neoplasm prevalence by gender and exposure, because of early mortality of many male rats, use of life tables that provide the numbers of rats that had died and the numbers of rats at risk at each time point would have been preferable. The investigators gave a detailed description of the multiplicity of neoplasms; however, a trend analysis also would have been informative. Finally, in the analysis of comparing the prevalence of neoplasms in the two genders, the investigators appropriately emphasized gender differences. Although logistic regression modeling did not demonstrate a significant difference between the genders, this was primarily due to large statistical errors, which were due, in turn, to the small number of surviving males during the last time period of the study. A clear gender difference was observed in occurrence of neoplasms. Overall, the statistical analysis of neoplasms was conducted thoroughly and is one of the strengths of this study.

Nonneoplastic Lesions

Evaluation of the effects of exposure and gender on nonneoplastic lesions was conducted by logistic regression analysis. This analysis had two problems. First, the investigators compared dichotomized severity scores rather than comparing the rats on the basis of the raw severity scores. Second, the points of division on the severity scale were different for each lesion and were not predetermined, but rather were selected to show best the progression from a low prevalence at early death times. Because the endpoints were defined after examining the raw data, this method has limitations. A more suitable method would have been to analyze the raw severity scale as a continuous endpoint, using analysis of variance (ANOVA). This approach would have solved the problem of data-dependency and provided more statistical power. However, even this method has potential limitations. Because severity scales are typically nonlinear, the assumptions of ANOVA could have been hard to defend. Over all, in the present analyses, the data-dependant dichotomizing of severity scores is acceptable because the logistic regression was executed clearly and proficiently.

Particle Clearance

After 3 and 18 months of exposure, clearance experiments were conducted on a subset of rats of each gender from each exposure group. Therefore, these were paired data on the same animals and should have been analyzed by a method that took into account that each animal was studied twice. Maximum statistical power for comparing exposure groups would have resulted from analyzing the changes in clearance kinetics between 3 months and 18 months within each animal. Instead the investigators addressed the differences among the five exposure groups at 3 months and at 18 months. No apparent recognition was made of the paired design.

The investigators also conducted whole-body measurements at intervals after the inhalation of radioactive tracer particles, and compared animals, genders, and exposure groups. Two different analytical methods were applied to generate a single clearance curve. First, a repeated-measures ANOVA was conducted. This method compared the pattern of successive whole-body measurements among animals, genders, and exposure groups. Repeated-measures analysis ties each animal's data together without assuming any particular mathematical form for the pattern of clearance, thus gaining value in generality although possibly losing value in statistical power. The repeated-measures analysis was performed on the combined genders, but separately for the 3- and 18-month data. As already mentioned, statistical power would have been improved by combining the 3-month and 18-month data and exploiting the paired design. Second, a biexponential function was used to describe clearance kinetics in terms of two "compartments," one fast-clearing and the other slow-clearing. The parameters of this mathematical model were the two rate constants and the respective percentages of particles cleared. In the construction of the biexponential curves, the data were inappropriately pooled across animals at each time point for the purpose of producing one composite curve for each exposure group. No precision estimates were provided for the fitted parameters on which to base comparisons between exposure groups. Despite the concerns mentioned above, the essential results of the clearance experiment can be inferred from the repeated-measures analysis. The plotted data in the Investigators' Report (see Figure 24) tell a clear story, even if, for the sake of illustration, some pooling has been done that would be inappropriate for statistical analysis.

RESULTS

Survival

Male rats in the treatment groups survived significantly shorter time periods than female rats; after 24 months, only 8% of the male rats survived in the group exposed to diesel exhaust at 6.5 mg/m^3, and 1% in the group exposed to carbon black at 6.5 mg/m^3, compared with approximately 35% survival for female rats in the same exposure groups. The fact that fewer male rats survived when exposed to carbon black at 6.5 mg/m^3 than to diesel exhaust at 2.5

mg/m^3, despite the fact that these two groups had similar particle lung burdens, suggests that survival depended on the particle exposure concentration rather than the particle lung burden.

Lung and Body Weight

Lung weights increased significantly and body weights reduced significantly in a dose-related manner by both carbon black and diesel exhaust exposures. Rats exposed to carbon black at 6.5 mg/m^3 exhibited a greater increase in lung weight and a greater decrease in body weight compared with rats exposed to diesel exhaust at 2.5 mg/m^3, again suggesting a dependance on exposure concentration rather than lung burden.

Particle Lung Burden

The investigators made two notable observations regarding particle lung burdens. First, accumulation of both diesel soot and carbon black particles in the lungs was related to exposure concentration. However, the two materials accumulated at different rates; diesel exhaust soot accumulated more rapidly than carbon black particles. This difference in particle accumulation may have been due to the bimodal particle size distribution. It is possible that carbon black particles, which had a greater proportion of the large-sized particles, deposited proximally in the pulmonary lobe, and, thus, were cleared more rapidly in the 0- to 7-day period after exposure to tracer particles, for which the data are not reported. In comparison, the diesel exhaust particles, which had a greater proportion of the small-size particles, may have deposited more deeply in the lung, requiring a longer time to be cleared. Second, when the carcinogenic response was evaluated as a function of exposure concentration, diesel exhaust soot and carbon black caused similar carcinogenic responses. However, when carcinogenicity was expressed as a function of lung burden, rats exposed to carbon black had a greater carcinogenic response than those exposed to diesel exhaust. The investigators report that, after 24 months of exposure, the mean particle lung burden for rats exposed to carbon black at 2.5 mg/m^3 was 21 mg, and at 6.5 mg/m^3 was 38.5 mg; the same mean particle lung burden for rats exposed to diesel exhaust at 2.5 mg/m^3 was 40.7 mg, and at 6.5 mg/m^3 was 85.4 mg. However, these data should be interpreted cautiously because, as discussed earlier, the measurements of lung burden may not have been sufficiently accurate to allow quantitative comparisons to be made.

The concentrations of diesel exhaust soot and carbon black in the lung-associated lymph nodes were similar throughout the exposure; the rate of accumulation decreased after 18 months at the higher exposure levels for both diesel exhaust and carbon black.

Particle Clearance

Both carbon black and diesel exhaust exposures caused dose-dependant and time-related delays in the clearance of [^{7}Be]CB tracer particles, and the patterns of clearance were similar for both exposure groups. However, a statistically significant difference was observed between male and female rats in the clearance of both diesel exhaust and carbon black particles, with clearance rates being slower for female rats than for male rats in all exposure groups at 3 months, but faster at 18 months. When the investigators analyzed differences among exposure groups, however, by combining data from female and male rats they found significant differences in clearance at both 3 and 18 months. At 3 months, particle clearance rates for rats exposed to diesel exhaust soot or carbon black at 2.5 mg/m^3 was faster than for the rats exposed to the same materials at 6.5 mg/m^3; however, at 18 months, the particle clearance for both exposure concentrations of both particles was nearly identical and markedly depressed. Despite the limitations in the statistical methods applied to this part of the study, the results suggest that for the particle concentrations used in this study, clearance essentially had stopped after three months of exposure to either type of particle.

Translocation and Sequestration of Tracer Particles

After 3 and 18 months of chronic exposure to either carbon black or diesel exhaust, inhaled fluorescent microspheres were localized in four primary regions of the lung categorized as alveolar, peribronchiolar, bronchiolar, and other. Overall, for all groups and at all times, the majority of the retained microspheres were found in macrophages in the alveolar region, the alveolar interstitium received a considerable exposure to fresh particles that were not enclosed in macrophages, and the pattern and kinetics of particle sequestration were the same for carbon black and diesel exhaust exposures.

DNA Adducts

The investigators demonstrated the presence of DNA adducts in type II alveolar epithelial cells isolated from the lungs of rats exposed for three months to carbon black particles or diesel exhaust soot at 6.2 mg/m^3 or to fresh air (Bond et al. 1990). The chromatographic patterns of individual adducts were similar among these three groups. The total levels of DNA adducts were similar in rats exposed to

diesel exhaust or carbon black, and these levels were significantly greater than in control rats. Also, both test atmospheres increased the levels of most individual DNA adducts in the type II cells above those observed in the control rats. None of these adducts were identified, and they did not comigrate with an authentic benzo[*a*]pyrene diolepoxide adduct standard.

Dr. Mauderly also measured DNA adducts in the lung tissues of animals that were killed at three months. A full discussion of these results will be reported in an Appendix of Part II of this Research Report, which will present Dr. Randerath's analyses of lung DNA adducts at multiple time points.

Nonneoplastic Responses

Markers of inflammation and cytotoxicity (white blood cells, selected cytoplasmic and lysosomal enzymes, and total protein) increased in a dose-related manner in rats exposed to diesel exhaust or carbon black. While the increase in the numbers of white blood cells was the same for rats exposed to carbon black at 6.5 mg/m^3 and rats exposed to diesel exhaust soot at 2.5 mg/m^3, the increase in protein and enzyme levels was greater in rats exposed to carbon black at 6.5 mg/m^3 than in rats exposed to diesel exhaust soot at 2.5 mg/m^3.

The investigators evaluated increases in the severity of nonneoplastic lesions, as determined by histopathologic scores, over time. Table 1 of this Commentary summarizes the occurrence of these lesions at different times.

At the early time points (three and six months), macrophage hyperplasia, alveolar epithelial cell hyperplasia, and chronic active inflammation were observed. The magnitude of severity and incidence was similar for each of these lesions. The portion of the lung volume occupied by single macrophages in the alveolar lumen increased in a dose-related manner. In the groups exposed to either carbon black or diesel exhaust at 6.5 mg/m^3, the macrophages were localized in the centriacinus, and the zone of involvement in this region was greater at six months than at three months.

At 12 months, the severity and the histologic characteristics of the lesions were generally similar to those observed at six months, except that the extent of each lesion increased for each exposure group. The volume of the lung occupied by single macrophages was greater in animals exposed to diesel exhaust soot at 6.5 mg/m^3 than in animals exposed to carbon black at 6.5 mg/m^3; however, the increase in aggregated macrophages was similar for both exposure groups. At 18 months, the incidence and severity of some of the lesions increased in both groups at 2.5 mg/m^3.

Table 1. Progression of Nonneoplastic and Neoplastic Lung Lesions in F344/N Rats Exposed to Diesel Exhaust or Carbon Black[a]

	Time of Observation (months)					
Lesions	3	6	12	18	23	More Than 23
Macrophage hyperplasia	+	+	+	+	+	+
Alveolar epithelial cell hyperplasia	+	+	+	+	+	+
Chronic active inflammation	+	+	+	+	+	+
Septal fibrosis	0	+	+	+	+	+
Alveolar proteinosis	0	+	+	+	+	+
Bronchiolar-alveolar metaplasia (alveolar bronchiolization)	0	0	+	+	+	+
Interstitial aggregation of macrophages containing particles	0	0	0	+	+	+
Focal fibrosis	0	0	0	0	+	+
Squamous metaplasia	0	0	0	0	+	+
Squamous cyst	0	0	0	0	+	+
Neoplasms	0	0	0	+[b]	+	+

[a] A + indicates the presence of lesions and a 0 indicates their absence.

[b] First tumor appeared at 15 months (see the Neoplastic Lung Lesions section of the Investigators' Report).

An increase in particle-containing aggregated macrophages in the interstitium also was apparent, as well as an increase in the amount of carbon black and diesel exhaust associated with alveolar proteinosis or debris.

At 24 months, focal fibrosis, squamous metaplasia, and squamous cysts were observed. In addition, the severity and the incidence of the lesions found at the earlier time points had increased slightly with dose; however, the severity scores were similar between the two exposure groups. Also, the lung alveoli were filled with particles, debris, proteinaceous material, macrophages, and inflammatory cells. This filling of the alveoli with particle debris and dead cells increased further by six weeks after the end of exposure (at the final scheduled necropsy). At that time point, the incidence of focal fibrosis increased in the groups exposed at 2.5 mg/m^3, and the incidence of squamous cysts increased in the group exposed to carbon black at 2.5 mg/m^3. Similar responses were observed in rats that died before the final scheduled necropsy.

Finally, the investigators reported differences in the distribution of alveolar macrophages in the lungs of rats exposed to diesel exhaust or carbon black. The independent pathologist engaged by the HEI Review Committee concurred with this observation, and noted that the lungs of rats exposed to diesel exhaust had more widespread involvement and more diffuse distribution of macrophages, and that the macrophages contained small amounts of pigment. Many of the alveoli in the lungs of rats exposed to carbon black were free of pigment-laden macrophages; however, the macrophages in peribronchiolar locations were heavily loaded with particles. These distributional differences were striking enough so that the nature of exposure for each rat was recognizable on microscopic examination. These observations support the view that the bimodal particle size distribution between animals exposed to diesel exhaust and those exposed to carbon black led to differences in initial deposition and distribution, and to the subsequent translocation and ultimate lung burden of particles, which is consistent with the more proximal deposition of the carbon black particles compared with the soot particles from the diesel exhaust.

Neoplastic Effects

Prolonged exposure (24 months) to either carbon black or diesel exhaust caused dose-related increases in the incidence of malignant and benign lung neoplasms in F344/N rats. No significant differences were noted in the neoplastic responses to these two exposure regimens as determined by logistic regression modeling. The neoplasms appeared late in life (15 to 18 months), and they appeared slightly earlier in rats exposed to carbon black than in rats exposed to diesel exhaust; however, this difference was not statistically significant. The crude incidences (percentage of rats with tumors), the lifetime incidences estimated by logistic regression analysis, and the multiplicity of neoplasms (neoplasm-bearing rats having one, two, three, or more neoplasms) were slightly higher among rats exposed to diesel exhaust than in rats exposed to carbon black, but again the differences were not statistically significant. A higher incidence of neoplasia occurred in female rats than in male rats, possibly due to the shorter life span of the male rats exposed to both concentrations of particles. In this study, lung neoplasm response was more closely related to particle exposure concentration than to measured lung burden.

The primary lung neoplasms were classified as adenomas, adenocarcinomas, squamous cell carcinomas, adenosquamous carcinomas, and one malignant neoplasm not otherwise specified. Adenomas and adenocarcinomas were the dominant neoplasms. They were further subclassified depending on their morphologic patterns. Of the squamous cell carcinomas, two arose from the epithelium of the squamous cysts and were subclassified as squamous cell carcinomas arising from squamous cysts. All the primary neoplasms appeared to arise from the parenchyma and not the conducting airways where gas exchange occurs. The morphologic features of the adenomas and adenocarcinomas suggest that they arose from the alveolar epithelium. No neoplasm type or structure was distinctive for carbon black or diesel exhaust exposure. The primary neoplasms occurred in all treatment groups, except that no squamous cell carcinomas were found in rats exposed to carbon black at 2.5 mg/m^3. The neoplasms occurred more often in females than in males. This gender difference was most striking at the exposure level of 6.5 mg/m^3 for both diesel exhaust soot and carbon black. In contrast, more squamous cell carcinomas occurred in male than in female rats.

When tumor tissues were transplanted subcutaneously into nude mice, the histologic appearance of the primary tumors did not differ between the two exposure atmospheres. Overall, 33% of the adenocarcinomas and 60% of the squamous cell carcinomas grew after transplantation, suggesting that the squamous cell carcinomas had more aggressive growth characteristics than the adenocarcinomas. None of the lesions classified as squamous cysts grew when transplanted, further supporting the investigators' view that these lesions should not be classified as neoplasms. In earlier studies, these lesions either have been reported as benign squamous tumors (Heinrich et al. 1986), have not been observed or reported (Iwai et al. 1986), or have been reported as benign tumors (Mauderly et al. 1987). The consensus to classify these lesions as cysts rather than

neoplasms is a majority opinion, rather than a unanimous one, among pathologists evaluating animal lung lesions. Some pathologists still prefer to consider these lesions as neoplasms, and others as nonneoplastic (Boorman et al. 1985; International Agency for Research on Cancer 1992).

The carefully documented results of this study suggest significant events relative to the pathogenesis of lung neoplasms associated with prolonged exposure to diesel exhaust and carbon black. Although a progression from squamous metaplasia to benign squamous neoplasm to squamous cell carcinoma was not clearly evident, the study showed an interesting sequence of cellular changes leading toward neoplasm (see Table 1 of this Commentary). First, with one exception, neoplasms developed only after 18 months of exposure. Second, bronchioalveolar metaplasia and interstitial aggregation of particle-containing macrophages were noted in the first neoplasm-bearing rat. This appeared to be a significant aspect of tumor induction. Third, the late appearance of neoplasms coincided with bronchioalveolar metaplasia, aggregation of particle-containing macrophages in the interstitium, and focal fibrosis. In the focal lesions, an accumulation of debris, comprised to a significant degree of dead macrophages was evident; these cells had disintegrated and released particulate matter permitting more extensive exposure of local tissues to the effects of particles, as well as to degradation products from the disintegrating macrophages.

INTERPRETATION

In Dr. Mauderly's study, chronic exposure to high concentrations of carbon black and diesel exhaust induced similar nonneoplastic and neoplastic responses in F344/N rats. Because the dose-response relation correlated to the particle concentration rather than the amount of mutagenic organic material associated with the carbon core, the results suggest that, in this rat model, the effects of the particles rather than the soot-associated mutagenic organic compounds most likely determine the pathogenesis of lung tumors associated with exposure to diesel exhaust. However, the data leave unanswered the questions of whether the particle-associated organic compounds play any role in rat pulmonary carcinogenicity and whether they are important in the pathogenesis of human lung cancer.

Dr. Mauderly's findings on particle-induced lung neoplasms in rats agree with the preliminary data published by Heinrich and coworkers (Heinrich et al. 1993; Heinrich 1994), who chronically exposed Wistar rats to high concentrations of diesel exhaust, carbon black (Degussa Printex-90), titanium dioxide (P 25), or to clean air (control group). As in the present study, the nonneoplastic disease of the lung was progressive and similar for rats exposed to diesel exhaust, carbon black, and titanium dioxide. A significant increase in the number of lung tumors was caused by all three exposures. The pulmonary carcinogenic potency of these materials, based on the incidence of tumors per unit of particle lung burden, were highest for titanium dioxide, intermediate for carbon black, and lowest for diesel soot. Both the Heinrich and Mauderly studies clearly demonstrate that carbon black and diesel soot are carcinogenic in rat lungs when inhaled chronically at high concentrations.

A diverse group of solid, poorly soluble, nonfiber particles also cause tumors in rats exposed to high concentrations by either chronic inhalation or intratracheal instillation (Pott et al. 1994; Pott and Roller 1994). The list includes several particles (quartz, carbon black, titanium dioxide, talc, and coal dust) that have no significant content of organic mutagens, some of which previously were thought to have little or no cytotoxicity.

The mechanism of particle-induced lung tumorigenesis in rats is unknown. It has been hypothesized that the tumorigenic effect of high particle exposure is associated with a phenomenon called particle overload, which occurs when insoluble particles are deposited in the lungs at a rate faster than they can be cleared. It has been speculated that particle overload causes inflammation, epithelial proliferation, and lung fibrotic reactions, and eventually leads to lung tumors if the particle exposure continues for a sufficiently long time (Morrow 1988, 1992).

One mechanism by which proliferative responses such as inflammation and hyperplasia might result in neoplasia is through the activation of oncogenes or the loss or inactivation of tumor suppressor genes. Expression of such genes is known to require proliferating cells (Sager 1992). Alternatively, genotoxicity and other forms of cell injury could result through the release of oxygen species by activated macrophages, which are a potential source of oxygen free radicals, such as superoxide anion, hydrogen peroxide, and hydroxide ion. Oxygen free radicals also can cause the formation of 8-hydroxyguanine–DNA adducts, which have been associated with some types of cancer in laboratory animals (Weiss 1989; Floyd 1990).

It is also possible that in a highly proliferative environment, such as that following particle overload, cytokines or growth factors are released by activated macrophages, phagocytes, and other cells; the cytokines and growth factors lead to an increase in cell division and ultimately to cancer. Several investigators have discussed the importance of chronic mitogenesis (increased cell division in the

target tissue) for many human cancers. Examples include the importance of hormones in breast cancer, hepatitis B or C viruses or alcohol in liver cancer, high salt or bacterial infection in stomach cancer, and papilloma virus in cervical cancer (Ames and Gold 1990; Preston-Martin 1992). It also has been suggested that increased cell proliferation, in addition to the genotoxic effects of various chemical exposures, may contribute to tumor development in humans. For example, cigarette smoking is known to cause bladder cancer in humans, perhaps due to the hyperplastic effect on the urothelium, in addition to the probable genotoxic damage that occurs (Cohen and Ellwein 1990).

Regardless of the mechanisms involved, the findings of this study and those of Heinrich and coworkers (Heinrich et al. 1993; Heinrich 1994) negate the hypothesis that the carbon matrix of the diesel soot particle simply acts as a vehicle that permits adsorbed mutagenic and carcinogenic organic compounds to come in contact with the respiratory tract and thus induce cancer in rats. They indicate that the small, respirable soot particles are primarily responsible for lung cancer developing in rats exposed to high concentrations of diesel exhaust. However, at this time, a role for the organic carcinogens found in diesel exhaust cannot be completely excluded from the etiology of lung cancer. It is possible that these compounds induce molecular changes that, in the presence of active cellular proliferation, subsequently result in neoplasia. However, it may not be possible to detect these changes in rats because they may be masked by the massive hyperplasia caused by large particle burdens, or because they may be below the detection limits of the animal bioassay. Finally, at lower exposure concentrations of diesel soot where massive epithelial hyperplasia is not the preponderant pathologic response, the mutagenic organic constituents could play an important role in a small fraction of the exposed population. Even though this population size may be too small to allow detection of an increase in lung neoplasms in an animal bioassay, such a response nevertheless would be of concern for human populations.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

The results of this study, rather than providing definitive answers about diesel carcinogenesis, evoke a series of investigative issues and questions. Although the findings clearly implicate particles as a causal factor in the induction of lung tumors in rats exposed chronically to high concentrations of diesel exhaust, the results have not been reproduced in another species. It is uncertain whether the initial stimulus for tumors is genetic or epigenetic, and the reason for the late occurrence of tumors has not been elucidated. It also is not clear what the association is, if any, between retarded clearance of the inhaled particles and resulting tumor induction.

Because the investigators did not study a dose range that included low levels of diesel exhaust comparable with environmental exposures, it is not known whether similar results would be observed under a scenario closer to human experience than the high exposure concentrations used in this study. The entry of particles into the rat lung initiates a chain of molecular and cellular events that results in chronic inflammation, epithelial proliferation, fibrosis, and ultimately tumors. Future studies are needed to provide information on the molecular and biochemical changes in laboratory animals exposed to diesel exhaust or other fine particles. Similar biochemical and molecular studies in a human population with known exposures to particles, such as coal miners, or in workers exposed chronically to carbon black or similar particles would provide a basis for extrapolating data on the basis of mechanisms of action.

REGULATORY IMPLICATIONS

This study demonstrates that the dose-response relation between exposure to diesel exhaust or carbon particles and the neoplastic and nonneoplastic response in rat lungs favors the particle rather than the adsorbed organic chemicals as the causative agent for pulmonary neoplasms in this species. The data call into question, and virtually disprove, the hypothesis that particle-associated mutagenic organic compounds alone induce these lung neoplasms in rats. Furthermore, the data raise the question of whether it is valid to extrapolate lung tumor incidence in rats exposed chronically to high concentrations of particles to estimate cancer risk in humans exposed to low levels of particles for prolonged time periods, using either the delivered dose of soot-associated organic compounds or the lung burdens of particles as the comparative dose term. Although the results of the Mauderly study point to particle dose as the relevant factor for extrapolation, this assumes that the mechanisms that operate in humans exposed to low doses are similar to those that operate in rats exposed to high doses; this assumption has not been validated.

Numerous attempts have been made to develop quantitative estimates of cancer risk for humans from diesel exhaust exposure (Smith and Stayner 1990; Pepelko and Chen 1993; U.S. Environmental Protection Agency 1993). A common approach for measuring carcinogenic potency is to

calculate unit cancer risk. This is an estimate of the excess or added probability that an exposed individual will develop cancer due to continuous exposure to one unit of dose of a chemical. It assumes a linear relation between dose and incremental probability at a low-dose level. One problem with assessing risk quantitatively for complex mixtures such as diesel exhaust is the difficulty of identifying the agent(s) responsible for the observed neoplasms. A second problem is the uncertainty inherent in extrapolating animal bioassay data to humans.

In the mid 1980s, cancer risk factors for diesel exhaust were based on the assumption that the soot-associated organic compounds were responsible for the lung tumors observed in rats exposed to diesel exhaust. They were calculated by comparing the mutagenic potencies of diesel exhaust extracts with other known human carcinogens (Albert et al. 1983; Lewtas et al. 1991). As preliminary data from the Mauderly and Heinrich studies became available, risk assessors abandoned this approach and used the carbon core of the diesel particles as the comparative dose term (Pepelko and Chen 1993; U.S. Environmental Protection Agency 1993; California Environmental Protection Agency 1994).

The results of Dr. Mauderly's study and recent findings from other laboratories indicate that the diesel soot particle, rather than the adsorbed organic carcinogens, cause pulmonary neoplasms in rats exposed to high concentrations of diesel exhaust. However, a number of uncertainties are associated with the use of particle exposure concentration as the comparative dose term.

It is not known whether the rat response to inhalation of high concentrations of fine particles is unique to that species. Long-term inhalation of high concentrations of diesel exhaust does not cause lung neoplasms in hamsters, and the results in mice are equivocal. The inflammatory response in the lungs of the rats exposed to high concentrations of irritant particles is more severe than that observed in the hamster, and this factor may be responsible for the observed differences in lung neoplasms in the two species. Because lung neoplasms induced by diesel exhaust have been observed in rats only under conditions where the lung particle burden has been so high that clearance mechanisms are greatly reduced or even cease completely, a major question is whether the rat or the hamster is a better predictor of the human response to inhaled particles. Additional questions are whether there is a threshold for the neoplastic response, and whether a linear extrapolation from high to low exposure concentrations is appropriate. Also, at this time, the possibility cannot be excluded that the carcinogens found in the diesel exhaust may play a role in cancer of the lungs or other organs.

As the mechanisms of lung carcinogenesis in different species become more thoroughly understood, a more rational approach can be taken to extrapolate from the high-dose data in animal experiments to low-dose natural exposures, and thereby to assess the risk faced by human populations exposed to diesel exhaust.

CONCLUSIONS

Dr. Mauderly and coworkers performed a carefully controlled animal bioassay, together with associated studies, to determine the relative contribution of soot-associated organic compounds and particles in the induction of lung tumors in rats exposed chronically to two levels of either diesel exhaust or carbon black. The investigators used carbon black particles that were similar to those of diesel exhaust soot; however, approximately one hundred times less organic material could be extracted from the carbon black than from the diesel exhaust particles, and the carbon black extracts produced little or no response in bacterial mutagenicity assays. Thus, the carbon black served as a surrogate for diesel exhaust carbon particles without the adsorbed organic constituents. The investigators exposed F344/N rats to either diesel exhaust or carbon black at two exposure levels (2.5 or 6.5 mg particles/m^3) for 24 months. These concentrations of diesel exhaust were known to cause lung cancer in this strain of rat. A number of other endpoints that are thought to be linked to the development and progression of lung cancer also were measured.

The investigators found that prolonged exposure to the same particle concentrations of carbon black or diesel exhaust produced nearly identical carcinogenic and noncarcinogenic effects. The neoplasms were predominantly adenomas and adenocarcinomas. No significant differences were noted in tumor incidence or histopathologic characteristics between rats exposed to diesel exhaust or carbon black. Both particles accumulated progressively, and clearance was impaired after three months in all exposure groups. Markers of cytotoxicity and nonneoplastic changes, such as inflammation, epithelial cell proliferation, and fibrosis, were progressive and related to dose. Changes in most of the above parameters were related to exposure concentration. The sequestration and translocation of tracer particles were similarly affected by both exposures. The investigators also measured DNA adducts in the rat lung after three months of chronic exposure to diesel exhaust and carbon black. These results will be discussed together with the results from studies by Dr. Randerath, who measured lung DNA adducts at multiple time points, in Part II of this Research Report.

The results of this study support the findings of other investigators that, at sufficiently high exposure concentrations, insoluble particles induce pulmonary tumors in the rat. Furthermore, they show that lung cancer induced by high concentrations of diesel exhaust in the rat can be attributed to the particle exposure rather than the soot-associated mutagens. However, the results do not completely refute a possible role for the mutagenic organic compounds found in diesel exhaust. The possibility exists that these compounds may contribute to lung cancer risk if exposure occurs at lower levels for longer periods of time. Interpreting the significance of these findings for human health assessments is difficult because it is not known whether the rat response to exposure to high concentrations of particles is unique to that species or if there is a threshold for the neoplastic response. Although the present results indicate the importance of particles rather than the amount of deposited organic material as the comparative dose term, caution should be exercised before either constituent of diesel exhaust is used for quantitative risk assessment. Additional studies of mechanisms are needed to provide an adequate scientific basis for extrapolating the data from lung tumors induced by diesel exhaust or particles in rats to potential risk for humans.

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REFERENCES

Adamson IY, Bowden DH. 1974. The type 2 cell as progenitor of alveolar regeneration. Lab Invest 30:35–42.

Albert RE, Lewtas J, Nesnow S, Thorslung TW, Anderson E. 1983. Comparative potency method for cancer risk assessment: Application to diesel particulate emissions. Risk Anal 3:101–117.

Ames BN, Gold LS. 1990. Chemical carcinogenesis: Too many rodent carcinogens. Science 249:970–971.

Ames BN, McCann J, Yamasaki E. 1975. Methods for detecting carcinogens and mutagens with the Salmonella/Mammalian-Microsome mutagenicity test. Mutat Res 31:347–364.

Bechtold WE, Dutcher JS, Mokler BV, Lopez JA, Wolff I, Henderson TR, McClellan RO. 1984. Chemical and biological properties of diesel exhaust particles collected during selected segments of simulated driving cycle. Fundam Appl Toxicol 4:370–377.

Belinsky SA, Foley JF, White CM, Anderson MW, Maronpot RR. 1990. Dose-response relationship between O^6-methylguanine formation in clara cells and induction of pulmonary neoplasia in the rat by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Cancer Res 50:3772–3780.

Bond JA, Johnson NF, Snipes BM, Mauderly JL. 1990. DNA adduct formation in rat alveolar type II cells: Cells potentially at risk for inhaled diesel exhaust. Environ Mol Mutagen 16:64–69.

Bond JA, Sun JD, Medinsky RK, Jones RK, Yeh HC. 1986. Deposition, metabolism, and excretion of 1-(^{14}C)nitropyrene and 1-(^{14}C)nitropyrene coated on diesel exhaust particle as influenced by exposure concentration. Toxicol Appl Pharmacol 85:102–117.

Bond JA, Wolff RK, Harekema JR, Mauderly JL, Henderson RF, Griffith WC, McClellan RO. 1988. Distribution of DNA adducts in the respiratory tract of rats exposed to diesel exhaust. Toxicol Appl Pharmacol 96:336–346.

Boorman GA. 1985. Bronchoeolar/alveolar adenoma, Lung, Rat. In: Respiratory Systems. Monograph on Pathology of Laboratory Animals (Jones TC, Mohr U, Hunt R, eds.) pp. 99–101. Springer-Verlag, New York, NY.

Brightwell J, Fouillet X, Cassano-Zoppi A-L, Berstein D, Gatz R, Duchosal F. 1986. Neoplastic and functional changes in rodents after chronic inhalation of engine exhaust emissions. In: Carcinogenic and Mutagenic Effects of Diesel Engine Exhaust (Ishinishi N, Koizumi A, McClellan RO, Stöber W, eds.) pp. 471–485. Elsevier Science Publishing Co., New York, NY.

Brightwell J, Fouillet X, Cassano-Zoppi A-L, Gatz R, Berstein D, Crawley F, Duchosal F, Gatz R, Perczel S, Pfeifer H. 1989. Tumors of the respiratory tract in rats and hamsters following chronic inhalation of engine exhaust emissions. J Appl Toxicol 9:23–31.

Brooks AL, Wolff RK, Royer RE, Clark CR, Sanchez A, McClellan RO. 1980. Biological availability of mutagenic chemicals associated with diesel exhaust particles. In: Health Effects of Diesel Emissions (Pepelko WE, Danner RM, Clark NA, eds.) pp. 345–358. EPA-600/9-80-057a. US Environmental Protection Agency, Cincinnati, OH.

California Environmental Protection Agency. 1994. Health Risk Assessment for Diesel Exhaust (Preliminary Draft). Office of Environmental Health Hazard Assessment, California Environmental Protection Agency, Sacramento, CA.

Cohen SM, Ellwein LB. 1990. Cell proliferation in carcinogenesis. Science 249:1007–1011.

DeLuchi MA. 1992. Greenhouse-Gas Emissions for the Use of Transportation Fuels and Electricity. Institute of Transportation Studies, University of California, Davis, CA.

Floyd RA. 1990. Role of oxygen free radicals in carcinogenesis and brain ischemia. FASEB J 4:2587–2597.

Grimmer G, Brune H, Deutsch-Wenzel R, Dettbarn G, Jacob J, Naujack K-W, Mohr U, Ernst H. 1987. Contribution of polynuclear aromatics and nitro-derivatives to the carcinogenic impact of diesel engine exhaust condensate evaluated by implantation in the lungs of rats. Cancer Lett 37:173–180.

Heinrich U. 1994. Carcinogenic effects of solid particles. In: Toxic and Carcinogenic Effects of Solid Particles in the Respiratory Tract (Mohr U, Dungworth DL, Mauderly JL, Oberdörster G, eds.) pp. 57–73. International Life Sciences Institute Press, Washington, DC.

Heinrich U, Muhle H, Takenaka S, Ernst H, Fuhst R, Mohr U, Pott F, Stöber W. 1986. Chronic effects on the respiratory tract of hamsters and rats after long-term inhalation of high concentrations of filtered and unfiltered diesel engine emissions. J Appl Toxicol 6:383–395.

Howard PC, Hecht SS, Beland FA, eds. 1990. Nitroarenes: Occurrence, Metabolism, and Biological Impact. Plenum Press, New York, NY.

Huisingh JL, Bradow R, Jungers R, Claxton L, Zweidinger R, Tejada S, Bumgarner J, Duffield F, Waters M, Simmon FVF, Hare C, Rodriguez C, Snow L. 1978. Application of bioassay to the characterization of diesel particle emissions. In: Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures (Waters MD, Nesnow S, Huisingh JL, Sandhu SS, Claxton L, eds.) pp. 381–418. Plenum Press, New York, NY.

International Agency for Research on Cancer. 1983. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol 32, Polynuclear Aromatic Compounds, Part I, Chemical, Environmental and Experimental Data. International Agency for Research on Cancer, Lyon, France.

International Agency for Research on Cancer. 1989. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 46, Diesel and Gasoline Exhausts and Some Nitroarenes. International Agency for Research on Cancer, Lyon, France.

International Agency for Research on Cancer. 1992. The rat: 1. Respiratory system. In: International Classification of Rodent Tumors, Part I (Mohr U, ed.). International Agency for Research on Cancer Scientific Publications Number 122. International Agency for Research on Cancer, Lyon, France.

Ishinishi N, Kuwabara N, Nagase S, Suzuki T, Ishiwata S, Kohno T. 1986. Long-term inhalation studies on effects of exhaust from heavy and light duty diesel engines on F344 rats. In: Carcinogenic and Mutagenic Effects of Diesel Engine Exhaust (Ishinishi I, Koizumi A, McClellan RO, Stöber W, eds.) pp. 329–348. Elsevier Science Publishing Co., New York, NY.

Iwai K, Udagawa T, Yamagishi M, Yamada H. 1986. Long-term inhalation studies of diesel exhaust on F344 SPF rats: Incidence of lung cancer and lymphoma. In: Carcinogenic and Mutagenic Effects of Diesel Engine Exhaust (Ishinishi N, Koizumi A, McClellan RO, Stöber W, eds.) pp 349–360. Elsevier Science Publishing Co., New York, NY.

Kawabata Y, Iwai K, Udagawa T, Tukagoshi K, Kazue H. 1986. Effects of diesel soot on unscheduled DNA synthesis of tracheal epithelium and lung tumor formation. In: Carcinogenic and Mutagenic Effects of Diesel Engine Exhaust (Ishinishi N, Koizumi A, McClellan R, Stöber W, eds.) pp. 213–222. Elsevier Science Publishing Co., New York, NY.

Kotin P, Falk HL, Thomas M. 1955. Aromatic hydrocarbons: III. Presence in the particulate phase of diesel-engine exhaust and the carcinogenicity of exhaust extracts. AMA Arch Ind Health 11:113–120.

Lewtas J. 1991. Carcinogenic Risks of Polycyclic Organic Matter (POM) from Selected Emission Sources. Deliverable Report Number 3128. Health Effects Research Laboratory, Research Triangle Park, NC.

Mauderly JL. 1992. Diesel exhaust. In: Environmental Toxicants: Human Exposures and Their Health Effects (Lippmann M, ed.) pp 119–162. Van Nostrand Reinhold, New York, NY.

Mauderly JL, Jones RK, Griffith WC, Henderson RF, McClellan RO. 1987. Diesel exhaust is a pulmonary carcinogen in rats exposed chronically by inhalation. Fundam Appl Toxicol 9:208–221

Morrow PE. 1988. Possible mechanism to explain dust overloading of the lungs. Fundam Appl Toxicol 10:369–384.

Morrow PE. 1992. Dust overloading of lungs: Update and appraisal. Toxicol Appl Pharmacol 113:1–12.

Nesnow S, Evans C, Stead A, Creason J, Slaga TJ, Triplett LL. 1982. Skin carcinogenesis studies of emission extracts. In: Toxicological Effects of Emissions from Diesel Engines (Lewtas J, ed.) pp. 295–320. Elsevier Science Publishing Co., New York, NY.

Nesnow S, Triplett LL, Slaga TJ. 1983. Mouse skin tumor initiation-promotion and complete carcinogenesis bioassays: Mechanisms and biological activities of emission samples. Environ Health Perspect 47:225–268.

Pepelko WE, Chen C. 1993. Quantitative assessment of cancer risk from exposure to diesel engine emissions. Regul Toxicol Pharmacol 17:52–65.

Pepelko WE, Peirano WB. 1983. Health effects of exposure to diesel engine emissions: A summary of animal studies conducted by the U.S. Environmental Protection Agency's Health Effects Research Laboratories at Cincinnati, Ohio. J Am Coll Toxicol 2:253–306.

Pott F, Dungworth DL, Heinrich U, Muhle H, Kamino K, Germann P-G, Roller M, Rippe RM. 1994. Lung tumors in rats after intratracheal instillation of dusts. Ann Occup Hyg (in press).

Pott F, Roller M. 1994. Relevance of nonpyhsiologic exposure routes for carcinogenicity studies of solid particles. In: Toxic and Carcinogenic Effects of Solid Particles in the Respiratory Tract (Mohr U, Dungworth DL, Mauderly JL, Oberdorster G, eds.) pp. 109–124. Internationl Life Sciences Institute Press, Washington, DC.

Preston-Martin S, Pike MC, Ross RK, Jones PA, Henderson BE. 1990. Increased cell division as a cause of human cancer. Cancer Res 50:7415–7421.

Sager R. 1992. Tumor suppressor genes in the cell cycle. Curr Opin Cell Biol 4:155–160.

Siak JS, Strom KA. 1981. Mutagenicity of Particles in Alveolar Macrophages from Diesel Exposed Animals. General Motors Research Publication Number GMR-3628. General Motors Research Laboratories, Warren, MI.

Smith RA, Stayner L. 1990. An Exploratory Assessment of the Risk of Lung Cancer Associated with Exposure to Diesel Exhaust Based on a Study in Rats. Report #45226. National Institute for Occupational Safety and Health, Cincinnati, OH.

Sun JD, Wolff RK, Kanapilly GM, McClellan RO. 1984. Lung retention and metabolic fate of inhaled benzo[*a*]pyrene associated with diesel exhaust particles. Toxicol Appl Pharmacol 73:48–59.

Takemoto K, Yoshimura H, Katayama H. 1986. Effects of chronic inhalation exposure to diesel exhaust on the development of lung tumors in di-isopropanol-nitrosamine–treated F344 rats and newborn C57BL and ICR mice. In: Carcinogenic and Mutagenic Effects of Diesel Engine Exhaust (Ishinishi N, Koizumi A, McClellan RO, Stöber W, eds.) pp. 3110–3327. Elsevier Science Publishing Co., New York, NY.

U.S. Environmental Protection Agency. 1993. Motor Vehicle-Related Air Toxics Study. EPA 420-R-93-005. Office of Mobile Sources, Ann Arbor, MI.

Vostal JJ. 1986. Factors limiting the evidence for chemical carcinogenicity of diesel emissions in long-term inhalation experiments. In: Carcinogenic and Mutagenic Effects of

Diesel Engine Exhaust (Ishinishi N, Koizumi A, McClellan RO, Stöber W, eds.) pp. 381–396. Elsevier Science Publishing Co., New York, NY.

Weiss SJ. 1989. Tissue destruction by neutrophils. N Engl J Med 320:365–375.

Wong DC, Mitchell CE, Wolff RK, Mauderly JL, Jeffrey AM. 1986. Identification of DNA damage as a result of exposure of rats to diesel engine exhaust. Carcinogenesis 7:1595–1597.

Yu CP, Xu GB. 1986. Predictive models for deposition of diesel exhaust particulates in human and rat lungs. Aerosol Sci Technol 5:337–347.

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