Effects of phosphorylation of chrysotile on pleuropulmonary fibrogenicity and carcinogenicity

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Abstract

As well as fibre size, fibre chemistry is a determinant of toxicity of mineral fibres. On these grounds, a few years ago, the asbestos industry in Québec developed a process to modify the surface chemistry of chrysotile asbestos by fixing phosphorous atoms. The pleuropulmonary fibrogenicity and carcinogenicity of the native and surface treated chrysotile, called chrysophosphate, were tested. Both products initiated similar pulmonary fibrogenicity in rats exposed by inhalation and sheep exposed by injection in the tracheal lobe. Tumour production in rats after long term inhalation or intrapleural injection was not different between chrysotile and chrysophosphate. These findings clearly show that modification of the phosphate surface of chrysotile fibres does not appear to alter the pleuropulmonary activities of the mineral, thus arguing against the earlier contention of less biological activity for chrysophosphate, a contention essentially based upon results of *in vitro* tests. This study strongly argues for *in vivo* testing of new fibrous materials before making definite conclusions about their biological activities.

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The biological importance of mineral fibre characteristics such as mineralogical type, fibre size, surface structure, and chemistry, and chemical stability is a subject of considerable research and controversy. Although there is some evidence to relate the biological effects to fibre geometry,¹ the chemical reactivity of the fibres has also been suggested to be of pathogenic significance.²⁻⁹ Some chemical alterations of chrysotile undoubtedly alter haemolytic activity,^{8,9}

cytotoxicity,⁹⁻¹³ and possibly affect carcinogenicity.^{13,14}

A few years ago, the Société Nationale de l'Amiante (SNA), a research centre for the asbestos industry in Québec, developed a process to modify the surface of chrysotile. The process aimed at fixing phosphorous atoms at the chrysotile surface, by adsorption of POCl₃ vapours and thermal treatment.¹⁶ It was hoped that the phosphorylated chrysotile (chrysophosphate), which had interesting technological properties, would also be less biologically active.¹⁶ Initial *in vitro* intraperitoneal and intrapleural studies supported this contention.^{13,14,17}

Because of the potential benefits for the chrysotile asbestos industry, the SNA provided us with native and treated chrysotile to assess the pulmonary fibrogenicity and carcinogenicity of these two products in animal experiments. Titanium dioxide (TiO₂; Chronos A/FDZ) was also tested as an inert dust. The three experiments were long term inhalation in rats, intrapleural injection in rats, and injection into the tracheal lobe of sheep.

Methods

TRACHEAL LOBE IN EXPERIMENTS IN SHEEP

Twenty four sheep weighing 25-40 kg were used. They were prepared and accustomed to the pulmonary techniques as previously reported.¹⁸ The flock was divided into three groups of eight sheep exposed either to 100 ml phosphate buffered saline (control group), 100 mg Canadian chrysotile fibres in 100 ml saline (chrysotile group) or 100 mg Canadian POCl₃ treated chrysotile fibres in 100 ml saline (chrysophosphate group). Exposures were carried out through bronchoscopic catheterisation and slow infusion in the tracheal lobe.

Bronchoalveolar lavage (BAL) was carried out before exposure (day 0) and 12, 24, and 60 days after exposure.

Procedures for BAL and analyses have been described elsewhere.¹⁸ The BAL effluent was passed through four layers of cheesecloth to remove mucus, and the cells were formed into a pellet by centrifugation. Cells were counted by haemocytometer and cytocentrifuge smears served to identify the cell

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Table 1 Criteria used for grading intensity and profusion of inflammation-fibrotic pulmonary reactions

Grades	Criteria
Intensity:	
0	Normal histology
1	Moderate cell accumulation
2	Greater cell accumulation with early organisation of the peribronchiolar area
3	Early peribronchiolar fibrosis extending in the adjacent interstitium
Profusion:	
0	Normal histology
1	Lesions occupying less than 25% of the lung tissue
2	Lesions occupying between 25% and 75% of the lung tissue
3	Pneumonia-like appearance

populations recovered with Wright-Giemsa stain. Albumin and fibronectin concentrations in the supernatant fluid were measured by the immunochemical method of Killingworth and Savoy¹⁹ with a Behring laser nephelometer (Hoerchst Behring, Frankfurt, Germany). For measurement of sheep albumin, specific antiserum raised in rabbits was obtained commercially (Cappell lab inc, Downington, PN). Sheep BAL fibronectin was purified on an affinity column followed by chromatography and antifibronectin antibodies were raised in rabbits as previously reported.²⁰ In the supernatant, the activity of lactate dehydrogenase (LDH) and β -glucuronidase (β -G) were measured as previously reported.¹⁸ All results were expressed per ml of BAL effluent as albumin did not vary significantly.

At day 60 of the study, all sheep were killed. Nine tissue samples of the tracheal lobe of each sheep were taken for microscopical examination. The samples were processed as routinely done for human lung tissues. In each sample, intensity and profusion of histological changes were graded by comparison with reference lesions.^{21, 22} Table 1 presents the grading criteria. For each sheep, a pathological score was obtained by multiplying intensity and profusion grades and averaging the nine values.

INTRAPLEURAL INJECTION IN RATS

Four groups of 40 Wistar (ICO, IOPS, AF/Han) rats (20 males, 20 females) were used. Weights at the start of the experiment were in the range 240–260 g for males and 180–200 g for females. Rats in the control group were housed without injection. Animals in the three other groups received a single intrapleural injection of 2 ml isotonic glucose in which 20 mg of either chrysotile, chrysophosphate, or titanium oxide (TiO₂; Chronos, Paris, France) had been suspended by ultrasonic treatment.

Animals were observed daily and weighed at monthly intervals. Those in poor condition or losing too much weight were killed in the course of the

experiments. One third of the survivors were killed 18 months after the injection, and the rest at 24 months.

At necropsy, the thoracic and abdominal cavities were examined and the left lobe of the lungs was fixed in inflation with 10% neutral buffered formalin for histological examination. Other lobes of the lungs and organs showing macroscopical changes were also sampled for histology. Three stains were used—namely, haematoxylin and eosin, Gordon and Sweet, and pictosirius.²³ To differentiate the three histological patterns of mesotheliomas, alcian blue staining was performed at variable pH.²⁴

INHALATION BY RATS

Three groups of 48 Wistar rats (24 females, 24 males) were exposed in inhalation chambers of 1300 l (whole body exposure) for five hours a day, five days a week during a period of 24 months. A modified Timbrell Mark D generator and a cyclone were used to produce clouds of respirable dust.²⁵ The gravimetric concentrations of the dust in the chambers were measured daily. The mean concentrations (mg/m³ (SEM)) of respirable dust were 5.8 (1.5) for chrysotile, 5.8 (1.4) for chrysophosphate, and 6.2 (3.2) for TiO₂. The size distributions of airborne particles were obtained after short term sampling on a membrane filter (Nucleopore, 0.2 μ m) and direct examination under a transmission electron microscope. Length measurements of airborne fibres were roughly log normally distributed, and did not differ significantly between chrysotile and chrysophosphate.

The control group was maintained in a similar inhalation chamber during the same period. The rats were observed daily and weighed at monthly intervals. Those in poor condition or losing too much weight were killed in the course of the experiment. All survivors were killed at 24 months. Histopathological examination was similar to that of the intrapleural experiments. Lung inflammation fibrosis was graded as explained for sheep.

STATISTICAL ANALYSES

Individual measurements were averaged in each group of animals. The standard error of the mean (SEM) was calculated as an index of dispersion. The method of analysis of variance for experiments with repeated measurements on the same subjects was used for BAL measurements in sheep together with the Kruskal-Wallis test. The pathological scores of inflammation fibrosis were analysed by Scheffé's procedure. Occurrence of lung tumours in groups of rats was compared using the χ^2 test with Yates' correction or Fisher's exact one tailed probability test²⁶; p values < 0.05 were considered significant.

Table 2 Tracheal lobe experiments in sheep: lung lavage measurements after exposure and pathological score in lung tissue (mean (SEM))

	Controls (n = 8)	Chrysotile (n = 8)	Chrysophosphate (n = 8)
Cellularity ($\times 10^4$ /ml)	20 (1)	40 (7)*	35 (5)*
Macrophages ($\times 10^4$ /ml)	16 (2)	23 (4)*	21 (2)*
%	76 (3)	58 (5)*	62 (5)*
Lymphocytes ($\times 10^4$ /ml)	3.8 (0.4)	5.0 (1)*	3.4 (1)
%	19 (3)	11 (2)*	10 (3)*
Neutrophils ($\times 10^4$ /ml)	0.6 (0.2)	11 (3)*	10 (3)*
%	3 (1)	25 (6)*	24 (5)*
Eosinophils ($\times 10^4$ /ml)	0.4 (0.1)	0.8 (0.3)	0.6 (0.3)
%	3 (1)	2 (1)	2 (1)
Albumin (μ g/ml)	85 (9)	110 (12)	108 (9)
Fibronectin (μ g/ml)	52 (10)	90 (5)*	88 (4)*
Lactate dehydrogenase (m I U/ml)	7 (2)	13 (2)*	14 (2)*
β -Glucuronidase (ng/min/ml)	0.9 (0.2)	1.9 (0.4)*	1.7 (0.4)*
Pathological score in lung tissue	—	2.86 (0.14)*	2.2 (0.5)*

*p < 0.05 for exposed v controls.

Results

TRACHEAL LOBE EXPERIMENTS IN SHEEP

In the control sheep, BAL total cellularity averaged $20 (1) \times 10^4$ cells/ml and did not vary significantly over the two months of the experiments. The differential cell counts also did not vary, with macrophages constituting 76 (3)%, lymphocytes 19 (3)%, neutrophils 3 (1)%, and eosinophils 2 (1)%. Albumin in BAL averaged 85 (9) μ g/ml, LDH 7.2 ml U/ml, and B-G 0.85 (0.16) ng/min/ml; none of these values varied significantly over time.

The BAL samples in the chrysotile group showed a significant sustained increase in total cells which averaged $40 (7) \times 10^4$ cells/ml. Macrophages, lymphocytes, and neutrophils were significantly increased. Mean concentrations of neutrophils were at 18 times the control values. Albumin was slightly increased at 110 (12) μ g/ml ($p > 0.05$). Fibronectin was significantly increased at 90 (5) μ g/ml ($p < 0.05$) and this increase remained significant after correction for albumin. The enzyme LDH in the BAL supernatant was increased at 13 (2) ml U/ml ($p < 0.05$); β -G was increased at 1.9 (0.4) ng/min/ml ($p < 0.05$).

In the chrysophosphate group, all BAL measurements were increased but at levels that did not differ significantly from those in the chrysotile group. In the absence of significant change with time in the post-exposure period, results were averaged in each group and compared (table 2).

The lungs of the control sheep, were normal at histopathological examination. A diffuse peribronchiolar fibrosing alveolitis was found in the chrysotile group. It was extending in the adjacent lung tissue as previously found with chrysotile tested in that model.¹⁸ The mean pathological score was 2.86 (0.14). In the chrysophosphate group, similar lesions were present, although somewhat less intense. The mean pathological score was 2.2 (0.5) lower but not significantly different from that in the chrysotile group.

INTRAPLEURAL INJECTION IN RATS

Table 3 gives the mortality in each group. It was always higher for rats injected with fibre, especially chrysotile in the period 18–24 months. In the chrysotile, chrysophosphate, and TiO_2 groups, an invasive thoracic tumour was present in 56.5%, 24.4%, and 2.8% of the animals respectively. A total of 28 mesotheliomas was produced. Table 3 reports their distribution by test group and period. One mesothelioma occurred in the TiO_2 group; none in the control group. The total numbers of mesotheliomas in the chrysotile and chrysophosphate groups were respectively 16 and 11.

In each of the three groups of rats exposed to dust particles, the proportion of animals with benign hyperplasia of the mesothelial lining averaged 8.5%. More than 90% of the animals exposed to fibres developed pleural thickening, with no difference between the chrysotile and chrysophosphate groups.

No primary pulmonary carcinoma was present in any of the animals examined. In 17% (chrysotile) and

Table 3 Mortality and occurrence of mesothelioma in intrapleural injection experiments in rats

	Months after intrapleural injection*				
	< 18	18	18–24	24	All
Number of rats dead or killed:					
Controls	2	14	3	12	31†
TiO_2	3	14	5	14	36†
Chrysotile	6	13	16	5	40
Chrysophosphate	6	12	8	14	40
Number of rats with mesothelioma:					
Controls	0	0	0	0	0
TiO_2	0	0	0	0	1
Chrysotile	2	3	10	1	16
Chrysophosphate	1	3	2	5	11

* < 18 = Early mortality before killing at month 18; 18 = killed at month 18; 18–24 = mortality between killing at month 18 and killing at month 24; 24 = killing at month 24.

†Some animals died in circumstances such that lungs were not available for examination.

Table 4 Mortality and occurrence of tumours in inhalation experiments in rats

	<i>Animals dead before month 24</i>	<i>Animals killed at month 24</i>	<i>All*</i>
Number of rats dead or killed:			
Controls	3	36	39
TiO ₂	10	34	44
Chrysotile	7	36	43
Chrysophosphate	13	29	42
Number of rats with adenomas:			
Controls	0	1	1
TiO ₂	0	1	1
Chrysotile	0	4	4
Chrysophosphate	1	3	4
Number of rats with adenocarcinomas:			
Controls	0	0	0
TiO ₂	0	0	0
Chrysotile	0	1	1
Chrysophosphate	0	2	2
Number of rats with squamous carcinomas:			
Controls	0	0	0
TiO ₂	0	0	0
Chrysotile	0	0	0
Chrysophosphate	0	3	3
Number of rats with lung tumours:			
Controls	0	1	1
TiO ₂	0	1	1
Chrysotile	0	5	5
Chrysophosphate	1	8	9

*Some animals died in circumstances such that lungs were not available for examination.

7% (chrysophosphate) of the rats exposed to fibre inflammatory granulomas were present. Granulomas were surrounded by collagen fibres and had a necrotic, sometimes calcified core. No granuloma was present in the TiO₂ group.

INHALATION BY RATS

Table 4 indicates the mortality before month 24. The highest rate (40%) of premature death was recorded in the chrysophosphate group. This excess was due to interstitial nephritis diagnosed as chronic progressive nephritis.

Benign tumours (adenomas) and malignant tumours (adenocarcinomas and squamous carcinomas) were found at histological examination. Table 4 presents their distribution in the four groups. Rats exposed to fibres developed more tumours than the controls. The difference was significant for the chrysophosphate group. The occurrence of tumours was not statistically different for chrysotile and chrysophosphate. No mesothelioma was detected.

In terms of inflammatory lesions, the control rats and the exposed to TiO₂ had essentially normal lungs, apart from a slight excess of alveolitis in the TiO₂ group. Most of the animals exposed to fibres had alveolitis, sometimes associated with alveolar proteinosis. Both interstitial and peribronchiolar fibrosis were found in animals exposed to fibres,

often associated with alveolar epithelial hyperplasia or metaplasia. The severity of these lesions was comparable between the chrysotile and chrysophosphate groups. Mean pathological scores in the chrysotile, chrysophosphate, and TiO₂ groups were respectively 2.08 (1.0), 1.96 (1.0), and 0.34 (0.2).

Discussion

This report presents the results of three sets of experiments that were conducted to evaluate the relative toxicity of native chrysotile fibres and POCl₃ modified fibres. These studies were carried out after earlier in vitro and in vivo studies had shown a relatively reduced cytotoxicity¹³⁻¹⁷ and a reduced carcinogenicity for the chrysophosphate in the rat intraperitoneal injection model.¹⁴

The present experiments were conducted in established animal models known to reproduce fibre related diseases. The sheep model reproduces the fundamental lesions of asbestosis^{18 21 22 27}; the rat intrapleural model is often used for studying the potency of fibres to induce mesothelioma²⁸⁻³⁰; the rat inhalation model is a well recognised model for asbestosis and lung carcinomas.^{31 32}

In our study, chrysophosphate and chrysotile exhibited similar pulmonary fibrogenicity in sheep and rats. During the final six months of the intrapleural rat experiment, mortality was higher in the chrysotile group and related to histologically identified mesotheliomas. The incidence of such tumours was lower in the chrysophosphate group than in the chrysotile group but the difference was not significant. Similar results were obtained by others using the intraperitoneal test.¹⁴ Our long term inhalation experiments, however, did not show less pulmonary carcinogenicity for chrysophosphate. The incidence of pulmonary carcinomas, especially squamous carcinomas, was greater with chrysophosphate than chrysotile, but again the difference was not statistically significant.

Previous attempts at modifying the chemistry or surface of asbestos fibres have been able to document some associated biological changes. Leaching the magnesium of chrysotile reduces its toxicity in vitro, whereas the same process enhances the toxicity of crocidolite.⁵ Haemolytic activity of asbestos fibres can be significantly reduced by serum, ethylenediaminetetraacetic acid (EDTA), and several other compounds, including metal oxides and metal micelles.^{8 12} Two independent studies have shown that 90% of magnesium depleted chrysotile asbestos has significantly reduced carcinogenicity, at least as determined by the intrapleural injection model.^{34 35} All these data strongly suggest that, as well as fibre size, surface chemistry can be a determinant of some biological activity.

Our results clearly show that the particular surface modifications induced by POCl₃ treatment were

ineffective in reducing the long term fibrogenicity and carcinogenicity of chrysotile in experimental animals. To reconcile the previous studies on chrysophosphate with the present observations, it must be considered that in vitro and intraperitoneal models may not always reflect entirely and completely the in vivo intrapleuropulmonary biological events and thus may give different results. Also it may be that in our studies the POCl_3 treatment did not reach all of the fibrils of chrysotile within the bundles often present in the raw chrysotile or chrysophosphate materials (B Pathak and P Sébastien, personal communication). Treatment with POCl_3 could have been more effective in the materials used in previous studies.

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