In vitro biodegradation of chrysotile fibres by alveolar macrophages and mesothelial cells in culture: comparison with a pH effect

M C JAURAND,¹ A GAUDICHET,² S HALPERN,³ AND J BIGNON¹

From the Groupe de Recherches sur les Affections Respiratoires et l'Environnement,¹ INSERM-U 139, ERA CNRS 845, CHU Henri Mondor, 94010 CRETEIL, the Laboratoire d'Etude des Particules Inhalées,² DASS de PARIS, 75013, Paris, and Laboratoire de Biophysique,³ Faculté de Médecine, 94010 CRETEIL, France

ABSTRACT The modification of the chemistry of asbestos chrysotile fibres ($\text{Mg}_3(\text{Si}_2\text{O}_5)(\text{OH})_4$) after their ingestion by cultured cells has been studied. Two types of cells involved in asbestos related pulmonary disease were used, rabbit alveolar macrophages (AM), recovered by bronchoalveolar lavage, and pleural mesothelial cells (PMC) obtained from the rat parietal pleura. Chemical characterisation of intracellular fibres was performed on unstained ultrathin sections by electron probe microanalysis. The results showed a progressive leaching of Mg, characterised by a time dependent decrease of Mg/Si. AM were more efficient than PMC at leaching intracellular chrysotile fibres since it took longer to obtain the same proportion of leached fibres with PMC than with AM. As in vitro Mg-leaching can be obtained by acid treatment, chrysotile fibres were incubated, either untreated or pretreated with cell membranes, at pH 4 or 7 for various times. The data show that the kinetic of leaching by AM was comparable with leaching at pH 4. The leaching by PMC was of the same order as leaching at pH 7. When membranes were adsorbed on to the fibres, a delayed leaching was observed. The results indicate that the solubilisation of chrysotile by AM could be an intraphagolysosomal event due to a pH effect. With PMC, however, it is not possible to draw this conclusion since nothing is known about the intracellular pH.

Chrysotile asbestos fibres, a hydrous magnesium sheet silicate ($\text{Mg}_3(\text{Si}_2\text{O}_5)(\text{OH})_4$), induce fibrosis and cancer in man and in animals. These fibres are unstable in a biological medium and previous studies have established that intrapulmonary fibres have a low magnesium content^{1,2} as compared with natural fibres. Such chemical modification is important since the carcinogenicity of Mg-depleted chrysotile was decreased as shown in animal experiments.^{3,4} We have observed, using a scanning electron microscope fitted with an energy dispersive x rays spectrometer, the solubilisation of magnesium by alveolar macrophages in culture.² The aim of the present work was to determine the kinetics of leaching in order to know if it is an early or late intracellular event. Two types of cells were used—alveolar macrophages (AM) which are mature cells specialised in

phagocytosis and the first to be in contact with inhaled asbestos fibres and pleural mesothelial cells (PMC) which are proliferative target cells for injury by asbestos fibres. It is well known that asbestos fibres are phagocytosed by AM; in the same way PMC phagocytose chrysotile fibres⁵ and a degranulation of lysosomes occurs in the phagocytic vacuole as shown in fig 1. As chrysotile fibres are unstable in acidic solution, it was additionally determined if the solubilisation of the magnesium from the fibres might be an intralysosomal process possibly related to the acidity of the intraphagolysosomal medium. Then the Mg/Si ratio of chrysotile fibres incubated at neutral or acidic pH was determined in order to compare these values with those obtained with intracellular fibres. As the phagocytic process contains a step of covering of the fibre by the membrane,⁵ and as membranes can be adsorbed on to the fibres, chrysotile fibres were, in addition, pretreated with red blood cell membranes in order to mimic the phenomenon occurring in the cells.

Received 14 February 1983
Accepted 20 June 1983

Fig 1 Acid phosphatase staining of PMC incubated for four hours with chrysotile. Positivity is observed close to two bundles of fibres (large arrow), indicating the degranulation of the lysosome into the membrane limited phagocytic vacuole (small arrow).

The chemical composition of the fibres was assessed by the determination of the Mg/Si ratio, which was carried out with an electron probe fitted to a transmission electron microscope.

Material and methods

ASBESTOS FIBRES

Rhodesian chrysotile fibres were obtained from the Union Internationale Contre le Cancer (UICC) (Dr Timbrell). Fibres were dispersed by sonication in the culture medium⁶ before their addition to the cells. To prepare control samples, fibres were dispersed in filtered distilled water and a drop put on a collodion coated grid.

TREATMENT OF CHRYSOTILE FIBRES WITH CELL MEMBRANES

Red blood cells (RBC) were lysed in ice cold phosphate buffer 5 mM according to Fairbanks *et al.*⁷ and the resulting ghosts were washed under centrifugation with the same buffer until the membranes became nearly white. Then the RBC pellet was brought to 10% v/v in 0.01 M citrate buffer. Fibres and ghosts were mixed together and incubated at 37°C for 20 minutes, the final concentrations being respectively 1 mg/ml and 2.5%; the ghost concentration was equivalent to 860 μg/ml of phosphatides; in these conditions the adsorption of RBC membranes was optimal.⁸ Then the mixture was underlayered on a 40% sucrose solution to eliminate the unadsorbed membranes.⁸ The fibres were finally washed three

times in an appropriate citrate buffer (pH 7 or 4). The same procedure was applied to the fibres not treated with membranes. Membrane treated and untreated fibres (1 mg/ml) were incubated at 37°C in Falcon flask for different times (60 hours and 12 days, pH 7; 70 hours and 8 days pH 4).

TREATMENT OF AM

AM were obtained by bronchoalveolar lavage from normal rabbits and cultured in a Falcon flask for 24 hours in a 199 medium supplemented with 0.32% bovine serum albumin (pH 6.9).⁶ Freshly sonicated chrysotile fibres were added to the culture (4 μg/cm²) for 45 min, four, or 20 hours. After incubation, the cells were treated using a standard electron microscope procedure as described elsewhere.⁹

TREATMENT OF PMC

PMC were obtained from normal rat parietal pleura and cultured as previously described.⁹ Briefly, the cells were cultured in NCTC 109 supplemented with 10% fetal calf serum (pH 7.3), and trypsinised every week. Freshly sonicated chrysotile fibres were added (2 μg/cm²) to 10 to 15 passages old confluent PMC. After 20 hours of contact with the fibres, PMC were washed with phosphate buffered saline to discard most of the extracellular fibres. The medium was replaced with fresh medium. PMC were then fixed two, four, six, and seven days after contact with fibres, as described with AM.

ELECTRON MICROSCOPE ANALYSIS

The electron microscopic analysis was performed on unstained ultrathin sections. Intracellular fibres were located on a carbon coated copper finder grid, with a transmission electron microscope (JEOL 100C); thereafter they were analysed by a microprobe equipped with a wavelength dispersive spectrometer (TAP cristal) fitted to a transmission electron microscope (CAMEBAX, MBX). Measurement of Si and Mg was performed on individual fibres of about 0.1 μm in diameter under the following experimental conditions: counting time, 10 s; beam voltage, 45 kV; beam current, 50 nA; probe diameter, 0.3 μm. For each point two counts were checked and only reproducible counts were taken into account. Thirty fibres were analysed in each series.

Results

The structural and ultrastructural morphology of the cells treated with chrysotile fibres is shown in figs 2 and 3. Intracellular fibres were observed in PMC as well as in AM; as has been shown previously, chrysotile fibres were ingested by PMC in culture and were located into phagolysosomes.⁵ It was

Fig 2 Rabbit alveolar macrophages treated with 50 µg/ml of chrysotile fibres (a) optical microscopy $\times 300$ and (b) transmission electron microscopy.

Fig 3 Rat pleural mesothelial cells treated with 5 µg/ml of chrysotile fibres (a) optical microscopy and (b) transmission electron microscopy.

Fig 4 Percentage of chrysotile fibres with $Mg/Si \leq 0.8$ versus time of incubation either at pH 4 with (Δ) or without ghosts ($\circ$) at pH 7 with ($\blacktriangle$) or without ghosts ($\bullet$). Kinetics obtained with AM ($\square$) and PMC ($\blacksquare$) are also shown.

Table 1 Chemical analysis of intracellular chrysotile fibres

Mg/Si values	Percentage of fibres			
	>0.8	0.8-0.6	0.6-0.4	≤ 0.4
Chrysotile	85	15	0	0
<i>Intracellular fibres</i>				
Macrophages:				
45 min	50	39	11	0
4 h	65	30	5	0
24 h	41	48	11	0
PMC:				
2 days	87	9	4	0
4 days	71	21	8	0
6 days	60	37	3	0
7 days	51	29	7	13

observed that fibres induced an intense vacuolation of PMC.

INTRACELLULAR CHRYSOTILE FIBRES

To describe the variation of the Mg/Si ratio inside each series, the values were ranged into four classes. Table 1 shows the percentage of fibres in each class. In control samples the Mg/Si ratio was always higher than 0.6; moreover 85% of the fibres had a Mg/Si higher than 0.8. By contrast, values lower than 0.6 were observed with intracellular fibres ingested either by AM or by PMC. Fifty per cent of intramacrophagic fibres were "leached" ($Mg/Si \leq 0.8$) after 45 min; seven days were necessary for intramesothelial fibres to reach the same proportion. Figure 4 shows the kinetics of the chrysotile biodegradation. The leaching was time dependent, Mg/Si

Table 2 Chemical analysis of treated chrysotile fibres

Mg/Si values	Percentage of fibres			
	>0.8	0.8-0.6	0.6-0.4	≤ 0.4
Chrysotile	85	15	0	0
pH without membrane				
60 h	63	37	0	0
12 days	39	52	9	0
pH 7 with membrane				
60 h	64	23	13	0
12 days	58	39	3	0
pH 4 without membrane				
70 h	0	16	42	42
8 days	3	6	13	78
pH 4 with membrane				
70 h	23	52	22	3
8 days	0	12	35	53

values lower than 0.4 were observed after seven days of contact with PMC.

CHRYSOTILE FIBRES TREATED AT pH 7 OR 4

To compare the present data with those obtained from cells in culture, the Mg/Si values were ranged in the same four classes. Results are reported in table 2 and in fig 4; they are compared with values obtained with control chrysotile fibres.

A leaching of Mg occurs both at pH 4 or 7; however, the effect was higher and faster at pH 4 than at pH 7. For example, at pH 7 there was no fibre with $Mg/Si \leq 0.4$ when, at pH 4, they were always present (table 2). At pH 4, the percentage of fibres with $Mg/Si \leq 0.4$ increased with the time of incubation

and depended on the pretreatment of the fibres.

The effect of membranes on leaching is illustrated in fig 4; it was characterised by a delay of the leaching process.

Discussion

These experiments have shown that cultured cells solubilise the magnesium from phagocytosed chrysotile fibres. This biodegradation appears to be an early process (fig 4). This was established from the comparison of the Mg/Si values between standard fibres and ingested fibres. The variability of the Mg/Si ratio, however, either along a fibre or from one fibre to another one inside a same given series must be noted. The former variations could be due to the instability of the analytical method or to the variation of the chemistry inherent in the natural chrysotile fibres; however, they were lower than in the range of the Mg/Si classes. The later variations might be due, in addition, to a difference in the time that the fibres are retained inside the cells.

The alveolar macrophages seemed to be more efficient than pleural mesothelial cells at leaching the Mg content of chrysotile fibres since 59% of the fibres were "leached" by AM after 24 hours and only 13% after two days of incubation with PMC.

The kinetics of leaching with PMC was linear after two days. By contrast, the kinetic of biodegradation was variable with AM. This is probably due to the different treatment of the cells: PMC were washed after 20 hours of incubation with fibres to eliminate extracellular fibres and to have a "zero time" of leaching; with AM, all the fibres stayed in the culture medium and they could be phagocytosed for the duration of the experiment.

From the morphological and chemical studies reported here, it is established that chrysotile biodegradation occurs inside the cells. Particulate matter ingested by AM is well known to sequester inside phagolysosomes; elsewhere, it has been shown that chrysotile fibres ingested by PMC are also found inside phagolysosomes.⁵ This suggests that leaching is an intralysosomal process, which could be related to the acidity of the phagolysosomal medium; indeed Mg leaching from chrysotile has been shown by acid treatment *in vitro*.¹⁰

These experiments have, in addition, established that leaching of chrysotile occurs in buffered solutions and is related to their acidity. Present data are in agreement with studies showing a leaching of Mg from acid treated chrysotile fibres.^{11,12}

When chrysotile fibres were pretreated with RBC membranes, they become covered with the membranes.⁸ The present results show that this adsorption induces a low protective effect and do not

inhibit Mg leaching. The failure of complete inhibition could be due either to a partial desorption or to the uncovering of some parts of the surface of the chrysotile fibres. Indeed the stability of the membranes adsorbed on the fibres is not known in the buffered solutions. It has been shown, however, that model membranes (liposomes of dipalmitoyl phosphatidyl choline) were still present at the surface after three days of incubation (at 4°C) in a KCl-NaCl 0.145 M solution, followed by 24 hours at room temperature (unpublished data). Moreover, after nine or 12 days of incubation phosphorus was present in the membrane treated series as determined by electron probe microanalysis.

Figure 4 shows the rate of leaching of intracellular chrysotile fibres in AM and PMC. It can be seen, firstly, that the values of Mg/Si obtained with PMC were in the range of these obtained at pH 7 and, secondly, that with AM they were close to the values found at pH 4. This discrepancy could be due to a different intraphagolysosomal pH in the two types of cells. Indeed, phagocytic vacuoles are well known to have an acidic pH and are numerous in macrophages and polymorphonuclear cells.¹³ The acidification of the phagocytic vacuole could be due to the action of a membrane bound ATPase proton pump as demonstrated with other types of cell.¹⁴ As PMC have few lysosomes compared with macrophages¹⁵ and are not "scavenger" cells, they may have a different membrane function and the PMC intraphagolysosomal pH could be less acid than in AM. Nevertheless, this could also be due to a cell damage related to the presence of chrysotile inside the vacuole.

The decreased or delayed Mg leaching resulting from a membrane pretreatment could be related to a "barrier effect" of the membrane since in our experimental conditions the whole surface of the fibres is supposed to be covered by the ghosts. The dissolution of chrysotile fibres resulting from an acid treatment has been studied elsewhere.¹⁶ It appeared that the two possible rate limiting steps were diffusion of Mg²⁺ through an external silical gel or protonation at the reaction site. Then the membrane could be a rate limiting barrier to the diffusion of cation through surface layer and interface.

Conclusion

These experiments confirm the solubility of chrysotile fibres in biological medium. The lower Mg content found in fibres isolated from human lungs could be partly due to an attack by the pulmonary cells. It also appears that chrysotile fibres are versatile compounds and their toxicity may be related not only to their physical or physicochemical state

when inhaled but also to transformation in the lung. The solubilisation of chrysotile fibres in AM may be an intraphagolysosomal event due to a pH effect; however, with PMC it does not correlate with the acid solubilisation; it is necessary to know more about the intracellular pH of PMC in order to conclude either to a cell impairment or to a normal specific state of the cell.

We are extremely grateful to Professor Galle, the director of the Department of Microanalysis, University of Paris XII, for the facilities offered to us, and to L. Keuhang and A. Renier for technical help.

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