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INTRACELLULAR SURFACTANT REMOVAL FROM PHAGOCYTIZED MINERALS: Development of a Fluorescent Method Using a BODIPY™-Labeled Phospholipid

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Lung surfactant serves as a protective coating when adsorbed on particle surfaces, so its removal or rate of removal in vivo may affect expression of mineral cytotoxicity. Removal of phospholipid surfactant components from the surface of mineral particles ingested by alveolar macrophages (AM) was measured using fluorescence microscopy. Dipalmitoylphosphatidylcholine with a fluorescent label (BODIPY™) substituted for C1–C4 on the second acyl chain (DPPC), was mixed with dioleoylphosphatidylcholine(DOPC) to coat respirable quartz and kaolin particles. Fluorescence from quartz or kaolin particles of 3–4, 5–6 and 8–9 μm size decreased in intensity with increasing ratios of DOPC/DPPC* for the same DOPC concentration of 0.4 mg/ml. There was a direct correlation between fluorescence and residual phospholipid surfactant remaining on particles using phospholipase A2 (PLA_2) digestion in a cell-free system, indicating that the presence of the fluorophore on DPPC did not hinder enzymatic recognition. Lavaged primary AM obtained from male Fischer rats were challenged in vitro with DOPC/DPPC* (10:1 mol:mol) coated particles at 50 μg particles/10^6 cells. In contrast to the biexponential response seen in cell-free experiments, the rate of fluorescence decay from ingested coated quartz or kaolin particles over 7 days was monoexponential, with the same $t_{1/2}$ (41 h) for each dust. This study suggests that the rate of phagolysosomal digestion and removal of the adsorbed surfactant is not a determinant of the different mineral-specific pathogenicities or toxicities of quartz and kaolin, although residual fluorescence remained on particles even after 7–8 days.*

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Alveolar macrophages (AM) are implicated in the initiation of pneumoconioses because they are involved in the initial response of the lung to inhaled particles. Dust particles depositing in the lung acinus first contact an aqueous medium containing surfactant biomolecules, the primary components of which include diacyl lecithin phospholipid, and subsequently are phagocytized by AM. Surfactant adsorption onto the dust particle surface acts as a protective coating, suppressing prompt membranolytic activity and delaying release of free radicals and possibly other inflammatory mediators from AM (Allison et al., 1966; Davis, 1986; Fantone & Ward, 1982; Jaurand et al., 1979; Richards & Curtis, 1984; Takemura et al., 1989). For example, Jaurand et al. (1979) showed that phospholipid coating suppressed hemolysis by asbestos, and previous work in this laboratory has shown that a dipalmitoylphosphatidylcholine (DPPC) coating of freshly sheared or stale quartz dusts reduces superoxide production from alveolar macrophages (Fantone & Ward, 1982; Takemura et al., 1989). When the coated dust particles are sequestered within the phagolysosomes of AM or other pulmonary alveolar cells, lysosomal enzymes can digest adsorbed surfactant and other adsorbed pulmonary alveolar hypophase biomolecules from the dust surface. The exposed dust particle surface is then available to interact with the interior of the phagolysosomal membrane, which is postulated to result in loss of integrity of the phagolysosome and consequent cell stimulation or killing (Allison et al., 1966; Davis, 1986; Fantone & Ward, 1982; Jaurand et al., 1979; Richards & Curtis, 1984; Takemura et al., 1989).

Details of the expression of cytotoxicity by phagocytized mineral particles are not completely understood, and in vitro cytotoxicity bioassays are used to attempt the prediction of in vivo mineral-specific pathogenicity. Wallace et al. (1985, 1988, 1989, 1992) compared respirable quartz (a fibrogenic mineral dust) with kaolin in erythrocyte hemolysis assays and in lavaged AM cytosolic and lysosomal enzyme release assays after dust treatment with DPPC, a primary phospholipid component of pulmonary surfactant. Both dusts were rendered inactive after treatment. Membranolytic activity was partly to fully restored after treatment with phospholipase A_2 (PLA_2), an enzyme normally associated with cellular plasma membranes and lysosomes (Wallace et al., 1989). The results indicated that quartz was more rapidly cleared of DPPC and its lysolecithin hydrolysis product, with a consequently more rapid restoration of hemolytic activity (Wallace et al., 1989, 1992).

This report extends in vitro studies of quartz and kaolin interactions with phospholipid surfactant and phospholipase enzymes, and subsequent particle cytotoxicity (Wallace et al., 1985, 1988, 1989, 1992; Liu et al., 1998), to direct observation of the rates of phospholipid surfactant digestion *within* cellular phagolysosomes in vitro. The objective was to determine if such early interactions might be a factor in the etiology of mineral dust-induced disease. The experiments in this study were divided

into three parts: measurement of fluorescence intensity on coated quartz and kaolin as a function of particle size and incorporation ratio; intracellular surfactant digestion from the surfaces of phagocytized particles; and cell-free surfactant digestion experiments on coated particles using PLA_2.

The experimental approach used was as follows. Respirable crystalline quartz and kaolin were incubated in an aqueous dispersion of fluorescently labeled DPPC (DPPC*) and host phospholipid DOPC. The unsaturated acyl chains of DOPC allowed the accommodation of DPPC* due to their natural fluidity. Fluorescence microscopy was used to visualize the surfactant coated particles and to measure the fluorescence emission from the labeled surfactant. Fluorescence was measured by focusing on individual particles of different sizes in the cell-free experiments or on ingested particle(s) within cells exposed to dust in vitro.

Fluorescence intensity measurements were made in cell-free tests as a function of different ratio loadings of host to labeled phospholipid in order to determine the best ratio to minimize self-quenching of fluorescence on the particle surface. At this ratio, intensity was measured on coated quartz and kaolin particles that had been exposed in vitro to alveolar macrophages for 7 days, or that had been subjected for the same length of time to cell-free PLA_2 digestion. In cell-free experiments, digestion products remaining on particles were determined by inorganic phosphate assay using thin-layer chromatography (TLC).

MATERIALS AND METHODS

Mineral Dusts

Quartz and kaolin clay samples used were from stock samples of respirable-size dusts previously characterized (Wallace et al., 1985). Min-U-Sil, obtained from U.S. Silica Corporation (Berkeley Springs, WV), was quantified by automated x-ray diffractometry and found to be 99.5% respirable α-quartz. About 98% of the particles were <5 μm, with an average specific surface area of 3.97 m^2/g as determined by nitrogen adsorption isotherm measurements. Respirable kaolin dust obtained from Georgia Kaolin Mill (Augusta, GA) was similarly analyzed. About 99% of the particles were <5 μm, with an average specific surface area of 13.25 m^2/g. Particles used were stale; that is, they were aged at least 2 wk prior to preparation for use in these experiments.

Chemicals

(2-(4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (BODIPY™-DPPC, DPPC*) was obtained from Molecular Probes (Eugene, OR). The BODIPY label was attached to the C1–C4 position on the second acyl chain. This fluorescent chromophore has a high quantum yield (0.9) and is not signifi-

cantly pH sensitive in the range 3–10 (Johnson et al., 1991). Dioleoylphosphatidylcholine (DOPC) obtained from Sigma Chemicals (St. Louis, MO) was dissolved in a stock solution of 20 mg/ml in $CHCl_3$. Phospholipase A_2 (PLA_2) was a neutral pH optimum porcine phospholipase obtained from Sigma Chemicals. It was made up of 6.3 mg protein/ml in a suspension of 3.2 *M* $(NH_4)_2SO_4$ with 4788 U enzyme/ml.

Coating of Dusts

The techniques used to coat the dusts were adopted from Wallace et al. (1985, 1988, 1989, 1992). Stock DOPC (200 µl) was diluted using $CHCl_3$:CH_3OH (20:1 by volume) to 0.4 mg/ml. $CHCl_3$:CH_3OH (20:1 by volume, 200 µl) was added to 100-µg vials of DPPC* to make final concentrations of 0.5 mg DPPC*/ml. Different amounts of DPPC* solution were added to aliquots of 1.25 ml DOPC solution (0.4 mg/ml) to make mixtures of 1:10, 1:50, 1:100, 1:400, and 1:1000 (mol:mol) DPPC*/DOPC. The solutions were dried under nitrogen and then vacuum dried. Then 0.165 *M* NaCl solution was added to the dried lipids to make a concentration of 1.0 mg (DPPC* + DOPC)/ml. Nitrogen was bubbled through the emulsion and sonicated with an ultrasonic horn at a power level of 75 W for 10 min. Dry quartz (5 mg) or kaolin (2.5 mg) was vortexed in 0.5 ml emulsion to make surfactant-to-dust ratios of 100 mg (DPPC* + DOPC)/g quartz and 200 mg (DPPC* + DOPC)/g kaolin. These concentrations provided total surfactant in excess of the measured adsorption isotherm values for the 2 dusts of approximately 60 mg DPPC/g quartz and 150 mg DPPC/g kaolin (Wallace et al., 1985, 1988). The dispersions were incubated for 24 h at 37°C, with continuous agitation of the samples in a rotary drum. After incubation, the dusts were washed twice by centrifugation for 10 min at 1500 × g and resuspended in minimum essential medium (MEM) with 10% fetal calf serum (FCS) at a final concentration of 1 mg coated dust/ml.

Fluorescence Particle Calibration Measurements

Quartz and kaolin particles prepared as already described, with different ratio loadings of DOPC/DPPC* (10:1, 50:1, 100:1, 400:1, and 1000:1 mol:mol), were used immediately to determine the optimal loadings and settings for fluorescence microscopy as well as to calibrate the system. A 500-µl dispersion of 50 µg coated dust/ml was added to a chamber mounted on the stage of a Nikon Diaphot TMD microscope connected to a Spex Fluorolog spectrometer. Using a 40× oil immersion objective and ordinary bright-field illumination, the particles were brought into focus. Selected single particles of approximately 3–4, 5–6, or 8–9 µm (or aggregates of smaller particles), as calibrated by a stage micrometer scale, were identified in turn by stepping down the aperture to 10 µm using an adjustable iris (window) to discriminate the region of interest for measurement. The bright-field illumination was then switched off, and excitation light at 488 nm (through a monochrometer diffraction grating) was

directed to the particles using a 510-nm dichroic mirror. The light emitted from the sample was further restricted using a narrow-bandpass filter (530DF30, Omega Optical, Dallas, TX) and directed to a Hamamatsu 552U Opaque photomultiplier tube and photocathode (Hamamatsu Co., Tokyo). Fluorescence intensity was recorded as counts per second for the duration of sample illumination (30 s) and expressed as a percentage above background for 10 randomly selected particles at each size and different loading of DOPC/DPPC*; 3 sets of runs were made. The background was measured separately on similar dispersions of single uncoated quartz and kaolin particles.

In Vitro Cell Fluorescence Experiments

Alveolar macrophages were obtained from male Fischer 344 rats (125–150 g) using pulmonary lavage (Castranova et al., 1979; Myrvik et al., 1961). The animals were obtained from Charles River Breeding Laboratories (Wilmington, MA) and were kept under controlled conditions in AAALAC approved animal quarters for at least 1 wk prior to use. Animals were anesthetized by an intraperitoneal injection using sodium pentobarbital in an amount not exceeding 0.1 g/kg body weight.

Cells obtained from 6 animals for each experiment were pooled and rinsed with Hanks balanced salt solution (HBSS) containing penicillin/streptomycin added at 2% v/v. Cells were counted in a hemocytometer and resuspended in MEM containing 10% FCS to a final concentration of 10^6 cells/ml. Twenty-five millimeter glass cover slips were autoclaved and placed in 35 × 10 mm petri dishes. Approximately 10^6 cells in 1 ml MEM + 10% FCS were placed in each dish and were allowed to plate onto the cover slips for at least 1 h. A 50-µl dispersion of 10:1 (mol:mol) DOPC/DPPC* coated quartz or kaolin particles (1 mg coated dust/ml MEM + 10% FCS) was introduced into each dish to obtain a concentration of 50 µg coated particles/10^6 cells, and the dishes were incubated at 37°C in an environment of 5% CO_2. At time intervals of 6, 24, 48, 72, 120, 144, and 168 h after exposure to particles began, cover slips were removed, mounted in a Teflon dish on the stage of the fluorescence microscope, and bathed in 500 µl of MEM + 10% FCS. Fluorescence intensity was measured by focusing on an area of a cell using a window size to discriminate 3–4 µm size particles only; in some cases the region of interest may have contained a few agglomerated smaller particles.

Trypan blue exclusion tests were used to determine cell viability of the initial total cell population, and then on adherent cells remaining on cover slips at 72 and 168 h after exposure to the dusts. Fresh medium was introduced every 72 h to maintain cell viability and to remove unphagocytized particles. Mean intensity was expressed as percent above background for at least 20 cells selected at random from different areas of the cover slip at each time point. The background consisted of similar readings taken on *uncoated* particles within 20 cells under similar conditions at

24, 48, and 72 h in order to account for possible autofluorescence effects; these readings were virtually identical. Five sets of experiments were performed and data reported as mean ± SE.

Cell-Free PLA_2 Digestion Experiments

DOPC/DPPC* (10:1, mol:mol) coated quartz or kaolin particles were incubated in test tubes containing PLA_2 dissolved in 0.165 *M* NaCl with 2 m*M* $CaCl_2$, at a pH between 6.5 and 7.0 and an enzyme concentration of 0.147 U PLA_2/24 mg quartz or 7.87 U PLA_2/24 mg kaolin. Approximately 50 times the PLA_2 activity was applied to kaolin as to quartz (about 20× on a surface area basis) to obtain comparable rates of digestion. Each test tube, containing 5 mg coated dust, was incubated for periods of 2, 24, 72, and 144 h in a rotary drum incubator. The supernatant was removed by vacuum suction and the samples were washed twice with equal volumes of 0.165 *M* NaCl supplemented with 2 m*M* ethylenediamine tetraacetic acid (EDTA) to quench enzymatic digestion. Particles were separated by centrifugation for 10 min at 1000 × g. Sterile H_2O (5 ml) was added after removing the supernatant to make a final concentration of 1 mg coated dust/ml. An aliquot of the sample diluted to 50 μg coated dust/ml was prepared for fluorescence measurements, as described for cell fluorescence experiments, and the remainder was used for analysis of residual surfactant on the particle surface as described later.

Inorganic Phosphate Analysis of Surfactant Digestion Products

The amount of phospholipid still adsorbed to the samples was quantified as DPPC* plus DOPC (undigested products) or as lyso-DPPC* plus lyso-DOPC (digested products). The samples (three separate runs for each dust at each time) were vacuum dried overnight and eluted with 250 μl chloroform:methanol (2:1 by volume). Twenty-microliter aliquots were separated by thin-layer chromatography (TLC) on silica gel G, 250-μm plates using 60:35:5 (v:v:v) chloroform:methanol:water. The bands for (DPPC* + DOPC) and (lyso-DPPC* + lyso-DOPC), verified by DPPC, DOPC, and lyso-DPPC standards, were scraped from the plates and quantified for inorganic phosphate content by the method of Bartlett (1959). Positive controls for inorganic phosphate analysis (corresponding to eluted residual surfactant on 5 mg dust per sample) consisted of 10:1 (mol:mol) DOPC/DPPC*-coated quartz and kaolin not exposed to PLA_2 digestion, after incubation times of 0, 24, and 144 h. Negative controls were inorganic phosphate measurements on blank silica gel lanes from the TLC plates and on uncoated quartz or kaolin subjected to the same treatments.

RESULTS

Mixtures of DOPC/DPPC* were used as a surrogate for pulmonary surfactant to analyze phagolysosomal digestion of respirable particle-adsorbed

surfactant. In contrast to radiolabeling studies, using a fluorescent probe permitted noninvasive observation of measurements of the digestion process in cultured cells. The reason for using DOPC as a host phospholipid was twofold. DOPC being an unsaturated phospholipid allows the accommodation of the BODIPY-DPPC molecule when adsorbed to a surface, due to its fluidity (lower viscosity) at physiological temperatures. In addition, DOPC was useful, as a host phospholipid because the fluorescence of BODIPY-DPPC adsorbed on a surface at high concentrations would be self-quenched if used alone. In calibration experiments fluorescence intensity as a function of the ratio of DOPC/DPPC* coated on the surface of quartz and kaolin particles was measured in cell-free experiments for three different particle size ranges (3–4, 5–6, and 8–9 μm). For both quartz and kaolin, immediately after particles were prepared, fluorescence decreased with a corresponding increase in the ratio of DOPC/DPPC* on the surface (Figure 1). For quartz or kaolin particles of 3–4 and 5–6 μm, the intensity decreased from 25–30% above background at a loading of 10:1 (mol:mol) DOPC/DPPC* to only about 1% above background at a loading of 1000:1. However, for the 8–9 μm size particles the response was significantly different. At 10:1 (mol:mol) DOPC/DPPC* the fluorescence for kaolin (109%) was much higher than for quartz (12%). The much higher fluorescence for kaolin particles was likely due to kaolin being mostly aggregates of smaller particles. Due to the significant difference between 8–9 μm quartz and kaolin particles, all other measurements were taken on particles in the size range of 3–4 μm only, at a 10:1 loading only, in order to ensure initial particle uniformity in fluorescence. The intensity did not change with time for particles stored in solution for up to 2 wk, and vortexing or mechanical agitation did not cause the lipid to dissociate from the particle surface.

Fluorescence decay associated with intracellular digestion of coated quartz and kaolin particles over 7 days is shown in Figure 2. For quartz, the intensity decreased from approximately 25% above background at 6 h to 1.4% at 168 h. For kaolin, the intensity decreased similarly from approximately 33% above background at 6 h to 2% at 168 h. Fitting a single exponential curve to the data for each type of dust resulted in essentially identical rates of fluorescence decay, with a half-time of about 41 h for both quartz and kaolin. To determine the fate of residual DPPC* at longer times, a few fluorescence measurements were made at 8 and 10 days after particles were fed to cells. For quartz particles within AM at 192 h and 240 h, the intensity decreased to 1.0 and 0.8% above background, while for kaolin particles, values of 2.0 and 1.8% above background were similar to the 7-day value.

Trypan blue exclusion assay in conjunction with cell counts using a hemocytometer showed that 95% of the initial pooled cell population (5 runs) was viable, with approximately 78% of the adherent population remaining viable after either 72 or 168 h.

In order to correlate surfactant digestion from the particle surface with

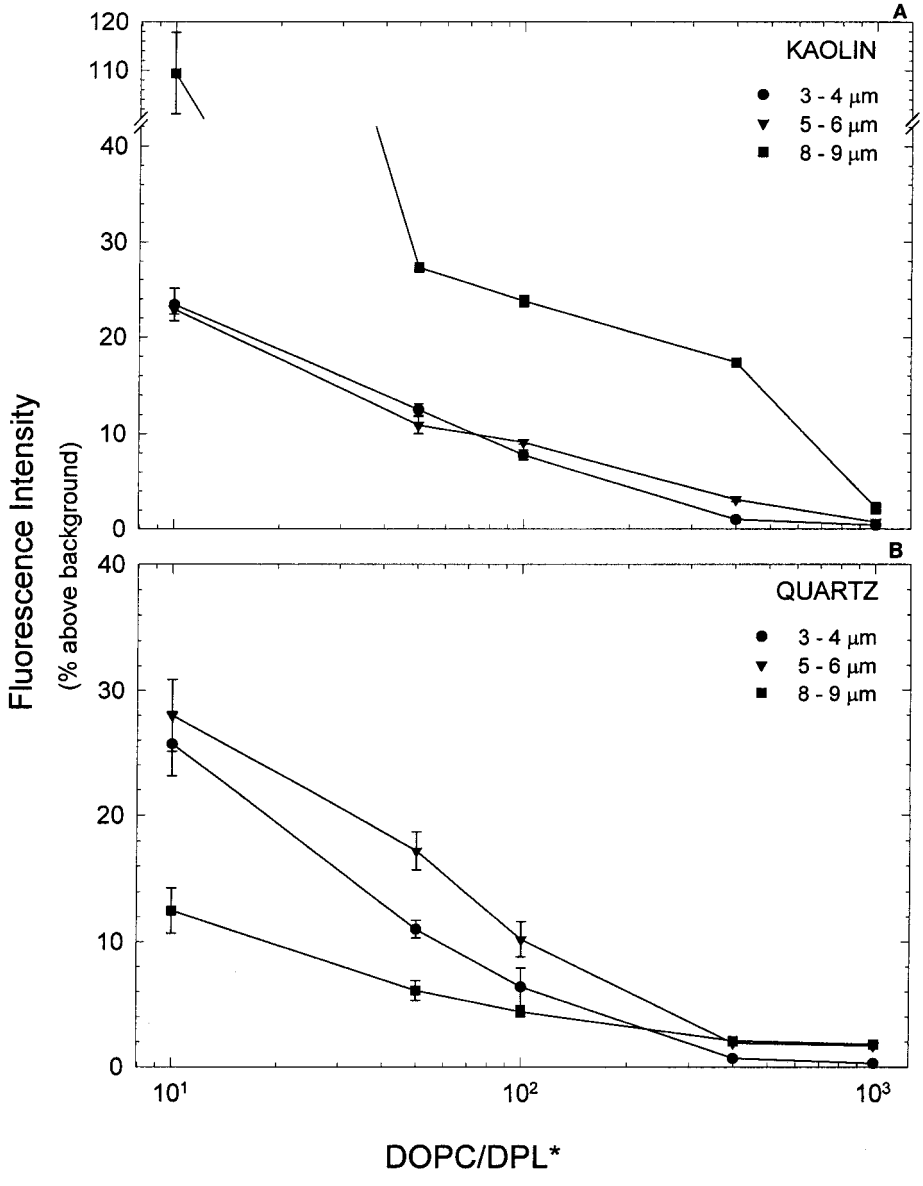

FIGURE 1. Mean fluorescence intensity (± SE) as a function of particle size and ratio loading (mol:mol) of unlabeled dioleoylphosphatidylcholine (DOPC) to fluorescently labeled dipalmitoylphosphatidylcholine (DPPC*) on the surface of (A) kaolin and (B) quartz particles. Measurements were taken just after coating of particles with surfactant; each data point represents at least 10 particles.

fluorescence decay, cell-free digestion experiments were performed on 10:1 (mol:mol) DOPC/DPPC*-coated quartz and kaolin particles exposed to PLA_2. The enzyme concentrations used corresponded to those determined by Wallace et al. (1989, 1992) to allow sufficient quantifiable surfactant to remain on the particle surface up to at least 48 h after incuba-

tion. Data were normalized to the amount of initial adsorbed surfactant per unit surface area of the minerals.

Residual surfactant (nanomoles) on particle surfaces was analyzed as either undigested (DPPC* + DOPC) or digested (lyso-DPPC* + lyso-DOPC) products using inorganic phosphate analysis of TLC bands of eluted residual surfactant (Figure 3). At 2, 24, 72, and 144 h after digestion with PLA_2, approximately 70, 53, 40, and 33% of the initial surfactant coating remained on quartz particles (Figure 3). There was a concomitant large increase in the residual lyso-products eluted from particles. As a percentage of initial surfactant, there was a 43% increase at 2 h and a 55% increase at 24 h after digestion. Thereafter the eluted products were about 40% of the initial surfactant at 72 and 144 h (Figure 3). The analysis of residual surfactant on kaolin showed that 41% of the initial amount of undigested surfactant remained on the surface after 2 h of incubation with PLA_2 (Figure 3). This was followed by a slower phase of digestion, with 19, 9, and 6% of the initial undigested surfactant remaining on the

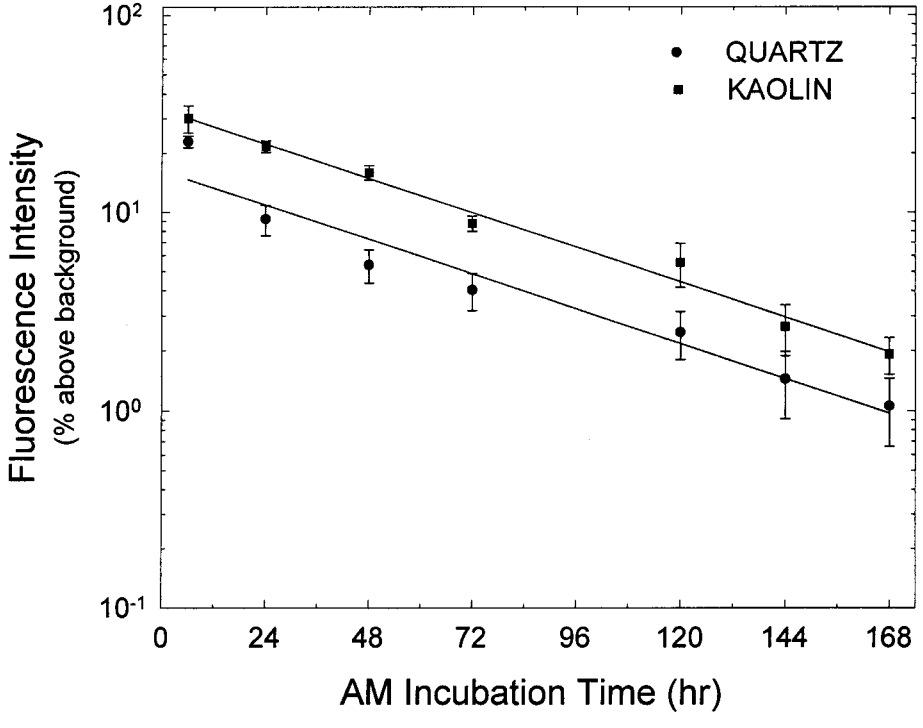

FIGURE 2. Rate of cell digestion of 10:1 (mol:mol) DOPC/DPPC* coatings from particles within phagolysosomes for a dust loading of 50 μg per million cells. Fluorescence intensity (data points) as a function of AM incubation times were fit to exponential decays (solid lines); r^2 values were 95% for quartz and 99% for kaolin. The rate constants (h^{-1}) were –0.01677 ± 0.002 for quartz and –0.01683 ± 0.001 for kaolin. Each point represents the mean (± SE) for at least 20 cells containing particle(s) from 5 different experiments of 6 animals each. The 6-h time point for quartz might suggest a more rapid initial removal of surfactant.

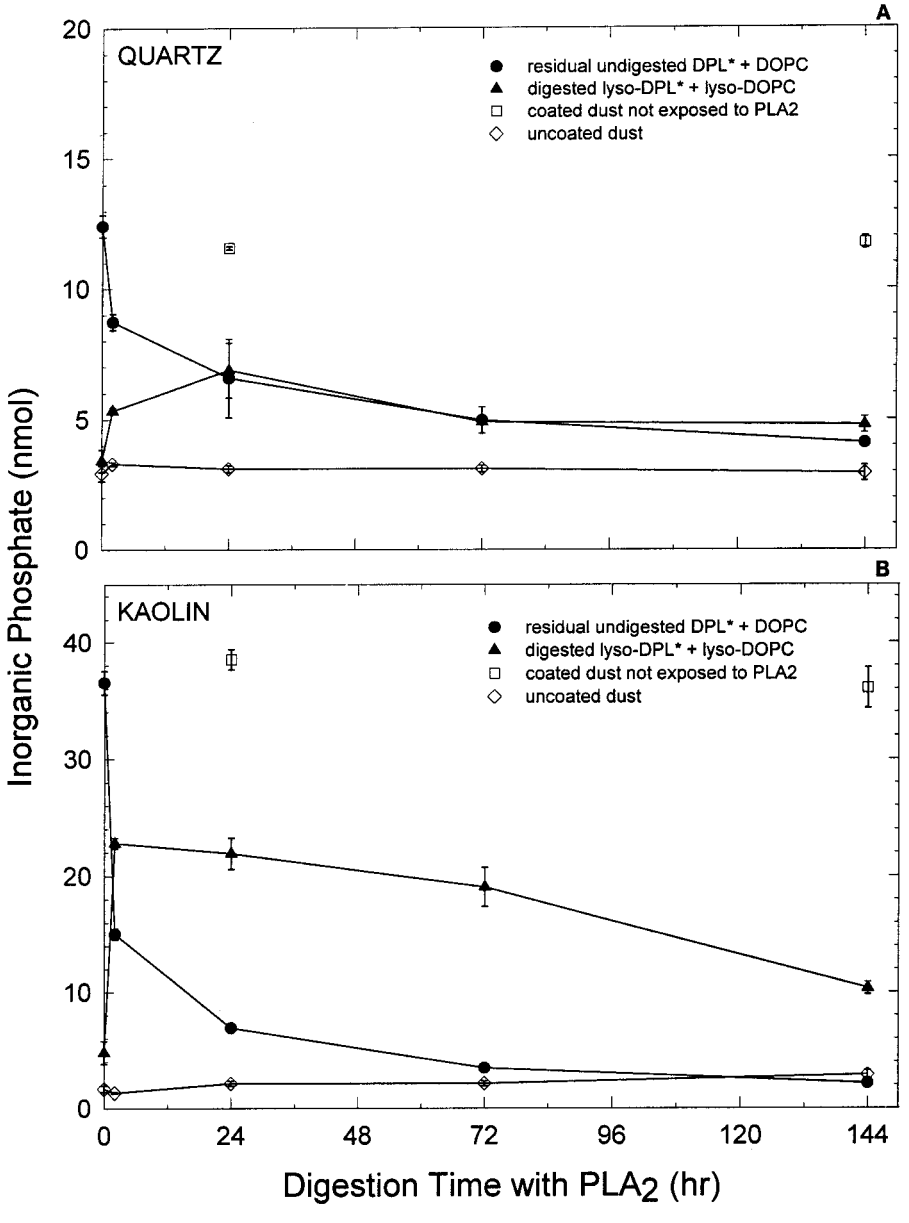

FIGURE 3. Inorganic phosphate analysis of eluted surfactant from TLC bands following cell-free digestion of 10:1 (mol:mol) DOPC/DPPC* coating from (A) quartz using PLA_2 (0.147 U PLA_2/24 mg) or (B) kaolin using PLA_2 (7.87 U PLA_2/24 mg). Products in nmol phosphate (mean ± SE) are identified as residual undigested (DPPC* + DOPC) or digested (lyso-DPPC* + lyso-DOPC). Positive controls (□) are eluted surfactant from coated particles not exposed to PLA_2; negative controls (◇) are blank silica gel, uncoated particles, and lyso-product bands corresponding to the positive controls.

surface at 24, 72, and 144 h, respectively. There was a concomitant very large increase in the lyso-phospholipid digestion products measured on surfactant eluted from kaolin particles. At 2 and 24 h after incubation with PLA_2, the lyso-products corresponded to increases of 62 and 60% of the initial surfactant on the surface, thereafter decreasing to 52 and 28% at 72 and 144 h, respectively.

Positive (dust not exposed to PLA_2 digestion) and negative (dusts without surfactant or silica gel lanes alone) control data measured at different times also are shown in Figure 3. The mean (± SE) of the positive controls measured in nanomoles Pi from inorganic phosphate analysis were 11.7 ± 0.1 and 37.3 ± 1.2 on quartz and kaolin, respectively. The average combined negative control values for each particle were 3.1 ± 0.1 and 2.0 ± 0.3, respectively. These controls clearly showed that digestion (or elution) of phospholipid species from particles did not occur in cell-free systems unless PLA_2 was present.

Fluorescence measurements were made concurrently on aliquots of particle suspensions used for the surfactant inorganic phosphate analysis. Readings were taken on single particles of quartz and kaolin of 3–4 μm size (Figure 4). The intensity of quartz particles decreased very rapidly to

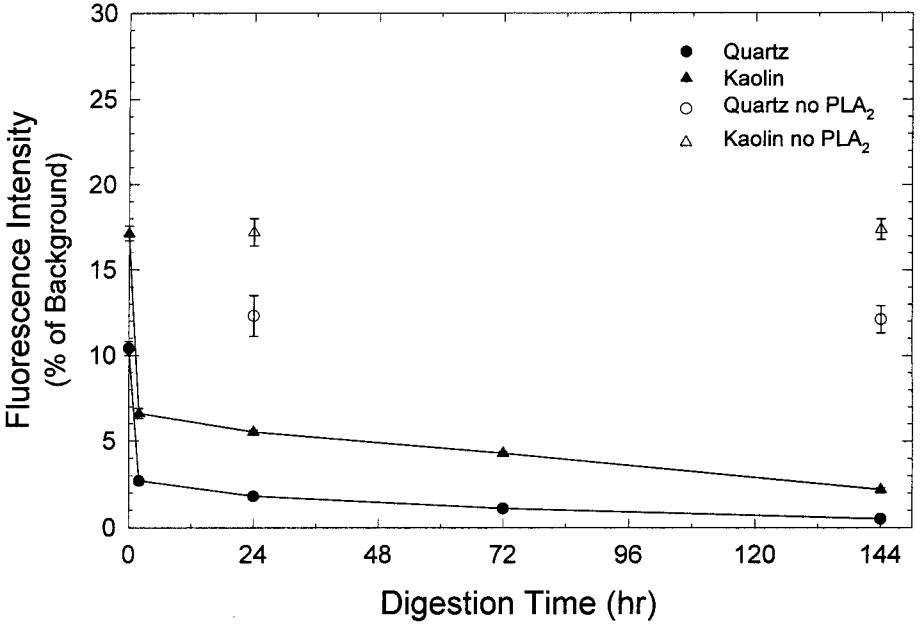

FIGURE 4. Mean fluorescence intensity (± SE) of single 3–4 μm size quartz and kaolin particles taken from the same aliquots used for inorganic phosphate analysis in Figure 3. Each point represents at least 20 particles. There was a very rapid decrease after cell-free digestion started, followed by a much slower decay afterward. The intensity of particles without PLA_2 digestion did not change with time.

only 26% of the initial value 2 h after incubation with PLA_2, again followed by a slower rate of decay corresponding to measured values of 17, 11, and 5% at 24, 72, and 144 h, respectively. On kaolin particles, the fluorescence decreased rapidly to 39% of the initial value within 2 h after incubation with PLA_2, followed by a slower rate of decay corresponding to values of 32, 25, and 13% at 24, 72, and 144 h, respectively. The initial fluorescence for either quartz or kaolin was unchanged after 24 or 72 h without digestion (Figure 4).

When combined with inorganic phosphate analysis, the results showed there was a greater retention of fluorescence on kaolin particles compared to quartz for a given retention of undigested surfactant (Figure 5). By 144 h, only 5% of the initial undigested phospholipid remained on the kaolin surface, corresponding to an intensity approximately 13% above background. In contrast, corresponding values for quartz were approximately 34% undigested phospholipid remaining with an intensity only 4% above background.

DISCUSSION

Dust-induced lung diseases involve a series of processes that originate with inhaled mineral particle deposition in the pulmonary respiratory

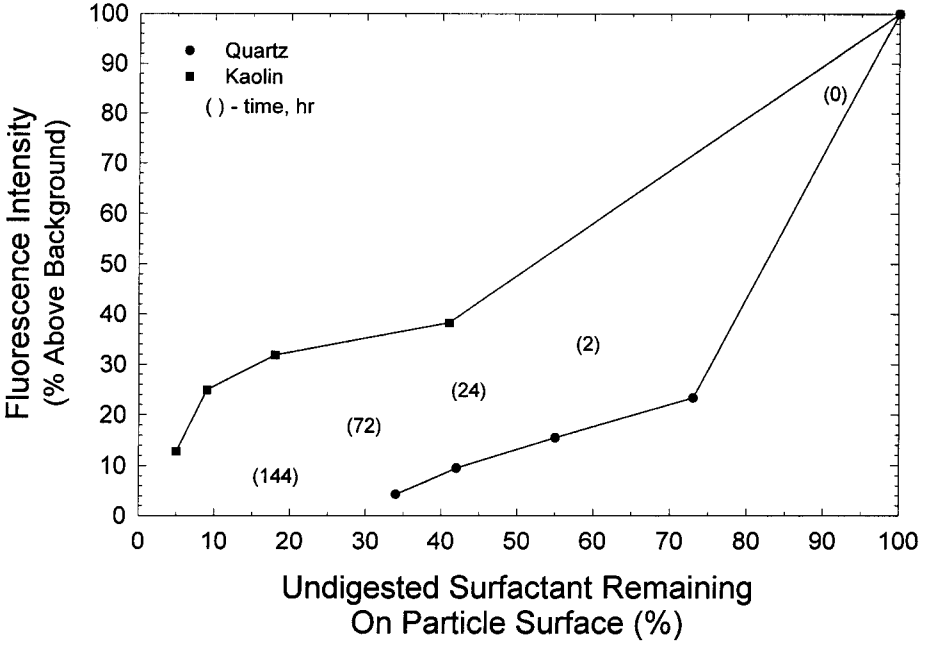

FIGURE 5. Relationship between mean fluorescence intensity and residual surfactant remaining on particle surfaces over 144 h of PLA_2 digestion. Data are expressed as a percentage of initial fluorescence intensity or undigested surfactant adsorbed to the particles; values in parentheses are the incubation time in hours.

bronchioles and alveoli, and subsequent interaction with defense mechanisms of the lung (Allison et al., 1966; Davis, 1986; Reiser & Last, 1979). Silica and silicate particles can adsorb components of the alveolar hypophase, including phospholipid surfactant, suppressing the potential prompt membranolytic activity of the particles. Particles are phagocytized by AM or otherwise cleared up the airways or into the lymphatic system, or are sequestered in the interstitium beneath the alveolar epithelium. After phagocytosis, particles may be contained in phagolysosomes or secondary lysosomes within the cell, and be subjected to contact by lysosomal enzymes. These lysosomal enzymes include phospholipases that can digest components of pulmonary surfactant. Removal of the particle surface-adsorbed surfactant and other biomolecules is postulated to restore surface bioavailability and cytotoxic activity to the particle now within the cellular phagolysosome, resulting in damage or cell death (Allison et al., 1966; Takemura et al., 1989). Thus, removal or perhaps the rate at which this coating is digested from dust surfaces within phagolysosomes of AM might be a factor in the expression of dust cytotoxicity. This phenomenon may explain why the cytotoxicity of kaolin (a layered aluminosilicate clay) is comparable to silica (α-quartz) measured under the same conditions in vitro but does not approach the potency of quartz (a fibrogenic mineral dust) for disease induction in vivo (Wallace et al., 1985).

The central objective of this study was to develop a method to visualize and quantify surfactant digestion from the surfaces of mineral particles, specifically quartz and kaolin, within the phagolysosomes of alveolar macrophages. Pulmonary surfactant is composed of various types of lipids. Phospholipids constitute 78% by weight of pulmonary surfactant, with saturated phosphatidylcholine (45 wt%) and unsaturated phosphatidylcholine (20 wt%) being the primary components (Hagwood, 1991). To achieve the objective, fluorescently labeled DPPC*, a primary phospholipid component of surfactant, and an unsaturated host phospholipid (DOPC) were used to coat the surfaces of respirable quartz and kaolin particles that subsequently were fed to alveolar macrophages.

DOPC/DPPC is considered a reasonable approximation to natural lung surfactant. Importantly, there were several notable characteristics of the surrogate surfactant that were useful in this study. The natural fluidity of the DOPC acyl chains allowed the DPPC* molecules to coexist in a typical bilayer arrangement on the particle surface, and the dilution effect of using DOPC prevented self-quenching of the fluorescence by the local association of a high concentration of DPPC* molecules (Johnson et al., 1991). Also, the partition coefficient for the fluorescent probe (BODIPY) in fatty acids is much higher for the more fluid DOPC bilayers than in gel-phase DPPC alone; the approximate threefold preference for fluid-phase lipids is typical of amphophilic probes with aromatic fluorophores (Johnson et al., 1991). Finally, the characteristics of the parent fluorophore, green fluores-

cence emission with high quantum yield and low environmental sensitivity, were retained when incorporated in the phospholipid (DPPC*).

In initial development work with the BODIPY probe using liposomes (0.01–0.1 μm), Johnson et al. (1991) and Pagano et al. (1991) observed progressive replacement of the usual green emission by a distinct red emission, probably due to excimer formation, at high incorporation ratios of 10:1 and 5:1 (mol:mol) DOPC/DPPC*. As the amount of DPPC* making up the liposomes was decreased, the intensity increased and was particularly noticeable at ratios of 1000:1 (mol:mol) DOPC/DPPC* (Johnson et al., 1991). However, in this study, using incorporation ratios of 1000:1 to coat quartz and kaolin particles (~5 μm), the initial fluorescence intensity before any treatment was only about 1–2% above background. The reason for this observation was that, for the same incorporation ratio, the DPPC* molecules could distribute over a considerably larger particle surface area compared to liposomes. Thus the larger surface area of the particles, coupled with the fluidity of the DOPC, allowed a higher number of DPPC* molecules to be accommodated without local self-quenching effects. This was especially true with the larger kaolin particles because they were agglomerates of smaller ones. Therefore, an incorporation ratio of 10:1 (mol:mol) DOPC/DPPC* was used, since the initial fluorescence on single surfactant-coated quartz and kaolin particles was the highest (~25–30% above background) (Figure 1).

Intracellular digestion experiments showed that the 10:1 (mol:mol) DOPC/DPPC*-coated quartz and kaolin particles were readily phagocytized by primary lavaged alveolar macrophages. Internalized particles were well-defined fluorescent green spots against a somewhat paler green background within the cell cytoplasm. The fluorescence intensity decreased with time following phagocytosis with respect to controls consisting of uncoated phagocytized particles in AM (Figure 2). At later times, fluorescence was observed to increase throughout the cytoplasm of the cells as well. One possibility for this may be that fluorescent lipid digestion products from the particle surfaces were being transported from the phagolysosomal compartments to other areas of incorporation within the cell. Similar intracellular lipid transport has been observed by Pagano et al. (1991) using a BODIPY-labeled ceramide to study cell trafficking. No release of fluorescence external to the macrophage was apparent, unless there was cell lysis, but this was not quantified in this study.

Since mineral specific differences might be expected based on known biological responses, faster removal might be anticipated from quartz surfaces than from kaolin surfaces. However, the equivalent rates of decrease of fluorescence from quartz and kaolin in AM over a 7-day period indicated that the labeled surfactant removal by intracellular processes is not mineral surface specific (Figure 2). This was in contrast to the mineral-specific differences seen in cell-free enzymatic digestion measurements (Wallace et al., 1985, 1988, 1989, 1992) as well as in the cell-free studies

reported here, which showed a double-exponential behavior. This effect has been attributed to the bilayer structure of adsorbed surfactant (Wallace et al. 1989, 1992). While this behavior was not apparent in the in vitro intracellular kaolin tests, this is not as clear in the short-time quartz data. Specifically, while a single exponential curve fits the quartz data overall very well, the first time point at 6 h is much higher than predicted (Figure 2). Thus a biexponential surfactant removal, characteristic of a bilayer, can not be ruled out completely for quartz without further measurements at very early times.

Thin-layer chromatography of the digestion products eluted from the surface of the particles at various times up to 6 days after incubation with PLA_2 showed a clear differentiation of residual undigested and digested products. As enzyme digestion progressed, particle-adsorbed undigested surfactant decreased, so that after 144 h most but not all of the residual surfactant appeared to have been sloughed off the surface. Corresponding fluorescence measurements showed a gradual decay as the digestion progressed, indicating the loss of fluorophore-containing molecules. These cell-free experiments indicated that enzymatic digestion of DPPC* by PLA_2 is not hindered by the presence of the fluorophore, since the decay in fluorescence intensity approximately tracked the amount of surfactant remaining on the particles, as measured by the inorganic phosphate assay. Thus, the labeled surfactant was indicative of the removal of non-fluorescent-labeled phosphate-bearing surfactant.

The assay of TLC bands for inorganic phosphate adapted from Bartlett (1959) does not account for nonphosphate fluorescent species, such as the free-fatty-acid digestion products without the phosphate head group (but containing the fluorophore), that might still be associated with the surface of the particles after enzymatic digestion. Thus, while the PLA_2 digests phospholipid species coated on a particle surfaces, the overall phosphate mass balance did not close exactly because other residual lipids were inherently excluded using this analysis. More refined technologies, such as nuclear magnetic resonance (NMR) analysis, could help to identify and quantify species more accurately.

Even 6–10 days after digestion, some residual fluorescence was still detected on the coated particles within AM (Figure 5). There appeared to be a slightly greater retention of nonphosphate fluorescent species on kaolin after cell-free digestion with PLA_2 at 144 h. This indicated the possible presence of BODIPY-labeled free fatty acid still adhering on the surface of the particles.

The higher fluorescence intensity observed on kaolin than on quartz particles (Figure 5) was explained as follows. The surface area/volume ratio is about three times greater for kaolin. It is possible, therefore, that DPPC* on the quartz surface was self-quenched (at 144 h) due to DOPC digestion causing sequestration of DPPC* molecules on the more angular (crystalline) surfaces of quartz. However, while a lower percentage of undigested sur-

factant remained on kaolin, it was more easily spread over a larger surface area on the more amorphous particle. Therefore, less quenching and higher intensity were observed on the kaolin particles.

The time course of intracellular digestion using BODIPY-labeled DPPC in situ has been corroborated in other experiments examining the relative cytotoxic expression of phospholipid surfactant-coated or native quartz on primary lavaged pulmonary alveolar macrophages. Cytotoxic effects on DNA damage were measured from whole-cell lysis using a gel electrophoresis "comet" assay (Liu et al., 1998). Fluorescence activity was shown to decrease to background levels within a 1-wk period. This was similar to the time course of digestion seen with radiolabeled DPPC from quartz particles in a P388D1 cell line (Hill et al., 1995), and to quartz cytotoxicity observed in rat macrophages treated with Survanta, a commercially available surfactant (Antonioni et al., 1994). Results from these studies suggested that the prophylactic effect of adsorbed surfactant is related directly to its rate of removal from the quartz surface as a function of phagolysosomal digestion (Liu et al., 1998).

This study has examined an early step in the interaction of alveolar macrophages with surfactant-coated quartz and kaolin particles using a fluorescently labeled surfactant molecule (DPPC*). The purpose was to identify events that may control mineral-specific differences in the pulmonary disease-producing potential between silica and silicate dusts. It appears that the initial step of removal (or rate of removal) of adsorbed phospholipid surfactant by enzyme digestion might not be critical to the recognition of toxicologic differences between quartz and kaolin. However, it is possible that residual free fatty acid digestion products adhering to the surface of the particles even after long-term phagolysosomal digestion might still act as a protective coating, possibly delaying the expression of cytotoxicity.

In summary, this study suggests that there does not appear to be any differences in time of removal or toxicity between dusts in AM. However, a more in-depth examination is needed of the nature of the residual lipid species remaining associated with particle surfaces after digestion, to determine whether surfactant–particle surface interactions provide a basis for the ultimate difference in cytotoxic expression between quartz and aluminosilicates.

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