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ScienceDirect
Toxicology 230 (2007) 90–104
TOXICOLOGY
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Pulmonary toxicity study in rats with three forms of ultrafine-TiO_2 particles: Differential responses related to surface properties

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Received 1 August 2006; received in revised form 3 November 2006; accepted 3 November 2006
Available online 10 November 2006

Abstract

Surface properties are critical to assess effects of ultrafine-TiO_2 particles. The aim of this study was to assess lung toxicity in rats of newly developed, well characterized, ultrafine-TiO_2 particles and compare them to TiO_2 samples in two different size ranges and surface modifications. Groups of rats were intratracheally instilled with doses of 1 or 5 mg/kg of either two ultrafine rutile TiO_2 particles (uf-1 or uf-2); rutile R-100 fine-TiO_2 (F-1); 80/20 anatase/rutile P25 ultrafine-TiO_2 (uf-3); or α-quartz particles. Phosphate-buffered saline (PBS) solution instilled rats served as vehicle controls. Following exposures, the lungs of PBS and particle-exposed rats were evaluated for bronchoalveolar lavage (BAL) fluid inflammatory markers, cell proliferation, and by histopathology at post-instillation time points of 24 h, 1 week, 1 and 3 months.

The ranking of lung inflammation/cytotoxicity/cell proliferation and histopathological responses was quartz > uf-3 > F-1 = uf-1 = uf-2. Exposures to quartz and to a lesser degree, uf-3 anatase/rutile TiO_2 particles produced pulmonary inflammation, cytotoxicity and adverse lung tissue effects. In contrast, exposures to F-1 fine-TiO_2 particles or to uf-1/uf-2 ultrafine-TiO_2 particle-types produced transient inflammation. We conclude that differences in responses to anatase/rutile uf-3 TiO_2 particles versus the rutile uf-1 and uf-2 TiO_2 particles could be related to crystal structure, inherent pH of the particles, or surface chemical reactivity. Thus, based on these results, inhaled rutile ultrafine-TiO_2 particles are expected to have a low risk potential for producing adverse pulmonary health effects. Finally, the results demonstrate that exposures to ultrafine-TiO_2 particle-types can produce differential pulmonary effects, based upon their composition, and crystal structure. Thus, the lung toxicity of anatase/rutile uf-3 should not be viewed as representative for all ultrafine-TiO_2 particle-types.

Keywords: Titanium dioxide particles; Ultrafine particles; Titanium dioxide nanocrystals; Pulmonary toxicity; Rutile crystal structure; Particle surface treatments; Nanomaterials

1. Introduction

Titanium dioxide (TiO_2) particles are regarded as poorly soluble particulates (PSP) by virtue of their low solubility and low toxicity (Hext, 1994; Hext et al., 2005; ILSI, 2000; Donaldson, 2000; Bermudez et al.,

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doi:10.1016/j.tox.2006.11.002

2002). TiO_2 particles, in either the fine or ultrafine sizes, are widely used commercially as white pigments or in cosmetic applications, respectively. From a toxicology perspective, fine-sized titanium dioxide particulates are often utilized as negative control reference particle-types (Tran et al., 2000; Hext et al., 2005). However, following chronic exposures in rats to high particle concentrations resulting in substantial particle overload conditions (e.g., 250 mg/m^3), inhaled pigment-grade TiO_2 (fine- or F-1 TiO_2) particles resulted in benign lung tumors, a response unique to that rodent species (Hext, 1994; ILSI, 2000; Warheit and Frame, 2006). Moreover, the limited toxicological data base demonstrates that P25 ultrafine anatase/rutile TiO_2 particles (henceforth referred to as uf-3) are, on a mass basis, significantly more potent than fine-sized TiO_2 particles in producing adverse lung effects (Bermudez et al., 2002, 2004; Warheit et al., 2006). In this regard, the results of 90-day and 2-year inhalation studies with uf-3 anatase/rutile ultrafine-TiO_2 or F-1 rutile fine-TiO_2 particles (average primary particle sizes ~25 and ~300 nm, respectively) have demonstrated that approximately 1/5 the inhaled mass concentrations of the ultrafine anatase/rutile (uf-3) TiO_2 particles, when compared with the fine-TiO_2 particles (F-1), produced equivalent numbers of pulmonary inflammation, fibrosis, or lung tumors in rats; effects occurring only at particle overload concentrations and unique to this species (Bermudez et al., 2002, 2004; Lee et al., 1985; Heinrich et al., 1995). The results of shorter-term pulmonary toxicity studies with ultrafine-TiO_2 particles in rats have supported the notion of enhanced lung inflammatory potency of the ultrafine particles when compared to exposures of fine-sized particulates of similar composition. This was considered to be associated with a greater surface area of the ultrafine particles when compared to fine-sized particles (Bermudez et al., 2002, 2004).

However, a closer examination of the physicochemical properties of the two TiO_2 particle-types indicates that the crystal structures of the fine-size, pigment-grade (100% rutile) and uf-3 ultrafine-TiO_2 particles (80/20 anatase/rutile) are different. Accordingly, the two forms of TiO_2 previously tested also represent different compositions as well as size characteristics. This would imply that the inherent differences are not simply particle size-related. Furthermore, recent *in vitro* studies suggest that TiO_2 particles with different crystal structures (i.e., anatase or rutile) produce different toxicological responses. Indeed, nano-TiO_2 particles in the anatase crystal phase were reported to be superior catalysts, better generators of reactive species, and more cytotoxic when compared to the rutile particle-type tested (Uchino et al., 2002; Sayes et al., 2006). These effects were considered to be due to differences inherent in the crystal structures of the two phases, and not due to differences in surface area. Thus, comparing the toxicological effects of anatase versus rutile TiO_2 particle-types may be more analogous to comparisons of crystalline silica versus amorphous silica particles than simply examining particle size and surface area differences.

This study was designed to determine whether ultrafine-TiO_2 particles impart significant toxicity in the lungs of rats, and more importantly, how the activity of different TiO_2 formulations compares with other reference particulate materials, such as anatase/rutile ultrafine-TiO_2 particles. Thus, the aim was to assess in rats, using a well-developed short-term pulmonary bioassay, the pulmonary toxicity effects of two intratracheally instilled, ultrafine-TiO_2 particle samples and to compare the lung toxicity responses of these samples with (1) a low toxicity particulate-type (negative control, F-1 TiO_2 particles); (2) a cytotoxic particulate-type (positive control, α-quartz particles); (3) an ultrafine-TiO_2 particle reference sample in a similar agglomerated size range (uf-3 TiO_2); (4) vehicle control (PBS).

2. Methods

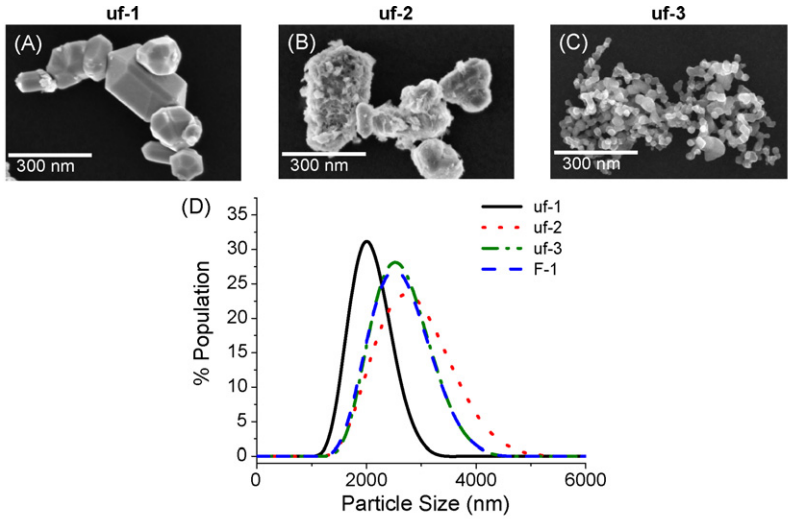
2.1. Animals

Groups of male Crl:CD®(SD)IGS BR rats (Charles River Laboratories, Inc., Raleigh, North Carolina) were used in this study. The rats were approximately 8 weeks old at study start (mean weights in the range of 210–280 g). All procedures using animals were reviewed and approved by the Institutional Animal Care and Use Committee. The animal program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

2.1.1. Particle-types and physicochemical characterization (see Fig. 1)

An ILSI Research Foundation/Risk Science Institute Nanomaterial Toxicity Screening Working Group has developed a screening strategy for the hazard identification of engineered nanomaterials (Oberdorster et al., 2005a). The report concludes that adequate characterization of nanomaterials represents one of the key aspects of toxicity screening strategies. In this regard, particle or test substance characterization is recommended for both the supplied material as well as the administered material. Accordingly, we have measured the physicochemical characteristics of the various TiO_2 particle-types in both a water suspension (representing the supplied material) as well as in the vehicle, phosphate-buffered saline (representative of the administered material).

An ultrafine particle-type is defined herein as a particle of average primary size approximating 100 nm and exhibiting a



(E)

Sample	Crystalline phase	Median size and width distribution (nm)		Surface area (m^2/g)	pH		Chemical reactivity
		in water*	in PBS		deionized water	in PBS	delta b*
F-1	rutile	382.0 ± 36%	2667.2 ± 35%	5.8	7.49	6.75	0.4
uf-1	rutile	136.0 ± 35%	2144.3 ± 45%	18.2	5.64	6.78	10.1
uf-2	rutile	149.4 ± 50%	2890.7 ± 31%	35.7	7.14	6.78	1.2
uf-3	80/20 anatase/ rutile	129.4 ± 44%	2691.7 ± 31%	53.0	3.28	6.70	23.8

Fig. 1. The characterization of the F-1, uf-1, uf-2, and uf-3 samples. (A–C) High resolution scanning electron micrographs (HR-SEM) of samples uf-1, uf-2, and uf-3, respectively. Micrographs depict variations in surface treatment and particle size within each ultrafine-TiO_2 particle-type. (D) Plot of size distribution of F-1, uf-1, uf-2, and uf-3 particle samples in PBS (vehicle) taken from DLS measurements. (E) Table of physical properties of the samples used in the study, including size and size distribution, surface area (from the dry state), pH, and chemical reactivity. Measurements were taken in both water (*aqueous solution of 0.1% tetrasodium pyrophosphate) and phosphate-buffered saline (PBS) solution.

property that is uniquely different than that of its bulk counterpart. Because both the uf-1 and uf-2 particulates have an average particle size of ∼140 nm, with a substantial size distribution that falls below 100 nm AND are much more effective UV-light stabilizers (a property that pigmentary or fine size TiO_2 does not have), they are termed ultrafine under the definition described above.

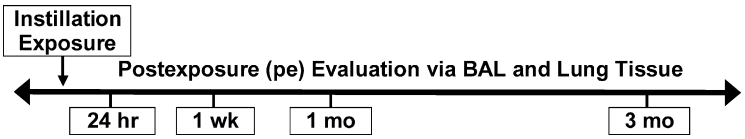
Two different rutile-type, ultrafine-TiO_2 particles, designated as uf-1 and uf-2 were obtained from the DuPont Company. DuPont uf-1 is composed of a titanium dioxide core with an alumina surface coating (∼98% titanium dioxide and ∼2% alumina) and possesses an average particle size of 136 nm in water (aqueous solution buffered in 0.1% tetrasodium pyrophosphate) using dynamic light scattering (in aqueous suspension) and an average BET surface area (in the dry state) of 18.2 m^2/g. DuPont uf-2 is composed of a titanium dioxide core with a silica and alumina surface coating (∼88 wt% titanium dioxide, ∼7 wt% amorphous silica and ∼5 wt% alumina) and possesses an average particle size of ∼149.4 nm in water (aqueous solution buffered in 0.1% tetrasodium pyrophosphate) using dynamic light scattering and an average BET surface area of 35.7 m^2/g (Brunauer et al., 1938).

R-100 rutile-type, fine-sized TiO_2 particles (henceforth referred to as F-1) were obtained from the DuPont Company. F-1 is composed of a titanium dioxide core with an alumina surface coating (∼99% titanium dioxide and ∼1% alumina) and possesses an average particle size of 382 nm in water (aqueous solution buffered in 0.1% tetrasodium pyrophosphate) using dynamic light scattering and an average BET surface area of 5.8 m^2/g. All rutile samples underwent a neutralization process during production, which neutralizes residual acidic chloride groups on the particle surface.

P25 ultrafine-TiO_2 particles, consisting of 80% anatase/20% rutile TiO_2 particles (henceforth referred to as uf-3) (average reported primary particle size of ∼25 nm primary particle size) were purchased from Degussa Corporation. Uf-3 is composed of 100 wt% titanium dioxide (80/20 anatase/rutile) and possesses an average particle size of 129.4 nm in water (aqueous solution buffered in 0.1% tetrasodium pyrophosphate)

Table 1
Protocol for ultrafine-TiO_2 particle bioassay study

Exposure groups
- PBS (vehicle control)
- Particle-types (1 and 5 mg/kg)
  - Rutile-type uf-1 TiO_2
  - Rutile-type uf-2 TiO_2
  - Anatase/rutile-type uf-3 TiO_2
  - Rutile-type F-1 fine-TiO_2 (negative control)
  - α-Quartz particles (positive control)



using dynamic light scattering and an average BET surface area of 53.0 m^2/g (Fig. 1).

Particles were dispersed in an aqueous solution buffered with 0.1% tetrasodium pyrophosphate solution for DLS analysis to sufficiently disperse the particles for sizing analyses. Tetrasodium pyrophosphate provides a basic (pH) solution for dispersion of TiO_2 particles. With this solution, the polyphosphate anions act as a dispersant at a pH which charge stabilizes the suspension, thus resulting in a sizing measurement of individually dispersed particles.

Quartz particles (crystalline silica (α-quartz), Min-U-Sil 5) with reported size range of 0.2–2 μm were obtained from U.S. Silica Company (Berkeley Springs, WV). Min-U-Sil 5 is composed of ∼100% silicon dioxide (α-quartz) and possesses an actual average particle size of ∼480 nm in water (aqueous solution buffered in 0.1% tetrasodium pyrophosphate) and an average surface area of 5.2 m^2/g.

Each of the TiO_2 samples were characterized to identify its crystallinity and surface area in its dry native state and its size, size distribution, pH, and chemical reactivity in water and buffered solutions. X-ray fluorescence was used to measure purity/composition. X-ray diffraction (Philips X'PERT automated powder diffractometer, Model 3040) was used to determine crystal structure, and crystallite size (Otwinowski and Minor, 1997). XRD patterns do provide information on the primary crystallite size, but do not, however, accurately represent the particle size of the dispersed agglomerates—therefore, sizing data was obtained using two separate methods: dynamic light scattering (DLS) and BET surface area analysis (Brunauer et al., 1938). DLS measurements (Malvern Zetasizer Nano-S, model Zen1600) were taken on each particle sample in solution: water (aqueous solution buffered in 0.1% tetrasodium pyrophosphate) and phosphate-buffered saline (PBS) solution. BET (Micromeritics ASAP 2405), to measure specific surface area, was taken on each particle sample in its dry state under nitrogen.

Chemical reactivity was measured using a Vitamin C yellowing assay (Association of Vitamin Chemists, 1951; Rajh et al., 1999). This assay measures the chemical reactivity of the sample toward an anti-oxidant, specifically a Vitamin C derivative. With greater chemical reactivity, the yellowing of the test sample will increase providing a higher Δb^*. Briefly, a standard solution of 6.25% ascorbic acid palmitate (L-ascorbic acid 6-palmitate, 99%, Alfa Aesar) in octyl palmitate (hexadecanoic acid 2-ethylhexyl ester, VanDyk) was prepared. Using a spatula and glass plate, 1.90 ± 0.05 mL of the solution was thoroughly mixed with 0.40 ± 0.01 g of titanium dioxide. The mixture was applied onto a white lacquered 3 in. × 5 in. card using a 6 mL Bird film applicator to form the test film. The color of the test film was measured using a hand-held spectrocolorimeter (Byk-Gardner, Model CB-6805), calibrated, and set to D65/10° (illuminant/observer). In the same manner, a blank film was prepared using neat octyl palmitate and titanium dioxide. The color of the blank film was then measured. The Δb^* value was determined by comparing the color of the test and blank films. The Δb^* value was a measure of chemical activity.

2.2. General experimental design (see Table 1)

The fundamental features of this pulmonary bioassay are dose–response evaluations and time-course assessments to determine the sustainability of any observed effect. Thus, the major endpoints of this study were the following: (1) time-course and dose/response intensity of pulmonary inflammation and cytotoxicity; (2) airway and lung parenchymal cell proliferation; (3) histopathological evaluation of lung tissue.

For the bronchoalveolar lavage studies, groups of rats (5 rats/(group dose time point)) were intratracheally instilled with single doses of either 1 or 5 mg/kg ultrafine-TiO_2 particle-type 1 (uf-1); ultrafine-TiO_2 particle-type 2 (uf-2); F-1 fine-TiO_2 particles; ultrafine uf-3 TiO_2 particle-types; or quartz (crystalline silica) particles (see Table 1). The intratracheal instillation method of exposure can be a reliable qualitative screen for assessing the pulmonary toxicity of inhaled particles (Warheit et al., 2005). All particles were prepared in a volume of phosphate-buffered saline (PBS) solution and subjected to ultrasonic probe sonication for 15 min at 60 Hz. Groups of PBS-instilled rats served as controls. The lungs of PBS and

particle-exposed rats were evaluated by BAL fluid analyses at 24 h, 1 week, 1 and 3 months postexposure (pe).

For the lung tissue studies, additional groups of animals (4 rats/(group high dose time period)) were instilled with the particle-types listed above plus the vehicle control, i.e., PBS. These studies and corresponding groups of rats were dedicated to lung tissue analyses but only the high dose groups (5 mg/kg) and PBS controls were utilized in the morphology studies. These studies consisted of cell proliferation assessments and histopathological evaluations of the lower respiratory tract. Similar to the BAL fluid studies, the intratracheal instillation exposure period was followed by 24-h, 1-week, 1-, and 3-month recovery periods.

2.2.1. Pulmonary lavage

The lungs of sham and particulate-exposed rats were lavaged with a warmed phosphate-buffered saline (PBS) solution as described previously. Methodologies for cell counts, differentials and pulmonary biomarkers in lavaged fluids were conducted as previously described (Warheit et al., 1991, 1997). Briefly, the first 12 mL of lavaged fluids recovered from the lungs of PBS or particulate-exposed rats was centrifuged at 700 g, and 2 mL of the supernatant was removed for biochemical studies. All biochemical assays were performed on BAL fluids using a Roche Diagnostics (BMC)/Hitachi® 717 clinical chemistry analyzer using Roche Diagnostics (BMC)/Hitachi® reagents. Lactate dehydrogenase is a cytoplasmic enzyme and is used as an indicator of cell injury. Alkaline phosphatase activity is a measure of Type II alveolar epithelial cell secretory activity, and increased ALP activity in BAL fluids is considered to be an indicator of Type II lung epithelial cell toxicity. Increases in BAL fluid microprotein (MTP) concentrations generally are consistent with enhanced permeability of vascular proteins into the alveolar regions, indicating a breakdown in the integrity of the alveolar-capillary barrier.

2.2.2. Pulmonary cell proliferation studies

Groups of particulate-exposed rats and corresponding controls were pulsed 24 h after instillation, as well as 1 week, 1 and 3 months postexposure, with an intraperitoneal injection of 100 mg/kg body weight of 5-bromo-2′-deoxyuridine (BrdU) dissolved in a 0.5N phosphate-buffered saline solution at a dose of 100 mg/kg body weight. The animals were euthanized 6 h later by pentobarbital injection. Following cessation of spontaneous respiration, the lungs were infused with a neutral-buffered formalin fixative at a pressure of 21 cm H_2O. After 15 min of fixation, the trachea was clamped, and the heart and lungs were carefully removed *en bloc* and immersion-fixed in formalin. In addition, a 1-cm piece of duodenum (which served as a positive labeling tissue control) was removed and stored in formaldehyde. Subsequently, parasagittal sections from the right cranial and caudal lobes and regions of the left lung lobes, as well as the duodenal sections were dehydrated in 70% ethanol and sectioned for histology. The sections were embedded in paraffin, cut, and mounted on glass slides. The slides were stained with an anti-BrdU antibody, with an AEC (3-amino-9-ethyl carbazole) marker, and counter-stained with aqueous hematoxylin. A minimum of 1000 cells/animal were counted each in terminal bronchiolar as well as alveolar regions. For each treatment group, immunostained nuclei in airways (i.e., terminal bronchiolar epithelial cells) or lung parenchyma (i.e., epithelial, interstitial cells or macrophages) were counted by light microscopy at 1000× magnification (Warheit et al., 1991, 1997).

2.2.3. Lung histopathology studies

The lungs of rats exposed to particulates or PBS controls were prepared for microscopy by airway infusion of formalin fixative under pressure (21 cm H_2O) at 24 h, 1 week, 1 and 3 months postexposure. Sagittal sections of the left and right lungs were made with a razor blade. Tissue blocks were dissected from left, right upper, and right lower regions of the lung and were subsequently prepared for light microscopy (paraffin embedded, sectioned, and hematoxylin-eosin stained) (Warheit et al., 1991, 1997).

2.2.4. Statistical analyses

For analyses, each of the experimental values were compared to their corresponding sham control values for each time point. A one-way analysis of variance (ANOVA) and Bartlett's test were calculated for each sampling time. When the F-test from ANOVA was significant, the Dunnett's test was used to compare means from the control group to each of the groups exposed to particulates. Significance versus PBS controls was judged at the 0.05 probability level.

3. Results

3.1. Physicochemical characterization of particles

The average particle size and size distributions in water and PBS, surface area measurements, crystal structures, pH in water and PBS, and chemical reactivity measurements for titanium dioxide test samples are presented in Fig. 1. The pH measurement for uf-3 shows it was much more acidic in deionized water than the other samples, but it was neutralized to approximately the same pH as the other samples in the phosphate-buffered saline solution.

The particle size distribution (PSD) results for all of the TiO_2 particle-types were highly agglomerated following dispersion in the phosphate-buffered saline solution – the vehicle utilized for the intratracheal instillation exposures – although the uf-1 sample showed a significantly smaller average particle size. The high degree of agglomeration in general arises from the proximity of the isoelectric point for all samples to the near-neutral pH of the buffer solution.

The PSD results in water (0.1% tetra sodium pyrophosphate, pH ∼9) provide an understanding of the size

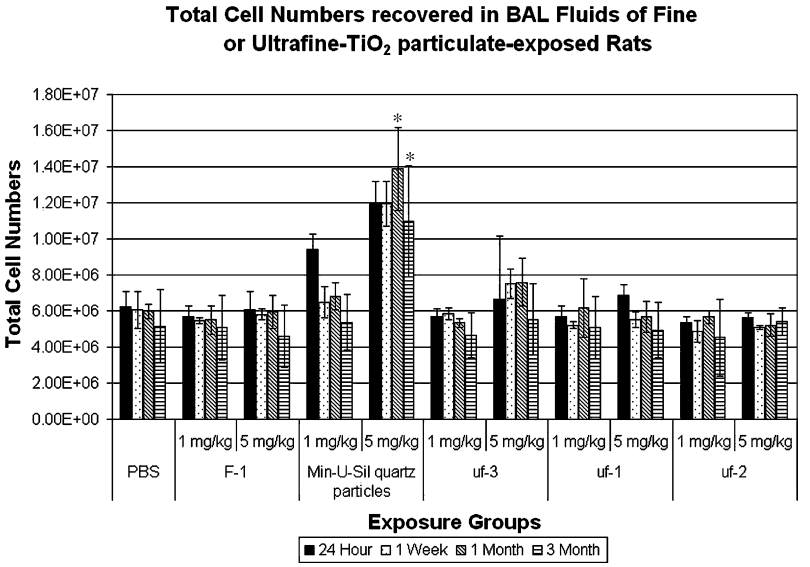


Fig. 2. Numbers of cells recovered in BAL fluids from particulate-exposed rats and corresponding controls at 24 h, 1 week, 1 and 3 months pe. Values given are means ± S.D. The numbers of cells recovered by bronchoalveolar lavage from the lungs of high dose quartz-exposed (5 mg/kg) groups were higher than any of the other groups for all postexposure time periods.

distribution for dispersed particle agglomerates. These results indicate a much larger d_{50} for the fine-TiO_2 (F-1) sample compared to the ultrafine-TiO_2 products. Further, some differences are evident in the PSD of the ultrafine-TiO_2 products, with uf-3 showing the finest distribution of the group. The three ultrafine-TiO_2 samples have surprisingly similar PSD results when dispersed in water, considering the primary particle size for uf-3 (∼25 nm) is much smaller compared to uf-1 and uf-2 (∼100 nm).

In addition, the uf-3 sample demonstrated the greatest chemical reactivity when compared to the other titanium dioxide samples. Sample F-1 gave very low chemical reactivity, due to relatively large primary and/or agglomerate PSD and alumina surface coating. Ultrafine-TiO_2 sample uf-2 was passivated with both silica and alumina coatings, as described above, to the extent that it nearly matched the low chemical reactivity of fine particle size TiO_2 F-1. Sample uf-1 was passivated to a lesser degree than uf-2, since uf-1 had only an alumina coating compared to the silica and alumina coating on uf-2.

3.2. Bronchoalveolar lavage (BAL) fluid results

3.2.1. Pulmonary inflammation

The numbers of cells recovered by bronchoalveolar lavage (BAL) from the lungs of high dose quartz-exposed (5 mg/kg) groups were significantly higher than any of the other groups for all postexposure time periods (Fig. 2). Intratracheal instillation exposures of several particle-types produced short-term, pulmonary inflammatory responses, as evidenced by increases in the percentages of BAL-recovered neutrophils, measured at 24 h postexposure (Fig. 3). However, exposures to quartz particles (1 and 5 mg/kg) resulted in sustained pulmonary inflammatory responses, as measured through 3 months postexposure (Figs. 2–3). In addition, high dose exposures to uf-3 TiO_2 particles produced sustained inflammation through 1 month postexposure and was still evident at 3 months postexposure (Fig. 3).

3.2.2. BAL fluid parameters

BAL fluid LDH activities were measured as indicators of cytotoxicity in the lungs of particle-exposed rats. No significant cytotoxic effects, relative to PBS controls, were measured in the lungs of rats exposed to F-1 fine-TiO_2, uf-1, or uf-2 ultrafine-TiO_2 particles at any time point post-instillation exposure. In contrast, exposures to 1 or 5 mg/kg quartz particles produced significant increases versus controls in BAL fluid LDH values through 1 week (1 mg/kg) and through all four postexposure time period (5 mg/kg) demonstrating a sustained cytotoxic effect on the lungs. Exposures to 5 mg/kg uf-3 TiO_2 particles produced significant increases in BAL fluid LDH values at 24 h and 1 month postexposure, indicating a longer lasting effect when compared to the other TiO_2 particle-types (Fig. 4).

BAL fluid microprotein (MTP) values were measured as indicators of enhanced cellular permeability in the lungs of particle-exposed rats. No significant increases

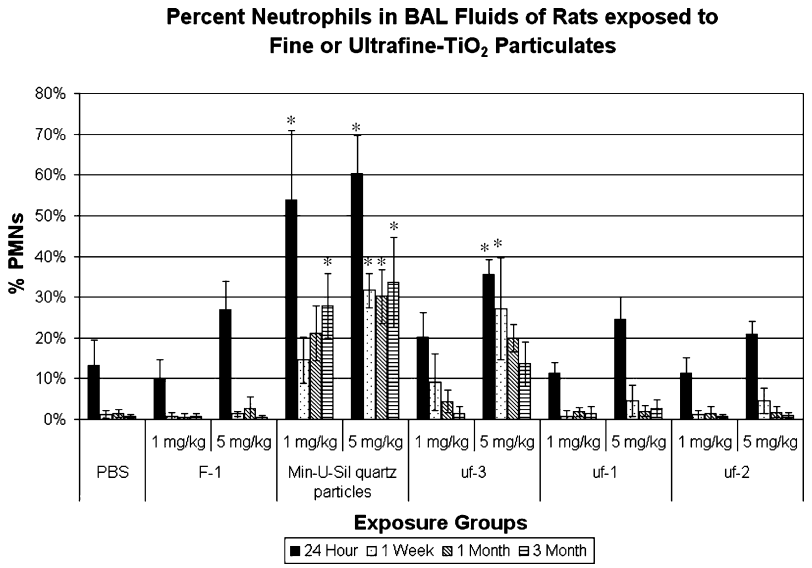


Fig. 3. Pulmonary inflammation in particulate-exposed rats and controls as evidenced by % neutrophils (PMN) in BAL fluids at 24 h, 1 week, 1 and 3 months postexposure (pe). Values given are means ± S.D. Intratracheal instillation exposures of several particle-types produced a short-term, pulmonary inflammatory response, as evidenced by an increase in the percentages/numbers of BAL-recovered neutrophils, measured at 24 h postexposure. However, the exposures to quartz particles (1 and 5 mg/kg) produced sustained pulmonary inflammatory responses, as measured through 3 months postexposure. $^{*}p < 0.05$ vs. PBS controls. Similarly high dose exposures to uf-3 particles produced significant inflammation through 1 week postexposure.

in BAL fluid MTP values relative to controls were measured in the lungs of rats exposed to F-1 fine-TiO_2, uf-1, or uf-2 ultrafine-TiO_2 particle-types at any postexposure time period. In contrast, exposures to 5 mg/kg quartz particles produced significant increases versus controls in BAL fluid MTP values at all postexposure time periods (i.e., 24 h, 1 week, 1 and 3 months) demonstrating a persistent effect on the alveolar/capillary membrane leading to leakage of proteins from the vasculature into the alveolar regions (Fig. 5). In addition, high dose exposures to

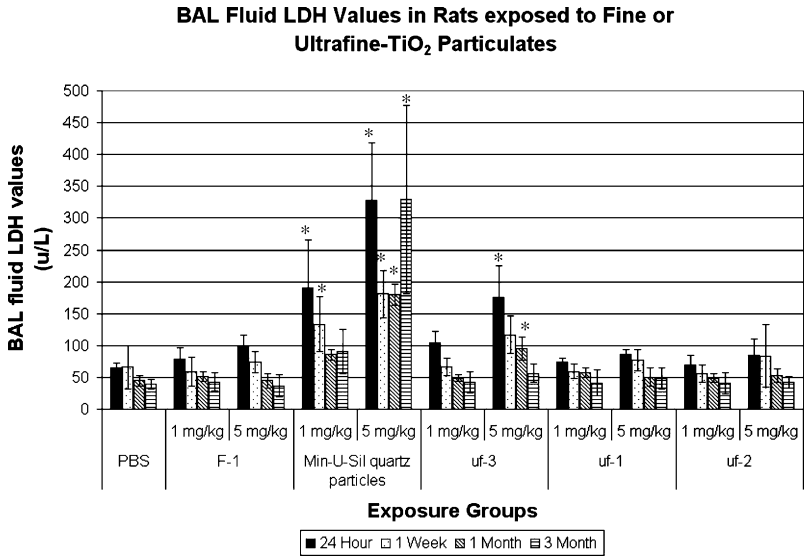


Fig. 4. BAL fluid LDH values for particulate-exposed rats and corresponding controls at 24 h, 1 week, 1 and 3 months postexposure (pe). Values given are means ± S.D. Increases in BAL fluid lactate dehydrogenase values were measured in the lungs of high dose (5 mg/kg) uf-3 at 24 h, 1 week, and 1 month postexposure. Exposures to 5 mg/kg quartz particles produced a sustained increase in BAL fluid LDH values through the 3-month postexposure period ($^{*}p < 0.05$ vs. PBS controls). Exposures to F-1, uf-1 or uf-2 did not produce any differences when compared to vehicle (PBS) controls.

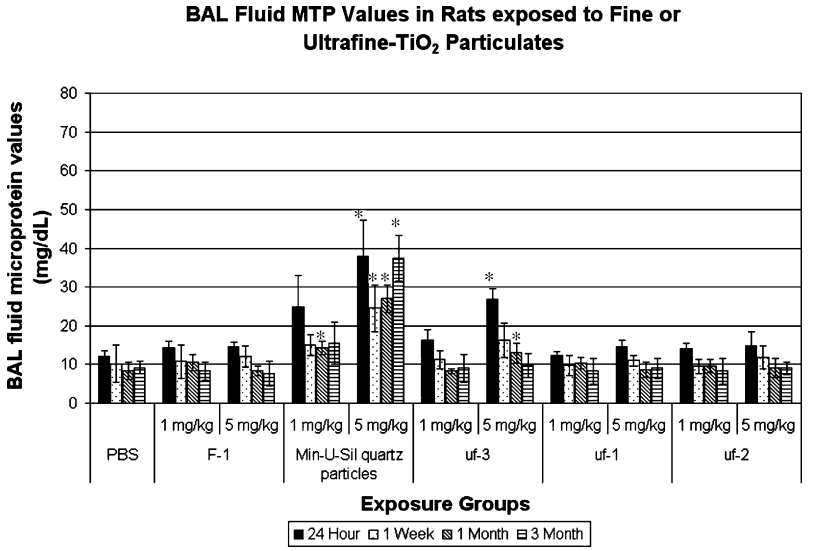


Fig. 5. BAL fluid protein (MTP) values for particulate-exposed rats and corresponding controls at 24 h, 1 week, 1 and 3 months postexposure. Values given are means ± S.D. Increases in BAL fluid microprotein values were measured in the lungs of high dose (5 mg/kg) uf-3-exposed rats at 24 h postexposure, but were not different from controls at 1 week postexposure. Exposures to 5 mg/kg quartz particles produced a sustained increase in BAL fluid microprotein values at 24 h, 1 week, 1 and 3 months postexposure ($^{*}p < 0.05$ vs. PBS controls). Exposures to F-1, uf-1 or uf-2 did not produce any differences when compared to vehicle (PBS) controls.

uf-3 TiO_2 particles produced significant increases versus PBS controls in BAL fluid MTP values at 24 h and 1 month postexposure, as a parallel effect to the measured BAL fluid LDH data (Figs. 4 and 5).

BAL fluid alkaline phosphatase (ALP) values were measured as indicators of alveolar epithelial Type 2 cell toxicity in the lungs of particle-exposed rats. No significant increases in BAL fluid alkaline phosphatase values were measured in any groups at any exposure time (data not shown).

The results from BAL fluid biomarker studies demonstrated that pulmonary exposures to quartz particles produced sustained, dose-dependent, lung inflammatory responses, concomitant with cytotoxic effects, measured from 24 h through 3 months postexposure. The ranking of pulmonary inflammation/cytotoxicity responses

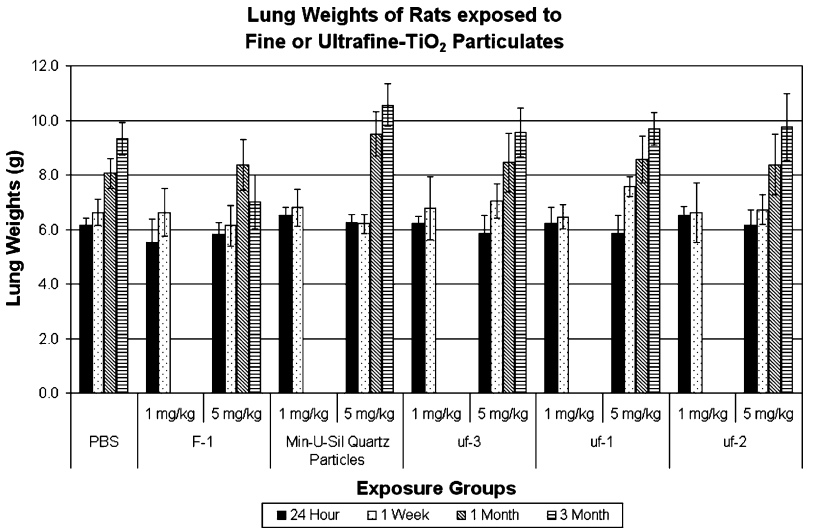


Fig. 6. Lung weights of particulate-exposed rats and corresponding controls at 24 h, 1 week, 1 and 3 months postexposure. Values given are means ± S.D. Increased lung weights were measured in quartz-exposed rats at 1 and 3 months postexposure. No significant effects were measured vs. PBS controls at any postexposure time points.

for the particles tested was the following: quartz > uf-3 > F-1 = uf-1 = uf-2. Uf-3 anatase/rutile TiO_2 particles produced significant pulmonary inflammation and cytotoxic effects lasting through 1 month postexposure.

4. Lung cell tissue studies

4.1. Lung weights

Lung weights of rats were enhanced with increasing age on the study (i.e., increased postexposure time periods following instillation) (Fig. 6). Lung weights in high dose quartz-exposed rats were slightly increased versus controls at 1 and 3 months postexposure (Fig. 6).

4.2. Cell proliferation results

Tracheobronchial cell proliferation rates (% immunostained cells taking up BrdU) were measured in low dose (1 mg/kg) exposed rats at 24 h and 1 week and in high dose (5 mg/kg), particulate-exposed rats and corresponding controls at 24 h, 1 week, and 1 and 3 months postexposure (pe). Increases in cell labeling indices compared to controls were noted in rats exposed to high dose uf-3 TiO_2 particles at 24 h postexposure and in high dose quartz-exposed animals at 24 h and 1 week pe, but these effects were not sustained. Exposures to particle-types F-1, uf-1 or uf-2 did not produce any significant cell labeling differences when compared to vehicle controls (Fig. 7).

Lung parenchymal cell proliferation rates (% cells immunostained for BrdU) were measured in high dose (5 mg/kg), particulate-exposed rats and corresponding controls at 24 h, 1 week, and 1 and 3 months pe. Significant increases in lung parenchymal cell proliferation indices were measured in rats exposed to high dose quartz particles at all times postexposure and increased labeling was measured in high dose uf-3-exposed rats at 24 h and 3 months postexposure. Exposures to particle-types F-1, uf-1 or uf-2 TiO_2 particles did not produce any significant differences when compared to vehicle controls (Fig. 8).

4.3. Histopathological evaluation

Histopathological evaluations of lung tissues revealed that pulmonary exposures to F-1 fine-TiO_2 particles, or to uf-1 or uf-2 in rats produced no significant adverse effects when compared to PBS-exposed controls (Fig. 9A–C). In this regard, evaluations of lung tissue responses in 4 rats/group per postexposure time point of high dose uf-1 or uf-2 TiO_2 particles represented normal pulmonary responses to low solubility particles and were similar to F-1-exposed lung tissue responses at each postexposure time period. Representative light micrographs of lung tissue sections of rats instilled with 5 mg/kg uf-1 (Fig. 9A), uf-2 ultrafine-TiO_2 (Fig. 9B) as well as F-1 fine-TiO_2 particles (Fig. 9C) at 3 months post-instillation exposure are presented and

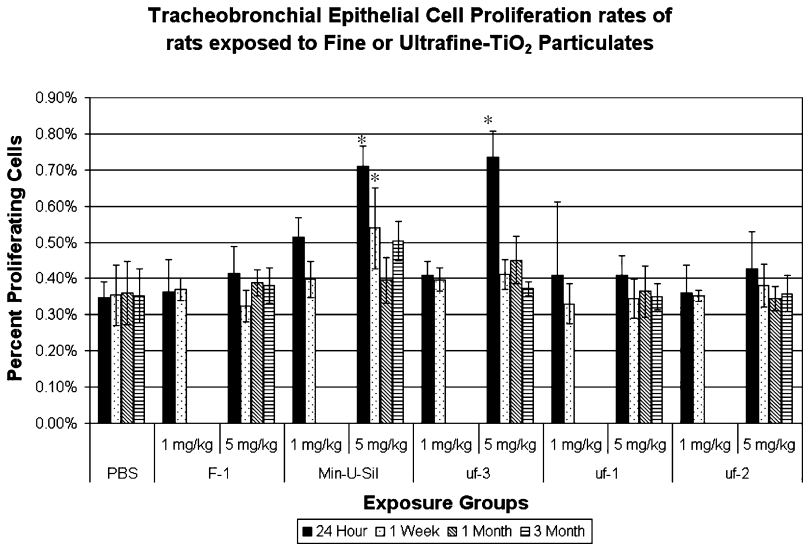


Fig. 7. Tracheobronchial cell proliferation rates (BrdU) in particulate-exposed rats and corresponding controls at 24 h, 1 week, 1 and 3 months postexposure (pe). Values given are means ± S.D. Cellular proliferation rates were measured in 1 mg/kg particulate-exposed rats only at 24 h and 1 week postexposure. Significant increases in airway cell proliferation indices were measured in high dose quartz-exposed rats at 24 h and 1 week postexposure and in high dose uf-3-exposed rats at 24 h pe. Exposures to F-1, uf-1 or uf-2 did not produce any differences when compared to vehicle (PBS) controls ($^{*}p < 0.05$ vs. PBS controls).

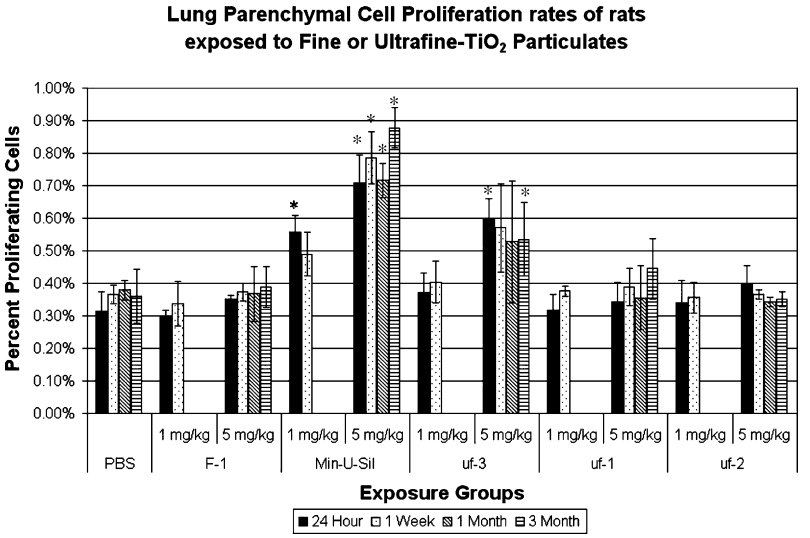


Fig. 8. Lung parenchymal cell proliferation rates (BrdU) in particulate-exposed rats and corresponding controls at 24 h, 1 week, 1 and 3 months postexposure (pe). Values given are means ± S.D. Cellular proliferation rates were measured in 1 mg/kg particulate-exposed rats only at 24 h and 1 week postexposure. Significant increases in lung parenchymal cell proliferation indices were measured in rats exposed to high dose quartz particles at all times postexposure and increased labeling was measured in high dose uf-3-exposed rats at 24 h and 3 months postexposure. Exposures to F-1, uf-1 or uf-2 did not produce any differences when compared to vehicle (PBS) controls ($^{*}p < 0.05$ vs. PBS controls).

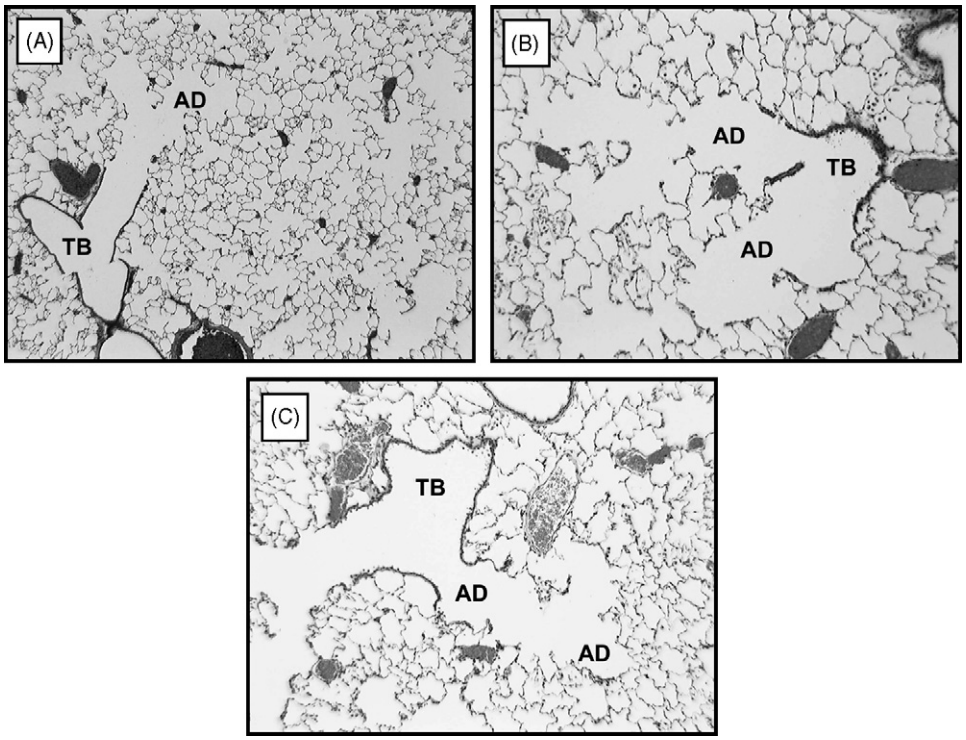


Fig. 9. Light micrographs of lung tissue of rats exposed to rutile-type (A) uf-1 and (B) uf-2 ultrafine-TiO_2 particles as well as (C) F-1 fine-TiO_2 particles (all at 5 mg/kg) at 3 months post-instillation exposure. The micrographs illustrate the terminal bronchiole (TB) and corresponding alveolar ducts (AD), and demonstrate normal lung architecture, indicating that exposures to F-1, uf-1 or uf-2 particles produced no adverse pulmonary effects (magnification = 40×).

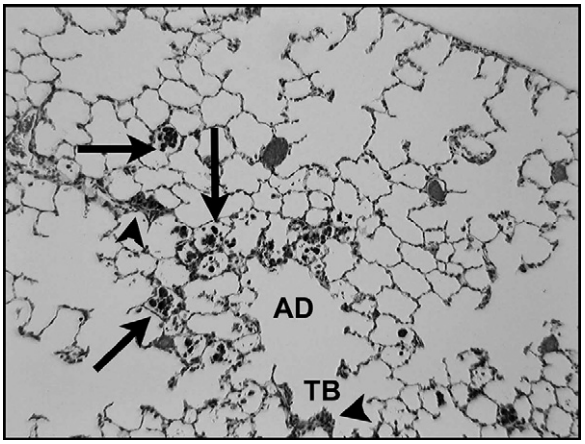


Fig. 10. Light micrograph of lung tissue of a rat exposed to anatase/rutile-type uf-3 TiO_2 particles (5 mg/kg) at 3 months post-instillation exposure. This micrograph illustrates the terminal bronchiole (TB) and corresponding alveolar duct (AD), and demonstrates accumulation of uf-3 containing macrophage aggregates (arrows) along with some occasional tissue thickening (arrowheads) (magnification = 100×).

demonstrate normal pulmonary architecture following particle exposures to low toxicity particulates.

Morphological assessments of rats exposed to uf-3 TiO_2 revealed a vigorous macrophage accumulation response to the instilled particles, concomitant with a sequestration of the aggregated macrophages within the alveolar regions of the lung. Subsequently, an occasional tissue thickening response was evident throughout lung sections evaluated at 3 months postexposure. The slight thickening was usually noted in association with uf-3 containing macrophage aggregates (Figs. 10 and 11).

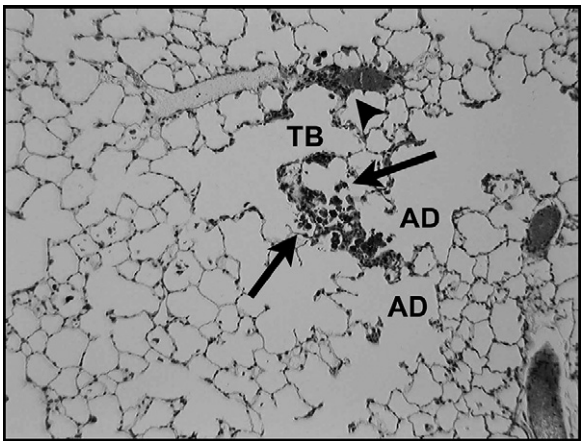


Fig. 11. Light micrograph of lung tissue of a rat exposed to anatase/rutile-type uf-3 TiO_2 particles (5 mg/kg) at 3 months post-instillation exposure. This micrograph illustrates the terminal bronchiole (TB) and corresponding alveolar ducts (AD), and demonstrates accumulation of uf-3 containing macrophage aggregates (arrows) along with some occasional tissue thickening (arrowhead) (magnification = 100×).

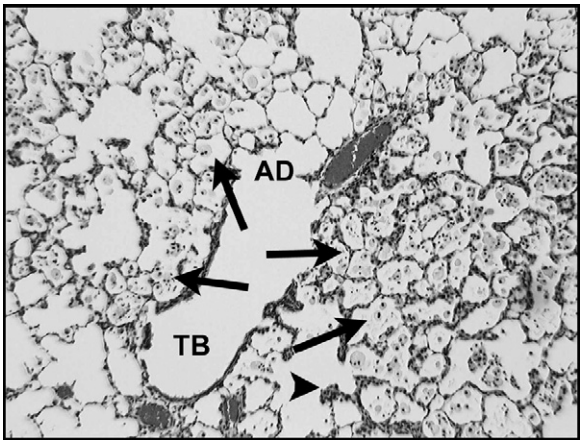


Fig. 12. Light micrograph of lung tissue from a rat exposed to quartz particles (5 mg/kg) at 3 months post-instillation exposure. This micrograph illustrates the terminal bronchial and corresponding alveolar ducts. Note the substantial accumulation of foamy quartz-containing alveolar macrophages which fill many of the alveoli (arrows) concomitant with the prominence of tissue thickening along the junctions at the terminal bronchiole and alveolar duct (arrowhead) (magnification = 100×).

The lung tissue responses to uf-3 TiO_2 particles clearly were different in degree and intensity from the persistent and progressive effects observed following exposures to quartz particles (Fig. 12).

Histopathological analyses of lung tissues revealed that pulmonary exposures to quartz particles in rats produced dose-dependent, persistent and progressive lung inflammatory responses characterized by inflammatory cells (primarily neutrophils and foamy (lipid-containing) alveolar macrophage accumulation). In addition, progressive septal lung tissue thickening responses as a prelude to the development of fibrosis were evident (Fig. 12).

5. Discussion

The objective of this study was to assess lung toxicity of intratracheally instilled, rutile-type ultrafine-TiO_2 particles (uf-1 and uf-2) versus reference particle-types in rats. Using pulmonary bioassay methodology, the pulmonary toxicity of instilled ultrafine uf-1 and uf-2 particles were compared with a positive control particle-type (α-quartz), a negative control particle-type (F-1 fine-TiO_2 particles), ultrafine uf-3 TiO_2 particle control, and vehicle controls (PBS).

Ultrafine-TiO_2 uf-1 or uf-2, or F-1 fine-TiO_2, did not produce sustained adverse pulmonary effects in any of the endpoints utilized in this study (i.e., BAL inflammatory indicators, cell proliferation, or histopathology). These passivated particle-types produced only transient

pulmonary inflammatory effects, i.e., measured at 24 h, but resolved by 1 week postexposure.

In contrast, quartz particles, particularly at the higher dose (5 mg/kg), produced significant adverse effects in pulmonary inflammation, cytotoxicity and lung parenchymal cell proliferation endpoints, all of which continued through the 3-month postexposure study period. Histopathological evaluation demonstrated that quartz particles produced pulmonary inflammation, foamy alveolar macrophages, and tissue thickening (as a prelude to the development of fibrosis). In addition, exposures to uf-3 TiO_2 particles produced intermediate pulmonary toxicity effects, i.e., producing inflammation and cytotoxicity responses through 1 month postexposure as well as enhanced cell proliferative labeling and histopathologically adverse lung tissue effects when compared to PBS vehicle controls.

The production of commercialized uf-3 does not appear to include an aqueous or wet surface treatment step to neutralize and remove the acidic chloride ions from the surface of the particles. Both uf-1 and uf-2, however, are subjected to this neutralization process. Further, wet treatment of these materials consisted of alumina or silica/alumina coatings to passivate the chemical and photo-chemical activity of the TiO_2. Uf-3 particles are produced pyrogenically via flame hydrolysis of titanium tetrachloride with hydrogen and oxygen and it is likely that residual chloride remains on the surface of the product. In deionized water, these particles are very acidic (pH 3.28), however, in the PBS buffer, the sample is neutralized to pH of 6.70. Interestingly, both uf-1 and uf-2 are substantially less acidic in deionized water (pH 5.64 and 7.14, respectively). Although PBS buffers the acidity of uf-3, it is conceivable that following instillation exposure/deposition in the lungs of rats, the acidic nature of the uf-3 particle-type could play an important role in the interaction with phagocytic or epithelial cells in alveolar regions. Therefore, the differences in lung responses between the uf-3 and the rutile ultrafine-TiO_2 particles (uf-1 and uf-2) in BAL fluid cytotoxicity and inflammation indices could be due, at least in part, to the neutralization and removal of chloride during the production of the ultrafine-TiO_2 particles uf-1 and uf-2.

In addition to differences in production, differences in chemical reactivity were measured among the various ultrafine-TiO_2 particle-types. In this study, chemical reactivity, an indicator of the particle's oxidation potential, was measured using the Vitamin C yellowing assay. Results indicated that the uf-3 particle sample showed substantially more chemical reactivity (higher Δb^*) than either of the uf-1 or uf-2 ultrafine-TiO_2 samples. Moreover, this assay represents an indirect measure of the number of active sites (sites on the particle's surface which can initiate the chemical transformation of molecules). Given this reactivity, uf-3 TiO_2 particles are likely to produce more reactive species than either of the uf-1 or uf-2 TiO_2 particle-types, thus possibly contributing to the sustained *in vivo* cytotoxicity and inflammation measured through 1 month postexposure. This is no surprise, given that uf-3 is among the most photochemically active of commercially available ultrafine-TiO_2 products (Ohno et al., 2001; Wahi et al., 2005; Canevali et al., 2006). The finding provides insight into the physiochemical differences, and by extension, the toxicological differences, between the ultrafine-TiO_2 samples tested.

Accordingly, based on the results of this study, the following hazard rank order of toxicity could be established (in descending order): Min-U-Sil quartz > uf-3 TiO_2 particles > F-1 fine-TiO_2 particles = uf-1 = uf-2 TiO_2 particles.

A variety of inhalation and instillation pulmonary toxicity studies have demonstrated that ultrafine particles produce increased inflammatory and particle translocation effects in rats when compared to fine-sized particulates of similar chemical composition at equivalent doses or mass concentrations (Ferin et al., 1992; Donaldson et al., 2001; Oberdorster et al., 2000, 2005b). Particle number and surface area indices appear to play important roles in the development of ultrafine particle lung toxicity (Donaldson et al., 2001; Oberdorster et al., 2000). A few inhalation toxicity studies have been reported which allow for comparisons of lung effects of ultrafine and fine titanium dioxide particles in rats at high particle concentrations (Lee et al., 1985; Heinrich et al., 1995; Bermudez et al., 2002, 2004). Analyses of these studies suggest that, on a mass basis, the ultrafine particle-types were 5–10× more potent than fine-TiO_2 particles, while on a surface area basis, the inflammatory effects were roughly equivalent. However, it should be noted that the crystal structures of fine (i.e., rutile) and ultrafine (80% anatase/20% rutile) TiO_2 particles in these studies were different, thus the size difference was not the only variable in these studies.

In a recently reported study, we tested the nanoparticle versus pigment (fine) size hypothesis of pulmonary toxicity, i.e., particle size and surface area strongly influence toxicity. Groups of rats were exposed via intratracheal instillation either to (1) fine-TiO_2 particles in the rutile phase, (2) nano-TiO_2 rods (100% anatase, ~200 nm × 40 nm), or (3) nano-TiO_2 dots (100% anatase, ~10–20 nm) at doses of 1 or 5 mg/kg body weight. The results demonstrated that following exposures in the lungs of rats, TiO_2 particle-types produced transient, and short-lived pulmonary inflam-

matory responses, with no corresponding adverse lung tissue effects. Moreover, no significant differences were measured in the inflammatory or cell injury responses among any of the groups (F-1 fine-TiO_2 particle-types, nano-TiO_2 dots or nano-TiO_2 rods) at any postexposure time periods. Thus, the findings of this study served to challenge the common perception that ultrafine- or nano-TiO_2 particle-types are more toxic when compared to pigment sized particles of similar chemical composition (Warheit et al., 2006).

Bermudez et al. (2004) assessed selected pulmonary responses of female rats, mice, and hamsters to inhaled uf-3 TiO_2 particles (80%/20% anatase/rutile), in order to determine if responses of the rat differed from those of two other rodent species, namely mice and hamsters. Accordingly, female rats, mice, and hamsters were exposed to aerosol concentrations of 0.5, 2.0, or 10 mg/m^3 uf-3 particles for 6 h per day and 5 days per week for 13 weeks. Animals were held for postexposure evaluation periods of 4, 13, 26 or 52 weeks (49 weeks for the uf-3-exposed hamsters). The responses studied were selected to assess a variety of pulmonary parameters, including inflammation, cytotoxicity, lung cell proliferation and histopathologic alterations. Mice and rats had similar retained lung burdens at the end of the exposures, when expressed as mg uf-3/mg dry lung, whereas hamsters had retained lung burdens that were significantly lower. 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 was evident in rats and mice exposed to 10 mg/m^3. Progressive epithelial and fibroproliferative changes were observed in rats of the 10 mg/m^3 group. 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 retention time postexposure. Epithelial, metaplastic, and fibroproliferative changes were not noted in either mice or hamsters.

To summarize these findings, there were significant species differences in the pulmonary responses to inhaled uf-3 particles. Under conditions wherein the lung uf-3 burdens were equivalent, rats developed a more severe inflammatory response than mice. A severe, persistent neutrophilic inflammatory response in the rat lung was believed to result in the development of 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-3 particles, whereas clearance in hamsters did not appear to be affected at any of the administered doses. These data were consistent with the results of an earlier study using inhaled pigmentary (fine) TiO_2 (Bermudez et al., 2002).

The results of previously reported inhalation toxicity studies with other low solubility particulates such as diesel exhaust, carbon black, or talc particles has indicated that rats, but not mice or hamsters develop lung tumors following chronic overload exposures (Heinrich et al., 1986; Hext, 1994; Mauderly et al., 1987, 1994; NTP, 1993; Muhle et al., 1998). The pulmonary sequelae in rats exposed to ultrafine particulate concentrations likely to induce pulmonary overload-related effects (i.e., chronic inflammation, fibrosis, fibroproliferative changes, epithelial hyperplasia and lung tumors) are different from the effects measured in similarly exposed mice and hamsters (Hext, 1994; ILSI, 2000; Bermudez et al., 2002, 2004). These differences can be explained both by pulmonary responses and by particle dosimetry differences between these rodent species.

Recently, Sayes et al. (2006) studied the catalytic properties of anatase and rutile nano-TiO_2 and correlated these properties to the *in vitro* cytotoxic responses of different nano-TiO_2 crystal structures on both human dermal fibroblasts and human lung carcinoma cells using various biochemical endpoints. These investigators concluded that nano-TiO_2 particles in the anatase crystal phase were superior catalysts, generators of reactive species, and more cytotoxic when compared to the rutile particle-types tested. These effects were due to differences inherent in the crystal structures of the two phases, and not because of differences in surface area or particle sizes.

The issue of primary particle size can be complex, particularly when considering the impact of agglomeration of small particles (Warheit et al., 2006). Previously we noted that in two interspecies comparison inhalation studies of fine or ultrafine-TiO_2 particles (uf-3), the pigment-grade TiO_2 particles had a primary particle size of $\sim$ 300 nm (Bermudez et al., 2002), while the uf-3 TiO_2 particles had an average primary particle size of 25 nm (Bermudez et al., 2004). Yet, the measured mass median aerodynamic diameters (MMAD) for the two TiO_2 particle-types were similar ($\sim$1.4 μm), indicating agglomeration of both pigment-grade as well as uf-3 TiO_2 particles in the aerosol chamber. Yet, despite the agglomeration of particles, the potency of the uf-3 to cause inflammation and cytotoxicity in the exposed rats was approximately 5$\times$ greater (on a mass basis) compared to the pulmonary effects of the inhaled pigment-grade TiO_2 particles. Given that the uf-3 TiO_2 particles have a measured surface area of $\sim$53 m^2/g,

while the pigment-grade TiO_2 particles have a surface area of ~5.8 m^2/g; these results are consistent with the hypothesis that when the inhaled doses are expressed in terms of surface area, the lung inflammation responses of these ultrafine and fine-TiO_2 particles fall on the same dose–response curve. However, when we now superimpose the results of the current study, which demonstrated that the rutile-type uf-1 and uf-2 particles with measured surface areas of 18.2 and 35.7 m^2/g, respectively, were *not* more inflammogenic than the F-1, pigment-grade TiO_2 particles (5.8 m^2/g); it further demonstrates that additional factors (beyond surface area) play an important role in the pulmonary effects related to particle exposures. Clearly, based upon the surface area hypothesis, one would expect the lung inflammatory effects of uf-1 and uf-2 to be intermediate between the responses of F-1 and uf-3 TiO_2 particle exposures.

The particle physicochemical characteristics illustrated in Fig. 1 were different when measured in water (0.1 wt% tetrasodium pyrophosphate) versus phosphate-buffered saline (PBS). First, although the primary particle sizes are significantly smaller in the uf-3 samples compared to the uf-1 or uf-2 samples, there is little or no difference in particle size distribution (PSD) when measured in 0.1% TSPP via dynamic light scattering. Even greater agglomeration (i.e., >2 μm) was measured in the PBS, the vehicle utilized in these studies, without a significant difference in sample particle size distribution (PSD). The degree to which engineered nanoparticles agglomerate in the ambient aerosol or in the intratracheal instillate and subsequently do or do not de-agglomerate following inhalation/instillation and particle deposition in the lung will strongly influence particle deposition rates and patterns for inhaled materials as well as interactions with lung cells for both the inhaled and instilled particle-types. If the ultrafine particles de-agglomerate upon interaction with alveolar lung fluids at sites of particle deposition (i.e., alveolar duct bifurcations), they could behave as discrete individual nanoparticles and may stimulate enhanced inflammatory cell recruitment. The particles could also preferentially translocate to more vulnerable anatomical compartments of the lung (e.g., interstitium). Alternatively, agglomerated nanoparticle-types could behave as fine-sized particles. However, without additional studies it is difficult to draw any specific conclusions regarding the potential for particles of uf-3 to de-agglomerate and translocate compared to that of uf-1 and uf-2.

In conclusion, the lung toxicities of two rutile-type ultrafine-TiO_2 particle samples, uf-1 and uf-2, were compared with α-quartz, an anatase/rutile ultrafine titanium dioxide (uf-3 TiO_2), and rutile fine-sized TiO_2 (F-1). Instillation exposures in rats to either uf-1, uf-2 or F-1 at doses of 5 mg/kg produced transient (i.e., 24 h pe) and reversible pulmonary inflammatory responses, which recovered at 1 week postexposure. The results of this study demonstrate that surface characteristics are an important determinant of toxicity. The responses to uf-1, uf-2 or F-1 TiO_2 particles were substantially less active in terms of inflammation, cytotoxicity, and fibrogenic effects when compared to the quartz, or to the uf-3 TiO_2 particles. Thus, based on the findings of this study, inhaled uf-1 and uf-2 particles are expected to have a low risk potential for producing adverse pulmonary health effects. It has been suggested that surface properties may account for the differences in pulmonary responses between uf-3 versus uf-1 and uf-2 ultrafine-TiO_2 particles, which relate to possible differences in particle chemical and photo-chemical reactivity. Accordingly, the toxicity of anatase/rutile uf-3 particles should not be viewed as representative of all ultrafine-TiO_2 particle-types and the NIOSH Current Intelligence Bulletin for Occupational Exposure to Titanium Dioxide (NIOSH, 2005) should discriminate between the various forms of ultrafine-TiO_2 particle-types, based on hazard data and physicochemical characteristics.

Acknowledgments

This study was supported by DuPont Titanium Technologies. Denise Hoban, Elizabeth Wilkinson and William L. Batton conducted the BAL fluid biomarker assessments. Carolyn Lloyd, Lisa Lewis, John Barr prepared lung tissue sections and conducted the BrdU cell proliferation staining methods. Don Hildabrandt provided animal resource care. We thank Drs. Brian Coleman, Gerald Kennedy Jr., Scott Loveless, Gary Whiting, and Austin Reid Jr. for helpful comments on this manuscript.

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