Phagosomal pH and Glass Fiber Dissolution in Cultured Nasal Epithelial Cells and Alveolar Macrophages: A Preliminary Study

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The dissolution rate of glass fibers has been shown to be pH sensitive using in vitro lung fluid simulant models. The current study investigated whether there is a difference in phagosomal pH (pH) between rat alveolar macrophages (AM) and rat nasal epithelial cells (RNEC) and whether such a difference would influence the dissolution of glass fibers. The pH was measured in cultured AM and RNEC using flow cytometric, fluorescence-emission ratiometric techniques with fluorescein-labeled, amorphous silica particles. Glass fiber dissolution was determined in AM and RNEC cultured for 3 weeks with fast dissolving glass fibers (GF-A) or slow dissolving ones (GF-B). The mean diameters of GF-A were 2.7 μm and of GF-B, 2.6 μm, the average length of both fibers was approximately 22 to 25 μm. Dissolution was monitored by measuring the length and diameter of intracellular fibers and estimating the volume, assuming a cylindrical morphology. The pH of AM was 5.2 to 5.8, and the pH of RNEC was 7.0 to 7.5. The GF-A dissolved more slowly in RNEC than in AM, and no dissolution was evident in either cell type with GF-B. The volume loss with GF-A after a 3-week culture with AM was 66% compared to 45% for cultured RNEC. These results are different from those obtained using in vitro lung fluid-simulant models where dissolution is faster at higher pH. This difference suggests that dissolution rates of glass fibers in AM should not be applied to the dissolution of fibers in epithelial cells. — Environ Health Prospect 102(Suppl 5):97-102 (1994)

Key words: dissolution, glass, fiber, nasal epithelial cells, alveolar macrophages, phagosomes, pH

Introduction

The biological activity of fibrous materials in the lung depends on many factors, including how long the material is present in cells and the interstitium of the lung. The longer the material remains in the lungs, the longer it can interact with the lung tissue and exert a pathogenic effect. The residence time of fibrous materials in the lung depends on the mechanical clearance rate and the dissolution rate of the fiber. The dissolution rate is governed mainly by fiber chemistry and the tissue/cell environment in which the fiber is found. Fibers can be phagocytized by many cell types within the lung. In rats, the majority of inhaled fibers are found within alveolar macrophages (AM); however, a small fraction of inhaled fibers can be found within the epithelial cells lining the respiratory tract and the fibroblasts and macrophages within the interstitium (1). The interactions of fibers with epithelial cells can result in lung cancer, while fiber/AM interactions can give rise to pulmonary fibrosis. In addition, the latter reactions may also enhance the carcinogenic potential of fibers. The interior of the phagosome in AM is acidic with a pH 4 to 6 (2). Laman et al. (3) measured the intracellular and extracellular fluid pH of rabbit AM and found pH values of 7.17 for the intracellular fluid and 7.40 for the extracellular fluid. Little is known about the phagosomal pH in other respiratory tract cells. Jaurand et al. (4) found the mesothelial phagosome; pH to be approximately 7 by using the pH-dependent leaching of magnesium from chrysotile in cultured mesothelial cells.

As a result of the importance of dissolution and the difficulty of measuring it in vivo, many studies have been conducted with lung fluid simulant models (5). Porter and Mattson (6) used a physiological saline solution to show that dissolution rates for glass fibers increased with higher pH. Morgan et al. (7) administered to rats by intratracheal instillation, sized glass fibers that had similar diameters, and measured the dimensions of fibers extracted from lungs at times up to 18 months. They showed that fibers <10 μm in length dissolved relatively slowly, while fibers >10 μm dissolved much more rapidly. The dissolution of the smaller fibers was uniform, while the dissolution of the longer fibers was less uniform. These differences were attributed to small variations in intracellular and extracellular pH. The short fibers were completely engulfed by AM, while the longer fibers were not. The difference between extracellular and intracellular pH may not be sufficient to account for this differential dissolution. Other factors such as glass chemistry and pH-independent processes may be involved in fiber dissolution.

This preliminary study had three goals: to determine whether there is a difference in the phagosomal pH in AM and rat nasal epithelial cells (RNEC) cells obtained from the respiratory tract; to ascertain whether there is a difference in dissolution rate of fibers within AM compared to RNEC; and to compare in vitro cellular dissolution rates with those obtained by other investigators (6,8). How fibers persist in AM and epithelial cells is important in understanding lung clearance mechanisms and possible lung cancer induction.
Materials and Methods

Fiber Samples

The glass fiber samples designated as GF-A and GF-B were provided by Owens-Corning Fiberglas (Granville, OH). The glass fiber samples had been used in vitro acellular studies (6) and in vivo (8). The fibers in those studies were designated as X7753 and X7779, which correspond to GF-A and GF-B in this study. The major chemical difference between the fibers is their Al₂O₃ content (GF-A, 0.5%; GF-B, 8.06%) (6). Al₂O₃ decreases the dissolution rate (6). The JM Code 100 glass microfiber was obtained from Johns Manville Corp. (Denver, CO).

Cell Culture

Normal AM were obtained by lavage from healthy female Fischer 344/N (8 to 12 week old) rats that were bred at the Institute. The animals were killed with an overdose of sodium pentobarbital. The pulmonary vasculature was flushed with sterile phosphate buffered saline (PBS), and the lungs were lavaged with 5 × 10 ml of cold PBS (without magnesium and calcium ions). The lungs showed no gross evidence of disease. The lavaged cells were centrifuged, resuspended in RPMI medium (Sigma, St. Louis, MO) which contained 10% MacSrim (Collaborative Research, Inc., Bedford, MA), and counted using a hemacytometer. Their viability was established using a trypan blue dye exclusion method. A cytological preparation was made using a cytocentrifuge and staining with Diff-Quik (American Scientific Products, McGraw Park, IL). The AM suspension was adjusted to a concentration of 0.5 × 10⁶ cells/ml. Two ml of this cell suspension were added to glass chamber slides (Nunc Inc., Naperville, IL) and incubated at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air for 20 hr. The medium was removed, and the cultures were washed with three changes of PBS and replaced with medium containing 35.5 μm/ml of either GF-A or GF-B; control cultures were given medium alone. The cells were cultured for 20 hr in the presence of fibers and then washed three times with PBS. Fiberglass free medium was added, and the cells were cultured for periods of either 1, 2, or 3 weeks. During that time the medium was changed every 2 to 3 days. At the termination of the culture periods, the cells were fixed and stained with Diff-Quik.

RNEC (cuboidal-transitional epithelial cells) were obtained from the anterior lateral wall of the nasal cavity of a second group of female Fischer 344/N (8- to 12-week old) rats killed with an overdose of sodium pentobarbital. The RNEC were chosen because they were available from untreated animals from another experiment at the Institute. In addition, RNEC have similar sensitivities to fibers as the more commonly used rat tracheal cells and possessed similar phagosomal pH (NF Johnson, unpublished data). The lining of the lateral wall was stripped from the underlying tissue and placed in a pronase (type XIV) solution (3.3 mg/ml) in alpha MEM medium (Sigma) for 15 min at 37°C in a shaking water bath. After incubation, an equal volume of RPMI medium containing 10% fetal calf serum was added to arrest the action of the pronase solution. The resultant suspension was filtered through a 70-μm nylon mesh filter. The filtered material was centrifuged and resuspended in serum-free medium (9) containing 0.8% bovine serum albumin. The cells were counted using a hemacytometer, and

Table 1. Fiber dimension changes in alveolar macrophage cultures.

| Sample Type | Mean Diameter (μm) | Mean Length (μm) | Mean Volume (μm³) |
|-------------|--------------------|------------------|-------------------|
| GF-A        | 2.7 ± 0.01         | 24.6 ± 0.2       | 130.8 ± 0.5       |
| GF-B        | 2.6 ± 0.01         | 22.6 ± 0.7       | 118.9 ± 4.5       |

Table 2. Fiber dimension changes in rat-nasal epithelial cell cultures.

| Sample Type | Mean Diameter (μm) | Mean Length (μm) | Mean Volume (μm³) |
|-------------|--------------------|------------------|-------------------|
| GF-A        | 2.6 ± 0.01         | 23.5 ± 0.5       | 123.5 ± 5.6       |
| GF-B        | 2.6 ± 0.01         | 21.8 ± 0.4       | 116.7 ± 5.1       |

*p < 0.05.
their viability was assessed by a trypan blue dye exclusion method. The cell suspension was adjusted to a concentration of $0.5 \times 10^6$ cells/ml; $0.5 \times 10^6$ cells in 2 ml of medium were placed in glass chamber slides and incubated for 20 hr under the same conditions used for the AM. The medium was removed and thereafter the RNEC cells were treated by exactly the same procedures that had been used for the AM.

The AM and RNEC cell cultures were viewed using a light microscope, and random fields were photographed at a magnification of approximately $\times 200$. A calibration slide was also photographed on each occasion that a series of photographs were taken. The negatives were enlarged to give a final magnification of $\times 600$. The number of fibers per cell was noted, and the length and diameter of each fiber were measured with a $\times 6$ magnifying eyepiece and a reticle displaying 100-µm divisions. Only fibers associated with cells were counted and measured. The volume of the individual fibers was estimated assuming a cylindrical morphology. The significance of the results was determined using a paired Student's t-test; the criterion for significance was $p > 0.05$.

**Flow Cytometry**

The pH of the phagosome was determined using a flow cytometric procedure employing fluorescein-isothiocyanate (FITC)-labeled amorphous silica particles (10); the fluorescence of fluorescein is pH sensitive. The AM and RNEC were cultured as described. The cells were further cultured in the presence of the FITC-coated particles for 20 hr after a 20-hr plating period and after 20 days of culture. The pH was analyzed using a dual-laser flow cytometer (Becton Dickinson, San Jose, CA); the FITC was excited at wavelengths of 457 and 488 nm. The emissions from both lasers were measured using a 515 narrow-band pass filter. The fluorescence intensities were measured on a log scale. The stored fluorescence data from the cell studies were edited using forward and side-scatter signals to eliminate non-cell associated events. The pH was estimated by taking the difference between the mean fluorescence channel number of the 457/515 and 488/515 edited histograms. This value was used to obtain the pH of the phagosome from its intersection with the regression line obtained from the pH calibration curve. This curve was derived from determining the fluorescence ratios of coated particles measured in buffers of predetermined pH.

**Ancillary Experiments**

**Cell Survival.** A single cytoxicity assay was undertaken using triplicate cultures. The lung epithelial cell (LEC) line (11) was used because the cells are sensitive to the cytotoxic effects of fibers (12). LEC were plated in Ham's F-12 medium supplemented with 10% heat-inactivated newborn calf serum, at a density of $5 \times 10^4$ cells per 25-cm diameter culture flask, and cultured as described above. After 24 hr, the medium was replaced with RPMI containing various concentrations of GF-A, GF-B, or JM Code 100 glass microfiber (0, 5, 10, 15, 25, and 50 µm/ml). The cultures were incubated for 20 hr, after which the cells were washed in buffered saline. The JM Code 100 fiber acted as a positive control, as it has been shown to be active in...
this cell assay (12). The cultured cells were replated at a density of 1.25 × 10⁵ cells per well of a six-well plate. After 8 days, the number of colonies that formed in each well was determined. Relative colony-forming efficiency was calculated as the ratio of the number of colonies formed in treated versus control cultures.

**Phagocytic Activity.** A single study of duplicate cultures was undertaken to investigate the phagocytic capability of the cultured cells. Cultures of AM and RNEC cells were established as described above. Cells were cultured for 20 hr or 20 days, after which 20 × 10⁴ fluorescently labeled, polystyrene microspheres were added and cultured for a further 20 hr. The cells were fixed and then stained. The number of cells associated with microspheres and the number of microspheres per cell were counted.

**Results**

The original samples of GF-A and GF-B had similar physical dimensions and volume (Figure 1, Table 1). The physical dimensions of GF-A and GF-B after 20 hr of culture with AM and RNEC were not significantly different from the dimensions of the original samples (Tables 1, 2). The dimensions of GF-A changed with the length of time that the fiber-treated cells were cultured; the changes were more marked in the AM cultures (Figures 2, 3; Tables 1, 2) than in the RNEC cultures. There was a time-dependent decrease in the mean volume of the fibers retained within the cells (Figure 4) resulting, for AM, in an approximately 66% decrease in the mean fiber volume following the 3-week culture period; for RNEC the decrease was approximately 45%. This decrease in fiber volume was the result of changes in both length and diameter of the fibers. In contrast, the dimensions of GF-B were not significantly affected by the period of culture in either the AM or RNEC cultures (Figures 2, 3; Tables 1, 2).

The measurement of phagosomal pH showed that the value for the AM was acidic and the value from the RNEC was neutral. The pH value for the AM was 5.2 to 5.8 with a mean value of 5.4 ± 0.3 (standard deviation; n = 5) following a 20-hr culture after the plating period. After the 20-day culture period, two pH determinations were 5.2 and 5.4. Comparable experiments with RNEC yielded pH values of 7.0 to 7.5 with a mean value of 7.2 ± 0.3 (standard deviation; n = 3), following a 20-hr culture after the plating period. After the 20-day culture period two pH determinations were 7.0 and 7.5.

The culture of the fiber-sensitive LEC in the presence of GF-A and GF-B did not produce a cytotoxic effect in the colony-forming assay at any dose level used (Figure 5). The positive control, JM Code 100 fiber, produced a dose-dependent decrease in cell survival (Figure 5). These results show that toxicity was not a factor in the differential solubility observed.

The numbers of AM and RNEC that were associated with microspheres after 20 hr and 20 days were similar. The 20-hr AM culture contained 98% of the cells associated with particles, while the comparable figure for the 20-day culture was 86%. In the former case, 7% of the cells were associated with 1 to 5 particles, and in the latter case 40% of the cells were associated with 1 to 5 particles. The 20-hr RNEC culture contained 70% of the cells that were associated with particles, while the comparable figure for the 20-day culture was 61%. In both cases, 45% of the cells were associated with 1 to 5 particles. These results show that both cell types could phagocytize particles in vitro. In this study the cell-associated fibers were assumed to be within phagosomes by virtue of the cells phagocytic activity and light microscopic appearance. In addition, fibers or particles have been detected in alveolar macrophages (10), type I epithelial cells (1,13,14), type II epithelial cells (1,15), tracheobronchial cells (16,17), and pleural mesothelial cells (18).

**Discussion**

This study shows that fibers dissolve at different rates within cultured AM and RNEC. The more rapid dissolution occurred with the AM cultures, where the measured pH of the phagosome was 5.2 to 5.8. The measured phagosomal pH in RNEC cultures was 7.0 to 7.5. The RNEC have been used as surrogates for pulmonary epithelial cells because of their availability; sensitivity to fibers; and their phagosomal pH, which is similar to that of alveolar type II cells and tracheal cell (NF Johnson, unpublished data). The fibers in this study have been used in a detailed in vitro acellular study (6) and an in vivo study (8), thus allowing the three approaches to be compared.

In the in vitro acellular system (6), GF-A fibers (designated as X7753) dissolved at a rate of 600 ng/cm²/hr. In that system, dissolution of this fiber type was more rapid in neutral or alkaline pH than in an acid pH, which suggests that dissolution ought to be faster within the phagolysosome of RNEC, at their pH 7.2 to 7.5.

However, the reverse was the case with our culture studies. To compare the dissolution rate of fibers in solution and in cultured cells, the data were plotted as 1 minus the square root of the volume fraction remaining. If the dissolution rate was proportional to fiber surface area and all the fibers had similar diameters, the rate would be linear (W. Eastes, personal communication). These conditions exist in the in vitro acellular system, and accordingly the dissolution rate can be described as a linear function over almost its entire range (Figure 6). In comparison, the fiber dissolution rate in the cell culture system is not linear (Figure 6) and is much slower in both AM and RNEC.

This disparity between the in vitro acellular and cellular dissolution rates, and the rate laws they obey, may result from exposing the fibers to different intracellular and
extracellular environments. Fibers that are completely engulfed within the cell will experience the microenvironment of the phagosome and the cytoplasm, while the longer fibers that are partially engulfed will experience both the intra- and extracellular environments. The pH of the AM phagosome is acidic, while that of the epithelial cell is approximately neutral. The cytoplasmic pH has been determined to be approximately neutral (19), while the pH of the alveolar fluid has been estimated to be 7.4 (20). These microenvironments will not be static; there will be intracellular movement of organelles and fibers within the cell (21), which, especially if it is a macrophage, will also be mobile. Fibers and particles may also bind proteins (22) and that may inhibit the pH-dependent dissolution of fibers. These factors of protein binding and changing patterns of cellular and extracellular environments may account for the difference between the in vitro acellular and cellular systems. The majority of in vitro acellular systems use physiological salt solutions at a neutral pH, designed to simulate body fluids (5); however, they generally lack proteins that may inhibit fiber dissolution.

In addition to the in vitro acellular system, the two fibers used in this study have been investigated in vivo. Morgan et al. (8) instilled the fibers intratracheally into the lungs of rats and killed the rats at various times after instillation to recover the fibers. Changes in the less durable fiber (GF-A) were apparent at 14 days after exposure; at 1 year, only about 10% of these fibers remained in the lungs. The changes were more apparent with the long fibers than with the short fibers. This difference was attributed to the fact that the longer fibers would be partially exposed to both cellular and extracellular environments. The faster dissolution was expected in the extracellular fluid because of its higher pH compared to the macrophage phagolysosome. The calculated dissolution rate for this in vivo experiment was 470 ng/cm²/hr (W. Eastes, personal communication).

The difference between the dissolution rates in AM and RNEC may have important implications for the clearance of fibers from the lung and for fiber-induced neoplasia, with the caveat that RNEC may not be the ideal surrogate for pulmonary epithelial cells. The way the AM handles fibers is a major factor in the long-term clearance of fibers from the lung and hence the lung burden of fibers. The lung burden of fibers is frequently related to lung pathology, including pulmonary fibrosis and lung cancer. These in vitro cellular results suggest that an epithelial component of glass fiber clearance will have a longer half-life because of the slower dissolution of engulfed fibers. The possible longer residence time in epithelial cells allow the fiber to exert its pathogenic effect over a longer period than would be predicted from the AM dissolution data. In addition, as the fibers dissolve, the thicker fibers may go through a size range that is most critical for the induction of neoplasia. It is not clear, however, whether the partially dissolved fibers maintain their structural integrity to exert significant biological activity. In addition, it may be the nature of the dissolution process that is important rather than the physical dimensions of the fibers. Stanton et al. (23) reported that fibers with diameters <0.25 μm and lengths >8 μm are the most carcinogenic following intrapleural implantation. A small proportion of GF-A fibers that had undergone dissolution fell within this size range. However, inhalation studies with glass fibers have given uniformly negative results both for an excess risk of lung cancer, and of pulmonary fibrosis (24), possibly because rapidly dissolving fibers do not remain long in the critical size range. This lack of correlation between phagosomal pH and dissolution in AM and RNEC suggests that phagosomal pH may not be the determining factor and that some pH-independent chemical process plays a role in fiber dissolution. It may also be that fiber dissolution occurs as a result of a combination of pH-dependent and pH-independent mechanisms. The exact role of dissolution or fiber durability in the induction of lung cancer or pulmonary fibrosis has yet to be determined.

The in vitro cellular system described here may be a useful model system to study further fiber and cell interactions related to fiber dissolution, especially when combined with the putative target cells for lung cancer. The cultures of AM and RNEC retain their ability to engulf particles and maintain their phagosomal pH over a 3-week period. The cellular dissolution of glass fibers is a complex process that does not follow the simple physicochemical process that occurs in a typical in vitro acellular system.
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