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THE BIOLOGICAL EFFECTS OF MAGNESIUM-LEACHED CHRYSOTILE ASBESTOS

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Summary.—Chrysotile asbestos was leached in N hydrochloric acid for varying times to produce a range of magnesium-depleted samples. The protein adsorptive capacity, the haemolytic activity, and the capacity to cause selective release of acid hydrolases from macrophages were measured for the various samples *in vitro*. The carcinogenicity of the same materials was determined following intrapleural inoculation in rats. The adsorptive capacity for albumin decreased linearly with magnesium removal. The haemolytic activity also declined until about half the magnesium had been removed, after which there was little further change. The selective release of acid hydrolases from macrophages in culture increased up to the point at which half the magnesium had been removed but by 90% depletion had declined rapidly. The carcinogenicity of 50%-depleted chrysotile was similar to that of intact, but at 90% depletion the incidence of mesothelial tumours had fallen considerably. There was no evidence that the leached samples fragmented more than the unleached *in vivo*.

IT has been established that the structural magnesium of chrysotile asbestos is soluble, at least to some extent, in biological systems. This has been demonstrated by using the electron microprobe to measure the Mg/Si ratio of fibres isolated from human lungs (Langer, Rubin and Selikoff, 1970) and also by radio-active tracer techniques (Morgan, Holmes and Gold, 1971). It appears that magnesium on the surface of the fibre dissolves relatively rapidly but, as more and more of the magnesium is leached, so the rate of dissolution declines. More recent studies with the electron microprobe (Pooley, 1972) on fibres isolated from both human and animal lungs indicated that not more than half the magnesium content of any fibre examined had been leached.

Normally, chrysotile carries a relatively high positive electrokinetic potential at physiological pH values and the high haemolytic and cytotoxic activities of chrysotile in protein-free systems are attributed to this charge. However, due to the strong adsorptive capacity of chrysotile for protein (Morgan, 1974) the haemolytic and cytotoxic activities of chrysotile are reduced in systems containing soluble protein. As the effect of magnesium leaching will be to modify the surface charge of chrysotile, it is to be expected that there will be corresponding changes in its biological activity. Morgan and Cralley (1973) recommended that the effect of magnesium removal on the cytotoxicity and carcinogenicity of chrysotile should be investigated. In this paper we report the results of an investigation in which the protein adsorption, haemolytic activity, capacity to cause selective release of lysosomal hydrolases *in vitro* and also the carcinogenicity of chrysotile asbestos, leached to varying degrees, were tested.

MATERIALS AND METHODS

Preparation of leached chrysotile.—The material used in these experiments was the

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standard reference sample of Rhodesian chrysotile (Timbrell, Gilson and Webster, 1968) prepared following recommendations of the *Union Internationale Contre le Cancer* (UICC). The rate of dissolution of magnesium from fibres of chrysotile asbestos by N HCl at 25° was investigated by Morgan, Lally and Holmes (1973). The rates of leaching for both UICC standard reference samples of chrysotile under these conditions were reported. For most of the experiments described below, 1 g samples of chrysotile were suspended in 200 ml of N HCl. Following sonication to disperse the fibres, leaching was allowed to proceed at 25° for predetermined times, after which the fibre was separated by centrifuging and the supernatant retained for determination of magnesium. The leached fibre was washed twice with distilled water and finally suspended in 250 ml isotonic saline giving a concentration of 4 mg/ml (equivalent). Unleached samples were prepared by suspending chrysotile in distilled water and sonicating in the same way.

Protein adsorption.—The adsorption of protein by chrysotile was measured with iodine-131 labelled human serum albumin using the technique described by Morgan (1974).

Haemolysis.—The erythrocytes used in these experiments were all obtained from a single donor. Blood (20 ml) was collected by venepuncture and transferred to heparinized vials. After dilution with physiological saline the blood was centrifuged and the supernatant and buffy coat removed. The erythrocytes were washed 3 times with saline, the supernatant being discarded on each occasion, and diluted with saline to give an approximately 2% suspension. The optical density of a lysate, prepared by diluting 1 ml of suspension to 10 ml with distilled water, was measured at 541 nm and the concentration of the suspension adjusted to give a value of 0·50.

Aliquots (1 ml) of the suspensions of chrysotile were pipetted into tubes and 4 ml of Veronal-buffered saline (pH 7·4) added. The resulting suspensions were mixed by vigorous shaking by hand for 10 s, after which 5 ml of the standardized suspension of erythrocytes were added to each tube. Controls containing 5 ml of buffer and of the erythrocyte suspension were also prepared. The contents of the stoppered tubes were mixed at room temperature by gentle continuous inversion to keep both cells and fibre in suspension. After 10 min, the contents of the tubes were centrifuged at 3500 rpm for 3 min. Aliquots (2 ml) were removed from the experimental samples after centrifuging and from the controls, both before and after centrifuging, to provide a correction for erythrocyte fragility. After dilution, optical densities were measured against an appropriate blank. Where necessary, adjustments were made to the amount of chrysotile used to ensure that haemolysis was always less than half that of the lysed control. Up to this point there is an approximately linear relationship between haemolysis and weight of chrysotile. The results were expressed as means of 4 or 5 replicates per sample and standard errors were calculated from the pooled variation between replicates within samples, assuming a constant variant after a logarithmic transformation.

Selective release of acid hydrolases from mouse peritoneal macrophages.—The effect of leaching on the capacity of chrysotile asbestos to cause selective release of acid hydrolases from mouse peritoneal macrophages was examined using the methods described previously (Davies *et al*., 1974) for unleached chrysotile asbestos.

Macrophage cultures were prepared as follows. Peritoneal exudates were collected by lavage with 5 ml M199 containing 100 u/ml penicillin, 100 μg/ml streptomycin and 10 u/ml heparin from outbred Swiss mice of either sex, 3 days after i.p. injection of 2 ml of proteose–peptone. Cell suspensions (5 ml) containing 0·8 to $1{\cdot}4 \times 10^6$ cells/ml were plated in 60-mm Petri dishes. After culture for approximately 1 h at 37° in 5% CO_2 in air, the medium containing nonadherent cells was removed and the remaining cell sheet washed 4 times with 4-ml aliquots of phosphate-buffered saline. The cells were then cultured overnight in M199 containing 10% swine serum (heat-inactivated at 56° for 30 min) (Biocult Laboratories Ltd., Glasgow, Scotland). Asbestos suspensions were sonicated for 2 min (MSE ultrasonic disintegrator) in serum-free M199. M199 and swine serum were added subsequently to give the desired concentration of asbestos and final swine serum concentration of 10%. Asbestos was usually preincubated in medium overnight before addition to cultures. At the end of experiments the culture medium was removed and retained. Cells were then detached by scraping with silicone bungs in the presence of 0·9% w/v sodium chloride containing 0·1% w/v Triton X-100 and bovine serum albumin. Early experiments concentrated on establishing the dependence of enzyme release from macrophages on time and dose. As a result of these, in the experiment comparing leached samples, peritoneal cells (plated at 7×10^6/plate) were exposed to 5 ml of medium containing asbestos at a concentration of 50 μg/ml for 9 and 24 h.

Six samples of asbestos were used, two unleached samples and samples with 11, 19, 55 and 93% of magnesium leached. In addition a control culture, not exposed to asbestos, was included. There were 5 replicates for each sample. The enzymes were assayed as follows: β-glucuronidase by the method of Talalay, Fishman and Huggins (1946), β-galactosidase by the method of Conchie, Findlay and Levvy

(1959), N-acetyl-β-D-glucosaminidase by the method of Woollen, Heyworth and Walker (1961) and lactate dehydrogenase by determination of the rate of oxidation of reduced adenine dinucleotide at 340 nm and 37°. The results were expressed in terms of the means of the 5 replicates and standard errors were calculated from the pooled variation between replicates with samples.

Carcinogenicity in vivo.—In this investigation samples of chrysotile with 50 and 95% of magnesium leached were used in addition to the untreated material. The experimental animals were barrier-protected Caesarian-derived rats of the Wistar strain, bred at the Pneumoconiosis Unit from a stock donated by Imperial Chemical Industries, Pharmaceutical Division, Alderley Edge, Cheshire, in 1968. Thirty-two rats (16 of each sex) were allocated at random to each of three treatment groups. At inoculation the rats were between 6 and 12 weeks old and a similar age distribution was achieved for the three groups by stratifying for age before randomization.

The samples of chrysotile were suspended in physiological saline (50 mg/ml). The dose was 20 mg per rat injected into the right pleural cavity, using the technique described previously by Wagner and Berry (1969). Following injection, rats were kept 4 to a cage isolated in a special unit and fed on a proprietary brand of autoclaved cubes with water *ad libitum*. Each rat was allowed to live until it died, unless it appeared to be distressed when it was killed by exposure to chloroform. A full necropsy examination was carried out.

The differences in incidence of mesothelioma between the groups have been analysed in two ways. First, we have applied the method using the Weibull distribution as advocated by Pike (1966), which we have previously used for experiments of this type (Wagner, Berry and Timbrell, 1973). Secondly, we have used the conditional likelihood method of Cox (1972), which we have also used previously (Berry and Wagner, 1976). In both methods it is assumed that, at any given time after injection, the mesothelioma incidence rates in the three groups differ only by constant factors of proportionality. There was no untreated control group in this experiment but our previous experiments have given no mesotheliomas in controls (Wagner, Berry and Timbrell, 1973).

In order to compare the *in vitro* results with the *in vivo* results, an index of the effect relative to the unleached sample and eliminating the control value was constructed as follows:

Let x_0 be the result for the unleached sample, x_c the control result, and x the value for a leached sample. Then the relative effect was defined as $(x - x_c)/(x_0 - x_c)$. This takes the value 1 for the unleached sample and 0 for the control. Limits of this index were calculated by the method of Fieller (1940).

RESULTS

Protein adsorption

Measurements of the adsorption of human serum albumin by various samples of chrysotile with magnesium depletion up to 93% are given in Table I. These were made at an equilibrium albumin concentration of 0·1 mg/ml. Fully dispersed chrysotile tends to a maximum of 70 mg/g at this concentration (Morgan, 1974) and the lower value obtained for unleached chrysotile in the present investigation indicates that its fibres are not fully dispersed into their constituent fibrils by the ultrasonic treatment. There does not appear to be much loss of adsorptive capacity when the superficial magnesium is removed from the fibre but, with further leaching, there is a progressive decline. Fully leached chrysotile has little affinity for albumin.

Haemolysis

The haemolytic activities of the same samples which were used for measurement of protein adsorption are given in Table I. The haemolytic activity was measured by adding equal volumes of suspensions of leached chrysotile (which originally contained the same weights of unleached fibre) to suspensions of erythrocytes. The haemolytic activities are expressed in terms of % haemolysis/mg equivalent. First results are given in terms of the weight of unleached chrysotile and the loss in weight due to removal of magnesium is not taken into account. Secondly, the results have been corrected for this loss in weight and represent % haemolysis/mg of leached fibre. Thirdly, the uncorrected results for the leached samples have been expressed as values relative to the mean of the unleached samples. The uncorrected results show that removal of the superficial magnesium produces little change in haemolytic activity but when 55% has

TABLE I.—*Results of Measurements of Protein Adsorption and Haemolytic Activity*

Treatment	Treatment time (h)	Mg leached (%)	Albumin adsorbed (mg/g)	Haemolytic activity: Uncorrected (%/mg)	Haemolytic activity: Corrected (%/mg)	Haemolytic activity: Relative to unleached uncorrected (95% limits)
H_2O	0·25	<1	27	19·8	19·8	
	601	<1	27	17·3	17·3	
HCl	0·25	11	26	18·9	20·1	1·02 (0·91, 1·13)
	4	19	23·5	11·6	13·0	0·62 (0·56, 0·69)
	28	36	19·5	10·5	14·0	0·57 (0·51, 0·63)
	70	55	15	5·5	8·5	0·29 (0·26, 0·33)
	162	79	7	4·7	9·6	0·25 (0·23, 0·28)
	431	93	2	4·3	10·8	0·23 (0·20, 0·25)
Standard error of each mean				4·4% of mean		

been leached the haemolytic activity has fallen to one third of its original value. From the corrected values it can be seen that when the results are corrected for loss in weight the haemolytic activity corresponding to 55% depletion is half that of the unleached material and there is no subsequent change with further removal of magnesium. Rahman *et al.* (1974) have also reported that treatment of chrysotile with dilute HCl results in a reduction of haemolytic activity. In a recent paper by Light and Wei (1977) it was shown that the zeta potential of chrysotile is reduced by leaching in dilute HCl and that there is a corresponding reduction in haemolytic activity.

Selective release of lysosomal enzymes from mouse peritoneal macrophages

In preliminary experiments we established that chrysotile asbestos causes a dose- and time-dependent selective release of acid hydrolases from mouse peritoneal macrophages. It is clear from Table II that the water-treated and all the acid-leached samples of chrysotile asbestos caused selective release of the three acid hydrolases assayed in significantly greater amounts than in control cultures. However the extent of selective release is markedly dependent on the degree of leaching. It is clear that when up to 55% of the magnesium is removed there is no diminution of acid hydrolase release. Indeed the 55% leached sample caused significant increases in the selective release of all three enzymes at both 9 and 24 h compared with the water-leached material. However, there is a highly significant decrease in the selective release of acid hydrolases caused by the 93% leached sample compared to the water-leached sample.

In Table III the values of the index expressing the results relative to the unleached sample are given for the percentage of cells released into the culture medium for the three enzymes. When assessed in terms of this index the three enzymes showed a similar pattern of results at 9 and 24 h. However, there was some evidence of an interaction between the size of the effect and the time the cultures were removed. For all three enzymes the effect relative to the unleached sample was greater at 9 h than 24 h up to 55% leached, but the opposite was the case for the 93% leached sample. This interaction was significant ($P < 0{\cdot}01$) for all three enzymes.

To establish that the release of acid hydrolase was selective in nature and that the macrophages remained viable, measurements of the cytoplasmic enzyme lactate dehydrogenase in the cells and culture medium were made. Table IV shows that in no instance was there an increase in the activity of lactate dehydrogenase in the medium of cultures exposed to any of the asbestos preparations. Also

TABLE II.—*Lysosomal Enzyme Levels and Distribution between Cells and Culture Medium in Macrophage Cultures Exposed to Various Samples of Leached Chrysotile Asbestos for 9 and 24 h. nmol Product per Plate/h*[1]

	N-acetyl-β-D-glucosaminidase				β-galactosidase				β-glucuronidase			
	9 h		24 h		9 h		24 h		9 h		24 h	
Sample	Total	% in medium	Total	% in medium	Total	% in medium	Total	% in medium	Total	% in medium	Total	% in medium
Unleached (H_2O for 0·25 h)	3710	37·9	3380	57·2	109	38·3	109	65·9	185	49·4	201	74·5
Unleached (H_2O for 601 h)	4091	38·5	3382	58·5	116	40·6	118	68·3	195	50·8	203	75·5
11% leached	3985	39·4	3644	59·3	114	43·1	121	65·1	192	50·9	212	74·0
19% leached	3895	42·2	3900	63·5	110	43·2	126	67·0	197	54·8	228	75·0
55% leached	3689	48·2	3067	68·8	103	50·2	113	74·2	187	60·6	211	80·2
93% leached	3558	15·2	4040	26·7	103	12·2	127	26·3	165	17·6	194	36·0
Control	3707	12·0	3803	16·0	109	9·4	129	15·5	164	11·6	192	19·7
Standard error of each mean	97	0·8	140	0·6	2·9	1·4	3·4	0·7	3·5	0·8	5·1	0·7

TABLE III.—In vitro *Experiment Results. Effects Relative to Unleached Sample* (95% *limits in brackets) for the Percentage of Lysosomal Enzyme Released into the Culture Medium*

Sample	N-acetyl-β-D-glucosaminidase		β-galactosidase		β-glucuronidase	
	9 h	24 h	9 h	24 h	9 h	24 h
11% leached	1·05 (0·97, 1·13)	1·03 (1·00, 1·07)	1·12 (1·01, 1·25)	0·96 (0·93, 1·00)	1·02 (0·97, 1·08)	0·98 (0·95, 1·01)
19% leached	1·15 (1·07, 1·24)	1·14 (1·10, 1·17)	1·12 (1·01, 1·25)	1·00 (0·96, 1·03)	1·12 (1·07, 1·18)	1·00 (0·97, 1·03)
55% leached	1·38 (1·30, 1·48)	1·26 (1·22, 1·30)	1·36 (1·23, 1·50)	1·14 (1·10, 1·18)	1·27 (1·22, 1·33)	1·09 (1·06, 1·13)
93% leached	0·12 (0·04, 0·20)	0·26 (0·22, 0·29)	0·09 (<0, 0·21)	0·21 (0·17, 0·25)	0·16 (0·10, 0·21)	0·29 (0·26, 0·33)

TABLE IV.—*Lactate Dehydrogenase (mU/plate) in Control Cultures and Cultures Exposed to Various Asbestos Preparations*

Sample	9 h		24 h	
	Cells	Medium	Cells	Medium
Unleached (H_2O for 0·25 h)	503	107	606	63
Unleached (H_2O for 601 h)	497	91	606	61
11% leached	494	71	574	33
19% leached	488	94	584	54
55% leached	450	114	512	83
93% leached	415	74	450	81
Control	396	100	368	57
Standard error of each mean	10	7	14	8

after 24 h exposure the cellular levels of lactate dehydrogenase were elevated in cells exposed to asbestos. Clearly the cells were viable and were responding to contact with the preparations of asbestos by showing increased lactate dehydrogenase activity, probably resulting from increased synthesis of this enzyme.

Carcinogenicity

One rat in the 95% leached group died after only 12 days and is not considered further. The mean survival times after injection and the number of rats with mesotheliomas are given in Table V. The 50% leached sample produced one more mesothelioma than the unleached sample. However, after allowing for the longer survival, by either method of analysis, the mesothelioma rate was slightly lower in the 50% leached group but the difference did not approach significance. Only 2 mesotheliomas occurred with the 95% leached sample, and compared with the other two samples the difference was significant ($P < 0{\cdot}01$). One of the mesotheliomas in the 95% leached group occurred early, only 347 days after injection. All the other mesotheliomas occurred after more than 500 days.

It has been suggested that the leaching might cause weakening of the fibres, possibly leading to fragmentation in tissue. This does not appear to be so. Examination under the transmission electron microscope of ashed lung sections from pleural granulomata of animals injected with leached and unleached chrysotile showed similar pictures (Fig. 1).

DISCUSSION

It is clear that leaching chrysotile with N HCl, resulting in the removal of a large proportion of the structural magnesium, produces marked changes in its biological activity both *in vitro* and *in vivo*. The initial effect of leaching will be to remove the brucite layer on the surface of the fibre. As the positive charge of chrysotile is due to the presence of magnesium ions there will be a marked reduction in surface charge. As the dissolution of the structural magnesium proceeds, leaving a skeleton of amorphous silica, the surface charge will eventually become negative. It has been shown by Morgan (1974) that whereas intact chrysotile adsorbs ablumin

TABLE V.—*Animal Experiment Results*

Sample	Number of rats with a mesothelioma out of 32	Mean survival (days)	Relative mesothelioma rates	
			Pike's method	Cox's method (95% limits in brackets)
Unleached	12	694	1·00	1·00
50% leached	13	738	0·95	0·92 (0·42, 2·13)
95% leached	2	717	0·15	0·17 (0·03, 0·68)

FIG. 1. Electron micrographs of chrysotile fibre in lung tissue; (a) rat dying 28 months after injection of unleached sample; (b) rat dying 24 months after injection of 95% leached sample.

very strongly, the opposite is the case for crystalline silica. The steady fall in adsorptive capacity for albumin with magnesium depletion is to be expected therefore.

With haemolytic activity, however, there was an initial decline up to the point at which half the magnesium had been leached after which there was little further change. The mechanism of chrysotile haemolysis has been discussed by Harington, Allison and Badami (1975), who suggested that it is due to interaction between positively charged magnesium ions on the fibre surface and negatively charged sialic acid groups of glycoproteins within the cell membrane. The haemolytic effect of silica on the other hand is attributed to hydrogen bonding of silicic acid groups with constituents of the red cell membrane. As magnesium is removed from chrysotile it seems likely that there will be transition from a chrysotile type interaction to a silica type which would explain the observed effect. If similar mechanisms are involved in the interaction of asbestos and macrophages it is clear that the optimal density of reactive magnesium groups on the surface of the asbestos fibres is higher for the induction of haemolysis than it is for the induction of the selective release of lysosomal enzymes from macrophages. Marked qualitative and quantitative differences in the known membrane components of erythrocytes and macrophages makes such a difference plausible. It should be noted, however, that the leached chrysotile was subjected to additional sonication before assessing its effect on the macrophage. If the partially

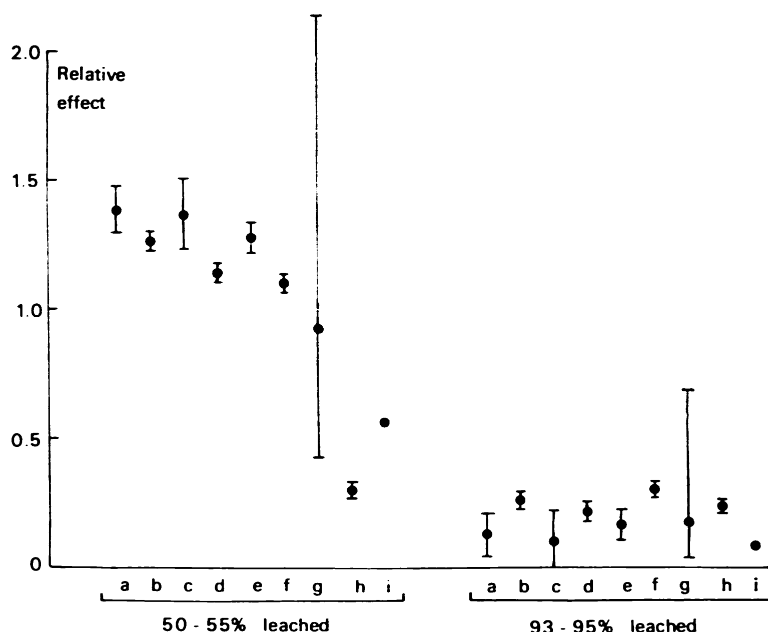

FIG. 2. Comparison of the capacity to cause selective release of acid hydrolases from macrophages carcinogenicity, haemolytic activity and protein adsorption in terms of effect relative to the unleached sample. The estimates and 95% limits are shown. (a) N-acetyl-β-D-glucosaminidase, 9 h, (b) as (a), 24 h, (c) β-galactosidase, 9 h, (d) as (c), 24 h, (e) β-glucuronidase, 9 h, (f) as (e), 24 h, (g) mesothelioma effect in rats, (h) haemolytic activity, (i) albumin adsorbed.

leached fibre can be opened more readily than the intact by sonication, then the observed increase in activity could be due to an increase in available fibre surface. It is clear from Fig. 1 that the original sonication left many fibres unopened. The importance of characterizing the surface area of suspensions of chrysotile used in biological investigations has been stressed by Morgan (1974).

The *in vivo* and *in vitro* effects of leaching chrysotile asbestos which we have described cannot be strictly compared in view of the different duration of the respective studies. It is probable that there was a loss of magnesium from chrysotile asbestos *in vivo*, as has been previously shown (Morgan *et al.*, 1971), whereas the duration of the *in vitro* experiments was short with less likelihood of any significant depletion of magnesium. Nevertheless, when the precision of the results is taken into account, the *in vivo* and *in vitro* results are compatible. In Fig. 2 the results are summarized for the two degrees of leaching used in both experiments, *i.e.* approximately 50 and 95%. This shows that the limits for the animal experiment results include the observed results of the *in vitro* experiment. The animal experiments with 32 rats per group were less accurate than the *in vitro* results with 5 replicates. The *in vitro* results were less expensive to produce and also were available more quickly, *i.e.* in just a few days compared with about 900 days. The relative haemolytic activity with 55% leached chrysotile was significantly different from the effects on selective release of lysosomal enzymes from macrophages and carcinogenicity (Fig. 2).

The increase in selective release of acid hydrolases caused by partially depleted samples of chrysotile asbestos was surprising, but may be explained by the mechanisms involved in its interaction

with specific groups on the macrophage plasma membrane as mentioned above. In retrospect it is unfortunate that samples leached more than 55% and less than 93% were not tested since maximum activity in causing acid hydrolase release would be expected in this range.

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