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The Effect of Lecithin Surfactant and Phospholipase Enzyme Treatment on Some Cytotoxic Properties of Respirable Quartz and Kaolin Dusts

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Two *in vitro* systems are being developed to model initial stages in the interaction of respired dusts with the acinar region of the lung. Incubation of quartz and kaolin with dipalmitoyl glycerophosphorylcholine, a major component of pulmonary surfactant, suppresses the prompt cytotoxicity of both dusts as measured by *in vitro* enzyme release from pulmonary macrophages or assay by erythrocyte hemolysis. Additional data is presented to quantitate the detoxification of quartz and kaolin by dipalmitoyl lecithin. Subsequent incubation of lecithin treated dusts with phospholipase enzymes results in partial to complete restoration of the assayed cytotoxic potentials, dependent on the lipase activity applied, the incubation time, and the mineral. Restored cytotoxic potential is due to removal of the prophylactic lecithin surface coating and/or due to residual lysolecithin which is produced by the partial enzymatic digestion of lecithin. Data is presented on the contribution of each mechanism to the restored toxicity. Again, this is dependent on the mineral used, the lipase activity applied, and digestion time. Additionally, a system is under development to challenge pulmonary macrophages in culture with native and surfactant treated dusts. A preliminary test of the dipalmitoyl lecithin – quartz – rabbit pulmonary macrophage system is presented to illustrate the method.

Introduction

Respirable quartz dust exposure is known to cause fibrotic lung disease; respirable clay dust exposure is less likely to do so as indicated by epidemiologic data on occupational exposures and by animal model studies.[1-7] The hypothesis that damage to pulmonary macrophages in the acinar region of the lung by respired dusts is an early event in the disease process requires consideration because kaolin clay and quartz dusts are comparably cytotoxic to pulmonary macrophages *in vitro*.[8,9]

Simulating dust interaction in the lung, quartz and kaolin dusts have been assayed for their cytotoxicity both in their native state and after incubation with an emulsion of dipalmitoyl glycerophosphorylcholine (DPL), called dipalmitoyl lecithin.[8,9] Lecithins are a major component of pulmonary surfactants which stabilize the surface properties of the hypophase coating of pulmonary alveoli.[10] This surfactant adsorbs to kaolin and to quartz from emulsion in physiologic saline. Adsorption isotherms for these dusts have been measured.[11,12] The cytotoxicity of both dusts is suppressed by such DPL adsorption at 37°C[8,9,12] as assayed *in vitro* by macrophage release of cytosolic and lysosomal enzymes or erythrocyte hemolysis. Lecithins are also major components of cellular membranes. Interactions of quartz with the bilayer lipid membrane have been proposed as a mechanism of cell lysis; and effects of dust surface crystallinity and coatings on this lysis have been studied.[13-15] Our simulation of pulmonary surfactant interaction with respired dusts replaced a false positive toxicity assay for kaolin with a false negative assay for quartz.

Removal of serum protein from phagocytized silica has been observed.[16] As a research hypothesis, we presume that the prophylactic surfactant coating adsorbed on a respired particle following contact with pulmonary surfactant is subject to enzymatic digestion following phagocytosis by pulmonary macrophages. We have been exploring this hypothesis by *in vitro* studies using dipalmitoyl lecithin in physiologic saline for alveolar hypophase surfactant and phospholipase enzymes A2 and C for two lysosomal enzymatic digestion processes. We used pulmonary macrophage cytosolic or lysosomal enzyme release or erythrocyte hemolysis assays to monitor the cytotoxicity of the quartz and kaolin dusts treated in different experimental protocols.[8,9,12]

In this report we provide quantitative data showing suppression of hemolytic potential of quartz and kaolin dusts as a function of DPL dose. We provide more data on the effects of phospholipase enzymes A2 and C on treated dusts. We discuss the bases for enzymatic restoration of cytotoxicity of treated dusts by providing initial data on the time course of enzymatic digestion and retoxification.

Although this simulation provides for detailed analyses of some proposed steps in the interaction of a mineral particle with pulmonary surfactant and macrophages, the adequacy of the selected parameters to model the complex *in vivo* situation must be questioned. We are developing a complementary method which may model that reality more closely, but which thereby poses more problems of interpretation. In this system, pulmonary macrophages are challenged *in vitro* with surfactant-treated or native dusts. The

attempt is then made to maintain their viability in culture for a sufficient period of time to detect macrophage attrition due to the surfactant treated dusts at levels above those for negative controls, i.e., macrophages which have not been challenged with dust. We discuss the method using preliminary data for rabbit pulmonary macrophages challenged with DPL treated and untreated quartz.

Materials and Methods

Alpha quartz used is at least 98.5 percent pure as determined by x-ray energy spectrometric analysis. It has a specific surface area of 3.97 m^2/g as determined by nitrogen adsorption. Kaolin used is at least 96 percent pure, contains no crystalline silica, and has a specific surface area of 13.25 m^2/g.

emulsion and the mixtures incubated for one hour at 37°C in a shaking water bath. Dusts were then centrifuged for ten minutes at 990 g and resuspended in calcium and magnesium free Dulbecco's phosphate-buffered saline (PBS).

Commercially supplied Phospholipase A2 was obtained from: porcine pancreas (Sigma) or from *Crotalus adamanteus* venom (Cooper Biomedical), as indicated. Phospholipase C from *Clostridium perfringens* was obtained from Sigma. Enzymes were dissolved in phosphate-buffered saline (PBS) containing 2.0 m*M* calcium chloride, to provide specific activities for designed digestion steps. Amounts of native or DPL-treated dusts were resuspended in PBS with 2.0 m*M* $CaCl_2$, desired amounts of the enzyme solution added, and the samples incubated for specified times at 37°C in a shaking water bath. Following incubation, the dusts were resuspended in PBS containing 2.0 m*M* EDTA to stop the

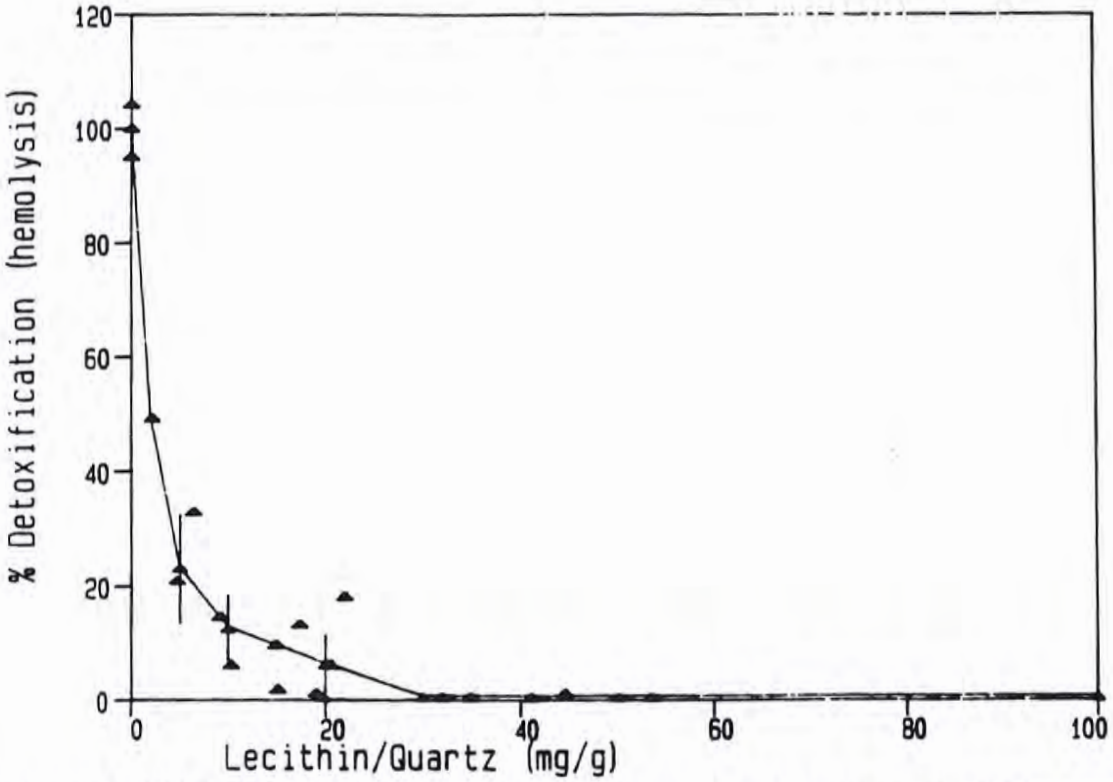

FIGURE 1. Hemolytic potential of quartz versus dipalmitoyl lecithin treatment. Erythrocyte hemolysis values are given as percentages of the value for untreated quartz. Open triangles connected by line segments show the diminution as a function of DPL added to the system. Line segments are connected between average values; and the bars show the ranges measured. The solid triangle scatter graph shows values from additional measurements where abscissa values are the amounts of lecithin actually adsorbed as measured by elution from the particles.

Erythrocytes were obtained for hemolysis assay from a sheep maintained on a fixed diet. Pulmonary macrophages were obtained by lavage of rats or rabbits, as indicated, using Hank's Balanced Salt Solution (HBSS). Cells were washed twice and resuspended in HBSS.

Commercially obtained L-alpha-dipalmitoyl glycerophosphorylcholine (DPL) (Calbiochem) was ultrasonically dispersed in 0.165 *M* sodium chloride to produce emulsions of the desired DPL concentration. Dry quartz or kaolin dust at the ratio of mass of dust to mass and concentration of DPL desired were dispersed into DPL

enzymatic digestion, and then resuspended in PBS alone. For conceptual purposes, we have expressed phospholipase activity in terms of "activity equivalents" which equal 0.0817 units of phospholipase. Using the equilibrium amounts of DPL adsorbed on kaolin from the adsorption isotherm study,[9] one activity equivalent would ideally remove that amount of DPL within one hour at 37°C.

Hemolytic potential of variously treated dusts was assayed by the erythrocyte hemolysis assay of Harington *et al.*[17] Suspensions of erythrocytes (2% by volume) and 1.0 mg dust per ml were incubated in PBS at 37°C for one hour.

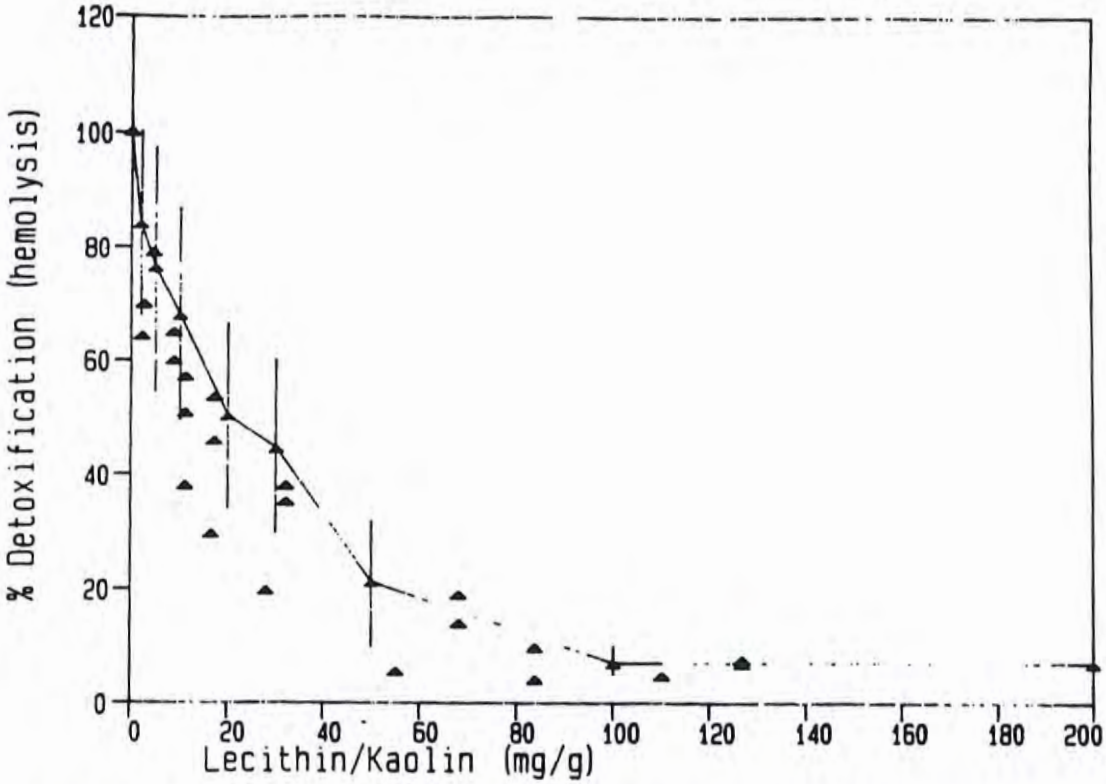

FIGURE 2. Hemolytic potential of kaolin versus dipalmitoyl lecithin treatment. Open triangles connected by line segments have as their abscissa values the amounts of lecithin added. The solid triangle scatter graph shows the potentials versus DPL eluted from the particles.

After centrifugation at 500 g, the optical density of the supernatant was spectrophotometrically determined at 540 nm wavelength to measure the hemoglobin concentration.

The release of lactate dehydrogenase from pulmonary macrophages was determined by the method of Reeves and Fimignari;[18] beta-N-acetyl glucosaminidase by the method of Lockart and Kennedy;[19] and beta-glucuronidase by the method of Sellinger *et al.*[20]

DPL or lysolecithin (L-alpha-lecithin, beta palmitoyl) adsorbed to dusts was measured by freeze drying the dust, eluting phospholipids from the dust with methanol/chloroform (2/1 by volume), separating by thin layer chromatography, and phosphate quantitating by the method of Bartlett.[21]

Suppression of the hemolytic cytotoxicity of quartz and of kaolin by DPL was measured after incubation of each dust in DPL emulsion at concentrations calculated to provide coatings from 2 to 200 mg lecithin per gram dust, assuming total adsorption of the lecithin in emulsion. Some experiments were run with parallel samples which were then analyzed for amount of DPL adsorbed as measured by the phosphate assay.

Combined effect of DPL and lysolecithin (which is itself a lytic agent) adsorbed to quartz and to kaolin on their hemolytic potentials was similarly measured, except that the dusts were incubated with DPL emulsion containing an equal molar concentration of dissolved lysolecithin.

Time course experiments for the effect of PLA2, from porcine pancreas, on DPL-treated dust hemolytic potential was determined for dusts incubated at 7.5 mg dust per ml of DPL emulsion. The emulsion concentration was 10 mg DPL per ml saline. This is sufficient to provide saturation as determined by the adsorption isotherm measurements. Enzyme activity at levels from 10 to 300 activity equivalents was applied for periods of 2 to 216 hours. Following the incubation with enzymes and washing procedures, the hemolytic strengths of the dusts were measured and the amounts of residually adsorbed DPL and lysolecithin measured.

For tests in a simulated long-term system, pulmonary macrophages were obtained from rabbits and maintained in tissue culture for different periods of time. Cells were washed twice with HBSS and resuspended in Medium 199 containing 10% fetal calf serum and penicillin/streptomycin 100 ug/ml. Approximately 4×10^6 cells were placed in individual culture wells, permitted to stabilize for 24 hours at 37 C, 5% CO_2, and 90% relative humidity, and then challenged with native or DPL-treated quartz at levels of 400 ug dust per 4×10^6 cells. Cells which were not challenged with dust were also maintained to determine the background rate of macrophage damage. Cytotoxicity was assayed by LDH and beta-N-acetyl glucosaminidase release.

Results

Hemolytic potentials of DPL-treated quartz are shown with respect to untreated quartz in Figure 1. The highest abscissa value is around 50 mg lecithin per gram quartz, in concert with adsorption isotherm values.[12] The hemolytic strength of quartz is reduced by adsorbed lecithin, reaching

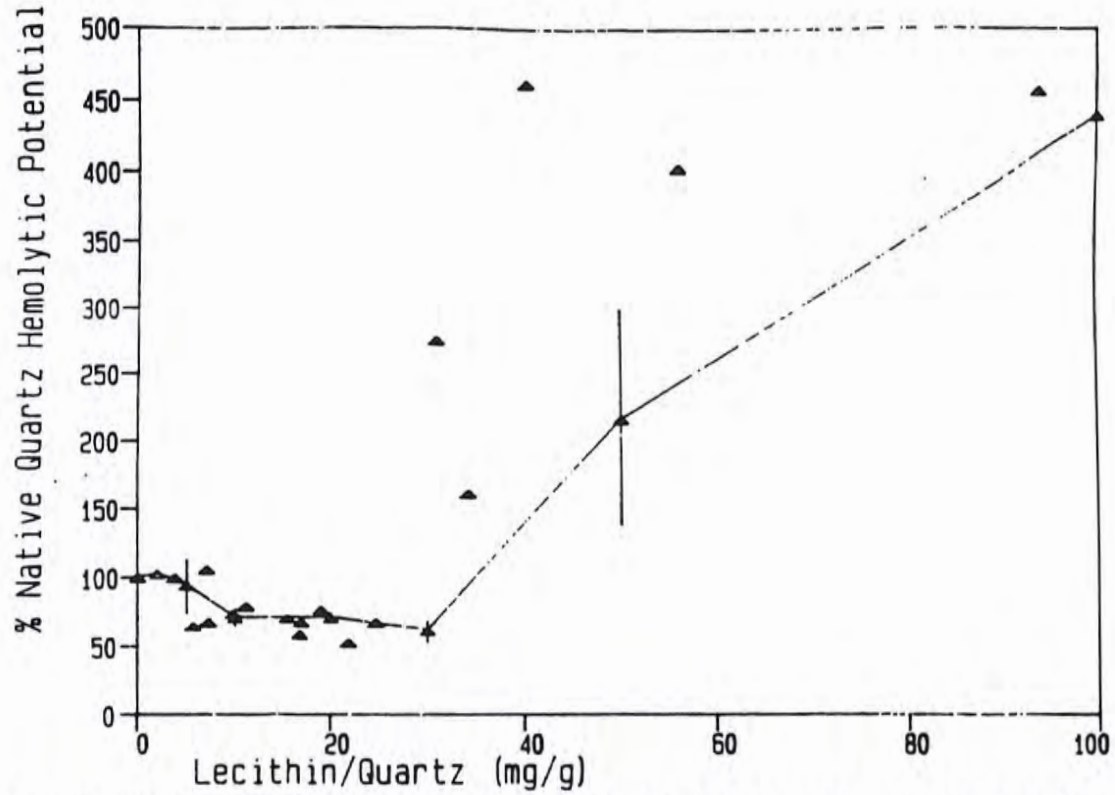

FIGURE 3. Hemolytic potential of quartz versus treatment by lysolecithin and dipalmitoyl lecithin added in equal molar amounts. Ordinate values are expressed as percentages of the value for untreated quartz. Open triangles with line segments have DPL added as their abscissa values. Solid triangle abscissa values are amounts of DPL eluted.

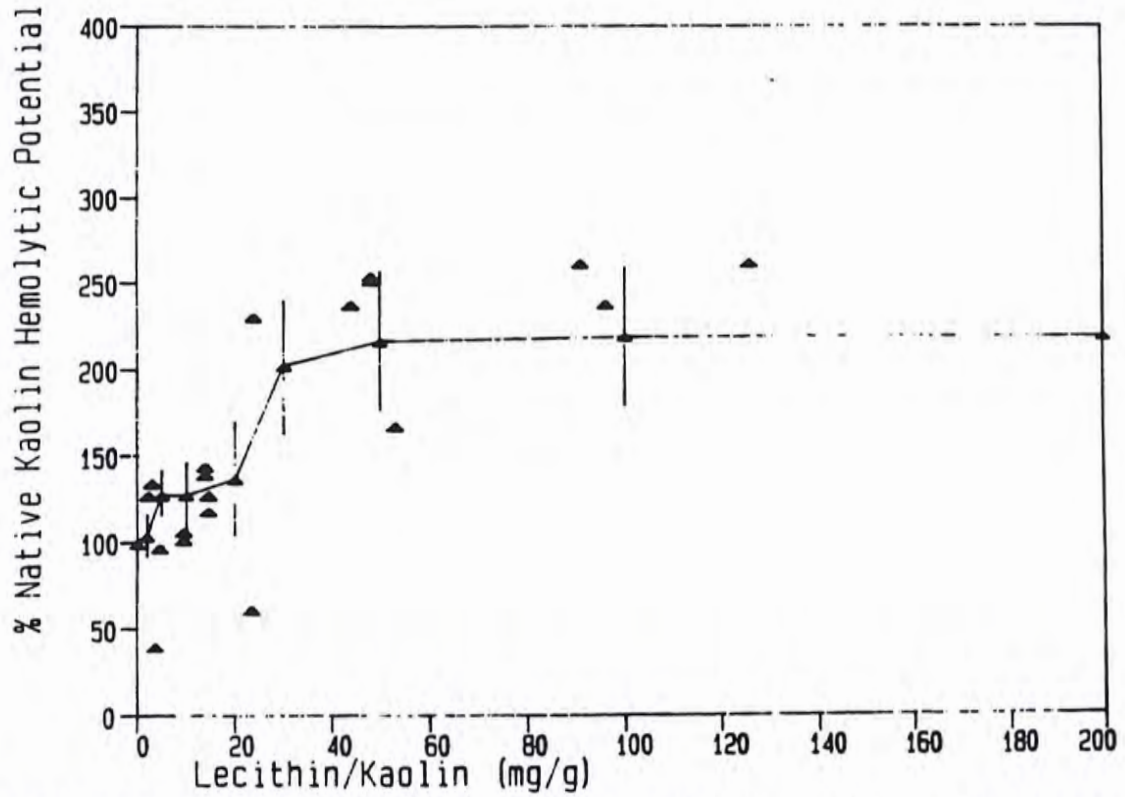

FIGURE 4. Hemolytic potential of kaolin treated with lysolecithin and dipalmitoyl Lecithin in equal molar amounts. Ordinate values are expressed as percentages of the value for untreated kaolin.

background levels between 20 and 30 mg/g adsorbed lecithin. Figure 2 similarly quantitates the hemolytic potential of kaolin. Maximum specific lecithin adsorption is about 130 mg/g; and values approach background between 80 and 100 mg/g. When comparing values between the quartz and kaolin dusts used, one should note the ratios of their specific surface areas, the kaolin having about three times the value of the quartz for surface area per gram.

Hemolytic potentials of quartz and kaolin treated with DPL and lysolecithin in combination were also measured. We have previously reported that the retoxification of dusts treated first with DPL and then with phospholipase enzymes can be due to a combination of functionally available particle lecithin isotherms have been presented elsewhere for these dusts.(12)

The hemolytic potential of DPL-treated quartz after phospholipase A2 digestion *in vitro* is shown in Figure 6. Hemolysis is shown as a function of enzyme activity and digestion time. With the exception of one point at the highest enzyme activity, the potential rises with digestion time to approach untreated quartz levels between 44 and 72 hours. Parallel samples were eluted with organic solvent, the eluted material separated by thin layer chromatography, and measured by phosphate analysis. Figure 7 shows the amount of DPL remaining on the quartz following digestion. This is shown as a function of applied enzyme activity and time of

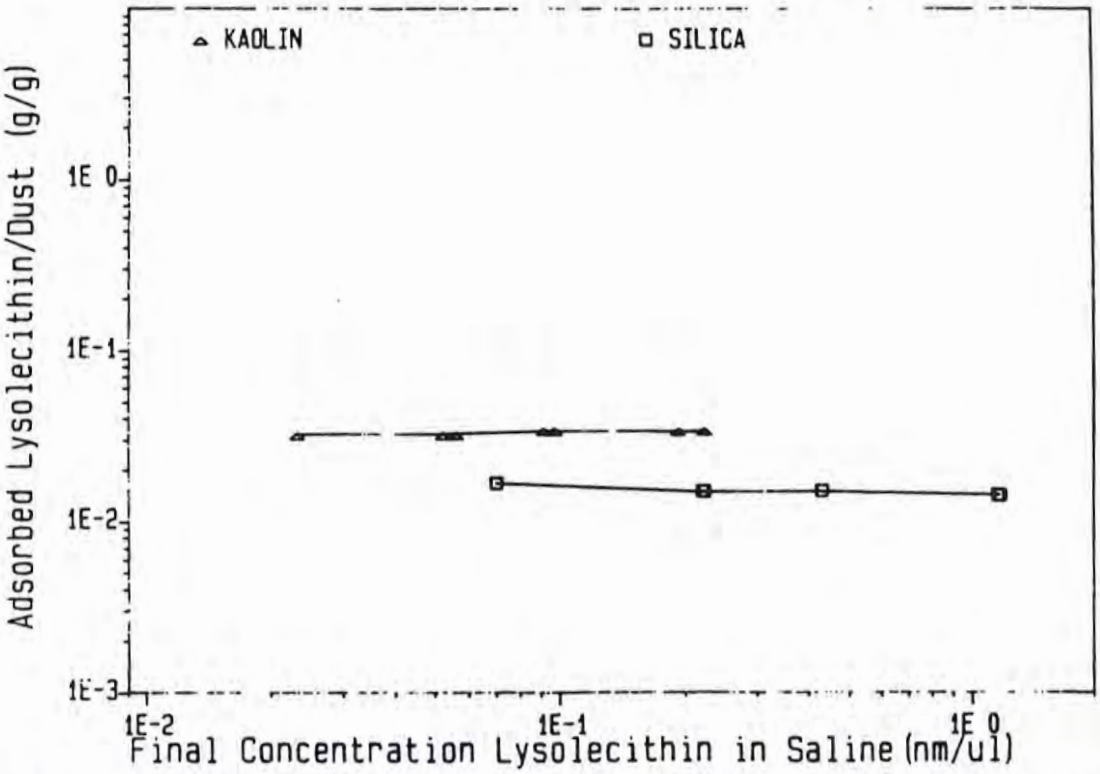

FIGURE 5. Adsorption isotherm for lysolecithin adsorption on kaolin and on quartz from saline at 37C. Ordinate values are mass specific adsorption as measured from initial and final concentration of lysolecithin in solution.

surface activity and to adsorbed lysolecithin produced by the enzymatic digestion of lecithin.[8,12] Figure 3 shows the hemolytic potential for quartz treated with equimolar concentration of lecithin and lysolecithin together in physiologic saline. Figure 4 presents comparable data on the hemolytic potentials of lecithin and lysolecithin-treated kaolin. In both Figures 3 and 4, the maximum values of hemolytic potential reached are limited by the measurement conditions; that is, all the erythrocytes have been lysed. The hemolytic potentials above the untreated dust levels are presumed to be due to lysolecithin adsorbed to the dust or adsorbed to lecithin on the dust. In general the amount of lysolecithin eluted was less than the lecithin eluted, the lysolecithin being substantially more soluble in saline than the lecithin. Figure 5 presents the 37°C adsorption isotherm for lysolecithin on quartz and kaolin in physiologic saline; the corresponding

enzymatic digestion. There is a rapid decrease in the amount of adsorbed DPL by the end of two hours, the shortest digestion time used. At 72 hours the levels detected are within the background for this method of separation and quantitation. Figure 8 shows similar measurements of the lysolecithin which was separated from DPL by the thin layer chromatography of eluted material. Much lower amounts of lysolecithin are seen, which decrease to background levels around 44 hours. The hemolysis and DPL retention data are overlaid on the maps of hemolytic potential of DPL and lysolecithin treated quartz in Figure 9. Values typically fall in the region indicating contributions to the cytotoxicity from both mechanisms, with the dust surface contribution increasing with digestion time.

The hemolytic potential of DPL-treated kaolin after digestion with phospholipase A2 is shown in Figure 10. Unlike the quartz results, the kaolin potential generally is sig-

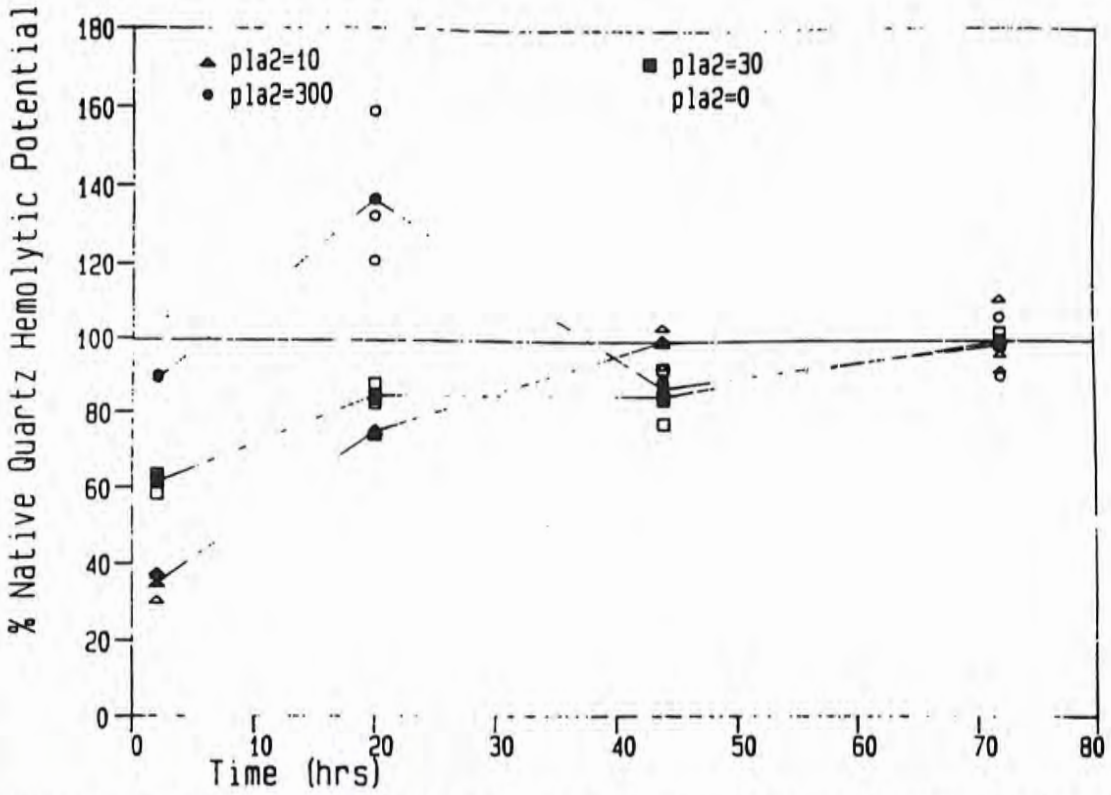

FIGURE 6. Erythrocyte hemolysis by dipalmitoyl lecithin and phospholipase A2 treated quartz versus time of incubation with the lipase. Data is shown for different levels of applied enzyme activity, expressed in terms of "activity equivalents" defined in the text.

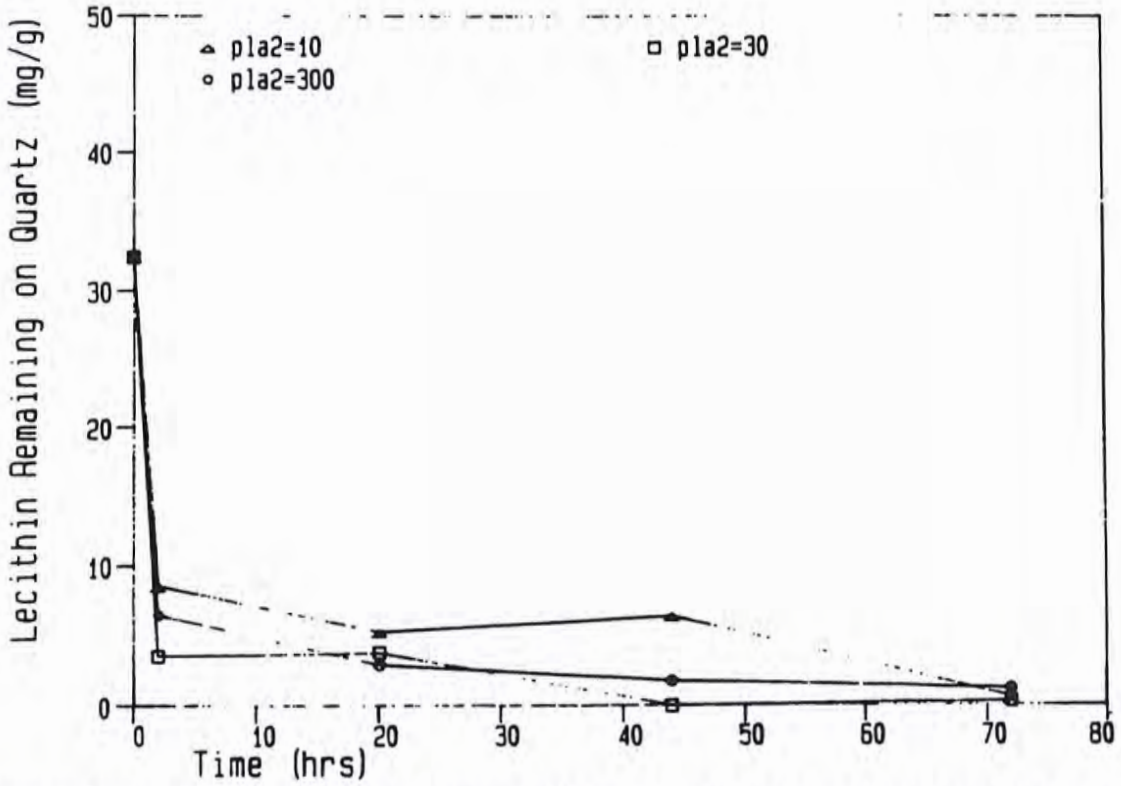

FIGURE 7. Dipalmitoyl lecithin retained on quartz following treatment with phospholipase A2 versus time of incubation with the lipase. Data is shown for different levels of applied enzyme activity expressed as "activity equivalents."

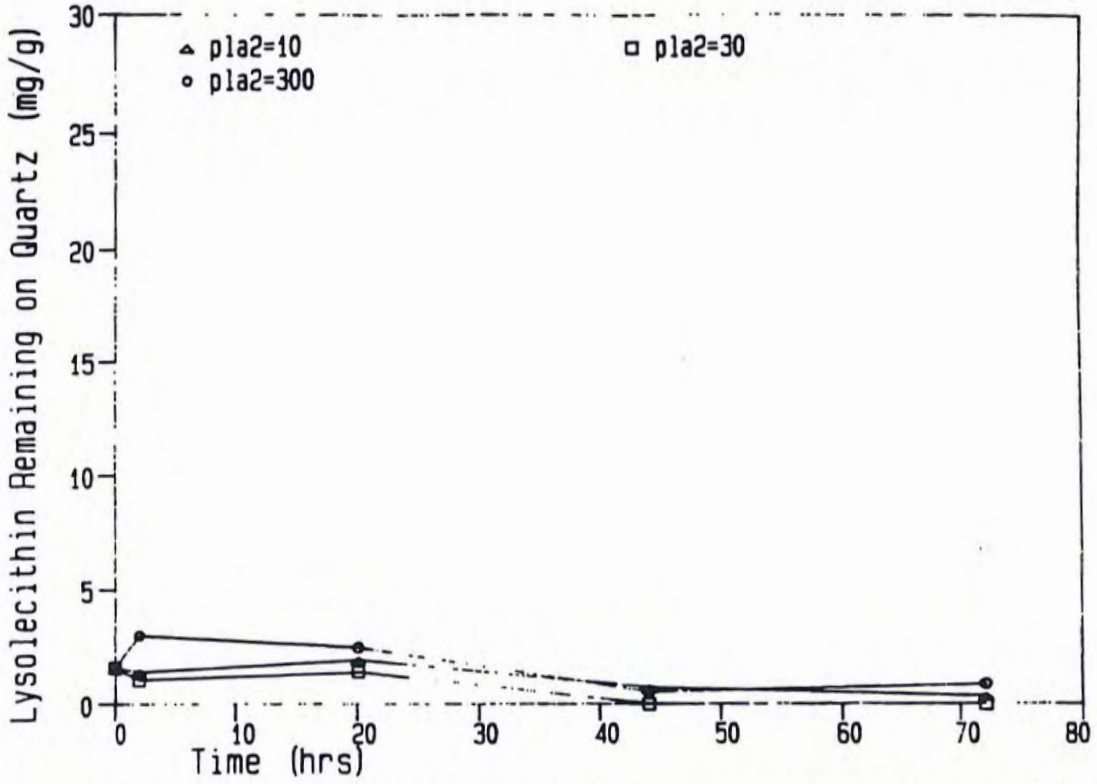

FIGURE 8. Lysolecithin retained on quartz following treatment with phospholipase A2 versus time of incubation with the lipase. Data is shown for different levels of applied enzyme activity expressed as "activity equivalents."

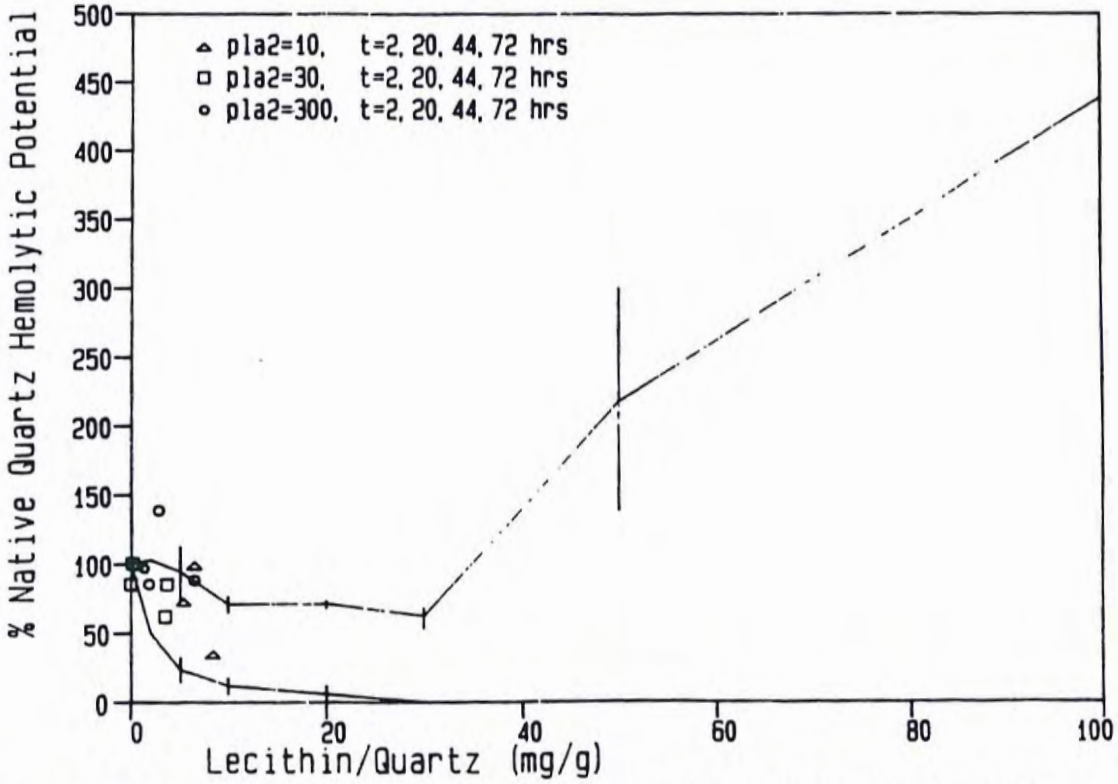

FIGURE 9. Erythrocyte hemolytic potential and retained dipalmitoyl lecithin for lecithin and phospholipase A2 treated quartz. Data from Figures 6 and 7 are presented on the map of quartz hemolytic potential versus lecithin and lysolecithin treatment from Figures 1 and 3.

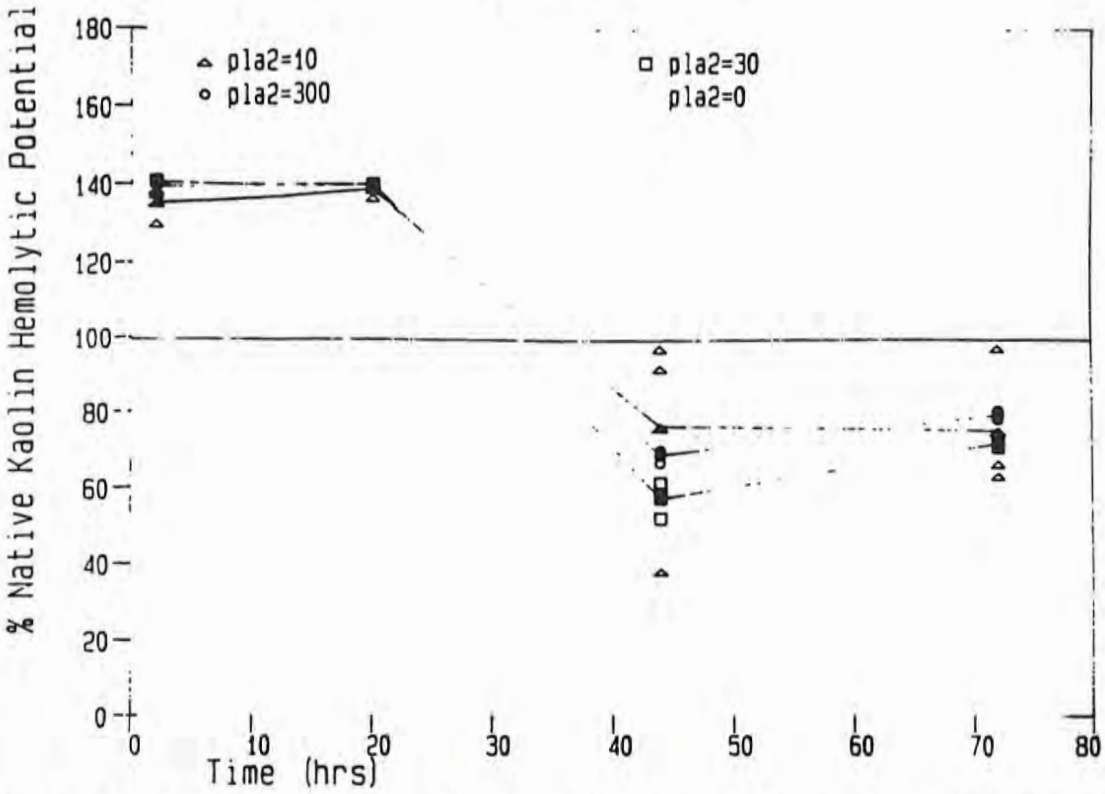

FIGURE 10. Erythrocyte hemolysis by dipalmitoyl lecithin and phospholipase A2 treated kaolin versus time of incubation with the lipase. Data is shown for different levels of applied enzyme activity, expresseed in terms of "activity equivalents" defined in the text.

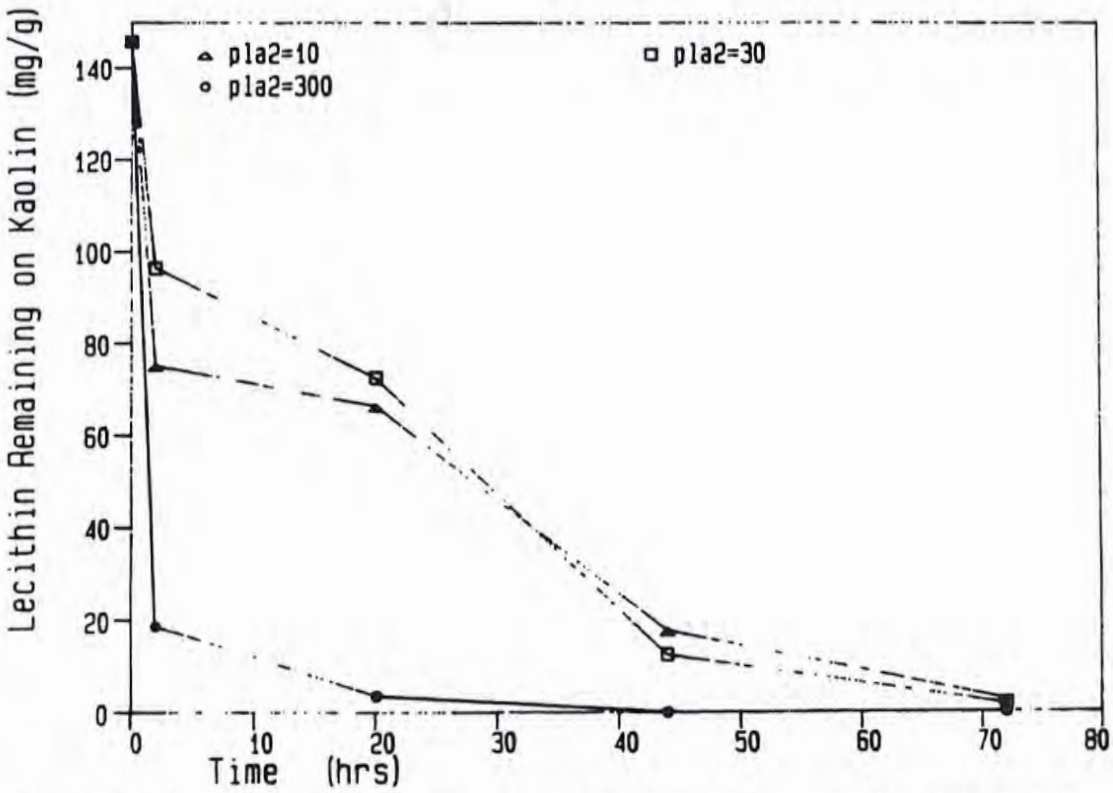

FIGURE 11. Dipalmitoyl lecithin retained on kaolin following treatment with phospholipase A2 versus time of incubation with the lipase. Data is shown for different levels of applied enzyme activity expressed as "activity equivalents."

nificantly greater than that of untreated kaolin. Somewhere between 20 and 44 hours these potentials drop to values below that of native kaolin. At 72 hours, the potential is rising back toward the native dust levels. Figure 11 shows the amounts of DPL remaining on the kaolin as a function of enzyme activity and of time of digestion. This is measured by elution, chromatography, and phosphate analysis as for the quartz. Relatively high levels of DPL are retained on the kaolin to 44 hours as shown, in contrast to the quartz behavior. Figure 12 shows the corresponding lysolecithin retention data. Again, in contrast to quartz, high levels are retained in general out to the 44-hour data point. Figure 13 displays the hemolytic potential and retained DPL data for kaolin on the map of potential versus adsorbed DPL and lysolecithin for kaolin. Lower enzymatic activities and digestion times are seen to result in potentials which can be attributed to enzymatically produced lysolecithin. Longer digestion times result in potentials which might be attributed at least in part to surface effects. That the high hemolytic potentials above those of native kaolin can be attributed to adherent lysolecithin has been demonstrated by additional mixed enzyme studies to those previously reported,[12] comparing the effects of one hour digestions with 300 activity equivalents of phospholipase A2, from Crotalus adamanteus venom, and mixtures of it with equal activities of phospholipase C. The double enzyme treatment reduces the hemolytic potentials and adsorbed DPL levels from system saturation values for kaolin to values consistent with fractional exposed surface toxicity with little lysolecithin contribution. The quartz potential after double lipase treatment is partially reduced from levels manifesting some combined effects of surface and lysolecithin to values consistent with activity due principally to exposed surface activity.

Development is underway in our lab on a system to challenge pulmonary macrophages *in vitro* with dusts treated with pulmonary surfactant, radiolabeled components of pulmonary surfactant, or proposed prophylactic agents. The attempt will then be to maintain the cells in culture long enough to measure any restoration of cytotoxic effects above those displayed by negative controls, that is, by cells not dosed with dusts. Figures 14 and 15 demonstrate, in part, the method and its major difficulty – maintaining viable control cells. The figures show the percentage of lactate dehydrogenase and beta-N-acetyl glucosaminidase which is released from the cells as a function of time since dust exposure. The top line is the release from cells challenged with untreated quartz. Cell damage or death is presumed to be indicated if both enzymes are released. The partial suppression of quartz hemolytic potential for the first 3 days is attributed to prophylactic effects of various biochemicals in the culture nutrient media. The bottom line shows the attrition of cells treated identically except that they have not been challenged with quartz. The data for cells challenged with DPL-treated quartz are initially at the levels for the negative controls. This may be due principally to the prophylactic effects of DPL on phagocytized dusts; we have observed that the DPL treatment also slows uptake of quartz by the cells. The high attrition rates in this experiment do not permit un-

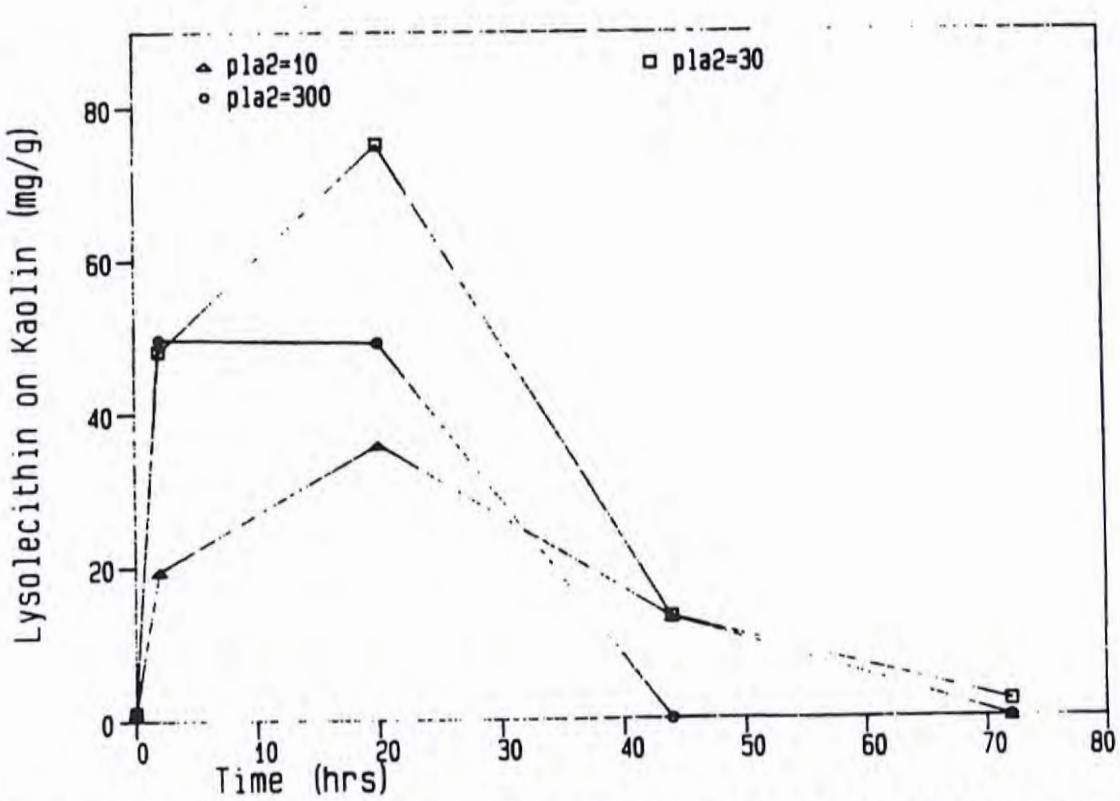

FIGURE 12. Lysolecithin retained on kaolin following treatment with phospholipase A2 versus time of incubation with the lipase. Data is shown for different levels of applied enzyme activity expressed as "activity equivalents."

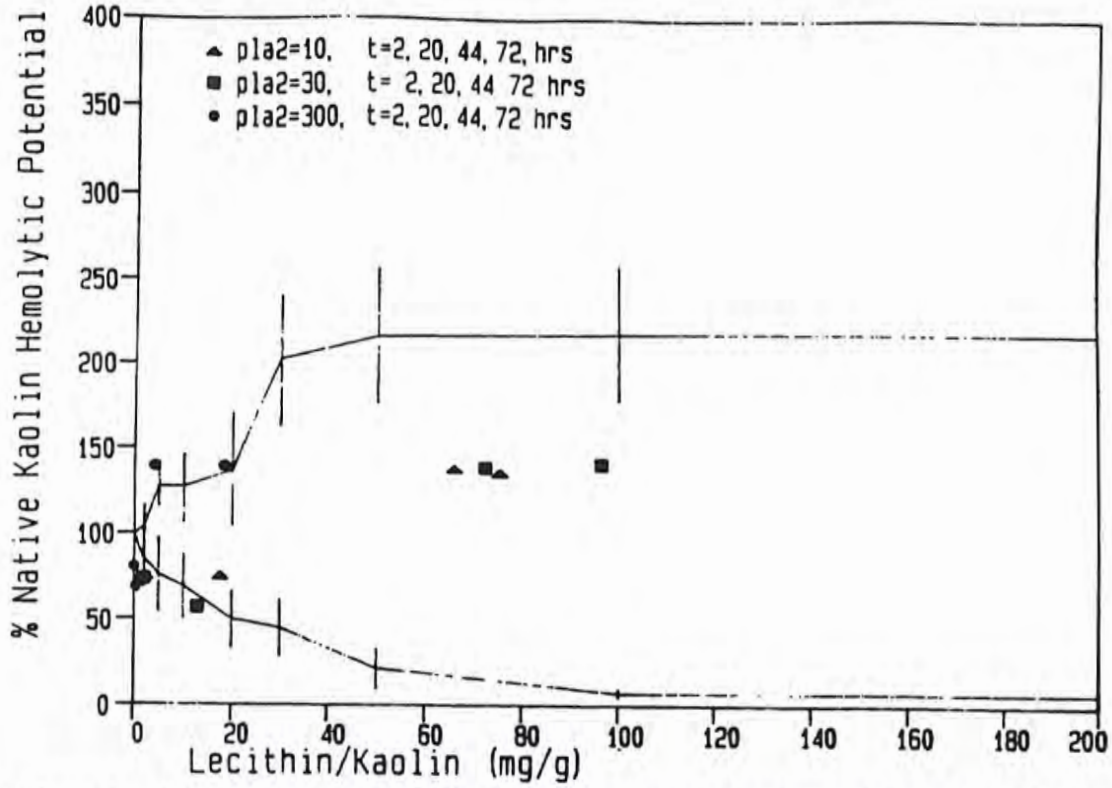

FIGURE 13. Erythrocyte hemolytic potential and retained dipalmitoyl lecithin for lecithin and phospholipase A2 treated kaolin. Data from Figures 10 and 11 are presented on the map of kaolin hemolytic potential versus lecithin and lysolecithin treatment from Figures 2 and 4.

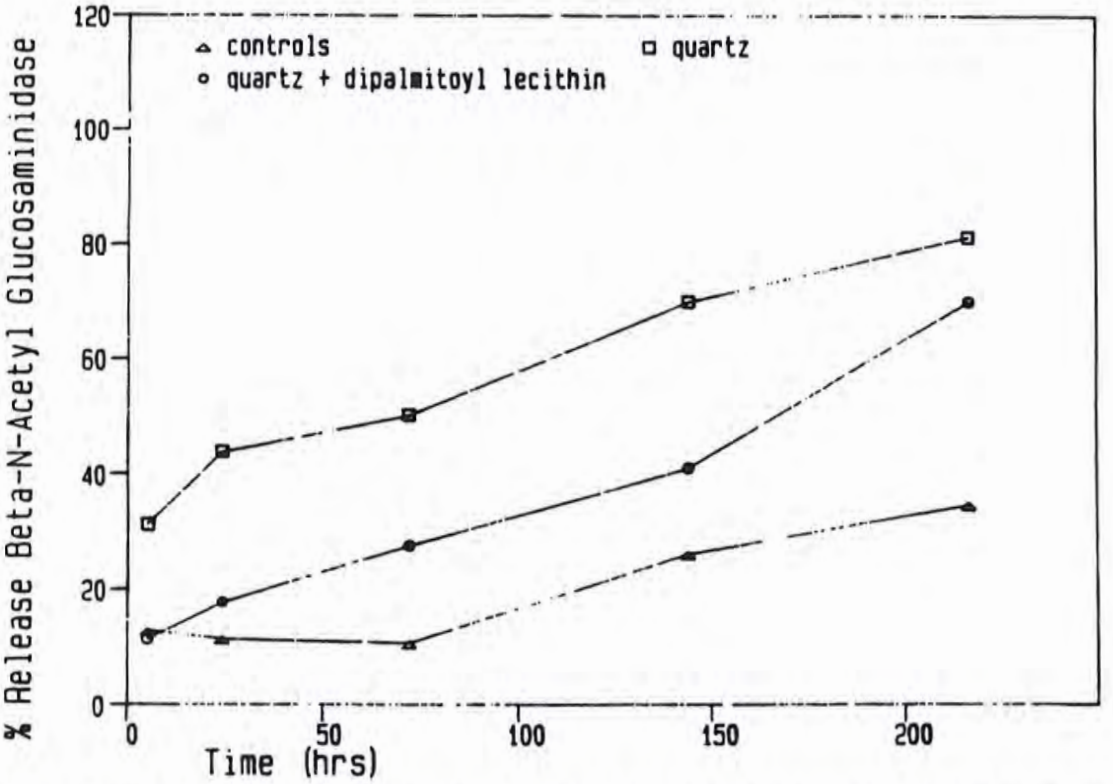

FIGURE 14. Release of lysosomal enzyme versus time following quartz dust challenge for rabbit pulmonary macrophages in culture. Data is shown for negative controls, i.e., macrophages not challenged with quartz; for positive controls, i.e., for challenge with untreated quartz; and for challenge with lecithin treated quartz.

equivocal interpretation of the longer time values. We currently are testing modifications of the system to improve the viability of the controls.

Discussion

Adsorption of pulmonary surfactant by a respired particle may suppress prompt cytotoxic damage to alveolar macrophages and epithelial cells in the lung. Dipalmitoyl lecithin, a major component of pulmonary surfactant, suppresses the *in vitro* hemolytic potential of both quartz and kaolin, and also suppreses their potential to cause major damage to pulmonary macrophages *in vitro* as measured by cytosolic and lysosomal enzyme release. Comparison of estimated amounts of surfactant in a healthy lung and DPL adsorption data indicate *in vivo* pulmonary surfactant levels are adequate to provide a defense against the prompt lytic effects of silicate or aluminosilicate dusts at typical occupational exposure levels.[11] Instillation of quartz and kaolin in animal lungs does not produce prompt permanent damage;[22] acute but transient inflammatory cellular response *in vivo* which is observed for both dusts[22] indicate some non-mineral specific effect of respired dusts. The effect or lack of effect of surfactant on dust induced release of antigenic factors by pulmonary macrophages has not been investigated.

Phospholipase A2 enzymatic digestion in vitro of DPL-coated quartz or kaolin can remove all or most of the adsorbed DPL over the period of a few days for the enzymatic activities used. There appear to be mineral specific differences in the rates of digestion and in the degree of adherence of lipase-produced lysolecithin to the DPL-dust complex at intermediate stages of digestion. Mineral specific rate differences might result from differing free energies of adsorption or from conformational differences in adsorbed DPL which provide different levels of steric hinderance to the lipase action. Phospholipase activity is affected by the physical state of its phospholipid substrate.[23]

Adsorption of lecithin to aluminosilicate dust surface may involve electrostatic and hydrogen bonding. Alumina surface is a better catalyst than silica for hydrolysis of lecithin in organic media,[24] indicating more effective interaction of alumina surface hydroxyls with the ester bonds to the glycerol moiety of lecithin. Lecithin is a zwitterion with the choline head group cationic and the phosphate group anionic. Silanol groups on the surface of quartz in aqueous media partially dissociate to produce negatively charged surface sites, with an isoelectric point around pH 1.5;[25] that is, part of the adsorption of DPL to quartz may be attributed to electrostatic interaction between the choline group at the tip of the DPL and the dissociated silanol groups on the quartz surface.[26] Kaolin has a structure of alternate layers of silica tetrahedra and alumina octahedra. Hydroxyl groups on the surface of alumina have an isolectric point of about pH 9.4,[25] the surface of alumina bearing a net positive charge. Adsorption of DPL to kaolin should involve in part electrostatic interaction between the alumina octahedra positively charged surface sites and the negatively charged phosphate

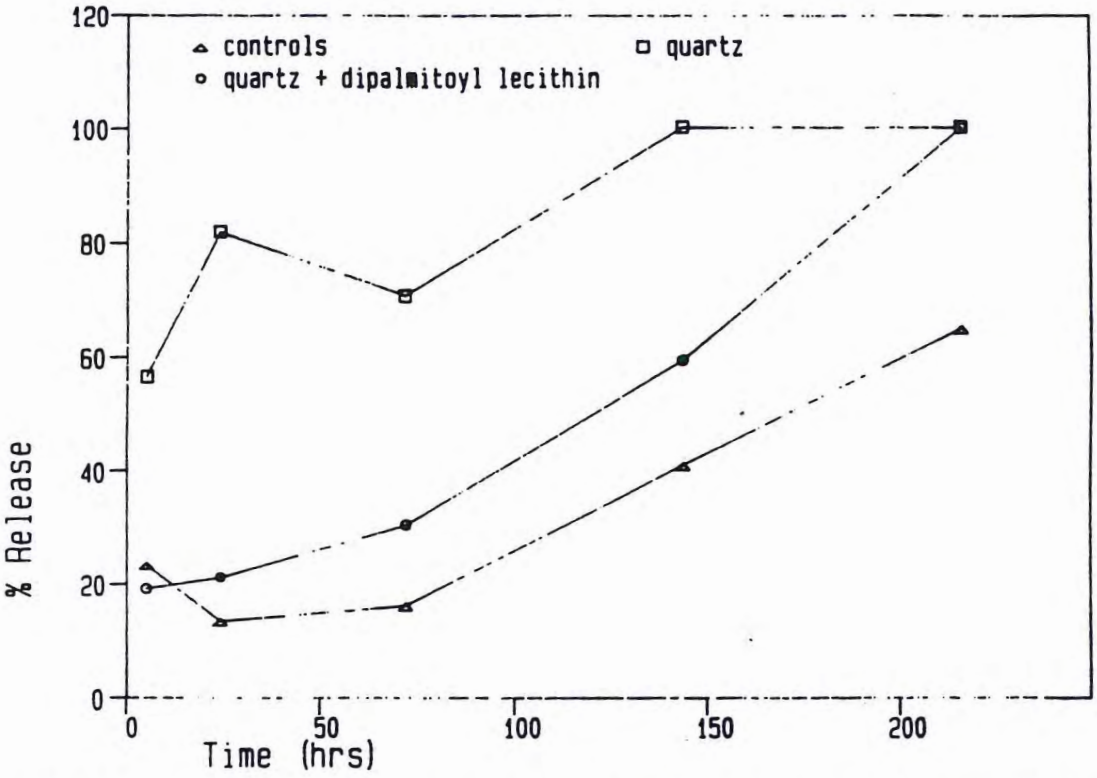

FIGURE 15. Release of cytosolic enzyme versus time following quartz dust challenge for rabbit pulmonary macrophages in culture. Data is shown for macrophages not challenged with quartz, challenged with untreated quartz, or challenged with lecithin treated quartz.

moiety of DPL. The phosphate-glycerol bond is hydrolyzed under the enzymatic action of Phospholipase C and the adjacent ester linkage of glycerol to a fatty acid acyl group is hydrolyzed by the action of Phospholipase A2. An adsorbed phospholipid conformation in which phosphate groups are directed toward alumina surface groups of aluminosilicate dust might hinder enzymatic hydrolysis of the target ester linkages.

Lysolecithin is adsorbed to a more limited extent than DPL by both quartz and kaolin in single compound adsorption isotherm measurements. Adsorption of lysolecithin at intermediate stages of the phospholipase A2 digestion of DPL on dusts may be due in part to lysolecithin association with adsorbed DPL. Lysolecithin on quartz and kaolin decreases as DPL is digested away. We have not determined yet the degree of mineral adsorption of hydrolysis produced fatty acids or their contribution to cytotoxicity suppression. In the phagolysosome of a pulmonary macrophage, a number of enzymes would act upon a surfactant coated particle. Our limited experiments with phospholipase A2 and C in combination indicate that the mixed activity can significantly diminish the lysolecithin contribution to the hemolytic potential compared to the single enzyme treatment result. To provide a useful basis for analysis of physiologic effects, these *in vitro* digestion studies must be extended to use lavaged surfactants, full lysosomal enzyme applied at acidic conditions representative of the phagolysosomal interior, and representative relative amounts of surfactant, enzyme activity, and dusts. We are currently measuring the DPL-digesting activity of full lysosomal enzyme, which we obtain by density gradient centrifugation methods[28] from rat liver homogenate.

Complementary to these *in vitro* digestion experiments, the long-term macrophage challenge system is an attempt to obtain time course information on the suppression of dust cytotoxic properties after challenge and phagocytosis of surfactant treated dusts. If macrophages can be maintained viable in culture long enough to unequivocally distinguish dust induced effects, then the possible correlation of induced effects with the fate of the initial coating of labeled surfactant will be tested. Comparing the time course for retoxification of kaolin with that of quartz should indicate whether or not these interactions are significant in distinguishing the pathogenicity of respirable quartz dust.

Acknowledgment

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