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SUPPRESSION OF INHALED PARTICLE CYTOTOXICITY BY PULMONARY SURFACTANT AND RE-TOXIFICATION BY PHOSPHOLIPASE: DISTINGUISHING PROPERTIES OF QUARTZ AND KAOLIN*

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Abstract—Inhaled particle contact with pulmonary surfactant in the hypophase lining pulmonary alveoli was modelled *in vitro* by exposing two respirable sized dusts, kaolin and silica quartz, to lecithin, a major component of pulmonary surfactant, emulsified in physiological saline. Lecithin adsorbs to the dusts upon incubation at 37°C and suppresses their cytotoxicity as measured by pulmonary macrophage and erythrocyte assays, suggesting that pulmonary surfactant provides a defence system against prompt cell membrane lysis by inhaled dusts. Subsequent pulmonary macrophage phagocytosis and lysosomal enzyme digestion of the coated dusts was modelled *in vitro* by incubation with phospholipase A_2 for one hour. With increasing lipase activity, silica haemolytic potential was restored to native silica levels. Kaolin was retoxified to levels far in excess of native kaolin haemolytic potential. Elution, thin layer chromatography and phosphate assay of treated dusts indicate that most lecithin on silica is digested to lysolecithin and desorbed, silica retoxification being due to surface and adherent lysolecithin effects. Most lecithin on kaolin is not digested in the same time; that which is, results in adherent lysolecithin which is responsible for the kaolin retoxification. Digestion with phospholipases A_2 and C together produces only weakly retoxified kaolin. Results suggest that surface adsorption properties which control the adherence of prophylactic surfactant distinguish the pathogenic potentials of quartz, kaolin and mixed dust.

INTRODUCTION

ERYTHROCYTE HAEMOLYSIS and pulmonary macrophage enzyme release assays show quantitatively comparable high cytotoxicities for both silica quartz and kaolin respirable sized dusts (BROWN, *et al.*, 1980; DANIEL and LE BOUFFANT, 1980.) This contrasts with the strong potential for respirable quartz dust to induce pulmonary fibrosis and the much more limited potential of kaolin dust to do the same (MORGAN and SEATON, 1975).

In an attempt to understand this anomalous situation, assays were performed on quartz and kaolin dusts incubated with dipalmitoyl lecithin emulsified in physiological saline, to simulate the initial contact of a respired dust with the pulmonary alveolar hypophase. The ability of kaolin dust to adsorb dipalmitoyl lecithin from physiological saline has been quantified (WALLACE, *et al.*, 1975). Such lecithin treatment suppresses

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the cytotoxicity of both silica and kaolin to background levels (WALLACE, *et al.*, 1984).

Surfactant coated de-toxified dusts should then be phagocytised by pulmonary macrophages, incorporated into a macrophage secondary lysosome and exposed to hydrolytic lysosomal enzymes. Among these is phospholipase A_2, which hydrolyses diacyl lecithin to lysolecithin. This was modelled by incubating lecithin treated dusts with phospholipase A_2 *in vitro* to determine if the enzyme can digest the dust-adsorbed lecithin, and if the retoxification differs between the two dusts in a manner which might distinguish their pathogenicity.

MATERIALS AND METHODS

The silica used was at least 98.5% pure as determined by X-ray energy spectrometric analysis and was alpha quartz as determined by X-ray diffraction. Its N_2 adsorption specific surface area was 3.97 m^2/g. The kaolin used was at least 96% pure, contained no crystalline silica, and had a specific surface area of 13.25 m^2/g.

L-α-dipalmitoyl glycerophosphoryl choline (lecithin) (DPL) was ultrasonically dispersed in 0.165 *M* NaCl saline to produce an emulsion of 10 mg DPL per ml saline. Dry silica or kaolin dusts were mixed into a concentration of 7.5 mg dust per ml emulsion. Mixtures were incubated for one hour at 37°C in a shaking water bath, centrifuged for ten minutes at 990 xg, and the dusts resuspended in calcium and magnesium free phosphate buffered 0.165 *M* NaCl saline (PBS). This washing procedure was repeated to remove unadsorbed lecithin, the lecithin coated dusts being suspended finally in PBS.

Aliquots of native or lecithin-treated dusts in PBS with 2.0 m*M* $CaCl_2$ were incubated with Phospholipase A_2 (PLA_2), obtained from *Crotalus adamanteus* venom, at 37°C for one hour. The concentrations used covered a range of activities from that needed to digest from one-tenth to 1000 × the amount of kaolin-adsorbed lecithin had the digestion been for a homogeneous phase. 'Phospholipase activity' is given in terms of 'activity equivalents' which are equal to 0.0817 units of phospholipase. Following digestion, the samples were centrifuged and the dusts resuspended in PBS containing 2.0 m*M* EDTA to stop the enzymatic digestion. This washing procedure was repeated, with the dusts suspended in PBS alone. All procedures were done under sterile conditions.

Suspensions of 2% by volume sheep erythrocytes and 1.0 mg native or treated dust per ml PBS were incubated in a shaking water bath at 37°C for one or two hours, as indicated, to assay the dusts' haemolytic potential (HARINGTON, *et al.*, 1971). Samples were then centrifuged at 990 xg and the optical density for haemoglobin read at 540 μm on a spectrophotometer. Haemolysis assays were also performed using kaolin incubated with L-α-lecithin, β palmitoyl (lysolecithin) using the lecithin treatment procedure.

Macrophages lavaged from male Sprague Dawley rats were mixed with native or treated dusts to produce concentrations of 2×10^6 cells per ml and one mg dust per ml. These were incubated for two hours at 37°C in a shaking water bath. Released enzyme activities were determined for lactate dehydrogenase (LDH) (REEVES and FIMIGNARI, 1963), beta-glucuronidase (B-GLUC) (LOCKART and KENNEDY, 1976), and beta-N-acetyl glucosaminidase (B-NAG) (SELLINGER, *et al.*, 1960). Percentages of enzymes released were calculated relative to Triton X-100 lysed samples.

Parallel samples of dusts were eluted with methanol/chloroform solvent (2:1 vol/vol) following their lecithin and phospholipase treatments. The eluted material was quantitated for lecithin and for lysolecithin by wet phosphate assay of thin layer chromatograph separated materials (BARTLETT, 1959).

Adsorption by silica and by kaolin of lecithin from physiological saline emulsion at 37°C was measured over a range of final (unadsorbed) supernatant concentrations of 0.02 to 10.0 nanomoles per ml emulsion, as quantitated by wet phosphate assay.

RESULTS

Native quartz and kaolin dusts produce comparable high haemolysis cytotoxic response (Fig. 1). On a surface area basis the two dusts were also comparable with haemolytic strengths of 22.6% for kaolin and 24.4% for silica per square decimeter dust surface area in the dispersion.

Lecithin treatment of dusts suppressed their haemolytic strengths to background levels (Fig. 1). This is due principally to lecithin interaction with the dust rather than with the cells (WALLACE, *et al.*, 1985). Lecithin treatment also suppresses the cytotoxicity of both silica and kaolin for pulmonary macrophages (Fig. 2). The kaolin and silica used in this study had specific adsorptions of around 150 mg lecithin/gram kaolin and 50 mg lecithin/gram silica over the supernatant concentrations of interest (Fig. 3).

Incubation of lecithin-treated quartz with PLA_2 results in increasing retoxification of the silica with PLA_2 activity, up to native quartz haemolytic potential (Fig. 4). Data are fitted by non-linear regression to a first order kinetics curve. Incubation of quartz with PLA_2 (alone) in physiological saline produces dusts of comparable cytotoxicities to native silica. Incubation of lecithin-treated kaolin with PLA_2 results in increasing retoxification of the kaolin with PLA_2 activity (Fig. 5). However, unlike the quartz, kaolin retoxifies to an anomalously high level, well above (of the order of twice) the

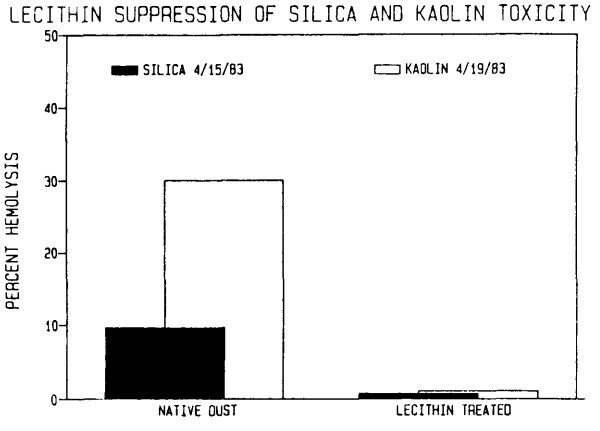

FIG. 1. Silica and kaolin haemolytic strength. Percent haemolysis of sheep erythrocytes induced by incubation for one hour with 1 mg/ml of native or lecithin-treated kaolin or silica.

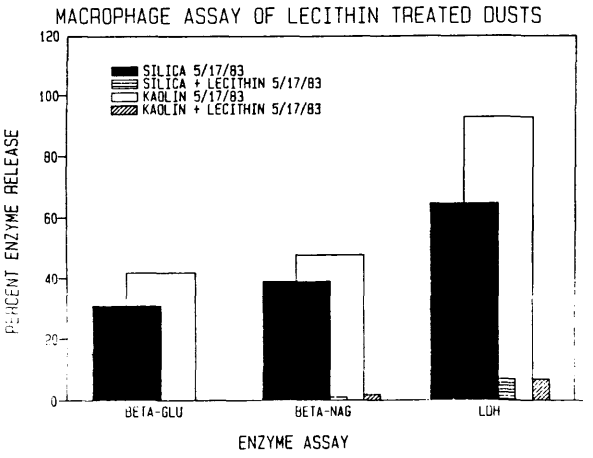

FIG. 2. Macrophage assay of lecithin-treated dusts. Release of beta-glucoronidase, beta-n-acetyl-glucosaminidase, and lactate dehydrogenase from rat pulmonary macrophage induced by two hour incubation with 1mg/ml of native or lecithin-treated kaolin or silica.

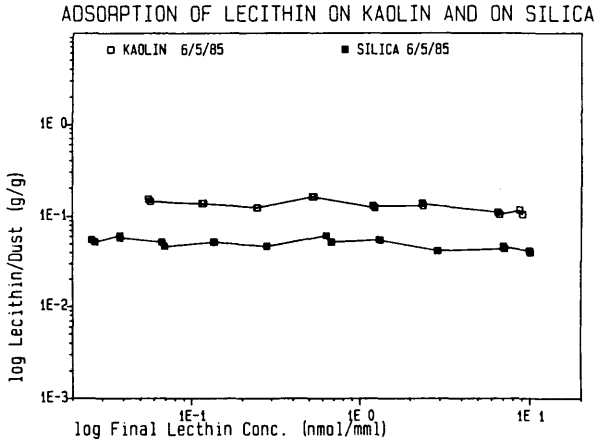

FIG. 3. Adsorption of lecithin on kaolin and on silica. Adsorption isotherm data for mass lecithin adsorbed per mass of kaolin or silica versus concentration of lecithin remaining in the supernatant.

cytotoxicity of native untreated kaolin. Incubation of kaolin with lysolecithin alone dispersed in physiological saline reproduces this high cytotoxicity (Fig. 5). Incubation of kaolin with PLA_2 alone in physiological saline produces dust of comparable cytotoxicities to native kaolin.

After PLA_2 digestion a high percentage of adsorbed lecithin is retained on the kaolin surface (Fig. 6). A significant amount of lysolecithin produced by the lecithin digestion is also associated with the lecithin-kaolin complex. A much smaller fraction of the initially adsorbed lecithin remains on the silica as lecithin or lysolecithin following PLA_2 digestion. Haemolysis assays of parallel samples are shown in Fig. 7.

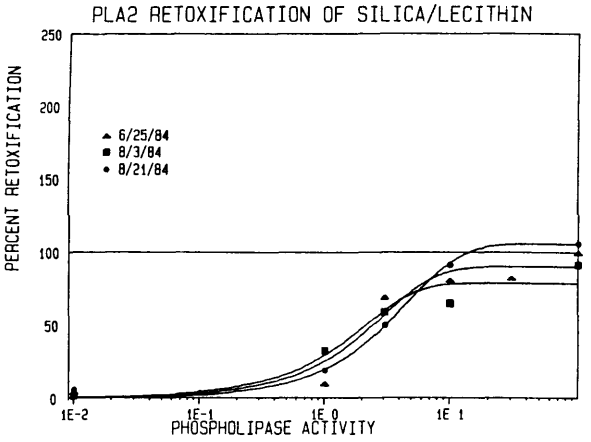

FIG. 4. PLA_2 retoxification of silica/lecithin. Erythrocyte haemolysis induced by two hour incubation with silica treated with lecithin for one hour, and then with differing amounts of phosphoslipase A_2 for one hour. Ordinate values are expressed as percent of the haemolysis induced by untreated silica. Abscissa values are the number of activity equivalents of phospholipase A_2 applied. Haemolysis by dusts treated with lecithin only is shown at the smallest activity value.

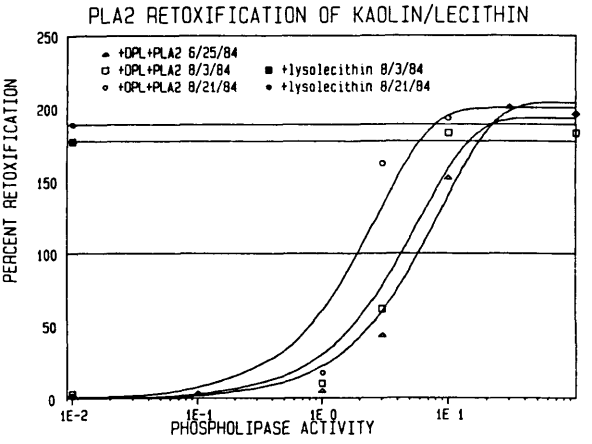

FIG. 5. PLA_2 retoxification of kaolin/lecithin. Erythrocyte haemolysis induced by kaolin treated with lecithin for one hour and then with differing amounts of phospholipase A_2 for one hour, and haemolysis by kaolin treated with lysolecithin only.

COMMENT

A major component of pulmonary surfactant, dipalmitoyl lecithin, can adsorb to quartz and to kaolin and suppress their cytotoxicity. This is consistent with lipidosis associated with large pulmonary dust burdens. Subsequent treatment with phospholipase A_2, a known component of pulmonary macrophage lysosomal enzyme, restores haemolytic ability to both treated dusts. However, this restoration differs quantitatively and qualitatively between the two dusts. This may be a basis for their differing pathogenicities.

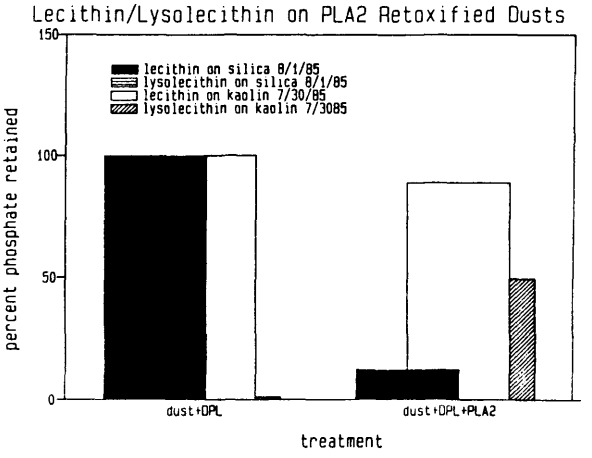

FIG. 6. Lecithin/lysolecithin on PLA_2 retoxified dusts. Lecithin and lysolecithin eluted by organic solvent from silica or kaolin treated with lecithin or treated first with lecithin and then with 1000 activity equivalents of phospholipase A_2. Ordinate values are percent of the lecithin eluted from lecithin-treated dusts.

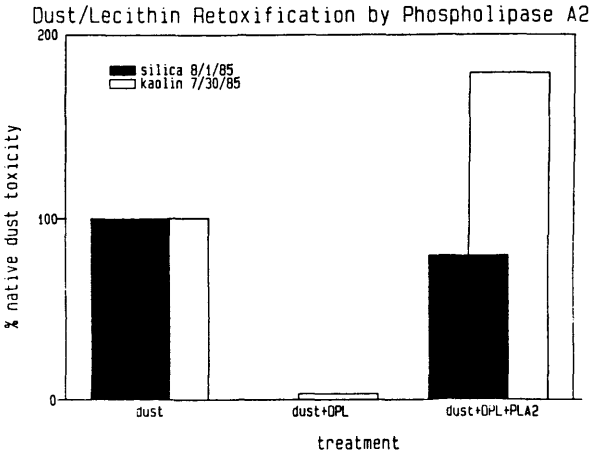

FIG. 7. Dust/lecithin retoxification by phospholipase A_2. Erythrocyte haemolysis induced by the dusts assayed for lecithin/lysolecithin shown in Figure 6.

Retoxification data indicate that the quartz surface is functionally bared by the digestive action of the PLA_2. Retention of most lecithin by kaolin after PLA_2 treatment indicates that kaolin is not functionally bared of lecithin for the short digestion times used here. The presence of lysolecithin on PLA_2 treated kaolin-lecithin and the simulation of kaolin retoxification by kaolin-lysolecithin indicate that kaolin retoxification is due to adherent lysolecithin.

The lysolecithin-based retoxification of kaolin would seem to be a bioassay result contrary to the differences in pathogenicity of the dusts. One must ask: in the normal course of digesting bacteria or other lipid-containing material, is the produced

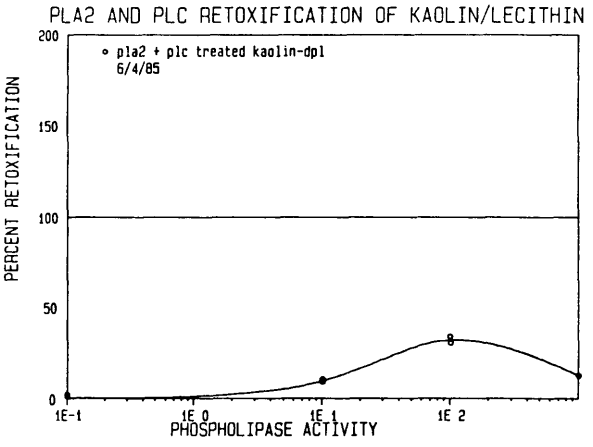

FIG. 8. PLA_2 and PLC retoxification of kaolin/lecithin. Erythrocyte haemolysis induced by kaolin treated with lecithin and then with phospholipase A_2 and phospholipase C together in equal activity equivalents.

lysolecithin further digested by lysosomal lysophospholipase enzymes? Or is the interior of the phagolysosome immune to lysis by lysolecithin? Figure 8 shows the cytotoxicity of kaolin which first has been incubated with lecithin, then retoxified by incubation with PLA_2 and PLC together for one hour. Data are fitted to a first order kinetics model of lecithin conversion to lytic lysolecithin by PLA_2 and subsequent lysolecithin conversion by PLC to non-lytic phosphoryl choline and glycerol palmitate.

The mineral surface properties responsible for these differences in retoxification could involve different strengths of sorption or conformations of the adsorbed lecithin. Kaolin is a mineral of layered alumina octahedra and silica tetrahedra. Alumina catalyses the hydrolysis of lecithin in organic solvent (RENKONEN, 1962); this does not occur for lecithin dispersion in saline. Surface properties would be expected to be more important to disease pathogenesis than the bulk non-biologically available mineral content of an inhaled particle. Occlusion or surface contamination of quartz by alumino-silicate or other coatings in mixed dust atmospheres should affect the biological activity expected of a respirable dust of a given silica concentration, by affecting the retention of surfactant during clearance *in vivo*. And the effectiveness of additive prophylactic agents for quartz-bearing dusts might be affected by their ability to be retained under the action of pulmonary surfactant solubilization and lysosomal enzyme digestion. Tests using lavaged surfactant with isolated lysosomal enzyme in longer term macrophage incubations should determine if such simple initial events in the interaction of mineral dusts with the lung are determinants of the potential for dust-induced pulmonary fibrosis.

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REFERENCES

BARTLETT, G.R. (1959) Phosphorus Assay in Column Chromatography. *Biolog. Chem.* **234**, 466–468.

BROWN, R.C., CHAMBERLAIN, M., DAVIES, R., MORGAN, D.M.C., POOLEY, F.D. and RICHARDS, T. (1980) A comparison of 4 *In Vitro* Systems Applied to 21 Dusts. In: *The In Vitro Effects of Mineral Dusts*, (Ed. BROWN, R.G., GORMLEY, I.R., CHAMBERLAIN, M. and DAVIES, R.) Academic Press, London, pp. 47–52.

DANIEL, H. and LE BOUFFANT, L. (1980) Study of a Quantitative Scale for Assessing the Cytotoxicity of Mineral Dusts. In: *The In Vitro Effects of Mineral Dusts* (Ed. BROWN, R.C., GORMLEY, I.R. CHAMBERLAIN, M. and DAVIES, R.) Academic Press, London, pp. 33–39.

HARINGTON, J., MILLER, K. and MACNAB, G. (1971) Hemolysis by Asbestos. *Env. Res.* **4**, 95–117.

LOCKART, V.G. and KENNEDY, R.E. (1976) Alterations in Rabbit Alveolar Macrophages As a Result of Traumatic Shock. *Lab. Invest.* **35**, 501–506.

MORGAN, W.K.C. and SEATON, A. (1975) *Occupational Lung Diseases*. W.B. Saunders, Philadelphia.

REEVES, W.J. and FIMIGNARI, G.M. (1963) An Improved Procedure for the Preparation of Crystalline Lactic Dehydrogenase from Hog Heart. *Biolog; Chem.* **238**, 3853–3858.

RENKONEN, O. (1962) Breakdown of Lecithin on Aluminium Oxide Columns. *Lipid Res.* **3**, 181–183.

SELLINGER, O.Z., BEUFAY, H., JACQUES, P., DOYAN, A. and DEDUVE, C. (1960) The Intracellular Distribution of (Beta)-N-Acetylglucosaminidase and of (Beta)-Galactosidase in Rat Livers. *Biochem. J.* **74**, 450–456.

WALLACE, W.E., JR., HEADLEY, L.C. and WEBER, K.C. (1975) Dipalmitoyl Lecithin Surfactant Adsorption by Kaolin Dust *In Vitro*. *J. Coll. Interf. Sci.* **51**, 535–537.

WALLACE, W.E., KEANE, M.J., VALLYATHAN, V., ONG, T-M and CASTRANOVA, V. (1984) Pulmonary Surfactant Interaction With Respirable Dust. In: *Generic Mineral Technology Center—Coal Mine Dust Conference Proceedings*, (Edited by PENG, S.), pp. 180–187. Available from National Technical Information Service, Report #PB86 169380/AS.

WALLACE, W.E., VALLYATHAN, V., KEANE, M.J. and ROBINSON, V. (1985) *In Vitro* Biologic Toxicity of Native and Surface-Modified Silica and Kaolin. *J. Tox. and Environ. H.* **16**, 415–424.