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Dispersion of Nanoparticles in Pulmonary Surfactants for In Vitro Toxicity Studies: Lessons from Ultrafine Diesel Exhaust Particles and Fine Mineral Dusts

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INTRODUCTION

Nanostructured materials including nanoparticles (NP) are generally defined as having at least one dimension smaller than 100 nm (1). Ultrafine particles are similarly defined as having diameters less than 100 nm; the general convention being that NP are manufactured or engineered materials in contrast to incidental or natural ultrafine particles. NPs are of special interest to the health effects researcher. They are not merely smaller forms of particulate matter; they can profoundly differ in their toxicological properties from fine-sized respirable particles, i.e., particles between 0.1 and 2.5 μm

in size. For example, fine-sized respirable TiO_2 particles are typically inert when studied in vitro and in vivo and are typically used as particle negative controls; while in contrast, nanoparticulate TiO_2, when used in animal model inhalation studies, causes lung injury (2). The basis for change in health effects associated with decreasing particle size is not necessarily size per se. There are other physical factors that change very strongly with decreasing particle size: one is particle number per unit mass; another is specific surface area (surface area per unit mass).

Because of their submicrometer sizes, NP or ultrafine particles have high specific surface areas. For example, ultrafine diesel exhaust particulate materials (DPM) can have specific surface areas in the range of 100 to $1000\,m^2/g$, in contrast to many fine-sized mineral dusts with values in the $10\,m^2/g$ range. In some cases, ultrafine particle surface area has provided an effective metric-relating exposure and response: TiO_2 ultrafine particles and carbon black dust were active for tumor induction in the rat with toxicity increasing with dust surface area (1). And surface area in some cases provides a measure of comparability between different ultrafine dusts, e.g., for some clearance or inflammation processes in vivo (3).

However, not all respirable particulate materials are equally toxic when concentration or exposure dose are normalized by surface area. The composition or structure of the particle surface can greatly affect toxicity. For fine-sized respirable particles, surface structural properties which are submicrometer or ultrafine in dimension can be determinants of health hazard and disease risk. For instance, unexpected morbidity and mortality in the workforce of a hard metal fabrication plant using a new process were related to subtle surface structural features of the generated fine respirable dusts: ultrathin cobalt coatings on the tungsten carbide particles were strongly catalytic in aqueous media for toxic reactive oxygen species generation (4–6). Anomalous differences in silicosis risk between two worker cohorts in China, identified in a 20,000 worker medical registry, were largely resolved by quantitatively normalizing exposures to respirable silica dust that was free from submicrometer aluminosilicate surface contamination (7–9). Such surface ultrafine structural effects on health hazard, albeit for fine-sized respirable particles, suggest the need for a thorough characterization of surface physicochemical and toxicological properties of new NP respirable materials with their very large specific surface areas.

Expression of toxic activities associated with respirable particle surfaces can be significantly modified by the initial interaction of particles depositing in the acinar region of the lung. After inhalation, the first contact of respired particles is with the lung's rich surfactant-coated hypophase lining of the air–tissue interface of the alveoli or respiratory bronchioles. Insoluble particles can adsorb components of that pulmonary hypophase surfactant, resulting in fundamental changes in the particles' biological disposition and expression of toxicity. In vitro toxicology studies can be

designed to retain particle surface structure and composition and to model the conditioning of those surfaces upon particle deposition in the deep lung in order to analyze consequent effects on toxicity. Ultrafine or fine respirable particulate materials have been dispersed into phospholipid components of lung surfactant as preparation for in vitro cytotoxicity and genotoxicity studies. Pragmatically, this overcomes laboratory handling problems innate in attempting aqueous system preparations of insoluble hydrophobic particulate materials, by permitting their dispersion in a physiologically reasonable manner. More profoundly, this provides a model of their surface conditioning as would occur upon deposition in the lung; of their in vivo biological availability; and of potential nanostructural synergisms for the expression of surface-associated toxicant activity. Some in vitro studies of phospholipid surfactant-conditioned ultrafine or fine respirable particles are reviewed here to illustrate testing procedures and their limitations for identification of NP toxic activities and potential respiratory hazard.

LUNG SURFACTANT AND INSOLUBLE RESPIRABLE PARTICLE IN VITRO TESTING

Lipid and lipoprotein surfactants synthesized and secreted by pulmonary alveolar II cells coat the air interface of the hypophase surface of the deep lung. Phospholipids constitute about three-quarters of the mass of pulmonary surfactant, with diacyl phosphatidylcholines (DAPC) accounting for over half of them (10,11). When spread upon the aqueous–air interface, phospholipids lower surface tension about 50-fold, down to the order of 1 dyne/cm; and they make surface tension a function of surfactant surface concentration, closely modeling those physiologically important properties of the pulmonary alveolar hypophase (12). A specific DAPC, dipalmitoyl phosphatidylcholine (DPPC), dispersed into physiologic saline has been used frequently as a simple model of lung surfactant in physiology studies; and more complex mixtures derived from animal lung lavage containing DAPC have been used in research, and have been used clinically, e.g., as therapy for infants' respiratory distress.

Hydrophobic or hydrophilic respirable fine or ultrafine particles can adsorb components of lung surfactant from aqueous dispersion. That results in prompt modification of the expression of some particle surface-associated toxicities; and subsequent cellular enzymatic digestive processing of that particle surface-adsorbed surfactant can determine the evolution of a particle's expression of toxicity. These surfactant interactions can significantly affect the biological availability or activity of particle-surface-borne toxicants or surface chemical functional groups. Such particle surface– surfactant interactions have been researched to address two general concerns for respirable particulate material toxicity: (1) the question of the physiological

significance and interpretability of conventional in vitro assays of diesel exhaust or other hydrophobic ultrafine particulate genotoxicants as conventionally performed on organic solvent extracts of collected DPM; and (2) the question of the failures of in vitro cytotoxicity assays to predict and distinguish pneumoconiosis hazard of fine-sized respirable mineral dusts.

Genotoxicity of Ultrafine Diesel Exhaust Particulate Materials

Diesel exhaust has been evaluated to be a potential or probable human carcinogen by the Centers for Disease Control and Prevention—National Institute for Occupational Safety and Health (13), the International Agency for Research on Cancer (14), and the U.S. Environmental Protection Agency (15). DPM is ultrafine carbonaceous hydrophobic particulate material, frequently containing complex polycyclic aromatic hydrocarbon (PAH) compounds which, separately, are known carcinogens, and sometimes containing a number of proven toxic species, including polar hetero- and polycyclic aromatics, radical species, entrained metal species, and other organic species (16). Being hydrophobic and insoluble in water, DPM typically have been prepared for chemical study or in vitro bioassay by dissolution and extraction in organic solvents. It has long been observed that in vitro genotoxic activities are expressed by organic solvent extracts of some filter-collected DPM, e.g., using acetone or dichloromethane (DCM) solvent (17–24). In vitro mutagenicity of solvent extract of DPM can vary systematically with operating conditions for a given engine, e.g., with engine speed, torque, and fuel (25).

One question of the role of DPM genotoxicants for in vivo tumorogenicity is this: are hydrophobic particle-borne genotoxicants biologically available for activity under conditions of particle deposition in the lung? Testing organic solvent extracts of DPM does not provide, a priori, a physiologically reasonable model of genotoxicant biological availability from intact DPM particles deposited in the lung aqueous hypophase. It was found that attempting to extract DPM with principal components of lung surfactant released few or no organic genotoxicants from the particles, and the surfactant extract expressed little or no in vitro genotoxic activity (26–29). That is, the hydrophobicity of the organic genotoxicants carried by the carbonaceous DPM particles prevented their extraction or release from the particles under conditions modeling their deposition on the surface lining of the deep lung.

Instead, a different paradigm—distinct from an extraction mechanism—was observed that could result in biological availability and activity of hydrophobic ultrafine particle-borne genotoxicants: if DPM were simply mixed into aqueous phospholipid surfactant dispersion, then the resultant dispersion expressed genotoxic activity in vitro. Those activities were shown to be associated with the nondissolved but surfactant-dispersed particulate phase material (29–34). That is, phospholipid surfactant adsorbs to the DPM surface, providing a hydrophilic coating, and permitting dispersion of the

surfactant-coated DPM as particles in aqueous media; and those particles can express genotoxic activities.

Such dispersion of DPM into components of lung surfactant in part models possible in vivo particle surface conditioning, disaggregation, and particulate-bound toxicant bioavailability, while avoiding the destruction of particle size, structure, and compositional properties that would result from organic solvent extraction of DPM. Surfactant dispersion also provides a convenience in preparatory handling of DPM for aqueous in vitro test systems or in vivo studies using instillation of collected NP materials.

The basis for the ability of DPPC to disperse DPM or other hydrophobic particles is the combination of hydrophobic and hydrophilic moieties of the DPPC molecule. One end of the molecule consists of hydrophilic choline and phosphate: a trimethyl ammonium cationic group is bound through a two-carbon chain to an acidic phosphate, forming a zwitterionic dipole and providing a hydrophilic end of the molecule. The phosphate then is esterified to the first carbon of a glycerol, which is esterified at the other two carbons to two long-chain fatty acid residues; palmitate in the case of DPPC. These provide two hydrophobic, lipophilic long tails to the molecule. When dispersed into aqueous media, the phospholipid molecules aggregate into multimolecular structures such that the hydrophilic zwitterionic head groups of the molecules are oriented to face into the surrounding water, while the hydrophobic fatty acid tails cluster among themselves, minimizing contact with water or with the hydrophilic heads of other phospholipids. This gives rise to spherical or lamellar structures made up of bilayers of surfactant molecules. The zwitterionic head groups are on the outer aqueous-side surfaces of the bilayer, with the lipid tails "sandwiched" between in order to minimize hydrophobic lipid tail contact with water. This structure also is the general basis for the bilayer phospholipid underlying all cell membrane structure.

Dry, waxy DPPC can be dispersed into aqueous media by ultrasonication, forming a pale milky and relatively stable dispersion. When dry or oily filter-collected DPM is mixed into this aqueous DPPC dispersion then the agglomerates of soot particles are observed to disperse. The DPM is "solubilized," that is, dispersed as small surfactant-coated particles, rather than dissolved. In this state, long-chain lipophilic/hydrophobic tails of the DPPC molecule associate with the organic DPM particle surfaces, while the zwitterionic hydrophilic trimethyl ammonium and phosphate head of the DPPC molecule orients outward to face the surrounding aqueous medium. A simplified picture is that of a DPM particle as a tar "pin-cushion" covered by DPPC soap molecule "pins" with their tails adsorbed to the tarry DPM particle and their heads oriented outward, providing a hydrophilic outer coating, in turn permitting the structure to act as a water-wet but nondissolved particle, which disperses in water. Filter-collected DPM mixed into an aqueous DPPC dispersion then can challenge cells effectively to express genotoxic activities for mammalian cell DNA and clastogenic damage, as well as for bacterial cell mutagenicity.

Order of magnitude estimate of the amounts of DPPC needed to fully disperse DPM or other ultrafine or NP material, and so to permit bioassay of full activity, can be estimated from the specific surface areas of the particulate materials. For fine mineral particulate matter a measure was made of the amount of DPPC mass adsorption per unit dust surface area as necessary to fully passivate the membranolytic toxicity of two silicate dusts (see below). Using that so-derived value of about 5 mg DPPC/m^2 surface area as an approximate general measure independent of particle composition, then ultrafine particles with specific surface areas of 100 to 1000 m^2/g would require, respectively, on the order of 500 mg DPPC to 5000 mg DPPC/g of particulate material. As a caveat to this estimate for hydrophobic NP materials: the minimal coating may be a monolayer of DPPC for hydrophobic particles, e.g., diesel soot, in contrast to a bilayer for mineral dusts. On the other hand, if the sample is collected on a filter from the aerosol state or otherwise aggregate collected, then agglomeration in the sample may require surfactant multilayers for physical disaggregation to proceed to completion. Bacterial mutagenic activity was measured versus DPPC concentration for three concentrations of DPM from a diesel truck exhaust-pipe deposit over a range from 1/1 to 10/1 mass ration DPPC/DPM. At any DPM concentration the mutagenic activity increased with DPPC concentration up to a DPPC/DPM ratio of about 7/1, i.e., 7000 mg DPPC/g DPM (29).

In Vitro Genotoxicity Assays of DPM Dispersed in Surfactants

In vitro genotoxic activities have been compared on organic solvent extraction versus surfactant dispersion preparations of parallel DPM samples. Several assays were performed upon DPM that had been filter-collected from the exhaust of a 1980-commercial 5.7 liter V-8 diesel engine operated on a dynamometer test stand under a Federal Test Procedure urban duty cycle, the material graciously supplied by the Lovelace Inhalation Toxicology Institute. Organic solvent extraction was prepared by dissolution of DPM in DCM or acetone and evaporative exchange into dimethylsulfoxide (DMSO). In some experiments, DMSO was used as the organic solvent for extraction. For the surfactant dispersion sample, the surfactant was prepared by ultrasonically dispersing DPPC into physiological saline solution (PSS); then DPM was mixed (not sonicated) into that dispersion. The Ames *Salmonella typhimurium* histidine reversion assay was used for the detection of gene mutation in bacteria. The assays for mammalian cells include sister chromatid exchange (SCE), micronucleus induction (MN), unscheduled DNA synthesis (UDS), chromosomal aberration (CA), gene mutation, and the single cell gel electrophoresis for single- or double-strand DNA damage. These comparison studies have been recently reviewed (35).

Both solvent and surfactant total preparations showed positive activity increasing with DPM concentration, for gene mutation in *S. typhimurium*

TA98 without microsomal S9 activation, with the 1980-engine soot somewhat more active as a surfactant dispersion compared to its preparation in DMSO. The extract of the solvent preparation was mutagenic, while the particulate residue of the solvent extraction was not. For the surfactant preparation of the DPM, the dispersion of whole DPM into surfactant was mutagenic, while its filtrate was not. When the surfactant-dispersed DPM was centrifuged rather than filtered, then some mutagenic activity was expressed by the supernatant suggesting a very fine particulate-active fraction, which could be filtered out but not centrifuged out of the surfactant dispersion (29–31).

The Chinese hamster pulmonary fibroblast-derived cell line (V79) was used for the SCE and UDS assays. Both DMSO organic solvent and DPPC surfactant dispersion total preparations of the 1980-diesel DPM expressed comparable activity, increasing SCEs with DPM concentration. Induction of SCE activity was found to reside in the supernatant fraction of the DMSO solvent-extracted samples, and in the sedimented (particulate) fraction for DPPC surfactant-dispersed samples (31). Using a DPM collected from a diesel truck exhaust-pipe deposit, both DPPC surfactant dispersion and DMSO solvent total preparations were active for the induction of SCE, with the DMSO solvent preparation about twice as strong as the surfactant preparation (30). Both DPPC dispersion and DMSO solvent preparations of the 1980-diesel DPM were also active in the UDS assay with the dispersion preparation about 50% stronger per mass of soot extracted. Induction of UDS was found in the supernatant fraction of the DMSO-extracted sample and in the sediment (particulate) fraction of the surfactant-dispersed sample (33).

In the study of MN induction in V79 cells by the 1980-diesel DPM, samples in DMSO solvent or DPPC surfactant preparation showed increasing positive concentration-response activity with the surfactant preparation about twice the strength of the solvent preparation. For CA induction by the same DPM and cells, DPPC surfactant-dispersed DPM was active, increasing with DPM concentration; CA comparison was not made with solvent extract samples. The 6-thioguanine-resistant gene mutation assay for a forward gene mutation using this DPM and V79 cells did not show a significant positive response as either DMSO solvent or DPPC dispersion preparations (34). In a separate MN study of the same DPM, the DPPC surfactant sediment and DMSO supernatant of preparations were comparably active in Chinese hamster ovary (CHO) cells; however, in V79 cells the DMSO solvent extract was active while the DPPC surfactant sediment was only marginally active (32). In contrast, MN assay of a National Institute of Standards and Technology standard DPM obtained from a fork-lift diesel exhaust expressed significantly greater activity for DPPC surfactant sample preparation versus that expressed by acetone solvent extract of an equal mass of soot. The same sample showed similar behavior of heightened activity for surfactant-dispersed particles in

single cell gel electrophoresis assay for single- or double-strand DNA break; but both preparations were comparably active in YG1024 bacterial mutagenicity assays (36). That is, in this case there appeared to be a synergistic increase in mammalian cell genotoxic activity for genotoxicants carried by ultrafine particles.

Surfactant Effects on Mineral Particle Toxicity

Fine-sized respirable mineral particle surface interactions with pulmonary surfactants significantly affect the dusts' expression of toxicities in vitro; and those findings can provide some semiquantitative design information for similar studies of ultrafine or NP respirable materials. Respirable fine-sized crystalline silica dust, e.g., quartz dust, is an exposure agent for pulmonary fibrosis; and it is promptly cytotoxic or membranolytic in numerous short-term in vitro bioassays (37). However, in vitro cellular bioassays cannot be used with specificity to distinguish quartz-associated fibrosis hazard in many mixed dusts. That is because it was found that kaolin dust, a common aluminosilicate clay dust, is as cytotoxic as quartz dust (38), despite the fact that kaolin dust exposures are associated with only limited risk of pneumoconioses in contrast with quartz dust (41–43). The two dusts expressed comparable in vitro cytotoxic activities on a surface area basis, as measured by mammalian cell release of lactate dehydrogenase (LDH) and lysosomal enzymes or by erythrocyte membranolysis (38–40). This fails to distinguish the strong pathogenic potential of quartz dust for fibrosis from kaolin dusts' far weaker hazard.

In vitro investigations of possible bases for the anomaly of equal cytotoxicities but different disease risks found that dust surface adsorption of components of lung surfactant can be prophylactic against otherwise prompt cytotoxic activities of both quartz and clay dust; and subsequent cellular enzymatic processes can modify that prophylaxis and permit expression of particle surface toxic interactions, in some cases with mineral specificity. Respirable particles can be conditioned by pulmonary surfactants (44). Adsorbed phospholipid can suppress silica particle cytotoxicity (45,46). Kaolin can adsorb surfactant (47) with suppression of membranolytic and cytotoxic activity in a number of short-term in vitro bioassays (48). That is, short-term in vitro assays of native quartz or kaolin dusts provide a "false-positive" prediction for native kaolin dust; but they result in a "false-negative" prediction for quartz dust when the dusts are surfactant-coated.

Assays of membranolytic activity versus adsorption of DPPC surfactant indicate that the amount of DPPC needed for full suppression of toxicity of quartz or kaolin dusts is proportional to the dust surface area. It was found that about 4 to 5 mg DPPC adsorbed per square meter of quartz surface and about 5 to 6 mg DPPC adsorbed per square meter of kaolin surface were the minimum amounts needed to provide complete

prophylaxis of otherwise prompt membranolytic activity (40,49,50). For comparison, a computational model of the surface area of DPPC at the aqueous interface of a bilayer suggests a value of about 62 square angstroms per molecule, which would be about 4 mg DPPC per square meter for a bilayer (51).

Significant quartz dust fibrogenic activity is observed following in vivo animal model or human exposures, suggesting loss with time of the surfactant prophylaxis. Cellular lysosomes contain enzymes, which digest components of pulmonary surfactant, including phospholipases. Quartz or kaolin dusts passivated by surface-adsorbed DPPC can be taken into the cell, e.g., by phagocytosis, and subjected to phagolysosomal phospholipase activity. In some cases the dust particles can be subjected in the extracellular environment to phospholipase released by cells.

In acellular studies, dusts were incubated in DPPC dispersion, rinsed to remove loosely held surfactant; and then were incubated with three different levels of porcine pancreatic neutral pH-optimum phospholipase A2 (PLA2). DPPC and lysolecithin remaining adsorbed and erythrocyte membranolytic activity of the preparations were measured at times out to 72 h of PLA2 incubation. Half the DPPC was rapidly hydrolyzed to lysolecithin within the 1-h first time point; and the second half of the DPPC was digested much more slowly, and with mineral specificity. Kinetics of the cell-free process were well-modeled mathematically with a two-exponential function for DPPC remaining adsorbed with time under enzymatic digestion; the exponential rate constant was fivefold greater for removal of DPPC from quartz than from kaolin. Membranolytic activity of the dusts was restored in parallel with the removal of the DPPC (50,52).

The same free energy considerations responsible for phospholipid bilayer conformation in cell membranes also suggests that DPPC surfactant is adsorbed to some hydrophilic-surfaced mineral dusts as such a bilayer, with one surface of the bilayer in contact with the particle mineral surface, and the other side oriented to the surrounding aqueous medium. PLA2 hydrolyzes the ester linkage of the fatty acid at the middle carbon of the glycerol moiety that links the hydrophilic "head" of the molecule to the two long-chain fatty acid hydrophobic "tails." The rapid loss of half the surfactant from both dusts, the slower removal of the second half, and the mineral specificity for the rate of digestive removal of the second half of the surfactant can be modeled as enzymatic digestion of an adsorbed bilayer of surfactant: A surfactant bilayer remains on the particle after DPPC incubation and rinsing. Subsequent incubation with PLA2 permits rapid hydrolysis of the outer aqueous-side face of the bilayer. The inner DPPC layer, in direct contact with the mineral surface, is digested more slowly due to more restricted access of the PLA2 enzyme. The mineral-specificity of the rate of enzymatic digestion of the second half of the adsorbed surfactant suggests a further mineral-specific hindrance to activity of the PLA2 for the

DPPC in contact with the particle mineral surface. The silica surface consists of silanol groups with their surface hydroxyls weakly acidic. The kaolin surface has both silanol and aluminol groups; and the surface aluminol hydroxyl groups are weakly basic or amphoteric. Thus, different interactions can occur on the surfaces of the two dusts with differences in the strengths or conformation of the adsorbed DPPC. The additional aluminol groups on the kaolin surface provide sites distinct from the quartz surface for interactions e.g., with DPPC phosphate or carbonyls. Consequent mineral-specific steric hindrance to PLA2 enzymatic activity and rates of digestive removal of prophylactic DPPC could then result in distinct rates or levels of expression of particle toxicity. There is limited infrared and nuclear magnetic resonance spectroscopic data indicating quartz versus kaolin mineral-specific differences in the adsorption of cationic trimethylammonium at the hydrophilic end of the DPPC molecule and of the phosphate near the carbonyl ester bonds in the DPPC (49,52–56).

Quartz or kaolin loss of adsorbed surrogate lung surfactant has been measured for cellular systems. Silica dust preincubation with a commercial multicomponent surrogate lung surfactant derived from bovine lung surfactant was used for in vitro challenge of lung macrophages lavaged from male Fischer 344 rats. It protected cell viability at 1 h; but the prophylaxis was significantly reduced at 24 h. Preincubation with Survanta of a high dose of silica instilled in vivo in the animals resulted in significant reduction in biochemical and cellular response parameters in bronchiolar lavage at 1 day but not at 14 days after challenge (37,57). Digestion of C-13 radiolabeled DPPC on quartz or kaolin after in vitro challenge of the P388D1 macrophage-like cell line found half the surfactant was digested for both dusts in the first 3 days; and approximately half of that remainder was digested at 9 days for both dusts. That is, no mineral specificity was seen for this system with cellular acidic phagolysosomal digestion conditions. In addition to digestion within the cell, phospholipase exudate of the cells was identified, which was active at pH 7 but not at pH 5 in the extracellular medium. Incubation of the DPPC-coated dusts with cellular-conditioned culture medium containing this pH-neutral phospholipase resulted in digestion of DPPC on quartz at the same rates effected by cellular digestion; however, half of the original amount of DPPC remained undigested on the kaolin after 14 days (58). In vitro cellular digestion was measured over 7 days for a fluorescent probe-labeled analog of DPPC from quartz and kaolin dust by pulmonary macrophages lavaged from male Fischer 344 rats. No difference was observed between quartz and kaolin for the decay of fluorescence intensity from labeled surfactant on cell-ingested particles; a single exponential fit was fit with a $T_{½}$ of about 40 h for both dusts (59). A similar fluorescent-labeled phospholipid preparation of quartz and kaolin dust was used to challenge alveolar macrophages lavaged from

Sprague–Dawley male rats. Quantitative fluorescence microscopy of label intensity on particles within cells at times from 1 to 10 days showed two exponential behavior with half or more surfactant removed at 1 day; and loss was more rapid from quartz compared to kaolin (60).

A SUMMARY OF METHODS AND CAVEATS

Different genetic endpoints in bacteria and in mammalian cells all showed genotoxic activity for DPM dispersed into DPPC surfactant for cases where the DPM also express activity as solvent extract. The activities in surfactant dispersion were associated with the nondissolved particulate phase material that were coated and solubilized, i.e., given a hydrophilic coating, by a major component of lung surfactant. In this way, DPM inhaled into the lung may be made bioavailable by virtue of the solubilization and dispersion properties of pulmonary surfactant components. This suggests that other insoluble hydrophobic ultrafine or NP materials depositing in the deep lung may effectively carry and deliver genotoxicants as particulate phase materials, which can there express their genotoxic activities as lung surfactant-dispersed particles.

In vitro assays, in general, are inexpensive and useful test systems for detection of genotoxic agents and potential carcinogens. Surfactant conditioning appears to permit the extension of such in vitro bacterial and mammalian cell testing to insoluble NP materials. DPM studies indicate dispersion in phospholipid surfactant of 5 to 10 mg DPPC/m^2 particulate material surface area can permit full expression of genetic toxicity in mammalian cell assays. In vivo, pulmonary surfactant is well in excess of amounts needed to provide such adsorption and solubilization of respired NP. By calculation from the amount of lavagable lung surfactant from the rat of about 7 ug phospholipid per mg dry lung weight (61), and the half life time of surfactant replacement of about 14 h (12), lung surfactant is in sufficient quantity to disperse some 100 times or more of the DPM that would be respired per workday under occupational exposures at the NIOSH recommended exposure limit of 0.05 mg DPM/m^3 (34).

A concern for the interpretation from in vitro genotoxic activity to disease risk, including the case of surfactant-dispersed DPM, is that DPM induction of lung tumors in animal models has been reported for conditions of "particle overload" exposures rather than under conditions representative of occupational exposures (62–64). Conditions of in vitro cell challenge leading to induction of genotoxic activities have usually been at DPM-to-cell concentrations far exceeding doses estimated for 1 day of exposure at the NIOSH recommended DPM exposure limit (13). However, incomplete lung clearance or sequestration of DPM within pulmonary cells can lead to increasing lung loads of DPM with increasing exposure times. This has been

observed in inhalation exposures of the rat to 0.25 to 6 mg DPM/m^3 of air, which resulted in residual lung burdens after 7 to 112 days of 0.2 to 12 mg retained DPM (65). These would be modeled for in vitro study by concentrations in the range of 1 to 10 μg DPM/cm^2 of plated cells, which are within concentrations used for in vitro mammalian cell studies of surfactant-dispersed DPM showing clastogenic and DNA damage (34).

Short-term in vitro cytotoxicity assays fail in the simplest comparison of quartz versus kaolin dusts to predict the great difference seen in vivo, e.g., for pulmonary fibrosis. In vitro assays for cytotoxicity risk false-positive interpretation for native particles and false-negative interpretation for surfactant-treated particles. Research implicates but does not clearly establish digestive removal of particle-adsorbed surfactant as a basis for distinguishing mineral-specific expression of toxicity that predicts disease risk with specificity. In vitro cellular systems using phospholipid surfactant and phagocytic cell systems are limited in several ways.

Firstly, DPPC is a limited model of lung surfactant. Other surfactants and other biological molecules are found in the lung alveolar hypophase; and mineral specificity of level of prophylaxis or of rate of restoration of toxicity may differ for them. Lipoprotein fractions of cell test system media serum can reduce the expression of crystalline silica cytotoxicity (66–68), with reactivation following trypsin digestion (69). Quartz and kaolin dust prompt in vitro induction of LDH release from macrophage was suppressed in 10% fetal bovine serum medium; however, quartz but not kaolin activity was restored at 6 h (70–72). This cautions that short-term in vitro results can be affected by assay system nutrients that are not necessarily representative of in vivo pulmonary hypophase exposures. Some typical components of nutrient serum, e.g., albumin, are increased in the alveolar hypophase by in vivo acute inflammatory reactions causing increased permeability of the microvasculature with transudation of plasma protein (73–75) into the lung alveoli. Thus, nonmineral specific retoxification of surfactant-conditioned particles by acidic processes in alveolar macrophages with subsequent inflammatory response might evoke a secondary round of prophylaxis, by plasma protein leaked into the alveolar hypophase interactions.

Secondly, the use of macrophage or phagocytic cell lines may not well-model the phagolysosomal digestive processes for primary cells involved in lung fibrosis. Some research has indicated that epithelial or interstitial cells of the pulmonary alveolus rather than macrophages are the target cells for silica dust-induced in vivo interactions that signal the pulmonary fibroblasts to upregulate collagen synthesis and produce lung fibrosis (76). Interstitial cells have pH neutral phagolysosomal conditions (77), similar to the pH conditions of the cell-free PLA2 tests, in contrast to lung macrophages and cell lines with pH acidic-optimum lysosomal phospholipases. The difference in rates of surfactant removal and restoration of dust toxicity seen for these in vitro macrophage-like cell

systems with acidic phagolysosomal conditions for enzymatic digestion does not appear sufficient to account for the differences of in vivo induction of fibrosis between quartz and clay dusts.

That is, development of in vitro cytotoxicity assays for endpoints other than genotoxic effects, e.g., for fibrosis, might require additional features to better model in vivo conditions, including (1) the use of additional constituents of the deep lung hypophase; (2) other cells or cell lines representative of structural cells of the lung acinus; and (3) incubation times consistent with rates of cellular metabolic processing of NP surface-adsorbed prophylactic biomolecules. Validity of resultant assays might then be tested by complementary in vivo measures of labeled surfactant loss from particles in tissue (60) correlated with histopathology measures of the onset of fibrogenic activity.

Conventional in vitro cellular assays for first-tier screening of potentially genotoxic compounds can be extended to ultrafine or NP materials by surrogate lung surfactant solubilization of the particulate sample, using adequate amounts of surfactant based on sample-specific surface area, as demonstrated for diesel exhaust particulate samples. In vitro cytotoxicity assays for particulate material performed without consideration of surfactant conditioning of particle surfaces can give false-positive results for the prediction of some lung disease, as demonstrated by aluminosilicate kaolin dust samples. But in vitro cytotoxicity assays of surfactant-treated mineral dusts require further development and validation to clearly distinguish disease hazard with specificity, as seen in comparisons of quartz and clay surfactant modified in vitro toxicities versus their in vivo fibrogenic activities. In vitro short-term assays for genotoxic or cytotoxic activities by ultrafine or nanoparticulate materials must be interpreted with caution until such time as bioassay systems considering NP surface properties and physiological conditioning are demonstrated to clearly distinguish respirable particulate materials of known differing disease risk.

DISCLAIMER

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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NANOTOXICOLOGY

CHARACTERIZATION, DOSING AND HEALTH EFFECTS

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