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IN VITRO BIOLOGIC TOXICITY OF NATIVE AND SURFACE-MODIFIED SILICA AND KAOLIN

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An in vitro study of the biologic responses of surface-modified and native silica and kaolin was made to provide comparative information on the supression of cytotoxicity by pulmonary surfactant. The release of alveolar macrophage cytoplasmic enzyme, lactate dehydrogenase (LDH), and lysosomal enzymes β-N-acetylglucosaminidase (β-NAG) and β-glucuronidase (β-GLUC) and sheep blood-cell hemolysis were monitored as indicators of cell membrane damage and cytotoxicity. Surface modification of silica and kaolin with dipalmitoyl lecithin (DPL) resulted in complete abrogation of cytotoxicity of both minerals. These findings indicate that surface modification of minerals with different adsorption properties by pulmonary surfactant generally lessens their prompt adverse effects.

INTRODUCTION

Acute and chronic silicosis are well-characterized clinical and pathologic entities resulting from the inhalation of crystalline silica (Morgan and Seaton, 1984). Kaolin, a layered aluminosilicate clay frequently mined and used in manufacturing, contains tetrahedral silica alternately layered with alumina octahedra. Kaolin is relatively inert biologically, and distinctly greater pulmonary burdens are seen in patients with kaolinosis than are seen in patients with silicosis. Crystalline silica (quartz) induces a significantly greater degree of fibrosis in exposed animals in vivo than does kaolin (Hamilton and Hardy, 1974; Parkes, 1982; Hunter, 1978; Sheers, 1964; Warraki and Herant, 1963; Lynch and McIver, 1954; Lapenas et al., 1984).

This difference in pulmonary disease associated with the two types of dust is not reflected by in vitro cytotoxicity as determined by pulmonary macrophage bioassays or erythrocyte hemolysis. Cytotoxicity studies using crystalline silica and kaolin of similar size fractions show comparable bioassay response on a mass basis. These cellular bioassays, therefore, do not correlate with the relative in vivo pathogenicity of silicates.

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The reasons for this discrepancy between in vitro and in vivo biologic behavior is not understood but may be related to biologic reactions associated with surface properties of dust.

Several studies have evaluated the effect of phospholipids on dust cytotoxicity, determining that lysis might result from dust surface interaction with the lecithin of the bilayer lipid cell membrane (Jurand et al., 1979; Emerson and Davis, 1983; Adamis and Timar, 1980). It should be noted that the pulmonary alveoli and respiratory bronchioles are coated with a hypophase surfactant, the primary constituent of which is the phospholipid diacyl glycerophosphoryl choline or lecithin (Clements et al., 1970). The adsorption of dipalmitoyl lecithin from physiological saline emulsion by respirable sized kaolin dust has been demonstrated and quantified (Wallace et al., 1975).

A more appropriate bioassay system to determine the fibrogenic potential of silicate dusts might include a representation of the contact and coating of pulmonary surfactant on a respired particle. Questions posed by pulmonary surfactant interaction with respired dusts are: (1) does such exposure modify the direct and immediate cytotoxicities of the dusts, and (2) do such modified cytotoxicities better correlate with known pathogenicities; or (3) do such coated dusts exhibit further modified cytotoxicities after pulmonary macrophage phagocytosis and possible phagolysosomal enzyme digestion of the surfactant coating, and (4) do those cytotoxicities correlate with the disease-producing potential of dusts?

This report addresses the first two questions, providing detailed information on the suppression of silica and kaolin cytotoxicity by a component of pulmonary surfactant.

MATERIALS AND METHODS

Crystalline silica (Min-U-Sil) used in this study was obtained from Pennsylvania Sand Glass Corporation and was fractionated using a Donaldson classifier. Eighty percent of the fraction collected for this study was < 5 μm particle diameter as estimated by automated image analysis (Stettler et al., 1983). The silica was at least 98.5% pure as determined by X-ray energy spectrometric (XES) analysis (Stettler et al., 1983). The crystalline form of silica used was determined to be alpha quartz by X-ray diffractometry using a Phillips automated X-ray diffraction system. It had a median area equivalent diameter of 1.24 μm. The specific surface area of the size fractionated silica was 3.97 m^2/g as determined on 1.0044 g silica by standard Bruneur-Emmett-Teller (BET) nitrogen adsorption isotherm measurements (Bruneur et al., 1938) using a Digisorb 2500 specific surface area/pore volume distribution unit. Kaolin used was obtained from Georgia Kaolin Mills, Augusta, Ga. It was similarly size-fractionated to obtain a fraction of which 90% was < 5 μm particle diameter. This kaolin was at least 96% pure, had a surface area of 13.25

m^2/g, and contained no crystalline silica, as determined using the same methods and units used for the silica.

Stock dipalmitoyl lecithin (DPL) emulsions were made by sonicating synthetic L-α-lecithin, β,γ-dipalmitoyl, $>99\%$ pure (Calbiochem Inc.) in 0.165 *M* NaCl saline at a concentration of 10 mg DPL/ml saline. Sonication was performed 3 times for 5 min each, using a Heat Systems-Ultrasonics, Inc. microprobe ultrasonic horn at a power level of 40 W. The emulsion was then centrifuged to remove debris and nonemulsified DPL. Dry silica and kaolin dusts were mixed with DPL emulsion by vortexing the sized, untreated dusts to complete dispersion at a concentration of 7.5 mg dust/ml emulsion and incubating the mixtures for 1 h at 37°C. This provided a nominal DPL/dust mass ratio of 1.33 and optimal kaolin surface coverage as estimated from the adsorption isotherm data (Wallace et al., 1975). Silica and kaolin controls were similarly incubated in physiological saline without DPL. Following incubation, the mixtures were centrifuged for 10 min at 990 × g, and the supernatant fluid was discarded. For hemolysis assays each dust was resuspended in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (PBS) (GIBCO). The dusts were centrifuged and again resuspended in PBS to a final working concentration of 2 mg dust/ml PBS. This stock suspension was diluted to make sample dust suspensions from 0.2 to 2.0 mg/ml. Dust suspension and DPL emulsification did not change the osmolarity of 296 ± 1mOsm or the pH of 7.3 of the saline. For macrophage assays the same preparation procedure was used, except HEPES buffer was used instead of PBS. HEPES buffer contains 10.0 m*M* *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid, 140 m*M* NaCl, 5 m*M* KCl, and 5 m*M* glucose.

As one index of cytotoxicity, hemolysis by the native and treated dusts was measured according to the method of Harington et al. (1971) with minor changes. Fresh sheep blood erythrocytes were prepared as a 4% by volume suspension in PBS after 3 washes in PBS and centrifugation at 990 × g. Erythrocyte preparation and the dust suspensions were mixed in equal volumes to make samples of 2% by volume erythrocytes with treated or native dust in concentrations from 0.1 to 1.0 mg/ml. Native silica and kaolin and DPL-treated silica and kaolin suspensions with erythrocytes were incubated at 37°C for 1 h, and then centrifuged at 990 × g for 10 min. Negative controls were made with erythrocytes in PBS and positive controls were made by lysing erythrocytes in PBS containing 0.5% Triton X-100. All samples were read at 540 nm using a Gilford spectrophotometer, and percent lysis was calculated from the optical density of positive controls. The released heme from lysed erythrocytes shows an absorption maximum at this wavelength.

Alveolar macrophage enzyme release studies were carried out using macrophages harvested from male Sprague-Dawley rats weighing 250-275 g. Animals were anesthetized with an intraperitoneal injection of sodium pentobarbitol (150 mg/kg rat weight) and exsanguinated by

severing the abdominal aorta. The lungs were then lavaged repetitively 10-12 times with ice-cold calcium- and magnesium-free Hanks balanced salt solution (GIBCO). Macrophages were sedimented (990 × g) from the pooled lavages and suspended in HEPES buffer at pH 7.4. Cell counts were made using a hemocytometer. By this procedure, approximately 95% of the cells obtained by lavage were alveolar macrophages.

Suspensions of native or DPL-treated silica or kaolin were mixed with the macrophage suspension to produce 2×10^6 cells/ml and dust concentrations of 1 mg/ml. All samples were incubated for 2 h at 37°C in a shaking water bath. For estimation of total enzyme released, one set of controls of cells without dust were lysed with 0.2% Triton X-100 at the end of incubation. Following incubation all samples were centrifuged at 500 × g for 10 min, and total and released activities of three enzymes were determined in duplicate tests. Lactate dehydrogenase (LDH) activity was determined according to the method of Reeves and Fimignari (1963). β-Glucuronidase (β-GLUC) activity was measured according to the method of Lockard and Kennedy (1976). β-*N*-Acetyl glucosaminidase (β-NAG) was assayed according to the method of Sellinger et al. (1960). Percentages of enzymes released were calculated relative to the Triton X-100-lysed samples.

One set of experiments was performed to determine the effect on hemolysis of varying the DPL/kaolin dust ratio. Samples of 1 mg/ml kaolin suspensions with relative concentrations of DPL to kaolin ranging from 0.01 to 1.33 mg DPL/mg kaolin in the mixture were incubated for 16 h, centrifuged, and resuspended. These were mixed with erythrocyte suspensions to form final suspensions of 1 mg dust/ml and 2% erythrocytes by volume. Percent hemolysis was determined after 1 h incubation.

Another set of experiments was performed to determine the degree to which changes in kaolin cytotoxicity was due to DPL-macrophage interaction as contrasted to DPL-mineral surface interaction. Alveolar macrophage suspensions of 1×10^6 cells/ml HEPES solution were incubated with DPL emulsion of 5 mg/ml in HEPES solution for 1 h at 37°C. The suspensions were then centrifuged at 990 × g, the cells were resuspended in HEPES buffer, and these DPL-treated cells were then incubated with 1 mg kaolin/ml and lysosomal enzyme release was measured as above. Enzyme release following kaolin incubation with the DPL-treated cells was then compared with enzyme release following DPL-treated kaolin incubation with untreated cells.

Data were subjected to statistical analysis using the Student's *t*-test to compare the significance levels. Regression analyses were performed on the hemolysis data from three sets of experiments using five dust concentrations.

RESULTS

Incubation of kaolin with lecithin suppressed the erythrocyte hemolytic cytotoxicity of the kaolin to near background levels. Figure 1 shows

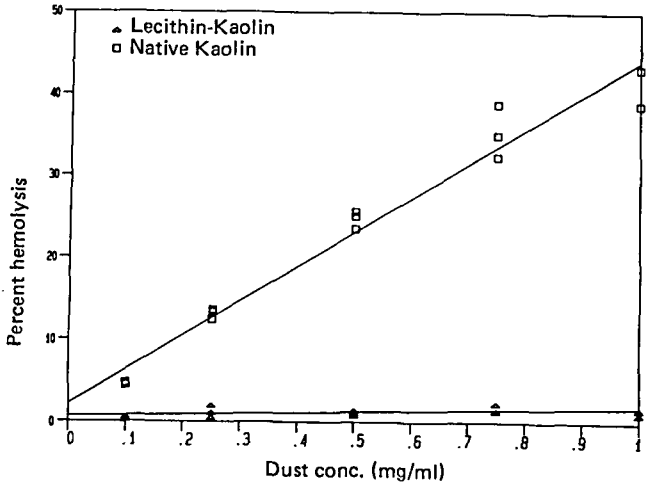

FIGURE 1. Effect of lecithin treatment on kaolin-induced hemolysis. Each data point represents the mean of five hemolysis tests.

the percent hemolysis following 1 h incubation of native or lecithin-treated kaolin with erythrocytes. For the untreated kaolin, the percent hemolysis shows a linear relationship with increased concentration of kaolin in suspension. At the maximum kaolin concentration used (1 mg/ml), the hemolysis value was 42%. The lecithin-treated kaolin induced only 2% hemolysis at the same concentration. Figure 1 illustrates the data points for each dust concentration for both the treated and native kaolin.

Similarly, silica data, shown in Fig. 2, also indicate suppression of the hemolysis by lecithin treatment. Hemolysis was linear with increasing

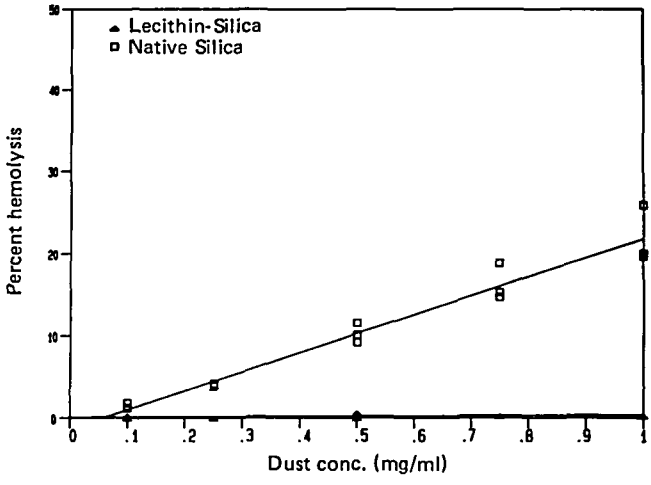

FIGURE 2. Effect of lecithin treatment on silica-induced hemolysis. Each data point represents the mean of five hemolysis tests.

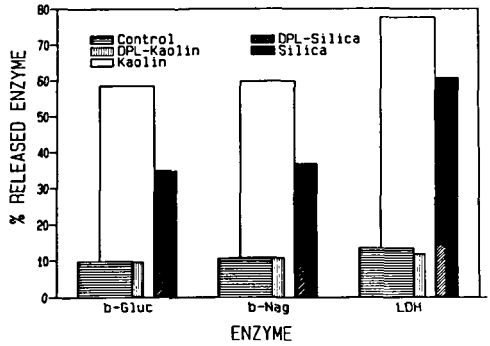

FIGURE 3. Comparative in vitro effect of native and lecithin-treated kaolin and silica on release of enzymes from rat alveolar macrophages.

native silica concentrations at 1 h incubation. At the maximum silica suspension used (1 mg/ml), the hemolysis value was 21.9% and lecithin pretreatment of the silica diminished hemolysis to 0.6% at this dust concentration (Fig. 2).

Results of pulmonary macrophage cytotoxicity assays were consistent with hemolysis results. Figure 3 displays the results of the cytosomal enzyme LDH, and lysosomal enzymes β-GLUC and β-NAG released from pulmonary macrophages following a 2-h incubation with native or lecithin-treated silica or kaolin. An optimal dust concentration of 1 mg/ml was used in all the experiments. Native silica induced 440% increase in release of LDH over the control value and lecithin treatment suppressed this enzyme release. Native silica-induced β-GLUC and β-NAG releases were 350% and 340% of control values, respectively, while lecithin-treated silica values did not exceed the controls. Native kaolin-induced LDH, β-GLUC, and β-NAG releases were 570%, 600%, and 570%

TABLE 1. Effect of Native and Lecithin-Treated Silica and Kaolin on Release of Enzymes from Rat Alveolar Macrophages

	Percent of enzyme released[a]		
Dust treatment	β-GLUC[b]	β-NAG[c]	LDH[d]
Control (no dust)	9.8 ± 1.3	10.5 ± 1.1	13.5 ± 1.3
Native silica	34.7 ± 8.8[e]	36.6 ± 8.0[e]	60.5 ± 7.9[e]
Lecithin-treated silica	9.0 ± 1.4	10.4 ± 0.6	14.7 ± 1.7
Native kaolin	58.5 ± 1.8[e]	59.8 ± 1.6[e]	77.6 ± 8.2[e]
Lecithin-treated kaolin	9.7 ± 1.6	10.8 ± 0.8	11.9 ± 1.9

[a] Mean ± standard error of mean of three experiments in five replicates.
[b] β-Glucuronidase.
[c] β-*N*-Acetyl glucosaminidase.
[d] Lactate dehydrogenase.
[e] Significantly different from control at $p < 0.05$.

TABLE 2. Percent Sheep Erythrocyte Hemolysis versus Mass Ratio of Lecithin to Kaolin Used in the Kaolin Treatment[a]

Mass ratio lecithin/kaolin (mg/mg)	% Hemolysis
0.00	44.8 ± 0.3
0.01	30.2 ± 0.9
0.025	23.0 ± 0.3
0.05	8.0 ± 0.5
0.075	2.5 ± 0.1
0.10	1.4 ± 0.1
0.125	1.6 ± 0.1
0.15	1.4 ± 0.1
0.42	0.9 ± 0.1
1.33	0.0 ± 0.1

[a]Mean ± standard error of mean of eight tests.

of control values, respectively, while lecithin treatment inhibited the release of these enzymes (Table 1).

The effect of lecithin concentration and the ratio of lecithin to kaolin on hemolysis is shown in Table 2. Native kaolin produced 44.8% hemolysis and treatment at 1.33 mg lecithin/mg kaolin fully suppressed hemolysis. From the adsorption isotherm data and specific surface area of kaolin used, it was estimated that 0.1 mg lecithin/mg kaolin would provide full surface coverage and suppress the hemolytic potential to 97% lower than the native kaolin.

The results of lecithin treatment of pulmonary macrophages and kaolin dusts is shown in Table 3. The results of β-GLUC and β-NAG release assays for 2 h incubation of macrophages with native kaolin and lecithin-treated kaolin, and of lecithin-treated macrophages with native kaolin

TABLE 3. Percentage of Rat Pulmonary Macrophage Release of Two Enzymes versus Lecithin Treatment of Kaolin and Lecithin Treatment of Cells

	% Enzyme release			
	Native macrophages		Lecithin-treated macrophages	
	β-GLU	β-NAG	β-GLU	β-NAG
Native kaolin	19.7[b]	28.9[b]	15.3[b]	23.6[b]
Lecithin-treated kaolin	4.8[a]	11.6[a]	4.1[a]	7.6[a]
Control	7.8 ($n = 2$)	15.1		

[a]Significantly different from native kaolin at $p < 0.05$.
[b]Significantly different from control at $p < 0.05$.

and lecithin-treated kaolin, are shown with negative controls. In all cases, lecithin-treated dust induced less enzyme release than control values. Native dust induced enzyme release above control values even with lecithin-treated cells. Lecithin treatment of the macrophages reduced the release of induced enzymes only to nonsignificant levels.

DISCUSSION

A significant release of cytosolic and lysosomal enzymes resulted when pulmonary alveolar macrophages were exposed to native silica or kaolin. On a mass basis the kaolin was more cytotoxic than silica for all three enzyme assays, and silica was more cytotoxic on a surface-area basis. On either basis the cytotoxicities differed by approximately a factor of two. Erythrocyte hemolysis assays were comparable for the two native dusts. These assay results do not parallel the major differences in fibrogenic potential of silica and kaolin.

Lecithin pretreatment of both dusts was performed to simulate a respired particle's contact with and adsorption of dipalmitoyl lecithin, the primary component of pulmonary surfactant. For both dusts such incubation with lecithin in physiological saline resulted in the near-total suppression of their cytotoxicity for pulmonary macrophage enzyme release and erythrocyte hemolysis for the 1- to 2-h cellular incubation periods used. While dust-erythrocyte interactions are not presumed to occur in the lung, erythrocyte hemolysis assays were used here, as in other studies, to measure external cell-membrane damage as an index of dust cytotoxicity. The results of the erythrocyte hemolysis assay using varying amounts of lecithin pretreatment indicate that a lecithin coating interferes with the ability of the native kaolin surface to lyse the cell membrane.

These results imply that although native silica or kaolin dusts can readily damage erythrocytes or pulmonary macrophages in vitro through a cell membrane-dust surface interaction, the primary component of pulmonary surfactant can adsorb to the mineral surface of silica or kaolin and eliminate this cytotoxicity for at least a period of 1-2 h. Depending on the amount of pulmonary surfactant available in a pulmonary alveolus or respiratory bronchiole and on possible competitive interactions of the mineral surface with other materials in the alveolar hypophase, the interaction of pulmonary hypophase lecithin with the surface of respired dusts may provide an initial short-term defense against direct lysis of macrophages or other pulmonary cells.

From limited data on the pulmonary surfactant load in the lung and the adsorption isotherm data for lecithin on kaolin, it has been previously estimated that only very high dust exposures should result in a significant depletion of pulmonary surfactant (Wallace et al., 1975). Thus for typical present-day occupational exposures, the ratio of lecithin to

surface area of respired dust should be adequate to provide total dust surface coverage with lecithin.

In vitro cytotoxicity assays of native kaolin result in a false strong positive prediction of pathogenic potential. With lecithin pretreatment the cytotoxicity of kaolin is reduced, reflecting its relatively benign in vivo behavior. However, lecithin pretreatment also reduced the cytotoxicity of crystalline silica, thus giving a false negative prediction of pathogenic potential. Therefore, distinction between the cytotoxic nature of silica and kaolin that correlates with their differing involvement in lung fibrosis is not possible using these short-term assays. This paradoxical situation can be resolved by postulating that the toxicity of quartz but not kaolin is reactivated following phagocytosis by the macrophage. After deposition in an alveolus, and presumably after adsorption of pulmonary surfactant or other hypophase constituents by the dust, the dust may be phagocytized by pulmonary macrophages. Phospholipase enzymes that can catalyze the hydrolysis of lecithins in homogeneous systems over a wide pH range have been identified in pulmonary macrophage lysosomes. The possibility exists for lysosomal enzymes, such as phospholipases, to digest surfactants adsorbed to dust particles. The degree of digestion may be affected by the nature of the adsorption and thus the properties of the dust surface. Thus for some dusts, the lecithin coating may be fully digested and the dust surface retoxified within the cell. For other dusts, a monolayer of lecithin or lecithin digestion products such as lysolecithin may remain adsorbed to the dust following exposure to lysosomal enzymes. We have observed restoration of the hemolytic ability of lecithin treated silica after subsequent treatment with phospholipase A_2. This treatment may model one in vivo reaction following phagocytosis and phagolysosomal digestion of lecithin-modified dusts in the lung. While such treatment restores lecithin-treated silica to native silica cytotoxicity levels, the restored kaolin hemolytic ability is qualitatively different from that of native kaolin (Wallace et al., 1984). Studies designed to explicate this retoxification process are continuing.

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