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The enzymatic removal of a surfactant coating from quartz and kaolin by P388D1 cells

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Abstract

The macrophage-like cell line, P388D1, was exposed to dipalmitoyl lecithin (DPL)-coated respirable quartz and kaolin, and the disappearance of the DPL was monitored for up to 9 days. The coating was removed rapidly at first (about 50% in the first 3 days) and then more slowly over the remaining 6 days, until about 30% remained on day 9. The rate of DPL digestion was independent of the type of dust and the amount of coated dust within the cell, indicating the existence of an extracellular phospholipase activity. This extracellular phospholipase activity was partially characterized. It was sensitive to temperatures above 56°C, the presence of EDTA, the action of the proteases trypsin and proteinase K, and pH, being active at pH 7 but not at pH 5. This is consistent with reports in the literature of the existence of an extralysosomal phospholipase which is active at pH 7 and dependent on the presence of divalent metal ions. There was a dust-dependent difference in the extracellular rate of DPL digestion from quartz and kaolin. The coating was removed more slowly from the kaolin than it was from quartz. The removal of the DPL coating seen in the presence of cells was presumably due to both an intracellular and an extracellular phospholipase.

Abbreviations: ATCC, American Type Culture Collection; BET, Bruneur–Emmet–Teller; CP, chlorphentermine; CQ, chloroquine; DPL, dipalmitoyl phosphatidyl lecithin; EDS, energy-dispersive X-ray spectrometry; I, imipramine; OsO_4, osmium tetroxide; PBS, phosphate-buffered saline; FBS, fetal bovine serum

Introduction

Exposure to respirable dusts is a major cause of occupational lung disease. Some dusts, such as crystalline silica (quartz) are extremely fibrogenic, causing impaired pulmonary function and increased mortality, whereas others, such as kaolin, are relatively nonfibrogenic. Although these diseases have been recognized for many years, the precise sequence of events between the inhalation of the dust and the development of fibrosis is poorly understood.

Most studies have centred on the interaction between the dust particles and alveolar macrophages since the dust is likely to be ingested by these cells after entering the alveolar region of the lung. Studies show that the highly fibrogenic dust, quartz, is toxic to macrophages *in vitro*, whereas dusts such as titanium dioxide, which are nonfibrogenic *in vivo*, are relatively nontoxic to macrophages *in vitro* (Style and Wilson, 1973; Davies, 1980). On the basis of

this, it has been suggested that the cytotoxicity of a dust is a good predictor of its fibrogenic potential (Marks and Nagleschmidt, 1959; Harington, 1972; Harington, 1974; Chamberlain and Brown, 1978). However, there are some dusts, for example the clay, kaolin, which are cytotoxic to macrophages *in vitro* (Brown et al., 1980; Daniel and LeBouffant, 1980) but which do not cause significant fibrosis *in vivo* (Lynch and McIver, 1954; Hamilton and Hardy, 1982; Lapenas et al., 1984; Vallyathan et al., 1988). It therefore seems likely that there are factors other than cytotoxicity involved in the initiation of fibrogenesis.

It has been proposed by Wallace et al. (1975, 1985) that prior to being ingested by alveolar macrophages the inhaled dust will come into contact with pulmonary surfactant and will become coated with it. Therefore, it would be more relevant to study the interaction between macrophages and surfactant-coated dust particles. Wallace et al. (1988a) have shown that coating both quartz and kaolin with dipalmitoyl phosphatidylcholine (DPL), the primary component of pulmonary surfactant, renders these dusts nontoxic to macrophages *in vitro*. They propose that after the alveolar macrophages have ingested the surfactant-coated dust particles, the lysosomal enzymes of the cell will digest away the protective coating, possibly retoxifying the dust. If this is the case, it may be the rate at or degree to which these cells are capable of removing the protective coating which is important in determining the fibrogenic potential of a particular dust (Wallace et al., 1985; Wallace et al., 1988b). The ease with which the cells are capable of removing the protective coating will presumably be related to the nature of the interaction between the dust and the surfactant, which will be determined by the nature of the dust surface (Keane et al., 1988).

The aim of this study was to ascertain whether macrophages *in vitro* are indeed capable of removing the DPL coating from the surface of quartz particles, and, if so, to determine whether the rate at which they do so differs between quartz and kaolin. Since primary macrophages are terminally differentiated cells, they do not survive for long in culture, and therefore the macrophage-like cell line P388D1 was used.

Materials and methods

Quartz

Crystalline silica or quartz (Min-U-Sil) was obtained from U.S. Silica (Berkeley Springs, WV, USA) and was fractionated using a Donaldson classifier. The fraction in which 80% of the particles had a diameter of less than 5 μm was collected and used throughout the project. The quartz was at least 98.5% pure as determined by energy-dispersive (EDS) X-ray analysis (Stetler et al., 1983). The specific surface area was 3.97 m^2/g as determined by Bruneur–Emmet–Teller (BET) N_2 adsorption (Bruneur et al., 1938).

Kaolin

Kaolin was obtained from Georgia Kaolin Mills (Augusta, GA, USA) and was similarly fractionated. The fraction in which 90% of the particles had a diameter of less than 5 μm was collected and used throughout the project. The kaolin was at least 96% pure as determined by EDS analysis (Stetler et al., 1983) and had a specific surface area of 13.25 m^2/g as determined by BET N_2 adsorption (Bruneur et al., 1938).

Cell culture

The cell line P388D1 was purchased from the American Type Culture Collection (Rockville, MD, USA) (ATCC # TIB 63). The cells were grown in RPMI 1640 medium plus 10% fetal bovine serum (FBS), penicillin and streptomycin, and were routinely maintained in 75 cm^2 flasks at 37°C in an atmosphere of 5% CO_2.

The cells were split whenever necessary by scraping them from the surface of the flask with a rubber policeman. The cells were replated at a density of $1–2 \times 10^5$ cells/ml.

Cell viability was assessed by the ability of the cells to exclude the dye, trypan blue. A sample of cells was added to half their volume of 4% trypan blue (Sigma), and left to stand at room temperature for 10 min. At this time, one tenth the volume of buffered formalin was added to fix the cells, and both viable and nonviable cells were counted using a hemacytometer.

DPL coating of quartz and kaolin

Synthetic DPL was obtained from Calbiochem (La Jolla, CA, USA) (#4295) and was supplied as a white powder. DPL, which was radiolabeled with ^{14}C on both carbonyl carbons of the palmitic acid chains, was obtained from Dupont (Boston, MA, USA) (#NEC 682).

An appropriate volume of [^{14}C]DPL was placed at the bottom of a test tube. The solvent was evaporated away under a stream of air for 20 min. Unlabeled DPL was dispersed in 0.165 mol/L saline by ultrasonication for 10 min and was added to the [^{14}C]DPL and sonicated for 2 min. This DPL was then added to the dust sample in a 15-ml test tube. The final DPL concentration was 40 mg/g quartz and 160 mg/g kaolin. The tubes were rotated in an incubator at 37°C for 1 h. The dust was separated from the supernatant by centrifugation at 1200*g* for 10 min and was then washed twice with 0.165 mol/L saline to remove any loosely bound DPL. It has previously been determined that, after washing, approximately 20 mg DPL/g dust remains attached to the quartz and about 80 mg DPL/g dust to the kaolin (Wallace et al., 1986).

DPL digestion

In all the DPL digestion experiments, the cells (P388D1) were grown in RPMI 1640 supplemented with FBS and streptomycin and penicillin. The cells were plated at a density of 4×10^5 cells/cm^2 in 6-well plates.

For the digestion experiments, [^{14}C]DPL-coated quartz or kaolin was added to the cells in 6-well plates which were incubated at 37°C in 5% CO_2. At various time intervals (0, 1, 3, 6 and 9 days), the wells were harvested by scraping the cells and dust from the surface of the well with a rubber policeman and washing the wells with 0.5 ml Triton X-100 (1% in PBS). The total lipids, and then the intact DPL, was extracted from each well as described below. A decrease in the amount of intact DPL recovered indicated that digestion had taken place. As controls, the same concentrations of dust were also incubated in the absence of cells.

Extraction of total lipids

The procedure used was a modification of the methods of Bligh and Dyer (1959) and Folch et al. (1957). The sample of interest was extracted with chloroform : methanol (2:1). The ratio of sample to solvent was no more than 0.7:10, such that the two phases were miscible. The two phases were mixed and left to stand at room temperature overnight. The following day, 2 ml ice-cold 0.1 mol/L KCl was added per 10.7 ml solvent plus sample. This was vigorously shaken for 1 min and then centrifuged at 1500*g* for 20 min to separate the phases. The KCl concentrates the lipids in the organic phase and the upper aqueous phase was removed. The sides of the tubes were washed 3 times with chloroform : methanol : H_2O (3:48:47, v:v:v). This mixture remains as a separate phase above the organic phase and was discarded. The lower organic phase contains the total lipids that were present in the sample. The intact DPL was then isolated as described below.

Isolation of DPL

The procedure used was that of Mason et al.

(1976). The total lipids which were dissolved in chloroform : methanol (2:1) were evaporated to dryness. The dried lipids were redissolved in 0.5 ml of 6.2 mg/ml OsO_4 (Sigma Chemical Co., St Louis, MO, USA) in CCl_4 (Sigma). The OsO_4 was stored as a stock solution of 100 mg/ml in CCl_4 in the dark at 4°C. The stock solution is stable for at least 2 years. The lipids were incubated in OsO_4 for 15 min at room temperature. The OsO_4 reacts with any double bonds to form complexes (Stoekenius and Mahr, 1965; Korn, 1967; Riesmersma, 1968), and the solution darkens as OsO_2 is formed. Since DPL has no double bonds, it is not affected by the treatment with OsO_4. The samples were evaporated to dryness and then redissolved in 1 ml chloroform : methanol (20:1, v:v). This was applied to neutral alumina columns. The columns were made by pouring 0.8 g aluminum oxide (neutral alumina 100-200 mesh, Biorad Laboratories, Richmond, CA, USA), which had been activated at 180°C overnight, onto a glass wool plug in a Pasteur pipette. The packing of the glass wool plug determines the flow rate of the column which should not exceed 0.8 ml/min. Neutral alumina binds to acidic lipids, resulting in both the DPL and the OsO_4–lipid complexes binding strongly to the column. The columns were first eluted with 10 ml chloroform : methanol (20:1, v:v). This eluted all of the neutral lipids and was discarded. The DPL was eluted from the column with 5 ml chloroform : methanol : 7 mol/L ammonium hydroxide (70:30:2, v:v). The OsO_4–lipid complexes remained on the column and were visible as a dark band near the top. The eluate was collected in scintillation vials, evaporated to dryness and counted in a Beckman LS9000 scintillation counter after the addition of 10 ml Instagel (Packard # 6013394).

Synthesis of DPL from free palmitic acid

The digestion products of DPL are the two palmitic acid chains containing the radiolabeled carbon atoms, the glycerol backbone and the phosphorylcholine head group. The method used to determine the rate of digestion of DPL assumes that the free palmitic acid chains are not used to resynthesize intact DPL. To ensure that this was not occurring, the synthesis of DPL from radiolabeled palmitic acid was measured. Palmitic acid, which was labeled on the carbonyl carbon, was obtained from Dupont and was supplied as a solution in ethanol. Unlabeled palmitic acid was obtained from Calbiochem (#5064). P388D1 cells were plated as before at a density of 2×10^5 cells/cm^2 in 6-well plates. One hundred mg or 1600 mg of DPL-coated quartz and an equivalent number of palmitic acid chains, of which a portion were radiolabeled, were added to the cells. The wells were harvested at 0, 1, 3, 6 and 9 days, and the amount of radioactive DPL present was measured as described previously. Any radioactive DPL present must have been synthesized from the palmitic acid chains supplied.

Extracellular phospholipase activity

Conditioned medium was prepared by growing P388D1 cells in the presence of 100 mg unlabeled DPL-coated quartz for 3 days. The cells were plated at a density of 2×10^5 cells/cm^2 and 2×10^5 cells/ml in 75-cm^2 flasks. After 3 days, the medium was removed from the flasks and the cells and dust were removed by centrifugation at 250*g* for 10 min. The resulting conditioned medium was used to measure the extracellular phospholipase activity.

The conditioned medium was incubated in 6-well plates in the presence of 80 mg [^{14}C]DPL-coated quartz in a total volume of 600 ml. The amount of dust used was equivalent to 100 μg/10^6 cells if there had been cells present. The conditioned medium and DPL-coated quartz were incubated at 37°C for 3 days. At the end of this time, the wells were harvested and the amount of DPL remaining was determined as described previously. As a

control, DPL-coated quartz was also incubated with RPMI, i.e. unconditioned medium.

In order to characterize the extracellular phospholipase activity, the conditioned medium was subjected to the following treatments prior to assaying for phospholipase activity: one cycle of freezing and thawing, heating to 56°C for 30 min, boiling for 10 min, incubation at 37°C overnight, incubation with 100 mg/ml proteinase K overnight at 37°C; incubation with 100 mg/ml trypsin overnight at 37°C and incubation with 100 mg/ml papain overnight at 37°C.

Conditioned medium was assayed for phospholipase activity on DPL dispersed in medium in the presence of 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5 and 5 mmol/L chlorphentermine (CP), 0.3 and 3 mmol/L imipramine (I), 10 and 50 mmol/L chloroquine (CQ), and at pH 5 and 7.

Phospholipase activity was measured after 3 days as described previously.

The rate of digestion of DPL from the surfaces of both quartz and kaolin by the extracellular phospholipase was determined over a period of 14 days. The dust concentrations used were 80 μg/well for quartz and 20 and 80 μg/well for kaolin. The wells were harvested at 0, 1, 3, 6, 9, and 14 days and the amount of DPL determined as described previously. As controls, the DPL-coated dusts were also incubated in the absence of cells.

Statistical analysis

The data were statistically analyzed by performing either the Student's *t*-test or an analysis of variance, using the general linear models procedure on mainframe SAS, followed by a least-square means test. All experiments analyzed by the latter method were randomized complete block designs and were analyzed as such. In all cases, the α level was set at 0.05.

Results

In order to determine which concentrations of quartz and kaolin should be used for the digestion studies, the viability of P388D1 cells in the presence of various concentrations of DPL-coated quartz and kaolin was measured. These results are presented in Figures 1 and 2.

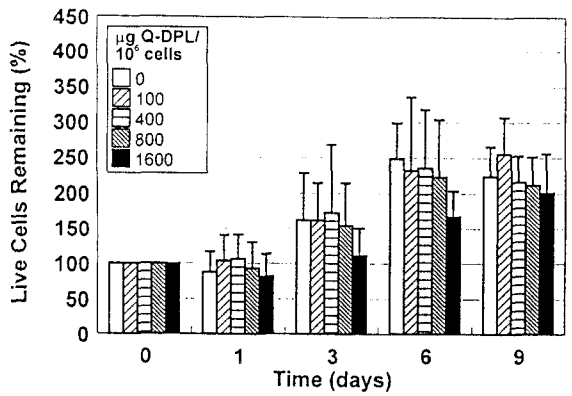

Figure 1. The viability of P388D1 cells in the presence of DPL-coated quartz (Q-DPL). Viability was assessed by trypan blue exclusion in conjunction with total cell counts. A decrease in cell number was assumed to represent cell death. The results are the means of four experiments and the error bars represent one standard deviation. The viability was statistically different from the control on days 3 and 6 for 1600 μg/10^6 cells.

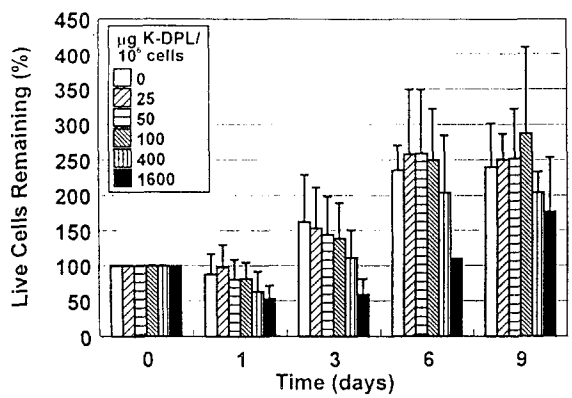

Figure 2. The viability of P388D1 cells in the presence of DPL-coated kaolin (K-DPL). Viability was assessed by trypan blue exclusion in conjunction with total cell counts. A decrease in cell number was assumed to represent cell death. The results are the means of four experiments and the error bars represent one standard deviation. The viability was statistically different from the controls on days 3 and 6 for 400 μg/10^6 cells, and days 3, 6, and 9 for 1600 μg/10^6 cells. ($\alpha = 0.05$).

In the case of DPL-coated quartz, the concentrations used were 100, 400, 800, and 1600 μg quartz/10^6 cells. The highest concentration used had a slightly deleterious effect on the viability of the cells. Statistical analysis indicates that, at a dust level of 1600 μg/10^6 cells, the viability was significantly different from the control on days 3 and 6.

The concentrations used for DPL-coated kaolin were lower since kaolin has a larger surface area and adsorbs more DPL per unit mass than quartz. The concentrations used were 25, 50, 100, 400, and 1600 μg/10^6 cells. The two highest concentrations used had an effect on the viability of the cells. Statistical analysis indicates that, at a dust level of 400 μg/10^6 cells, the viability was significantly different from the control on days 3 and 6, and, at a dust loading of 1600 μg/10^6 cells, the viability was different from the control on days 3, 6, and 9.

Comparing the data for the two dusts, it appears that 1600 μg quartz/10^6 cells is comparable to 400 μg kaolin/10^6 cells. Therefore, the concentrations chosen for the digestion studies were 100, 400, 800, and 1600 μg/10^6 cells for quartz and 25, 50, 100, and 400 μg/10^6 cells for kaolin.

The digestion of DPL from the surface of quartz and kaolin by P388D1 cells in tissue culture was measured by monitoring the disappearance of ^{14}C-labeled DPL over 9 days. It was assumed that the DPL which had disappeared had been digested by the phospholipases of the cells. Since the labeled carbon atoms would remain attached to the palmitic acid chains of the DPL after digestion by phospholipases A1 and A2, this method relies on the assumption that none of this palmitic acid was used by the cells to resynthesize DPL. In order to test this assumption, P388D1 cells were supplied with unlabeled DPL-coated quartz and ^{14}C-labeled palmitic acid, and the appearance of labeled DPL was monitored over a period of 9 days. The amount of palmitic acid used was the same amount which would have been present if all of the DPL supplied had been digested. There was no significant synthesis of DPL from the palmitic acid supplied.

Figures 3 and 4 show that DPL is indeed digested from the surface of both quartz and kaolin by P388D1 cells, and that the rate is

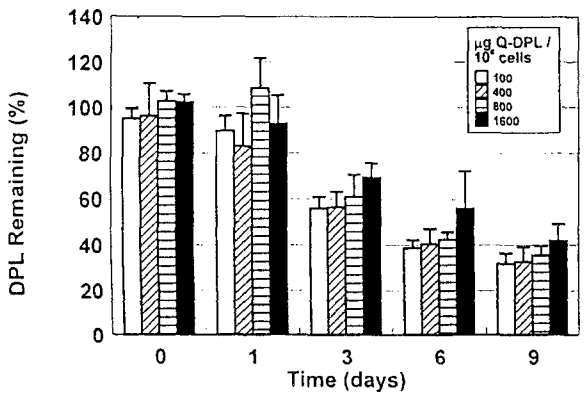

Figure 3. The digestion of DPL from the surface of quartz by P388D1 cells. The results are means of three experiments and the error bars represent one standard deviation. The group without cells was statistically different from the group with cells. The rate of digestion of the highest concentration was significantly different from the other three on day 6. ($\alpha = 0.05$).

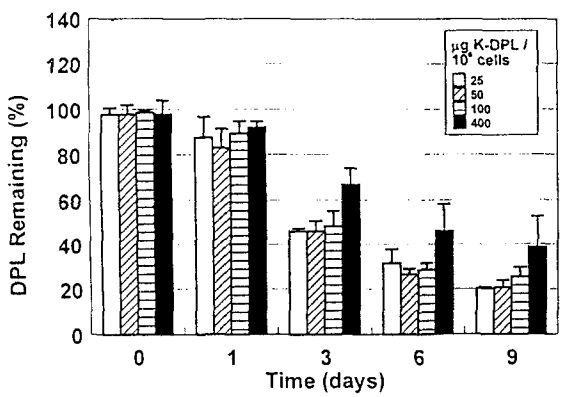

Figure 4. The digestion of DPL from the surface of kaolin by P388D1 cells. The results are means of three experiments and the error bars represent one standard deviation. The group without cells was statistically different from the group with cells. The rate of digestion of the highest concentration was significantly different from the other three on days 3, 6, and 9. ($\alpha = 0.05$).

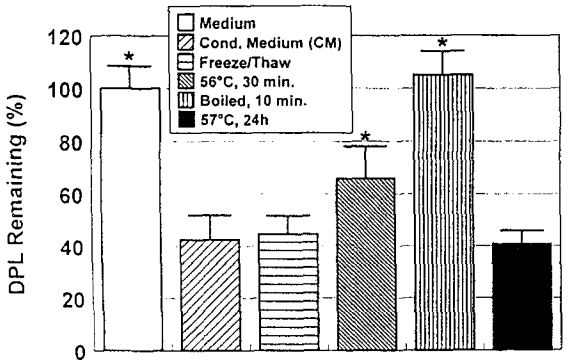

Figure 5. Demonstration of an extracellular phospholipase activity and the effect of temperature on this activity. Conditioned medium was prepared by growing P388D1 cells in the presence of DPL-coated quartz for 3 days. 400 ml conditioned medium (CM) or culture medium (RPMI) was incubated with 80 mg [^{14}C]DPL-coated quartz for 3 days at 37°C. The results represent the means of three experiments and the error bars represent one standard deviation. The symbol, *, indicates that the result is significantly different from that of CM. ($\alpha = 0.05$).

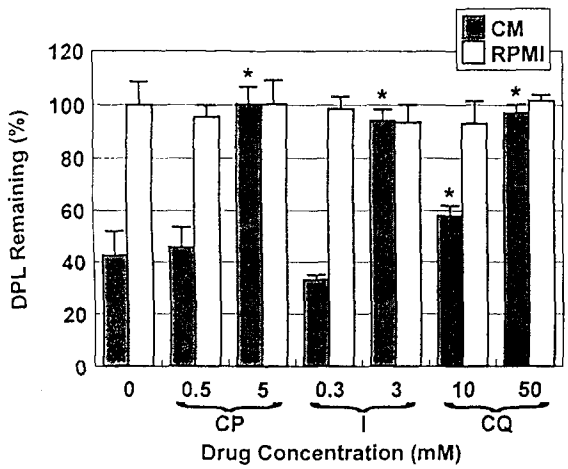

Figure 6. The effects of the drugs, chlorphentermine (CP), imipramine (I) and chloroquine (CQ) on the extracellular phospholipase activity. The conditioned medium (CM) or culture medium (RPMI) plus the drug was incubated with 80 mg [^{14}C]DPL for 3 days. The results represent the means of three experiments and the error bars represent one standard deviation. The symbol, *, indicates those samples, within the CM group, which were significantly different from the control. ($\alpha = 0.05$).

independent of dust concentration for the lower three concentrations used. At the highest concentration of dust used, the rate of digestion was slightly slower in both cases, possibly reflecting the lower cell viability at this concentration. The rate of digestion in the lower three concentration was independent of dust concentrations and independent of the amount of dust within the cells. Microscopic examination revealed that only at the lowest concentration was all of the dust within the cells.

An extracellular phospholipase activity is clearly recorded in Figure 5. The degree of DPL digestion by the conditioned medium was statistically significantly different from the degree of digestion by culture medium, which was essentially zero. This extracellular phospholipase activity is inhibited by boiling the sample for 10 min, slightly inhibited by heating the sample to 56°C for 30 min, and unaffected by freezing and thawing or incubation at 37°C overnight. Figure 6 shows the effect of the three drugs, chlorphentermine (CP), imipramine (I), and chloroquine (CQ), on the extracellular phospholipase activity. All three drugs were

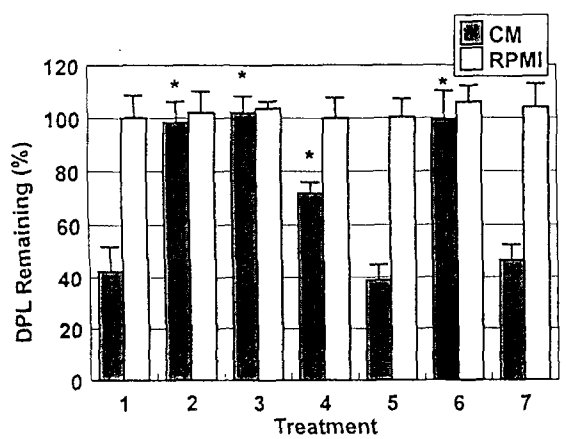

Figure 7. The effect of various treatments on the extracellular phospholipase activity. Conditioned medium (CM) or culture medium (RPMI) was incubated with 80 mg [^{14}C]DPL for 3 days. Treatments were: 1. control; 2. 2 mmol/L EDTA; 3. 100 μg/ml proteinase K; 4. 100 μg/ml trypsan; 5. 100 μg/ml papain; 6. pH = 5; 7. pH = 7. The results represent the means of three experiments and the error bars represent one standard deviation. The symbol, *, indicates those samples, within the CM group, which were significantly different from the control. ($\alpha = 0.05$).

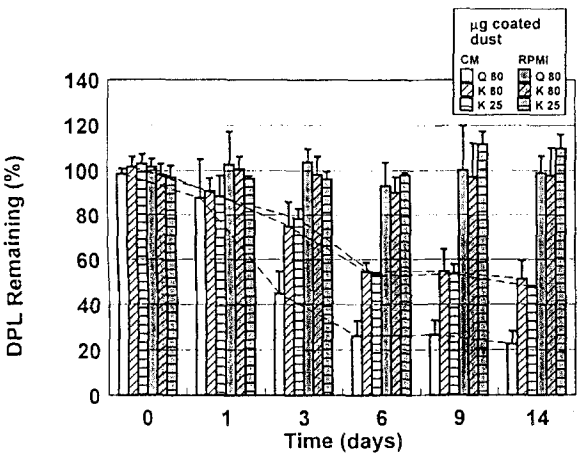

Figure 8. The rate of digestion of DPL from the surface of quartz and kaolin by an extracellular phospholipase. Conditioned medium (CM) or culture medium (RPMI) was incubated with [^{14}C]DPL-coated quartz or kaolin for periods of up to 14 days. The results represent the means of three experiments and the error bars represent one standard deviation. The rate of digestion of DPL from quartz was statistically different from the rate of digestion from kaolin.

capable of inhibiting this activity. The drugs themselves do not degrade the DPL. Figure 7 shows the effect of various other treatments on the extracellular phospholipase activity. This activity is inhibited by EDTA (2 mmol/L), pretreatment with proteinase K or trypsin, and pH 5.

Figure 8 shows the rate of removal of DPL from quartz and kaolin over a period of 14 days by the extracellular phospholipase. The concentrations of dust used were 80 µg/well quartz and 80 and 20 µg/well kaolin. Statistical analysis shows that the rate of DPL removal from kaolin was independent of the concentration and was slower than the removal of DPL from quartz. There was no spontaneous degradation of the DPL.

Discussion

This report appears to be the first demonstration that macrophages are capable of digesting DPL from the surface of a mineral dust, and lends some support to the theory that this coating has to be digested away before the dust has an effect on the macrophages. The rate of digestion of DPL from the surface of quartz is shown in Figure 3. Approximately 50% is removed by the end of the first day and by the sixth day only about 30% remains. Wallace et al. (1988b) measured the digestion of DPL from the surface of quartz by porcine pancreatic phospholipase A2 in a cell-free system. Their data indicates that there are two rates of digestion of the DPL, an initial very rapid rate over the first few hours followed by a slower rate of digestion extending over 3 days. These data have been interpreted in terms of bilayer adsorption of DPL on the dust surface, the inner layer being closely associated with the charged groups on the mineral surface. The outer layer may be digested rapidly and in a non-mineral-specific manner, with the inner layer being digested more slowly and in a mineral-specific manner. The data presented here do indicate that there may be two rates of digestion, the first being faster than the second (Figures 3 and 4). Wallace et al. (1992) also found that the DPL coating was removed from quartz at a far faster rate than it was removed from kaolin. It was therefore surprising that, in this system, there was no difference in the rates at which the coating was removed from the two dusts. However, in neither dust was the DPL completely removed within 9 days: about 30% remained undigested. It is possible that a difference may be seen between the dusts on removal of the last 30% of the DPL. This 30% may be tightly bound to the surface of the dust, and therefore removed slowly and in a dust-dependent manner. The rate of DPL digestion from both dusts was independent of the amount of dust which was within the cell, indicating that at least some of the digestion was occurring outside the cell. This was verified by demonstrating that the extracellular phospholipase activity was sensitive to two proteases and inhibited by three drugs which

have been shown to inhibit phospholipase *in vitro* (Hostetler and Matsuzawa, 1981). In addition to this, the extracellular phospholipase was active at pH 7 and not pH 5 and was inhibited by EDTA. In this regard it is similar to a phospholipase activity reported by Franson et al. (1973), in a preparation of disrupted alveolar macrophages. They found that there were two phospholipases present in disrupted macrophages. The first had a pH optimum of 5; its activity was enhanced by the presence of EDTA and it was associated with the lysosomal fraction. The second had a pH optimum of 7.0–8.5, required Ca^{2+} ions for activity and was distributed among several fractions. It is suggested that this phospholipase is associated with the plasmalemma although its precise intracellular location was difficult to determine because it lost activity during proliferation (Franson et al., 1973).

The extracellular phospholipase reported here was also found to digest a DPL coating from the surface of both quartz and kaolin but at different rates. This was not the case in the digestion seen when there were cells present, where presumably the digestion was due to both a lysosomal phospholipase and this extracellular phospholipase. It is interesting to note that Wallace et al. (1992) saw a difference in the rate of DPL removal from quartz and kaolin when using a purified phospholipase at pH 7. In both cases, the DPL was removed from the quartz more quickly than it was removed from kaolin. It is possible that this is related to the pH at which the digestion took place.

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