In Vivo Evaluation of Chemical Biopersistence of Man-made Mineral Fibers

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Techniques developed at the Harwell Laboratory for the determination of the biopersistence of man-made mineral fibers (MMMF) in vivo are described. Results obtained with samples of glass fiber with a range of compositions, and with a sample of rockwool, are summarized. With glass fibers the rate of dissolution of fibers in vivo depends not only on their chemical composition, but also on their length. Certainly, for all fibers exceeding 10 μm in length, the longer the fiber the more rapidly it dissolves. This effect is attributed to differences in the microenvironments to which long and short fibers are exposed. Although this phenomenon appears to operate with all glass fibers, it may not apply to other types of MMMF that dissolve more readily in environments with low pH. Finally, the article examines the validity of the intratraheal method of administration for studying the biopersistence of MMMF in vivo and the use of the rat for this purpose. — Environ Health Perspect 102(Suppl 5):127–131 (1994)

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Introduction

It is well established that exposure to airborne asbestos fibers can result in a variety of lung diseases including fibrosis (asbestososis), bronchial carcinoma and pleural mesothelioma. In recent years there has been concern that exposure to asbestos substitutes should not result in the same diseases and, in 1988, the International Agency for Research on Cancer (IARC) reviewed the available evidence on the toxicity of man-made mineral fibers (MMMF) and evaluated the carcinogenicity of the various types of fiber (1).

In the case of amphibole asbestos fibers, the principal determinant of carcinogenic potency appears to be fiber dimensions, although variations in surface properties cannot be excluded. In general, it is believed that fibers less than 0.2 μm in diameter and longer than 10 μm are most likely to induce tumors. The persistence of fibers deposited in the respiratory tract is another important determinant of toxicity, and fibers that dissolve rapidly in the lung are unlikely to cause any of the effects that result from exposure to asbestos. The fibrous amphiboles appear to be insoluble in lung tissue over the normal human lifespan, so for practical purposes their biopersistence is infinite. Chrysotile fibers on the other hand are subject to leaching of structural magnesium in vivo (2); this results in a change to their surface properties and, at least in studies with experimental animals, alterations to their carcinogenicity (3,4).

With MMMF, two approaches can be used to minimize carcinogenic potential. First, the fibers can be made relatively thick, so that they are essentially nonrespirable. However, because the diameters of commercial MMMF generally cover a considerable range, it is not always feasible to ensure that all the fibers in a product are totally nonrespirable. Second, the fibers’ composition can be designed so that they dissolve at a finite rate in the lung; this approach is being investigated by a number of manufacturers.

The solubility of asbestos and MMMF in vitro and in vivo has been reviewed (5). A number of investigations of fiber solubility have been conducted in vitro under physiological conditions thought to resemble those in the lung (6–8). These studies have demonstrated that the chemical composition of glass fibers has a strong influence on their dissolution rates. However, as it is known that microenvironments in the lung may vary considerably with respect to pH and to other factors that influence solubility, it is important that the results of such studies be validated by measurements of the dissolution rates of the same fibers in vivo. This article summarizes measurements of the biopersistence of MMMF in vivo carried out at the Harwell Laboratory in recent years (9,10) and justifies the methods used.

Materials and Methods

Chemical Composition and Size of MMMF Used in In Vivo Studies

The composition of the sized glass fibers used in the first investigation is shown in Table 1. The material from which they were made has the code JM-753. The fibers were prepared as single filaments with a nominal diameter of 1.5 μm. Bundles of fibers were embedded in an acrylic block with methacrylate resin and the block was cut with a microlathe to give sections of 5, 10, 30 and 60 μm. The acrylic resin was then dissolved with methyl ethyl ketone and residual contaminants removed by low-temperature ashing to give fibers with these nominal lengths.

The compositions of the experimental glass fibers (X7753 and X7779) used in the second investigation are included in Table 1. They represent the extremes of dissolution rates of a range of glass fibers measured in vitro (8). These samples were prepared from a single filament with diameter about 2 μm. Bundles of fiber were embedded in resin and 50 μm slices were cut with a rotary end mill. The fiber diameter was unaffected by this treatment and the count median lengths of the products

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Table 1. Composition of MMMF used in studies of biopersistence in vivo.\textsuperscript{a}

| Oxide    | Sized glass JM-753 | Experimental glass fibers X7753 | X7779 | Rockwool |
|----------|---------------------|---------------------------------|-------|----------|
| SiO\textsubscript{2} | 63.2                | 65.7                            | 60.1  | 45.5     |
| Al\textsubscript{2}O\textsubscript{3} | 5.5                 | 0.5                             | 8.1   | 13.1     |
| B\textsubscript{2}O\textsubscript{3} | 5.4                 | 8.0                             | 1.8   |          |
| CaO      | 6.0                 | 5.7                             | 9.2   | 14.9     |
| MgO      | 3.1                 | 3.0                             | 4.3   | 10.8     |
| Na\textsubscript{2}O | 14.8               | 15.8                            | 15.3  | 2.4      |
| K\textsubscript{2}O | 1.1                 | 0.8                             | 0.8   | 1.0      |
| FeO      | —                   | —                               | 8.0   |          |
| Fe\textsubscript{2}O\textsubscript{3} | 0.1                | 0.3                             | 0.3   | —        |
| TiO\textsubscript{2} | —                   | 0.1                             | 0.1   | 1.7      |

\textsuperscript{a}(% by mass)

Table 2. Strains of rodents used and the concentrations and amounts of fiber suspensions administered.

| Material   | Rodent | Strain | Fiber suspension | Concentration, mg/ml | Volume, ml |
|------------|--------|--------|------------------|----------------------|------------|
| Sized glass Fiber | Rat    | Wistar | 2.0              | 0.5                  |
| X7753      | Hamster| Syrian | 2.0              | 0.5                  |
| X7779      | Rat    | Fischer 344 | 4.0 | 0.5         |
| Rockwool   | Rat    | Wistar | 1.0              | 0.5                  |
|            | Hamster| Syrian | 1.0              | 0.5                  |

were in the range 15–20 \(\mu\)m, although a small fraction of fibers exceeded 50 \(\mu\)m in length.

Table 1 also presents the composition of the rockwool fibers used. Unlike the samples of glass fiber, the diameter of the product was not uniform and ranged from approximately 0.3 to 5 \(\mu\)m: the count median diameter was approximately 1.1 \(\mu\)m and the count median length approximately 27 \(\mu\)m.

Administration of MMMF

All the samples of MMMF used in these investigations were administered to adult rats or hamsters by intratracheal instillation of a suspension of fibers in physiological saline. The strains of animals used, and the concentrations and volumes of suspensions instilled, are given in Table 2. With rats, either 1 or 2 mg of fiber was administered under light Halothane anesthesia, via a cannula inserted into the trachea through the mouth. A similar technique was used with hamsters except that smaller volumes of suspension were administered.

Recovery of Fibers from Lung

Animals were anesthetized with Halothane and killed by severing the brachial artery. The lungs, excluding the trachea and external airways, were excised and placed in polypropylene centrifuge tubes with 50 ml of commercial bleach at 4°C. The tubes were rotated on a roller unit in a cold room; under these conditions, the lungs were completely digested in about 4 h. Aliquots of the digest were filtered through 2.5 cm diameter mixed cellulose ester filters (Millipore HA; pore size 0.45 \(\mu\)m) which were washed well with distilled water and dried. All reagents were pre-filtered before use.

Characterization of Fibers

For examination by phase-contrast optical microscopy (PCOME), the Millipore filters were cleared using the technique of Le Guen and Galvin (11). With the materials used in these investigations, all the fibers were thick enough to be detected by PCOME at an overall magnification of either \(\times500\) or \(\times1000\). In the earlier studies fiber dimensions were measured manually, but in the more recent investigations (12) an image analysis system (Magiscan, Joyce Loeb, Gateshead) was used. With the latter, 30 randomly-selected fields were scanned on each cleared filter at a magnification of \(\times500\) using a pointset size of either \(140 \times 140 \mu\)m or \(170 \times 170 \mu\)m superimposed on each field. The number of fibers within each pointset was scored manually using the modified counting rules described by the World Health Organization (WHO) (13) and by the National Institute of Occupational Health and Safety (NIOSH) (14). Fiber lengths and diameters were measured using the same optics. With the experimental glass fibers, energy dispersive X-ray analysis (EDX) measurements of fiber composition were made at the Owens-Corning Technical Center, Granville, OH, using a Kevek Delta Class V analyzer, interfaced to a quantum light element X-ray detector.

Results

Sized Glass Fibers (JM-753)

A detailed account of the results of this study has been reported elsewhere (9). In brief, they showed that the 1.5 \(\mu\)m diameter fibers with lengths of 5 and 10 \(\mu\)m were cleared mechanically from the lung and dissolved rather slowly and uniformly over 18 months. In contrast, fibers of the same diameter, but with lengths of 30 and 60 \(\mu\)m, dissolved quite rapidly and in rather an irregular manner. Some of these long fibers dissolved more rapidly in the middle, ultimately giving rise to fragments, as observed by other researchers (15); other fibers dissolved more rapidly at the ends (Figure 1B).

Experimental Glass Fibers (X7753 and X7779)

A detailed account of the results of this study has been reported elsewhere (12). It

![Figure 1](image-url)

Figure 1. Phase contrast optical micrographs of 1.5 \(\times\) 80 \(\mu\)m sized glass fibers. (A) as administered; (B) after 18 months in rat lung. Bar equals 60 \(\mu\)m. (Published with permission Annals of Occupational Hygiene).
was found that the long fibers of the X7753 material, which dissolved more rapidly in vivo, also dissolved quite rapidly in vitro. Fibers longer than 30 μm had all dissolved completely after two months and, after one year, virtually all the fibers remaining in the lung were <15 μm in length. Analysis of the length distribution of fibers in the <15 μm category showed that fibers in the 10–15 μm length range dissolved more rapidly than those in the 5–10 μm range but more slowly than longer fibers. Figure 2 is a scanning electron microscopy (SEM) photomicrograph of fibers of this material recovered after 28 days in lung. This illustrates the fact that some fibers had already dissolved substantially by this time but that the diameter of the shorter fibers was comparatively unaffected. With the more durable X7779 fiber, there was no significant change in either length or diameter after more than one year in lung. Estimates of the total numbers of fibers remaining in the lungs at various times after administration show that the numbers of the more durable fibers stayed relatively constant (Figure 3). The numbers of the less durable material declined rapidly however, and after one year, less than 10% of those administered remained in the lung.

**Rockwool Fibers**

A detailed account of this study has been reported elsewhere (10). Because the lengths and diameters of the rockwool fibers used covered such a wide range, the count median lengths (CMLs) and diameters (CMDs) of fibers recovered from the lungs at various times were measured by PCOM. There was no apparent change in these parameters for fibers recovered up to 18 months after administration. However, by that time, some fibers were getting thinner at the ends than in the middle. Because the diameters of fibers were measured at their centers, this change was not apparent from the measurements of CMDs.

**Discussion**

These studies show that for glass fibers, the rates of dissolution in vivo vary considerably with the chemical composition of the material. Although the sized glass fibers had a different nominal diameter than the experimental fibers, their dissolution rate in vivo was greater than that of the X7779 and less than that of the X7753 material. This indicates that the aluminium content of glass fibers is a major determinant of their solubility in vivo.

The in vivo solubilities of the experimental glass fibers appear to follow the same ordering as that observed in vitro. The results also show that, for the less durable glass fibers, the longer the fiber the more rapidly it dissolves. For such fibers, there does not appear to be any length below which they are insoluble in the lung, but those that are less than 10–15 μm in length dissolve much more slowly than longer fibers. This tends to confirm the hypothesis that fibers that are short enough to be contained within an alveolar macrophage (diameter about 12 μm) are exposed to a different, more acid, microenvironment than longer fibers. In our investigations, macrophages recovered by bronchoalveolar lavage at more than one year after administration of the more durable fiber, contained many relatively short fibers up to 20 μm in length. It appears (Figures 1, 2) that the rate of dissolution of longer, less durable, glass fibers is quite variable, which may be due to different microenvironments. For example, fibers that penetrate into the interstitium may be exposed to less acid conditions than those that remain in the alveolar spaces where parts of the fiber surface may be within macrophages for at least some of the time.

In the case of rockwool, the fibers appeared to be very durable in the lung and there was no apparent effect of length on durability.

These results broadly agree with investigations by Bellmann et al. (15), who administered a range of asbestos and MMMF to Wistar rats by intratracheal instillation. The fibers were recovered byashing the lungs at low temperature, a procedure thought to result in breakage of the longer and more brittle fibers due to the rapid shrinkage of the tissue (16). The only material common to that investigation and the current one is the JM-753 glass fiber, which, according to Bellmann et al., has an intermediate solubility in vivo. This is in agreement with the findings reported here.

**Morphological Changes**

In my studies with experimental glass fibers, those of less durable material were analyzed by EDAX. Three types of morphology were observed. Generally, long fibers were highly leached and consisted of thin siliceous cores, most of the Na, Mg and Ca having been removed. Some of the aluminum appeared to be retained, however, when all the other cations had been lost. The second type of fiber was less degraded and consisted of a leached gel layer on an otherwise intact core. Finally, fibers were also present that did not appear to have been attacked significantly and that had diameters similar to those of the administered fibers. The chemical composition of some of the degraded fibers was similar to that reported for fibers recovered post mortem from the lungs of glass fiber production workers (17).

**Comparison of in Vivo and in Vitro Studies**

Because of the effect of fiber length on solubility, it is difficult to compare in vitro dissolution rates with those observed in vivo. However, it appears that the ordering of glass fiber solubility measured in vitro is the same as that determined in vivo, but for the less durable fibers, a wide range of dissolution rates can be obtained in vivo, depending on the fiber length selected. Although the acid milieu of the alveolar macrophage appears to increase the durability of short glass fibers, the opposite may be true for fibers made of materials whose low pH favors dissolution. Clearly, much more work is required to assess the relevance of in vitro determinations of bioper-
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Figure 4. Showing (A), an alveolar macrophage in metaphase containing a glass fiber and (B), a binucleate macrophage containing a fiber. Cells recovered from rat lung by bronchoalveolar lavage one year after administration. Bar equals 20 μm.

sistence and how best they can be related to dissolution rates measured in vivo.

Role of Macrophages

Another advantage of the in vivo method is that information on the biological effects of the administered fibers can be obtained at little additional cost. For example, bronchoalveolar lavage can be used to recover a sample of free cells from the alveolar spaces.

Differential measurements of such cell preparations can give information on the inflammatory response of the lung to the administered fibers. Inflammatory mediators also can be determined in the cell-free supernatant obtained by centrifuging lavage fluid. Genotoxic effects of fibers on the alveolar macrophage population also can be investigated. Macrophages containing glass fibers are still able to progress into the metaphase stage of the cell cycle (Figure 4A), but many more binucleate macrophages recovered by lavage contain fibers than do normal macrophages (Figure 4B). This suggests that intracellular fibers interfere with cytokinesis. It has been proposed that the physical interaction of asbestos fibers with the chromosomes or structural proteins of the spindle apparatus may cause mis-segregation of chromosomes during mitosis, resulting in aneuploidy (18). Thus, the incidence of nuclear aberrations in macrophages may provide a useful index of the genotoxicity of fibers.

Binucleate macrophages have a phagocytic capacity similar to that of normal macrophages (19). If the mobility of these aberrant cells is similar to that of smaller, normal macrophages, this may explain the presence of relatively long (20–30 μm) glass fibers, which can be recovered by tracheal lavage for at least several months after their administration to rats by intratracheal instillation (unpublished data).

Validity of Intratracheal Instillation Techniques

Several aspects of the techniques developed at the Harwell Laboratory for determining the biopersistence of MMMF in vivo should be considered, starting with the validity of the intratracheal instillation technique for administration of fibers. Most of the fibers used in the investigations described here are essentially nonrespirable in the rat so that they could not have been administered by inhalation. A study of the distribution of nonfibrous radioactive particles in the rat lung following their administration in a single intratracheal instillation showed that the particles were not distributed uniformly throughout the lung parenchyma, as would have been the case following inhalation. Rather, they were concentrated in the central region of each lobe with little or no penetration to the periphery (20). The delivery of particles to the alveolar region of the lung by instillation is incomplete; generally about 20% of the administered material is deposited in the conducting airways of the lung and cleared rather rapidly to the GI-tract. However, using radioactive tracer techniques, one can estimate the number of fibers initially retained in the alveolar region of the rat lung (21). MMMF containing sodium can be activated in a nuclear reactor before administration inducing 24Na (half life, 15 h). Two days after instillation of the fiber, the residual radioactivity can be measured in vivo by gamma counting, which enables the number of fibers retained in the alveolar region of the lung to be estimated for comparison with the number recovered at later times. Only short irradiation times are required for this purpose and no significant differences were found between the dissolution rates of irradiated and unirradiated glass fibers in vitro (JA Davis, personal communication).

The inhalation route may be preferred for the administration of MMMF, provided the fibers to be studied are respirable. However, the fate of fibers administered by instillation is similar to that of fibers administered by inhalation, except that the distribution in the lung parenchyma is not as uniform.

Choice of Experimental Animal

A recent study showed (22) that there are interspecies differences in the rates at which nonfibrous particles dissolve in the lung, and also in the ability of fibers to become coated in the lung. This raises the question of the suitability of the rat for studies of the biopersistence of MMMF. For example, in the hamster and guinea pig, asbestos fibers form golden-yellow bodies not dissimilar to those observed in human lung; in the rat lung, however, very few fibers become coated and the coating is white rather than yellow. If the coating of MMMF prevents their dissolution, then measurements of biopersistence in rodents other than the rat may be more relevant to the situation in man. The formation of pseudoasbestos bodies on sized glass fibers following their administration to hamsters by intratracheal instillation has been investigated (23). The time of appearance of coated glass fibers was similar to that observed with fibers of anthophyllite asbestos (24). However, the proportion of glass fibers that were coated appeared to increase over 3–4 months and then to decline. The time of onset of body formation decreased with increasing fiber length and fibers <10 μm in length did not become coated. Measurements of fiber diameter showed that the glass fibers, particularly the longer ones, dissolved rather more rapidly in hamster than rat lung. Thus, it does not appear, therefore, that the fiber coating phenomenon prevented the dissolution of glass fibers in the lung.

Conclusion

The techniques developed at the Harwell Laboratory are the most appropriate yet devised for studying the biopersistence of MMMF. The advantages may be summarized as follows: The method may be applied to fibers that are respirable in the rat lung, to those that have limited respirability, and to those that are nonrespirable. The number of fibers retained in the lungs of individual rats following their administration by intratracheal instillation
can be estimated. These values can be compared with the number remaining in the lung at sacrifice, providing useful information on the mechanical clearance of short fibers and the total removal of long fibers by dissolution. Because fibers administered by this route all have had the same residence time in the lung, it is much easier to determine the temporal effects on fiber dissolution than with the inhalation route. Fibers recovered from the lung can readily be prepared for sizing by PCOM, SEM, or TEM, or for chemical analysis by EDXA. Information on the inflammatory response to different fibers in vivo can be obtained by analysis of the cell types recovered by bronchoalveolar lavage and the determination of inflammatory mediators in lavage fluid. Analysis of nuclear aberrations in macrophages recovered by bronchoalveolar lavage can be used to compare the interaction of different types of fiber with the chromosomes and spindle mechanism of cells at mitosis.

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