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Biological *in Vitro* and *in Vivo* Responses of Chrysotile Versus Amphiboles

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Although all commercial forms of asbestos have been demonstrated to be carcinogenic in animals, so far epidemiological data are controversial concerning what asbestos types are the most carcinogenic and fibrogenic in humans. In order to understand the early cellular events induced by fibrous particles, different *in vitro* studies (hemolysis, release of enzymes by macrophages, assays on cell culture systems) have been carried out in several laboratories; most of these studies have shown that cell and subcellular *in vitro* responses were different depending on fiber types: chrysotile versus amphiboles. This presentation compares the results of different laboratories with our data obtained by using a model which modifies the chemistry of the fibers by acid treatment. The acid-leached chrysotile and acid-treated amphibole fibers showed different biological responses in several *in vitro* systems used in comparison to unleached fibers. These differences in the *in vitro* reactivity were related to the chemical state of the fibers and might explain the differences in their effects in animals after intrapleural injection as assessed by the percentage of mesothelioma, the latency period, the survival time and the degree of pleural fibrosis. The carcinogenic effect of the fibers is discussed in relation of their *in vitro* inflammatory or cytotoxic responses.

Introduction

Although the fibrogenic and carcinogenic properties of asbestos dusts are universally accepted, there is still considerable debate regarding on the one hand the mechanisms of fibrogenesis and carcinogenesis and on the other, the gradient in pathogenicity of different types of fibers.

With regard to the mechanisms of asbestos-related diseases, over the past 10 years considerable emphasis has been placed on the experiments of Stanton et al. (*1, 2*) and Pott et al. (*3, 4*) which demonstrated that the carcinogenic potential of fibers was mostly related to the fiber size, the most carcinogenic fibers in the pleura being those more than 4 μm in length and less than 0.25 μm in width. Actually, the Stanton hypothesis, based on the concept of the "solid state" or "foreign body" carcinogenesis, put the role of physicochemical parameters far behind (*5*). However, we will see later on that the role of chemical and physical constituents and particularly those available at the surface of the fibers must also play a role, but have been insufficiently assessed.

From human and animal data, there is strong evidence that the three commercial types of asbestos, the serpentine chrysotile and the amphiboles, crocidolite and amosite, are all responsible for lung and pleural fibrosis and for lung and mesothelial cancers (*6*). However, there is still controversy about the gradient of pathogenicity of these three types of asbestos. Several epidemiological studies on human populations which have been exposed to one type of fiber have persuaded many people that crocidolite (*7-9*), and perhaps also amosite (*10, 11*), is much more carcinogenic towards the pleura than chrysotile (*12*). Previously, a group of experts at the 1976 IARC meeting (*6*) concluded that occupational exposure to chrysotile was more likely to cause lung fibrosis and lung cancer than exposure to amphiboles. Occupational exposure to crocidolite and amosite, however, was more often associated with pleural and peritoneal mesotheliomas than exposure to chrysotile. In a recent editorial, however, Liddell (*13*) gave another opinion, pointing out that amphiboles were not only the most carcinogenic fibers in the mesothelium, but were also more fibrogenic and carcinogenic in the lung. Many authors are not convinced by this assertion, especially after the epidemiological demonstration by Peto that the incidence of pleural mesothelioma was almost as high in a cohort of workers ex-

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posed mostly to chrysotile as in cohorts exposed only or mostly to amphiboles (*14, 15*).

Experiments in animals have also shown discrepancies in the fibrogenic and/or carcinogenic potential of asbestos according to the type of fiber, but in all studies other factors intervened, such as doses, mode of dust introduction (inhalation, intratracheal instillation, intrapleural implantation of injection, intraperitoneal injection), type of diseases induced, animal strain, age and survival time. However, the results showing a modification in the carcinogenic effects of chrysotile after acid treatment (*16, 17*) raised the question that other factors, besides shape and size (chemical composition, surface physicochemistry), may play a role in the induction of fibrosis and cancer.

The controversial position of scientists concerning such an important point needed a critical review in an attempt to evaluate significant information from the comparison of the biological responses of chrysotile and amphiboles *in vitro* as well as *in vivo*. The effect of acid treatment of the fibers will also be taken into account, since it can lead to a better understanding of the mechanisms of fiber carcinogenesis. Our provisional conclusions will be derived from concordant results obtained in our laboratory and in others during the last decade.

In Vitro Studies

Reactivity with Red Blood Cells

The hemolytic assay provides a rapid way for investigating the interaction between dusts and biological membranes. Using this system, several authors (*18-22*) found different responses with chrysotile and the amphiboles, the former being more hemolytic than the latter. Generally speaking, after acid treatment chrysotile was less hemolytic (*16, 21, 22*), whereas acid-treated amphiboles were found to be more hemolytic (*21, 22*). Thus, if hemolysis explores the interaction between fibers and cell membranes, chrysotile appeared as the most reactive fiber type in these experiments. This discrepancy was also found when studying the adsorption of phospholipids on fibers, and this was greater with chrysotile than with the amphiboles (Jaurand et al., unpublished data) (Fig. 1). Jaurand et al. (*24*), when studying the kinetics of hemolysis by chrysotile, have shown a self inhibition of the reaction due to adsorption of the membranes. This correlates with the observation of a decreased hemolytic activity after incubating fibers with phospholipids (*24*) and probably relates to a decrease in the zeta potential (*26*). Indeed, Light and Wei (*21*) have demonstrated that the hemolytic activity was related to the absolute value of the zeta

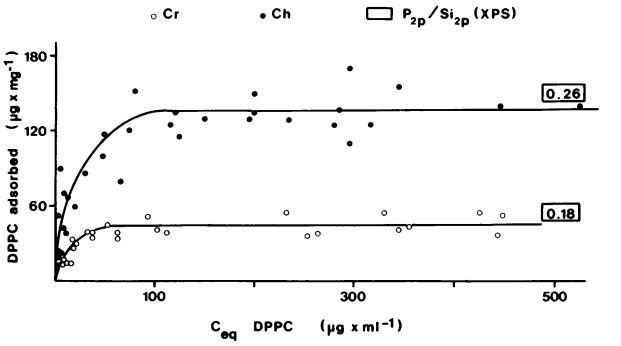

FIGURE 1. Adsorption isotherms of liposomes of dipalmitoyl phosphatidyl choline (DPPC) on chrysotile (Ch) and crocidolite (Cr) fibers. Variation in the amount of DPPC adsorbed on the fibers with the equilibrium concentration C_{eq}. In the squares are indicated the values of the ratio P_{2p}/Si_{2p} determined by photoelectron spectrometry analysis (XPS) (*23*).

potential of fibers. With chrysotile it decreased during leaching, whereas with crocidolite it increased during the same treatment.

Reactivity with Macrophages

Studies carried out in different laboratories over the last five years, describing the release of lysosomal acid hydrolases from peritoneal or alveolar macrophages maintained in culture, have clearly shown differential responses between chrysotile and the amphiboles.

Davies et al. (*27*) and, more recently in our laboratory, Jaurand et al. (*22*) have clearly shown that chrysotile works as an inflammatory stimulus, inducing a selective release of lysosomal acid hydrolases. However, there was no release of cytoplasmic enzymes such as lactate dehydrogenase (LDH), which, in the case of peritoneal macrophages, showed higher intracellular levels, suggesting an enhanced protein synthesis (*27*). Similar responses have been obtained with other particles such as zymosan which we know to elicit inflammation (*28*).

This type of inflammatory response could be related to the physicochemical surface properties of the fibers, since acid-treated chrysotile, which has lost most of its Mg, did not release lysosomal enzymes (*28*) and even released LDH, indicating a cytotoxic effect (*22, 29*).

In contrast, untreated amphiboles (crocidolite and amosite) seemed to be cytotoxic, releasing both lysosomal acid hydrolases and cytoplasmic LDH. Acid-treated amosite and crocidolite, however, enhanced the release of lysosomal hydrolases, but this was associated with the release of LDH (*22*) (Fig. 2).

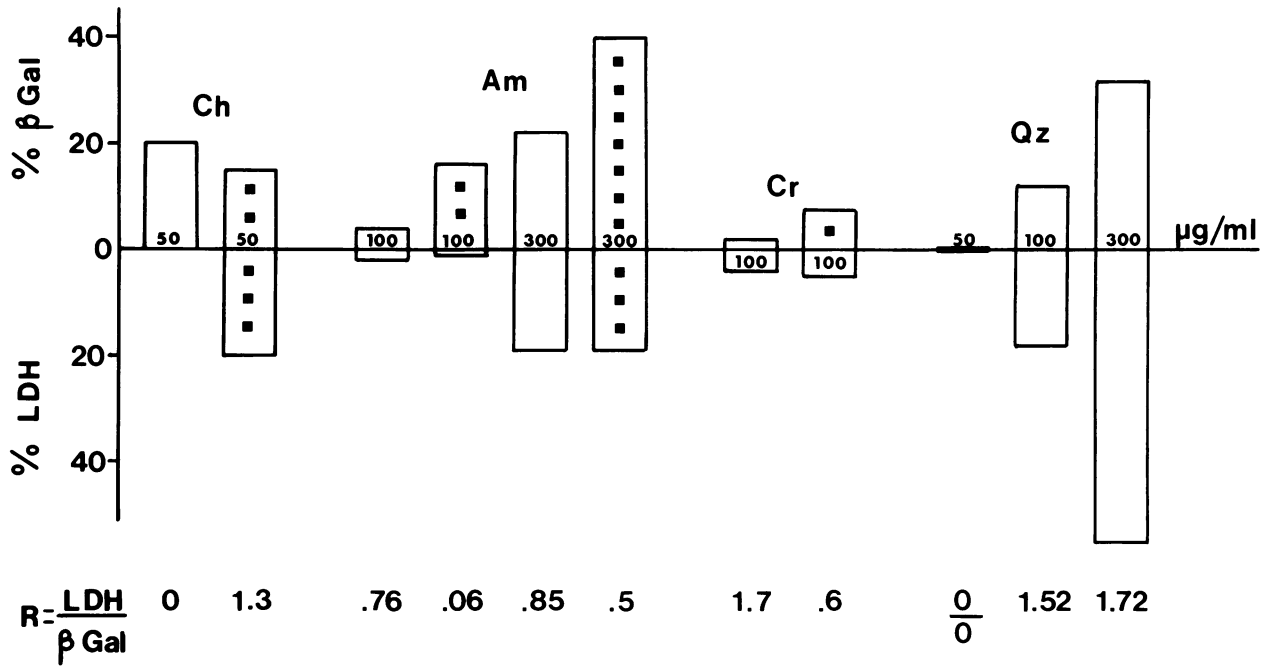

FIGURE 2. Release of enzymes from rabbit alveolar macrophages cultured with chrysotile (Ch), amosite (Am), crocidolite (Cr) or quartz DQ_{12} (Qz) either unleached (□) or oxalic acid-leached (■). Percentage of LDH and β-galactosidase (β Gal) released in the culture medium, following 20 hr of contact with the particles (concentration 50, 100 or 300 µg/mL).

It has also become clear that macrophages secrete a number of products (enzymes, mediators) after incubation *in vitro* with asbestos and that several of these molecules may be directly involved in chronic inflammatory responses. Chrysotile has been shown to elicit a highly significant increase in macrophage phospholipase activity and prostaglandin synthesis (*30*) and to induce the secretion by alveolar macrophages, of a chemotactic factor which attracts polymorphonuclear leukocytes (*31*). Macrophages from mice given intraperitoneal injections of chrysotile asbestos secrete considerable amounts of plasminogen activator when cultured *in vitro*, whereas latex particles do not yield such an increase (*32*). All these *in vitro* studies indicate clearly that chrysotile works as a very potent activating factor on alveolar macrophages. However, other studies did not confirm the greater stimulatory effect of chrysotile. Thus, White and Kuhn (*33*) found an increased secretion of elastase by peritoneal macrophages under the action of chrysotile and crocidolite, but the high doses used in this experiment do not allow a comparison of the effect. Moreover, in a recent experiment exploring the oxidant production by guinea pig alveolar macrophages *in vitro* (release of O^{2-} and H_2O_2) an opposite pattern was shown, e.g., the amphibole asbestos were more effective than chrysotile to cause macrophage oxidase activation (*34*). Other *in vitro* experiments are needed, taking into account most of the parameters involved (animal species, types of macrophage, culture conditions and so on). This will allow us to better understand the significance of the *in vitro* responses of macrophages in different biological pathways according to fiber types, in relation to the type and intensity of fibrogenesis and carcinogenesis *in vivo*: however the relationship between the *in vitro* macrophage response to fibers and pulmonary or pleural carcinogenesis, however, is far from being clearly understood.

Reactivity with Proliferative Cells

Several authors have used cell lines in short-term studies of asbestos cytotoxicity. Most of these studies are summarized in Table 1 which indicates the cell lines used by the authors, the cytotoxicity assays, the doses of asbestos tested and the gradient of toxicity according to the type (chrysotile versus amphiboles) of fiber (*35-41*). In most studies chrysotile was more "toxic" than the amphiboles. Moreover, the acid treatment decreased the cytotoxicity of chrysotile and increased the cytotoxicity of crocidolite and amosite (*41*).

Up to now, few experiments have been conducted with normal tissue or cells in culture. Some of them were carried out with normal tracheal tissue explants cultured *in vitro* (*42*). Only amphiboles have

Table 1. Asbestos cytotoxicity on cell lines.

Epithelial-like cell lines	Cytotoxicity assays	Asbestos doses, μg/mL	Gradient toxicity[a]	Reference
Macrophagelike P 388 D1 cells	Growth inhibition	10-100	Ch > Cr	(35, 36)
Human lung fibroblasts W 38 cells	Morpho changes	100	Ch > Am	
Rat liver-derived K 22 cells	Growth inhibition	10	Ch >> Cr>$_{Am}$	(37)
Chinese hamster ovary CHO cells	Colony efficiency Trypan blue exclusion			
Chinese hamster lung V 79-4 cells	Colony efficiency	10-50 (cells seeded with fibers)	Am > Cr > Ch	(38)
Human alveolar lung A 549 cells	Growth inhibition		Ch > Cr> glass fibers > LCh	
Chinese hamster lung-derived CHL 39 cells	Colony efficiency	10	Ch > Am >> Cr	(39)
Chinese hamster ovary CHO cells	Growth inhibition	10	Ch > Cr, Am	(40)
Human intestine-derived I 407 cells	Colony efficiency	250	Ch 10 times > Am-Cr	
Adult rat liver-derived ARL 6 cells			I 407 > ARL 6	(41)
Mouse colon-derived MCE 1 cells			LCr ↓ cytotoxicity LAm & Cr ↑ cytotoxicity Cr > Ch, Am	

[a]Ch = chrysotile; Cr = crocidolite; Am = amosite,LCh,LCr, LAm = leached chrysotile, crocidolite, amosite.

been tested in this model. In our laboratory, we have developed a model using cultures of normal rat mesothelial cells for testing the reactivity with different types of fiber (43). This test studied the morphology and the growth characteristics of mesothelial cells treated with chrysotile and crocidolite which were either oxalic-acid leached or unleached (44). When the samples are compared weight to weight, the results agree with those obtained by others who used epithelial-like cell lines (37, 41). Thus, chrysotile seems to be more reactive and cytotoxic with epithelial-like cell lines than crocidolite; leaching of chrysotile fibers decreased the reactivity; conversely, leaching the crocidolite increased the cytotoxic effects on the cells.

In vitro studies have also been carried out with cultures of lung fibroblasts which were stimulated to produce fibrous collagen under the action of different types of asbestiform minerals (45). In these experiments, chrysotile was the most reactive, followed by anthophyllite and amosite/crocidolite. This effect was dose-dependent, but the response was not constantly the same. In contrast, the acid-leached chrysotile, particularly when 80% of the magnesium was depleted, was much less active on collagen synthesis.

Subcellular Effects

It is still controversial as to whether or not asbestos can bind to DNA and induce damage and mutations. No mutagenicity was demonstrated by means of the Ames tests on bacteria (46). However, tests carried out on mammalian cells in culture have shown that asbestos fibers may interact with DNA, since they gave a weak mutagenic response with the HGPRT mutant phenotypic test (39), induced chromosomal damage (45) and slightly increased sister chromatid exchanges (38). However, no difference was noted between chrysotile and the amphiboles.

Although it has been demonstrated that chrysotile asbestos was much more active than the amphiboles in binding IgG (48), no difference was noted between chrysotile and the amphiboles for the activation of the classical and alternative pathways of complement (48, 49). Apparently, complement activation was not related to reactive sites at the fiber surface, since there was no difference between chrysotile and the amphiboles or between chrysotile and leached chrysotile (49, 50).

Nevertheless, surface properties seem important for the adsorption of macromolecules by asbestos fibers, as suggested by the results obtained in our laboratory with chrysotile and oxalic acid-leached chrysotile. The adsorption of albumin or dipalmitoyl phosphatidyl choline on Mg-depleted chrysotile fibers was characterized by a bulk incorporation of the macromolecules into the fibers. However, these results are different from those of others (51, 52) who found that albumin had a decreased affinity for Mg-depleted chrysotile.

Animal Studies

Differential Fibrogenesis

The early animal experiments did not clearly define the relative importance of asbestos fiber types in the production of lung or pleural fibrosis (53, 54). However, since the work of Wagner et al. (55) and more recently of Davis et al. (56), it appears that

chrysotile given by inhalation causes far more lung fibrosis than crocidolite, which in turn is more fibrogenic than amosite. This fibrogenic gradient was still found to be the same when the number of fibers was adjusted to an equivalent number in dust samples (*56*). This gradient may be related to fiber size discrepancies between asbestos types as suggested by many authors. Davis et al. (*56*), who used an extensive fiber-length distribution, showed that the chrysotile clouds in the chamber had many more fibers over 20 μm in length than either of the amphibole clouds in their experiment. It seems that short fibers, less than 5 μm in length, are phagocytosed without causing fibrosis, while fibers longer than 5 μm in produce foreign body granuloma with fibrosis. In a recent, well-controlled animal experiment using inhalation, Lee et al. (*57*) found that amosite was at least 10 times more fibrogenic than potassium octatitanate (Fybex) fibers, although concentrations and lengths of these man-made organic fibers were many times higher in the clouds than those of amosite. These findings suggest that physicochemical properties of the surface of the fibers must play an important role in fibrogenesis.

Differential Carcinogenesis

Several experiments, some of them large-scale, have been carried out in different species in order to study the differential effect of fibers introduced into the pleural or peritoneal cavities, either by injection or by implantation. The intrapleural or intraperitoneal inoculation of dusts has the advantage that experiments can be conducted with small amounts of material which allow the comparison of various samples of specially prepared or modified dusts. However, the experiments using the inhalation of dusts through the airways are more realistic when compared with human exposure: the ideal is chronic inhalation in a special chamber.

Most early animal inhalation studies did not find differential results in the production of bronchial carcinomas and mesotheliomas with different asbestos types (*58-60*). Wagner et al. (*55*), in a series of experiments in rats using amosite, anthophyllite, crocidolite and two varieties of chrysotile, found that the shortest mean survival time after first exposure was observed with chrysotile, particularly the Canadian one, followed by crocidolite and amosite. In the same way, the highest number of malignant tumors was observed in animals treated with Rhodesian chrysotile and the lowest in those treated with amosite. Anthophyllite, crocidolite and Canadian chrysotile gave about the same number of tumors. The more carcinogenic effects of chrysotile versus the amphiboles were observed even though much less dust was retained in the lungs exposed to chrysotile. Davis et al. (*56*) found clear-cut results after inhalation studies in rats comparing UICC chrysotile A, crocidolite and amosite. UICC chrysotile A was more fibrogenic and carcinogenic than UICC crocidolite and UICC amosite, since all the malignant lung tumors were found in animals that had inhaled chrysotile dust. Only two mesotheliomas were found in this study, one with crocidolite, and one with chrysotile.

Regarding pleural carcinogenesis, it also appears that chrysotile is the most carcinogenic—or at least as carcinogenic as the amphiboles. As early as 1969, Wagner and Berry (*61*), in a large-scale experiment comparing the effect of chrysotile, crocidolite and amosite on specific pathogen-free (SPF) and standard rats, found clear-cut results, in that all types of asbestos produced mesotheliomas. Chrysotile and crocidolite produced about the same percentage of tumors, the percentage in SPF animals with mesotheliomas being 61% for chrysotile and 59% for crocidolite, while in standard animals the corresponding percentages were 69% and 68%. The fewest mesotheliomas were produced by amosite (40% of the SPF and 31% of the standard animals). Moreover, when comparing the mean survival times for SPF and standard rats with mesotheliomas, after eliminating the effect of mortality due to other causes, the authors found that chrysotile exposure led to the shortest survival times (598 days for SPF and 621 days for standard rats). This was significantly less than crocidolite (718 and 655, respectively) and much less than amosite (811 and 801 days, respectively). In contrast, the survival of SPF and standard rats without mesothelioma, after elimination of the effect of mortality due to mesothelioma, was not different. Wagner et al. (*55*), however, using intrapleural inoculation of various dusts in rats, found that among the UICC standard reference samples (experiment 3), UICC crocidolite was the most carcinogenic, being three times as active as UICC chrysotile. But, in this very paper, the results of experiment 1, where SFA chrysotile was compared to crocidolite in a dose-effect relationship, were in contradiction with the above conclusion. There was a relationship between the number of mesotheliomas and the dose (from 0.5 to 8 mg) for both SFA chrysotile and crocidolite, but if we total the number of rats with a mesothelioma, there were 21 out of 59 animals with mesotheliomas in the SFA chrysotile group while there were 11 out of 59 animals with mesotheliomas in the crocidolite group.

In a recent study carried out in our laboratory, after intrapleural injection of different dusts in the rat, chrysotile and crocidolite produced about the

same number of mesotheliomas; the most striking difference was a more marked initial inflammatory reaction of the pleura with chrysotile, with a greater percentage of animals dead from other causes than cancer. Moreover, the latency period was shorter in the chrysotile group than in the crocidolite group (*17*).

Conclusion

Obvious discrepancies exist between the biological effects of chrysotile and the amphiboles either *in vitro* or *in vivo*. Chrysotile seems to be the most reactive *in vitro* as well as *in vivo*. These findings question whether or not it is scientifically correct to apply the Stanton theory generally to carcinogenesis induced by fibers, since it takes into account only the fiber size parameters, length and diameter. In this respect, it is odd that the paper in memory of Staton (*62*) takes into account only amphiboles and amphibole-like fibers, excluding chrysotile fibers, which according to our results and to those of other laboratories, appear as the most potent inflammatory stimulus. The striking modification in the biological response of acid-treated asbestos suggests that reactive sites at the surface of the fibers could also play a role in the pathogenic effects of fibers, particularly in relation to cancer. Thus the difference in the survival time between chrysotile and crocidolite in rats whose pleural cavity had been injected with fibers might be due to the fact that chrysotile was immediately reactive in inducing inflammation and subsequently cancer, whereas crocidolite needed some *in vivo* modification to become inflammatory and carcinogenic.

This puzzling biological problem makes the interpretation of human data difficult. First, humans have usually been exposed to mixed fibers associated with different cofactors. Peto et al. (*14*), analyzing epidemilogical data, observed that the risk of developing mesothelioma was substantially lower in humans whose exposure to chrysotile was reduced or ceased than in those where exposure was maintained; by contrast, even brief exposure to crocidolite could produce a substantial incidence of mesothelioma many years later (*8*). Peto et al. (*14*) suggests that this difference could be due either to the fact that chrysotile was largely eliminated from the lung whereas amphiboles remained almost indefinitely (*63*) or to the fact that chrysotile fibers are leached *in vivo* (*64*) and thus cease to be biologically active in the body, while crocidolite fibers remain active or even become more active.

Recent experiments in animals have shown that other fibrous minerals such as erionite-zeolite were also carcinogenic, even more than asbestos, although fibers were short (*65*). This underlines the necessity of pursuing basic research on the mechanisms of the biological effect of fibers because it seems that the Stanton hypothesis does not explain all situations.

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