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Effects of Chrysotile on a Lysosomal Enzyme Preparation and on the Hydrolytic Enzyme Activity of Cultured Alveolar Macrophages

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The interaction between chrysotile and three lysosomal enzymes (acid phosphatase, acid RNase and acid protease) in isolated lysosomal enzyme-rich preparations (LEP), from sheep alveolar macrophages maintained in the presence and absence of serum components or pulmonary surfactant at pH 5.0 and pH 7.0 for up to 22 days, is investigated. It is concluded that chrysotile does not inhibit or enhance lysosomal enzyme activity at either pH but may preferentially absorb specific enzymes and that the binding reaction between any given enzyme and mineral can be dependent on the presence of other organic compounds. The release of three hydrolytic enzymes (β-galactosidase, acid RNase and protease) from cultured rabbit alveolar macrophages, in the presence of different concentrations of bovine serum (5-20%) and in the presence and absence of chrysotile for 72 hr, was also studied. Chrysotile enhances early differential release of each hydrolytic enzyme, but after 72 hr both control and chrysotile-treated cultures (maintained in 10-20% serum) have very similar intra- and extracellular levels of hydrolytic activity. The apparent differential release of lysosomal enzymes by untreated macrophages, which is dependent on serum concentration and time *in vitro*, is discussed.

Introduction

Following the early studies of Allison et al. (*1*) on the increased permeability of lysosomes in cultured peritoneal exudate cells exposed to silica, a number of investigators have shown that a variety of agents, often capable of inducing granulomas *in vivo*, can induce selective release of lysosomal enzymes from these cells *in vitro*.

One such agent is chrysotile asbestos (*2-5*), which seems equally effective in inducing a dose-response selective hydrolytic enzyme release in cultured peritoneal or alveolar macrophages incubated in the presence or absence of serum. The work reported here attempts in part to investigate this phenomenon in more detail. We therefore posed a series of questions such as whether different hydrolytic enzymes (β-galactosidase, acid RNase and acid protease) are released in equivalent amounts from alveolar macrophages treated with chrysotile and whether altering the amount of serum components in the culture medium would affect the release pattern. Further studies were carried out to investigate the interaction between chrysotile and lysosomal enzyme preparations under a variety of conditions such as in the presence of serum components and pulmonary surfactant. In order to simulate possible dust/enzyme reactions intralysosomally, these studies were carried out at pH 5.0,and in order to study dust/enzyme interactions following possible hydrolytic enzyme release from cells, the studies were carried out at pH 7.0.

The studies are of importance when considering the possible intra- and extracellular activities of lysosomal enzymes in *in vitro* investigations. In addition, they may bear relevance to the effects of chrysotile *in vivo* where it has been shown that inhalation (*6, 7*) and instillation (*8*) promote the accumulation of alveolar surface protein and pulmonary surfactant and raise the intracellular specific activity of lysosomal enzymes in both free cells (mostly macrophages) and lung tissue.

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Materials and Methods

Ribonucleic acid (from yeast), *p*-nitrophenyl phosphate and *p*-nitrophenyl-β-D-galactopyranoside were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Bovine hemoglobin (Type II) was purchased from the Sigma Chemical Co., Ltd., Kingston, Surrey, U.K.

Isolation of Lysosomes from Alveolar Macrophages

Fresh sheep lungs were obtained from the slaughterhouse with a maximum post-mortem delay of 1 hr. Alveolar macrophages were obtained by standard lung lavage procedures as initially described by Myrvik et al. (*9*) and separated by centrifugation at 300*g* for 20 min at 4°C. The cell pellet containing $2\text{-}3 \times 10^9$ cells were resuspended in 0.25*M* sucrose (20 mL), homogenized, and fractionated to obtain a lysosomal-rich preparation based on the procedure of deDuve et al. (*10*).

Reaction of Lysosomal Enzyme Preparations with Chrysotile Asbestos

The lysosomal-rich fraction from sheep alveolar macrophages was suspended in 20 mL of 0.2 *M* sodium acetate-buffered iso-osmotic saline (pH 5.0) or 0.2 *M* sodium phosphate-buffered saline (pH 7.0) ultrasonicated at 4°C for 2 min and sterilized by passage through a 0.45 μm membrane filter. The enzyme preparation (LEP) was incubated at 35°C with UICC chrysotile A (at a final concentration of 1 mg/mL) in the presence or absence of 20% fetal bovine serum (FBS) + Waymouths' medium (protein concentration 9.2 mg/mL) and in the presence and absence of sheep pulmonary surfactant [final concentration 0.5 mg/mL; for preparation see Harwood et al (*11*)]. In vessels where serum or surfactant was present, the chrysotile was preincubated in these biological media for 60 min at 37-C. The reaction mixtures were maintained for up to 22 days at 35°C under sterile conditions with continuous gentle shaking. Periodically, aliquots were removed and either centrifuged at 1300*g* for 20 min at 4°C prior to assay, or used directly in assays for lysosomal enzymes. Control mixtures consisting of LEP in buffered saline, chrysotile and serum medium, and chrysotile and surfactant, the latter two in the absence of LEP, were treated identically in each case.

Effects of Chrysotile on Lysosomal Enzyme Levels in Cultured Rabbit Alveolar Macrophages

Rabbit alveolar marcophages, collected by lavage using sterile technique, were pelleted by centrifugation at 300*g* for 20 min at 4°C. The cells were washed twice with sterile antibiotic solution (100 units penicillin, 100μg streptomycin/mL 0.15 *M* NaCl) and cultured in Leighton tubes with 1×10^6 cells/culture in 5%, 10%, 15% or 20% FBS × Waymouths' medium (1 mL) at 37°C and gassed with 95% air plus 5% CO_2. Cultures were maintained for 3 days with or without heat-sterilized UICC chrysotile A (100 μg/culture). At 24 hr intervals, the supernatant fluid of each culture was centrifuged at 800*g* for 20 min to collect "floating" cells which were suspended in 0.15 *M* NaCl and returned to the culture bottle. Hydrolytic enzyme activity was measured in the cell fraction (following sonication in 0.15 *M* NaCl) and in the culture medium.

Enzyme Assays

Acid phosphatase was measured as described by Richards and Wusteman (*12*) by using nitrophenylphosphate as the substrate, and activity was expressed as μmole liberated nitrophenol/hr. Acid ribonuclease was assayed according to the modified method of Josefsson and Lagerstedt (*13*) and activity expressed as units activity/hr. Acid protease was measured by using partially denatured hemoglobin as substrate and activity expressed at μmole tyrosine liberated/hr (*12*). β-Galactosidase was assayed by using the method of Beck and Tappel (*14*), and results are expressed as μmole nitrophenol liberated/hr.

Table 1. Total acid protease and acid RNase activity in a lysosomal enzyme preparation (LEP) maintained at pH 5.0 for 22 days at 35°C in the presence and absence of chrysotile (UICC, A).

	Acid protease, μmole tyrosine liberated/hr/mL		Acid RNase, units/hr/mL	
Day of assay	LEP alone	LEP + chrysotile	LEP alone	LEP + chrysotile
0	0.38 ± 0.08	0.40 ± 0.11	1.76 ± 0.09	1.69 ± 0.31
6	0.24 ± 0.04	0.29 ± 0.10	1.10 ± 0.05	1.24 ± 0.17
9	0.22 ± 0.01	0.21 ± 0.06	1.04 ± 0.15	1.09 ± 0.10
12	0.18 ± 0.07	0.16 ± 0.09	1.02 ± 0.12	0.92 ± 0.19
17	0.15 ± 0.05	0.14 ± 0.02	1.02 ± 0.09	0.99 ± 0.21
22	0.09 ± 0.04	0.10 ± 0.05	1.02 ± 0.26	1.05 ± 0.11

Results and Discussion

The results from experiments which attempt to simulate conditions for investigating the intralysosomal reaction of hydrolytic enzymes with chrysotile in the presence of organic materials (serum components, pulmonary surfactant) are shown (Table 1 and Fig. 1).

The enzymes measured show remarkable stability when maintained at pH 5.0 at 35°C for periods of up to 22 days. However, the loss of activity from the LEP alone is different with different enzymes, in that acid ribonculease shows a total reduction of activity over 22 days of 42%, acid protease a reduction of 76% (Table 1) and acid phosphatase 71%. With this latter enzyme most of the activity is lost within 4-6 days (data not shown), as opposed to the more gradual loss of acid protease activity. Chrysotile asbestos does not inhibit, destroy or enhance hydrolytic enzyme activity as total enzyme activity decreases in an identifical manner in control and treated preparations (Table 1). However, analysis of the enzyme activity remaining in the supernatant fraction following centrifugation of the complete preparations suggests that chrysotile does absorb some enzyme protein from solution (Fig. 1*a*). It appears that ribonuclease is more readily removed from solution than acid protease following centrifugation in the presence of chrysotile (Fig. 1*a*), whereas the mineral does not absorb acid phosphatase from the LEP. Such results suggest differential absorption of hydrolytic enzymes to chrysotile and equate with previous findings that proteins may be selectively absorbed by different mineral dusts (*15*). The presence of FBS + Waymouths' medium in the reaction mixture tends to reduce the loss of enzyme activity over 22 days from LEP (Fig. 1*b*), an effect most noticeable with acid protease activity. In addition, the presence of serum components seems to reduce the absorption of hydrolytic enzymes by chrysotile, this effect being most noticeable with acid RNase (Fig. 1*b*). Presumably, there is competition between serum components and hydrolytic enzymes in the LEP for available binding sites on the mineral surface. The presence of pulmonary surfactant in the reaction mixture tends to reduce the loss of

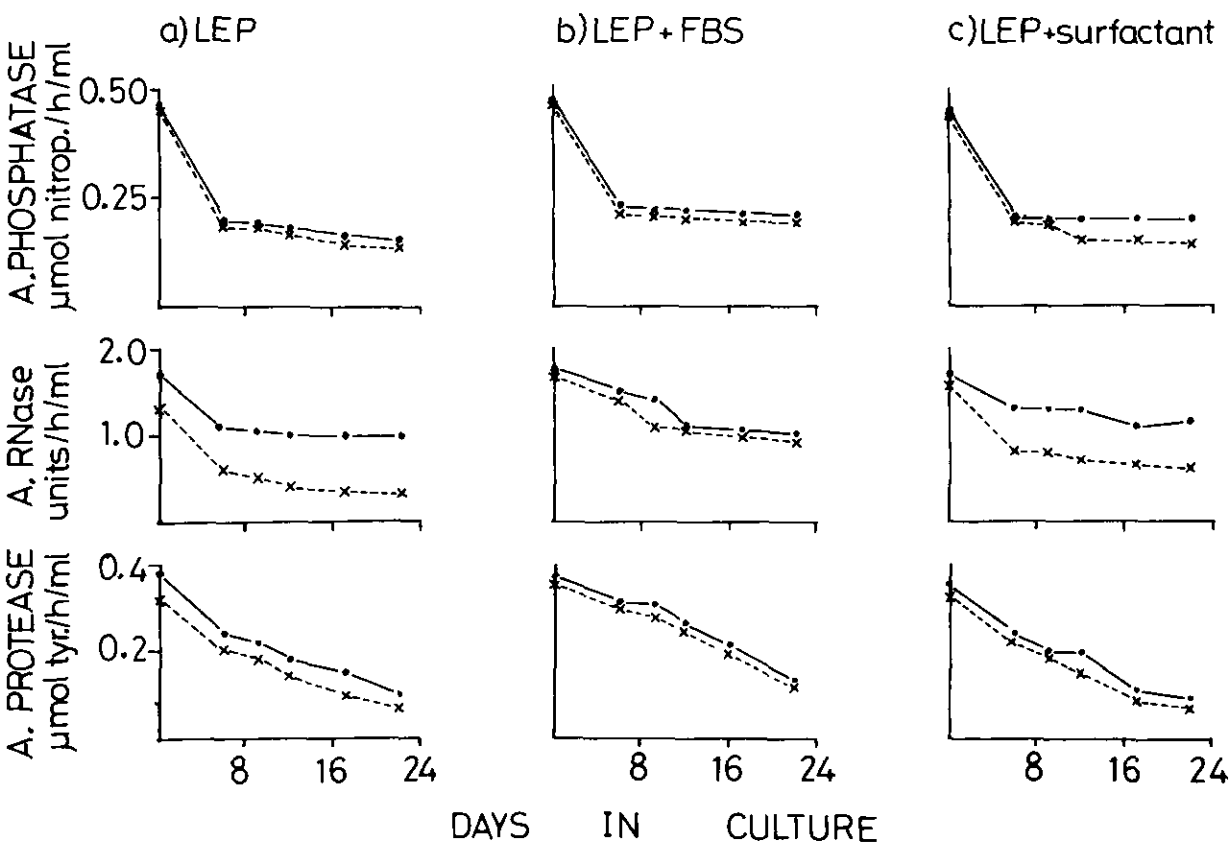

FIGURE 1. Lysosomal enzyme activity remaining in the supernatant fraction of a lysosomal enzyme preparation (LEP) maintained in fetal bovine serum (FBS) or pulmonary surfactant media and incubated at pH 5.0 for 22 days at 35°C in (×) the presence or (•) absence of chrysotile asbestos.

acid-RNase over 22 days (like serum components) but not acid protease or phosphatase (Fig. 1*c*). Interestingly, however, the surfactant does not interfere with the binding of acid RNase by chrysotile (Figs. 1*a* and 1*c*) to the same extent as that found when serum components were present (Fig 1*b*). In summary, these results suggest that chrysotile does not enhance or inhibit lysosomal enzymes at pH 5.0 but may preferentially absorb specific enzymes and that the binding reaction between enzyme and mineral can be dependent on the presence of other organic components.

All of the above studies were repeated at pH 7.0 in an attempt to simulate hydrolytic enzyme/chrysotile interactions which may occur following extracellular release of lysosomal enzymes into different culture media containing serum components or pulmonary surfactant. The levels of enzyme activity decrease more rapidly at pH 7.0 than at pH 5.0 (again differentially for the three enzymes studied; data not shown), although the presence of serum components is clearly beneficial in preventing loss of enzyme activity and particularly for acid protease (Fig. 2). Loss of acid protease in the presence of surfactant is considerably less than in LEP alone when maintained at pH 7.0, an effect which was not observed in preparations maintained at pH 5.0. As was found with the studies conducted at pH 5.0, chrysotile does not enhance or destroy enzyme activity when the preparation is maintained at pH 7.0. At this latter pH, less acid RNase and protease was apparently bound to the mineral than found at pH 5.0.

Total (extracellular plus intracellular) hydrolytic enzyme activities, in cultures of normal rabbit alveolar macrophages and those treated with chrysotile, remain relatively constant for a period of 3 days in concentrations of FBS between 10-20% (data not shown except by reference to Figs. 3 and 4). However, in cultures maintained in 5% serum, total β-galactosidase and acid RNase activities are elevated by 35% and 22%, respectively, after 3 days, whereas acid protease activity is elevated 12% after 24 hr, whereafter it remains constant.

The distribution of extra- and intracellular levels of two enzymes—β-galactoside and acid protease—in these cultures is shown in Figures 3 and 4. Extracellular release of each enzyme by normal cells is different, and both intra- and extracellular activities seem dependent on serum concentration and time in culture.

In control cultures maintained in 10-20% serum, the intracellular level of β-galactosidase is elevated slightly at 24 hr and then gradually decreases up to 72 hr, at which time an elevated level is found in the medium (Fig. 3). In cultures maintained in 5% serum, the intracellular level of this enzyme only increases after 72 hr, after which time the activity in the medium has not increased from that found at the start of the experiment. Ribonuclease activity (data not shown) exhibits a similar pattern with a high activity measurable in the medium of control cultures after 72 hr corresponding to reduced levels detectable within the cells. The extracellular release of this enzyme is not so great (as a percentage of total activity) as found with β-galactosidase. Acid

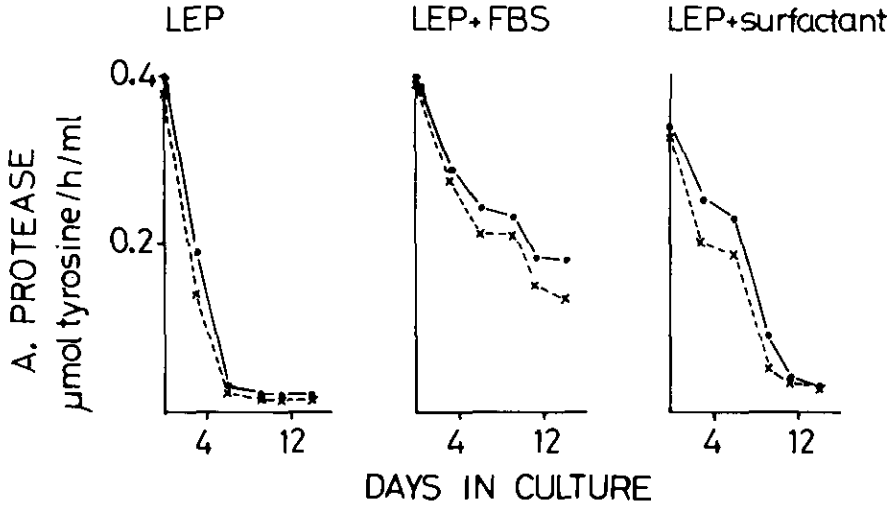

FIGURE 2. Acid protease activity remaining in the supernatant fraction of a lysosomal enzyme preparation (LEP) maintained in fetal bovine serum (FBS) or pulmonary surfactant media and incubated at pH 7.0 for 14 days at 35°C in (×) in the presence or (•) absence of chrysotile asbestos.

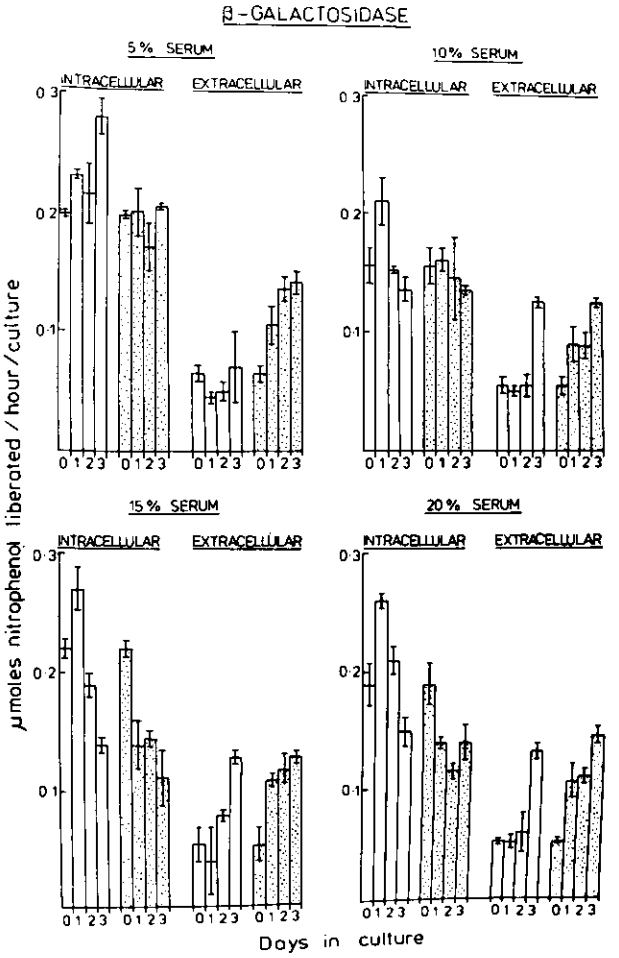

FIGURE 3. Intracellular and extracellular β-galactosidase activity in (□) control and (⊠) chrysotile-treated rabbit alveolar macrophage cultures maintained in different concentrations of serum for 3 days.

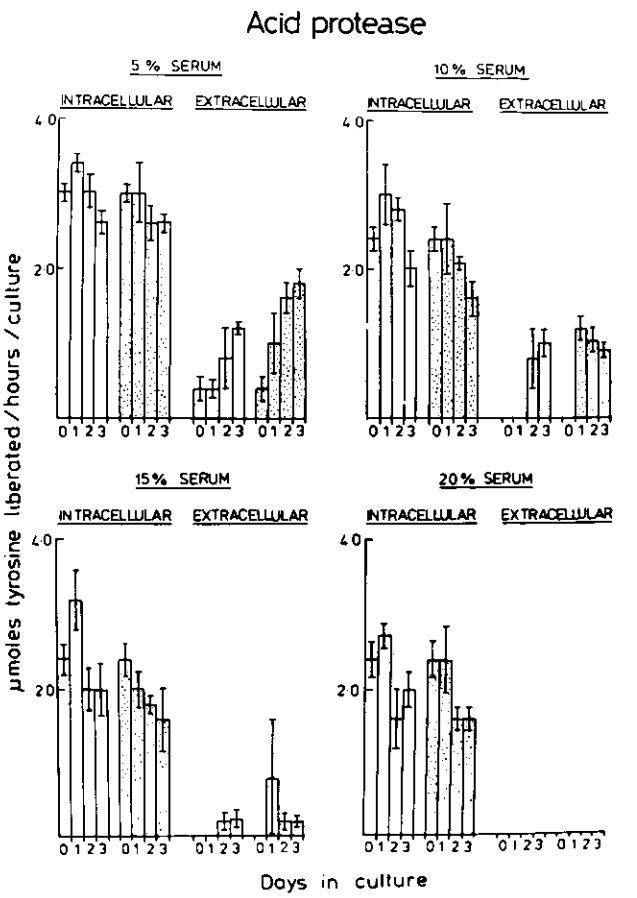

FIGURE 4. Intracellular and extracellular acid protease activity in (□) control and (⊠) chrysotile-treated rabbit alveolar macrophage cultures maintained in different concentrations of serum for 3 days.

protease is detectable only in the medium of control cultures maintained in 5% serum at all time intervals or after 2 days in normal cultures maintained in 10% and 15% serum (Fig. 4).

The presence of chrysotile initiates an early release of hydrolytic enzymes, an effect practically independent of serum concentration and increased cellular levels within the dust-treated macrophages. With the exception of the cultures maintained in 5% serum the extracellular levels of all enzymes studied are no higher in the chrysotile-treated cultures than in control cultures after 72 hr. Acid protease activity is not detected in the extracellular medium of control or chrysotile-exposed cultures in 20% serum throughout the time period of experimentation.

Chrysotile clearly stimulates an early release of lysosomal enzymes from rabbit alveolar macrophages *in vitro* without causing cell death (cell viability was greater than 98% throughout these experiments as assessed by eosin dye exclusion). Similar findings have been reported for peritoneal macrophages (*2, 3*) and rabbit alveolar macrophages (*4, 5*) for periods of experimentation ranging between 18 and 24 hr. The present study raises two further points not examined in these previous investigations. First, control macrophages in the absence of any dust stimulus will release lysosomal enzymes after 72 hr in amounts comparable to that released in the presence of chrysotile. The question is thus posed as to whether chrysotile merely accelerates a natural process of lysosomal enzyme release in this *in vitro* system. Previous ultrastructural studies on the effect of chrysotile on lung fibroblasts in culture suggested that sublethal doses of the mineral induce an early "maturation" process in these cells (*16*). Perhaps the addition of chrysotile to alveolar macrophages induces early 'maturation' and hence premature release of lysosomal enzymes which is

dependent on serum concentration and time of experimentation *in vitro*. For example, after 72 hr, approximately 50% of total β-galactoside, 25% of RNase and 0% of acid protease activity is detectable in the extracellular medium of macrophage exposed to 20% serum-containing media. Such differential release may reflect different intralysosomal arrangement or attachment of the enzymes to the enclosing membrane. Higham et al. (*17*) have shown that β-galactosidase may be wholly released from lysosomal pellets at pH 5.0 over 21 hr, whereas β-xylosidase is retained.

The presence of chrysotile within the lysosome, while not enhancing or retarding hydrolytic enzyme activity, probably provides an excellent surface for the absorption of enzymes and their degradation products (*18*). This may result in specific alterations in the attachment of membrane-bound enzymes and in the event of lysosomal instability a selective, differential release of hydrolytic enzymes. Such suggestions clearly require a greater knowledge of the lysosomal structure and information on the integration of the enclosed hydrolytic enzymes.

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