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Response of Rodents to Inhaled Diluted Diesel Exhaust: Biochemical and Cytological Changes in Bronchoalveolar Lavage Fluid and in Lung Tissue

R. F. HENDERSON, J. A. PICKRELL, R. K. JONES, J. D. SUN, J. M. BENSON, J. L. MAUDERLY, AND R. O. MCCLELLAN

Inhalation Toxicology Research Institute, Lovelace Biomedical and Environmental Research Institute, Albuquerque, New Mexico 87185

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Response of Rodents to Inhaled Diluted Diesel Exhaust: Biochemical and Cytological Changes in Bronchoalveolar Lavage Fluid and in Lung Tissue. HENDERSON, R. F., PICKRELL, J. A., JONES, R. K., SUN, J. D., BENSON, J. M., MAUDERLY, J. L., AND MCCLELLAN, R. O. (1988). *Fundam. Appl. Toxicol.* **11,** 546–567. The effect of long-term (24 months) inhalation of diesel exhaust on the bronchoalveolar region of the respiratory tract of rodents was assessed by serial (every 6 months) analysis of bronchoalveolar lavage fluid (BALF) and of lung tissue from F344/Crl rats and CD-1 mice (both sexes) exposed to diesel exhaust diluted to contain 0, 0.35, 3.5, or 7.0 mg soot/m^3. The purpose of the study was twofold. One was to assess the potential health effects of inhaling diluted exhaust from light-duty diesel engines. The second was to determine the usefulness of BALF analysis in detecting the early stages in the development of nononcogenic lung disease and differentiating them from the normal repair processes. No biochemical or cytological changes in BALF or in lung tissue were noted in either species exposed to the lowest, and most environmentally relevant, concentration of diesel exhaust. In the two higher levels of exposure, a chronic inflammatory response was measured in both species by dose-dependent increases in inflammatory cells, cytoplasmic and lysosomal enzymes, and protein in BALF. Histologically, after 1 year of exposure, the rats had developed focal areas of fibrosis associated with the deposits of soot, while the mice, despite a higher lung burden of soot than the rats, had only a fine fibrillar thickening of an occasional alveolar septa in the high-level exposure group. Higher increases in BALF β-glucuronidase activity and in hydroxyproline content accompanied the greater degree of fibrosis in the rat. BALF levels of glutathione (GSH) and glutathione reductase activity increased in a dose-dependent fashion and were higher in mice than in rats. Lung tissue GSH was depleted in a dose-dependent fashion in rats but was slightly increased in mice. This depletion may have played a role in the greater fibrogenic response observed in rats. Other tissue changes in enzymatic activity were small compared to changes observed in BALF. The exposure did not increase the cytochrome *P*-450 content of the lung in either species. The results suggest that, for the noncarcinogenic health effects reported in this paper, there is a threshold of exposure below which adverse effects were not observed. This threshold was well above environmentally relevant levels of diesel exhaust but may be in the range of some occupational exposures. The analysis of BALF proved a useful adjunct to the chronic toxicity study to quantitate the inflammatory changes accompanying the development of pulmonary disease.

The potential for increased use of diesel engines in light duty vehicles has raised a concern for the health risk to man of increased diesel emissions. Data on the health effects of inhaled diesel engine emissions obtained from the study of human populations suggest the potential for carcinogenicity (Garshick *et al.*, 1987), but are not adequate for predicting risk. Human studies have also not allowed assessment of the potential for diesel emissions to induce nononcogenic lung disease. Therefore, an international effort has been directed at determining the health effects of long-term inhalation of diluted diesel exhaust in ani-

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mals (Ishinishi *et al.*, 1986a). These include studies by the U.S. National Institute of Occupational Safety and Health (Lewis *et al.*, 1986), the U.S. Environmental Protection Agency (Albert and Chen, 1986), the Southwest Research Institute (White *et al.*, 1983), General Motors Research Laboratories (Vostal *et al.*, 1982), the Japan Automobile Research Institute (Ishinishi *et al.*, 1986b), the Fraunhofer Institute for Aerosol Research (Stöber, 1986), the Battelle-Geneva Research Center (Brightwell *et al.*, 1986), and the Lovelace Inhalation Toxicology Research Institute (Mauderly *et al.*, 1987b).

Our laboratory has completed a 2-year study in which F344/Crl rats and CD-1 mice were exposed to various levels of diluted exhaust from diesel engines and the health effects observed at 6-month intervals. Biochemical, physiological and histopathological responses were monitored. This is a report of the biochemical and cytological responses observed in bronchoalveolar lavage fluid and the biochemical response observed in lung tissues of the exposed rodents. The accumulation of diesel soot in the lungs of the rats (Wolff *et al.*, 1987), the effect of the exposures on pulmonary immune responses (Bice *et al.*, 1985) and respiratory function (Mauderly *et al.*, 1987a), and the tumorigenic response of the rats to the soot (Mauderly *et al.*, 1987b) have been reported.

The use of bronchoalveolar lavage (BAL) as a probe to detect acute lung injury in toxicological studies is now well established (Henderson *et al.*, 1985a; Henderson, 1984; Beck *et al.*, 1982; Henderson *et al.*, 1981). One purpose of the present study, in addition to determining the health effects of long-term inhalation of diesel emissions, was to determine the usefulness of bronchoalveolar lavage fluid (BALF) analysis in detecting the early stages of developing chronic lung disease and differentiating them from the normal repair processes. If BALF analysis can indicate changes in the bronchoalveolar milieu that precede and predict development of chronic pulmonary pathology, the usefulness of the technique in animal toxicologic research and in human diagnostic procedures would be greatly enhanced.

METHODS

Animals. Male and female F344/Crl rats and CD-1 mice, specific pathogen-free, from the Institute's colony were housed continuously in whole-body exposure chambers (Hazleton 2000) and exposed 7 hr/day, 5 days/week for up to 30 months to clean air as controls (C) or to concentrations of diluted diesel exhaust containing nominally 7.0 (high = H), 3.5 (medium = M), or 0.35 (low = L) mg particles/m^3. Exposures were initiated when the animals were 17 weeks old. The animals were from a colony maintained in specific-pathogen-free status. Sera from selected animals were collected at the end of the 30-month exposure and no positive titers to pathogenic viruses were found. This report includes data collected through 24 months of exposure for rats and 18 months of exposure for mice. There were insufficient numbers of animals for these assays at later termination times.

Exposure system. The diesel exhaust exposure system (Mokler *et al.*, 1984) and the chemical and physical characterization of the diesel exhaust (Cheng *et al*, 1984) have been reported. Exhaust was generated by 1980, 5.7-liter Oldsmobile engines operating on the Federal Test Procedure urban driving cycle and burning a standardized certification fuel (D-2 Diesel Control Fuel, Phillips Chemical Co.). Exhaust was diluted serially with clean air to provide the desired concentrations. Particle concentrations were measured by filter samples taken from one chamber per level daily; bag samples for gas analysis were taken from one chamber per level weekly. A summary of the characteristics of the exposure atmospheres is given in Table 1.

Bronchoalveolar lavage fluid analyses. The techniques used in the BALF and the lung tissue analyses have been reported (Henderson *et al*, 1978, 1979a,b; DeNicola *et al.*, 1981). Briefly, for BALF analysis, groups of 16 rats or mice (8 of each sex per group) were killed after 6, 12, and 18 months of exposure and groups of 16 rats were killed after 24 months of exposure (insufficient surviving mice at 24 months for observations) by cervical dislocation while the animals were under halothane anesthesia. The heart–lung block was removed and weighed and the bronchoalveolar airways were washed two times with 7 ml (male rats), 5 ml (female rats), or 1 ml (mice) of 0.15 M saline. Lavage fluids from 2 mice (1 of each sex) were pooled to give adequate material for analysis. The lavaged lung lobes were removed and the heart and trachea were removed by dissection and weighed to determine the original wet weight of the lung lobes by difference. The cells were removed from the lung washings by centrifugation (300*g* for 10 min) and were resuspended in 1 ml of saline. Total cell counts were made on a Coulter counter and differential cell counts were made on cyto-

TABLE 1

EXPOSURE ATMOSPHERES THROUGH 24 MONTHS OF EXPOSURE[a]

		High		Medium		Low		Control	
		$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
Particles	$\mu g/m^3$	7080	810	3470	450	350	70	13	6
Carbon dioxide	ppm	6640	1320	4360	590	2280	370	2010	390
Carbon monoxide	ppm	30	13	17	7	3	1	1	0.7
Hydrocarbons	ppm	13	8	9	5	4	0.9	3	0.6
Nitrogen dioxide	ppm	0.7	0.5	0.3	0.2	0.1	0.1	0	0
Nitrogen oxide	ppm	10	3	6	1.5	0.7	0.3	0	0
Ammonia	ppm	0.7	0.6	0.9	0.9	1.4	1.3	1	3

[a] Mean of weekly mean values taken during the exposures.

centrifuge preparations stained by a Wright–Giemsa-type stain (Diff-Quik, Harleco).

Biochemical parameters chosen for measurement in the BALF included lactate dehydrogenase and glutathione reductase (LDH and GSH-R, cytoplasmic enzymes used as a measure of cell injury), β-glucuronidase and acid phosphatase (BGLU and AcP, measures of hydrolytic enzymes released from phagocytic cells by active processes or by cell death), protein (measure of increased permeability of alveolar/capillary barrier), total and differential cell counts (measure of influx of inflammatory cells), alkaline phosphatase (potential measure of Type II cell secretions), and hydroxyproline (measure of release of small collagen-derived peptides or monomers). At 18 months, reduced glutathione (GSH) was measured in BAL to test the hypothesis that GSH in BAL might be increased in exposed animals as a protective measure against oxidants released into the lumen as a result of exposure to the diesel emissions.

The parameters measured in BALF are reported as concentrations in the total recovered lavage fluid. This is based on the fact that the differences in wash volumes used provides the required adjustment for the difference in lung size between species and sexes. Recovery of lavage fluid was ~75% on the first wash and ~100% on the second wash, giving an overall recovery of ~88%. Percentage recovery of lavage fluid was consistent throughout the study for both species. The data were also analyzed by calculating the total amount of each parameter recovered in the lavage fluid and dividing this value by the mean wet lung weight of the control animals to allow comparisons among species and sexes. The control wet lung weight was used to avoid underestimating responses due to the increased lung weights of animals exposed to the higher concentrations of diesel exhaust. There were no major differences in the results as analyzed by the two methods and results are reported only as concentrations in BALF.

Lung tissue analyses. For analyses of lung tissues, the lavaged lungs were homogenized in 0.1 M Tris buffer containing 0.25 M sucrose, pH 7.4, for 30 sec at 50,000 rpm in a Tissumizer (Tekmar Co.). Lungs from two mice (one of each sex) were pooled prior to homogenization. The homogenate was centrifuged at 300*g* for 10 min and the supernatant used for the analyses.

In lung tissue, LDH was used as a measure of increased cytoplasmic mass; BGLU, acid phosphatase, and acid proteinase activities were a measure of increased numbers of phagocytic cells coming into the lung or the stimulation of such cells already present, and alkaline phosphatase and glutathione reductase were measured for comparison with results of lavage fluid analyses.

Lung tissue acid proteinolytic activity was measured as release of ^{14}C from globin labeled with [^{14}C]leucine, at pH 4.2, and the activity expressed as milligrams of protein released per hour (Schapira *et al.*, 1968; Barrett, 1980; Pickrell *et al.*, 1987). Total lung collagen was measured as hydroxyproline by an automated procedure (Grant, 1965), and expressed as milligrams of collagen. The methods of enzyme analysis were standard (Bergmeyer, 1974) and have been reported (Henderson *et al.*, 1978, 1979a,b; DeNicola *et al.*, 1981). GSH analyses were done as reported earlier (Sun *et al.*, 1985) using a modification of the method of Cohn and Lyle (1966) which is specific for GSH. Finally, lung cytochrome *P*-450 content was measured by the method of Miyake *et al.* (1974) to determine if the exposure induced elevated levels of this key component of xenobiotic metabolizing enzymes.

For quantitation of materials measured in the supernatant of the tissue homogenate, the amount in an aliquot of the fluid was determined and this was multiplied by the appropriate factor to determine the amount present in the total lung. Total values in lung tissue were normalized both to the wet weight of the individual lungs and to the mean wet lung weight of the control animals. This allowed comparisons between sexes (rats only) and species and a determination of the extent to which increases in parameters paralleled increases in lung wet weight due

TABLE 2

LUNG BURDENS OF DIESEL SOOT (mg/g CONTROL LUNG; $\bar{X} \pm$ SE)[a]

	Exposure time (months)	Exposure concentrations		
		Low	Medium	High
Mice[b]	6	0.21 ± 0.02	3.7 ± 0.3	5.3 ± 0.4
	12	0.47 ± 0.03	6.3 ± 0.2	8.8 ± 0.6
	18	0.57 ± 0.04	9.5 ± 0.4	14.3 ± 1.0
Rats[c]	6	0.18 ± 0.02	0.73 ± 0.16	3.4 ± 0.8
	12	0.18 ± 0.01	1.6 ± 0.18	5.4 ± 0.2
	18	0.22 ± 0.01	4.5 ± 0.2	9.0 ± 0.3
	24	0.42 ± 0.01	7.8 ± 0.4	14.0 ± 0.2

[a] Total milligrams of soot in lungs was normalized to the mean wet weight of lungs from control animals killed at the same time as the exposed animals to allow comparisons among species and sexes and to avoid underestimating lung burdens due to increased lung weights in animals with inflammatory responses.

[b] $n = 8$ samples of 2 lungs each.

[c] $n = 16$ samples, except for 6 months time point when $n = 4$.

to the influx of fluid and cells in the inflammatory process.

Statistical analyses were by one-way ANOVA and t tests using BMDP software and applying the Bonferroni correction for multiple comparisons as appropriate.

RESULTS

Diesel soot in lung. The amount of diesel soot present in the lungs of the exposed rats at time of termination has already been reported in detail (Wolff *et al.*, 1987; Henderson *et al.*, 1987) and are briefly summarized in Table 2 to facilitate interpretation of the biochemical data reported here. The lung burdens of soot in the mice are also shown in Table 2. Mice consistently had greater concentrations of soot in their lungs (reported as mg soot/g control lung) than did the rats.

Lung wet weight. The lung wet weights were increased in the high-level and in medium-level exposed mice at all time points and in high-level exposed rats at all time points after 6 months (Fig. 1). Female rat lung weights were increased in the medium-level exposed group only after 18 months of exposure and in male rats after 24 months. No effect on lung wet weights was observed at any time in either species from the exposure to the low level of diesel exhaust. Consistent with the greater lung burdens of soot in mice, the increases in lung wet weight as a percentage of chamber control lung weights were higher in mice than rats (data not shown).

BALF analyses. An inflammatory response in the lung was observed by the increase in inflammatory cells (Figs. 2 and 3) and in the protein content of the BALF (Fig. 4). These changes were observed only in the rodents exposed at the high or medium concentrations of diluted diesel exhaust and increases at most times were greater in mice than in rats. The degree of increase in neutrophils was much greater than the increase in macrophages, particularly in the rats. As with the lung weight, significant increases were observed in mice after 6 months of exposure at both the high and medium exposure levels, but in rats, only the high-level exposed animals showed an inflammatory response at 6 months.

The indicator of cytotoxicity, LDH, was elevated in BALF in a dose-dependent manner (Fig. 5). The LDH content of BALF from rats increased with time in the two highest exposure groups up to 18 months and then decreased in the high exposure group. The LDH content of BALF from mice remained relatively stable during the 6-, 12-, and 18-month observation times but was elevated only in the lavage fluid from the two highest exposure groups. The largest increases were approximately eightfold in both species.

The other cytoplasmic enzyme assayed, glutathione reductase, was elevated in BALF in a dose-dependent manner in both species but was much higher in the mice (Fig. 6). The highest increase in rats was 6-fold, while the highest in mice was 16-fold.

Of the two lysosomal enzymes monitored in BALF, there was a large (up to 70-fold) dose-dependent increase in the amount of the BGLU activity in both species (Fig. 7), but no change in acid phosphatase (data not shown). Because control levels of BGLU were approximately twice as high in mice as in rats, the

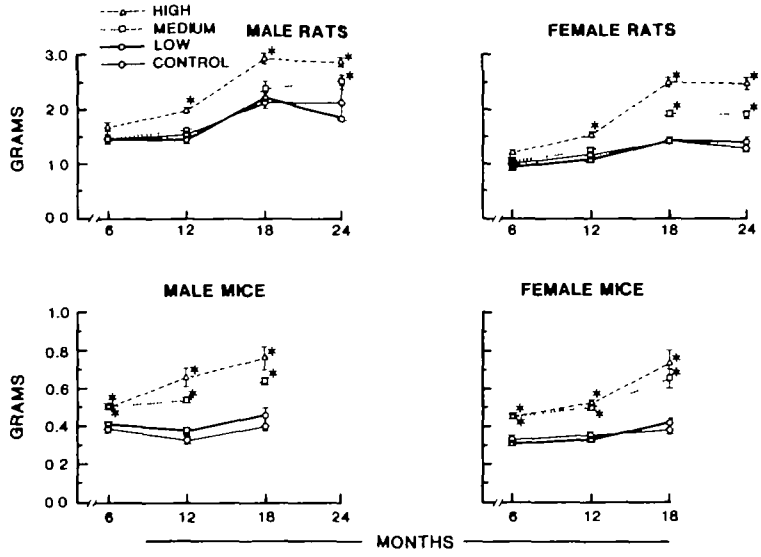

FIG. 1. Changes in wet lung weights in rodents exposed to low (0.35 mg soot/m^3), medium (3.5 mg soot/m^3), and high (7.0 mg soot/m^3) concentrations of diluted diesel exhaust over a 24-month (rats) or an 18-month (mice) period. Values are $\bar{X} \pm$ SE, $n = 8$. *Indicates values statistically different from control values, $p < 0.05$.

degree of increase was up to 70-fold in rats and up to 25-fold in mice.

The glutathione content of BALF was found to be elevated in a dose-dependent manner in both species at the 18-month observation point (Fig. 8). This parameter was not measured earlier. The GSH concentration in BALF was higher in mice than in rats.

Alkaline phosphatase activity in lavage fluid was not affected by the exposure (data not shown). However, chamber control rats had much higher activities of this enzyme than mice (~20 mIU/ml in rats versus ~2 mIU/ml in mice).

The hydroxyproline content of the BALF protein was elevated in the mice at all observation times for the two highest exposure groups, but was increased in BALF from rats only after 12 months at the highest exposure level and after 18 months at the medium ex-

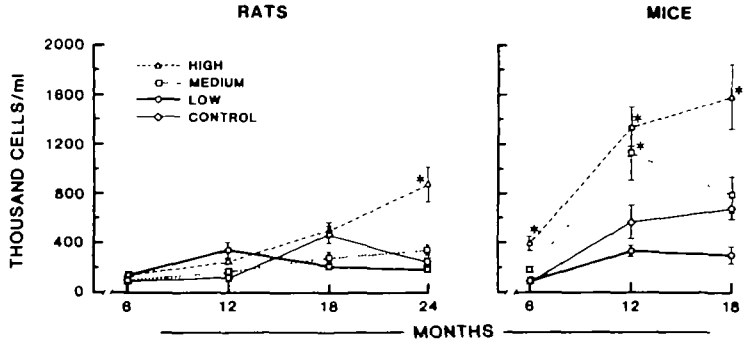

FIG. 2. Macrophages present in BALF from rodents exposed to low (0.35 mg soot/m^3), medium (3.5 mg soot/m^3), and high (7.0 mg soot/m^3) concentrations of diluted diesel exhaust over a 24-month (rats) or an 18-month (mice) period. Values are $\bar{X} \pm$ SE, $n = 16$ (rats) or 8 (mice). *Indicates values statistically different from control values, $p < 0.05$.

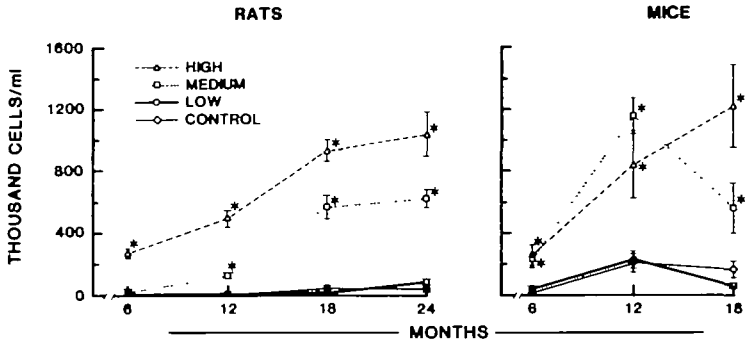

FIG. 3. Neutrophils present in BALF from rodents exposed to low (0.35 mg soot/m^3), medium (3.5 mg soot/m^3), and high (7.0 mg soot/m^3) concentrations of diluted diesel exhaust over a 24-month (rats) or an 18-month (mice) period. Values are $\bar{X} \pm$ SE, n = 16 (rats) or 8 (mice). *Indicates values statistically different from control values, $p < 0.05$.

posure level (Fig. 9). Levels of hydroxyproline were slightly higher in control mice than control rats, so that the increase in BALF hydroxyproline as a percentage of chamber control values was higher in rats (fourfold) than mice (twofold) (data not shown).

Tissue analysis. Both the cytoplasmic enzymes (LDH and GSH-R) and the lysosomal enzymes (BGLU, acid proteinases, and AcP) were increased in activity in lung tissue from the exposed rodents (Tables 3 and 4). The pattern of the increases in relation to exposure level and species followed the pattern seen in BALF but the degree of increase was less in the tissue than in BALF.

Between 18 and 24 months of exposure, total lung collagen per gram of control lung weight increased sharply in rats (Fig. 10). At 24 months, total lung collagen per gram of control lung weight at the two highest exposure levels was significantly elevated relative to those of control rats. No exposure-related changes were noted at the lowest exposure level in either species.

Lung tissue levels of GSH were higher in control mice than in control rats after 18 months of exposure (Fig. 11). There was a dose-dependent decrease in lung GSH content in rats, but, in mice, the tissue GSH was slightly increased over controls in all exposure groups.

A reduction in cytochrome *P*-450 content was observed in the lungs of rats at all exposure concentrations (Table 5). There was no

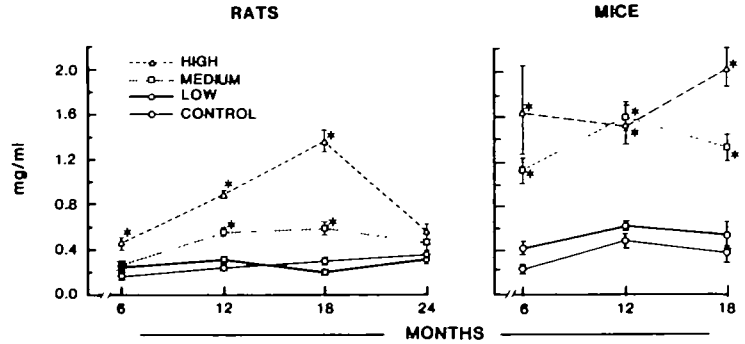

FIG. 4. Protein content of BALF from rodents exposed to low (0.35 mg soot/m^3), medium (3.5 mg soot/m^3), and high (7.0 mg soot/m^3) concentrations of diluted diesel exhaust over a 24-month (rats) or an 18-month (mice) period. Values are $\bar{X} \pm$ SE, n = 16 (rats) or 8 (mice). *Indicates values statistically different from control values, $p < 0.05$.

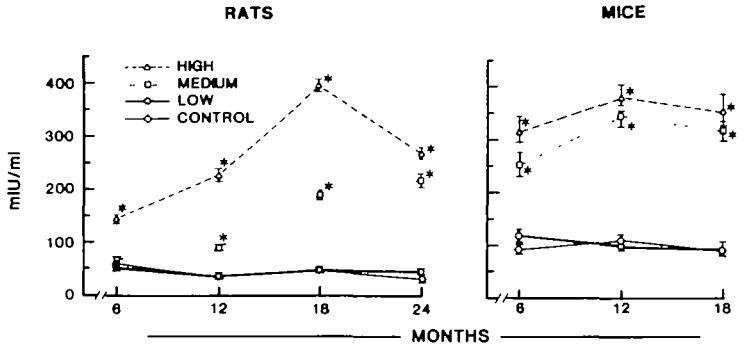

FIG. 5. Lactate dehydrogenase activity in BALF from rodents exposed to low (0.35 mg soot/m^3), medium (3.5 mg soot/m^3), and high (7.0 mg soot/m^3) concentrations of diluted diesel exhaust over a 24-month (rats) or an 18-month (mice) period. Values are $\bar{X} \pm$ SE, $n = 16$ (rats) or 8 (mice). *Indicates values statistically different from control values, $p < 0.05$.

consistent, dose-dependent effect on the cytochrome *P*-450 content of mouse lungs.

Histological changes. The chronic lung disease that developed in the rats on this study has been reported separately (Mauderly *et al.*, 1987a,b; McClellan, 1987; McClellan *et al.*, 1986) and are briefly reviewed here for comparisons with the biochemical and cytological changes. Histologic and biochemical evidence of fibrosis were detectable in rats after 12 months of exposure to the high level of diesel exhaust. The amount of fibrosis increased with increasing time of exposure and was observable in the medium exposure group at 18 months. In rats, the areas of fibrosis were focal in nature and associated with dense deposits of black soot. The soot was present within both macrophages and the interstitium of alveolar septa, small conducting airways, and blood vessels. Most of the more dense accumulation of collagen was adjacent to terminal bronchioles or around subpleural soot deposits. The severity of inflammatory reaction and fibrosis was directly related to the inhalation exposure concentration as shown in Figs. 12A, 12B, and 12C. At the lowest exposure concentration, there was no inflammation or fibrosis and the only evidence of exposure to diesel exhaust was the presence of an occasional intraalveolar macrophage containing black soot. Although mouse lungs contained more

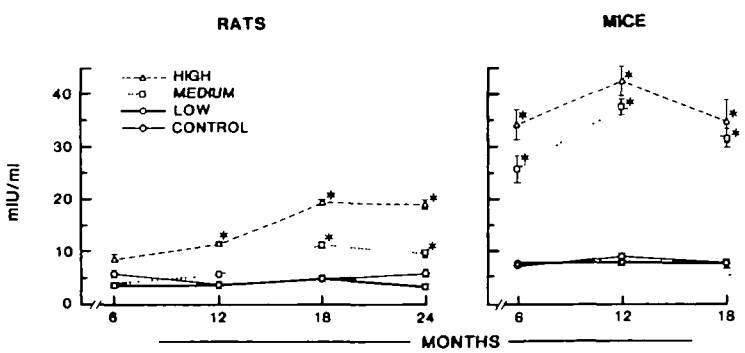

FIG. 6. Glutathione reductase activity in BALF from rodents exposed to low (0.35 mg soot/m^3), medium (3.5 mg soot/m^3), and high (7.0 mg soot/m^3) concentrations of diluted diesel exhaust over a 24-month (rats) or an 18-month (mice) period. Values are $\bar{X} \pm$ SE, $n = 16$ (rats) or 8 (mice). *Indicates values statistically different from control values, $p < 0.05$.

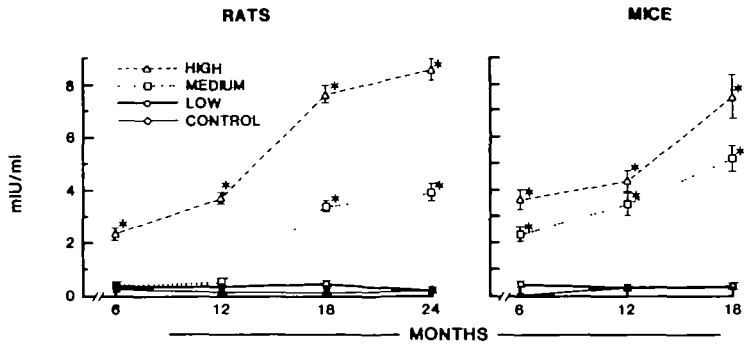

FIG. 7. β-glucuronidase activity in BALF from rodents exposed to low (0.35 mg soot/m^3), medium (3.5 mg soot/m^3), and high (7.0 mg soot/m^3) concentrations of diluted diesel exhaust over a 24-month (rats) or an 18-month (mice) period. Values are $\bar{X} \pm$ SE, $n = 16$ (rats) or 8 (mice). *Indicates values statistically different from control values, $p < 0.05$.

soot than rats at each equivalent exposure concentration, there was substantially less inflammatory reaction and fibrosis than was the case in rats (Figs. 13A, 13B, and 13C). Fibrosis was seen only at the highest exposure level and consisted of fine fibrillar thickening of an occasional alveolar septa. Also, mouse total lung collagen did not increase significantly after the exposure while rat lung collagen did.

DISCUSSION

The 2-year exposure of rats or 18-month exposure of mice to 0.35 μg/m^3 of diluted diesel exhaust had little effect that could be detected by parameters in BALF that are sensitive indicators of inflammation. No significant differences in any of the parameters studied were observed in either species exposed to this level. This finding is important because it indicates that inflammatory responses were not induced by exposures considerably higher (approximately 10- to 20-fold) (Cuddihy *et al.*, 1984) than projected environmental levels of diesel exhaust if substantial use were made of diesel engines in light-duty vehicles. Earlier reports from this study also indicated no effect of this level of exposure in rats on pulmonary function (Mauderly *et al.*, 1987a), clearance of either the deep lung or upper respiratory tract (Wolff *et al.*, 1987), pulmonary immune responses (Bice *et al.*, 1985), or tumor induction (Mauderly *et al.*, 1987b).

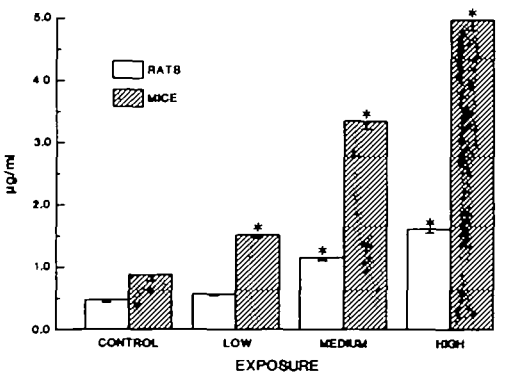

FIG. 8. Glutathione content of BALF in rodents exposed to low (0.35 mg soot/m^3), medium (3.5 mg soot/m^3), and high (7.0 mg soot/m^3) concentrations of diluted diesel exhaust for 18 months. Values are $\bar{X} \pm$ SE, $n = 16$ (rats) or 8 (mice). *Indicates values statistically different from control values, $p < 0.05$. Rat values differed from mouse values for all exposure groups, $p < 0.05$.

It should be noted, however, that interest in the health effects of diesel exhaust exposures also extends to situations where individuals may be exposed occupationally to exhaust from heavy-duty engines. For example, Cantrell (1987) has recently reported preliminary data from a single coal mine using heavy-duty diesel equipment suggesting exposure concentrations for diesel soot particles in the vicinity of the diesel equipment in the range of 0.9 to 1.9 mg/m^3. Such levels are higher than the lowest level in this study,

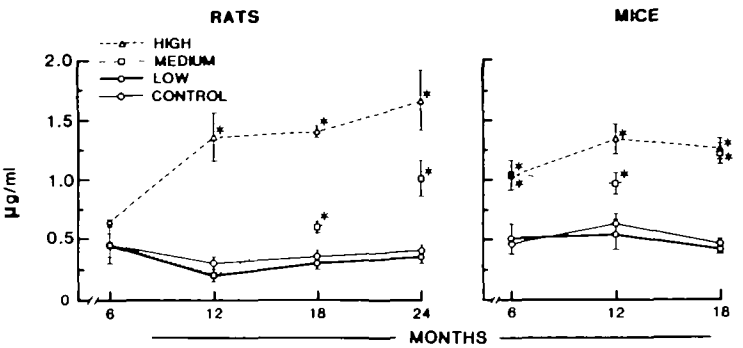

FIG. 9 Hydroxyproline content of BALF from rodents exposed to low (0.35 mg soot/m^3), medium (3.5 mg soot/m^3), and high (7.0 mg soot/m^3) concentrations of diluted diesel exhaust over a 24-month (rats) or an 18-month (mice) period. Values are $\bar{X} \pm$ SE, n = 16 (rats) or 8 (mice). *Indicates values statistically different from control values, $p < 0.05$.

which did not produce observable effects and are only half the lowest level which produced substantial effects in this study.

One objective of this study was to determine if biochemical and cytological changes in BALF could detect early indications of the development of chronic nononcogenic lung diseases. The initial observation of the fibrotic response in rats corresponded with the initial increase in hydroxyproline content of the BALF. Fibrosis was first observed in the high-level exposed rats after 12 months of exposure and this corresponded with a fourfold increase in BALF hydroxyproline observed in rats. This parameter was increased only twofold in mice, in parallel to their lesser fibrosis. Undoubtedly, many as yet unknown factors are involved in determining the observed species differences in fibrogenic response to the inhaled particles. A similar species difference in development of pulmonary fibrosis after intratracheal instillation of silica particles in rats and mice has been reported (Hatch *et al.*, 1984).

BALF hydroxyproline is present in both ultrafilterable collagenous fragments and larger collagen monomers. Its presence in normal lavage fluid is thought to represent turnover of the extracellular collagenous matrix, both by the depolymerizing and collagenolytic action of cathepsin B and by the activity of macrophage collagenase, and polymorphonuclear leucocyte elastase (Barrett, 1980). The fourfold increase in hydroxyproline in BALF in rats, relative to the ~twofold increase in mice indicated that rats had undergone considerably more disruption and subsequent turnover of their lung's extracellular collagenous matrix than had the mice. Evidence of disruption of the extracellular matrix and interruption of the basement membrane has been shown to be important to the subsequent development of irreversible pulmonary fibrosis (Pickrell *et al.*, 1983). The results of the current study suggest that large increases (4×) in BALF hydroxyproline may be a potential early marker for developing pulmonary fibrosis. The value of BALF hydroxyproline as such a marker remains to be validated by additional studies in which the appearance of increased BALF hydroxyproline is compared to radiographic methods for detection of early developing fibrosis.

A dose-dependent chronic inflammatory response was detectable by analysis of BALF and lung tissue from both species of rodents exposed to the two higher concentrations of diluted diesel exhaust. The inflammatory response, as measured by protein content and neutrophil and macrophage cell counts in the BALF, was qualitatively associated with the amount of diesel soot in the lung. That is, the inflammatory response appeared sooner and was greater in the mice, the species with the higher lung burden of soot, than in the rats. It should be noted that the increases in lung

TABLE 3

BIOCHEMICAL CHANGES IN LUNG TISSUE OF RATS IN RESPONSE TO INHALED DIESEL EXHAUST[a] (UNITS/g LUNG ± SE, $n = 16$)[a]

Parameter (Units)	Months of exposure											
	6			12			18			24		
Lactate dehydrogenase (IU/g)												
Control	34	± 1		35	± 1		31	± 2		42	± 3	
Low	34	± 1		39	± 1		30	± 2		38	± 3	
Medium	35	± 1		44	± 1*		36	± 2	(45)*	48	± 2	(63)*
High	40	± 1*		49	± 1*	(63)*	36	± 1	(57)*	60	± 5*	(97)*
Acid proteinase (mg solubilized/hr·g)[b]												
Control	16	± 3		19	± 2		20	± 2		17	± 1	
Low	17	± 2		30	± 4		19	± 2		15	± 2	
Medium	24	± 3		44	± 6*		34	± 3*	(44)*	21	± 1	(30)*
High	28	± 3*		44	± 8*	(61)*	30	± 2*	(55)*	15	± 2	(31)*
β-Glucuronidase (IU/g)												
Control	0.13	± 0.01		0.03	± 0.01		0.17	± 0.02		0.19	± 0.03	
Low	0.11	± 0.01		0.04	± 0.01		0.26	± 0.02*		0.17	± 0.01	
Medium	0.13	± 0.01		0.18	± 0.01*		0.18	± 0.01	(0.22)	0.38	± 0.03*	(0.51)*
High	0.19	± 0.02*		0.25	± 0.01*	(0.33)*	0.44	± 0.01*	(0.68)*	0.87	± 0.05*	(1.40)*
Acid phosphatase (IU/g)												
Control	2.5	± 0.1		2.0	± 0.1		1.8	± 0.1		2.2	± 0.1	
Low	2.2	± 0.1		2.3	± 0.1		3.5	± 0.1*		2.4	± 0.1	
Medium	2.7	± 0.1		2.3	± 0.1		2.3	± 0.2	(2.9)	3.0	± 0.1*	(3.7)*
High	2.5	± 0.1		3.0	± 0.1*	(3.9)*	3.6	± 0.1*	(5.7)*	3.5	± 0.2*	(5.5)*
Alkaline phosphatase (IU/g)												
Control	2.9	± 0.1		2.5	± 0.1		2.7	± 0.2		3.1	± 0.4	
Low	3.0	± 0.1		2.4	± 0.1		2.9	± 0.1		3.4	± 0.2	
Medium	1.7	± 0.1*		2.9	± 0.2		4.1	± 0.2*	(5.1)*	5.2	± 0.3*	(6.3)*
High	2.5	± 0.1		4.0	± 0.1*	(5.2)*	3.2	± 0.1	(5.0)*	3.7	± 0.4	(5.8)*
Glutathione reductase (IU/g)												
Control	1.7	± 0.1		1.8	± 0.1		1.8	± 0.1		1.9	± 0.1	
Low	1.5	± 0.1		2.0	± 0.1		1.5	± 0.1		1.9	± 0.1	
Medium	1.7	± 0.1		2.0	± 0.1		1.7	± 0.1	(2.1)	3.2	± 0.2*	(4.3)*
High	1.8	± 0.1		2.3	± 0.1*	(2.9)*	1.9	± 0.1	(2.9)*	2.7	± 0.2*	(4.4)*

[a] Values are shown as the total International Units (IU) in the lung normalized to the wet weight of the lung in grams. For values from animals with statistically significant increases in lung weight (see Fig. 1), the value of the total IU in the lung normalized to the mean wet weight of lung from control, unexposed animals killed at the same time is given in parentheses to show the value if there had been no increase in lung weight.

[b] $n = 4$–9.

* Value differs from control value, $p \leq 0.05$.

weight could not be accounted for by the weight of the deposited soot, which ranged up to 27 mg in the highest exposed rat group, compared to increases in lung weight of approximately 1 g. Thus, the increased lung weights can be associated with the edema accompanying the inflammatory response and with cell proliferation.

The largest change in any of the observed enzymatic parameters was the increase in BGLU activity in BALF. The relative increase over controls was approximately 2-

TABLE 4

BIOCHEMICAL CHANGES IN LUNG TISSUE OF MICE IN RESPONSE TO INHALED DIESEL EXHAUST[a] (UNITS/g LUNG ± SE, $n = 8$)

Parameter (Units)	Months of exposure		
	6	12	18
Lactate dehydrogenase (IU/g)			
Control	35 ± 2	43 ± 3	33 ± 4
Low	33 ± 2	38 ± 2	40 ± 3 (44)
Medium	34 ± 1 (44)*	43 ± 1 (64)*	48 ± 2* (79)*
High	45 ± 2* (59)*	42 ± 2 (71)*	46 ± 2* (86)*
Acid proteinase (mg solubilized/hr·g)[b]			
Control	15 ± 1	13 ± 2	20 ± 2
Low	13 ± 1	16 ± 2	26 ± 4
Medium	13 ± 1 (17)	39 ± 8* (47)*	25 ± 3 (40)*
High	15 ± 1 (28)*	24 ± 3* (38)*	23 ± 2 (47)*
β-Glucuronidase (IU/g)			
Control	0.04 ± 0.01	0.04 ± 0.01	0.07 ± 0.01
Low	0.04 ± 0.01	0.21 ± 0.01*	0.08 ± 0.01
Medium	0.06 ± 0.01* (0.08)*	0.09 ± 0.01* (0.13)*	0.17 ± 0.02* (0.28)*
High	0.07 ± 0.01* (0.10)*	0.14 ± 0.01* (0.23)*	0.04 ± 0.02 (0.07)
Acid phosphatase (IU/g)			
Control	1.7 ± 0.1	1.9 ± 0.1	2.0 ± 0.1
Low	2.3 ± 0.1*	1.9 ± 0.2	2.0 ± 0.1
Medium	2.7 ± 0.1* (3.5)*	2.4 ± 0.2 (3.5)*	3.1 ± 0.2* (5.2)*
High	3.0 ± 0.1* (3.9)*	2.7 ± 0.1* (4.6)*	3.9 ± 0.3* (7.3)*
Alkaline phosphatase (IU/g)			
Control	0.23 ± 0.05	0.16 ± 0.06	0.26 ± 0.06
Low	0.12 ± 0.04	0.15 ± 0.08	0.33 ± 0.07
Medium	0.07 ± 0.03 (0.11)	0.13 ± 0.03 (0.19)	0.41 ± 0.10 (0.53)
High	0.23 ± 0.04 (0.30)	0.07 ± 0.02 (0.11)	0.20 ± 0.05 (0.38)
Glutathione reductase (IU/g)			
Control	2.5 ± 0.1	3.4 ± 0.2	3.3 ± 0.2
Low	2.7 ± 0.2	3.4 ± 0.2	2.8 ± 0.2
Medium	2.6 ± 0.1 (3.4)	3.8 ± 0.1 (5.6)*	3.8 ± 0.1 (6.4)*
High	3.3 ± 0.1* (4.4)*	3.5 ± 0.2 (5.9)*	3.1 ± 0.2 (5.9)*

[a] Values are shown as the total International Units (IU) in the lung normalized to the wet weight of the lung in grams. For values from animals with statistically significant increases in lung weight (see Fig. 1), the value of the total IU in the lung normalized to the mean wet weight of lung from control, unexposed animals killed at the same time is given in parentheses to show the value if there had been no increase in lung weight.

[b] $n = 6$–8.

* Value differs from control value, $p \leq 0.05$.

fold higher in rats than in mice. Several investigators (Weissman *et al.*, 1980; Bentwood and Henson, 1980; Henson *et al.*, 1981; Keeling and Henson, 1982) have found that phagocytic cells, both macrophages and neutrophils, will release β-glucuronidase or *N*-acetyl-β-glucuronidase upon stimulation by various materials. The extremely large (up to 70-fold) increase in BGLU activity in BAL fluid is most likely associated with the activation of phagocytic cells by the particles in the lung. The BGLU could also have come from lysed phagocytic cells. Exposure of rats to α-quartz particles (known to be toxic to macrophages) in our laboratory also resulted in high levels of BGLU in BALF and a decrease in total number of macrophages recovered in BALF (Henderson *et al.*, 1985b). However, a

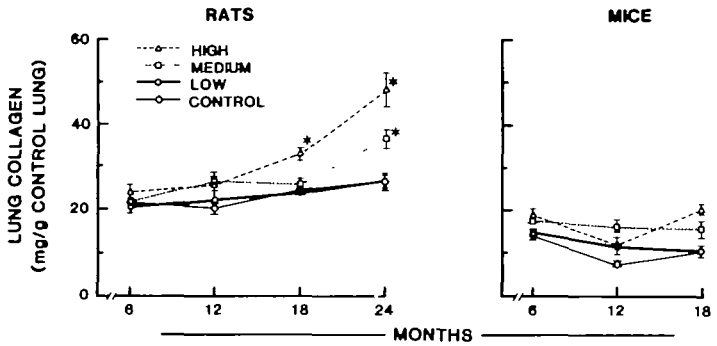

FIG. 10. Lung collagen content in rodents exposed to low (0.35 mg soot/m^3), medium (3.5 mg soot/m^3), and high (7.0 mg soot/m^3) concentrations of diluted diesel exhaust over a 24-month (rats) or an 18-month (mice) period. Values are $\bar{X} \pm$ SE, $n = 16$ (rats) or 8 (mice) and are normalized to the mean wet weight of lungs from control animals to allow comparisons among sexes and species and to avoid underestimating the collagen content due to the increased lung weight of animals in the higher exposure groups. *Indicates values statistically different from control values, $p < 0.05$.

large portion of the β-glucuronidase must be released by stimulation of intact cells, since the increases in β-glucuronidase were 20- to 25-fold while the increases in LDH were only 4- to 8-fold.

The ability of particles to cause the release of BGLU from intact macrophages may prove to be a useful *in vitro* screen for the fibrogenicity of particles. A comparison of the results of such a study based on the *in vivo* response of rats to inhaled particles versus the *in vitro* response of pulmonary macrophages to the same particles has been reported (Henderson *et al.*, 1985b). The relative amounts of BGLU in BALF from rats exposed to the different types of particles corresponded well with that observed in culture media *in vitro* and, in both systems, the level of BGLU released corresponded well with the degree of pulmonary fibrosis induced by the particles *in vivo*. The use of increases in lysosomal enzymes such as BGLU in BALF as a tool to

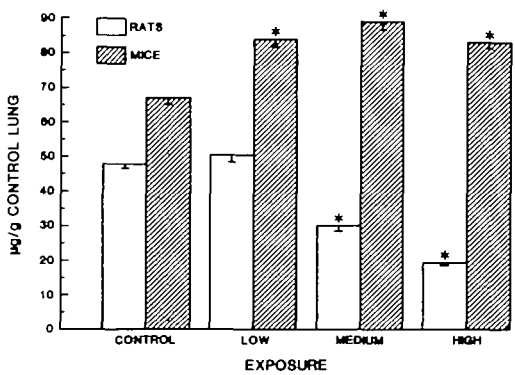

FIG. 11. Lung tissue content of GSH in lungs of rodents exposed to low (0.35 mg soot/m^3), medium (3.5 mg soot/m^3), and high (7.0 mg soot/m^3) concentrations of diluted diesel exhaust for 18 months. Values are $\bar{X} \pm$ SE, $n = 16$ (rats) or 8 (mice) and are normalized to the mean wet weight of lungs from control animals to allow comparisons among sexes and species and to avoid underestimating the GSH content due to the increased lung weight of animals in the higher exposure groups. *Indicates values statistically different from control values, $p < 0.05$. Rat values differed from mouse values for all exposure groups, $p < 0.05$.

TABLE 5

LUNG MICROSOMAL CYTOCHROME *P*-450 CONTENT IN RODENTS EXPOSED FOR 1 YEAR TO DILUTED DIESEL ENGINE EXHAUST[a] (pmol/mg PROTEIN)

Exposure group	Rats	Mice
Control	23.1 ± 8.3	32.1 ± 8.4
0.35 mg/m^3	11.7 ± 3.3[b]	32.8 ± 7 1
3.5 mg/m^3	10.1 ± 5.6[b]	11.2 ± 4.4[b]
7.0 mg/m^3	6.5 ± 1.7[b]	24.4 ± 14 4

[a] Results represent the mean ± SD of values obtained from at least six individual animals.

[b] Values for exposed animals are significantly different from those of the controls.

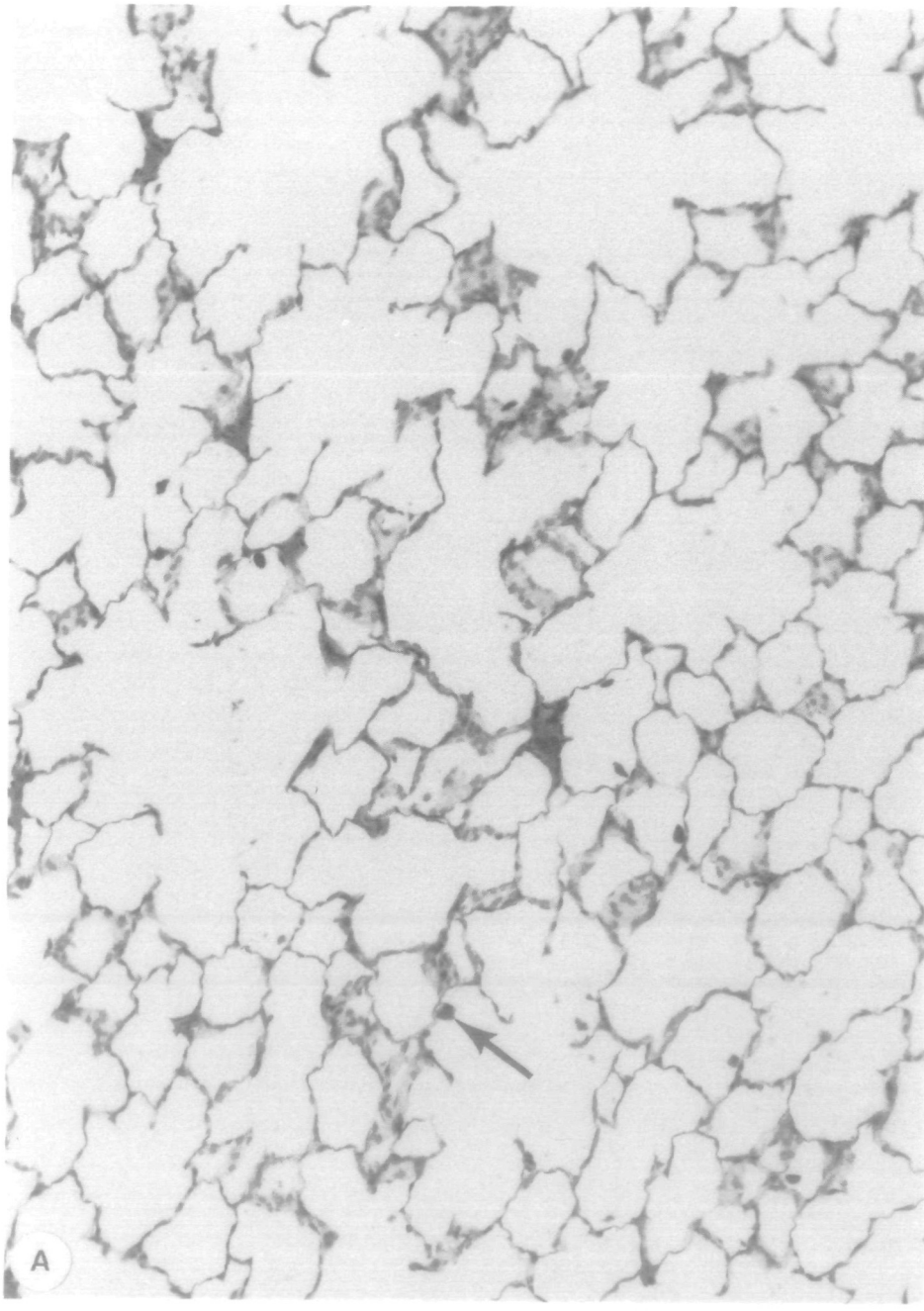

FIG. 12. (A) Photomicrograph of rat lung exposed to 0.35 mg soot/m^3 for 18 months. The only evidence of exposure is the presence of an occasional soot-laden intraalveolar macrophage (arrow). H and E stain × 200. (B) Photomicrograph of rat lung exposed to 3.5 mg soot/m^3 for 18 months. Note clusters of soot-laden intraalveolar macrophages and focal thickening of alveolar septa (arrow). H and E stain × 200. (C) Photomicrograph of rat lung exposed to 7.0 mg soot/m^3 for 18 months. Alveoli contain clusters of soot-laden macrophages, cellular debris, and occasional neutrophils. Alveolar septal thickening is more extensive than in lower exposure level rats (arrow). H and E stain × 200.

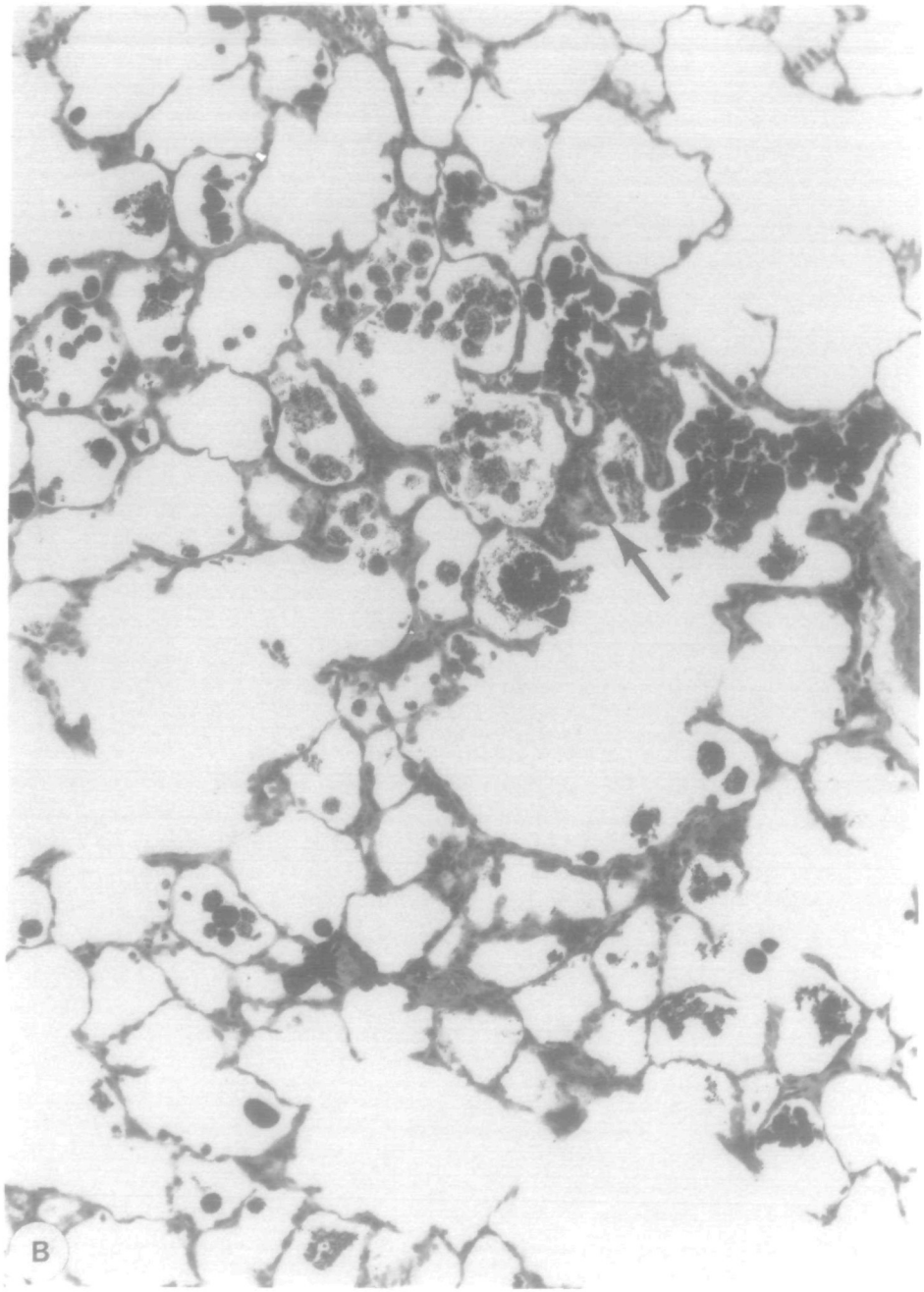

FIG. 12—*Continued.*

rank the fibrogenicity of inhaled particles has also been suggested by the work of Beck *et al.* (1982), who reported on the use of β-*N*-acetylglucosaminidase for this purpose.

It is noteworthy that the lysosomal enzyme, AcP, was not elevated in the BALF from rodents exposed to diesel exhaust. This suggests that BGLU and AcP either come

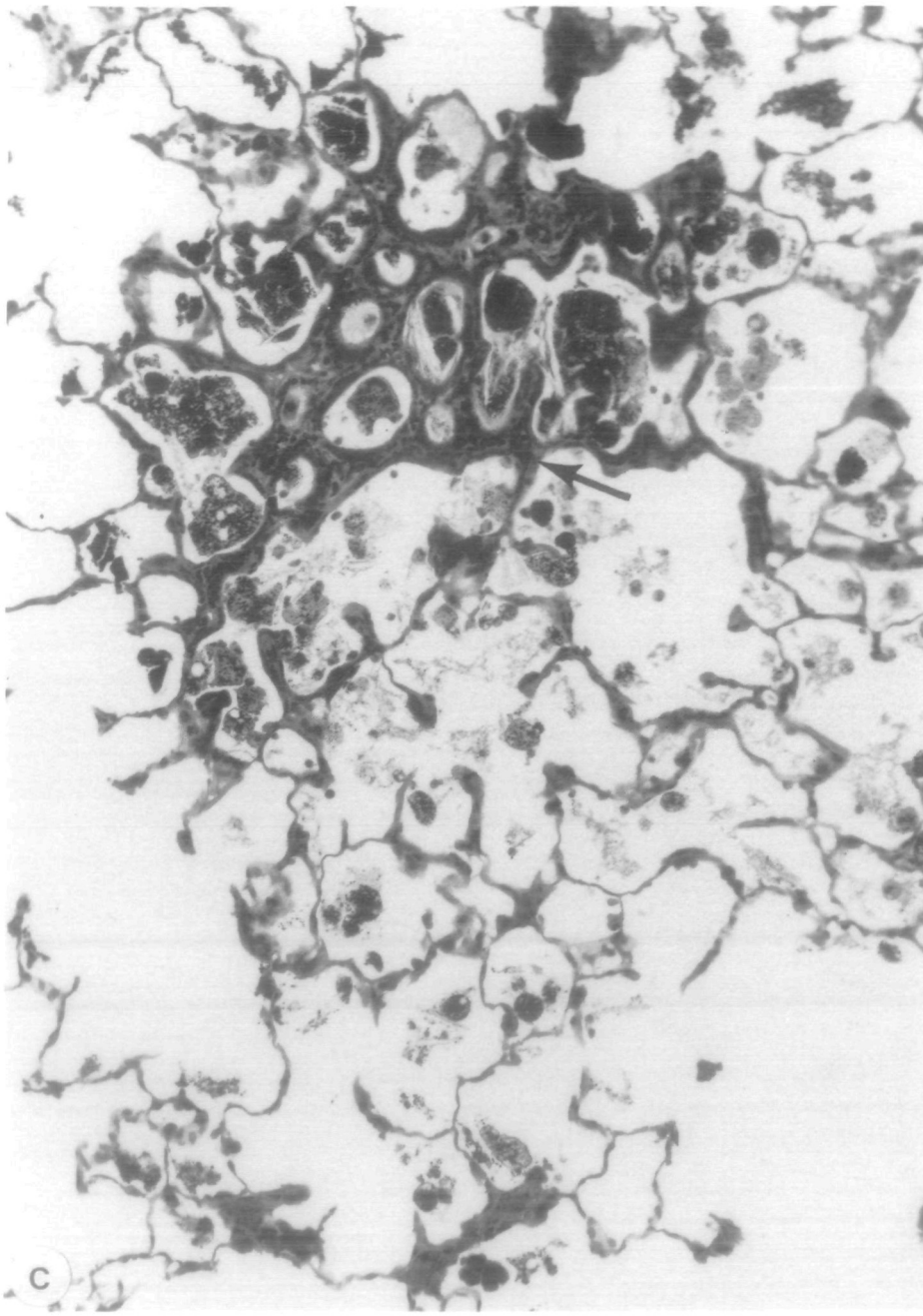

FIG. 12—*Continued.*

from different sources or have different half-lives in the airways where they are released. The differential release of lysosomal constituents from inflammatory cells has been reported (Bentwood and Henson, 1980).

The dose-dependent increase of GSH in BALF suggests that the GSH might be released as a protective measure against damage caused either by the oxidants in the diesel exhaust or by the oxygen radicals released by

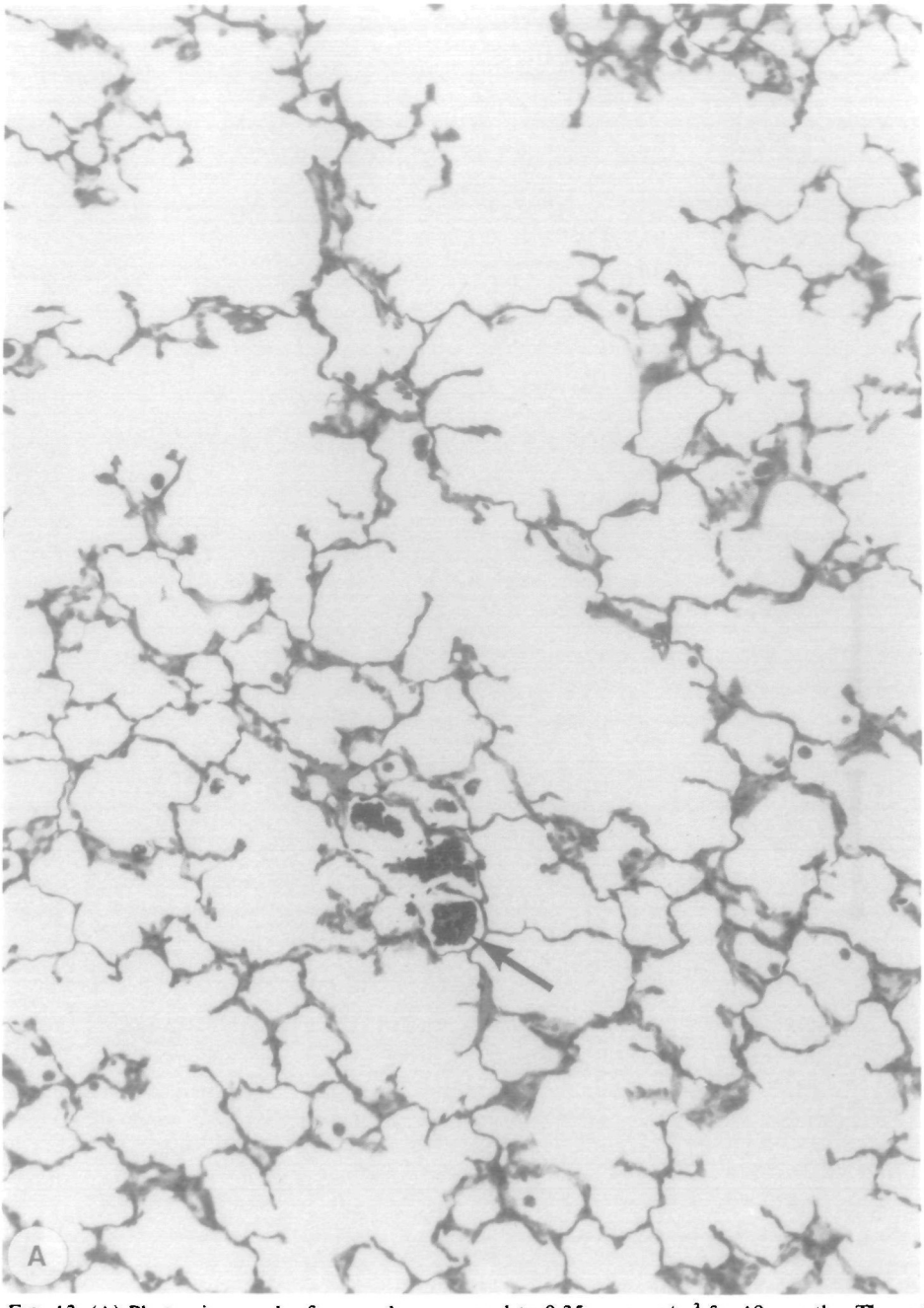

FIG. 13. (A) Photomicrograph of mouse lung exposed to 0.35 mg soot/m^3 for 18 months. The only evidence of exposure is the presence of occasional clusters of large soot-laden intraalveolar macrophages (arrow). H and E stain × 200. (B) Photomicrograph of mouse lung exposed to 3.5 mg soot/m^3 for 18 months. Amount of retained soot is greater than in equivalently exposed rats with most in large intraalveolar macrophages. Note absence of alveolar septal thickening. H and E stain × 200. (C) Photomicrograph of mouse lung exposed to 7.0 mg soot/m^3 for 18 months. Soot accumulation is greater than in lower exposure level mice and rats exposed to the same level, but there is no inflammatory reaction or thickening of alveolar septa. H and E stain × 200.

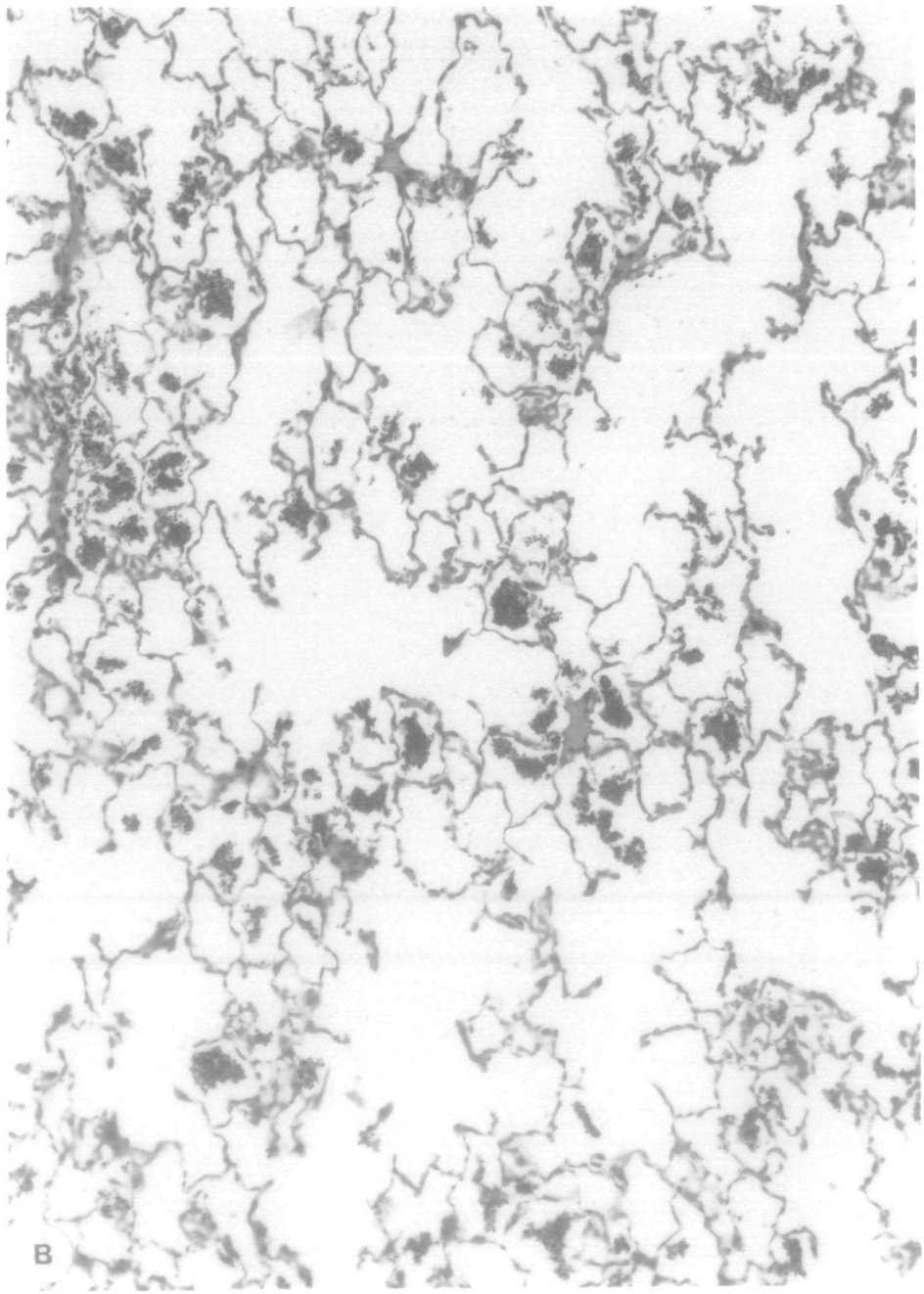

FIG. 13—*Continued.*

the stimulated phagocytic cells (Weissman *et al.*, 1980). However, the source of the GSH is unknown. Both the BALF and the lung tissue from mice had greater amounts of GSH than those from rats. Also, mouse BALF had much higher increases in the enzyme, glutathione reductase, which is necessary to maintain the GSH in the reduced state. The rat

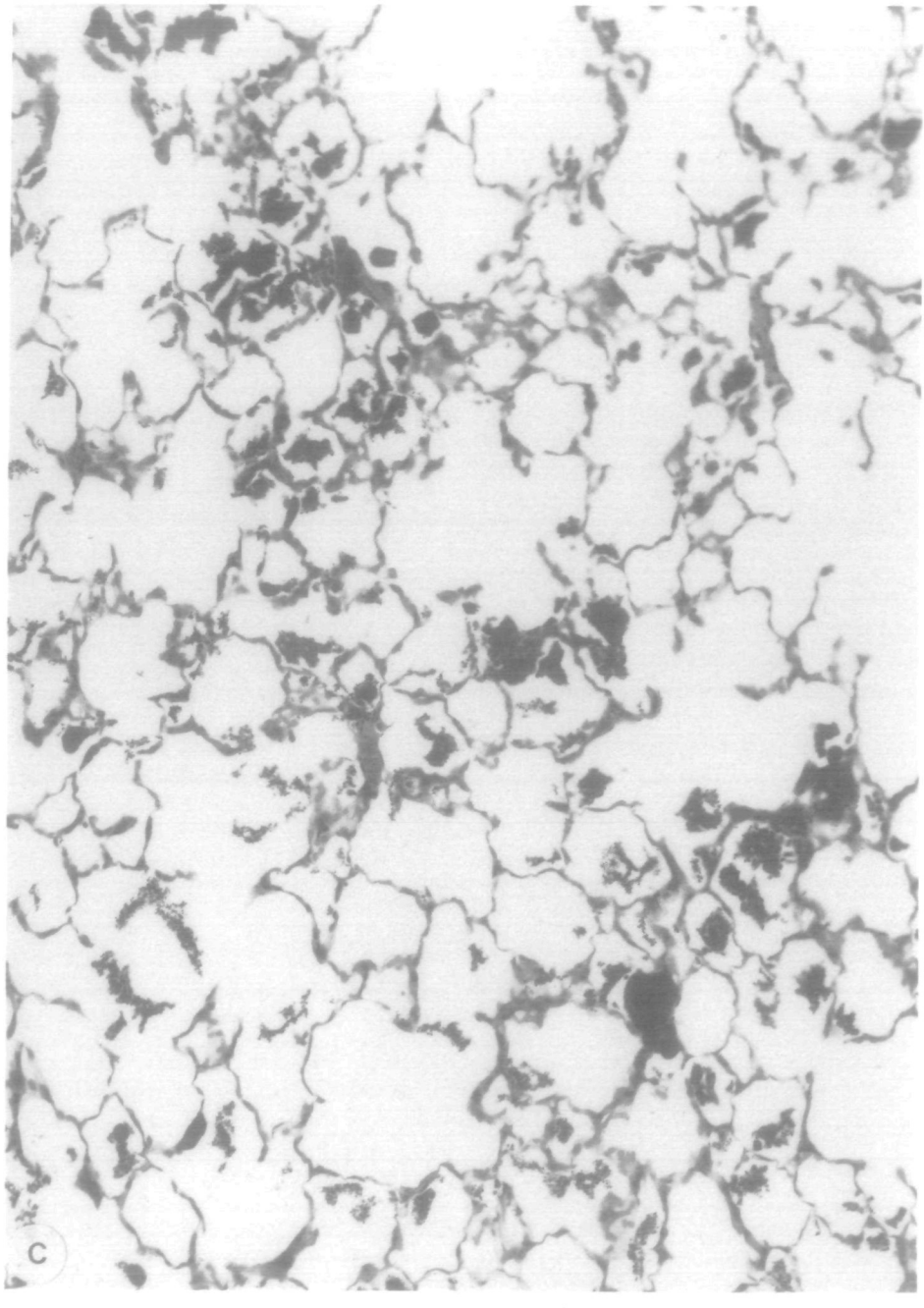

FIG. 13—*Continued.*

lung tissue had a dose-dependent decrease in GSH, indicating that the lung was depleted of GSH by the exposure, while the mouse lung GSH increased slightly. If GSH is required to protect against the damaging oxygen radicals known to be produced by both stimulated neutrophils and macrophages (Weissman *et al.*, 1980), then the depletion of lung GSH

may be an important factor in the development of fibrosis in the rats.

Researchers at the Fraunhofer Institute for Aerosol Research also used BALF analysis to follow the health effects of long-term inhalation of diesel exhaust (Heinrich *et al.*, 1986) in Wistar rats (females) and Syrian hamsters (both sexes). Animals were exposed to exhaust containing 4 mg soot/m^3, 19 hr/day, 5 days/week, for 2 years, giving an exposure concentration × time value of approximately 1.5 times our highest exposure regimen. In the rats, the percentage increases they observed in lung weight and in BALF LDH and protein were also 1.5 to 2 times what we observed. They observed approximately the same percentage increases in BALF hydroxyproline and in cell content as reported here.

The results of this study and of earlier work by others suggest the following pathogenic sequence of events during chronic inhalation exposure of rodents to diesel exhaust. As the soot accumulates in the lung, the activated macrophages release chemotactic factors that attract neutrophils (Fels and Cohn, 1986). Both the neutrophils and the macrophages are stimulated to release mediators of inflammation as well as injurious oxygen radicals. Some of the phagocytic cells die and release more hydrolytic enzymes. The macrophages turn over more rapidly than in unexposed animals. In the mouse, sufficient GSH is available to counteract the oxygen radicals and, while cells are injured (as evidenced by LDH release), repair can keep pace with injury and fibrosis does not result. In the rat, the smaller GSH stores are depleted, leaving more oxygen radicals available for causing cell injury. The epithelial lining and its supporting extracellular collagenous matrix is disrupted to a sufficient degree and for sufficient time to reduce or eliminate the likelihood of normal repair (Vracko, 1974; Pickrell *et al.*, 1983). This leads to the release and phosphorylation of fibronectin, the release of alveolar macrophage-derived growth factor (a factor promoting growth of fibroblasts), the proliferation of fibroblasts, and the development of pulmonary fibrosis (Ali, 1983; Bitterman *et al.*, 1983; Martin *et al.*, 1983). This hypothesized scenario is consistent with our observed data.

This study indicates that analysis of BALF can be a useful component in chronic toxicity studies to follow the development of chronic pulmonary disease. In this instance, the presence of greatly elevated β-glucuronidase activity and concentrations of hydroxyproline in rat BALF corresponded with the developing fibrosis. The higher concentration of GSH in BALF and lung tissue of mice might have been a factor in reducing the cytotoxicity and fibrosis in the mice compared to the rats.

In conclusion, a long-term study of the health effects of inhaled diluted diesel exhaust in rodents indicated no adverse effects from exposure to atmospheres containing 0.35 mg soot/m^3 air, a level that is directly relevant to some occupational exposures and is 10 to 20 times higher than any current or anticipated environmental exposures. This indicates that a low level of risk for chronic nonneoplastic lung disease is associated with current levels of environmental exposure to exhaust from diesel engines in light-duty vehicles (Cuddihy and McClellan, 1983; McClellan, 1986; McClellan *et al.*, 1985). For the noncarcinogenic health effects reported in this paper, there appears to be a threshold of exposure below which adverse effects were not observed. The possible association between these nonneoplastic changes and the observed increases in neoplasms in the rats is unknown. However, if some of the processes leading to the nonneoplastic changes also have a role in the neoplastic process, then the observation of a threshold may be of added significance in the extrapolation of the neoplastic results to low-level exposures. These results were based on exposures to exhaust from well-tuned, light-duty diesel engines in good repair, running on a standard cycle. Caution should be used in extrapolating the results of the current study to likely results from occupational exposures to exhaust from heavy-duty diesel engines, for which fuel type, combustion efficiency,

and load cycle may vary considerably from those used in this study.

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