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Respirable Quartz and Kaolin Alumino-silicate Induction of *In Vitro* Cytotoxicity and Apoptosis in the Presence of Surfactant or Serum: Caveats to Bioassay Interpretation

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Respirable sized quartz and kaolin dusts at concentrations from 0.25 to 1.0 mg/ml were comparably cytotoxic measured as lactate dehydrogenase (LDH) release from lavaged rat lung macrophages challenged for 2 h in serum-free medium. Kaolin was about twice as active as quartz on a mass basis and about half as active on a surface area basis. Use of fetal bovine serum (FBS) in the medium reduced this activity for both dusts in a serum concentration-dependent manner. Using rat alveolar macrophage-derived NR8383 cells in medium containing 10% FBS, quartz dust challenge for 6 h at dust concentrations from 50 to 400 µg/ml induced significant and dust concentration-dependent necrosis, as measured by LDH release, and apoptosis, as measured by the terminal deoxynucleotidyl transferase-mediated dUTP–fluorescein nick end DNA labeling assay. Under these same conditions, kaolin dust was significantly active only at the highest dust concentration. Challenge at an intermediate concentration of 100 µg/ml over time periods of 6 h to 5 days produced significant LDH release with quartz at all times, while kaolin-induced activity was significant only at 3 and 5 days and was not as strong as quartz-induced activity at those times. Pretreatment of quartz with dipalmitoyl phosphatidylcholine (DPPC), to model conditioning of respired dust surfaces by interaction with a primary phospholipid component of the pulmonary surfactant, further suppressed quartz activity in the FBS system over 3 days, with no additional DPPC prophylactic effect seen at 5 days. No additional prophylactic effect of DPPC was seen for kaolin in the FBS system. *In vitro* assays of respirable particulate necrotic or apoptotic activities can be significantly affected by non-physiologically meaningful surface modifications of dusts occurring in a cellular test system, as observed here for serum in cell culture medium. At the same time, interpretation of *in vitro* assays may be limited if *in vivo* physiological modification of particle surfaces, such as adsorption of prophylactic components of lung surfactant as studied here, is not modeled in the experimental design.

Keywords: quartz; kaolin; NR 8383 cells; apoptosis; TUNEL; lactate dehydrogenase; fetal bovine serum; DPPC surfactant.

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INTRODUCTION

Several anomalies must be explained by any unified theory of pneumoconiosis. One is the comparable *in vitro* cytotoxicity of respirable quartz and some clay dusts, in the face of the much different risk they present for pulmonary disease (Wallace *et al.*, 1985; Vallyathan *et al.*, 1988; Schulz, 1993; IARC, 1997). Presuming that both the *in vitro* and *in vivo* toxic activities of both silica and silicate insoluble dusts are associated with their surface properties, the design of *in vitro* studies should consider modeling the surface conditioning which may occur upon particles depositing on the bronchio-alveolar surface hypophase in the lung. Experimental design should also be alert to inadvertent and non-physiological particle surface conditioning which may occur due to materials added to the cell suspension or culture medium.

We have been studying the use of dipalmitoyl phosphatidylcholine (DPPC) to model pulmonary surfactant adsorption by silica and silicate dusts, their consequent passivation and the ability of subsequent

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phospholipase or cellular digestion to remove the surfactant coating and restore particle toxicity. We have suggested that mineral-specific aspects of such restoration of surface toxicity may distinguish the disease-inducing characteristics of different silica and silicate dusts (Wallace *et al*., 1985, 1992; Liu *et al.*, 1998). Barrett *et al.* (1999) have reported the suppression of chrysotile dust cytotoxicity by components of fetal bovine serum (FBS) in culture medium. The current study: (i) considers the effect of FBS in the medium on quartz- and kaolin-induced prompt necrosis of lavaged rat pulmonary macrophages; (ii) reviews recent results (Gao *et al.*, 2001) on the effects of FBS in the medium and the combined effect of this with DPPC pretreatment of quartz and kaolin on the induction of necrosis and apoptosis of a rat macrophage-derived cell line over a 5 day period. Quartz is known to be active for apoptosis induction and some substances induced by silica exposure may induce apoptosis (Leigh *et al.*, 1997).

MATERIALS AND METHODS

The mineral dusts used were: (i) Min-U-Sil 5 respirable quartz dust (US Silica Corp., Berkeley Springs, WV), 99.5% α-quartz by X-ray diffraction, 98% of particles <5 µm area equivalent diameter, specific surface area 3.97 m^2/g as measured by BET N_2 gas adsorption; (ii) a sized fraction of respirable kaolin dust (Georgia Kaolin Mills, Augusta, GA), at least 95% alumino-silicate with no crystalline quartz detected by X-ray diffraction, 99% of the fraction <5 µm area equivalent diameter, specific surface area 13.25 m^2/g.

The surfactant used was DPPC (Calbiochem, San Diego, CA), ultrasonically dispersed in 0.165 M NaCl physiological salt solution (PSS) at 5 mg DPPC/ml PSS, followed by centrifugation at 1500 *g* for 10 min to remove non-dispersed DPPC. Quartz and kaolin were mixed in this dispersion at a ratio of 0.1 g DPPC/g quartz and 0.2 g DPPC/g kaolin, centrifuged at 1500 *g* for 10 min and the dusts resuspended in complete RPMI 1640 medium (Sigma, St Louis, MO) to the desired concentrations. Approximately 20 mg DPPC/g quartz and 80 mg DPPC/g kaolin provides a bilayer covering which is stable to rinsing and fully suppresses hemolytic activity (Wallace *et al.*, 1992).

Pulmonary macrophages were obtained by lavage of male Sprague–Dawley rats and prepared as previously detailed (Gao *et al.*, 2000).

NR8383 (American Type Culture Collection, Manassas, VA) is a rat alveolar macrophage cell line derived by lung lavage of a normal adult male Sprague–Dawley rat. NR8383 cells were maintained as a monolayer culture in RPMI 1640 culture medium supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 10% heat-denatured FBS (Sigma, St Louis, MO) and 1% penicillin/streptomycin solution. Five million cells were seeded in each well of a 6-well plate for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP–fluorescein nick end labeling (TUNEL) assay. Cells were challenged for 6 h with untreated and DPPC-treated quartz and kaolin concentrations of 50, 100, 200 or 400 µg/m, and were incubated at a dust concentration of 100 µg/ml for selected times of 6 h to 5 days.

Cell damage was determined by measuring lactate dehydrogenase (LDH) activity in the culture medium using a LDH assay kit (Roche Diagnostics, Indianapolis, IN).

Apoptosis was determined by the TUNEL assay, which measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein–12-dUTP at 3′-OH DNA ends using the enzyme TdT (Leigh *et al.*, 1997). The fluorescein–12-dUTP-labeled DNA can then be visualized directly by fluorescence microscopy. A 75 µl cell suspension aliquot (1×10^6 cells/ml) was placed on a cytospin slide, centrifuged, fixed, permeabilized with Triton X-100 and incubated with 50 µl of TdT incubation buffer for 60 min at 37°C. Cells were stained with propidium iodide and analyzed immediately under a fluorescence microscope using a standard fluorescein filter set to view the green fluorescence of fluorescein at 520 ± 20 nm, while the red fluorescence of propidium iodide was viewed at >620 nm.

RESULTS

Quartz and kaolin caused comparable dust dose-dependent release of LDH from lavaged pulmonary macrophages after 2 h incubation in serum-free medium for dust concentrations of 0.25, 0.5 and 1.0 mg/ml. Kaolin induced 59, 104 and 117 U LDH/l, respectively, at those dust concentrations; corresponding quartz values were 29, 49 and 98 U/l; the no-dust control value was 21 U/l. The three masses of dust correspond, respectively, to kaolin surface area concentrations of 0.0033, 0.0067 and 0.013 m^2/ml, and to quartz surface area concentrations of 0.00099, 0.0020 and 0 .0040 m^2/ml. Thus, the kaolin was about twice as active as the quartz on a mass basis and about half as active on a surface area basis, using the BET N_2 gas adsorption measured surface area. Using a dust concentration of 1 mg/ml and using increasing percentages from 0, to 1, 3 and 10% FBS in the medium, the necrotic activity decreased with increasing FBS. For kaolin the corresponding values of LDH release were 117, 58, 34 and 26 U/l; for quartz the corresponding values were 98, 48, 24 and 25 U/l.

Using NR8383 cells in medium containing 10% FBS, concentrations of quartz dust of 50–400 µg/ml used for a 6 h challenge resulted in statistically

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significant LDH release above the control at all quartz concentrations, with a monotonic increase in LDH release with increasing quartz concentration (Gao *et al.*, 2001). Cell challenge for 6 h with native kaolin resulted in significant LDH release at the two highest kaolin concentrations of 200 and 400 µg/ml. The responses at these kaolin concentrations were significantly lower than for quartz and the response was not significant at lower kaolin concentrations. Challenge of cells over a 5 day period with quartz or kaolin at 100 µg/ml in the system containing 10% FBS caused a time-dependent increase in LDH release which was significant at $P < 0.01$ for quartz at all time points and was significant for kaolin at $P < 0.01$ at 3 and 5 days. DPPC treatment delayed significant quartz-induced LDH activity until 3 days; by 5 days the effect of DPPC treatment was no longer seen. Using NR8383 cells in 10% FBS-containing medium and exposures for 6 h at dust concentrations of 50–400 µg/ml, quartz challenge at all concentrations caused statistically significant cell apoptosis as measured by the TUNEL assay, increasing with dust concentration. Kaolin induced significant apoptosis only at the highest concentration (Gao *et al.*, 2001).

DISCUSSION AND CONCLUSIONS

Native quartz and native kaolin are known to be comparably cytotoxic, in the absence of prophylactic adsorbates, to lavaged pulmonary macrophages and comparably membranolytic to red blood cells (Vallyathan *et al.*, 1988; Wallace *et al.*, 1985, 1992). However, testing these same dusts in serum-containing medium found quartz to be almost twice as active as native kaolin in a 'live–dead' fluorescence assay for cell viability (Gao *et al.*, 2000). Components of serum are known to affect cristobalite induction of cytotoxicity *in vitro* (Barrett *et al.*, 1999). In the current study, using primary macrophages, there was an FBS dose-dependent suppression of quartz- and kaolin-induced LDH release from lavaged rat alveolar macrophages *in vitro*. Using NR8383 cells in culture medium containing 10% FBS, quartz caused a statistically significant increase in cell-released LDH and in apoptotic activity with increasing dust concentration, while kaolin effects were not seen at the two lowest concentrations. This may indicate a mineral-specific passivation effect in this *in vitro* system, e.g. prophylactic components of serum in limited amounts may preferentially deactivate kaolin, until higher kaolin concentrations deplete the components. Additional research is needed to determine if this mechanism is involved and to determine the role of separate constituents of serum. DPPC suppression or diminution of quartz early necrotic and apoptotic activity with restoration of quartz activity at 5 days is consistent with the results of our other studies on the *in vitro* rates of surfactant digestion from quartz particles and associated restoration of toxicity (Wallace *et al.*, 1992; Hill *et al.*, 1995; Liu *et al.*, 1998). *In vitro* experiments on particle toxicity should consider potential inadvertent effects of the cell test system, e.g. serum, which may confound the interpretation or physiological relevance of the results. At the same time, the potential prophylactic effects of *in vivo* particle contact with surfactants lining the lung brochio-alveolar surface should be considered in the design and interpretation of *in vitro* studies of respirable insoluble particle toxicity.

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REFERENCES

Barrett EG, Johnston C, Oberdorster G, Finkelstein JN. (1999) Silica binds serum proteins resulting in a shift of the dose–response for silica-induced chemokine expression in an alveolar type II cell line. Toxicol Appl Pharmacol; 161: 111–22.

Gao N, Keane MJ, Ong T, Wallace WE. (2000) Effects of simulated pulmonary surfactant on the cytotoxicity and DNA-damaging activity of respirable quartz and kaolin. J Toxicol Environ Health; 60A: 153–67.

Gao N, Keane MJ, Ong T, Ye J, Miller WE, Wallace WE. (2001) Effects of phospholipid surfactant on apoptosis induction by respirable quartz and kaolin in NR8383 rat pulmonary macrophages. Toxicol Appl Pharmacol; 175: 217–25.

Hill CA, Wallace WE, Keane MJ, Mike PS. (1995) The enzymatic removal of a surfactant coating from quartz and kaolin by P388D1 cells. Cell Biol Toxicol; 11: 119–28.

IARC. (1997) IARC monographs on the evaluation of carcinogenic risks to humans: no. 68: silica, some silicates, coal dust, and para-aramid fibrils. Lyon, France: IARC. pp. 41–242.

Leigh J, Wang H, Bonin A, Peters M, Rean X. (1997) Silica-induced apoptosis in alveolar and granulomatose cells *in vivo*. Environ Health Perspect; 105 (suppl. 5): 1241–5.

Liu XR, Keane MJ, Harrison JC, Cilento EV, Ong T, Wallace WE. (1998) Phospholipid surfactant adsorption by respirable quartz and *in vitro* expression of cytotoxicity and DNA damage. Toxicol Lett; 96/97: 77–84.

Schulz CO. (1993) Silicon and silicates including asbestos. In Clayton GD, Clayton FE, editors. Patty's industrial hygiene and toxicology 1993, Vol. II, Part A. New York, NY: John Wiley & Sons.

Vallyathan V, Schwegler D, Reasor M, Stettler L, Clere J, Green FHY. (1988) Comparative *in vitro* cytotoxicity and relative pathogenicity of mineral dusts. Ann Occup Hyg; 32: 279–89.

Wallace WE, Vallyathan V, Keane MJ, Robinson V. (1985) *In vitro* biologic toxicity of native and surface-modified silica and kaolin. J Toxicol Environ Health; 16: 415–24.

Wallace WE, Keane MJ, Hill CA, Vallyathan V, Regad ED. (1992) Contrasting respirable quartz and kaolin retention of lecithin surfactant and expression of membranolytic activity following phospholipase A_2 digestion. J Toxicol Environ Health; 37: 391–409.

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