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SOME BIOCHEMICAL EFFECTS OF ASBESTOS ON MACROPHAGES

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Summary.—The *in vitro* effects of chrysotile, crocidolite and amosite asbestos, and silica and rutile dust, on hamster peritoneal macrophages were assessed by estimating the release of acid phosphatase into the culture medium and by changes in the composition of phospholipids in the cells.

Chrysotile was as toxic as silica, while crocidolite, amosite and the control dust, rutile were inert. The toxicity of chrysotile and silica is evidently due to the surface interaction of the dusts with phagosomal membranes, leading to the release of lytic enzymes from the damaged phagosomes into the cytoplasm. Considerable amounts of acid phosphatase were released into the medium 28 hours after the phagocytosis of both dusts, whereas very little was found when crocidolite, amosite and rutile were used.

Further evidence of cytopathic effects was seen by changes in lipid components of cells which had ingested silica and chrysotile. A decrease in total lipid content and an increase in lysolecithin apparently indicate specific, secondary and toxic effects following the release of lysosomal enzymes.

THE fibrogenic and carcinogenic activities of certain forms of asbestos in man and animals have attracted considerable interest in recent years. With regard to the part played by the macrophage, there is general agreement that particles are engulfed by macrophages and then taken up into phagosomes (secondary lysosomes) (Dourmashkin and Dougherty, 1961; Cohn and Wiener, 1963; Davis, 1963; de Duve, 1963; Harington and Allison, 1965; Allison, 1967). While particles such as rutile, carbon and diamond dust have negligible effects, silica rapidly destroys the macrophage by damaging the membrane of the phagosome in such a way that hydrolytic enzymes are released into the cytoplasm, so destroying the cell by autolysis (Allison, Harington and Birbeck, 1966; Koshi and Sakabe, 1966; Comolli, 1967).

Particles released after the cell has been destroyed are then taken up by other macrophages (Marks and Nagelschmidt, 1959) which are in turn affected. This repeated destruction, during which materials such as lipids, lipoproteins, polysaccharides and others are released (Harington, 1963; Harington and Allison, 1965; Heppleston, 1969), apparently stimulates fibroblasts to lay down collagen.

Unlike what has been found for silica, there is no clear-cut correlation between the cytotoxicity of different forms of asbestos and their fibrogenicity (Allison, 1967), the toxic effect being disproportionately high in the case of chrysotile. There is also a certain amount of contradictory evidence concerning the cytotoxicity of this particular form of asbestos. In the hands of Koshi (1965), Parazzi *et al.* (1968),

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Koshi, Hyashi and Sakabe (1968), a marked toxic effect on macrophages has been reported. More recently, it has been found (Bey and Harington, 1971; Miller, 1971) that the cytotoxic effects of chrysotile are similar in speed and extent to those of an active form of silica. On the other hand, other workers (Pernis *et al.* 1966; Beck, 1970; Beck, Holt and Nasrallah, 1971; Pernis and Castano, 1971) did not find any pronounced activity for chrysotile.

The discrepancies could be due to differences in cell culture techniques, methods of estimating cytotoxicity and types of chrysotile used (Harington, 1972). With regard to this last feature, Beck *et al.* (1971) found Rhodesian fibre to be inactive. Pernis and Castano (1971) reported that Balangero chrysotile was without effect, although in an earlier study (Pernis *et al.*, 1966), this form (and crocidolite) were shown to be toxic. Bey and Harington (1971) used U.I.C.C. chrysotile, the same as that used in the present study, and found it to be as toxic as silica. The results of Pernis and his colleagues could possibly be explained by changes in cell culture conditions.

The way in which active forms of chrysotile might react with the phagosomal membrane of the macrophage also requires clarification. While there is some suggestion that mechanical rupture of phagosomes might occur (Davis, 1967), it seems more likely that a chemical reaction takes place between the fibre and the membrane of the phagosome. It is known that chrysotile and other forms of asbestos can haemolyse erythrocytes (Macnab and Harington, 1967; Secchi and Rezzonico, 1968; Schnitzer and Pundsack, 1970; Harington, Miller and Macnab (1971), and magnesium in its ionic form has been suggested as being the principal reactant in this phenomenon (Macnab and Harington, 1967; Harington *et al.*, 1971).

MATERIALS AND METHODS

Mineral dusts.—The asbestos used was standard, finely-ground U.I.C.C. chrysotile, amosite and crocidolite, which together with rutile (titanium dioxide), were kindly supplied by the National Research Institute for Occupational Diseases, Johannesburg. Alkali-etched Fransil silica (an amorphous form), with a particle size range from 0·1 to 0·5 μ, and a specific surface area of 32·82 m^2/g, was provided by the Safety in Mines Research Establishment, Sheffield. All dusts were dry-sterilized at 160° for 2 hours before being suspended in Eagle's minimum essential medium and ultrasonicated for 20 seconds immediately before use. For every million cells in culture, 300 μg mineral dust were added in 0·5 ml MEM, 6½ hours after the cells had been planted (see " Macrophage cultures " below).

Cell culture media.—Eagle's minimum essential medium (MEM), containing 0·1 mmol $CaCl_2$, 0·1 mmol glycine, 0·1 mmol serine (Freeman *et al.*, 1967) and 200 units/ml penicillin and 100 units/ml streptomycin, was used throughout. Medium A consisted of MEM supplemented with 40% filter-sterilized bovine serum inactivated at 56° for 30 minutes. Medium B consisted of MEM supplemented with 10% heat-inactivated bovine serum but pre-conditioned by a 24-hour contact with a growing culture of SA7 virus-transformed hamster skin fibroblasts (Whitcutt and Gear, 1968) or one of transformed baby hamster kidney fibroblasts (Virolainen and Defendi, 1967).

Macrophage cultures.—Peritoneal exudate cells were collected from young adult male golden hamsters 5–6 days after the intraperitoneal injection of glycogen (6 ml of 0·5% suspensions in physiological saline). The animals were killed by cervical fracture and 20 ml of MEM medium and 1 ml of heparin (5000 units/ml) were injected into the peritoneum. After 20 minutes the abdomen was opened and the washings aspirated with a Pasteur pipette. Each experiment was carried out on pooled cells from the hamsters, thus obviating biological variation. The nucleated cell count averaged 16×10^6 of which a minimum of 75% were macrophages.

Cell suspensions were seeded directly into 60 × 15 mm Cooper dishes (Falcon Plastics Co., Los Angeles) or Falcon flasks, (25 cm^2 or 75 cm^2) and left at 37° for 20 minutes, by

which time the macrophages had adhered firmly to the floor of the flasks. The medium was then decanted, the cells washed and fresh medium, consisting of one half medium A and one half medium B, was added. All cultures were gassed briefly with 5–10% CO_2 and incubated at 37°. After 4 hours the majority of macrophages had spread out and showed the typical pseudopodia and ruffled membranes of healthy mature cells.

As cell damage is considerably reduced or delayed if dust particles are coated with serum (Allison *et al.*, 1966), the medium used was replaced by MEM alone, 2 hours before the addition of 300 μg of mineral dust (in 0·5 ml medium) per million cells. Macrophages were allowed to phagocytose for 1 hour, after which they were washed and returned to the earlier growth medium, namely, half medium A and half medium B. Cultures were maintained for up to 72 hours after the addition of dust although most experiments were terminated after 4, 16 and 28 hours.

Morphological examination.—Stimulated peritoneal macrophages aspirated from single young male hamsters were equally distributed into 3 Cooper dishes and maintained in 4 ml of medium under conditions described above. In a few experiments, 5 cm diameter petri dishes containing 7 plastic coverslips (20 × 20 mm) were used. The coverslips were attached to the floor of the dish with DPX (Gurr), diluted 1 to 5 with xylene, before sterilization at 160° in a hot air oven. Both Cooper and petri dishes were kept at 37° in desiccators containing 1–2 g dry ice (Bey and Whitcutt, 1969).

Coverslips or Cooper dishes were removed at 0, 4, 16 and 28 hours after the addition of dust, rinsed with isotonic solution and fixed with Bouin's fixative for a minimum of 1 hour and then stained with Ehrlich's haematoxylin (Culling, 1963). An aqueous mounting medium, Burstone's P.V.P. (Pearse, 1961) was used.

Cell counts were made in 14 different areas equally distributed over the entire coverslip. Light microscope photography (Zeiss photomicroscope II) of fixed and stained preparations was performed using Pan F film (Ilford).

Chemical estimations.—The acid phosphatase activity in medium containing macrophages cultured in Falcon flasks (25 cm^2) was determined, using *p*-nitrophenylphosphate as substrate (Sommer, 1954; Bergmeyer, 1963). Aliquots of about 0·5 ml of medium were removed 16, 20, 24, 28, 40 and 60 hours after the addition of dusts and carefully centrifuged to remove any cells or cell debris. Volumes of 0·2 ml were then removed for estimation of enzyme activity. After 30 minutes incubation in an acid-substrate solution (0·05 mol/l citrate buffer, $5{\cdot}5 \times 10^{-3}$ mol/l *p*-nitrophenylphosphate; pH 4·8), the enzyme was inhibited by the addition of 4 ml of 0·1 N sodium hydroxide. The intensity of the colour of the liberated *p*-nitrophenol was measured at 405 nm in a Beckman DU spectrophotometer. The results, expressed as mmol units of acid phosphatase activity, were calculated from a standard curve.

DNA content of cells (in 75 cm^2 Falcon flasks) was estimated by the method of Burton (1956), in which the colour reaction between diphenylamine and DNA (Dische, 1930) was modified by addition of acetaldehyde, and by allowing colour development to take place over several hours at 30° rather than heating the solution to 100°. This modified method is more sensitive and specific than the original.

Cells in single flasks were taken 4, 16 and 28 hours after the addition of dusts. After the cells had been washed 3 times with an isotonic solution at 37° they were scraped off with a " rubber policeman ", using ice-cold 0·5 N perchloric acid. After centrifugation, the supernatant solution was decanted and the DNA content of the cell plug determined.

Determination of phospholipids in cells was made 4, 16 and 28 hours after the addition of dusts. The cells were washed 3 times with isotonic solution at 37° to remove any traces of serum and were then scraped off with a " policeman " in cold isotonic saline. The suspension was centrifuged, the supernatant solution decanted and the cell pellet weighed. The lipids were then immediately extracted (Folch, Lees and Sloane Stanley, 1957), weighed, and redissolved in chloroform : methanol 2 : 8 v/v (Gottfried, 1967). After this they were transferred to small pyrex tubes and stored under nitrogen at −20° for not longer than 2 weeks. Individual phospholipid components were separated by thin layer chromatography (Skipski, Peterson and Barclay, 1964). After development with solvent (chloroform : methanol : glacial acetic acid : water; 50 : 26 : 10:5 v/v), the separated components were rendered visible with iodine vapour. The plate was then removed from the vapour, the spots marked, and after all traces of iodine had disappeared the test, standard and blank areas were scraped off and the phosphorus content of each determined (Parker and Petersen, 1965).

RESULTS

Morphological examination

After 16 hours chrysotile and silica-treated macrophages showed marked degenerative changes consisting of vacuolation of the cytoplasm, rounding of the cell and finally destruction, as shown by pyknosis and the disappearance of the cell membrane (Fig. 1, 2). These changes were not seen in macrophages given rutile, crocidolite, amosite or in untreated (control) cells.

A marked decrease in cell number was also found when silica and chrysotile were used (Table I), and again this was not so with the other dusts nor in the control cells.

TABLE I.—*Comparison of Cell Number at Different Times After Addition of Various Mineral Dusts to Macrophages*

Dusts	Cell number after various times after addition of dusts (hours) 4	16	28	Per cent decrease in cell number from 4 to 28 hours
" Fransil " silica	500	240	160	68
Chrysotile	490	233	140	71
Amosite	506	485	482	5
Crocidolite	513	460	472	8
Rutile	510	490	486	5
Control cells*	500	400	412	18

* Untreated cells, that is, non-phagocytosing macrophages.

Changes in levels of acid phosphatase in the medium

This enzyme was released into the medium within 16 hours of uptake of silica and chrysotile and reached a peak at 28 hours. After this, the levels rapidly dropped, probably reflecting destruction of the cells as well as inactivation of the enzyme. By contrast, cultures treated with amosite and crocidolite released very small amounts of enzyme at 28 hours (Fig. 3).

Low values for acid phosphatase were also given by control cultures and those treated with rutile (0·07 and 0·14 mmol phosphatase units, respectively, at 28 hours).

Changes in phospholipid composition of macrophages

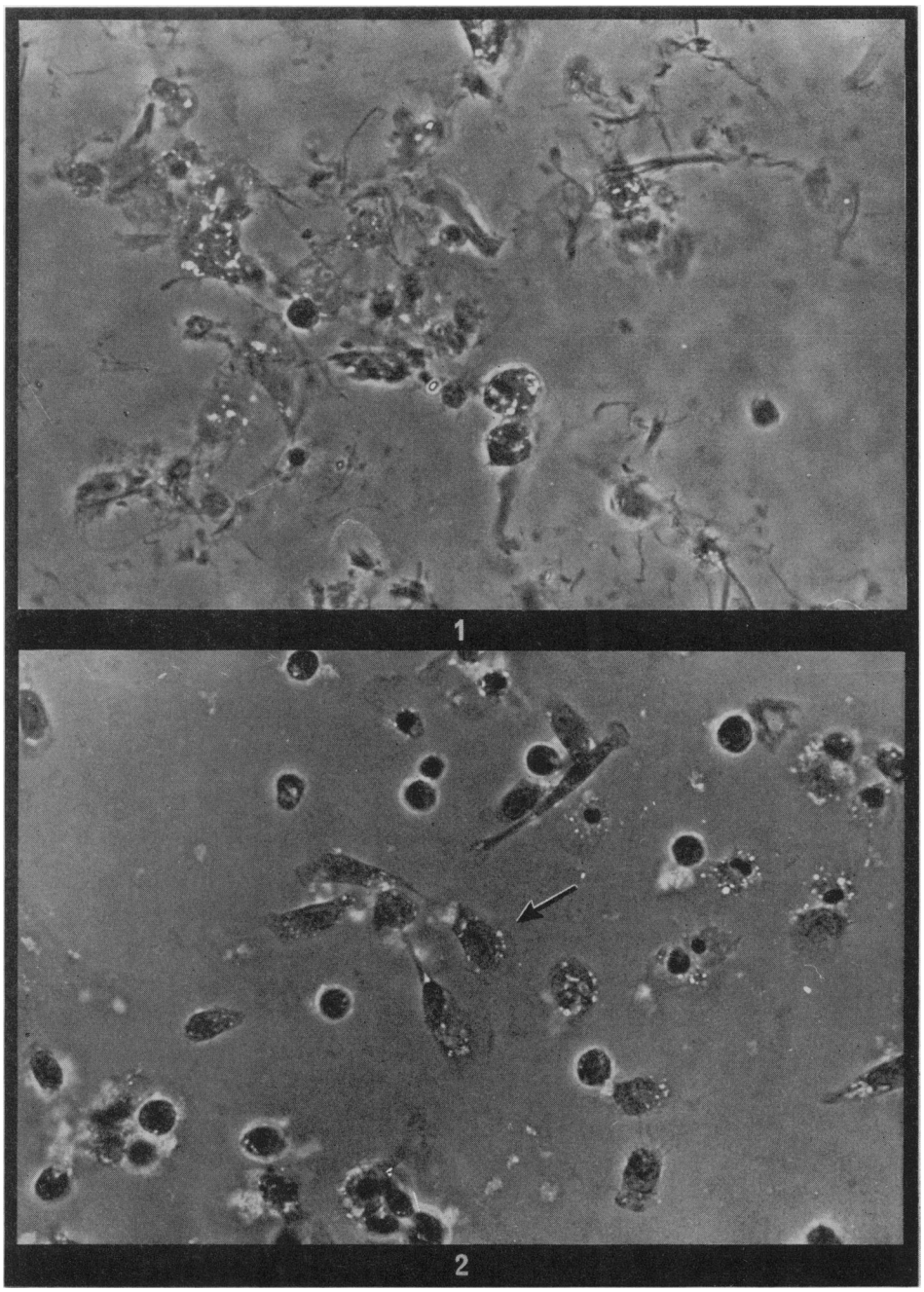
In 2 separate experiments on macrophages taken from 60 and 40 male hamsters and analysed 4, 16 and 28 hours after administration of chrysotile and silica, a 10-fold increase of lysolecithin, at the expense of lecithin and cephalin, was seen. Control cells and those given rutile were unaffected (Table II and III).

These results emphasize clearly the similarity between the effect of chrysotile and silica on macrophages. In both cases, the morphological and biochemical changes after administration of the dusts are characterized by decrease in cell number, release of acid phosphatase into the medium and changes in phospholipid composition.

EXPLANATION OF PLATES

FIG. 1.—Marked destructive effect of chrysotile asbestos on hamster peritoneal macrophages in culture 16 hours after administration of dust.

FIG. 2.—Effect of silica on hamster peritoneal macrophages in culture 16 hours after administration of dust. Arrow shows cells still healthy and in active phagocytosis; remaining cells show toxic effects of vacuolation, rounding up, pyknosis and lysis.



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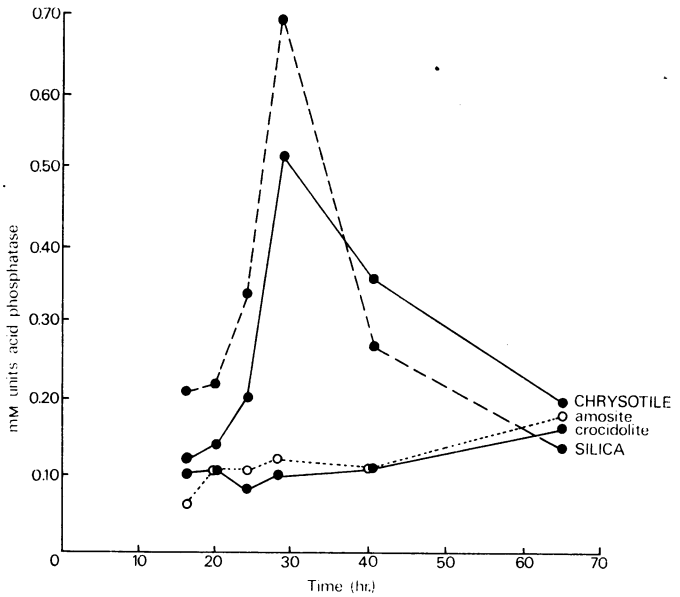

FIG. 3.—Release of acid phosphatase into cell culture medium after macrophages have been exposed to various mineral dusts over 65 hours.

TABLE II.—*Phospholipid Composition of Macrophages at Different Times After Addition of Various Dusts*

Dusts	Time (hours)	Phospholipid components (μg/100 μg DNA) Lysolecithin	Sphingomyelin	Lecithin	Phosphatidyl ethanolamine	Total*
Silica	4	12·3	86·4	208·0	122·3	470
	16	80·5	123·0	216·2	160·0	557
	28	164·8	182·0	252·0	176·2	767
Chrysotile	4	10·7	66·9	153·0	98·9	324
	16	90·4	145·9	218·0	155·8	600
	28	129·0	129·8	147·2	110·0	526
Rutile	4	15·1	148·1	368·0	224·0	732
	16	20·9	156·8	290·2	194·6	608
	28	27·1	107·0	206·0	145·2	469
Control cells	4	8·9	92·2	190·2	130·1	427
	16	11·3	109·5	188·0	141·2	505
	28	48·5	148·0	300·0	180·0	634

* Estimated by separate chemical determination.

DISCUSSION

Asbestos, like silica, is taken up into phagosomes of macrophages after phagocytosis, although fibres are frequently found scattered and lying free in the cytoplasm (Davis, 1967; Allison, 1969). From this point onwards, the mode of action of asbestos is much less well understood than that of silica, although there is clear proof that at least one form, chrysotile, can be as toxic as silica.

There has hitherto been no evidence that chrysotile damages phagosomes in the way silica does, although it is known from work on haemolysis that this fibre

is membrane-active (Macnab and Harington, 1967; Harington *et al.*, 1971). The marked release of acid phosphatase into the culture medium—as shown in the present study—now strongly indicates that some forms of chrysotile act directly on the phagosomal membrane, so causing the death of the cell. Nevertheless, the release of lysosomal enzyme as seen here requires confirmation by histochemical studies using, for example, the technique of Nadler and Goldfischer (1970).

TABLE III.—*Phospholipid Composition of Macrophages at Different Times After Addition of Various Dusts*

Dusts	Time (hours)	Phospholipid components (per cent of total phospholipid)				
		Lysolecithin	Sphingomyelin	Lecithin	Phosphatidyl ethanolamine	Others*
Silica	4	2·5	20·2	46·2	29·8	1·3
	16	14·2	22·1	38·6	25·1	—
	28	21·2	23·5	32·2	22·6	0·5
Chrysotile	4	2·6	18·3	44·0	31·9	2·2
	16	15·0	24·2	35·1	26·0	—
	28	24·5	24·6	27·0	20·9	3·0
Rutile	4	2·1	19·2	48·2	30·3	0·2
	16	3·2	19·8	46·0	31·0	—
	28	5·8	20·8	43·3	31·0	—
Control cells	4	2·1	21·6	44·6	30·4	1·3
	16	2·2	22·9	43·8	26·9	4·2
	28	7·7	22·4	42·4	28·4	—

* Components not determined.

Experiments mentioned above (Harington *et al.*, 1971) have already shown that magnesium ions are probably the active agents in inducing haemolysis of erythrocytes, and it is possible that this metal could react with negatively charged components (possibly glycolipids) on the phagosomal membrane of the phagosome. In this way a lytic effect, similar to that resulting from the hydrogen-bond interaction between the surface of silica and the phagosomal membrane of the macrophage (Nash, Allison and Harington, 1966) could be induced, so leading to a similar release of lytic lysosomal enzymes.

It is also known that the ingestion of silica by macrophages leads to a breakdown of the diacylphosphatides, lecithin and cephalin, and an increase in lysolecithin (Munder and Fischer, 1965; Munder *et al.*, 1966; Modolell, Munder and Fischer, 1967; Munder *et al.*, 1967, 1969). The present study shows that chrysotile behaves identically. It is, however, not yet possible to judge whether the increase in lysolecithin is a primary or secondary event after chrysotile or silica has been used. There is good evidence that phospholipases are present in phagosomes (Mellors and Tappel, 1967) where, with other enzymes, they may be attached by electrostatic binding to the membrane (Koenig, 1967), and from where they could possibly be freed after chrysotile (or silica) has reacted with the phagosomal membrane. On the other hand, it has been suggested that phospholipases are present in an inert form which can be activated by silica when both are present in the phagosome (Munder *et al.*, 1967, 1969)

We consider that the former alternative is the more likely one and that lipolytic and other enzymes are released from charged sites on the membrane after lysis by toxic mineral dusts. If this is so, the discharge of phospholipase, and the subsequent increase in lysolecithin, would be secondary events attendant upon the primary

damage to the phagosomal membrane by physico-chemical interaction with both chrysotile and silica. This would explain the similarity of the morphological and biochemical changes which occur in macrophages after the ingestion of both dusts, even though the primary damage to the phagosome may differ, one (silica) being due to hydrogen bonding and the other (chrysotile) probably to magnesium ions.

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