Inhalation Toxicology, 20:53–62, 2008

ISSN: 0895-8378 print / 1091-7691 online
DOI: 10.1080/08958370701758742

informa
healthcare

Concordance Between *In Vitro* and *In Vivo* Dosimetry in the Proinflammatory Effects of Low-Toxicity, Low-Solubility Particles: The Key Role of the Proximal Alveolar Region

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We previously demonstrated the importance of the surface area burden as the key dose metric in the elicitation of inflammation in rat lungs by low-solubility, low-toxicity particles (LSLTP). We have now explored the dosimetry of LSLTP *in vitro* using epithelial cell interleukin (IL)-8 gene expression as a surrogate for potential of particles to cause inflammation. The proximal alveolar region (PAR) of the lung has been identified as a key site for the retention of respirable particles, as it receives high deposition but has slow clearance compared to the larger airways. For these reasons, a few days after exposure to particles the residual dose is concentrated in the PAR region. Re-expressing our rat lung data as particle surface area burden per unit of PAR surface area we obtained a threshold value for onset of inflammation of 1 cm^2/cm^2. We carried out dose responses *in vitro* for onset of IL-8 gene expression with the same particles as we had used *in vivo*. When we expressed the *in vitro* dose as surface area dose per unit A549 cell culture surface area, we obtained a threshold of 1 cm^2/cm^2. This concordance between proinflammatory effects *in vivo* (PMN in BAL) and *in vitro* (epithelial IL-8 gene expression) confirms and supports the utility of the particle surface area metric and the importance of the PAR. These studies also open the way for future *in vitro* approaches to studying proinflammatory effects of a range of toxic particles based on sound dosimetry that complemenst animal use in particle toxicology.

Received 13 June 2007; accepted 7 September 2007.

K. E. Pinkerton acknowledges Dr. Bob Mercer (NIOSH) as an important influence in PAR and ventilatory unit reconstructions in his laboratory, including the original reconstructions presented in this article. The component of this work from K. E. Pinkerton was supported in part by U.S. EPA grants R829215 and R832414 and NIOSH grant OH07550. K. Donaldson acknowledges the support of the Colt Foundation. G. Oberdorster acknowledges support from NIEHS grant ESO 1247.

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Low-toxicity, low-solubility particles (LTLSP) including TiO_2, carbon black, and diesel soot have been extensively studied as to their effects in rat lungs. At high exposure they produce rat lung overload, a condition characterised by slowed clearance, accumulation of lung burden, inflammation, proliferation, fibrosis, and cancer (ILSI Workshop, 2000). LTLSP have not been extensively studied *in vitro*, and in fact LTLSP, like TiO_2, are more often used as controls for comparison with toxic particles in *in vitro* studies.

We previously demonstrated that surface area drives the initiation of overload inflammation and retention for two very different LTLSP (Tran et al., 2000). In seeking to carry out a study on *in vitro* effects of LTLSP we considered dosimetry, and this forms the basis of the present report. Target sites of particle deposition in the lungs under ambient exposure conditions include bifurcations along the conducting airway (Balashazy et al., 2003) and the centriacinar region or proximal alveolar region (PAR) (Chang et al., 1992), and these can serve as important drivers of effect. These include localized focal effects at sites of particle deposition with subsequent inflammatory effects and tissue changes in the lungs, but also translocation from these sites to other target tissues and organs.

Inhaled particles, depending on their size, deposit in the upper airways, the tracheobronchial region, or the terminal bronchiolar/alveolar region of the lung (Miller, 2000). If they deposit in the upper airways or the ciliated airways of the bronchi then clearance of deposited particles is relatively rapid, with most of the particles being removed within 24 h (Schlesinger et al., 1997). If the particles deposit in the terminal bronchiolar/alveolar region, then clearance is much slower, with half-times of the order of 65 days for rats, and months to years for human (Snipes et al., 1988; Yeh et al., 1996). Immediately following exposure to respirable particles in different species, the particles can be seen on the centriacinar surfaces (Warheit et al., 1984).

This slowed clearance means that the terminal bronchiolar/alveolar region accumulates particle dose. This is evident in the lungs of smokers and coal miners, which show pigment accumulations in this region (Churg & Green, 1999; Girod & King, 2005). High dose in this fragile region is a major concern for chronic health effects caused by LTLSP, as well as other fine particles. No doubt as a consequence of this, prolonged exposure to low-toxicity dusts, such as coal-mine dust, leads to formation of "macules." These are defined as "non-palpable and non-fibrotic collections of pigmented dust and dust-laden macrophages around small airways and vessels in the centre of the pulmonary lobule" (Churg & Colby, 1998). For more toxic dusts the inflammatory reaction set up in this region, terminal bronchiolitis/ alveolitis, leads via a complex series of events, to the lesions of fibrosis (Churg & Green, 1999) or centriacinar/ centrilobular emphysema(Churg & Colby, 1998). This sequence of events includes epithelial changes that include the loss of type I cells and an increase in type II cells (Albrecht et al., 2001) and increased macrophage trafficking via chemotaxis to sites of particle deposition, and both of these processes could favor particle retention.

In coal workers' pneumoconiosis the emphysema characteristically forms in the respiratory bronchiole in the vicinity of the macules and is termed centriacinar or centrilobular emphysema(Gibbs, 1995; Green & Vallyathan, 1998). Experimental studies with diesel exhaust and coal-mine dust confirm the centriacinar region to be the principal site of retention, although there are differences in exact anatomical situation between primates and rats (Nikula et al., 1997). In the case of rats the particles are located mainly in macrophages in the centriacinar alveoli and also interstitially in the walls, while in monkeys the pigment is predominantly interstitial in the walls of terminal and respiratory bronchioles. A recent study confirmed the importance of the this region in rats exposed to coal fly ash, showing that this region was the target for adverse effects with focal septal thickening and increased cellularity in alveoli immediately beyond terminal bronchioles (Smith et al., 2006). Although some particles may penetrate further than the PAR, the retention is greatest at this region.

In all instances, the PAR continues to represent the site for the highest deposition of particles entering into the lung parenchyma. The PAR is also likely to be a site for preferential particle retention due to a high initial particle deposition within this region, based on its architectural makeup, leading to unique aerodynamic characteristics for enhancing particle deposition. This can be understood in view of the nature of airflow in the pulmonary acinus, with its anatomical transition from a relatively narrow conducting airway to an exponential increase in volume and surface area (beyond the terminal brochiole [TB]) with zero airflow.

Up to this point the term "alveolar deposition" has been used without elaborating of the exact location of the retained particles. For example, alveolar deposition efficiency (i.e., the fraction of the total inhaled mass that deposits beyond the ciliated airways) is utilized to calculate the retained mass dose (lung burden) accumulating in the lung (Tran et al., 2000). However, the lack of homogeneity with respect to pulmonary retention is readily apparent anatomically (Nikula et al., 1997). We therefore contend that for insoluble particles a refinement of the dose metric would be the expression of the dose per unit area of the principal site of retention, the PAR. Expression of the dose in such a way should more accurately describe the dose but could also allow direct extrapolation to the *in vitro* situation, where dosimetry is notoriously difficult to equate to the *in vivo* circumstance.

We examined the impact of LTLSP on human alveolar type II epithelial cells, as represented by the A549 alveolar epithelial cell line. Epithelial cells are the first cells in the respiratory tract to come into contact with inhaled particles. We along with others have hypothesized that activation of these cells can serve as a direct and sensitive predictor of particle-induced inflammation. Inflammation is mediated by a variety of soluble factors including the chemokines, and interleukin (IL)-8 is representative of these and is especially important in acute inflammation

caused by particles (Driscoll et al., 1997), and the epithelium is a potent source of IL-8 after particle exposure (Driscoll, 1994, 2000).

Although particles also encounter macrophages and in fact are actively sought by them, the surface area of the centriacinar region is predominantly epithelial cells. Therefore most particles must first make contact with epithelial cells, and this may be a key interaction. Although most of this surface is type 1 epithelial cells, there is no reliable *in vitro* model for these cells and type 2 alveolar epithelial are commonly used as a surrogate. The ability of particles to stimulate A549 cells to release inflammatory cytokines and chemokines has been a staple test used to discriminate between pathogenic and non-pathogenic particles such as stone dust and other mineral particles (Ovrevik et al., 2005) with low and high amounts of biologically available iron (Aust et al., 2002), different types of minerals (Hetland et al., 2000), TiO_2 versus quartz (Schins et al., 2000), TiO_2 versus quartz, residual oil fly ash (ROFA), and PM_{10} (Stringer & Kobzik, 1998), and TiO_2 versus asbestos. The A459 IL-8 release assay is therefore validated for discriminating between pathogenic and non-pathogenic particles. The epithelial cells lining the PAR are postulated to play a key role in the initiation and progression of particle-induced pulmonary inflammation by releasing chemokines that are central to the recruitment of polymorphonuclear neutrophils (PMN) by chemotaxis (Driscoll et al., 1997). We hypothesize that this interaction between particles and epithelial cells in the PAR initiates a cascade of events that underlie the terminal bronchiolitis and alveolitis associated with harmful exposures to inhaled particles. This cascade involves oxidative stress, activation of redox-responsive transcription-regulating proteins, histone acetylatio,n and inflammatory gene transcription (Donaldson et al., 2003, 2004; Donaldson & Tran, 2002).

In vitro effects of particles are known to be difficult to relate to their *in vivo* equivalents, because of a lack of understanding of the correspondence between the dosimetry *in vivo* compared to the doses experienced *in vitro*. This is usually perceived as an enormously greater exposure *in vitro* to see increases in an endpoint than is calculated to plausibly occur *in vivo*. There is also the difficulty that some types of *in vivo* response cannot be reproduced *in vitro*; for example, the number of PMNs in the bronchoalveolar lavage fluid (BAL), which represents the gold standard for the degree of pulmonary inflammation, cannot be replicated *in vitro*.

This article sets out to attempt to relate *in vivo* and *in vitro* dosimetry for LTLSP in terms of an *in vitro* endpoint, IL-8 gene expression in a human epithelial cell line, that corresponds *in vivo* to PMN accumulation in rat lungs, a hallmark of inflammation. Such an analysis necessarily needs to take account of the species difference, in that rats possess macrophage inflammatory protein (MIP)-2 as a homologue of human IL-8 (Driscoll et al., 1995), so IL-8 was the endpoint used in the cell studies.

The data used here emanate from studies published in the past (Tran et al., 2000; Monteiller et al., 2007) but we present a novel interpretation of these data here, having recalculated the data in terms of surface area of particles and surface area of lung in order to demonstrate new relationships not previously reported. Ideally we would repeat these studies in an experimental design that would allow us to fully test the hypotheses we make here in relation to *in vitro* tests and *in vivo* tests. However, these inhalation tests were very expensive and in the present climate are unlikely to be repeated. This has necessitated resorting to testing our hypothesis based on the existing peer-reviewed and published studies, however imperfect the approach.

No other chemokines were measured in the Monteiller study (Monteiller et al., 2007), although oxidative stress showed a similar pattern of surface area dose-related effect, confirming the importance of oxidative stress in IL-8 gene expression caused by particles (Gilmour et al., 2003; Shukla et al., 2000).

MATERIALS AND METHODS

The *in vivo* data are from the studies carried out for the UK Health and Safety Executive by Tran et al. (2000), which were subchronic and chronic inhalation exposures in Wistar rats with two LTLSP particles, TiO_2 and $BaSO_4$. The exposure clouds had differing mass median aerodynamic diameter (MMAD), 2.1μm for TiO_2 and 4.1μm for $BaSO_4$. Therefore, in order to achieve similar lung burdens, different airborne mass concentrations were used: 25 and 50 mg/m^3 for TiO_2 and 37.5 and 75 mg/m^3 for $BaSO_4$. Lung burdens of particles in the lung were measured 3 days after the rats were removed from exposure, to allow airway clearance and at 6 timepoints up to 200 days. Polymorphonuclear leukocyte (PMN) response in the bronchoalveolar lavage was assessed at the 6 timepoints, being carried out 18 h after removal from exposure. The detailed methods and full results are given in Tran et al. (2000), with other relevant data in its companion paper (Cullen et al., 2000), while the present article confines itself to the PMN data and the lung burdens.

The *in vitro* data emanate from a study carried out for the UK Health and Safety Executive that is currently in press (Monteiller et al., 2007). The study set out to investigate whether the surface area of LTLSP is a better descriptor than mass of their ability to stimulate proinflammatory responses *in vitro*. The study utilized the human alveolar epithelial type II-like cell line, A549, and measured interleukin-8 (IL-8) mRNA, among other proinflammatory and oxidative stress endpoints, after treatment with a range of LTLSP (fine and nanoparticles) at between 10 and 250 μg/ml, surface-area doses of up to 200 cm^2. Of importance to the present study was a clear relationship between the surface-area dose of the LTLSP particles and gene expression for IL-8. Similar surface-area relationships were seen for IL-8 protein (somewhat complicated by adsorption of protein to the particle surface) and oxidative stress. Full details of the methodology and results can be obtained in the original paper (Monteiller et al., 2007)

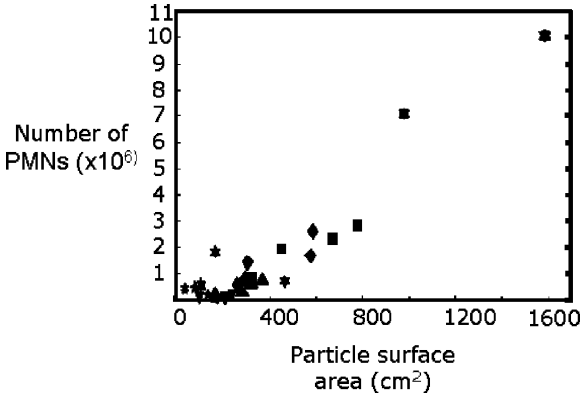

FIG. 1. The *in vivo* dose-response relationship between inflammation (PMN number in BAL) and the surface area dose of particulate administered per animal. The treatments include TiO_2 at two exposure concentrations (rectangle and diamond) and $BaSO_4$ at two exposure concentration (triangles) (figure modified from Tran et al., 2000). Also plotted are data from Oberdörster et al. (1994b) for fine and ultrafine TiO_2 (stars).

Particle Surface Area Dose and PMN Accumulation in BAL

Tran et al. (2000) established a dose-response relationship for two LTLSP (TiO_2 and $BaSO_4$) with respect to their abilities to evoke PMN influx into the lung (Figure 1). The vertical axis of Figure 1 represents the level of pulmonary inflammation, as quantified by the number of PMNs in the bronchoalveolar lavage (BAL) fluid, while the horizontal axis represents the lung particle dose (lung burden), described in surface area units (cm^2). The figure clearly indicates a threshold lung surface area dose at approximately 200–300 cm^2, beyond which inflammation is elevated.

Defining the Proximal Alveolar Region

Recently the anatomical site of interest here has been defined as the proximal alveolar region (PAR), that, is the terminal bronchiole (TB) and the first 400 to 600 μm beyond the bronchiole–alveolar duct junction (Pinkerton et al., 2004). This same region has also been referred to as the opening into the pulmonary acinus, a unit of gas exchange defined as the composite set of all alveoli arising from a single terminal bronchiole, also known as the ventilatory unit of the lungs (Mercer & Crapo, 1987). The PAR is the transitional zone between the terminal airways and the alveoli where gas exchange occurs. The epithelial cells in this region are mainly Clara cells and bronchial epithelial cells in the terminal airways and type 2 and type 1 alveolar epithelial cells in the alveoli.

Studies to measure the relative size of the PAR versus the entire pulmonary acinus use critical anatomical landmarks for analysis. These include the boundaries formed proximally by the anatomical junction formed between the terminal bronchiole and the first alveolar outpocketings of the first generation

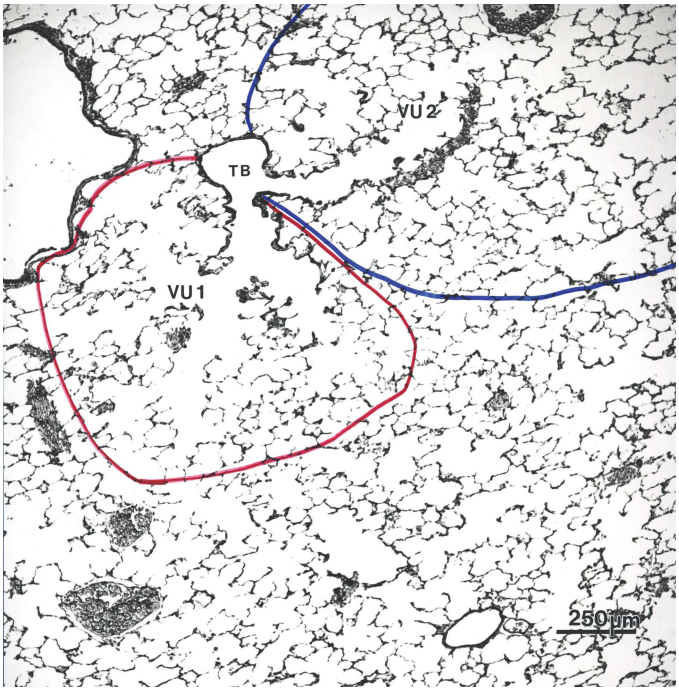

FIG. 2. Light micrograph of two ventilatory units (VU) arising from short but distinct terminal bronchioles (TB) in the lungs of a Sprague-Dawley rat. The boundary of each ventilatory unit is circumscribed in color (red or blue). The proximal alveolar region (PAR) is designated as all alveoli from the first and second generations of alveolar ducts arising from the TB. The PAR extends 400 to 600 μm from the TB into the VU.

alveolar duct and continuing beyond the first alveolar duct bifurcation down to the alveoli into second generation alveolar ducts (Figures 2–4). The volume circumscribed by these anatomical boundaries represents approximately 2 to 5% of the total volume of the pulmonary acinus. This measure can be determined by morphometric methods to estimate the volume fraction (V_f) of the parenychma encompassed by the PAR or by three-dimensional reconstruction of the pulmonary acinus using serial sections prepared from inflation-fixed, embedded lung tissue blocks. Mercer and colleagues found the volume of the pulmonary acinus in the rat can vary by threefold (Mercer et al., 1991), thus directly influencing the relative volume of air passing through the PAR region as the critical orifice to the gas exchange regions of the lungs.

RESULTS

Calculating the Rat PAR Surface Area

For the reasons given earlier, we hypothesize that it is more accurate to express the particle dose in terms of particle surface area dose per unit surface area of the PAR.

Table 1 summarizes the values of the parameters needed to calculate the PAR area. The surface area of each terminal

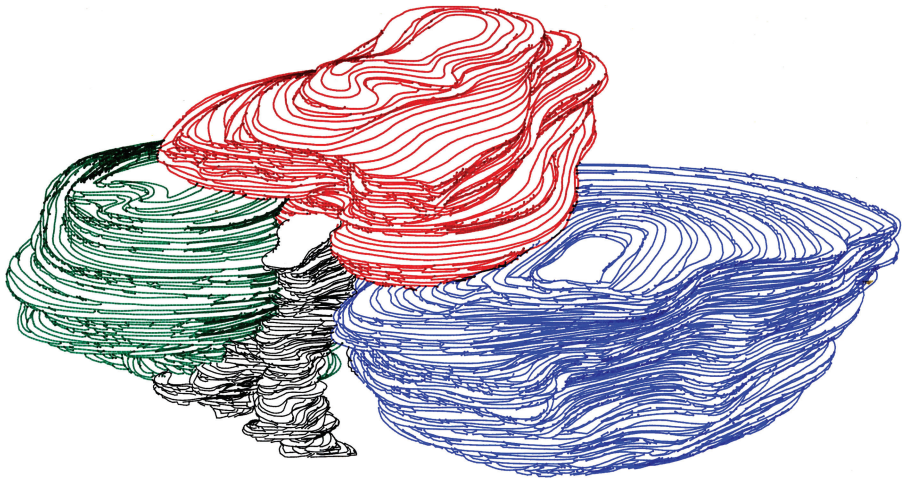

FIG. 3. Serial three-dimensional reconstruction of three distinct ventilatory units arising from adjacent terminal bronchioles (shown in panel). Each ventilatory unit is designated by a different color (red, blue, or green) with the proximal conducting airway giving rise to each terminal bronchiole shown in black.

bronchiole may be estimated by taking its shape as approximately cylindrical, with length l_{TB} and diameter d_{TB}. The total surface area of the terminal bronchioles, where N_{TB} is the number of terminal bronchioles, is then estimated, using the values in Table 1, and the equation:

$$\text{TB surface area} = N_{TB} \times \pi \times l_{TB} \times d_{TB}$$

The volume of the PAR region in the rat is approximately 5% of the total volume of the lung (Pinkerton, personal communication). The surface area to volume ratio for the rat lung parenchyma is approximately 500 to 600 cm^2/ml (Mercer & Crapo, 1987).

$$\text{PAR surface area} = \frac{\text{Vol} \times \text{pc} \times \text{cf}}{100}$$

where Vol is the parenchymal lung volume of the rat, pc is the PAR volume as a percentage of the parenchymal lung volume, and cf is the surface area to volume ratio for the rat lung parenchyma.

From the values in Table 1, the centriacinar surface area, defined as the terminal bronchiole surface area plus PAR area, is calculated as 300 cm^2 approximately.

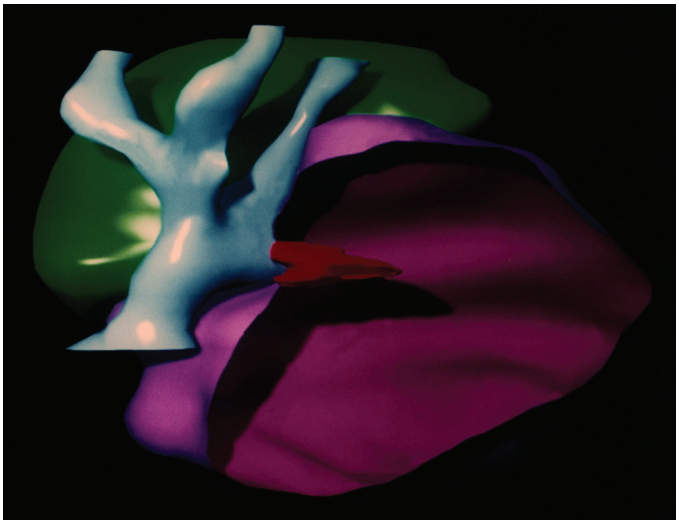

FIG. 4. Three-dimensional reconstruction of the ventilatory unit arising from a terminal bronchiole. The cut-away of the ventilatory unit reveals the presence and relative size of the PAR relative to the entire VU. Note that a second VU shown in green is located immediately adjacent, but arises from a separate terminal bronchiole.

Threshold for Inflammation in Rat Lungs Expressed as Particle Surface Area Dose per Unit PAR Area

Figure 5 illustrates the *in vivo* data taken from Figure 1, modified so that the horizontal axis values are reexpressed as the

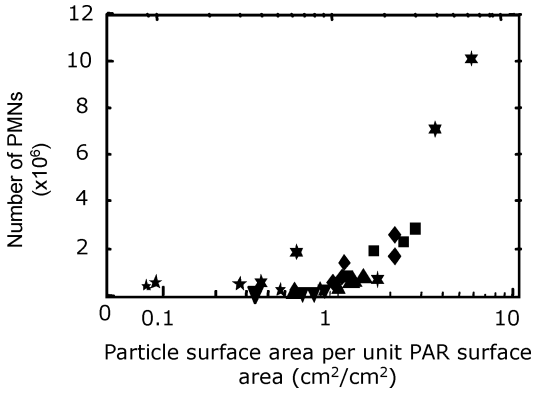

FIG. 5. The dose response between particle surface area and the number of PMN in BAL 18–24 h after inhalation exposure (Tran et al., 2000) in the rat using the proposed normalized dose metric (cm^2/cm^2). $BaSO_4$ (■), TiO_2 fine (♦), TiO_2 ultrafine (✱).

TABLE 1
Parameters values used to estimate the centriacinar surface area: For l_{TB} and cf midpoint values were used for calculations, and the range of quoted values is given in brackets

Parameters in the rat	Value	Units	Reference
Number of terminal bronchioles			
N_{TB}	2500		Pinkerton et al. (1997)
Length of terminal bronchiole			
l_{TB}	450 (range 400 to 500)	μm	Pinkerton et al. (1997)
Diameter of terminal bronchiole			
d_{TB}	180	μm	Pinkerton et al. (1997)
Parenchymal lung volume			
VOL	10.9	ml	Gehr et al. (1981)
PAR volume as a percentage proportion of the parenchymal lung volume			
pc	5	Percent	Pinkerton, personal communication
Surface area to volume ratio			
cf	550 (500–600)	cm^2/ml	Mercer and Crapo (1987)

particulate surface area dose per animal divided by the calculated PAR surface area, i.e., particle lung burden cm^2/PAR surface area cm^2. The graph show a critical value of approximately $1cm^2/cm^2$ as the threshold for the onset of inflammation.

Threshold for IL-8 Gene Expression *In Vitro* Expressed as Particle Surface Area Dose per Unit A549 Epithelial Cell Culture Area

Figure 6 shows the *in vitro* results for IL-8 mRNA production by A549 cells, obtained on treatment with the same particles (fine and ultrafine TiO_2 and $BaSO_4$). In this case the dose is expressed as particle surface area per unit A549 culture surface area, assuming confluence. A clear threshold of 1 cm^2/cm^2 is evident. In both Figures 5 and 6 the particle dose is expressed relative to surface area of the cells exposed: in the former case for cells lining the lung, and in the latter case for the epithelial cell line in culture. Most importantly, the threshold dose identified in each data set appears to be the same when considered as particulate surface area per unit surface area (i.e., $\sim$1 cm^2/cm^2). Thus, for LTLSP, knowledge of the threshold dose for stimulation of IL-8 gene expression *in vitro* predicts the threshold dose for stimulation of PMN influx into the lung *in vivo*. There was no threshold relationship when the particle mass was used as the metric, as shown in Monteiller et al. (2007).

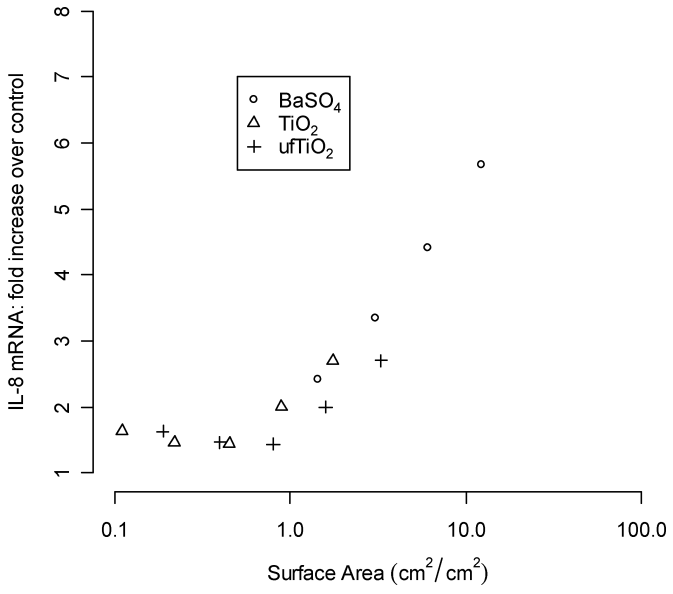

FIG. 6. The dose-response relationship between IL-8 mRNA production by A549 epithelial cells and normalized particle surface area dose(cm^2/cm^2) measured *in vitro*. Data from Monteiller et al. (2007f).

DISCUSSION AND CONCLUSIONS

In this article we describe the *in vitro* dosimetry of LTLSP for proinflammatory gene expression (IL-8) in the context of previously published data on *in vivo* dosimetry (Tran et al., 2000). In considering the *in vitro* effects, we built on our finding that surface area of LTLSP is the metric governing threshold for the pro-inflammatory effects of LTLSP *in vivo* (Tran et al., 2000).

We have demonstrated that nanoparticles/ultrafine patricles have considerable activity in causing inflammation in rat lungs and proinflammatory effects *in vitro* (Brown et al., 2001; Gilmour et al., 2004; Renwick et al., 2004). We hypothesized that this is a result of the high surface area of NP per unit mass and in fact have shown a straight-line relationship between the surface area dose of a range of fine and NP LTLSP delivered to the lung and the extent of the inflammatory response (Duffin et al., 2002). This suggests that even low-toxicity surface area, if there is sufficient dose of it, can produce oxidative stress (Brown et al., 2001; Wilson et al., 2002) by unknown mechanisms.

For our *in vitro* studies we therefore chose to express the dose to our cells as units of surface area, and this in turn led us to consider the divisor for the expression of dose in the lungs.

We originally expressed the *in vivo* data as the surface area dose divided by the total surface area of the lung airspaces. Reflection on the focal nature of retention in the PAR caused us to revise this based on the extensive knowledge of one of us (KP) of the morphometry of the lungs. When we divided the surface area lung burden by the calculated PAR area we contend that we moved much closer to the actual dosimetry that pertains in the lungs. This reflects the sum of efficient deposition plus slow clearance that leads to dose retention in the PAR. It is important to note that in the estimations carried out here the *in vivo* dose is the lung burden obtained 3 days after the cessation of exposure, to ensure that upper airways clearance has indeed taken place. We mean that the lung burden, within a few days after cessation of exposure, is in fact the PAR burden. The graph so produced gave us a threshold for the initiation of inflammation of about 1 cm^2 particle surface area burden per square centimeter of PAR. A threshold of 1 cm^2/cm^2 is clear from the data based on the mean PMN count and lung burden in surface area terms. A statistical analysis including interanimal variations estimated the threshold to be between 1 and 2 cm^2 (Tran et al., 2000).

This model clearly represents an oversimplification, as it is not feasible that all of the particle dose is concentrated in the epithelium, nor that the only target cells are the type II cells alone. Dealing first with former point, particles not immediately removed from epithelial surfaces are likely to be taken up into the underlying interstitium, and studies by one of the authors (GO) with fine and ultrafine TiO_2, show distribution of the particle burden between alveolar macrophages, bronchus-associated lymphoid tissue (BALT), interstitium, type I cells, and lymph nodes (Oberdorster et al., 1994a). We therefore advance the explanation that the relationship seen here with the type 2 epithelial cell line may be representative of the response of PAR cells in general (macrophages, epithelial cells, interstitial cells, etc.) to oxidative stress and IL-8 production. The preceding argument, of course supports the use of the type II cells when they are usually outnumbered by type 1 cells, but another important consideration is the marked increase in type II cell prevalence within this region consistently seen with PAR-associated inflammation and injury. Type I cells may enhance particle retention in this region, due to the fact that type I cells present an extremely thin barrier to the underlying interstitium. Should type I cells by virtue of their morphology permit greater access of particles to the interstitium and thereby enhance their retention in the region, this would further lead to type II cell proliferation and their greater ability to influence the local inflammatory response.

In vitro we chose IL-8 as the target endpoint because of the key role that epithelial IL-8 has in recruiting PMN following contact with particles (Driscoll et al., 1993; Driscoll et al., 1995; Stone V et al., 2001). Although we measured IL-8 protein, we have encountered problems with high surface areas of particles adsorbing the IL-8 protein and distorting the dose response (Kim et al., 2003). As the intracellular precursor of IL-8, mRNA for IL-8 has no such technical problem in its measurement. When the surface area dose was placed on the horizontal axis and IL-8 mRNA on the vertical axis a clear threshold of 1 cm^2 particle surface area dose per square centimeter of epithelial cell culture was evident for IL-8 gene expression.

These results then suggest that, there is good agreement that surface area of particles is the common metric for effective dose *in vivo* and *in vitro*. However the caveats that apply are (a) that in the lungs of rats the PAR surface area is the target surface area over which the dose is applied and (b) that *in vitro* IL-8 expression by A549 epithelial cells is the endpoint and surface area of culture the equivalent target surface.

The model derived here is based on data from epithelial cells alone, and it is necessary to also consider the macrophage. In dust-exposed lungs, by 3 days postexposure, particles are readily seen inside macrophages, although it is impossible to discount interstitial transfer of particles, which is not as easily seen. The role that the macrophage plays is therefore potentially twofold. For LTLSP it may be that the macrophage phagocytosis "neutralizes" LSLTP and that they do not stimulate macrophages to any appreciable extent; this is borne out by many studies that use TiO_2, for example, to be a control non-stimulatory particle. Therefore LSLTP in macrophages may elicit no response that adds to that of the epithelial response, in which case the primary target cell for the effects of LSLTP may be the epithelial cell. This is entirely consistent with our results showing the inflammogenicity of LSLTP to be explained by their effects on epithelial cells. On the other hand, in response to a particle burden, macrophages may secrete mediators like tumor necrosis factor (TNF)-α that can activate epithelial cell IL-8 gene expression. Our results, however, suggest that if this is indeed true, the effect may be pro rata with the particle /epithelial effect and so impossible to untangle in the *in vivo* studies carried out here. This is possible, and indeed we have seen modest increases in TN-Fα secretion by macrophages exposed to our LTLSP. TNF-α protein production at 4 h increased steadily in response to doses of LSLTP of up to 5 cm^2/cm^2. Such an effect could well have occurred *in vivo*, but we have no way to relate the extent of IL-8 mRNA production *in vitro* to the total PMN response seen *in vivo*. In either case, the data show that for LSLTP the ability to induce expression of the IL-8 gene in epithelial cells *in vitro* has the same threshold surface area dose as occurs *in vivo*.

Notwithstanding the preceding arguments, the proinflammatory response may be different for other cell types, such as primary type II cells, type I cells, Clara cells, or bronchial-epithelial cells. We do not necessarily anticipate that all cells will have the same threshold and respond similarly. Moreover, different particulate material with different surface chemistries, reactivities, and crystallinities will likely give different thresholds in terms of the dose metric surface area.

In summary, we present the first data showing a concordance between surface area dose of LSLT particles that produces inflammation *in vivo* and the surface area dose of LSLTP that

produce a key proinflammatory effect in epithelial cells *in vitro*. Importantly, this relationship is only seen if the surface area dose that elicits inflammation *in vivo* is expressed per unit PAR area. This highlights the importance of the PAR, which has long been known to be the site of high particle retained dose due to high deposition efficiency in this region coupled with slow clearance. The concordance between inflammatory effects *in vivo* (PMN in BAL) and proinflammatory effects *in vitro* (epithelial IL-8 gene expression) using surface area to express particle dose confirms and supports the utility of the particle surface area metric. This has special importance for the nanoparticle (NP)/ultrafine issue, where the greater surface area per unit mass of material in the form of NP is associated with greater inflammogenicity (Renwick et al., 2004). However, interstitial access can be very high for ultrafine/nanoparticles, and this may lead to a shift of inflammatory response away from the alveolar space toward the interstitium such that there is an apparently paradoxical decrease in lavageable PMN at higher delivered doses compared to lower doses (Oberdorster et al., 1992).

In conclusion, we believe that the concordance between *in vitro* and *in vivo* dosimetry of LTLSP based on surface area dose, described here for the first time, is a rational extension of the available data. The emphasis on surface area dose makes this model highly applicable to the testing of nanoparticles, which have a high surface area that is considered to play a key role in their effects (Oberdorster et al., 2005). We have recently investigated the role of surface area and surface reactivity in the dosimetry of low solubility particles dose *in vivo* and found that the sum potential of any low-solubility particle to induce inflammation is the product of the total surface area and the "reactivity" (e.g., oxidative stress potential) (Duffin et al., 2007). The model described here readily allows comparison of NP samples at the same surface area dose, enabling the "surface reactivity," and therefore the total hazard, to be determined. These studies also open the way for future *in vitro* approaches to studying proinflammatory effects of a range of toxic particles that are based on sound dosimetry enabling *in vitro* studies as a valuable complement to animal studies.

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