An Experimental Approach to the Evaluation of the Biopersistence of Respirable Synthetic Fibers and Minerals

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The biopersistence of fibers and minerals in the respiratory tract is an important parameter in the toxicity of those materials. The biopersistence of respirable synthetic fibers and minerals in man can be most closely evaluated in an animal model. While acellular and in vitro systems are important for initial evaluation of solubility and durability, they cannot simulate the dynamics of inhalation deposition and clearance and the subsequent systemic reaction to fibers and minerals that occurs in the animal. To evaluate the biopersistence of synthetic fibers, male rats were exposed to a well defined respirable aerosol of man-made vitreous fibers (MMVF), 6 hr/day for 5 days. Following exposure, subgroups were sacrificed at intervals ranging from 1 hr to 52 weeks. Following sacrifice, the lungs were removed, weighed, and immediately frozen at 20°C for subsequent digestion by low temperature plasma ashing. The number, size distribution, and chemical composition of the fibers in the aerosol and lung were determined. With this animal model the role of biopersistence in altering the geometry and clearance of fibers can be systematically evaluated. The model also can be applied for the evaluation of the biopersistence of nonfibrous minerals. — Environ Health Perspect 102(Suppl 5):15-18 (1994)

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Introduction

Recent studies of the oncogenic potential by inhalation of crocidolite asbestos, refractory ceramic fibers (RCF) and man-made vitreous fibers (MMVF) in rats have shown that fibers of different composition and size can elicit notably different pulmonary reactions (1–6). Earlier work (7–9) suggests that there were at least three important criteria for a fiber to produce a pathogenic response. The fiber must have a diameter small enough to penetrate to the terminal bronchial or alveolar regions of the lung. Fibers must be long enough to impair the function of the macrophages that attempt to phagocytose and clear them from this region and must have a sufficient biopersistence in the distal airways and alveoli so that they do not dissolve or degrade into shorter pieces that could then be cleared.

To evaluate these criteria, an inhalation model of biopersistence of fibers in the rat was developed, based in part on the work of Hammad et al. (8) and in part on the fiber aerosol generation and inhalation exposure system developed for the inhalation oncogenicity studies (Bernstein et al., manuscript in preparation). Rats were exposed for 6 hr/day for 5 days, then sacrificed at predefined time intervals thereafter. Following sacrifice the numbers, dimensions, and chemical composition of the fibers in the lung were determined. All work was performed in accordance with Good Laboratory Procedures.

Methods

The fibers evaluated thus far in this model have been, except for the asbestos, derived from commercially produced fibers. Most commercially produced insulation fibers contain a wide range of diameters and lengths. However, only a small subset of these fibers are thin or short enough to remain suspended in the occupational work environment. Simulated industrial hygiene exposures with commercially available RCF fiber, performed at Schuller International, found that the fibers that remained suspended in the air had mean diameters of approximately 1 μm and mean length of approximately 20 μm. This subset is of most interest in the evaluation of biopersistence.

The use of the rat as the model for man in these studies superimposes an additional although parallel consideration. Man can inhale into the distal airways and alveoli fibers of up to approximately 3 μm in diameter. This corresponds at a mass median aerodynamic diameter (MMAD) of approximately 9 μm. However, the MMAD also is influenced by fiber length and density (10) and the method of extrapolation. The rat, however, due primarily to its more restrictive nasal passages, can only inhale into the distal airways and alveoli smaller fibers generally of less than approximately 1 μm diameter (50% cut-off). The approximate size for this cut-off has been determined in rat inhalation studies with RCF and MMVF. This fraction is referred to as "rat respirable."

To obtain fibers for evaluation that meet these criteria, fibers were preselected using a water-based separation process, which selected fibers of nominal dimensions of ≤1 μm diameter and ≥20 μm length. This process also eliminated most of the "shot" present in the commercial product.
Aerosol Generation

The fiber aerosol generation system was designed to loft the fibers without breaking, grinding, or contaminating them. The system (Figure 1) uses a Teflon-coated piston to push the fibers, which are gently and uniformly packed in the cylinder, onto a stainless steel brush. The brush is housed within a hardened stainless steel enclosure, with approximately 1 mm clearance between the brush and the housing; it turns in the range of 100 rpm. The fibers arriving from the cylinder are transported on the surface of the brush as it rotates to the top. At this point the fibers are entrained into the airstream by filtered compressed air moving tangentially across the top of the brush at approximately 140 l/min. The lofted fibers then pass through a Ni-63 charge neutralizer to reduce the electronic charge on the fibers to Boltzmann equilibrium (11).

Inhalation Exposure

The animals were exposed by the flow-past nose/snout-only inhalation exposure system (Figure 1). This system was derived from Cannon et al. (12) and is different from conventional nose-only exposure systems in that fresh fiber aerosol is supplied to each animal individually and exhaled air is immediately exhausted. The system was modular and had 16 ports per level; it was used, depending upon group size, with up to 10 levels. The internal volume for exposing 160 animals was 4 l with a T99 = 30 sec. The airflow supplied to each animal was approximately 1 l/min and was calculated to have been laminar prior to exiting. Oxygen measurements in the vicinity of the animal’s nose confirmed that this airflow was sufficient for proper oxygenation.

With this system the animals, which were restrained in open Battelle type polycarbonate restraint tubes (13), were found to be attracted to the supply tube airflow and kept their noses close to this opening. Successful operation of the system required either an animal or an equivalent resistance to be present at every port to provide a uniform pressure drop from the supply to the extraction. Prior to use, the system was tested for uniformity; port-to-port variation in fiber concentration was less than 10%.

Experimental Design

Groups of 10 weanling (100–150 g) male Fischer 344 rats (SPF quality) were exposed using the flow-past nose-only system 6 hr/day for 5 consecutive days to a fiber aerosol concentration of either 30 mg/m³ for MMVF or 10 mg/m³ for crocidolite asbestos. These concentrations corresponded to the highest concentration used for the fibers in inhalation oncogenicity studies. In addition, a negative control group was similarly exposed to filtered air. To be comparable with current and previous fiber inhalation studies, male Fischer 344 rats (CDF (F-344)/CrlBR) obtained from Charles River Laboratories (Kingston, NY) were used.

During the treatment period, animals were housed in wire cages which were contained in sealed stainless steel chambers (one per group) operated under negative pressure with approximately 20 air changes/hr. Following the treatment period, the animals were housed in polycarbonate cages with wood bedding. Temperature was maintained at 22 ± 3°C, relative humidity at 30 to 70%, and the room maintained on a 12-hr, light/dark cycle. When not exposed, the animals were provided pelleted Kliba 343 rat maintenance diet (Klingentalmuehle AG, CH4303 Kaiseraugst, Switzerland) and filtered fresh water ad libitum. All animals were observed for mortality, morbidity, and clinical signs; body weights were measured at regular, prescheduled intervals.

Sacrifice Schedule and Procedure

Groups of 10 animals per group were sacrificed at 1 hr, 1 day, 5 days and 4 weeks following the last (day 5) exposure and at 13, 26, and 52 weeks following the start (day 1) of exposure. All animals were anesthetized with an intraperitoneal injection of sodium pentobarbital and sacrificed by exsanguination. The lungs and the lower half of the trachea were removed, weighed, and immediately deep frozen at approximately −20°C to minimize dissolution of the fibers.

Fiber Analysis in the Lung

From five rats per group/time point the lungs were thawed, the accessory lobe removed for analysis, and the remainder of the lung tissue refrozen. The tissue was initially dehydrated and dried to constant weight to determine the dry weight of the tissue. The dry tissue was plasma ashed in a LFE LTA 504 multiple chamber plasma unit. Upon removal from the ashing unit, the ash from each lung was dispersed and mixed in distilled water. An aliquot was then removed and filtered onto a 25 mm Nuclepore membrane filter (0.2 mm), dried, mounted on a scanning electron microscopy (SEM) stub and gold coated. The fiber diameters and lengths were determined by SEM according to the World Health Organization (WHO) (14).
A minimum of 20 graticule fields were evaluated. If fewer than 100 fibers were present, additional graticule fields were counted until a minimum of 100 fibers were sized. The fiber diameters were measured using either a JOEL T 300 or JOEL 840 SEM equipped with the Videoplan Image Analysis System. The fiber lengths were measured by optical microscopy with the Videoplan Image Analysis System at either 150× or 185× to reduce the number of fibers truncated in the measurement.

**Aerosol Sampling and Analysis**

**Sampling.** All aerosol sampling was performed by connecting a Gelman in-line 47 mm stainless steel filter holder to the supply tube of an unused animal exposure port on the flow-past exposure system. The inlet of the in-line filter holder was machined to provide a snug metal-to-metal fit without obstruction. Sampling was performed at 1/ min, approximately the same flow rate as the aerosol supply stream to each animal. Thus, all sampling was isooxial with no isokinetic sampling bias.

**Aerosol Mass Monitoring and Analysis.** The gravimetric aerosol concentration (mg/m³) of the aerosol (fibers and particulates) was determined daily by filter sampling with Gelman membrane filters (0.45 mm pore size). Filters were weighed before and after sampling on a Mettler micro balance in a temperature- and humidity-controlled room. The sampling time was approximately 5 hr.

**On-Line Monitoring.** The relative aerosol concentration was monitored for uniformity of exposure using a RAS light scattering device (MEI Corp., Bedford, MA). This nonquantitative method was useful in controlling drift during exposure.

**Fiber Number Monitoring and Analysis.** The number of fibers/cm³ were determined by sampling onto Millipore membrane filters (0.45 mm pore size) and subsequent counting using phase contrast optical microscopy (PCOM) for the MMVF and transmission electron microscopy (TEM) for the crocidolite.

Following sampling for approximately 10 min, the filters were mounted on glass slides and cleared using acetone vapor. All aerosol samples were counted using the WHO Reference Methods (14); total and WHO fiber/cm³ counts were reported. The PCOM analysis was performed at a magnification of 400× using a Bausch and Lomb Balpan phase-contrast microscope. The TEM analysis was performed at 5000× using a Philips EM 400 TEM. Total fibers and nonfibrous particles (including shot) were also counted and recorded on selected samples.

**Fiber-Size Monitoring and Analysis.** The diameter and length distributions of the lofted fiber aerosol were also determined following filter sampling onto Millipore membrane filters. To avoid dispersion and loss of the fibers, the filters were placed in sealed glass bottles containing approximately 8 ml sodium azide in 100 ml distilled water. Prior to analysis the filter surface was washed into the bottle, the filters then were ashed and the ash was suspended with the fibers in the bottle. This suspension was diluted to 250 ml with distilled water and homogenized by sonication. An aliquot was then removed and filtered onto a 25-mm Nuclepore membrane filter (0.2 mm pore size), dried, mounted on SEM stubs and gold coated. The fiber diameters and lengths were determined following exactly the same procedure as that described for fiber analysis in the lung. The MMVF fiber lengths were measured by optical microscopy at lower magnification to reduce the number of fibers truncated in the measurement. The diameters and lengths of the crocidolite asbestos were measured by TEM using a Philips EM 400 microscope.

**Fiber Chemistry.** Stock fibers were analyzed by inductively coupled plasma (ICP) techniques, atomic absorption spectrophotometry (AAS), and gravimetric chemistry (GM) to yield a complete oxide analysis.

The chemistry was also determined on fibers from both aerosol and lung digestion samples. The aerosol was sampled by filtration using Gelman membrane filters (0.45 mm pore size). An aliquot of the fibers recovered from the lung digestion procedure were filtered on separate membrane filters.

A SEM mount was prepared from fibers sampled from the aerosol and energy dispersive spectrophotometry (EDS) spectra were collected on seven fibers with diameters ranging between 0.5 and 1.0 mm. The elemental intensities used by the SEM–EDS quantitative analysis program were then adjusted so that the mean values of the analysis of the seven fibers by EDS matched the composition of the stock fibers previously analyzed by ICP, AAS, and GM. These corrected elemental intensities were used during subsequent analyses of all fibers that were either sampled from the aerosol or recovered from the lungs. With this method the concentrations of SiO₂, Al₂O₃, TiO₂, MgO, CaO, FeO₃, Na₂O, K₂O, and SO₃ in the fibers were determined.

**Results and Discussion**

Presentation of the specific fibers used in this model and the results obtained have been reported by Muller et al. (15) and Musselman et al. (16). Those reports have shown the value of this model in the determination of the biopersistence of fibers and have enabled further precision in its use and interpretation.

**Measurement of Mass Loss of Deposited Fibers and Particulates.** Clearance studies with nonfibrous particles have indicated that the determination of the total mass of particulates deposited in the lung over time can be a useful parameter for correlation with fibrosis and tumors. The total retained mass of inhaled fibers and particles should be determined at each sacrifice interval to determine the importance of this parameter in fiber exposure and clearance.

**Reporting the Number of Rat Respirable Fibers.** The water selection process used to prepare the subset of fibers from the commercial bulk for use in the fiber aerosol generator came close to the intended objectives. Although a percentage of the fibers was greater than 1 μm in diameter. While that was not unexpected because of the difficulty in selecting these fibers from the commercial bulk (yields <2%), it is important in evaluating dose response in rat inhalation studies to report and take into account only the subset of fibers of less than approximately 1 μm diameter, which can be inspired by the rat into its distal airways. This is especially true in determining comparability of fiber exposure between different fibers as well as in modeling the exposure concentration in dose response evaluation.

**Measurement of the Bivariate Log-Normal Size Distribution.** The individual length and diameter distribution data provided thus far in the study design have been very useful and have allowed for a good deal of information to be obtained. This data base could be improved even further with the measurement of length and diameter for each fiber to determine the bivariate (length and diameter) log-normal distribution. Ideally, the bivariate distribution should be determined for the fiber aerosol and the fibers recovered from the lungs, and should be measured without truncation (17).

In the aerosol, the bivariate distribution would provide the information necessary to determine the length of the fibers that are
rat respirable, i.e., ≤1 μm diameter, which is not possible with the current study design. In addition, it would be useful to know the diameters of the fibers longer than 10 to 20 μm that can impair macrophage phagocytosis and therefore not be readily cleared from the lung. These fibers are thought to be potentially biologically active and to produce a pathological response in the lung. This could be important as long, thin fibers would have a higher probability of reaching the distal airways, where the fibrotic response originates; long, thick fibers would have a greater probability of depositing in the upper bronchial tree.

In the lung, the bivariate distribution would provide additional precision in the evaluation of the fibers as a function of time. Currently, analysis indicates that the MMVF tested all appear to clear similarly. As reduction in fiber diameter by dissolution is a relatively slow process (3–10 nm/day at 37°C), analysis of the evolution of fiber length within narrow ranges of fiber diameters could provide additional precision in differentiating fibers that are breaking or dissolving.

Chemical Composition. The change in chemical composition of the fibers over time seems to depend on whether they remain intact or dissolve or break apart. In particular, Hesterberg et al. (18) have reported that fibers from which significant amounts of calcium and sodium oxides have been leached, once deposited in the lung, show little or no biological response compared with fibers of similar length from which those elements have not been leached. However, chrysotile asbestos also undergoes rapid leaching (magnesium) in vivo, yet is still very active. The data suggest that additional research on the influence of leaching on the physical and biological activity of the fibers is important to the understanding of potential pathogenic response.

Conclusions

Use of the inhalation model for determining the biopersistence of fibers in rats provides a precise method for routinely evaluating the potential for fibers to remain intact in the distal airways and alveolar regions of the rat lung. Fibers that do not persist undergo significant changes in chemical structure which, it is thought, lead eventually to physical degradation of the fiber breaking or to complete dissolution. It is through such processes that it may be possible for long, "biologically active" fibers that reach the distal airways either to break into shorter pieces and be cleared from the lung, or to dissolve, eliminating them as pathogenic agents.

This model, in addition to being used with fibers, also has been applied to evaluation of the retention of spherical particulates, including the evaluation of the dissolved and translocated fractions (19,20).

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