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Pulmonary Responses of Mice, Rats, and Hamsters to Subchronic Inhalation of Ultrafine Titanium Dioxide Particles

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A multispecies, subchronic, inhalation study comparing pulmonary responses to ultrafine titanium dioxide (uf-TiO_2) was performed. Female rats, mice, and hamsters were exposed to aerosol concentrations of 0.5, 2.0, or 10 mg/m^3 uf-TiO_2 particles for 6 h/day, 5 days/week, for 13 weeks. Following the exposure period, animals were held for recovery periods of 4, 13, 26, or 52 weeks (49 weeks for the uf-TiO_2–exposed hamsters) and, at each time point, uf-TiO_2 burdens in the lung and lymph nodes and selected lung responses were examined. The responses studied were chosen to assess a variety of pulmonary parameters, including inflammation, cytotoxicity, lung cell proliferation, and histopathological alterations. Retained lung burdens increased in a dose-dependent manner in all three species and were at a maximum at the end of exposures. Mice and rats had similar retained lung burdens at the end of the exposures when expressed as mg uf-TiO_2/mg dry lung, whereas hamsters had retained lung burdens that were significantly lower. Lung burdens in all three species decreased with time after exposure, and, at the end of the recovery period, the percentage of the lung particle burden remaining in the 10 mg/m^3 group was 57, 45, and 3% for rat, mouse, and hamster, respectively. The retardation of particle clearance from the lungs in mice and rats of the 10 mg/m^3 group indicated that pulmonary particle overload had been achieved in these animals. Pulmonary inflammation in rats and mice exposed to 10 mg/m^3 was evidenced by increased numbers of macrophages and neutrophils and increased concentrations of soluble markers in bronchoalveolar lavage fluid (BALF). The initial neutrophil response in rats was greater than in mice, whereas the relative increase of macrophages was less than in mice. The neutrophilic response of rats, but not mice, declined in a time-dependent manner correlating with declining lung burdens; however, the fraction of recovered neutrophils at 52 weeks postexposure was equivalent in the two species. Consistent increases in soluble indicators of toxicity in the BALF (LDH and protein) occurred principally in rats and mice exposed to 10 mg/m^3 and diminished with time postexposure. There were no significant changes in cellular response or with markers indicating toxicity in hamsters, reflecting the capacity of these animals to rapidly clear particles from the lung. Progressive epithelial and fibroproliferative changes were observed in rats of the 10 mg/m^3 group. These lesions consisted of foci of alveolar epithelial proliferation of metaplastic epithelial cells (so-called alveolar bronchiolization) circumscribing aggregated foci of heavily particle-laden macrophages. The observed epithelial proliferative changes were also manifested in rats as an increase in alveolar epithelial cell labeling in cell proliferation studies. Associated with these foci of epithelial proliferation were interstitial particle accumulation and alveolar septal fibrosis. These lesions became more pronounced with increasing time postexposure. Epithelial, metaplastic, and fibroproliferative changes were not noted in either mice or hamsters. In summary, there were significant species differences in the pulmonary responses to inhaled uf-TiO_2 particles. Under conditions where the lung uf-TiO_2 burdens were equivalent, rats developed a more severe inflammatory response than mice and, subsequently, developed progressive epithelial and fibroproliferative changes. Clearance of particles from the lung was markedly impaired in mice and rats exposed to 10 mg/m^3 uf-TiO_2, whereas clearance in hamsters did not appear to be affected at any of the administered doses. These data are consistent with the results of a companion study using inhaled pigmentary (fine mode) TiO_2 (Bermudez *et al*., 2002) and demonstrate that the pulmonary responses of rats exposed to ultrafine particulate concentrations likely to induce pulmonary overload are different from similarly exposed mice and hamsters. These differences can be explained both by pulmonary response and by particle dosimetry differences among these rodent species.

Key Words: **ultrafine titanium dioxide; inhalation; lung response; rats; mice; hamsters.**

Titanium dioxide (TiO_2) is one of several dusts that are grouped into the category of poorly soluble particulates (PSP) by virtue of their low solubility and toxicity. TiO_2 has wide commercial utility in the production of white pigments and other applications, where it is used as either the fine or ultrafine mode of the mineral (Bingham *et al*., 2002).

Chronic ultrafine TiO_2 (uf-TiO_2) inhalation exposures have been conducted and the pulmonary toxicological ef-

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fects assessed. Wistar rats exposed to uf-TiO_2 for 24 months at an average concentration of 10 mg/m^3 developed pulmonary tumors (Heinrich *et al*., 1995). Similarly, chronic exposure to other PSP such as carbon black, diesel exhaust, pigmentary TiO_2, and talc led to the development of lung tumors in rats but not hamsters or mice (Heinrich *et al*., 1986; Hext, 1994; Lee *et al*., 1985; Mauderly *et al*., 1987, 1994; Muhle *et al*., 1998). The pulmonary response of rats to high, chronic doses of low solubility particles include findings of bronchoalveolar hyperplasia and metaplasia, fibrosis, and pulmonary tumorigenesis. These responses to the inhalation of PSP appear to be unique to the rat, relative to other rodent species, and limited to scenarios where there are substantial particle lung burdens and a concomitant impairment of alveolar macrophage-mediated lung clearance (pulmonary overload).

Impairment of pulmonary particle clearance and an attendant pulmonary inflammatory response have been shown to occur in rats exposed subchronically by inhalation to concentrations of uf-TiO_2 and other PSP known to induce pulmonary overload under a chronic exposure regimen (Cullen *et al*., 2000; Ferin *et al*., 1992; Warheit *et al*., 1997). Persistent inflammation has been hypothesized to occupy a central role in the pathogenesis of PSP-induced epithelial changes leading to lung tumorigenesis in rats (Donaldson, 2000). Notably, rats appear to mount greater inflammatory and epithelial responses than other laboratory rodent species following the inhalation of pigmentary TiO_2 and other PSP (Bermudez *et al*., 2002; Donaldson and Tran, 2002).

Ultrafine particles, defined as having a diameter of less than 0.1 μm, have been shown to have a greater capacity to induce inflammation of the lung than fine particles. Ferin and coworkers have shown that uf-TiO_2 has a greater capacity than fine mode TiO_2 to induce an inflammatory response of the lung in rats exposed to equivalent aerosol concentrations (Ferin *et al*., 1992). Although the properties of uf-TiO_2 underlying the heightened biological responses of rats relative to fine TiO_2 are unknown, it is hypothesized that increased surface area of this material and oxidative stress are important players (Donaldson *et al*., 2002).

Data regarding the pulmonary responses of rodents to ultrafine materials have been in large part collected using the rat. Studies examining the differences in species responses to ultrafine particulates, uf-TiO_2 in particular, are few. The present study was one of two, identical in design (Bermudez *et al*., 2002), conducted to carry out a systematic comparison of the pulmonary responses of laboratory rodent species to inhaled TiO_2 particles and to test the hypothesis that the pulmonary responses of rats to these particles differ from those of mice and hamsters. Subchronic inhalation exposures of mice, rats, and hamsters to equivalent aerosol concentrations of uf-TiO_2 were conducted and the pulmonary pathobiological responses during recovery compared.

MATERIALS AND METHODS

Animals. Female B3C3F1/CrlBR mice, CDF (F344)/CrlBR rats, and Lak:LVG (SYR) BR hamsters (Charles River Breeding Laboratories, Wilmington, MA), 6 weeks old and free of parasites, mycoplasma, bacterial, and viral pathogens, were used in these studies. Animals were housed in an AAALAC-accredited facility in 1 m^3 H-1000 stainless steel and glass inhalation chambers in suspended steel wire caging and were acclimated to this housing arrangement before beginning the exposures. Mice and rats were housed in similar stainless steel suspended caging in the same holding room during the postexposure period. Hamsters were housed in filtered, microisolated, polycarbonate cages on direct-contact cellulose bedding during the postexposure period. Animals were supplied an NIH07 cereal-based diet and water *ad libitum*. Animals were uniquely identified by implanted microchip transponders (Biomedic, Inc., Seaford, DE) in the subcutis and were randomly distributed to exposure groups using a computer-generated randomization algorithm. Room temperature was maintained at 17–26°C and humidity at 40–60% throughout the exposure and postexposure periods. All animals were acclimated for approximately 9 days prior to exposure. Body weights for each animal were recorded prior to exposure, weekly for the first 17 weeks, and biweekly thereafter.

Aerosol generation and monitoring. Uf-TiO_2 (P25, average primary particle size of 21 nm) was obtained from DeGussa-Hüls AG (Frankfurt, Germany). Aerosol generation was accomplished using a brush generator (CR-9100 Aerosol Generator, CR Equipment SA, Tannay, Switzerland) with dispersion of the particles by jet streams of air and passage through a mixing chamber prior to delivery to the exposure chambers. Inhalation exposures were conducted in 1 m^3 H-1000 stainless steel chambers (Lab Products, Maywood, NJ). During the exposures, particle concentrations were continuously monitored using light scatter (Model RAM-S, Monitoring Instruments for the Environment, Inc., Bedford, MA), and the time-averaged concentration was recorded at least six times over the 6 h exposure period. The particle size distribution of the aerosol was measured at least twice per exposure level per chamber (excluding the control chambers) during the course of the study. Measurements were made using a MOUDI (Micro-Orifice Uniform Deposit Impactor, model 100, MSP Corporation, Minneapolis, MN). Mean mass-median aerodynamic diameter was 1.37 μm (see Results).

Experimental design. Animals were exposed to 0.5, 2.0, or 10 mg/m^3 uf-TiO_2 for 6 h/day, 5 days/week, for 13 weeks. Controls were exposed to filtered air only. Hamsters were exposed separately from the mice and rats due to health considerations. Groups of 25 animals for each species and time point were used. Animals were sacrificed immediately following completion of the 13-week exposure periods and additional recovery groups were held for postexposure periods of 4, 13, 26, or 52 (49 for hamster) weeks in clean air. Following exposure and at each recovery time, the uf-TiO_2 burdens in the combined right lung lobes and in the lung-associated lymph nodes were determined. The left lungs of these animals were used to assess lung cell proliferation and histopathological end points. The inflammatory status of the lung was assessed at each time point using a separate group of animals subjected to bronchoalveolar lavage. The bronchoalveolar lavage fluid (BALF) was assayed for lactate dehydrogenase (LDH) and total protein levels and the differential cytology of the recovered cells was determined.

Titanium burden analysis. Burden analysis was performed according to the method of Levine *et al.* (2003). At necropsy, right lung and lymph node tissue samples were collected and stored frozen until ready for titanium burden analysis. Prior to burden analysis, the tissues were thawed, weighed, dried overnight in a muffle furnace at 37°C, desiccated, and weighed again. Dry tissue samples were digested overnight in a mixture of nitric acid and hydrofluoric acid and then further digested in a microwave oven. The sample digests were diluted with deionized water to 25 ml, and samples were analyzed using an inductively coupled plasma optical emission spectrometer (ICP-OES; Thermo Jarrell Ash AtomScan 16, Thermo Elemental, Franklin, MA). Data for lung tissue are expressed as milligrams of uf-TiO_2 per gram dry lung. For lymph node tissues, the data are expressed as the total micrograms of uf-TiO_2

present because the harvest of lymph node tissues was subject to collection of extraneous tissue to ensure collection of the nodes. The method quantitation limit (MQL) was 0.05 μg Ti/ml sample digest. The minimum detectable concentrations (MDC) of uf-TiO_2 in pulmonary tissues were calculated using the MQL in combination with the average control lung weight; for lung, these were 0.14, 0.03, and 0.03 mg uf-TiO_2/g dry weight for mouse, rat, and hamster, respectively, and, for lymph nodes, it was 2.09 μg uf-TiO_2/sample. Values above the MDC were observed in control mouse lung tissues at the end of the exposures and reflect an increase in the baseline for that run of the assay rather than the presence of uf-TiO_2 in control tissues. Retention half-times for uf-TiO_2 in lung were calculated using the best-fit equations to the lung burden data from all time points.

Bronchoalveolar lavage. Lungs were lavaged five times with equal volumes of phosphate-buffered saline. Fluid from the first two lavages was recovered, pooled, and placed on ice. The subsequent three lavages were pooled and also placed on ice. Recovered cells from all lavages were collected by centrifugation, resuspended in cell culture medium, and counted using an automated cell counter (Model ZM, Coulter, Marietta, GA). Cell differential counts were performed on Wright-Giemsa–stained cytocentrifuge slide preparations. LDH and total protein levels in cell-free fluid from the first two pooled lavages were quantitated spectrophotometrically using a COBAS FARA II automated analyzer (Roche Diagnostic Systems Inc., Montclair, NJ).

Lung cell proliferation. Five days prior to euthanasia, animals were subcutaneously implanted with osmotic pumps (Alza, Palo Alto, CA) containing bromodeoxyuridine (BrdU, Sigma Chemical Co., St. Louis, MO). Rats and hamsters were implanted with model 2ML1 (10 μl/h) pumps containing 5 mg/ml BrdU. Mice were implanted with model 2001 (1 μl/h) pumps containing 16 mg/ml BrdU. At necropsy, left lungs were pressure-infused intratracheally (20 cm H_2O for mice and 30 cm H_2O for rats and hamsters) with 10% neutral-buffered formalin. Lungs were fixed for approximately 48 h and then changed to 70% ethanol. Subsequently, the lungs were embedded in paraffin, sectioned at 5 μm, and stained for BrdU by established methods (Rutten *et al.*, 1994). Terminal bronchiolar and alveolar cell labeling indices were determined for each animal, and the mean labeling index was calculated for each group of five animals.

Histopathology. Paraffin-embedded left lung tissues were sectioned at 5 μm and stained with Masson's trichrome. The trichrome-stained lung sections were evaluated for particle-induced histopathological changes.

Statistical methods. All data were tested for normality and homogeneity of variance. If the hypotheses for these assumptions were rejected ($p < 0.01$), common transformations (e.g., log, square root, arc sine) were applied and the data were retested. Comparisons to controls were made using Dunnett's test. The software package JMP (SAS Institute, Cary, NC) was used for the statistical analysis.

RESULTS

Chamber Concentration

Target chamber concentrations of uf-TiO_2 aerosol were 0.5, 2.0, and 10 mg/m^3 and the actual particle concentration in each chamber was monitored using a Real Aerosol Monitor. Mean ($\pm$ standard deviation) particle concentrations over the exposure period were as follows: for mice, 0.54 $\pm$ 0.06, 2.2 $\pm$ 0.1, and 10.8 $\pm$ 1.0; for rats, 0.52 $\pm$ 0.03, 2.1 $\pm$ 0.1, and 10.5 $\pm$ 0.7; and for hamsters, 0.53 $\pm$ 0.03, 2.1 $\pm$ 0.1, and 10.7 $\pm$ 0.6 mg/m^3. The particle size characteristics for the 13-week exposure periods for each species are presented in Table 1.

TABLE 1
Particle Size Analysis of Ultrafine TiO_2 Aerosols

Species	Mass median aerodynamic diameter (μm)	Standard deviation	Geometric standard deviation	Standard deviation
Hamster	1.29	0.30	3.65	1.24
Mouse	1.45	0.49	2.46	0.31
Rat	1.44	0.57	2.60	0.38

Animal Mortality and Clinical Observations

Treatment-related losses during the exposure phase of the study were limited to mice (4). In the postexposure phase, there were unscheduled losses of rats (7) and mice (4) distributed over the various treatment groups. The hamsters had greater morbidity and mortality (35 animals during the postexposure phase of the study) than the mice and rats, presumably due to the occurrence of age-related spontaneous conditions such as chronic renal disease, with losses distributed among the treatment groups. Gross evidence of renal disease (bilateral, pale, small, firm kidneys with irregular pitted cortical surfaces) was observed in hamsters that died or were removed unscheduled from the study. A few of these animals had evidence of ascitic fluid and subcutaneous edema suggestive of nephrotic syndrome. Many of the hamsters scheduled for sacrifice at the 49-week time point also had evidence of severe chronic renal disease at necropsy. These findings contributed to the decision to move up the terminal sacrifice so those hamsters under study would not be unduly compromised by potential renal dysfunction leading to pulmonary inflammatory changes from uremia.

Body Weights

Body weights were collected weekly for the exposure phase of the study and biweekly from week 17 through the end of the postexposure period. A depression in body weight gain was noted in all groups of mice and rats following the end of the exposure period with recovery occurring over the next 3 to 4 weeks (data not shown). Frank body weight loss was observed in hamsters at the end of the exposure (9–15%) with a slow recovery over the remainder of the study (data not shown). Body weight fluctuations were more frequent and abrupt in hamsters than in mice or rats (data not shown). A likely reason for the body weight effects in the three species was the need for a period of acclimation to changes in environmental conditions experienced by the animals during the postexposure recovery period, when they were no longer housed in inhalation exposure chambers and were moved to new housing.

Uf-TiO_2 Burdens

There were dose-related changes in uf-TiO_2 lung burdens for all three species. Following 13 weeks of exposure, rats and mice exhibited equivalent uf-TiO_2 lung burdens at all exposure

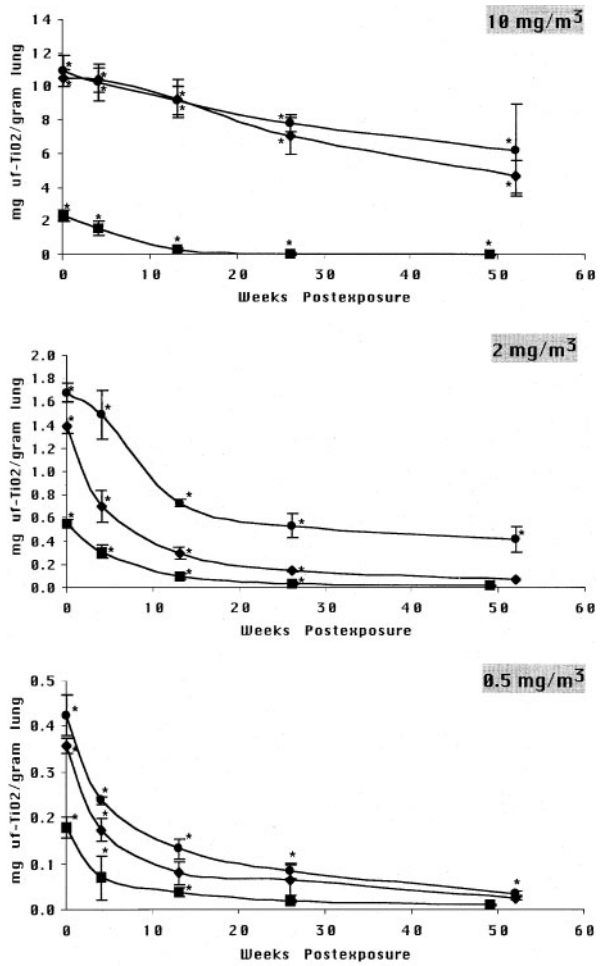

FIG. 1. Retained lung particle burdens in rodents exposed for 13 weeks to ultrafine TiO_2 and held unexposed for up to 52 weeks. Statistically significant differences from controls (Dunnett's, $p < 0.05$). Diamond, mouse; circle, rat; square, hamster.

concentrations (Fig. 1). Lung burdens of uf-TiO_2 in hamsters were approximately two- to five-fold lower than those of rats and mice. Particle burdens decreased in the lung with time postexposure in mice, rats, and hamsters (Fig. 1). Decreases in lung burdens with time after exposure were sharply different among exposure concentration groups in both rats and mice. In mice, the high-dose burdens decreased slowly to approximately 46% of the initial burden, whereas low- and mid-dose burdens were at undetectable levels by the end of the recovery period. Similarly, rats of the high-dose group retained approximately 57% of the initial lung burden, whereas the lung burdens in animals of the low- and mid-dose groups decreased to 10 and 25%, respectively, by the end of the recovery period. There were very significant differences among species in the pulmonary clearance kinetics of uf-TiO_2. Whereas the lung burdens of uf-TiO_2 in mice and rats of the high-dose group decreased in a linear fashion during the recovery period to approximately 50% of the lung burden at the end of the exposure, retained lung burdens in hamsters declined in a biphasic manner to just 3% of the initial burden. For animals of the mid- and low-dose groups, the change in lung burdens was also biphasic; however, in rats and not mice or hamsters, there were detectable concentrations of uf-TiO_2 at the end of the recovery period.

Burdens of uf-TiO_2 in the lymph nodes increased with time postexposure in rats of the mid- and high-dose groups and in mice of the high-dose group (Fig. 2). Concentrations of uf-TiO_2 in the lung-associated lymph nodes of hamsters did not exceed the MDC at any time or treatment dose.

Cytology

The number and types of cells recovered by lavage of the lungs were used to indicate the extent of pulmonary inflammation (for complete data for all doses, species, and time points, see supplementary Tables S1–S3). The mean number of

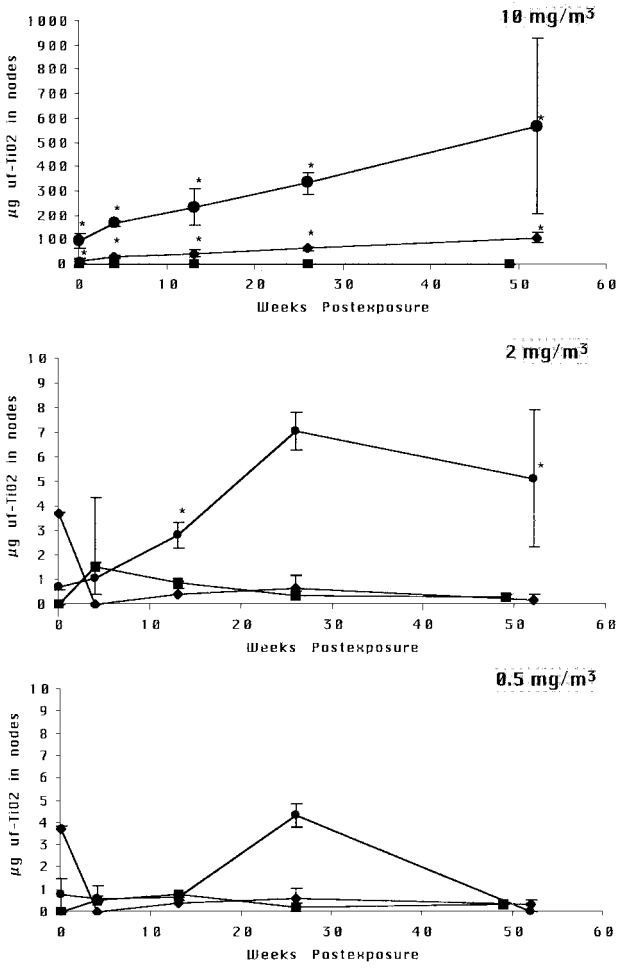

FIG. 2. Retained lung-associated lymph node particle burdens in rodents exposed for 13 weeks to ultrafine TiO_2 and held unexposed for up to 52 weeks. Statistically significant differences from controls (Dunnett's, $p < 0.05$). Diamond, mouse; circle, rat; square, hamster.

cells recovered by lavage from control animals was greatest in hamsters ($3.5 \times 10^6 \pm 0.6$) followed by rats ($1.8 \times 10^6 \pm 0.6$) and then mice ($1.8 \times 10^5 \pm 0.5$) and was consistent with our previous studies with these species. The cytological profile of control mice and rats was similar in that the majority (≥99%) of the recovered cells were macrophages. In contrast, the population of macrophages in control hamsters comprised approximately 81–96% of the cells and neutrophils ranged from 2 to 8%.

Significant increases in the total number of cells recovered (number ± one standard deviation) were observed at the end of exposures in rats ($5.3 \times 10^6 \pm 1.2$) and mice ($4.7 \times 10^5 \pm 1.1$) of the high-dose group. These elevations in the number of cells recovered from uf-TiO_2–exposed rats and mice declined with time after exposure to control levels in rats by 26 weeks postexposure but remained significantly ($p < 0.05$) different ($3.8 \times 10^5 \pm 0.11$) from concurrent controls in mice at 52 weeks postexposure.

Statistically significant changes in the cytological profile of the cells recovered by BAL were limited to the mice, rats, and hamsters of the high-dose group. Mice had significantly elevated numbers of macrophages, neutrophils, and lymphocytes at the end of the exposure period (Fig. 3). Over the recovery period of 52 weeks, the numbers of these cell types and the percentages of the populations that they comprised remained relatively constant and were significantly different from concurrent controls at the 52 week time point (Figs. 3 and 4). Rats also had significantly elevated numbers of macrophages, neutrophils, and lymphocytes at the end of the exposure period. Unlike mice, in rats the numbers of these cell types had returned to control levels by 26 weeks of recovery (Fig. 3), although the proportion of the population that was made up of macrophages and neutrophils remained significantly different from concurrent controls at 52 weeks of recovery (Fig. 4). Significant changes in recovered cell populations from the hamsters were limited to an increase in the number of neutrophils ($0.3 \times 10^6 \pm 0.1$) at the end of the exposures. At this same time point, the percentage of neutrophils was increased but not statistically significant, mostly due to the large variation among animals (10.2% ± 14.7).

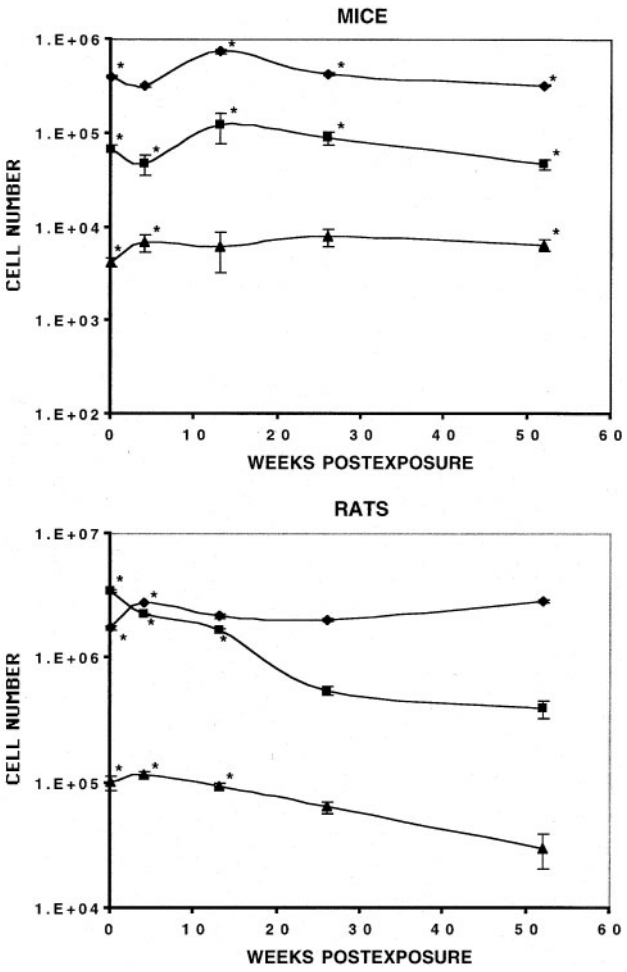

FIG. 3. Cytology of cells recovered by lung lavage from animals of the 10 mg/m^3 exposure concentration. The data represent the number of cells recovered by lung lavage. Each data point represents the mean of five animals. Statistically significant differences from controls (Dunnett's, $p < 0.05$). Diamond, macrophages; square, neutrophils; triangle, lymphocytes.

Total protein and LDH

Hamsters did not show any significant changes from controls at any time point or dose (Table 2). Responses in mice of the low- and mid-dose groups were not significantly elevated over corresponding controls at any time point, and in rats only the 4-week protein levels of the mid-dose group were greater than corresponding controls (Table 2). Significant increases over corresponding controls of total protein and LDH were measured in BALF of rats and mice of the high-dose group. Mouse LDH levels were greater than corresponding controls at 4 and 13 weeks postexposure, whereas the total protein levels were significantly elevated at all time points examined (Table 2). LDH levels in rat BALF were significantly increased through 13 weeks postexposure, whereas the concentrations of total protein had returned to control levels by 4 weeks postexposure (Table 2).

Lung Cell Proliferation

Control terminal bronchiolar cell replication was approximately the same across species with labeling indices ranging from 1 to 3% (Table 3). Significant terminal bronchiolar cell replication was evident at the end of the exposure period in mice and hamsters of the high-dose group and in rats of the mid- and high-dose groups (Table 3). At 4 weeks postexposure, the labeling indices of bronchiolar cells in all species were no longer greater than those of control animals (Table 3). Labeling indices of hamsters in the low-dose group were significantly decreased at 4 weeks postexposure but, considering subsequent values, was probably not biologically significant (Table 3).

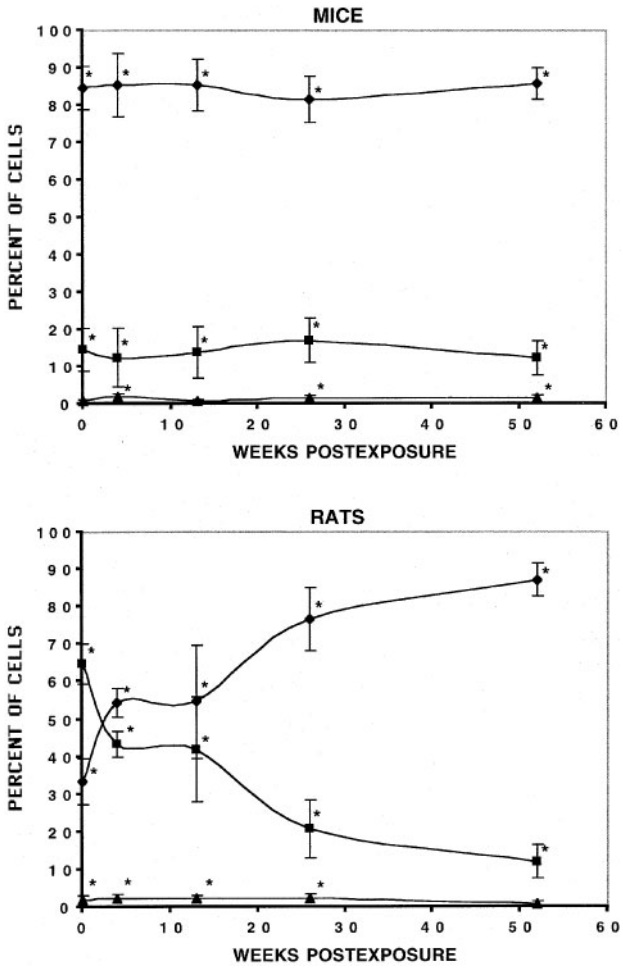

FIG. 4. Cytology of cells recovered by lung lavage from animals of the 10 mg/m^3 exposure concentration. The data represent the percentage of cells counted (200 per slide) identified as macrophages, neutrophils, or lymphocytes. Each data point represents the mean of five animals. Statistically significant differences from controls (Dunnett's, $p < 0.05$). Diamond, macrophages; square, neutrophils; triangle, lymphocytes.

TABLE 3
Terminal Bronchiolar Cell Replication

		Mice		Rats		Hamsters	
Weeks postexposure	Dose group (mg/m^3)	Labeling index[a] Mean	SD	Labeling index Mean	SD	Labeling index Mean	SD
0	0	2.16	0.69	2.09	1.15	0.91	0.68
	0.5	3.47	1.58	2.32	1.80	1.22	0.29
	2	3.93	1.74	3.79*	1.52	1.52	0.86
	10	5.27*	1.55	5.45*	0.95	2.46*	1.25
4	0	2.82	0.27	1.36	0.55	2.54	0.95
	0.5	3.64	2.55	1.24	0.97	0.79*	0.46
	2	2.10	0.70	1.23	0.74	2.16	1.15
	10	4.41	0.94	1.92	0.78	1.82	0.70
13	0	2.53	0.64	1.30	0.91	2.96	1.75
	0.5	2.05	1.25	1.87	0.73	2.68	0.68
	2	2.67	1.30	2.14	0.42	3.37	2.05
	10	2.57	3.05	2.19	0.85	2.60	1.42
26	0	1.31	0.47	1.27	0.64	2.58	2.46
	0.5	2.17	0.50	1.11	0.24	1.98	1.77
	2	2.20	1.82	1.87	0.75	2.08	1.17
	10	3.44*	2.33	1.06	0.69	2.55	0.69
52(49)[b]	0	1.38	0.76	1.15	0.55	1.42	1.01
	0.5	1.49	0.81	2.28	1.12	1.37	0.59
	2	2.06	1.23	1.21	0.47	1.10	0.63
	10	1.59	0.85	1.12	0.53	1.58	1.37

[a]Labeling indices are reported as the percentage of BrdU-labeled cells of the cells counted (minimum of 400 cells counted).
[b]Hamsters were sacrificed at 49 weeks postexposure.
*Significantly different from the concurrent control ($p < 0.05$).

Similarly, a statistically significant increase of the labeling indices for mice of the high-dose group was measured at 26 weeks postexposure but was probably not biologically significant (Table 3).

Control alveolar cell replication was similar in mice and rats

TABLE 2
Lactate Dehydrogenase (LDH) and Total Protein Concentrations in BAL Fluid from Mice, Rats, and Hamsters

	Mice					Rats					Hamsters				
	Weeks postexposure					Weeks postexposure					Weeks postexposure				
	0	4	13	26	52	0	4	13	26	52	0	4	13	26	49
LDH (U/l) (SD)															
Control	53 (35)	38 (10)	37 (16)	35 (10)	28 (9)	24 (2)	29 (1)	29 (3)	34 (16)	30 (8)	26 (5)	25 (3)	26 (5)	18 (5)	6 (5)
0.5 mg/m^3	42 (14)	46 (18)	41 (14)	60 (54)	45 (17)	26 (2)	32 (11)	29 (16)	27 (7)	28 (4)	26 (4)	27 (7)	29 (5)	21 (2)	11 (5)
2 mg/m^3	38 (20)	48 (17)	45 (17)	45 (14)	35 (8)	29 (7)	36 (17)	26 (6)	25 (7)	25 (5)	27 (8)	26 (5)	28 (3)	20 (1)	14 (7)
10 mg/m^3	87 (18)	103* (24)	120* (81)	63 (10)	72 (55)	122* (18)	112* (8)	83* (14)	50 (5)	33 (5)	24 (5)	27 (3)	22 (3)	17 (4)	9 (6)
Protein (μg/ml) (SD)															
Control	92 (19)	91 (12)	69 (15)	68 (27)	115 (15)	83 (15)	79 (5)	88 (9)	97 (33)	125 (32)	95 (20)	100 (20)	102 (50)	142 (21)	145 (35)
0.5 mg/m^3	92 (9)	82 (5)	80 (12)	97 (27)	129 (24)	111 (25)	80 (7)	81 (15)	98 (33)	116 (12)	106 (10)	91 (32)	94 (53)	138 (20)	119 (72)
2 mg/m^3	67 (25)	85 (17)	89 (5)	92 (26)	98 (23)	104 (91)	102 (15)	90 (20)	100 (34)	89 (30)	86 (30)	104 (13)	155 (40)	132 (37)	191 (79)
10 mg/m^3	257* (31)	256* (42)	274* (126)	169* (31)	206* (44)	236* (28)	223* (12)	133 (54)	138 (26)	149 (21)	118 (39)	113 (17)	134 (40)	143 (7)	143 (54)

*Significantly different from concurrent control, $p < 0.05$.

TABLE 4
Alveolar Cell Replication

		Mice		Rats		Hamsters	
Weeks postexposure	Dose group (mg/m^3)	Labeling index[a] mean	SD	Labeling index mean	SD	Labeling index Mean	SD
0	0	6.20	1.93	4.53	1.78	9.67	0.74
	0.5	5.34	1.43	6.23	2.42	10.25	0.48
	2	5.51	2.39	7.81*	1.22	10.60	3.81
	10	8.98	3.63	12.18*	2.53	12.87	5.22
4	0	7.28	2.64	4.59	1.05	11.85	2.43
	0.5	5.36	1.02	6.30	1.18	12.51	9.06
	2	5.91	1.53	7.15	1.44	9.88	2.01
	10	9.21	2.18	10.06*	0.96	12.03	2.53
13	0	5.64	1.12	5.09	1.83	12.02	2.40
	0.5	7.06	2.36	5.48	2.06	12.77	1.43
	2	6.09	1.88	7.17	4.37	10.01	3.70
	10	10.21*	1.99	9.40*	2.66	11.89	3.48
26	0	4.91	1.17	5.44	1.68	10.13	2.45
	0.5	6.53	1.91	4.18	1.28	12.36	2.66
	2	5.92	1.81	5.49	0.33	7.71	2.54
	10	9.46*	2.45	7.48	2.37	10.95	2.39
52(49)[b]	0	5.34	0.77	6.29	0.98	17.04	3.76
	0.5	8.04	2.08	6.61	0.96	16.95	3.10
	2	7.28	1.64	3.97	0.67	13.89	3.11
	10	8.09	2.75	7.21	1.40	15.92	3.39

[a]Labeling indices are reported as the percentage of BrdU-labeled cells of the cells counted (minimum of 400 cells counted).

[b]Hamsters were sacrificed at 49 weeks postexposure.

*Significantly different from the concurrent control ($p < 0.05$).

with values of 5–7%, whereas in control hamsters values ranged from 10 to 17% (Table 4). Replication of alveolar cells at the end of the exposures was significantly increased in rats of the mid- and high-dose groups but not in mice or hamsters (Table 4). Postexposure, the labeling indices in alveolar cells of rats decreased to control values in the mid-dose group by 4 weeks and in the high-dose group by 26 weeks. In contrast, labeling indices in mice were transiently significant at 13 and 26 weeks postexposure before returning to control values at 52 weeks postexposure. Hamster labeling indices remained equivalent to concurrent controls throughout the recovery period (Table 4).

Histopathology

Rats developed particle-induced lesions that were both concentration and time dependent. Changes in the lungs of low concentration–exposed animals were restricted to the appearance of particles within alveolar macrophages and very minimal changes in the patterns of alveolar macrophage accumulation in the lungs. Lesions in the mid-dose group were minimal to mild in severity and consisted primarily of particle-laden macrophage accumulation and aggregation in subpleural regions and in centriacinar zones. These macrophage aggregations were associated with minimal hypertrophy and hyperplasia of type II alveolar epithelial cells. Over time with clearance, during the recovery phase of the experiment, these aggregations of cells became more focally concentrated and could be noted in interstitial areas (Fig. 5). In the high concentration–exposed rats, through 13 weeks postexposure, there were progressively more severe epithelial proliferative changes, including metaplastic changes in the centriacinar region (bronchiolization of alveolar epithelium) associated with particle and particle-laden macrophage accumulation. Most of these epithelial proliferative lesions regressed with time postexposure (13–52 weeks), although a few foci with associated mild fibroplasia were noted at the final sacrifice time point (Fig. 6B). These particular foci were believed to have progressed in severity. At the 52 week final sacrifice, metaplastic changes were minimal to mild and minimal to mild particle-induced alveolar septal fibroplasia was present.

No lesions were associated with the low and middle concentration–exposed mice. In these animals, change was limited to the findings of particles free and within alveolar macrophages and in alveolar septal regions. In the high concentration–exposed mice, there was little epithelial change and the lesions primarily consisted of aggregations of heavily particle-laden macrophages that were concentrated in central lobar centriacinar sites (Fig. 6A). Over time postexposure, there was evidence of concentration of these cell aggregates and movement to interstitial areas primarily around blood vessels and peribronchiolar interstitium. Perivascular lymphoid proliferation was a significant particle-associated lesion noted in the high concentration group.

Other than alveolar and interstitial macrophages containing particles and the occasional aggregation of particle-laden macrophages in the high concentration–exposed group, there was no pathology associated with uf-TiO_2 exposure in the hamsters (Fig. 6C).

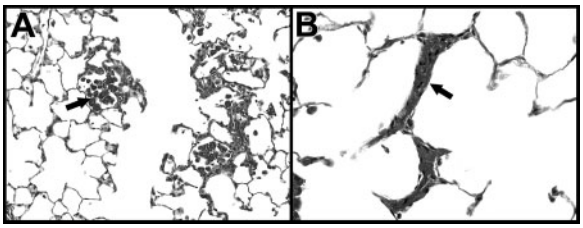

FIG. 5. (A) Photomicrograph of rat lung in high concentration–exposed group (10 mg/m^3) at 4 weeks postexposure. Numerous aggregates of particle-laden alveolar macrophages are noted clustered within alveolar lumina (arrow). Alveolar septal walls surrounding particle-laden macrophages are relatively normal with the exception of the occasional hypertrophied type II epithelial lining cell. Mallory's Trichrome, 150×. (B) High magnification photomicrograph of alveolar septal region from rat lung of high concentration–exposed group at 52 weeks postexposure. There is particle interstitialization noted as marked focal thickening of alveolar septal regions with interstitial accumulation of particle-laden macrophages (arrow). Mallory's Trichrome, 300×.

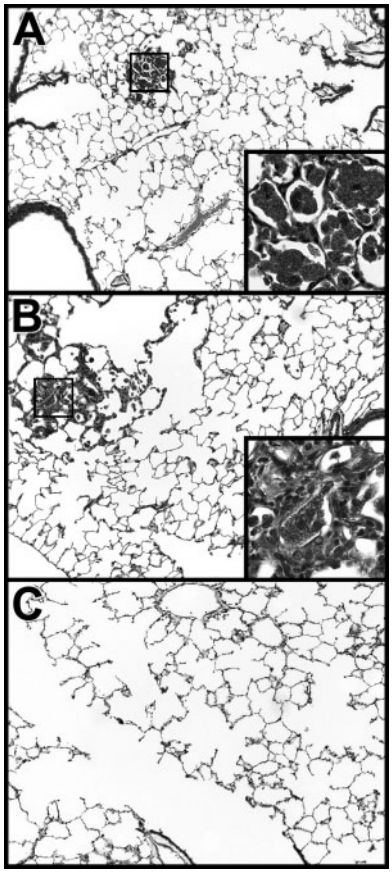

FIG. 6. Comparison of representative centriacinar regions of lungs from high concentration–exposed groups (10 mg/m^3) of all three species at 52 weeks postexposure. (A) Mouse lung depicting alveolar accumulation of particle-laden macrophages surrounded by relatively normal alveolar septal structures. (B) Lung tissue from exposed rat showing intra-alveolar accumulation of particle-laden macrophages, interstitial fibrosis and thickening, and alveolar epithelial metaplasia (bronchiolization) of lining epithelium. (C) Hamster lung showing lack of retained particle burden and associated lack of macrophage accumulation. Mallory's Trichrome, 75×; insets 300×.

DISCUSSION

Pulmonary overload is characterized by the impairment of particle clearance resulting in persistent lung particle burdens. Previous studies with ultrafine particles have shown that pulmonary overload can be achieved with relatively insoluble, low toxicity particles, including uf-TiO_2 (Creutzenberg *et al*., 1990). Extended impairment of clearance has been shown to lead to the development of pulmonary tumors with ultrafine particles in rats but not in mice or hamsters (Heinrich *et al*., 1986; Muhle *et al*., 1990). Uf-TiO_2 aerosol concentrations for the present study were chosen to result in retained lung burdens that would span from minimal accumulation in alveolar macrophages to pulmonary accumulations associated with pulmonary overload and the induction of lung tumors in chronic studies (Heinrich *et al*., 1995).

Particle concentrations in the aerosols for the exposures of each species were within 11% of the target concentrations of 0.5, 2, and 10 mg/m^3. The aerodynamic diameter of the particles was found to be in the range of 1.29 to 1.44 μm and did not significantly differ between exposures. Considering the primary particle size of approximately 0.02 μm, the aerosol generated was made up of particle aggregates, probably due to agglomeration. This is a common occurrence in the generation of aerosols of these types of particles, and it is assumed that once the particles are deposited there is disaggregation of the primary particles.

There were species differences in retained lung burdens. Lung burdens of uf-TiO_2 were increased in a concentration-dependent manner in rats, mice, and hamsters after 13 weeks of exposure. Initial lung burdens per gram of lung were similar in rats and mice; however, hamsters had approximately 23% of the rat and mouse burdens. The fact that hamsters had lower initial lung burdens is indicative of the ability of hamsters to efficiently clear particles from the lung during exposure (Bermudez *et al*., 2002; Creutzenberg *et al*., 1998). The retained lung burden in rats exposed to 10 mg/m^3 was 2.1 mg/lung and was comparable, adjusting for dose and daily length of exposure, to lung burdens found in other studies using this material (Ferin *et al*., 1992; Heinrich *et al*., 1995). Similarly, uf-TiO_2 lung burdens of 0.42 mg/lung in mice of the high-dose group were comparable to the mice in the study by Heinrich *et al*. (1995), when adjusted for dose and daily length of exposure.

Determinations of lung burdens subsequent to exposure showed a decrease with time. Uf-TiO_2 was detectable in the lungs of rats of all dose groups at the end of the postexposure period, whereas this was true only for mice and hamsters of the high-dose group. A comparison of the percentages of initial lung burden remaining in the lungs of the high-dose animals at the end of the postexposure period reveals that rats and mice were approximately equivalent with respect to particle retention (57 and 45%, respectively). In contrast, particle retention in hamsters was much lower (3%). The percentage of the initial burden retained in rats is in good agreement with that found in the study by Ferin *et al*. (1992). Calculation of retention half-times from the lung burden data of the low-, mid-, and high-dose groups showed the following, respectively: rats had half-times of 63, 132, and 395 days; mice had half-times of 48, 40, and 319 days; and hamsters had half-times of 33, 37, and 39 days. The evident prolongation of particle clearance from the lungs of the high-dose rats and mice, such that clearance of the particle burden would require more than the animal's lifetime, is indicative of pulmonary overload.

The overload of the lung noted in mice and rats was reflected in the translocation of particles to lung-associated lymph nodes. The lack of particle translocation to the lymph nodes in hamsters reflects the short clearance half-times/low lung burdens in these animals. Rats and mice of the high-dose groups had significant lymph node burdens that continued to increase with time postexposure, consistent with the declining lung burdens and indicating an ongoing translocation of particles to

these tissues. These results are consistent with other studies, using various particulates, showing that at lung burdens of 1 mg/g lung or greater there is a prolonged retention of particles (Morrow, 1992).

Pulmonary inflammation is a common response to the inhalation of various types of particles and has been closely associated with other chronic pathological outcomes, such as fibrosis and cancer, following extended exposure to these materials (Bajpai *et al*., 1992; Driscoll *et al*., 1990; Muhle *et al*., 1998; Oberdorster *et al*., 1992; Warheit *et al*., 1997). Characterization of the inflammatory response is usually accomplished by the assessment of changes in lung cell populations and biomarkers of inflammation in parenchymal lung tissue and BAL fluid. In the present study, histopathology of lung sections and cytological and biochemical markers of toxicity in BAL fluid (LDH and protein) were examined. Significant alterations in inflammatory parameters were observed only in those animals exposed to the high concentration (10 mg/m^3) of uf-TiO_2. In general, the magnitude of the responses was greatest in rats and least in hamsters.

Mice of the high-dose group had mild, persistent inflammation of the lung following 13 weeks of exposure. This inflammation was characterized by elevated concentrations of BALF protein and increased numbers of macrophages and neutrophils. With time postexposure, LDH transiently increased then returned to control levels, whereas BALF protein concentrations and inflammatory cell numbers remained relatively constant.

Inflammation in rats of the high-dose group following 13 weeks exposure was more severe than in mice in that all the measured parameters were significantly increased, particularly the number of neutrophils (65% of the recovered cells). Upon cessation of the exposure, there was a decline in all measured parameters with BALF LDH and protein and macrophage numbers returned to control levels by 26 weeks postexposure. The number of neutrophils remained elevated at 52 weeks postexposure and at the same level as mice (12%), indicating the presence of mild inflammation. These results are similar to those reported by Ferin *et al*. (1992), who observed a persistent increase in neutrophils in rats 52 weeks after a 12-week exposure to uf-TiO_2.

Pulmonary inflammation was absent in hamsters, as evidenced by control levels of biochemical and cytological parameters. This is not surprising in light of the relative low lung burdens noted at the end of the exposure and the short retention half-times of the particles for this species.

Bronchiolar cells of rats, mice, and hamsters of the high-dose group exhibited increased cell replication following the cessation of exposure. This response was transient and is consistent with the clearance of particles or inflammation of the airway and is believed to represent a lesion separate from cell proliferation of alveolar epithelial cells.

Rat alveolar cells had significantly elevated (two- to three-fold) cell replication in animals of the mid- and high-dose groups at the end of exposure. This increased cell replication persisted through 13 weeks postexposure and correlated well with proliferative lesions observed in these animals. These results are similar to the two- to three-fold elevations of alveolar cell replication noted in rats exposed to fine grade TiO_2 (Bermudez *et al*., 2002; Warheit *et al*., 1997). Quantitative differences in levels of cell replication noted in control populations between the present study and that of Warheit *et al*. (1997) are probably due to the differences in the methods of label administration (pulse vs. continuous administration of BrdU). Persistent alveolar cell replication in rats 1 year after exposure to 23.5 mg/m^3 uf-TiO_2, using the same exposure regimen as in the present study, has been reported (Baggs *et al*., 1997). The genesis of the observed alveolar cell replication is unclear, as there was a good correlation of this parameter with lung burdens and neutrophil numbers; however, persistent replication of parenchymal cells with attendant macromolecular damage and impairment of clearance may lead to the induction of pulmonary tumors.

Mice also had elevated cell replication of the alveolar cells, however the pattern was somewhat different than in rats. Significantly elevated alveolar cell replication was first observed 13 weeks after cessation of the exposure and had returned to control values by 52 weeks postexposure. Although cell replication correlated fairly well with neutrophil numbers ($r^2 = 0.717$), it did not correlate with lung burdens. This suggests that in mice the augmentation of cell replication is related to the particle-induced inflammatory response rather than being a direct effect of particles.

Unlike mice and rats, hamsters demonstrated no change in alveolar cell replication indices. This was consistent with the lack of an inflammatory response and low lung burdens in these animals.

Histopathological findings for mice, rats, and hamsters exposed to uf-TiO_2 differed. Rats exposed to 10 mg/m^3 exhibited septal thickening and slight proliferation of type II cells but little metaplasia at the end of the exposure period. With time postexposure, these lesions progressed such that by 13 weeks there was increased thickening of the interstitium, epithelial cell proliferation, and metaplasia of alveolar epithelium. At subsequent time points, there was a diminution of the epithelial response and focal clustering of particle-laden macrophages, mostly alveolar intraluminal but with increased aggregation in interstitial regions. At the final time point, tight clusters of particle-laden macrophages were seen in some alveoli and substantial interstitialization of such macrophages was apparent. Occasional small foci of epithelial hyperplasia and hypertrophy and minimal metaplasia were present. A minimal fibrotic response was noted as thickening of alveolar septae in centriacinar regions associated with particle aggregation. There was, in general, little epithelial or fibroproliferative reaction in regions of intraluminal or interstitial aggregation of particle-laden macrophages. In mice exposed to 10 mg/m^3 uf-TiO_2, particle-laden macrophages and aggregates of free particles

were present in centriacinar regions with no epithelial response. Interstitialization of these macrophages was apparent at the 13-week time point but again no epithelial response was observed. At the final time point, intraluminal accumulations of particle-laden macrophages were still present but, again, in the absence of an epithelial response. The rapid clearance of particles from the lungs of hamsters resulted in relatively few particle-associated lesions. Occasional particle-laden macrophages were present in alveoli adjacent to and along the alveolar ducts, and occasional particles were noted either free or cell-associated in alveolar and bronchiolar interstitium. Clearance of particles in hamsters continued into the postexposure period such that there were virtually no particles or particle-laden macrophages visible by 26 weeks.

Experimental exposure to fine and ultrafine modes of some particulates leads to differential pulmonary effects (Donaldson *et al*., 1998; Li *et al*., 1999). Titanium dioxide in the ultrafine mode has been shown to result in more lung injury and pathology than equivalent deposited mass concentrations of pigmentary TiO_2 (Ferin *et al*., 1992; Janssen *et al*., 1994). Comparisons between these two particle sizes of TiO_2 on a mass basis do not correlate well with the observed tissue responses; however, comparisons using the surface area per unit mass have yielded an improved correlation of the rat data for some end points (Oberdorster, 1996). Previous work in this laboratory examined the pulmonary effects of three concentrations of inhaled pigment grade TiO_2 in mice, rats, and hamsters using the same end points as in the present study (Bermudez *et al*., 2002). Of the three airborne concentrations used in that experiment, two (50 and 250 mg/m^3) resulted in pulmonary overload in rats and mice. A comparison of the lung burdens between the two studies, using surface area as the dose-metric, reveals that the lung burdens in those animals exposed for 13 weeks to 10 mg/m^3 uf-TiO_2 or to 50 mg/m^3 pigmentary TiO_2 were approximately the same for all three species. When the pulmonary responses of each species to 50 mg/m^3 pigmentary and 10 mg/m^3 uf-TiO_2 were compared, there was concordance in the BALF indicators of toxicity (i.e., LDH and total protein). This conclusion is based on an examination of the magnitude of the pulmonary responses in dosed animals relative to the concurrent controls.

In comparing the two 90-day interspecies inhalation studies, one with 0, 10, 50, and 250 mg/m^3 pigment-grade TiO_2 particles and the other with 0, 0.5, 2, and 10 mg/m^3 ultrafine TiO_2 particles, the results demonstrate many similarities. In the pigment-grade study, exposures to 50 and 250 mg/m^3 produced particle overload in rats and mice but not in hamsters. Lung inflammation was measured in all three species at 50 and 250 mg/m^3, but the ranking of sensitivity comparisons were (rats $>$ mice $>$ hamsters). Pulmonary lesions were most severe in rats, manifested by progressive epithelial metaplastic and fibroproliferative changes concomitant with enhanced alveolar epithelial cell proliferation measured in the group exposed to 250 mg/m^3. In the studies with ultrafine TiO_2 particles reported herein, particle overload was measured in rats and mice but not hamsters exposed to 10 mg/m^3. The ranking of severity of the lung inflammatory responses at 10 mg/m^3 was as follows: rats $>$ mice $>$ hamsters. Progressive epithelial and fibroproliferative changes were observed in rats but not in mice exposed to 10 mg/m^3. These lesions were characterized by foci of alveolar epithelial proliferation of metaplastic epithelial cells circumscribing the aggregated foci of heavily particle-laden macrophages. Thus, to summarize the findings of the two studies, clear species differences were observed and measured in pulmonary responses to both pigment-grade (50 and 250 mg/m^3) and ultrafine (10 mg/m^3) titanium dioxide particles. Moreover, there was consistency among the species in responses to both particle types. Clearly, the rat is the most sensitive species in the pulmonary responses to both pigment-grade and ultrafine TiO_2 particles.

In summary, inhalation of 10 mg/m^3 uf-TiO_2 for 13 weeks resulted in pulmonary overload in rats and mice but not in hamsters where the lung burdens were approximately 23% of the other species. While there were various responses in mice and rats, hamsters had very limited responses probably due to the low lung burdens and rapid clearance of particles in these animals. Responses in mice were limited to animals exposed to 10 mg/m^3, whereas in rats responses were also observed in animals exposed to 2 mg/m^3. The magnitude and spectrum of responses were, in general, equivalent in rats and mice. The extent and character of the inflammatory responses in rats differed from mice; in rats the responses had a greater neutrophilic component that diminished with time, whereas in mice significantly increased neutrophil and macrophage numbers remained relatively constant. Histopathological examination of rats and mice uncovered progressive fibroproliferative lesions in rats but not in mice. Taken together, the species differences observed in this study reflect the outcome of previously reported chronic exposures to poorly soluble particulates for each species and suggest that susceptibility of the rat, under pulmonary overload conditions, to the induction of lung tumors by these materials has underlying components of dosimetry and biological response.

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