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A Quantitative In Vitro Fluorescence Imaging Method for Phospholipid Loss from Respirable Mineral Particles

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Respirable quartz and kaolin particles were treated with fluorescent-labeled phospholipids to model contact of fibrogenic and nonfibrogenic particles with pulmonary surfactant in the alveolar regions of the lung. Particles were used to challenge rat pulmonary macrophages in vitro at times from 1 d to 10 d. The objective was to develop a quantitative method to track surfactant components that adsorb to respirable particles in the lung or inside cells. Confocal laser scanning microscopy was used to image and quantify surfactant remaining on particles internalized by cells. Results indicate that the fluorescent label is removed from quartz particles quickly, with the fluorescence intensity less than 15% of initial value at 3 d, and about 5% at 10 d. In contrast, the kaolin particle-associated fluorescence was still ∼39% of initial intensity at 3 d, and 10–15% at 10 d. Unchallenged cells showed a background of ∼5%, and noninternalized particles did not exhibit any loss of fluorescence over the 10-d exposure. The results indicate the method may be useful in label-removal rate studies of respirable particles in vitro, with some cautions and limitations. Results are discussed and compared with similar studies using nonimaging techniques.

Exposure to respirable-sized quartz or other crystalline silica dusts occurs in mining, construction, manufacturing, and agriculture. It has been estimated (NIOSH, 1983) that over 3 million workers are potentially exposed to crystalline silica in the United States. Silicosis resulting from occupational exposure to quartz dust continues to cause work-related deaths (NIOSH, 2002). Epidemiological studies have shown that the risk for respiratory cancer is increased in long-term respirable crystalline silica-exposed workers in the mineral industry (Bertazzi et al., 1986; Pairon et al., 1991).

Kaolin was selected as a silicate mineral with in vitro cytotoxicity comparable to crystalline silica (Vallyathan et al., 1988), but with low fibrogenic potential. Workers exposed to kaolin clay dusts, typically in pottery and brick manufacture, have an increased prevalence of pneumoconiosis; however, clay mineral-induced disease typically is not as severe as that induced by quartz. Kaolin has not been systematically studied for genotoxic or carcinogenic effects in vitro, in experimental animals in vivo, or in exposed workers (Schulz, 1993).

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After inhalation and deposition in pulmonary alveoli, respirable particles contact the surfactant-rich hypophase on the lung surface and adsorb pulmonary surfactant before interacting with epithelial cells or other lung cells. Pulmonary surfactant is a complex mixture of proteins and lipids which forms the tissue–air interface in alveoli and respiratory bronchioles. It reduces the surface tension at the air–liquid interface, stabilizing the lung from collapse. Phospholipids are a major component of pulmonary surfactant and can reproduce in vitro most of the surface tension-modifying effects of pulmonary surfactant, but protein components of pulmonary surfactant are needed to facilitate the spreading of phospholipids in vivo. Pulmonary surfactant also may modify otherwise toxic interactions of respired particles with cells, at least in short-term assays (Emerson & Davis, 1983). Antioxidants and other components of pulmonary surfactant may be important factors in the expression or suppression of some toxic effects of mineral dusts, but studies have shown that the phospholipid component can have a significant effect on the otherwise prompt membranolytic activity of respirable mineral dusts (Jaurand et al., 1979; Wallace et al., 1985). Phospholipids, such as dipalmitoyl phosphatidyl choline (DPPC), are adsorbed from a lipid dispersion in physiological saline by quartz and kaolin particles and suppress the otherwise prompt in vitro cytotoxicity of the dusts (Wallace et al., 1985). Cell-free studies have shown that an extracellular phospholipase enzyme, PLA2, can digest DPPC from

the dusts, with a subsequent restoration of cytotoxic activity (Wallace et al., 1988, 1992). In vitro studies have found that cellular phagolysosomal digestion and extracellular digestion processes can remove quartz- and kaolin-adsorbed DPPC (Hill et al., 1995), and that in vitro cellular digestive processes can restore quartz toxicity (Liu et al., 1998; Gao et al., 2000, 2001). An in vitro study by Das et al. (2000) used fluorescence measurements of labeled phospholipids to measure loss from mineral particles within cells; results were correlated with chemical analyses of phospholipids species after chromatographic separation.

The purpose of this study was to develop and evaluate a method for measuring the removal of a labeled simulated pulmonary surfactant from respirable quartz and kaolin dusts in cultured rat pulmonary macrophages, and to compare the results to similar studies using toxicological endpoints. Interactions of respired dusts with pulmonary surfactant and subsequent removal processes may be critical in determining whether inhaled particles are cleared from the lung or remain and initiate fibrosis.

MATERIAL AND METHODS

Respirable quartz dust (Min-U-Sil 5; U.S. Silica Corporation, Berkeley Springs, WV) was determined to be 99.5% alpha quartz by x-ray diffraction with 98% of particles smaller than 5 μm mass median equivalent diameter; this is the 50th percentile of measured aerodynamic particle diameters, weighted by mass, determined using the TSI (St. Paul, MN) aerodynamic particle sizer. Respirable kaolin dust (Georgia Kaolin Mills, Augusta, GA) used was at least 95% aluminosilicate with no crystalline quartz detected by x-ray diffraction, with 99% of particles <5 μm diameter. Specific surface areas of the dusts were 3.97 m^2/g for quartz and 13.25 m^2/g for the kaolin, by BET N_2 adsorption measurement. Both dusts were isolated as a $<5\mu$m fraction with a Donaldson particle classifier.

The simulated surfactant phospholipid component was prepared using dioleylphosphatidyl choline (Sigma-Aldrich) with 10% by weight of 2-(4,4-difluoro-5,7-dimethyl-4-bora−3*a*,4*a*-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (β-BODIPY FL-C_{12}-HPC; Molecular Probes, Eugene, OR) in an isotonic NaCl solution. Both lipids were dissolved in $CHCl_3$ and mixed; the $CHCl_3$ was evaporated under N_2, 0.165 *M* NaCl was added, and the mixture was sonicated 10 min at 50 W. The final concentration of mixed lipids was 1 mg/ml. The dusts were suspended in the lipid mixture at 100 mg phospholipids/g dust for quartz and 200 mg/g for kaolin; these are far in excess of measured adsorption isotherm values for these dusts for similar phospholipids such as DPPC (Keane et al., 1990). Incubation of dusts with lipids was done for 2 h at 37°C with continuous mixing. Dusts were centrifuged 10 min at 2000 × g, and the supernatant was discarded, which removes nonadsorbed phospholipids. The dusts were resuspended in complete medium (RPMI 1640, phenol red-free, Invitrogen), with 10% fetal bovine serum (Sigma) and 2% penicillin–streptomycin solution (Invitrogen). The final quartz concentration was 0.1 mg/ml, and 0.025 mg/ml for kaolin; this allowed exposure of cells to approximately the same number of particles, since the average particle size of the quartz was somewhat larger than the kaolin, although both were isolated as a <5-μm fraction with a Donaldson classifier. There were typically 8–20 particles visible within each cell, in the focal-plane recorded image; there were usually slightly more particles per cell in the kaolin-challenged cells. The 1:4 mass ratio approximates the surface area ratio (3.3:1), since the removal/hydrolysis rate would be expected to be surface area related. The 1:4 ratio selected was a compromise between the area ratios and the particle per cell number.

Primary alveolar macrophages were isolated by lavage of Sprague-Dawley male rats, 175–225 g. After injection with an overdose of sodium pentabarbitol, the renal artery was severed, the trachea was exposed and opened, and the lungs were lavaged repeatedly with 5- to 7-ml portions of ice-cold Hanks balanced salt solution, Ca^{2+}- and Mg^{2+}-free (Gibco). Cells were centrifuged 10 min at 600 × g and resuspended in complete medium. Cells were counted using a hemacytometer, and viability was determined by trypan blue exclusion. Sterile 12-mm glass coverslips were added to each well of a 24-well plate (Falcon), and 1 ml of the 4×10^5 cells/ml suspension in complete medium was added to each well. Cells were allowed to adhere to the coverslips for 1 h at 37°C, the medium was removed, and 1 ml of each challenge dust suspension was added, with separate slides for each time point; medium only was added to the control wells. Cells were incubated for times from 1 d through 10 d, and the medium was changed on d 4 and 7. At every time point, the coverslip was tilted with a needle and picked up with forceps, rinsed twice by dipping in phosphate-buffered saline (PBS), and fixed for 20 s in 10% buffered formalin. The coverslips were placed cell side down in a nail polish well 12 mm in diameter on a standard microscope slide containing 20 μl of PBS. The back of the coverslip was blotted dry, and the edges were sealed with clear nail polish.

Microscopic Examination

Slides were viewed as quickly as possible on the same day of preparation, using the Sarastro 2000 confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA). An argon ion laser operated at 20 mW with a 510-nm primary beamsplitter and a secondary 505-nm dichroic filter was used to obtain fluorescence images. A 50-μm confocal aperture was used for scanning, and the fluorescence, reflected, and transmitted images were saved for each field. Objectives were 40× and 60× oil-immersion lenses on a Nikon Axiophot microscope; transmitted images used almost-crossed polarizers to visualize cells, since the cells themselves were unstained. Images were saved as 1024 × 1024 pixel anaglyphs, with the cells and internalized

particles in gray scale and the fluorescent-labeled phospholipids as a green color overlay.

Image Analysis

Images were displayed in Image Space software (Molecular Dynamics), the contrast was optimized for both fluorescence and gray-scale images, and particles within the cells were located by their size and angularity. A region of interest within a particle boundary was selected with a rectangle tool, and a fluorescence intensity profile in that rectangle was saved as a column file for that particle. Thirty particles were measured for each treatment for each exposure day; only clearly visible particles were selected, and typically at least 5 cells were required to score 30 particles, sometimes substantially more. Files were merged, and an overall mean and standard deviation were computed for each treatment. Negative control (unchallenged cells) background levels were measured in the cell cytoplasm. Noninternalized particles were located on the lower focal plane beneath the coverslip, and particle intensity measurements were made to check for label fading and label hydrolysis over time. Results were compared at each time point, using Student's *t*-test, with a Bonferroni correction for overall familywise error rates.

RESULTS

Cell number decreased with time, especially for quartz-challenged cells, but sufficient cells were present for scoring, even at the 10-d time point. Cells were typically larger and more spread for the kaolin-challenged macrophages, and were more rounded and apparently less adherent for the quartz-challenged ones. No cells were scored that did not have an intact membrane and good morphology.

A typical CLSM image field is shown in Figure 1, which shows quartz-challenged macrophages after 3 d of incubation. Quartz particles are clearly evident inside the cells, and many of the particles have lost all or most of the phospholipid coating at this point. The arrows indicate quartz particles that show partial removal of the phospholipid coating; it is evident that these particles show areas of more complete removal than other areas. Cells show mostly a rounded form, suggesting diminished adherence after exposure to quartz, but with a continuous membrane without evident damage. In contrast, Figure 2, which shows kaolin-challenged cells at 10 d, shows cells with less rounded shapes, indicating possible better adherence, which is more typical of macrophages on glass coverslips. Kaolin particles are evident in the cells, with fluorescence still associated with discrete particles at the 10-d time point; quartz-challenged cells were much less numerous at this time point. The square in the lower left corner of both figures is a 2-μm marker.

Results of quantitative fluorescence measurements of phospholipids on particles are shown in Figure 3 for quartz- and kaolin-challenged pulmonary macrophages as well as unchallenged control cells. Plotted values are the average fluorescence intensities across the region of interest inside the boundaries

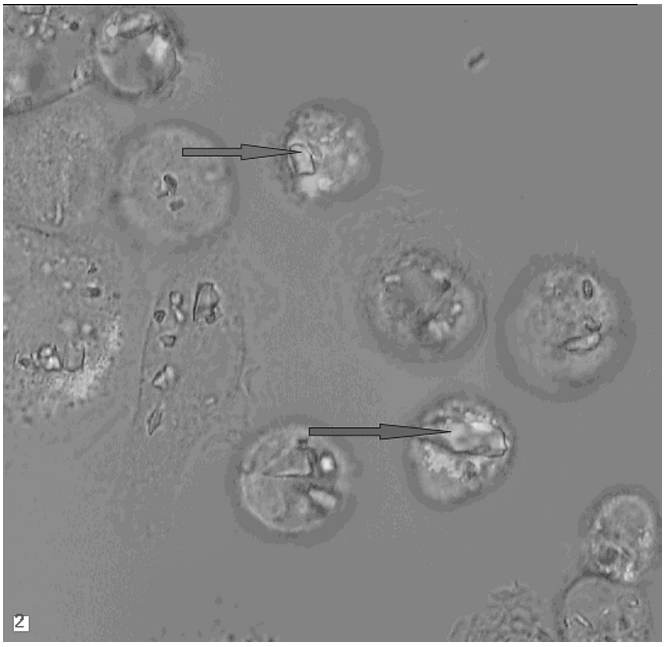

FIG. 1. Photograph (anaglyph) of fluorescent-labeled phospholipids on quartz particles within rat alveolar macrophages after 3 d of treatment in vitro. Green is proportional to fluorescent label intensity; cells are unstained, through almost-crossed polars, 200×. Arrows highlight partially cleared quartz particles. Marker in lower left corner is a reference 2 μm square.

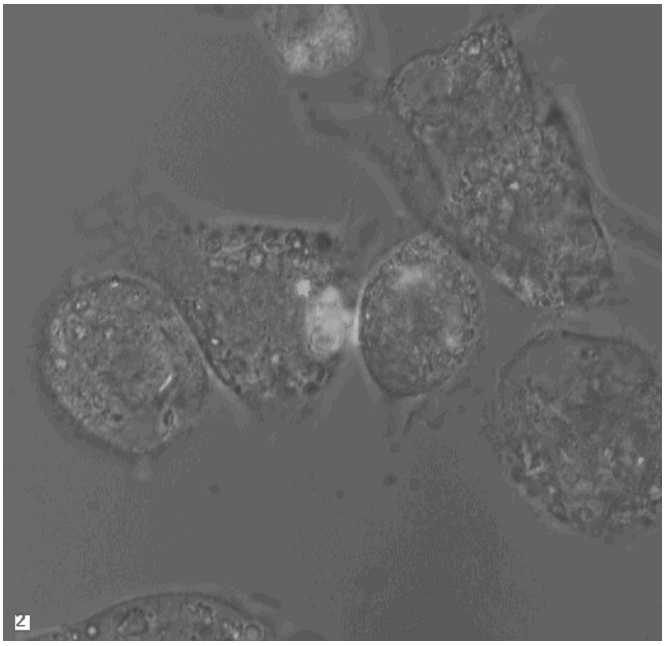

FIG. 2. Photograph (anaglyph) of fluorescent-labeled phospholipids on kaolin particles within rat alveolar macrophages after 10 d of treatment in vitro. Green is proportional to fluorescent label intensity; cells are unstained, through almost-crossed polars, 400×. Marker in lower left corner is a reference 2 μm square.

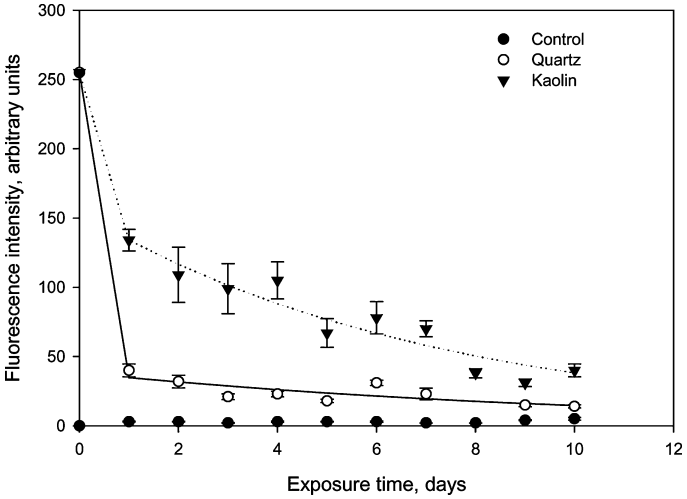

FIG. 3. Graph of fluorescence intensity versus time for phospholipids on mineral particles inside rat alveolar macrophages for times spanning 1 to 10 d in vitro. Controls represent unchallenged cells, with fluorescence measurements taken in the cell cytoplasm. Lines plotted without symbols are predicted values from best-fit four-parameter double exponentials of the form $I(t) = ae^{-bt} + ce^{-dt}$.

of 30 particles at each time point. Error bars represent the standard error of the mean for those observations. All quartz measurements were significantly different from the controls at each time point, as were the kaolin measurements; quartz and kaolin were also different from each other, $p < .001$. Results are also modeled as 4-parameter double-exponential equations, $I(t) = ae^{-bt} + ce^{-dt}$; model equations were $I(t) = 216.6e^{-52.9t} + 38.39e^{-0.097t}$, $R^2 = .995$, for quartz, and $I(t) = 100.95e^{-7.89t} + 154e^{-0.139t}$, $R^2 = .970$, for kaolin.

DISCUSSION

The objective of this study was to develop a study tool to examine how well surfactant-treated quartz and kaolin respirable particles retain adsorbed phospholipids in pulmonary macrophages, and to evaluate the method for possible use in future in vivo studies. The basic hypotheses that have been tested in prior studies in this laboratory are that (1) adsorbed pulmonary surfactant components passivate respirable dusts, (2) dusts are stripped of these substances in vivo, and (3) if surfactant removal rates are comparable to or faster than clearance mechanisms, dusts such as silica would manifest fibrogenic effects, while similar silicate mineral dusts that are not stripped of surfactant components as rapidly would be eliminated by mucociliary clearance or other clearance processes and not manifest fibrogenicity. The phospholipids are adsorbed strongly on the dusts, and are resistant to rinsing; we would expect a removal rate that is area related; exposures were selected to be a compromise between the same number of particles per cell and equivalent surface area per cell.

Previous studies have looked at restoration of mineral dust particle toxicity as a function of time. In a cell-free system using porcine pancreatic phospholipase A2, the rates of surfactant removal and restoration of cytotoxicity were much different for quartz and kaolin, with quartz being much more rapidly stripped of phospholipids and its in vitro cytotoxicity restored (Wallace et al., 1992). Using cultured cells in vitro, the results have been more complex. In a study by Hill et al. (1995), phospholipids were removed from quartz and kaolin at very similar rates inside $P388D_1$ cells, but the cells also expressed an extracellular phospholipase activity that removed phospholipids much more rapidly from quartz than from kaolin. Interestingly, the pH dependence of the two enzyme activities was different, with the activity in the conditioned medium being greater at neutral pH, and the intracellular activity being greater at acidic pH. Several studies in this laboratory have examined phospholipid hydrolysis in vitro and measured the hydrolysis products, lysophosphatidyl choline and fatty acids. Analysis included solvent extraction of lipids, separation using thin-layer chromatography, and quantification using both radiolabeling/scintillation counting and inorganic phosphate analysis (Hill et al., 1995; Wallace et al., 1992). In a study by Gao et al. (2000) using rat primary pulmonary macrophages in vitro, cytotoxicity was restored for DPPC-treated quartz and kaolin particles at about 3 d, but the ability to cause DNA damage, as determined by the single-cell gel electrophoresis (SCGE) assay, was restored for quartz in about 5 d and in about 7 d for kaolin. Labeled surfactant retention versus time was well fit by a double exponential for both dusts. On the order of half (40%) of the fluorescence is removed from kaolin at the more rapid rate, with much slower removal of the remainder as seen between 1 d and 9 d. A double-exponential rate model also fit the removal of DPPC from kaolin in a cell-free system using extracellular phospholipase A2 (Wallace et al., 1992). That model suggested that a bilayer structure of adsorbed DPPC resulted in a double exponential rate of DPPC removal, with toxicity restoration beginning with the slower removal of the second half or inner layer of DPPC from the mineral particle surface. In that cell-free study, a double-exponential behavior also was seen for DPPC removal from quartz dust; however, the second half of the DPPC was digested much more quickly from quartz than from kaolin, suggesting a mineral-specific hindrance for the enzymatic digestion of the DPPC molecules immediately adherent to the mineral surface.

In the current study the quartz behavior differs in that most (85%) of fluorescence is lost rapidly. In a study of apoptosis induction in NR8383 cells in vitro, Gao et al. (2001) demonstrated similar delays in the expression of toxicity by quartz, with DPPC-treated quartz expressing apoptosis after 3 d, but with apoptosis by kaolin only partially restored at about 7 d.

In comparison with toxicity testing for delayed expression of toxicity after surfactant treatment of dusts, the agreement is good for quartz, but kaolin results show a beginning of toxicity before the phospholipids are fully removed, as indicated by this study. This could be due to partial surface restoration that could

allow toxic effects before complete removal of phospholipids from the particle surfaces.

In the current study, the dust–surfactant association was studied directly, as a complementary approach to examining the subsequent toxicity of the dust on cells after the surfactant components (phospholipids) are removed from the dust surfaces. This approach may be important in understanding the initial events in fibrotic lung disease, since other agents and factors, such as the serum used as part of in vitro experiments, as well as surfactant, can suppress or delay toxicological effects (Barrett et al., 1999). That study demonstrated that a specific protein present in serum, apolipoprotein A_1, altered chemokine induction and cytotoxicity in MLE-15 cells in vitro, and that this protein bound selectively to silica. Although the current study used serum in the culturing of the macrophages, pretreatment of the dusts with phospholipids before cell culture might minimize direct serum–dust interactions.

The method just presented has a number of features:

1. Fluorescence intensity is stable with time, as exemplified by the noninternalized particles, which were observed at each time point to be approximately the same intensity as particles at the initial time point. These particles were in a significantly lower focal plane than the cells, and could not have been close enough to be in contact with any cells. Particles that are subjected to the same culture conditions as the internalized particles would additionally indicate losses from chemical hydrolysis or quenching of label in solution, as well as hydrolysis from extracellular phospholipases, which some cells produce (Hill et al., 1995).
2. There was little background fluorescence by the cells or reagents at the wavelength used in this study. It is possible that the low but nonzero fluorescence level in the quartz-treated cells is residual, unhydrolyzed or unquenched label, but the effect is very small and comparable to the negative controls.
3. There was no label fading evident in the single scans used in this study. During method development, however, it was evident that photobleaching was noticeable after three to four scans.
4. Data acquisition is simple and rapid, typically 1 min for a high-resolution image of a field, and only a few seconds to locate and acquire and store an intensity profile from a particle within a cell.
5. Cell staining is not required under the conditions of this study, nor is phase contrast or other specialized optics required.
6. Replicate observations are reasonably precise; typical coefficients of variation are 10–12% in this study.
7. The method makes it unnecessary to extract total lipids, separate polar lipids by chromatography, and analyze by chemical analysis or scintillation counting, as was done in prior studies in this laboratory.

One disadvantages is that at early time points, fluorescence is so strong that it is difficult to image individual particle fluorescence profiles; this makes some of these observations less numerically reliable than later observations, where there is little overlap of fluorescence between the particle and cell cytoplasm. It may be advantageous to reduce the number of particles per cell to address this problem. At time points after 5 or 6 d, the number of viable cells declined significantly for highly cytotoxic dusts such as silica, although there were still adequate scorable cells at 10 d. Cell viabilities were not measured in this study, but parallel wells could be added to quantitatively assess viability in future studies; viability could be determined using the same fluorescence filter set using appropriate fluorescent dyes for viability.

The method also shows promise for use in vivo. In a pilot study, rats were instilled with 20 mg labeled quartz and kaolin; the lungs were removed, inflated with a 1:1 polyethylene glycol/PBS solution, and quickly frozen in isopentane. Ten-micrometer sections were prepared using a cryostat, and unstained sections were examined by the same CLSM techniques. Quartz and kaolin particles were evident 1 d after instillation and were located within the alveolar septa and highly fluorescent. The thickness of the section and the unstained tissue made transmitted-light images of particles and cell boundaries difficult to observe. Additional improvements, such as thinner sections and staining techniques for cell boundaries, need to be perfected.

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