Biopersistence of Inhaled Organic and Inorganic Fibers in the Lungs of Rats

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Fiber dimension and durability are recognized as important features in influencing the development of pulmonary carcinogenic and fibrogenic effects. Using a short-term inhalation bioassay, we have studied pulmonary deposition and clearance patterns and evaluated and compared the pulmonary toxicity of two previously tested reference materials, an inhaled organic fiber, Kevlar para-aramid fibers, and an inorganic fiber, wollastonite. Rats were exposed for 5 days to aerosols of Kevlar fibrils (900–1344 f/cc; 9–11 mg/m³) or wollastonite fibers (800 f/cc; 115 mg/m³). The lungs of exposed rats were digested to quantify dose, fiber dimensional changes over time, and clearance kinetics. The results showed that inhaled wollastonite fibers were cleared rapidly with a retention half-time of <1 week. Mean fiber lengths decreased from 11 µm to 6 µm over a 1-month period, and fiber diameters increased from 0.5 µm to 1.0 µm in the same time. Fiber clearance studies with Kevlar showed a transient increase in the numbers of retained fibrils at 1 week postexposure, with rapid clearance of fibers thereafter, and retention half-time of 30 days. A progressive decrease in the mean lengths from 12.5 µm to 7.5 µm and mean diameters from 0.33 µm to 0.23 µm was recorded 6 months after exposure to inhaled Kevlar fibrils. The percentages of fibers >15 µm in length decreased from 30% immediately after exposure to 5% after 6 months; the percentages of fibers in the 4 to 7 µm range increased from 25 to 56% in the same period. These data suggest that both inhaled Kevlar and wollastonite fibers have low durability in the lungs of exposed rats, and this may be responsible for the measured differences in toxicity between Kevlar and wollastonite on the one hand, and durable dusts such as silica or crocidolite asbestos fibers on the other.

Key words: wollastonite, Kevlar, biopersistence, durability, rat lung, lavage, BrdU labeling

Introduction

Fiber dimensional characteristics and biopersistence are two of the most important factors in the development of fiber-induced lung disease. Several studies have shown that long, thin, durable fibers are more toxic in vitro and in vivo than short, thin fibers (1). A Syrian hamster embryo (SHE) in vitro cell system was used to compare normal glass fibers measuring 15 µm with a similar preparation of milled fibers, 2 µm in length (2). The longer glass fibers were cytotoxic to SHE cells and increased the transforming frequency, but the effects disappeared after milling. In contrast, both long and short crocidolite asbestos fibers were toxic to macrophages in vitro via an oxidant and iron-dependent mechanism (3).

In vivo studies have also demonstrated a dependence upon fiber length to induce pulmonary pathological effects. When long and short sample preparations of either fiberglass or asbestos were instilled into the lungs of guinea pigs, long fibers of either type produced severe pulmonary fibrosis, while the shorter sample preparations produced no significant effects (4). In a series of experiments using inhalation exposures, rats were exposed for 1 year to aerosols of specially prepared “short” amosite or chrysotile asbestos fibers <5 µm in length, and the pulmonary fiber-induced effects were compared with preparations of long amosite or chrysotile asbestos fibers, >20 µm long, at similar gravimetric concentrations. One-third of rats exposed to the “long” amosite fibers developed pulmonary tumors or mesotheliomas, and virtually all animals had pulmonary fibrosis. In contrast, the shorter fiber-types produced no significant pulmonary effects (5). Similar results were reported in the chrysotile study. Following a 1-year inhalation exposure, the long-fiber chrysotile produced a 3-fold increase in the numbers of pulmonary tumors and six times more advanced interstitial fibrosis, compared to the effects produced by the shorter fibers (6). In contrast to these results, cytotoxic effects were observed following repeated injections of short crocidolite fibers into the peritoneal cavities of mice, when the clearance of these fiber-types was prevented (3).

Fiber clearance studies following short-term exposures either to inhaled chrysotile or crocidolite asbestos fibers in rats, have been reported, in which fibers were recovered from digested lung tissue and analyzed for dimensional changes at several postexposure times. Following exposure to crocidolite, there was a progressive increase in mean fiber length over time postexposure, but no significant change in the mean diameter of fibers retained in the lung (7). In the chrysotile-exposed rats, there was a similar progressive increase in mean fiber length but a significant reduction in mean fiber diameter (8). It appeared that the longer fibers of chrysotile and crocidolite were selectively retained in the lungs of exposed rats, whereas longitudinal splitting, with a corresponding decrease in mean fiber diameter, occurred only with the chrysotile asbestos fibers. These results have been supported by the results of a 2-year intratracheal instillation/fiber clearance study, wherein the lung clearance of short crocidolite fibers was slow and the numbers of crocidolite fibers longer than 5 µm did not decrease over a period of one year. In contrast, the numbers of retained
Table 1. Kevar and wollastonite fiber exposure data and inhalated dose.

| Fiber type | Duration of exposure, days | MMAD (ug), um | Mean gravimetric concentration, mg/m³ | Mean fiber dose, /h | Number of retained fibers, /g SLT | Count median length, μm | Count median diameter, μm |
|------------|-----------------------------|---------------|--------------------------------------|---------------------|-----------------------------------|-------------------------|-------------------------|
| Wollastonite | 5                           | 4.3 (2.2)     | 59                                   | 123                 | ND                                | ND                      | ND                      |
|            | 5                           | 2.6 (2.0)     | 114                                  | 835                 | 1.3 x 10³                          | ND                      | ND                      |
| Kevar      | Experiment 1⁶              | 5             | 4.5 (2.7)                            | 4.4                 | 1073                              | 3.16 x 10⁶              | 9.9                     | 0.3                     |
|            | 5                           | 3.4 (2.7)     | 8.5                                  | 1344                | 3.5 x 10⁶                          | 9.9                     | 0.3                     |
|            | Experiment 2⁷              | 5             | 3.2 (2.9)                            | 2.9                 | 613                               | 1.4 x 10⁶              | 10.0                    | 0.3                     |
|            | 5                           | 4.7 (3.2)     | 11.1                                 | 877                 | 1.3 x 10⁶                          | 10.0                    | 0.3                     |

Abbreviations: MMAD, mass median aerodynamic diameter; 8g, standard geometric deviation; ND, not determined. ⁶ Number of retained fibers/gram dry lung tissue. ⁷ Used for BAL, cell-labeling and fiber clearance/retention studies.

chrysotile fibers >5 μm in length increased continuously over a 2-year period, due primarily to longitudinal splitting of the fibers. The current studies were developed to elucidate pulmonary clearance patterns and to evaluate the pulmonary toxicity of a selected organic fiber, Kevar para-aramid, and wollastonite, an inorganic fiber, relative to other reference materials, using a short-term inhalation bioassay. The results indicate that the low durability of these fiber types may be responsible for the observed transient pulmonary inflammatory effects.

Materials and Methods

General Experimental Design

Groups of 8-week-old male Crl:CD BR rats (Charles River Breeding Laboratories, Kingston, NY) were exposed 6 hr/day for 5 days to aerosols of Kevar in concentrations ranging from 877 to 1344 f/cc (9–11 mg/m³), or to wollastonite fibers at 835 f/cc (114 mg/m³). Following exposure, groups of 4 animals for each fiber were assayed for lung histology and BAL fluid analysis. Additional groups of three to four animals/time point were used for lung digestion studies.

Fiber Preparations

Ultrafine respirable-sized Kevar para-aramid fibrils (DuPont Fibers, E. I. du Pont de Nemours, Willmington, DE) were prepared for a 2-year inhalation study (17), utilized for this study. A preparation of Wollastonite NYAD-G fibers (NYCO, Willboro, NY), measured using scanning electron microscopy (SEM), had diameters ranging from 0.2 to 3.0 μm.

Inhalation Exposure

Dust generation techniques for Kevar and wollastonite exposures have been described in detail elsewhere (12,13). Atmospheres of Kevar fibrils or wollastonite fibers were generated with a K-tron bin feeder (K-tron Co., Glasboro, NJ) equipped with twin screws. Baffles were inserted into the generation apparatus and increased the respirability of the sample. Fibers or fibrils were metered into a plastic funnel connected to a cyclone where high-pressure air transferred the test material into a microjet apparatus. Chamber concentrations were...
maintained by controlling the dust-feed rate into the generation apparatus or by varying the air-flow rate. The methods for determining gravimetric concentrations, particle/fiber size and fiber numbers have been previously described (12,13).

**Pulmonary Lavage**

Bronchoalveolar lavage procedures (14) were repeated five times or until 50 ml of fluid was collected. Lavage fluids recovered from control and dust-exposed rats were centrifuged at 250g, and the supernatant was removed and concentrated for biochemical studies. The cell pellet was resuspended in Eagles Minimal Essential Medium (Eagles MEM F-11; pH 7.2, Gibco, Grand Island, NY) supplemented with penicillin and streptomycin. The methods of quantitation of cell numbers, viabilities and differential counts have been previously described (15).

**Biochemical Assays on Bronchoalveolar Lavage Fluid**

Lavage fluids from the first two washes were centrifuged and the supernatant concentrated 10-fold in an Amicon concentrator (cut-off mw=10,000). All biochemical assays were performed on concentrated bronchoalveolar lavage (BAL) fluids at 30°C, using a semiautomated clinical chemical analyzer (Encore II, Baker Instruments, Allentown PA). Lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and N-acetyl-beta-glucosaminidase (NAG) were measured using commercially available reagent kits (Baker Instruments, Allentown, PA, for LDH and ALP; Boehringer Mannheim Biochemica, Indianapolis, IN, for NAG). Lavage fluid protein was measured using a commercially available reagent kit based on Coomassie Blue dye binding (QuanTest, Quickmetrix, Hawthorne, CA). Statistical methods have been previously reported (15). For statistical analysis of the biochemical data, a one-way analysis of variance (ANOVA) and Bartlett’s test were calculated for each sampling time. Statistical analyses for fiber dimensions and 5-bromo-2’-deoxyuridine (BrdU) cell labeling were carried out using Student’s t-test.

**Pulmonary Cell-labeling Studies**

BrdU cell labeling (12) was designed to measure the effects of Kevlar or wollastonite fiber inhalation on airway and lung parenchymal cell turnover in exposed rats. Groups of Kevlar fiber-exposed rats and controls were pulsed immediately after exposure and one week or one month later with an intraperitoneal injection of BrdU dissolved in a 0.5 N sodium bicarbonate buffer solution at a dose of 100 mg/kg body weight. Groups of wollastonite-exposed rats and controls were similarly pulsed. The animals were sacrificed 2 hr later by pentobarbital injection. It has been shown that the single intraperitoneal pulse leads to efficient and sufficient labeling in the peripheral lung to differentiate significant differences between exposure groups (12).
All sonicated, high filters were recovered from Kevlar lungs, and fibers were digested in hypochlorite solution. Ethanol-digested samples were placed in a grinding tube, poured into a vacuum-filtered Millipore filter (pore size = 0.20 μm or 0.45 μm), and placed in an oven overnight. The fibers were then mounted and prepared either for phase contrast light microscopy (PCOM: for counting), or for scanning electron microscopy (SEM: for fiber dimensional analysis). It has been demonstrated that these techniques did not affect the dimensions of the retained fibers.

The numbers of fibers/area of filter were counted by PCOM using the National Institutes for Occupational Safety and Health (NIOSH) 7400B counting method (NIOSH Manual of Analytical Methods). Only fibers with an aspect ratio of 3:1 (length:width), and >5 μm in length were counted. Three animals per Kevlar exposure group/time period and four per wollastonite group/time period were measured in this manner and recorded. Fiber dimensional analysis was carried out by random selection by SEM of fibers ≥4 μm in length. Fiber mass concentrations were calculated using the formula of mass = volume × density.

**Results**

The Kevlar and wollastonite studies were carried out at several exposure concentrations and for several time periods. Only the data from the 5-day, high-exposure concentrations will be reported here.

**Chamber Atmosphere Analysis**

The exposure generation data for aerosolized Kevlar fibrils and wollastonite fibers are summarized in Table 1.

**Analyses of Cellular Constituents in BAL**

Exposures to Kevlar fibrils did not alter the total numbers of cells recovered by lung lavage; increased numbers of cells were recovered 1 week post-exposure from the lungs of wollastonite-exposed rats but the number of cells returned to control levels after one month. The viability of cells recovered in exposed or control rats was greater than 94% at all time periods.

Cell differential analyses of lavaged cells recovered from the lungs of exposed rats demonstrated that fiber inhalation produced an early but transient pulmonary inflammatory response, as evidenced by increased numbers of neutrophils in BAL fluids (Table 2). This effect was measured through a 1-week post-exposure period but was short-lived, since neutrophil numbers were counted. Three animals per Kevlar exposure group/time period and four per wollastonite group/time period were measured in this manner and recorded. Fiber dimensional analysis was carried out by random selection by SEM of fibers ≥4 μm in length. Fiber mass concentrations were calculated using the formula of mass = volume × density.

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were not significantly different from control values after 1 month or at any later time.

**Enzyme and Protein Analyses in BAL Fluids**

Transient increases in BAL LDH, protein, alkaline phosphatase and NAG values were measured in the rats exposed to fibers for 5 days. However, no significant increases in these parameters were measured after one week (Figures 1,2).

**Cell-labeling Studies and Histopathology**

No significant differences in the labeling index of lung parenchymal cells were detected between Kevlar or wollastonite-exposed rats and their corresponding controls at any time period (Table 2).

Increased BrdU-labeling of terminal bronchiolar cells in Kevlar-exposed rats was measured immediately after exposure. However, no significant differences were observed later, indicating that this effect was transient. Histopathologic analysis at 3 months postexposure indicated that neither fiber produced pulmonary lesions.

**Fiber Clearance Studies**

Clearance studies demonstrated a transient increase in the numbers of retained Kevlar fibrils at 1 week postexposure, with rapid clearance of fibers thereafter (Figure 3). The retention half-time was <30 days (Table 3). Mean fiber lengths and diameters decreased progressively with time over a 6-month postexposure period. Mean fiber lengths decreased from 12.5 μm to 7.5 μm and mean fiber diameters, from 0.33 μm to 0.23 μm (Table 3) (Figure 4A,B). Wollastonite fibers were cleared rapidly from the lungs of exposed animals with a retention half-time of <1 week (Figure 5). Mean fiber lengths decreased from 11 μm to 6 μm over a 1-month period, but mean fiber diameters increased from 0.5 to 1.0 μm (Table 3) (Figure 6A,B).

**Discussion**

The finding of increased numbers of retained fibers at 1 week postexposure in Kevlar-exposed rats may relate to fiber shortening during the first week after exposure. Mean fiber lengths recovered from digested lung tissue decreased from 12.5 μm to 7.5 μm, over a 6-month post exposure period, and mean fiber diameters,
from 0.33 μm to 0.23 μm. These results suggest that Kevlar fibrils are shortened in the lungs of exposed rats, indicating a different pulmonary clearance mechanism from that associated with either chrysotile or crocidolite asbestos; in both of these fiber-types the mean lengths of inhaled fibers was progressively increased (7,8).

The wollastonite clearance data presented here confirm the recently reported results of studies using intratracheal instillation methods of exposure (18). In that study, the durability of instilled wollastonite, crocidolite asbestos, and glass fibers were evaluated in lung tissue from exposed rats. The retention half-times for three wollastonite samples were 10, 11, and 12 days. The retention half-times for different glass fiber samples ranged from 38 to 238 days; and the clearance of crocidolite was insignificant, with a half-time rate of 1000 days. When a correlation between durability of fibers in the lung and carcinogenic potency was sought in the intraportal test, the data supported the hypothesis that long, thin, and durable fibers are capable of inducing tumors.

Wollastonite fibers are composed primarily of calcium silicates, which are soluble in lung fluids and within cells. Conceivably, the thinner calcium-containing wollastonite fibers were quickly solubilized and cleared following inhalation, while the thicker wollastonite “stumps” (Figure 5B) were more difficult to clear from the lung.

Fiber dimension and durability generally have been recognized as important features in influencing the development of carcinogenic and fibrogenic effects in the lungs of exposed animals. Stanton et al. (19) have proposed that fibers >10 μm in length and ≤0.25 μm in diameter have the greatest potential for producing lung tumors. Similar conclusions can be drawn from recent studies with silicon carbide whiskers and fibers (20,21). Inhalation of these durable materials produces severe pulmonary fibrosis and lung tumors, while nonfibrous silicon carbide particles are regarded merely as nuisance dusts (22).

A short-term inhalation bioassay has been developed to assess the potential for inhalated particles or fibers to produce pulmonary fibrosis. This screen has utilized a series of biomarkers that may predict the progression of fiber-induced pulmonary injury following chronic exposures. In previous studies, the efficacy of this short-term inhalation screen was tested by exposing rats to several concentrations of various reference materials, including known fibrogenic dusts such as α-quartz silica (15) and crocidolite asbestos (13), as well as materials with minimal or moderate biological activity such as titanium dioxide, carbonyl iron particles (15), or carbon fibers (23). Short-term exposure of rats to silica or crocidolite asbestos produced sustained pulmonary inflammatory responses. In contrast, exposures to wollastonite or Kevlar fibrils, which have low biopersistence, produced only transient inflammatory effects. In summary, short-term inhalation of durable fibers produces sustained pulmonary inflammatory effects along with consistently elevated indicators of cytotoxicity and consequent pulmonary lesions, while only transitory effects are produced by fibers of low biopersistence.

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