

Interaction of Amosite and Surface-Modified Amosite with a V79-4 (Chinese Hamster Lung) Cell Line

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We have been examining a number of chemically modified mineral fibers, derived from amosite asbestos, by *in vitro* methods to clarify the role of the fiber surface in determining biological activity. The various fibers have identical size distributions but differ in their affinities for components of the cell membrane. They were treated with boiling toluene or chemically modified by treatment with alkyltrimethylchlorosilanes ($R = C_8, C_{18}$) that react with free-surface hydroxyl groups to form the corresponding siloxanes.

Fibers in MEM supplemented with 15% fetal calf serum were added to a suspension of V79-4 cells labeled with tritiated thymidine and the mixture was incubated. Aliquots of this mixture were spun down on a density gradient to determine the degree of cell-fiber interaction. At 37°C native amosite (UICC standard) stuck to cells within 15 min of incubation, and the amount of sticking was maximum within 70 min. Decreasing the temperature decreased the amount of sticking, and at 20°C no sticking was observable. The chemically modified amosite and the amosite treated with boiling toluene did not stick to the cells even after 70 min. Soaking the toluene-treated amosite with aqueous solutions at room temperature for 48 hr produced a material that had the same sticking properties as the original untreated fiber. These results indicate that the silanol content, and possibly the degree of hydration of the fiber surface, is important for a fiber to stick to a cell surface.

Introduction

The inhalation of mineral fibers can lead to the development of a variety of diseases, particularly in the case of asbestos, pulmonary fibrosis, and neoplasms of the lung parenchyma or mesothelium (1). The full extent of the biochemical and cellular consequences of fiber inhalation remains uncertain; the properties responsible for the pathogenic activity of these otherwise chemically inert materials are unknown. Although there is a great deal of information on the human health effects of exposure to asbestos, the mechanisms of toxicity are undefined. Toxicological studies have shown the importance of fiber type, size, and shape (2), but conclusions concerning the role of these variables are limited by the heterogeneity of the material studied.

Ultimately there must be interactions between the fibers and their biological targets at the molecular level, and it is these interactions that are the least understood. The chemical reactions and physical interactions that occur when fibers meet cells are mostly unknown, in spite of the intensive investigation of the effects of asbestos fibers on cell cultures.

The present investigation extends earlier work (3) with surface-modified amosite asbestos, in which bovine serum albumin glutaraldehyde linked to a silylated surface was shown to reduce the cytotoxic effect of this mineral. Starting from a sample of UICC amosite (4), we have prepared a series of fiber samples by both physical and chemical treatments that were designed to alter the chemical structure of the surface. Using a fibroblast pulmonary cell line, which represents one of the cell types believed to be a potential target for asbestos *in vivo* (5), we studied the sticking of these cells to native and variously treated and chemically modified amosite fibers.

There are several reasons for undertaking these studies. The fibers to which humans are exposed are not necessarily similar to those in the UICC samples. Many uses for asbestos in insulation and friction materials

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involve their exposure to elevated temperatures that might result in some of the types of modification studied in this paper. While the effects of bulk heating on the phase transitions in these minerals have been commented on many times (6), the heating necessary to modify only the surface might be quite different. This could leave morphologically resilient fibers with altered surface chemistries. The health effects of these altered minerals might not be predictable from animal or other studies of native fibers.

Materials and Methods

Fibers

UICC-amosite (4) and samples derived from it were used. Samples of amosite were heated in vacuum (0.05 torr) at 110° or 200°C for 6 hr.

Samples of amosite were boiled under reflux in dry toluene alone or dry toluene containing octyldimethylchlorosilane (C8-silane), octadecyldimethylchlorosilane (C18-silane), or epichlorohydrin (ECH) for 6 hr. The samples were filtered, washed with dry toluene, and dried in a vacuum. Carbon/hydrogen analyses for the chemically modified materials are given in Table 1.

The fiber samples were weighed and autoclaved dry before use (121°C for 15 min in sealed Universal bottles). These fiber samples, including a sample of untreated amosite, were sonicated for a few minutes in an ultrasonic bath after adding dry acetone to the tube. This was to wet and separate the fibers (see "Discussion") and had no effect on the size distribution of the fibers. The medium was then added to give a fiber concentration of 2 mg/mL and an acetone concentration of 10%. Immediately before use the fibers were resuspended by sonication for 2 min and then added to a cell suspension, such that the final concentration of fiber was 200 µg/mL. Untreated amosite in medium without acetone was also examined in the system. To investigate the importance of hydration of the surface, samples of amosite that had been heat-treated, treated with boiling toluene alone, or chemically modified were suspended in medium for 48 hr before use. Changes produced by boiling in toluene, with and without subsequent rehydration, were investigated by differential scanning calorimetry (DSC).

Cells

The V79-4 Chinese hamster lung cell line was obtained from C. Arlett (MRC Cell Mutation Unit, Brighton, UK) and was cultured in minimum essential medium (MEM) supplemented with 15% heat-inactivated fetal calf serum, streptomycin (50 µg/mL), penicillin (50 IU/mL), and sodium bicarbonate (3.6 g/L), at 37°C in an atmosphere of 5% CO₂ in air. The tissue culture medium, serum, and sterile plastic were from Flow Laboratories (Irvine, Scotland, and Gibco Europe, Paisley, Scotland).

Density Gradients

A density gradient based upon a sodium diatrizoate-Ficoll mixture, Histopaque 1119 (Sigma, Poole, Dorset, UK) was designed so that amosite (density 3.4–3.5 g/cm³), alone or in a complex with cells, could move through it while cells were retained in the upper third of the gradient. The gradient medium was diluted with phosphate-buffered saline (PBS) (from 100% to 60% Histopaque in nine even dilution steps), 1 mL of each, with a top layer of 1 mL PBS, total 10 mL.

Method

V79 cells were grown to confluence in medium containing 2 µCi/mL ³H-thymidine. A single cell suspension (7.5 × 10⁵ cells/mL) was prepared from these by trypsinisation, and 10-mL aliquots were transferred to silanized flasks. Aliquots (1 mL) of the fiber samples in medium were added and the mixture incubated at a constant temperature in a shaking water bath. One-milliliter samples were removed at timed intervals, layered onto the density gradients, and spun at 1100g for 5 min. The spread of radioactivity through the gradient was measured by taking sequential 0.5-mL aliquots. These were placed in plastic insert vials, 4 mL liquiscint (National Diagnostic, Manville, NJ) was added, and the mixture was counted in a liquid scintillation counter.

Results

When amosite fibers are shaken with single-cell suspensions of V79 cells in medium containing serum, they may become attached to the plasma membrane of the cells under certain conditions. If such a cell fiber mixture is layered onto a density gradient and centrifuged as above, then the components of the mixture can be separated. Fibers and complexes of cells attached to fibers can move to the bottom of the gradient. Unattached cells will move to approximately one-third of the way through, and any cells lysing will result in counts remaining at the top. It has been found experimentally that in the absence of fiber a density gradient, constructed as described in the method above, does not lyse cells.

At 37°C, native amosite fibers stuck to V79 cells within 15 min, and the sticking was at a maximum within 70 min (Fig. 1). Some time after maximal adhesion, cell lysis occurred and could be detected by counts appearing at the top of the gradient. This was variable in extent but suggests that attachment was a prerequisite for lysis.

Table 1. Carbon/hydrogen microanalyses for chemically treated UICC amosite samples.

Treatment	Percent carbon	Percent hydrogen
None	0.00	0.00
ECH ^a	0.00	0.00
C8-silane	0.58	0.14
C18-silane	0.60	0.18

^aAs can be seen, the epichlorohydrin (ECH) had not reacted significantly with the fiber; the difference between the analyses for the C8- and C18-silane treated samples suggests that surface coverage is not complete for the latter compound.

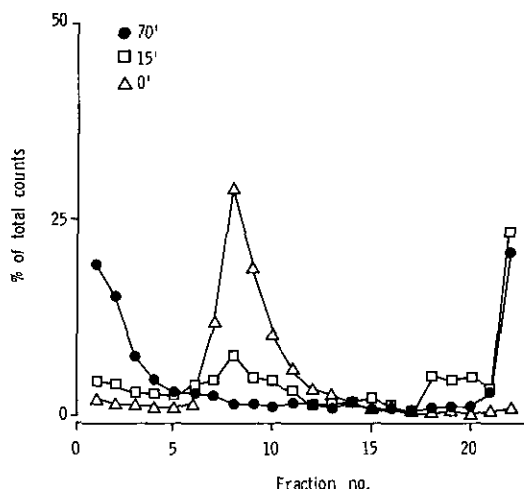


FIGURE 1. Adhesion of UICC amosite to V79-4 cells. Samples of cell-fiber mixture were layered onto density gradients at the times shown after incubation at 37°C. Most of the cells had attached to fibers at 15 min, and this resulted in counts appearing in fractions 21 and 22. At 70 min considerable lysis had occurred, and over 20% of the counts were in the first few fractions; the degree of lysis occurring at times over 1 hr was variable.

Some preliminary studies have been carried out on the conditions necessary for optimal fiber-cell interaction. The sticking of fibers to cells was not affected by the trypsinization procedure necessary to produce a cell suspension from a confluent cell layer. Cells allowed to recover from this procedure had identical sticking characteristics to cell suspensions used immediately. The presence of EDTA completely abolished fiber-cell adhesion, and similarly when the incubation temperature of the cell-fiber mixture was decreased, fewer cells attached to fibers; at 20°C the sticking of cells to fibers did not occur (Fig. 2).

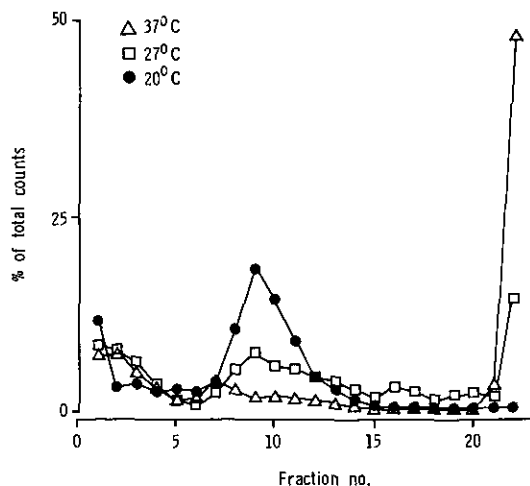


FIGURE 2. Adhesion of UICC-amosite to V79-4 cells at various temperatures after 70 min. Method as in Figure 1. Attachment was reduced at 27°C and abolished at 20°C.

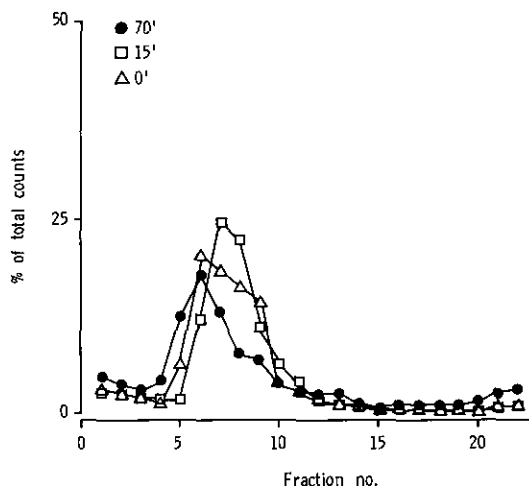


FIGURE 3. Adhesion of toluene-treated amosite to V79-4 cells. Method as in Figure 1. No significant adhesion or cell lysis occurred.

Modification of the surface of amosite drastically decreased its ability to stick to fibers. Heating under boiling toluene removed completely the ability of the fibers to stick to the cells (Fig. 3), as did heating to 200°C in a vacuum, while similarly heating to 110°C (the boiling point of toluene) did not. The wettability of the fiber by the medium was unaffected by this treatment. In contrast, when the chemically modified amosite fibers were examined they could not be wetted by medium prior to being added to the cell suspension. They could be disaggregated by sonicating in acetone before adding medium. This procedure produced a satisfactory fiber suspension at a concentration of 10% acetone, without visibly denaturing the serum proteins. The presence of 1% acetone in the final cell-fiber mixture was shown not to affect the binding behavior of amosite and heat-treated amosite to V79 cells. The behavior of amosite in medium alone and amosite sonicated in acetone before adding to medium were identical.

While at 37°C, neither the chemically modified samples nor the toluene-treated amosite stuck to V79 cells. When the toluene-treated amosite was soaked in medium for 48 hr before adding to the cell suspension, the sticking properties were restored. The same result was obtained whether or not the soaking medium contained serum. Examination by DSC showed that the bulk physical properties of the toluene-treated material and native amosite were different and that soaking the former in water for 30 hr produced a material with nearly identical properties to the original UICC amosite.

Discussion

Amosite asbestos is an amphibole mineral having a structure with a double chain of silica tetrahedra, cross-linked with bridging cations that are predominately Fe^{2+} with some Mg^{2+} and smaller variable amounts of

Fe³⁺ and other cations (6). The silica chains terminate in free silanol groups and these confer a hydrophilic surface to this mineral. They are responsible for the normal acidic surface of the mineral, and those at the surface of a fiber can undergo chemical reactions. With trialkylchlorosilanes they react to form a surface of the corresponding trialkylsilyl groups, bonded through siloxane linkages to the fiber.

Epichlorohydrin was expected to react to give a mixture of primary and secondary hydroxyethyloxy groups on the fiber surface as it reacts in this way under the experimental conditions above, with glass fiber (unpublished results). However, the carbon/hydrogen analyses show that little or no reaction occurred.

In order for a fiber to interact with a cell surface, it must be dispersed in the cell suspension medium. This is not possible for a material which is, as a result of its surface, equivalent to bulk hydrocarbon. Hydrophobic materials need some other agent, a detergent or water-soluble organic solvent, for dispersal in an aqueous medium. For our modified amosite samples, the problem of wetting was overcome by adding medium to an acetone suspension of the fibers. Fibers treated in this way remained in suspension without clumping and could be used in the experiments.

Conditions for Adhesion

When the incubation temperature of a fiber-cell suspension mixture was reduced below 37°C the number of cells stuck to fibers was reduced (Fig. 2). This effect suggests that the sticking might be an active process related to the cell attachment to other surfaces. Alternatively, Lackowicz and Hylden (7) have shown that liposome-fiber interactions are dependent on the melting point of the lipid involved. Thus, these results might be because of the reduction in the formation of attachment sites or to a phase change in the cell membrane. The chemical and physical conditions required for the fiber-cell interaction are being investigated further.

Chemical Treatment

Neither C8-silyl-amosite nor C18-silyl-amosite stuck to V79 cells. The fibers produced no lysis, unlike that found in experiments with UICC amosite. They were, so far as the cells were concerned, inert.

Physical Treatment

Amosite was subjected to a variety of physical treatments, often incidental to intended chemical treatment or as part of an experiment. The suspension of native amosite fibers by sonicating in acetone could be expected to remove water adsorbed onto the surface of the fibers. This treatment would not have any effect on surface silanol groups, and any modification that is effected by the acetone treatment would be expected to be transient and readily reversible. No effect of this treat-

ment was observed. The more drastic dehydration produced by boiling in toluene at 110°C, however, does have an effect, whereby water can be removed as an azeotrope. Material so treated does not stick to V79 cells within the time-course of the experiment. To achieve the same effect by simply heating UICC amosite in a vacuum requires temperatures well in excess of the boiling point of toluene to significantly alter its properties. If the toluene or 200°C-treated materials are allowed to rehydrate by soaking in medium for 30 to 48 hr, then their sticking properties are restored to those of native amosite. It seems probable that the dehydration/rehydration phenomena observed are purely a surface effect. Differential thermal analysis of crocidolite, for example, shows that loss of water occurs at around 420°C (6). This is presumably the loss of water of hydration from the linking cations between the silicate chains, rather than the loss of surface water or condensation of silanol groups. In the simpler structure of pure silica, it has been shown (8) that rehydration of this material after heating is essentially complete within 5 hr. Preliminary studies with DSC show that the material produced by boiling amosite in toluene has different thermal properties to those of the untreated UICC amosite and that upon rehydration in water for 30 hr, the properties of the original material are restored. Treatment with toluene might not only dehydrate the fiber surface, but it could also produce internal condensations through conversion of *ortho*- to *meta*-silicate structures.

Our experiments support a hypothesis that free silanol groups must be present at an amosite fiber surface for the interaction with a cell membrane to occur. If the carcinogenic effects of mineral fibers are related to their inappropriate stimulation of intracellular events, commonly triggered by the activation of surface receptors (9), then a study of fiber-cell interactions might reveal the mechanisms involved in this stimulation.

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REFERENCES

1. Wagner, J. C., Berry, G., and Pooley, F. D. Carcinogenesis and mineral fibres. *Br. Med. Bull.* 36: 53-56 (1980).
2. Asbestos and Other Natural Mineral Fibers. *Environmental Health Criteria* 53, WHO, Geneva, 1986.
3. Evans, P. H., Brown, R. C., and Poole, A. Modification of the *in vitro* activities of amosite asbestos by surface derivatization. *J. Toxicol. Environ. Health* 11: 535-543 (1983).
4. Timbrell, V., and Rendall, R. E. G. Preparation of the UICC standard reference samples of asbestos. *Powder Technol.* 5: 279-287 (1972).
5. Allison, A. C. Experimental methods of cell and tissue culture: effects of asbestos particles on macrophages, mesothelial cells, and fibroblasts. In: *Proceedings of a Working Conference*, October 1972. IARC Pub. No. 8 (P. Bogovski, J. C. Gilson, V. Timbrell, and J. C. Wagner, Eds.), International Agency for Research on Cancer, Lyon, France, 1973, pp. 89-93.

6. Speil, S., and Leineweber, J. P. Asbestos minerals in modern technology. *Environ. Res.* 2: 166–208 (1969).
7. Lackowicz, J. R., and Hylden, J. L. Asbestos mediated membrane uptake of benzo(a)pyrene observed by fluorescence spectroscopy. *Nature* 275: 446–448 (1978).
8. Gobet, J., and Kovats, E.sz. Preparation of hydrated silicon dioxide for reproducible chemisorption experiments. *Adsorp. Sci. Technol.* 1: 77–92 (1984).
9. Marsh, J. P., and Mossman, B. T. Mechanisms of induction of ornithine decarboxylase activity in tracheal epithelial cells by asbestiform minerals. *Cancer Res.* 48: 709–714 (1988).