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Thymidine Incorporation by Lung Fibroblasts as a Sensitive Assay for Biological Activity of Asbestos[1]

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Fibroblasts exposed to chrysotile B asbestos displayed an initial growth inhibition compared to controls during the first 24 hr of incubation. At 48 hr, however, there was resumption of cell proliferation as evidenced by elevated thymidine (TdR^3H) incorporation. UICC samples of chrysotile A, chrysotile B, crocidolite, amosite, and anthophyllite all altered thymidine incorporation by lung fibroblasts in a dose-dependent manner and to different maximal levels. Amphibole asbestos was either equally or less active than chrysotile. Silica had the lowest activity. Various other particles, including latex, zymosan, titanium dioxide, magnetite, and Mt. St. Helens volcanic ash, had no effect. Chemically modified chrysotile samples which retained their fibrous structure were also tested for possible alterations of fibroblast thymidine incorporation. The DNA synthesis response of human lung fibroblasts to asbestos correlates with its *in vivo* fibrogenic and tumorigenic potential and may prove useful in determining the pathological effects of asbestos.

INTRODUCTION

Many environmental pollutants cause lung disease characterized by connective tissue alteration and damage. Overactivity of connective tissue components such as fibroblast proliferation and/or collagen synthesis would cause fibrosis, while underactivity would lead to abnormal lung structure and function. Pulmonary fibrosis as observed in asbestosis is diffuse and histopathological studies show extensive fibrosis with marked emphysematous changes in asbestotic lungs (Haley, 1975). The mechanisms involved in asbestos-induced fibrosis are unknown and study of the effects of asbestos at the cellular level may be useful in elucidating the molecular basis of fibrogenesis. As has been suggested for silica (Heppleston and Styles, 1967a, b), interaction between macrophages and fibroblasts may play a crucial role in asbestosis although no direct experimental evidence clearly supports that assumption. *In vivo* studies have shown that asbestos fibers reach the connective tissue compartment of the lung (Brody *et al.*, 1979) and it is likely that direct interaction of asbestos with the pulmonary interstitium may also have determinant effects in asbestos-related diseases.

Different types of particles vary considerably in their *in vivo* effects. For example, all types of asbestos and silica have been shown to produce fibrosis in experimental animals (Harington *et al.*, 1975; Reiser and Last, 1979), while latex,

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titanium dioxide, and magnetite are almost inactive in these respects (Christie *et al.*, 1963; Davis, 1972; Shorlemmer *et al.*, 1977). Furthermore, chrysotile, crocidolite, and, to a lesser extent, amosite and anthophyllite produced mesothelioma in rats (Wagner *et al.*, 1973, 1974) whereas silica did not (Wagner and Berry, 1969). Although *in vivo* experimentation is the ultimate means of determining the biological activity of asbestos, it is time consuming and does not lend itself to the comparison of a large number of different pollutant particulates. For this purpose, *in vitro* assays are more suitable and in the past years, numerous studies of the effects of asbestos on various cell types and functions have been reported (Aravinda Babu *et al.*, 1980; Chamberlain and Brown, 1978; Harrington *et al.*, 1975; Huang, 1979; Lemaire and Lemaire, 1981; Neugut *et al.*, 1978; Reiss *et al.*, 1980; Richards *et al.*, 1971). In many instances, however, the observed *in vitro* effects of asbestos did not closely correlate with its fibrogenic and tumorigenic potential *in vivo*. In this regard, crocidolite and amosite have been shown to be poorly cytotoxic (Harrington *et al.*, 1975; Miller and Harington, 1972) or hemolytic (Macnab and Harington, 1967), although both types of amphiboles caused fibrosis and mesothelioma *in vivo* (Harington *et al.*, 1975; Kannerstein *et al.*, 1978; Wagner *et al.*, 1973, 1974).

In the present study, the effects of asbestos and various particles on *in vitro* DNA synthesis by lung fibroblasts were examined in order to define a parameter in fibroblast function which may be related to *in vivo* effects of these particles. Since adequate fibroblast proliferation and DNA synthesis are crucial for the maintenance of normal lung fibroblast population, modification of these functions by asbestos may result in connective tissue alterations and contribute to the pathogenesis of asbestos-related diseases.

MATERIALS AND METHODS

Particles and fibers. UICC standard asbestos samples (Canadian chrysotile B, Rhodesian chrysotile A, crocidolite, amosite, and anthophyllite) were obtained from the National Research Institute for Occupational Diseases, Johannesburg, South Africa. The asbestos fibers were uniform and well characterized, 92% having less than a 0.25-μm diameter and 20-μm length (Timbrell *et al.*, 1978). A commercial sample of Quebec grade 7D chrysotile (Paperbestos 7D, two passes B.O.P.; Berger and Oesper, 1963) was used in some experiments. Latex (1.1 μm) and zymosan were purchased from Sigma. Titanium dioxide (TiO_2) was a product of Aldrich Chemicals. Magnetite and atapulgite were generously provided by Dr. D. Nadeau, Department of Biology, Université de Sherbrooke. Mt. St. Helens volcanic ash was obtained through the courtesy of Dr. R. Bégin, Pneumology, Centre Hospitalier Universitaire, Sherbrooke. Silica (Min-u-sil; <5 μm) was obtained from Pennsylvania Glass Sand Corporation, Pittsburgh. Modified chrysotile fibers were prepared by the "Programme de recherche sur l'amiante, Université de Sherbrooke" from Quebec grade 4T30 chrysotile sample (Dunningan *et al.*, 1980). Very short chrysotile fibers were obtained after sedimentation in water for 24 hr. EM examination of this material indicates that more than 50% of the fibers are <1 μm and 100% <8 μm (Jolicoeur *et al.*, 1981). Heat-treated samples were obtained by heating this material at 325 and 500°C. Such treatment produced a

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dehydroxylation of chrysotile as evidenced by the disappearance of the OH peaks of the infrared spectrum 2.7 μm, while preserving the fibrous structure, as verified in electron microscopy (Dunningan *et al.*, 1980). Magnesium leaching from chrysotile was obtained by treatment of samples on fritted glass funnel (grade M) with 1% acetic acid. Acid washings were tested for presence of Mg^{2+} by flame photometry. Acid treatment was continued until values lower than 1 ppm were obtained.

Fibroblast cultures. Human embryonic lung fibroblasts WI-38 (Hayflick and Moorhead, 1961) were obtained from Flow Laboratories, Rockville, Maryland, at passage level 22–24. Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin (100 U per ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS) (Grand Island Biological Co., Grand Island, N.Y.). The cultures were maintained at 37°C in a humidified 95% air–5% CO_2 atmosphere in 75-cm² tissue culture flasks (Falcon). When confluent, the cells were dispersed by treatment with trypsin–EDTA solution (0.05%–0.02%) in phosphate-buffered saline (PBS) and split 1:2 twice weekly. For the *in vitro* analysis of proliferation, fibroblasts between passage 25–27 were trypsinized and replated into multiwell tissue culture plates (Linbro) at a density of 5×10^4 per 0.5 cm² in 1 ml DMEM containing 10% FBS. After an incubation of 4 hr to permit adherence, the medium was aspirated and the attached cells were washed with serum-free medium and cultured overnight in DMEM containing 0.5% FBS.

Incubation of fibroblasts with asbestos and various particles. The fiber or particle sample was autoclaved for 45 min, suspended at a concentration of 1 mg/ml in DMEM without serum with a Dounce glass homogenizer, and maintained under constant stirring. Zymosan was prepared in DMEM medium as described by Trush *et al.* (1977). The resulting suspension was added at varying concentrations (0–100 μg/ml) to 5×10^4 fibroblasts in a final volume of 1 ml of DMEM supplemented with 0.5% FBS. After the indicated incubation time, the fibroblast cultures were pulsed with 1 μCi/ml tritiated thymidine (TdR³H, sp. act. 2.0 Ci/mM, New England Nuclear, Boston, Mass.) for 4 hr. For determination of incorporated TdR³H, the medium was aspirated and the cells were exposed to trypsin (0.12%) and collected with 3 vol of 1 ml 10% trichloroacetic acid (TCA) on glass fiber filters (GF/B, Whatman) in a multiple-membrane filter apparatus (Yeda, Linca Lamon Inst., Tel-Aviv). The filters were washed three times with 10% TCA and three times with distilled H_2O and the radiolabeled TCA precipitable material was counted in Aquasol scintillation fluid (New England Nuclear). Each determination was done in triplicate. Data are expressed as thymidine incorporation index, which represents the ratio of thymidine incorporation in treated cultures over control cultures, as calculated by the formula E/C, where E represents cpm of fibroblasts exposed to asbestos and C represents cpm of fibroblasts in medium alone.

In additional studies, fibroblasts were not pulsed with TdR³H, but after treatment were washed with PBS to remove nonadherent cells, trypsinized, and counted in a hemocytometer chamber. Cell viability was determined by trypan blue exclusion. Four determinations of triplicate cultures were done and the data represent the mean ± SEM of these analyses.

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RESULTS

Effects of Chrysotile B Asbestos on Fibroblast Growth and DNA Synthesis

When human lung fibroblasts were exposed to chrysotile B asbestos for varying periods of time, alterations in their proliferation pattern were observed (Fig. 1). Fibroblasts incubated in the presence of 10 μg/ml of chrysotile displayed an initial growth inhibition compared to control cultures during the first 24 hr of exposure. At 48 hr, the control cultures reached stationary phase and displayed low levels of thymidine incorporation, whereas the treated fibroblast population started to grow exponentially as evidenced by cell counts (Fig. 1A). This recovery from growth

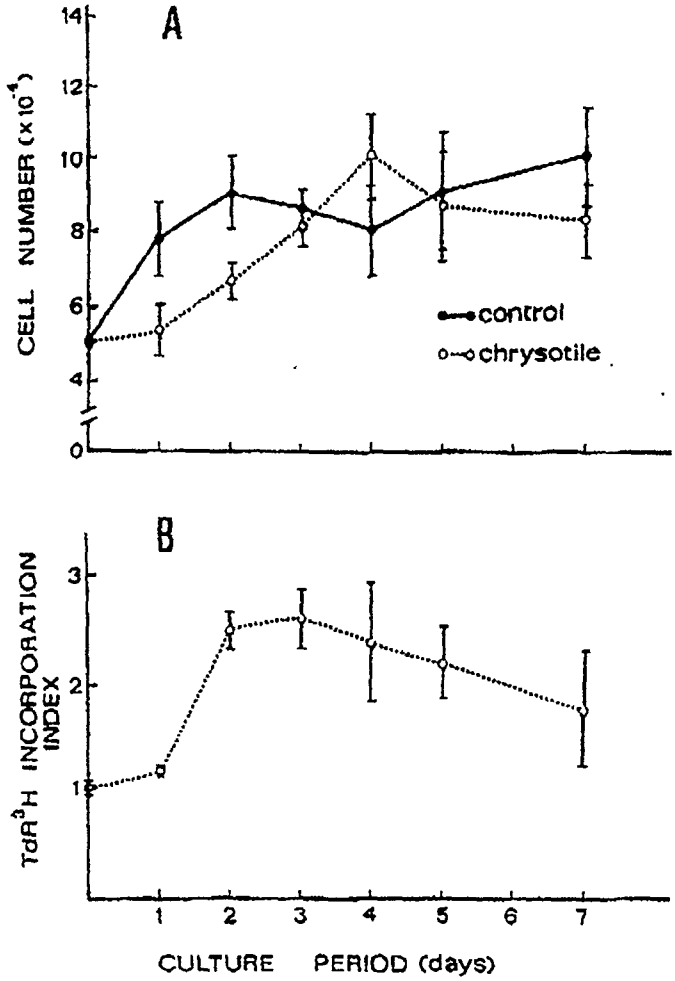


FIG. 1. Effects of chrysotile B asbestos on lung fibroblast growth (A) and DNA synthesis (B). WI-38 fibroblasts (5 × 10⁴) were incubated in the presence or absence of 10 μg/ml UICC chrysotile B for varying periods of time. Each value represents the mean ± SEM of at least two separate determinations of triplicate cultures.

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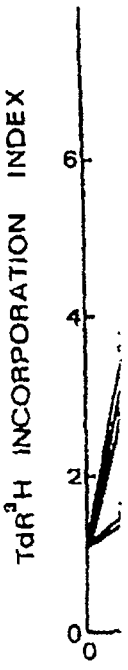


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inhibition and resumption of cell proliferation was more accurately reflected by the elevated TdR³H incorporation index observed at 48 hr in fibroblasts exposed to asbestos (Fig. 1B). Despite the continued presence of asbestos, TdR³H incorporation remained elevated and fibroblasts reached confluency within 4 to 5 days.

Dose–Effect Relationship of Different Types of UICC Asbestos Fibers

In order to further characterize the DNA synthesis response of lung fibroblast to asbestos, varying amounts (0–100 μg/ml) of different UICC standard samples of asbestos were incubated with 5×10^4 fibroblasts for 48 hr. As shown in Fig. 2, elevated TdR³H incorporation was observed in the presence of all types of asbestos in a dose-dependent fashion. Canadian chrysotile B, Rhodesian chrysotile A, and crocidolite were the most active in causing this response, with optimal levels obtained at approximately 25 μg/ml and half-maximal response at approximately 5 μg/ml. Amosite and anthophyllite were also active although to a lesser extent (relative potency: 50 and 33%, respectively). Maximal levels of TdR³H incorporation were reached at low concentrations of asbestos, indicating that the DNA

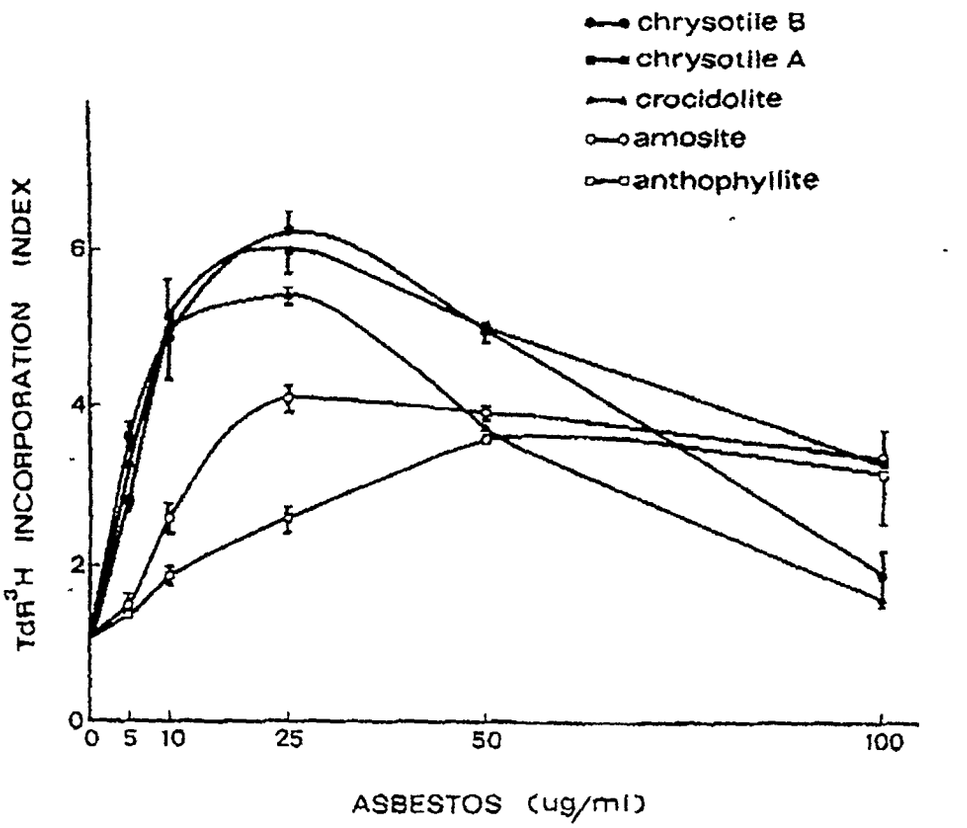


FIG. 2. Dose–effect relationship of different types of UICC asbestos fibers. Varying amounts of asbestos samples were incubated with 5×10^4 fibroblasts for 48 hr. Each value represents the mean ± SEM of at least three separate determinations of triplicate cultures.

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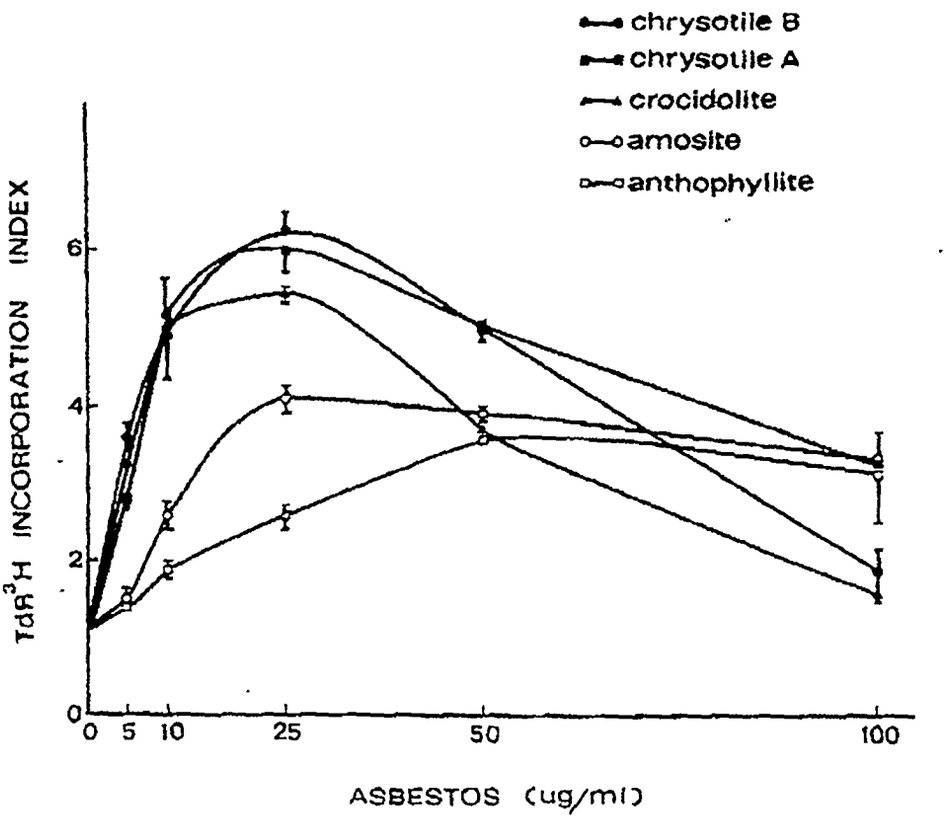


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synthesis response of lung fibroblasts is very sensitive. The order of potency of the various types of asbestos tested was as follows: chrysotile B, chrysotile A, crocidolite > amosite > anthophyllite.

Comparison of the Effects of Various Particles

Pollutant particles vary considerably in their *in vivo* effects. In an attempt to correlate the DNA synthesis response of lung fibroblasts with biological activity of particulates, we investigated the effects of various fibrogenic and nonfibrogenic particles on TdR^{3}H incorporation by fibroblasts. Table 1 shows that chrysotile had the highest activity. Silica was significantly less active with half-maximal response obtained at a dose of 26 μg/ml compared to 5 μg/ml for chrysotile (relative potency: 19%). However, the maximal effects of silica on TdR^{3}H incorporation were identical to those obtained with chrysotile asbestos. Latex, a noninflammatory compound, and titanium dioxide, a nonfibrogenic dust, had no activity. Zymosan, magnetite, and Mt. St. Helens volcanic ash also had no activity on lung fibroblasts.

Effects of Various Treatments of Chrysotile Fibers on TdR^{3}H Incorporation

Table 2 shows that incubation of chrysotile fibers in distilled water for 24 hr did not alter its activity. Furthermore, the leaching fluid had no effect by itself, suggesting that direct interaction of chrysotile with the cells is necessary for the DNA synthesis response of lung fibroblasts.

The chemical or physical surface characteristics of chrysotile are important for its interaction with cell membranes. We verified therefore if various modifications of chrysotile fibers would alter its effects on lung fibroblasts (Table 2). When chrysotile was coated with serum proteins for 24 hr, its maximal activity was

TABLE 1
COMPARISON OF THE EFFECTS OF VARIOUS PARTICLES ON TdR^{3}H INCORPORATION

Particle	ED_{50}[a] (μg/ml)	Relative potency (%)	Maximal incorporation index[b]
Chrysotile B	5	100	6.2
Chrysotile 7D Paperbestos	7	71	6.5
Zymosan		No effect	
Titanium dioxide		No effect	
Magnetite		No effect	
Atapulgite	8	63	3.3
Latex		No effect	
Mt. St. Helens volcanic ash		No effect	
Silica	26	19	7.0

Note. Varying amounts of particulate samples (0–100 μg/ml) were incubated with 5×10^4 fibroblasts for 48 hr. The experiments were repeated three times in triplicate.

[a] Concentration which gives half-maximal response. ED_{50} was evaluated by log probit plots of the data.

[b] Thymidine incorporation index was calculated as follows: cpm of fibroblasts exposed to asbestos/cpm of fibroblasts in medium alone.

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TABLE 2
EFFECTS OF VARIOUS TREATMENTS OF CHRYSOTILE FIBERS ON TdR³H INCORPORATION

Sample	ED_{50}[a] (μg/ml)	Relative potency (%)	Maximal incorporation index[b]
Chrysotile B	5	100	7.8
Chrysotile B washed H_2O	5	100	7.1
Chrysotile B coated with serum	16	31	6.7
Chrysotile 4T30 short (<1 μm)	7	71	7.5
Chrysotile 4T30 short, 325°C	11	45	5.4
Chrysotile 4T30 short, 500°C	24	20	5.6
Chrysotile 4T30 short, Mg-leached	49	10	5.1

Note. Chrysotile B was suspended in distilled water and serum under constant stirring for 24 hr. Preparation of short fibers, heat-treated samples, and Mg-leached samples was as described under Materials and Methods. Varying amounts of asbestos samples were incubated with lung fibroblasts for 48 hr. The experiments were repeated three times in triplicate.

[a] Concentration which gives half-maximal response. ED_{50} was evaluated by log probit plots of the data.

[b] Thymidine incorporation index was calculated as follows: cpm of fibroblasts exposed to asbestos/cpm of fibroblasts in medium alone.

decreased by 15% and its ED_{50} was increased threefold (ED_{50}: 16 μg/ml compared to 5 μg/ml for untreated chrysotile). A very short type (<1 μm) of chrysotile fiber was successful in altering TdR³H incorporation by lung fibroblasts but at a higher concentration than chrysotile of intermediate length (ED_{50}: 7 μg/ml; relative potency: 71%). Dehydroxylation of chrysotile sample by heat treatment at 325 and 500°C reduced its activity by 40 and 70%, respectively. Similarly, magnesium leaching from short chrysotile fibers caused an 85% reduction of its activity. By order of effectiveness, the most potent treatments performed to reduce the reactivity of the short chrysotile fibers were: acid-leached > heat treatment (500°C) > heat treatment (325°C).

DISCUSSION

Pollutant dusts as well as chemically defined particles were tested for their ability to alter fibroblast proliferation. Chrysotile B asbestos caused a growth inhibition during the first 24 hr of incubation followed at 48 hr by elevated thymidine incorporation and resumption of cell proliferation. Elevated thymidine incorporation appeared to be directly related to the effectiveness of chrysotile in inhibiting the growth of lung fibroblasts and may represent a sensitive measure of escape from growth inhibition. Resumption of cell proliferation has been previously noted with a fibroblast culture exposed to a high dose (250 μg/ml) of asbestos for several days (Richards and Jacoby, 1976). The DNA synthesis response of human lung fibroblasts in our study occurred at low concentrations of asbestos (3–10 μg/ml) in a relatively short period of time (48 hr) and was dose related.

All types of asbestos were active in our assay, chrysotile B, chrysotile A, and crocidolite being the most potent. Amosite and anthophyllite were about 50 and 33% as active as chrysotile B. A sample of Quebec commercial-grade chrysotile

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(Paperbestos 7D) was also active on human lung fibroblasts. The order of potency for various types of UICC asbestos samples (chrysotile B, chrysotile A, crocidolite > amosite > anthophyllite) was different from that observed in other *in vitro* assays but seemed to parallel the *in vivo* situation. Chrysotile A and B were reported to be highly toxic for macrophages in cultures, whereas crocidolite and amosite had little activity (Miller and Harington, 1972). Similarly, chrysotile was found to be highly hemolytic on red blood cells, while other forms of asbestos, notably crocidolite and amosite, were relatively inactive (Macnab and Harington, 1967). The ability of asbestos samples to cause DNA synthesis response of lung fibroblasts appears to follow their pattern of *in vivo* activity. Wagner *et al.* (1974) showed that chrysotile and crocidolite were more effective in producing mesothelioma in rats than amosite. These same authors also found amosite to be less fibrogenic than chrysotile in rats. However, there are discrepancies in the degree of fibrosis (Hiett *et al.*, 1978) and mesothelioma (Wagner *et al.*, 1973) induced in experimental animals by various types of asbestos, probably because of the differences in the assay procedures, and comparison of their relative pathogenic potential *in vivo* is difficult. Nevertheless, as summarized by Harington *et al.* (1975), all types of asbestos are fibrogenic in man and can induce mesotheliomas in animals.

Our results also demonstrate that particulates considered nonfibrogenic, including zymosan, latex (Shorlemmer *et al.*, 1977), and titanium dioxide (Christie *et al.*, 1963), or poorly fibrogenic, such as magnetite (Davis, 1972), had no effect in our assay. Mt. St. Helens volcanic ash which did not cause lung injury (Beck *et al.*, 1981) was also inactive. Surprisingly, silica, which is a potent fibrogenic particle (Reiser and Last, 1979), had little activity. As reviewed by Heppleston (1979), there are evident disparities in the cellular response to silica and asbestos and their mechanism of action may be different. In this respect, elaboration of a macrophage fibrogenic factor by silica has been reported (Heppleston and Styles, 1967a), whereas a similar effect has yet to be demonstrated for asbestos. Furthermore, chrysotile was shown to induce collagen formation after direct incubation with rabbit lung fibroblasts while silica had no direct effect (Richards *et al.*, 1971). The importance of the fibrous nature of the dust for stimulation of collagen synthesis (Davis, 1972) has been emphasized and it may be that the fibrous structure of the material, particularly a polyfilamentous structure (Gross and Harley, 1973), is determinant for its activity on lung fibroblasts.

Previous work has led to the concept that longer fibers of respirable length are more fibrogenic than shorter fibers (Harington *et al.*, 1975). Furthermore, the studies of Maroudas *et al.* (1973) suggest that fibers about 20–200 μm in length and 0.5–5 μm in diameter are more carcinogenic than larger or smaller fibers. However, there is disagreement on the minimum size of fiber required for pathogenicity, and very small fibers have been found to be very efficient in causing fibrosis (Holt and Mills, 1963) and mesothelioma (Wagner and Berry, 1969). Our results indicate that very short chrysotile fibers (<1 μm) (Jolicoeur *et al.*, 1981) are successful in altering fibroblast DNA synthesis. This raises the question of whether the effect of asbestos is exerted at the cell surface or within the cell since small fibers are more likely to be ingested.

The mechanisms involved in the DNA synthesis response of lung fibroblasts are

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unknown but our observation that deionized water previously treated with chrysotile for 24 hr did not modify fibroblast proliferation is consistent with an effect of the fibers per se on the cells. Interaction between fibers and cell membrane was reported to be dependent on the physical and chemical characteristics of asbestos in other systems. For example, Leight and Wei (1977) have reported that different forms of asbestos can be activated or inactivated by surface charge alterations, while Morgan *et al.*, (1977a) claimed that the hemolytic activity of asbestos was primarily related to specific surface areas of the fibers. Other experimental evidence indicates that magnesium on the surface of asbestos fibers plays an important role in their action with membranes (Morgan *et al.*, 1977b), and Reiss *et al.*, (1980) have demonstrated that leaching of chrysotile in acid, which results in release of substantial levels of Mg^{2+}, greatly decreased its cytotoxicity. Also, chemical modification of chrysotile has been reported to influence its biological effects. Thus treatment of asbestos with metal tungstate (Pezzoli, 1979) or with heat (500°C) (Dunningan *et al.*, 1980; Sakabe *et al.*, 1971) reduced its hemolytic activity. Our findings that magnesium leaching and heat treatment reduced the effects of chrysotile on fibroblast proliferation suggest that the Mg^{2+} ions on the surface of the fibers are important for their interaction with fibroblasts and that at least part of the effects of asbestos on lung fibroblasts may be associated with some chemical surface characteristics of chrysotile.

In conclusion, nonfibrous particles which are devoid of fibrogenic activity, such as latex and zymosan, were inactive in our assay. Fibrogenic nonfibrous dusts, such as silica, were less active. By contrast fibrous dusts which are both fibrogenic and carcinogenic *in vivo* greatly altered fibroblast proliferation *in vitro*. These results indicate that DNA synthesis is a parameter in fibroblast function which may be related to *in vivo* effects of asbestos. This *in vitro* response of lung fibroblasts is sensitive, rapid, and reproducible, suggesting that measurement of thymidine incorporation by lung fibroblasts may represent a sensitive means of assessing rapidly the biological activity of asbestos. The mechanisms involved in the DNA synthesis response of human lung fibroblasts are unknown but the potential relevance of this activity to fibrogenesis and tumorigenesis warrants further investigation.

ACKNOWLEDGMENTS

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