

Correlation between Particle Size, *In Vivo* Particle Persistence, and Lung Injury

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Dosimetry parameters such as deposition, clearance, retention, and translocation and dissolution of inhaled particles in and to different lung compartments may be important for the persistence of particles in the lung and may correlate with adverse pulmonary effects. We investigated such correlations using a model involving TiO₂ particles of two particle sizes (20 nm diameter, ultrafine; 250 nm diameter, fine) of the same crystalline structure (anatase). A 12-week inhalation experiment in rats resulted in a similar mass deposition of the two particle types in the lower respiratory tract. The ultrafine particles elicited a persistently high inflammatory reaction in the lungs of the animals compared to the larger-sized particles. In the postexposure period (up to 1 year) retention in the alveolar space per se was not different between fine and ultrafine TiO₂. However, the following differences between the particle types were noted: a significantly different total pulmonary retention, both quantitatively (significantly prolonged retention of the ultrafine TiO₂) and qualitatively (increased translocation to the pulmonary interstitium and persistence there of the ultrafine TiO₂); greater epithelial effects (Type II cell proliferation; occlusion of pores of Kohn) and the beginning of interstitial fibrotic foci with ultrafine TiO₂; significant sustained impairment of alveolar macrophage function after ultrafine TiO₂ exposure as measured by the clearance of test particles. A correlation between particle surface area and effects was observed. A comparison of the adverse reactions with dosimetric parameters of TiO₂ in different lung compartments in the postexposure period showed a correlation of the persistence of effects in both the alveolar and interstitial space with the persistence of particles in the respective compartment. — *Environ Health Perspect* 102(Suppl 5):173–179 (1994)

Key words: ultrafine particles, dosimetry, retention, lung clearance, particle surface area, inflammation

Introduction

When evaluating exposure–dose–effect relationships of inhaled particles the definition and determination of the relevant dose is crucial (Figure 1). The initially deposited dose may not be a decisive parameter, since particles probably clear at varying rates from different lung compartments. In contrast, the retained dose may be the more important parameter. Conventionally and conveniently, doses usually are expressed in terms of particle mass (gravimetric dose). However, when different types of particles are compared, doses may be more appropriately expressed as particle volume, particle surface area, or numbers of particles, depending on the effect in question. For example, the retardation of alveolar macrophage-mediated clearance due to particle overload appears to be better correlated with phagocytized particle volume rather than mass (*1*).

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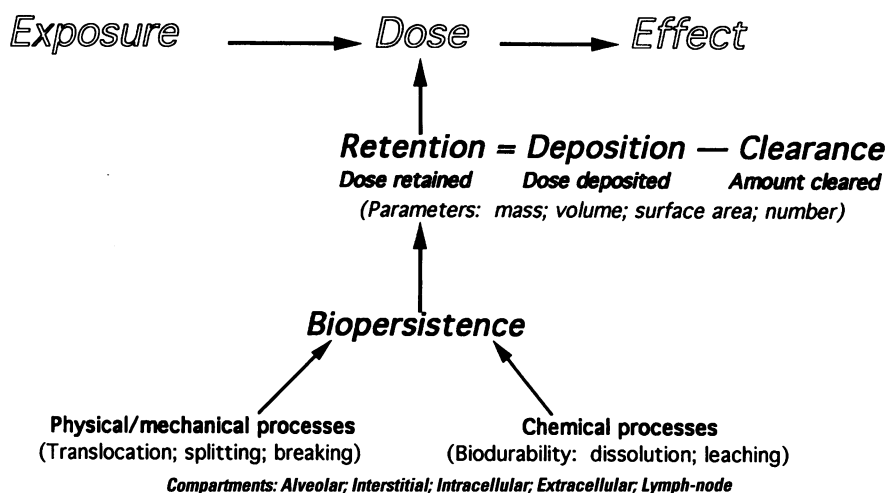


Figure 1. Biopersistence and biodurability in relation to dose parameters for exposure-dose-effect relationships of inhaled nonfibrous and fibrous particles.

The retained dose is a result of the biopersistence of a particulate compound, which is based on several clearance mechanisms. These can summarily be described by physical/mechanical and chemical processes. Although the terms retention, biopersistence, and biodurability often are used interchangeably, we suggest that “biopersistence” should refer to the *in vivo* behavior of a particle (fibrous or nonfibrous) and that “retention” should reflect a dosimetric term. The term “biodurability”

should be reserved for chemical processes occurring *in vivo* and contributing to biopersistence. Biopersistence mechanisms can be very different for different pulmonary compartments (e.g., alveolar vs interstitial, intracellular vs extracellular). The issue of biopersistence and the correlation between particle dose parameters and retention in individual lung compartments have been studied here by performing inhalation studies with TiO₂ particles of two sizes and evaluating resulting specific effects. Both

particle types were TiO₂ of submicronic particle size with the crystalline structure of anatase, one particle type with an average particle diameter of about 20 nm (ultrafine) and the other with an average diameter of about 250 nm (fine). Both are highly insoluble, so their biopersistence is mainly due to physical/mechanical processes. This study was based in part on our previous observation that the intratracheal instillation of ultrafine particles led to a significantly greater pulmonary inflammatory response than larger-sized particles (2,3).

Methods

Male Fischer 344 rats (bw 177 g ± 12 g) were exposed in whole body exposure chambers to either ultrafine TiO₂ (TiO₂-D) at a concentration of 23.5 ± 2.9 mg/m³ or fine TiO₂ (TiO₂-F), at 22.3 ± 4.2 mg/m³. The exposure was for 6 hr/day, 5 days/week for 12 weeks; there were 64 animals per group. Control animals were sham-exposed to filtered air. Upon aerosolization, both TiO₂ particle types formed agglomerates with mass median aerodynamic diameters of 0.71 μm (TiO₂-D) and 0.78 μm (TiO₂-F) and with geometric standard deviations of 1.9 for TiO₂-D and 1.7 for TiO₂-F. Since the aerodynamic diameters of the aerosols were essentially the same for the two particle types, the compartmental deposition in the respiratory tract of the animals was expected to be very similar. After deposition in the lung these particle agglomerates appear to disaggregate to smaller aggregates, as we and others (4) have observed by electron microscopy of lung sections. At 4, 8, and 12 weeks of the exposures, six animals per group were killed by an overdose of pentobarbital. Further animals were killed at 29 and 64 weeks after cessation of exposure.

The following measurements during and after exposure were performed: analysis of TiO₂ burdens in different compartments of the lung, i.e., lavagable versus nonlavagable particle burdens, and particle content in regional lymph nodes. An extensive lung lavage was performed, and lavagable cells were analyzed for macrophages, polymorphonuclear cells (PMNs), lymphocytes, and other types. Furthermore, lysosomal and cytosolic enzymes and total protein were determined in the lavage fluid, as additional parameters of toxicity. Light and electron microscopy were performed after respective preparation of fixed lung sections to evaluate epithelial and interstitial responses.

An important parameter of alveolar macrophage (AM) function, i.e., AM-mediated particle clearance, was deter-

Table 1. Lavagability of polystyrene particles from rat lungs by extensive lavage.

Day after administration	3.5-μm particles		10.3-μm particles	
	% lavagable	% nonlavagable	% lavagable	% nonlavagable
1	80	20	86	14
4	80	20	85	15
10	79	21	83	17
30	75	25	80	20
62	75	25	80	20
132	77	23	78	22
202	76	24	76	24
With inflammation (20–40% PMN)				
1	81	19		
1	78	22		
1	76	24		

Radioactively labeled polystyrene particles of two sizes were administered by intratracheal instillation to differentiate between alveolar lung burden, interstitial lung burden, and total lung burden. Interstitial dose = total lung burden – alveolar burden – lymph node burden. (Particle dose 20–100 μg). On average, 23% of alveolar burden was nonlavagable after day 30. (Alveolar burden = 1.3 × lavagable amount). This number was not influenced by inflammation induced by coinstilling ultrafine TiO₂ together with the polystyrene particles.

mined by measuring *in vivo* the retention kinetics of ⁸⁵Sr-labeled polystyrene test particles (3M Company) following inhalation via tracheal tubes. For this purpose, four rats of each group were anesthetized with halothane after 12 weeks of exposure (end of exposure) and another four rats per group after 7 months, and connected to a respirator system by intratracheal tubes (inserted via the mouth), which allowed groups of up to 16 rats to be exposed to the test particles simultaneously without any external fur contamination. An aerosol of the labeled particles was inhaled over a period of 15 min. The retained activity of ⁸⁵Sr was determined on subsequent days

(up to 200 days postexposure) by a double detector system collimated to the lung region of the rats. Short- and long-term effective clearance rates were determined from the data and the respective biological retention halftimes were calculated.

Results of these measurements were correlated with specific doses of the retained particles in individual lung compartments. To assess the burden of particles in the alveolar space versus the interstitium, the excised lungs of the animals were lavaged extensively 10 times with a volume of 5.0 ml saline per wash cycle. This yielded an average of approximately 1.1 to 1.2 × 10⁷ total cells in the lung

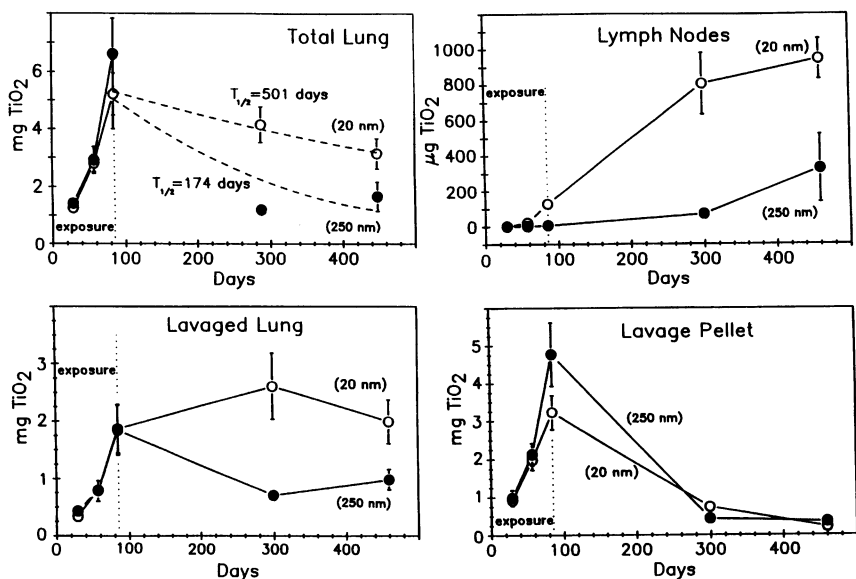


Figure 2. TiO₂ burdens in different lung compartments during and after 12 weeks of exposure of rats to fine and ultrafine TiO₂ particles (mean ± SD; n = 4).

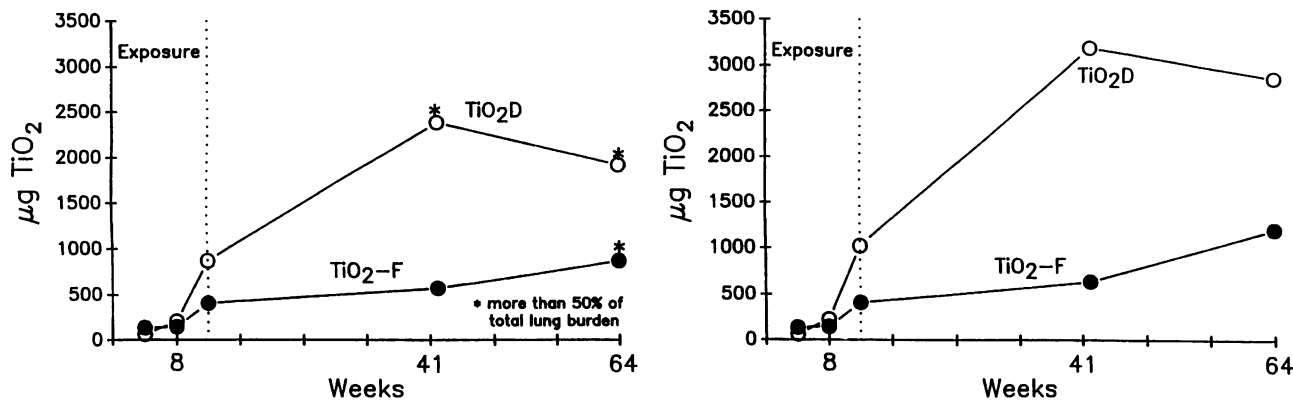


Figure 3. Estimated interstitial burden (left) and interstitial plus regional lymph node burden (right) during and after exposure of rats to ultrafine (TiO₂-D) and fine (TiO₂-F) particles.

washes. However, even an “extensive” lavage cannot completely deplete the alveolar space of macrophages and particles, and it cannot be assumed that the lavagable portion is equivalent to the retained dose in the alveolar space. To determine the alveolar as well as the interstitial dose more accurately, a pilot study was performed with instillation of very large (10.3 µm) and smaller (3.5 µm) polystyrene test particles labeled with two radioactive tracers (¹⁴¹Ce, ⁹⁵Nb). Groups of animals were lavaged during the subsequent 202 days and the lavagable and nonlavagable portions of the radioactively labeled test particles were determined (5). It was assumed that the larger particles would not be transferred to the interstitium and that, accordingly, the sum of lavagable and nonlavagable fractions would represent the burden in the alveolar space. Histological evaluations confirmed that none of the large test particles were in epithelial cells or the interstitium, whereas a very few of the smaller (3.5 µm) particles could occasionally be seen at those sites. On average, beyond day 30 after instillation, 22.9 ± 2.0 percent of the total retained lung burden was not lavagable (Table 1). Thus, the alveolar burden can be estimated from the lavagable fraction by multiplying this fraction by 1.3. Furthermore, the interstitial dose of other retained particulate material can be calculated by subtracting the alveolar burden and the lymph nodal burden from the total lung burden.*

To demonstrate that these very different polystyrene particles are an accurate model of TiO₂ interaction with AM, 500 µg TiO₂-F were coinstituted with the poly-

styrene particles; we found that, both shortly after instillation and 24 hr later, there was no difference in the lavagable and nonlavagable fraction of TiO₂ and polystyrene particles. Furthermore, since a high inflammatory response expected with the ultrafine TiO₂ might affect the lavagability of the lung, we also determined the lavagability of radioactively labeled test particles in the presence of such inflammation by coinstituting 500 and 1000 µg ultrafine TiO₂ with radioactive polystyrene particles. The nonlavagable fraction of the polystyrene particles was essentially the same as before, i.e., approximately 22%, demonstrating that the inflammatory response did not influence alveolar lavagability. In contrast, a larger fraction of the ultrafine TiO₂, compared to the polystyrene particles, was nonlavagable, indicating some translocation of the ultrafine TiO₂ to epithelial and interstitial sites. We conclude from this pilot study that for subsequent determinations of the alveolar and interstitial burdens of particles, a nonlavagable fraction of 23% (using our lavage technique) of the alveolar burden in rat lungs should be assumed (Table 1).

Results

Dosimetry

The total retained lung burden at the end of the 12-week exposure was 6.62 ± 1.22 mg for TiO₂-F and 5.22 ± 0.75 mg for TiO₂-D (Figure 2). Assuming first-order clearance kinetics, retention halftimes for the ultrafine particles were approximately 500 days and for the larger-sized particles approximately 170 days postexposure. The lavagable amount (lavage pellet) postexposure was generally similar in both particle groups, indicating no large differences in the overall clearance of the different particle sizes from the alveolar compartment.

However, large differences were observed in the fraction remaining in the lung. The ultrafine TiO₂ particles showed a significantly greater fraction being retained compared to the fine TiO₂ particles. A significantly larger fraction of the ultrafine particles was transferred to the regional lymph nodes, as compared with fine particles, indicating a greater ability of the ultrafine particles to enter interstitial spaces after alveolar deposition (Figure 2).

The interstitial dose of the retained TiO₂ particles was determined (see Methods). More than 50% of the total retained lung burden of the ultrafine particles could be attributed to interstitial localization at the two postexposure time points studied (Figure 3). For the fine particles, this was also the case at the latest time point examined. The interstitial dose decreased somewhat beyond week 41 for the ultrafine particles, whereas it continued to increase for the fine particles. This may indicate a faster clearance rate of the ultrafine particles from interstitial sites as opposed to the fine particles, perhaps, via the bronchus-associated lymphatic tissue (BALT) onto the mucociliary escalator of the conducting airways (6). This, however, needs to be investigated further.

The clearance of both TiO₂ particle types from the alveolar space showed similar kinetics as indicated in Figure 2 (lavage pellet). A more complete analysis of the data is given in Table 2 by comparing the amounts of TiO₂ in the alveolar and interstitial spaces 1 year after the end of exposure. In spite of the prolonged pulmonary retention of ultrafine TiO₂ (Figure 2), the overall clearance of both particle types from the alveolar space is virtually identical. Within the 1-year period postexposure, 93% of either particle type present at the end of exposure in the alveolar space was removed from this compartment.

*The term interstitial dose should not imply that all particles of this compartment are in the pulmonary interstitium; some also may be in epithelial cells. Regardless, they are no longer in the alveolar space per se.

Table 2. Clearance of TiO₂ particles from alveolar space during 1 year after cessation of 12-week exposure.

Time after exposure days	Alveolar space			Interstitial space (incl. LN)			Clearance to other sites (GI) % (of alveolar)
	µg	Clearance		µg	Input		
		µg	%		µg	% (of Alveolar)	
0	4192			1023			
TiO ₂ -D		3915	93		1841	44	50
365	277			2864			
0	6202			412			
TiO ₂ -F		5743	93		788	13	80
365	459			1200			

LN, lymph nodes.

However, clearance pathways for the two TiO₂ particles evidently differed markedly. A large fraction (44%) of alveolar burden of the ultrafine particles appeared in the interstitial space, compared to only 13% for the fine particles. Most of the fine particles presumably cleared to the GI tract, although fecal excretion of TiO₂ particles was not specifically evaluated in the present study. Clearance to the GI tract was much less for the ultrafine particles, indicating prolonged clearance of these particles from

the alveolar space via AM and up the mucociliary escalator.

Inflammatory Response

There was a progressive increase in total cells lavaged from the TiO₂-D exposed animals during the 12-week exposure period (Figure 4), due to an increase of both AM and PMNs. The inflammatory response to the TiO₂-D persisted for a long period after exposure. The numbers of these cell types had returned almost to normal levels 1 year after exposure. These effects were not (or

only modestly) observed with TiO₂-F. The lavagable protein (Figure 4), (as well as cytoplasmic and lysosomal enzymes—LDH and β-glucuronidase—data not shown), was also significantly elevated in the lavage fluid from animals exposed to the ultrafine particles during and at the end of the exposure and at week 41 after the start of exposure. Taken together, these data show the greater pulmonary inflammatory potency of inhaled ultrafine TiO₂ particles compared to fine TiO₂ particles, when lung doses are expressed in terms of particle mass (equal gravimetric doses). This confirms earlier results obtained with intratracheally instilled ultrafine and fine TiO₂ particles (3).

However, when the inflammatory influx of PMNs was correlated with the surface area of the retained particles (i.e., lavagable particles) both ultrafine and fine particle effects could be described by a common dose-effect curve. This underlines the importance of considering different dose parameters (e.g., mass vs surface area) when interpreting particle effects. These results also confirmed earlier findings with instilled particles (3).

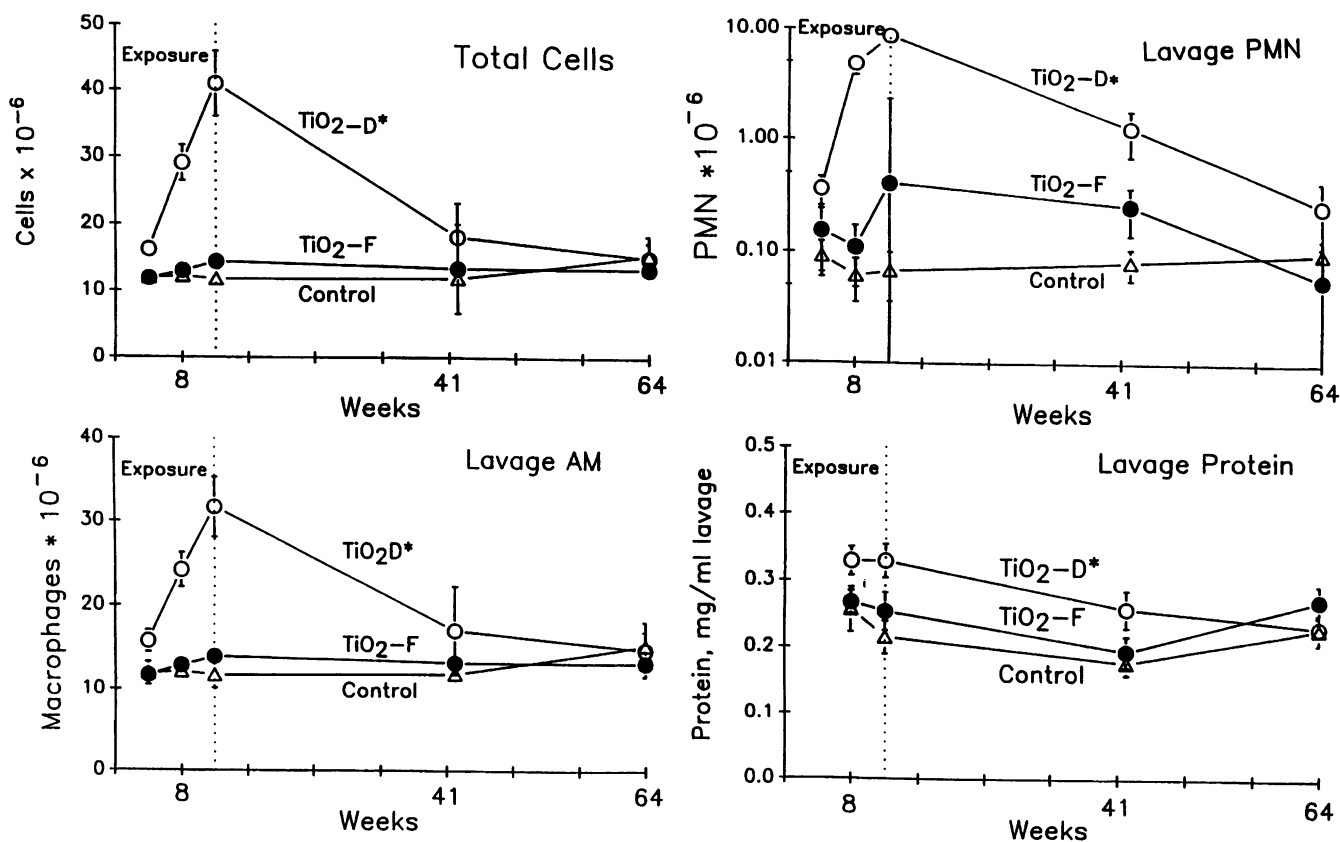


Figure 4. Lung lavage parameters during and after 12 weeks of exposure of rats to TiO₂-D (ultrafine) and TiO₂-F (fine) particles compared to sham exposed controls (mean ± SD; n=8).

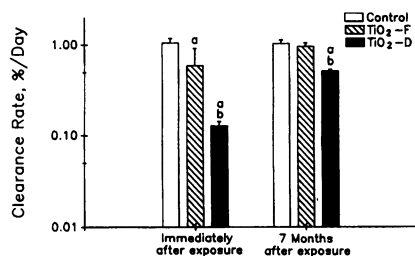


Figure 5. Pulmonary clearance rates of ^{85}Sr labeled test particles inhaled either immediately after 12 weeks of exposure to TiO_2 or 7 months later. (Mean and 95% confidence interval; a = significantly different from control; b = significantly different from $\text{TiO}_2\text{-F}$).

Alveolar Macrophage Clearance Function

Subchronic inhalation of ultrafine and fine TiO_2 particles resulted in a significant impairment of AM-mediated test particle clearance (Figure 5). Control animals had a clearance rate of the test particles of approximately 1% per day, for the animals exposed to the fine particles, the clearance rate was reduced to 0.6% per day; and for those exposed to the ultrafine particles, it was even further reduced, to 0.13% per day. Retention half-times were 66 days for the control animals, 117 days for those exposed to fine particles, and 541 days for those exposed to ultrafine particles. Seven months after exposure, when the TiO_2 burdens in the lung had decreased (Figure 2) the test particle clearance for animals exposed to fine particles was no longer impaired, whereas the animals exposed to ultrafine particles still showed a significantly diminished clearance rate of 0.52%.

Since prolonged AM clearance function in a particle overload situation may be correlated with the volumetric burden of the phagocytized particles in the AM (1), different dose parameters of the TiO_2 particles retained in the AM were correlated with the impaired particle clearance. Neither average gravimetric nor average volumetric burdens of both particle types correlate well with the observed effect on AM clearance function (Table 3). With fine TiO_2 particles, there was an AM volumetric load of 9%, greater than the 6% that Morrow hypothesized (1) to indicate a particle overload condition with subsequent effect on particle clearance; indeed, prolonged clearance was seen in the present study. Exposure to ultrafine particles, however, resulted in much lower average volumetric burdens, which possibly indicated an increased cytotoxicity of these particles. Expressing these doses as the retained particle surface area in the macrophages

shows that the effects on AM-mediated clearance function of the two different particle types can be expressed by a common dose-response curve (Figure 6). (Calculation of the phagocytized volume is based on an average rat AM volume of $1000 \mu\text{m}^3$ [7] and of a TiO_2 anatase density of 3.8 g/cm^3 . The specific surface area had been determined previously to be $50 \text{ m}^2/\text{g}$ for ultrafine TiO_2 and $6.4 \text{ m}^2/\text{g}$ for fine TiO_2).

Lung Morphology

Histological evaluation of lung sections at 41 weeks of the study showed early fibrotic reactions in the ultrafine TiO_2 -exposed animals and to a lesser degree also in the animals exposed to fine TiO_2 . However, 1 year after the exposure the early fibrotic reactions had shown signs of regression and were no longer as prominent (8). Type II cell hyperplasia, especially in alveoli that contained aggregates of particle-laden AM, was also observed in the animals exposed to ultrafine particles. The observation that such epithelial response resulted in the occlusion of the pores of Kohn may have important implications, since these pores are thought to serve as interalveolar migration pathways for AM (9-11). Interstitial inflammatory reactions, (i.e., PMN increases), were also observed to a higher degree at the end of the exposure period in the animals exposed to the ultrafine particles.

Discussion

This subchronic inhalation study with two sizes of TiO_2 particles confirmed the previous findings with these particles after intratracheal instillation. When the same gravimetric doses of ultrafine and fine TiO_2 particles were delivered to the lung, ultrafine particles produced significantly greater inflammation and interstitial translocation (2,3). The study also shows that the pulmonary clearance of the ultrafine particles was significantly slower, and

that this was due to an altered biopersistence in both the alveolar space and in the pulmonary interstitium. The overall retention in the alveolar space was no different for fine and ultrafine TiO_2 particles. However, compared to the fine TiO_2 , a larger fraction of the ultrafine TiO_2 was translocated to the pulmonary interstitium where it was retained for a longer time. Dissolution of TiO_2 particles in lung tissue has not been reported, and we can assume that pulmonary clearance of these particles is solely dependent on physical/mechanical processes (Figure 1).

Several inhalation studies by different laboratories in the past have shown that a large load of highly insoluble particles in the alveolar space results in severe retardation of AM-mediated particle clearance (12,13). This phenomenon was referred to as "particle overload," indicating an overloading of the AM by phagocytized particles with subsequent impairment of their clearance function. Morrow (1) suggested that this is correlated with the phagocytized particle volume and that AM function starts to be impaired when on average 6% of the normal AM volume is filled by phagocytized particles. The load of AM with fine TiO_2 particles in our study did indeed reach the value of 9% (Table 3), which was associated with an almost doubling of the retention of inhaled test particles. However, ultrafine particles only reached a phagocytized volume in the AM of 2.6%, yet they prolonged retention of the test particles by a factor of more than 4 (Table 3). Even after 7 months, test particle clearance continued to be retarded. In contrast, correlating the prolonged retention with the retained surface area of the particles in AM showed that the data could be expressed with a dose-effect relationship common to both particle types (Figure 6), independent of postexposure time.

Several points should be emphasized. One is that the overall retention of both the fine and ultrafine particles did not dif-

Table 3. Particle dose parameters and effects on AM-mediated particle clearance.

	Time postexposure, weeks	Retained dose/ 10^6 AM			AM-effect	
		μg	nl (% of AM volume)	cm^2	Particle no. $\times 10^9$	Test particle retention, control = 1
Control	0, 29	0	0 (0)	0	0	1.0
$\text{TiO}_2\text{-F}$	0	340	90 (9)	21.9	10.9	1.8 ^a
	29	32.5	9 (0.9)	2.1	1.1	1.1
$\text{TiO}_2\text{-D}$	0	99.8	26 (2.6)	49.9	5420	8.2 ^a
	29	43.8	11 (1.1)	21.9	2380	2.0 ^a

AM = alveolar macrophages. ^aSignificant change against controls $p < 0.05$.

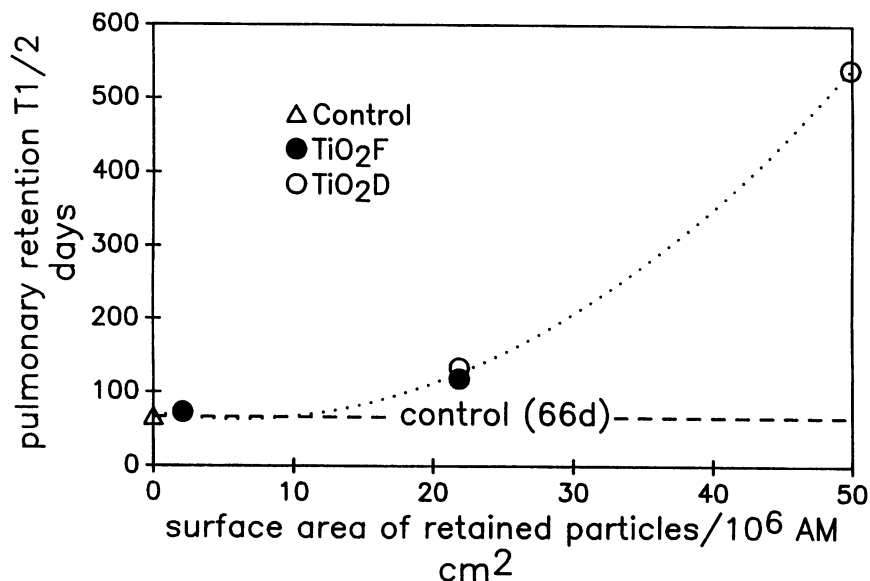


Figure 6. Correlation between surface area of TiO₂ particles phagocytized by AM and pulmonary retention half-time of inhaled polystyrene test particles.

fer in the alveolar space (Table 1), although the ultrafine particles were cleared via the mucociliary escalator into the GI tract at a significantly slower rate. This can be explained by the effect on AM function as demonstrated by the result of the test particle clearance. A second point is that the retention in the alveolar space—more precisely the dose retained in AM expressed as particle surface area—rather than the overall pulmonary retention of the TiO₂ particles, is important for the effect on AM clearance function. Third, the volumetric load of the AM is not a good predictor for the delayed clearance effect when ultrafine particles are involved. The surface area of the retained particles appears to be a better dose determinant. Although our data are limited at this point (four data pairs only, Figure 6), other studies have also demonstrated the importance of the dose parameter “surface area” for the adverse biological effects of both fibrous and spherical particles (14,15). This does not mean that AM volume load may not be important for AM clearance function, in particular for larger particles, as has been shown repeatedly (5,12,16). However, surface area may become a significant factor when particle size gets smaller, apparently gaining greater importance as dose parameter for particles in the submicronic range.

The mechanism(s) underlying the retarded removal of AM-phagocytized par-

ticles from TiO₂-D exposed lungs remain(s) to be elucidated. Green (9) and Ferin (10) speculated that the pores of Kohn may serve as a shortcut by which AM migrate from distal alveoli to more proximal airways, for subsequent transport up the conducting airways. It is therefore tempting to speculate that the prolonged AM-mediated particle clearance observed with the ultrafine particles was related to the Type II cell hyperplastic response and occlusions of the pores of Kohn in affected alveoli. These alveoli often contained large aggregates of particle-filled AM, which, in turn, appeared subjectively to contain the vast bulk of the TiO₂ present in the alveolar space compartment.

The dose parameter “surface area” of the particles also correlates better than other dose parameters with the inflammatory PMN influx into the alveolar space. This corroborates the earlier results with instilled particles of several types including ultrafine and fine TiO₂ rutile and anatase, and with ultrafine carbon black (3). In the present and earlier studies, the surface area of the particles retained in the alveolar space represented a better dose determinant than surface area of the particles retained in the lung *in toto*, which underscores the significance of determining particulate dose levels in individual lung compartments.

However, there may also be “interference” between lung compartments via released cell mediators affecting specific responses, depending on the particle load in each compartment. For example, the acute particle-induced inflammatory influx of PMN into the alveolar space was significantly reduced, when more than 50% of an acutely administered high dose of ultrafine TiO₂ particles was rapidly translocated to the interstitial space, thereby shifting inflammatory events (e.g., release of chemotactic factors) to the interstitium. This reduced PMN influx was not observed with ultrafine carbon black particles, which were not rapidly translocated to the interstitium (3).

The observed early fibrotic reactions in this study were more pronounced after TiO₂-D exposure than after TiO₂-F. This may also correlate with the larger interstitial dose of the ultrafine particles, although it is conceivable that the particle burden in the alveolar space also contributed to these events. It has been clearly demonstrated that particles, including TiO₂, will activate AM to release both proinflammatory cytokines and fibrogenic factors (17,18). In the present study it was observed that fibroblast growth factors are released in the alveolar space, partly due to release by AM (data not shown). Since we could not assess the fibrogenic activity occurring in the interstitial space, we cannot differentiate between the contribution of alveolar and interstitial burdens to the fibrogenic response. However, histological evidence from other studies points to the interstitial particle load as being most important (19).

We conclude from these studies that the greater pulmonary effects of ultrafine particles, compared to larger submicronic particles, can be explained by their larger specific surface area, the greater interstitial access, and their altered biopersistence, resulting in the increased retention of ultrafine particles. Pulmonary compartmental doses thus should be considered when evaluating effects (alveolar versus interstitial versus total dose), and the intrapulmonary kinetics and translocation of particles should be evaluated. We conclude further that when evaluating the biopersistence and related dose-effect relationships of inhaled particles of largely different sizes, the particle surface area rather than the mass of the retained particles appears to be the most relevant dose parameter.

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