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EFFECT OF FIBER LENGTH ON GLASS MICROFIBER CYTOTOXICITY

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Fiber length has been implicated as a determinant of fiber toxicity. Fibers of narrowly defined length can be generated by dielectrophoretic classifiers. Since the quantities of fibers produced are very small, we developed a rat alveolar macrophage microculture system to study the toxicity of these samples. The objective of this study was to examine the role of fiber length on the cytotoxicity of Manville code 100 (JM-100) fibers. Rat alveolar macrophages were cultured with 0–500 μg/ml of 5 lengths of JM-100 fibers on 96-well plates. After 18 h, well supernatants were removed and lactate dehydrogenase (LDH) activity was measured to assess cell damage. Chemiluminescence (CL), an assessment of macrophage function, was measured by adding lucigenin with or without zymosan, a particulate stimulus, to appropriate wells. For each fiber length the effects were concentration dependent: CL declined and LDH rose with increasing fiber concentration. Comparing the effects of different lengths showed the greatest toxicity from a relatively long fiber sample (mean length = 17 μm). Microscopic examination of the interaction of fibers with macrophages revealed multiple macrophages attached along the length of the long fibers. This suggests that frustrated, or incomplete, phagocytosis may be a factor in the increased toxicity of longer fibers. Overall the results demonstrate that length is an important determinant of toxicity for JM-100 fibers.

The term *asbestos* refers to a group of naturally occurring fibrous metallic silicates that have been used widely in construction and industry. The use of asbestos, however, has been limited because asbestos inhalation is associated with pulmonary fibrosis and cancer (Donaldson et al., 1993). This discovery led to the use of several types of man-made glass and ceramic fibers as substitutes for asbestos. While these substitutes are believed to be less toxic than asbestos (Bunn et al., 1993; Hesterberg et al., 1993), the ability of the man-made fibers to cause pulmonary disease has not been fully defined.

Various approaches have been used to determine the mechanism(s) of fiber toxicity. Characteristics of fibers such as chemical composition and size have been suggested to be important determinants of toxicity. Chemical composition can result in differences in the ability of fibers to generate toxic oxidant species or differences in durability. For example,

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the presence of iron in crocidolite has been related to its ability to generate hydroxyl radicals and damage lung cells (Vallyathan et al., 1992), while the dissolution of glass fibers has been reported to occur in vivo at rates much faster than asbestos (Morgan et al., 1982; Holmes et al., 1983). Among respirable fibers, long fibers have been reported to be more toxic than short fibers. For example, long asbestos fibers are more carcinogenic than short fibers when implanted in the pleural spaces of rats (Stanton et al., 1981). In addition, fiber length has been reported to influence fibrogenicity in guinea pigs (Wright & Kuschner, 1977). Fiber length has also been suggested to be an important determinant of the cytotoxicity of man-made fibers. For example, long thin glass fibers appear to exhibit in vitro cytotoxicity similar to that of asbestos (Wheeler, 1990).

Tilkes and Beck (1983) have shown that glass fibers with a median length of 10 μm exhibited greater cytotoxicity than fibers of 5 μm median length. However, extensive investigation of fiber length effects on toxicity has generally been limited by the inability to produce fibers of a specified length in quantities sufficient for biological studies. Recently a classifier has been developed to separate fibers by length using dielectrophoresis, the movement of neutral particles in a gradient electric field (Baron et al., 1994). Presently, however, the classifier has limited output. The quantities of length-selected fibers that have been produced are only a few milligrams, which precludes their use in inhalation models or even conventional culture systems. In a preliminary study, then, our laboratory developed a microculture system to study the effects of fiber length on rat alveolar macrophages (AM). AM were selected for this study because, as the principal lung phagocytes, AM attempt to ingest inhaled fibers. Furthermore, it has been demonstrated that asbestos can injure AM in vitro and it is also believed that at low concentrations asbestos stimulates AM secretion of oxidants and inflammatory cytokines (Donaldson et al., 1993).

The AM microculture system was first used to examine the effects of crocidolite asbestos and JM-100 glass fibers (Blake et al., 1996). Cultures were performed in 96-well plates using less than 5 mg of each fiber type. Two cytotoxicity endpoints were measured in this system: cell viability (lactate dehydrogenase release) and phagocyte function (zymosan-stimulated lucigenin chemiluminescence). Both fiber types caused concentration-dependent cytotoxicity, supporting the usefulness of the microculture system. The samples used in these preliminary experiments were polydisperse in terms of fiber length.

The goal of the present study was to examine more specifically than previously possible the role of fiber length in cytotoxicity. Specifically, JM-100 fibers of narrowly defined length were produced using the fiber classifier. These samples were then incubated with AM, and after 18 h the

cells were assessed for cytotoxicity. The hypothesis tested was that fiber length is an important factor in the cytotoxicity of JM-100 fibers.

METHODS

Fibers

Bulk samples of JM-100 glass fibers were first milled, aerosolized, and separated into five length categories using dielectrophoresis (Baron et al., 1994). The dielectrophoretic classifier was operated in a differential mode so that fibers with narrow length distributions were extracted in an air suspension at the end of the classifier. These fibers were collected on a polycarbonate (Nuclepore) filter at rates up to 1 mg/d, scraped off, and accumulated for the following experiments. Samples of the length-classified fibers were prepared for size and count analysis by adding weighed portions of the dusts to freshly filtered water. These samples were then sonicated, diluted, and filtered through polycarbonate filters. The filters were then sputter coated (gold palladium) and initially examined. If required, further dilutions were made to achieve optimum fiber density for final analysis.

Measurements of length, width, and fiber count/mass were made using a JEOL JSM-6400 scanning electron microscope. Measurements of both length and width were made on each fiber, which typically required switching between two magnification levels. Readings were made from the screen using digital calipers. Output from the calipers was dumped directly to a spreadsheet for later analysis and graphing. Measurements at each magnification were referenced to a National Bureau of Standards electron microscopy standard rule.

Alveolar Macrophages

Male Sprague-Dawley rats (200–300 g) were obtained from Hilltop (Scottdale, PA). Animals were anesthetized with sodium pentobarbital and exsanguinated. The trachea was cannulated and the lungs washed with 8-ml aliquots of Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (145 m*M* NaCl, 5 m*M* KCl, 9.35 m*M* Na_2HPO_4, 1.9 m*M* NaH_2PO_4, 5.5 m*M* glucose; pH 7.4). A total of 80 ml lavage fluid was obtained from each rat. Cells in the lavage fluids were pelleted by centrifugation and then resuspended in a small volume of Ca^{2+}/Mg^{2+}-free phosphate-buffered saline. Alveolar macrophages (AM) were counted with a Coulter counter and differentials determined with a cell sizing attachment (Coulter Instrument Co., Hialeah, FL); cell preparations were always >90% AM. AM (yield 4–8 × 10^6 cells/rat) were pelleted again by centrifugation and resuspended in Eagle's minimum essential medium (EMEM; pH 7.4) to yield 5 × 10^6 AM/ml. Usually AM from two rats were combined to make an adequate AM pool for each culture replicate.

Cell Culture

AM (100 µl = 5 × 10^5 AM) were plated in wells of 96-well white microplates. Fiber samples were heat-sterilized at 160°C for 1 h, suspended in EMEM, and sonicated before use in culture. Fiber suspensions were then added to wells; EMEM was added as needed to bring the total volume of each well to 200 µl. Cells in the presence or absence of fibers were incubated at 37°C for 18 h. (This 18-h exposure time was chosen as a result of preliminary time-course studies.) At the end of the culture period, each plate was removed from the incubator and centrifuged in a plate carrier to sediment fibers and any nonadherent cells. Well supernatants were removed and retained for biochemical analysis while the cells, still on the plate, were used for chemiluminescence and microscopy.

Chemiluminescence

Chemiluminescence (CL), or light production, was measured to assess macrophage function. Lucigenin (Aldrich Chemical Company, Milwaukee, WI), a light enhancer, was added to the cells at a final concentration of 125 µ*M*. Lucigenin has been shown to measure superoxide release (Gyllenhammar, 1987; Ischiropoulos et al., 1989). Saline or saline plus zymosan, a particulate stimulus, was added to the cells; final zymosan concentration was 2 mg/ml. CL was measured using a microplate chemiluminometer (ML 3000 microtiter plate luminometer, Dynatech Laboratories, Chantilly, VA) for 1 h and reported as area under curve per well per hour. For graphs, percent vehicle response was calculated. The value for baseline (unstimulated) CL was subtracted from the corresponding stimulated (i.e., with zymosan) value. Then for each culture the fiber-treated values were calculated as a percent of the control (vehicle) value, giving percent vehicle response to zymosan.

Lactate Dehydrogenase

Lactate dehydrogenase (LDH) is a cytosolic enzyme released when cells are damaged and was used as an index of AM cytotoxicity. LDH was measured in culture supernatants using a colorimetric assay based on the reduction of pyruvate and reported as U/ml. For graphs, percent maximum release was calculated as 100[(treated value) – (control value)]/maximum LDH release. Maximum LDH release was determined in a separate experiment after lysing freshly isolated AM by sonication.

Statistics

Data were compared on a micrograms per milliliter concentration basis by two-way analysis of variance (ANOVA). The Student–Newman–Keuls method was used to determine differences among groups. For all analyses, $n = 3$ or 4 and significant differences were determined at $p < .05$. Values are mean ± SD for µg/ml data and mean for log fibers/ml data.

RESULTS

Figure 1 contains scanning electron microscope images (all at the same magnification) and length and width distributions for the five glass fiber samples after sonication, that is, as used in the bioassay studies. Table 1 contains summary statistics of these results. Note that the samples are referenced as 33, 17, 7, 4, and 3 μm based on the measured average fiber lengths. Both the images and graphs show that the longer fiber sizes are more polydisperse in length than the shorter fiber samples. Coefficients of variation for particle length are 78 and 63% for the 33- and 17-μm samples, respectively, compared to 23 and 33% for the 4- and 3-mm samples.

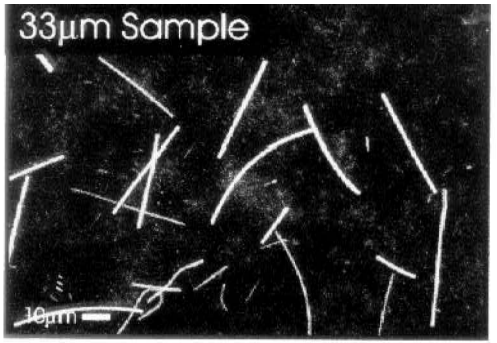
There is an association between particle width and length, with the longer fibers being thicker. The differences were not, however, large (average fiber width = 0.75 and 0.35 μm, respectively, for the longest and shortest samples). As expected, the highest fiber count was associated with the smallest fibers (5.9×10^8 fibers/mg). The lowest count was associated with the 33-μm sample (5.8×10^6 fibers/mg).

Figure 2 shows the length and width distribution and a scanning electron microscope image of the 33-μm glass fiber sample as measured directly from the fiber classifier, that is, without sonication. The distributions are tighter than those of the fiber samples used in cultures (Figure 1), which had been sonicated, but there appear to be agglomerates of smaller fibers on the surfaces of large fibers in this unsonicated sample.

Figure 3A shows that at concentrations of 250 and 500 μg/ml, each fiber sample significantly reduced zymosan-stimulated CL, indicating decreased release of reactive species. CL is an index of macrophage function; therefore, these data indicate an impairment of AM respiratory burst activity. Conversion of the data to log fibers/ml shows that the 33- and 17-μm samples appear to be most toxic, as they diminish CL at lower numbers of fibers than 7-, 4-, or 3-μm-length glass fibers (Figure 3B). For example, at 1×10^7 fibers/ml, CL is almost completely inhibited by longer fibers while inhibited 40% or less by the shorter fibers.

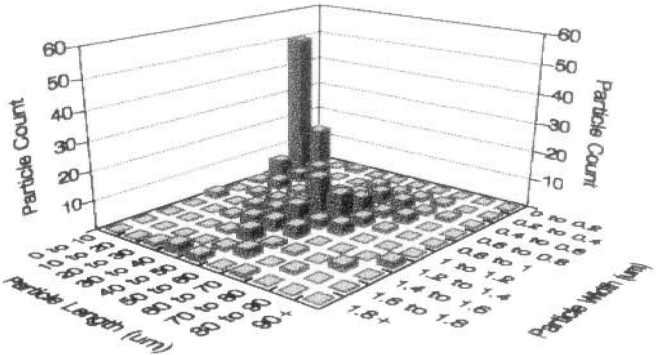
Figure 4A displays significant increases in LDH release at concentrations of 250 and 500 μg/ml for all fiber samples. The greatest increases occurred from the 17-μm sample. LDH is an index of AM cytotoxicity, so these data show a concentration-dependent increase in AM cytotoxicity for each fiber length. When the data are viewed as log fibers/ml, shown in Figure 4B, the 17-μm sample still appears to be the most cytotoxic, causing the highest maximal release of LDH and causing toxicity at a low number of fibers. The 33-μm sample was also highly toxic; the other fiber samples also increased LDH but only at substantially higher concentrations.

Figure 5 displays scanning electron microscope images showing the interaction between alveolar macrophages and the 33- and 3-μm glass fiber samples. The 3-μm fibers appear to be completely engulfed by


33µm Sample
10µm


17µm Sample
10µm


7µm Sample
10µm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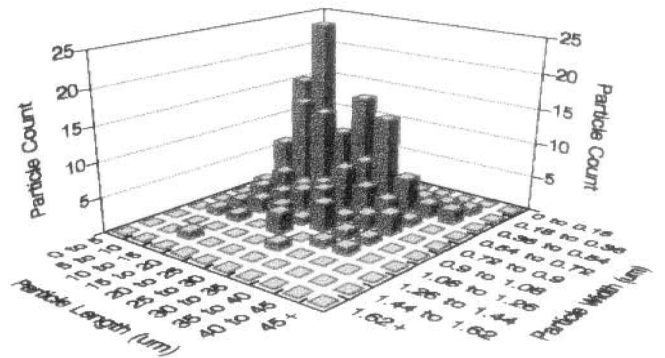
Particle Length (µm)
Particle Width (µm)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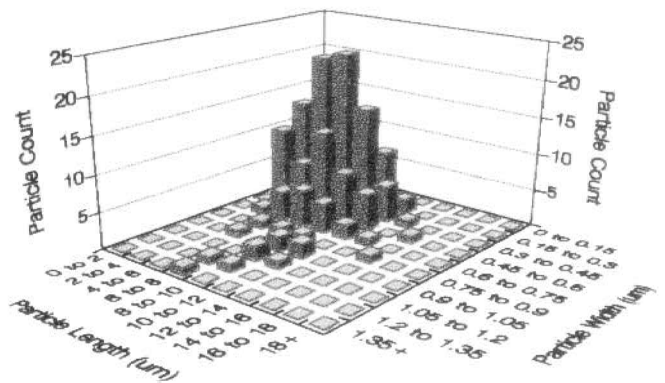

Particle Count
Particle Length (µm)
Particle Width (µm)

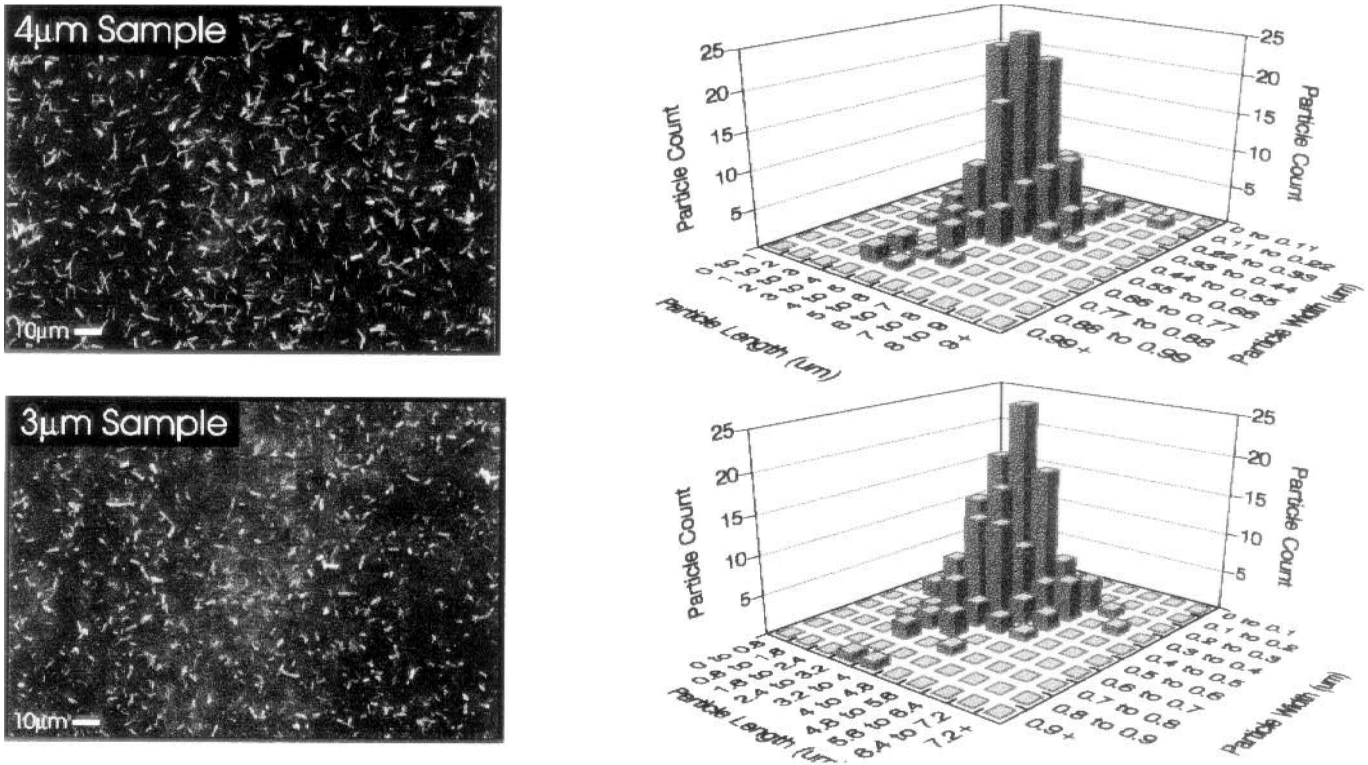


FIGURE 1. Scanning electron microscope images and length and width distributions of glass fiber samples as introduced to cell cultures, that is, after sonication. Each photograph was taken at a magnification of 600×. Fiber measurements were based on 200 randomly selected particles.

TABLE 1. Physical characteristics of sonicated glass fiber samples

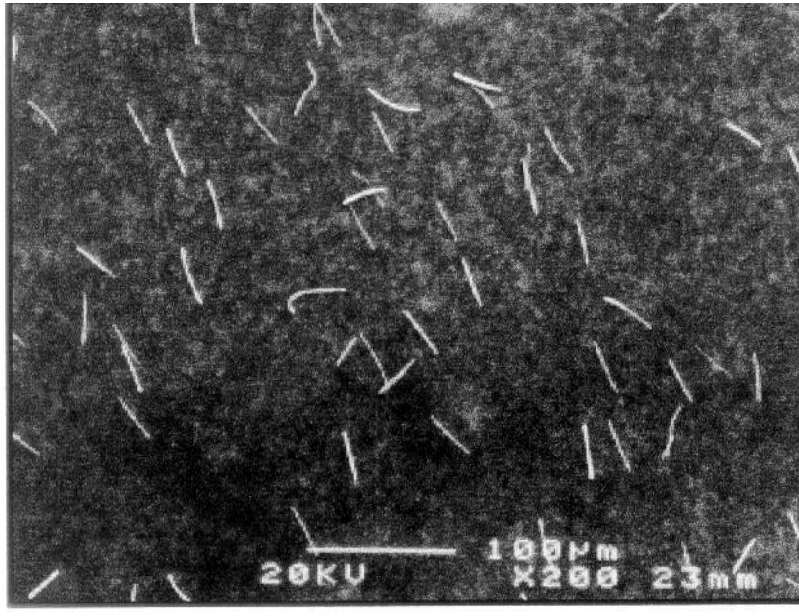
	Sample				
Measurement	33 μm	17 μm	7 μm	4 μm	3 μm
Length (μm), mean (SD)	32.7 (23.5)	16.7 (10.6)	6.5 (2.7)	4.3 (1.0)	3.0 (1.0)
Width (μm), mean (SD)	0.75 (0.50)	0.49 (0.27)	0.44 (0.22)	0.40 (0.15)	0.35 (0.14)
Fibers/mg, mean (SD)	5.8E6 (0.49E6)	2.0E7 (0.26E7)	3.0E8 (0.20E8)	5.5E8 (0.32E8)	5.9E8 (0.21E8)

macrophages, while the 33-μm fibers commonly show several macrophages aligned along a single fiber.

DISCUSSION

Fiber length has often been suggested to be an important determinant of fiber toxicity; however, the study of fiber length relative to toxicity has been impeded by the inability to obtain fibers of specified lengths. This difficulty has been lessened by the development of the fiber classifier. The goal of this study was to examine the effect of fiber length on the toxicity of JM-100 fibers to rat AM. Samples of JM-100 fibers were separated by dielectrophoresis into five length classifications and incubated with rat AM. All lengths of fibers increased AM cytotoxicity (measured by LDH release) and decreased AM phagocytic function (assessed by zymosan-stimulated lucigenin chemiluminescence). The 17-μm and 33-μm fiber samples appeared to be the most toxic; the 3-μm, 4-μm, and 7-μm samples were less damaging.

One possible explanation for the increased toxicity from longer fibers is frustrated phagocytosis. It has been proposed that when AM are unable to completely engulf fibers, oxidants and enzymes leak from the AM and cause cell damage (Archer, 1979). Figure 5 contains photomicrographs of the interaction between alveolar macrophages and the longest and shortest glass fiber samples used in this study. For the 33-μm sample, it was common to see multiple macrophages on individual fibers. In some cases (Figure 5, C and D) it appears as though the fibers were protruding through the macrophage membrane. Close-up examination also shows ruffled, therefore apparently activated, cells. This unsuccessful, or frustrated, phagocytosis might cause the release of injurious agents, accounting for the increased toxicity of the longer fibers. In this study there was a greater toxic response from 17-μm and 33-μm fibers than from shorter fibers but little excess toxicity from 33-μm fibers compared to the 17-μm sample. It may be that once fibers are too long to be engulfed (i.e., once the length exceeds the diameter of a macrophage), any excess length is less influential. The 33-μm sample also had a much lower fiber number



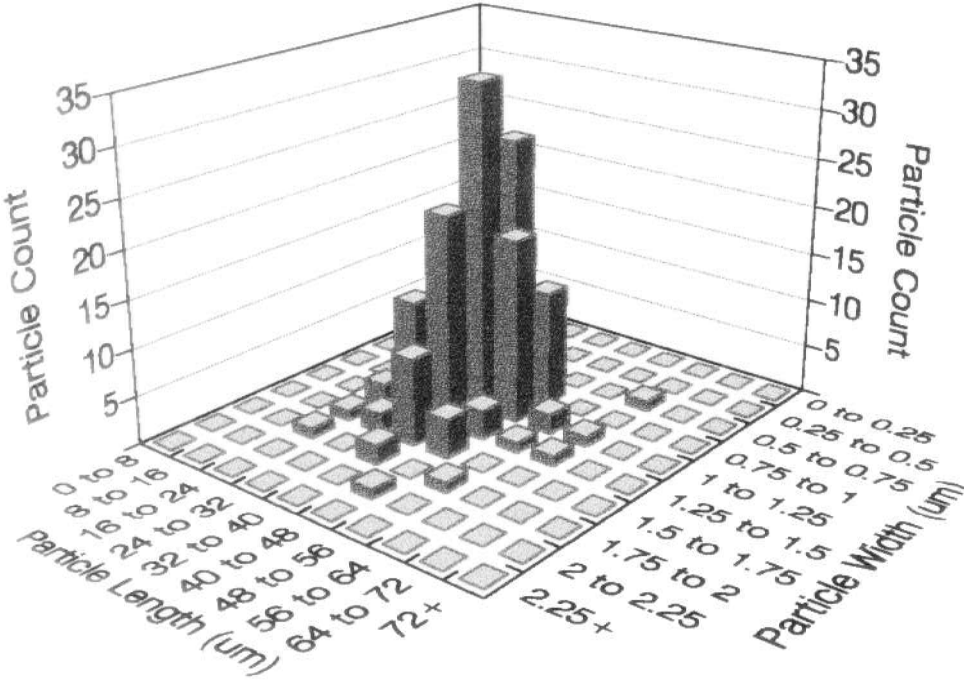


FIGURE 2. Scanning electron microscope image and length and width distribution of the 33-µm glass fiber sample as measured directly from fiber classifier, that is, without sonication. Fiber measurements were based on 200 randomly selected particles.

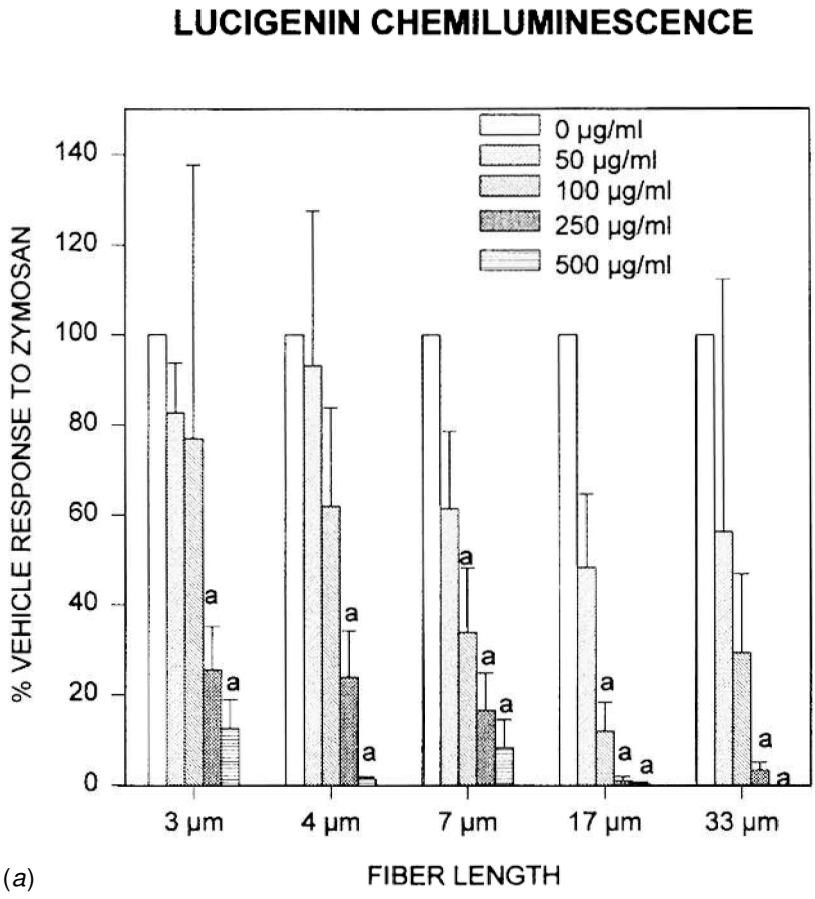


FIGURE 3. Chemiluminescence following zymosan stimulation of alveolar macrophages incubated with different samples of JM-100 fibers. Data are presented as µg/ml (A). The µg/ml data are means ± SD, while points represent calculated means for the log fibers/ml data (n = 3–4). The µg/ml data were compared by two-way ANOVA and Student–Newman–Keuls method. There is a statistically significant effect of both length and concentration, but no interaction between the two. Within a given fiber sample, "a" indicates a significant decrease from the control value for that length. The log fibers/ml data were not subjected to statistical analysis.

count (due to its much longer length), and the reduction in fiber number in those cultures may have somewhat reduced the toxic response relative to the 17-µm sample. A similar size cutoff has been reported for the clearance of sized glass fibers after intratracheal instillation of rats; that is, 90% of 5- and 10-µm-length fibers were cleared by 1 yr postexposure, while almost no clearance was reported for glass fibers 30 and 60 µm in length (Morgan et al., 1982).

The generalized impairment of AM function by JM-100 fibers is of additional interest. In this study the decrement in phagocytic function was measured by lucigenin chemiluminescence, which reportedly assesses

superoxide release. Other studies have shown that in vitro asbestos exposure increases the release of superoxide from AM; in contrast, AM removed from rats that had inhaled asbestos produced significantly less superoxide when challenged with zymosan than AM from sham-exposed rats (Petruska et al., 1990). The reason that the results of the present in vitro study with JM-100 do not mimic the previous in vitro work with asbestos is not clear. The preliminary studies conducted in our laboratory with polydisperse JM-100 and crocidolite showed that both fiber types decreased lucigenin chemiluminescence; therefore, the discrepancy appears not to be due to fiber type. It may be, however, that the time

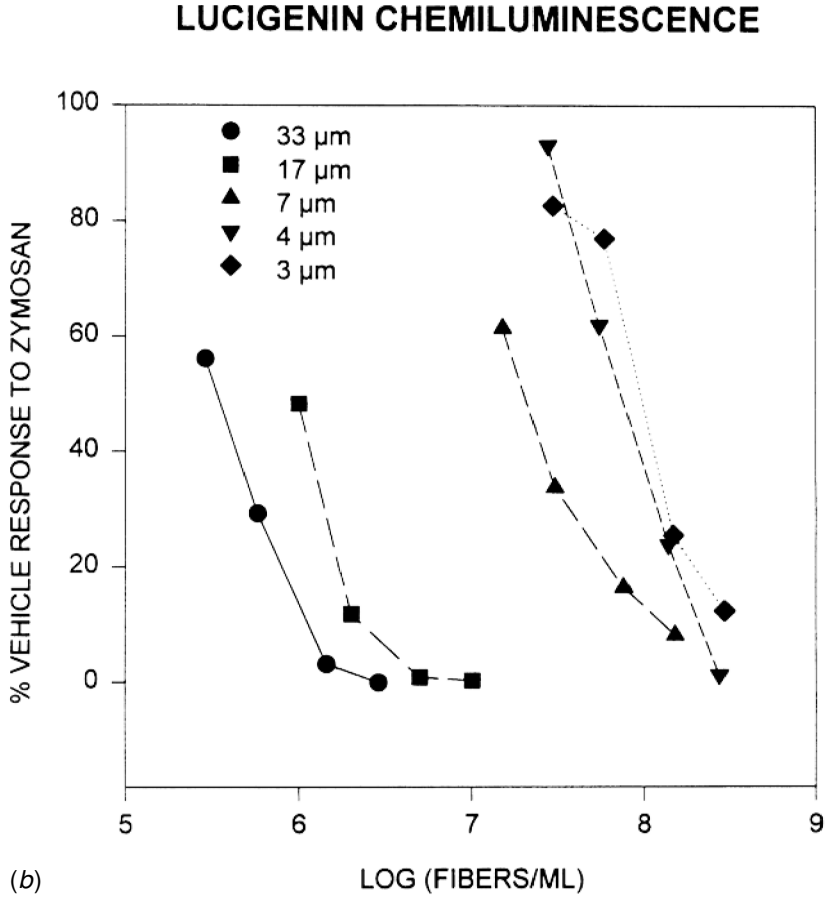


FIGURE 3. (*Continued*) Chemiluminescence following zymosan stimulation of alveolar macrophages incubated with different samples of JM-100 fibers. Data are presented as log fibers/ml (B). The µg/ml data are means ± SD, while points represent calculated means for the log fibers/ml data (n = 3–4). The µg/ml data were compared by two-way ANOVA and Student–Newman–Keuls method. There is a statistically significant effect of both length and concentration, but no interaction between the two. Within a given fiber sample, "a" indicates a significant decrease from the control value for that length. The log fibers/ml data were not subjected to statistical analysis.

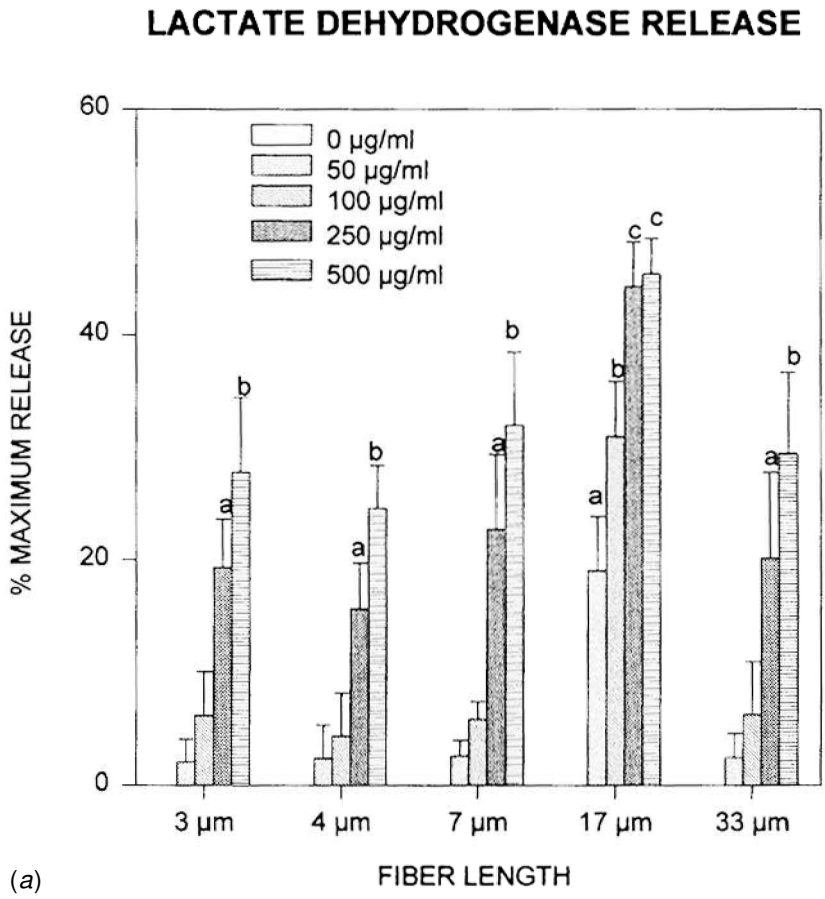


FIGURE 4. Lactate dehydrogenase release from alveolar macrophages cultured with different samples of JM-100 fibers. Data are presented as µg/ml (A). The µg/ml data are means ± SD, while points represent calculated means for the fibers/ml data (n = 3–4). The µg/ml data were compared by two-way ANOVA and Student–Newman–Keuls method. There is a significant effect of both length and concentration and a statistically significant interaction between the two. For a given fiber sample, different letter denotations indicate groups that are significantly different from one another. The log fibers/ml data were not subjected to statistical analysis.

course of the experiments is important. In the present study, AM were incubated with fibers for 18 h, a relatively long period of time. Perhaps there is an initial burst of superoxide release, followed by a longer term decrease in the ability of AM to release superoxide when challenged with a further particle stimulus (i.e., zymosan).

The size measurements reported here are from samples that were hydrosolized, sonicated, and redeposited on filters. This was done to match the conditions of the culture experiments. When fiber samples are collected onto a filter directly from the fiber classifier and sized without any particle transfer steps, the size results show narrower length distribu-

tions (coefficient of variation around 20%). Figure 2 contains a scanning electron microscope image and length and width distribution for one such sample. Examination of individual fibers collected from the classifier shows that agglomeration of smaller fibers on the surface of large fibers is common. It is conceivable that when the fibers are placed in solution and sonicated, these agglomerates are broken up. This would explain the relatively broad distribution of fiber lengths especially evident for the 33- and 17-μm samples. Deagglomeration probably occurred for all samples but is most evident for longer samples, since these fibers would presumably carry more agglomerates. Also, for the shorter fiber samples, the released

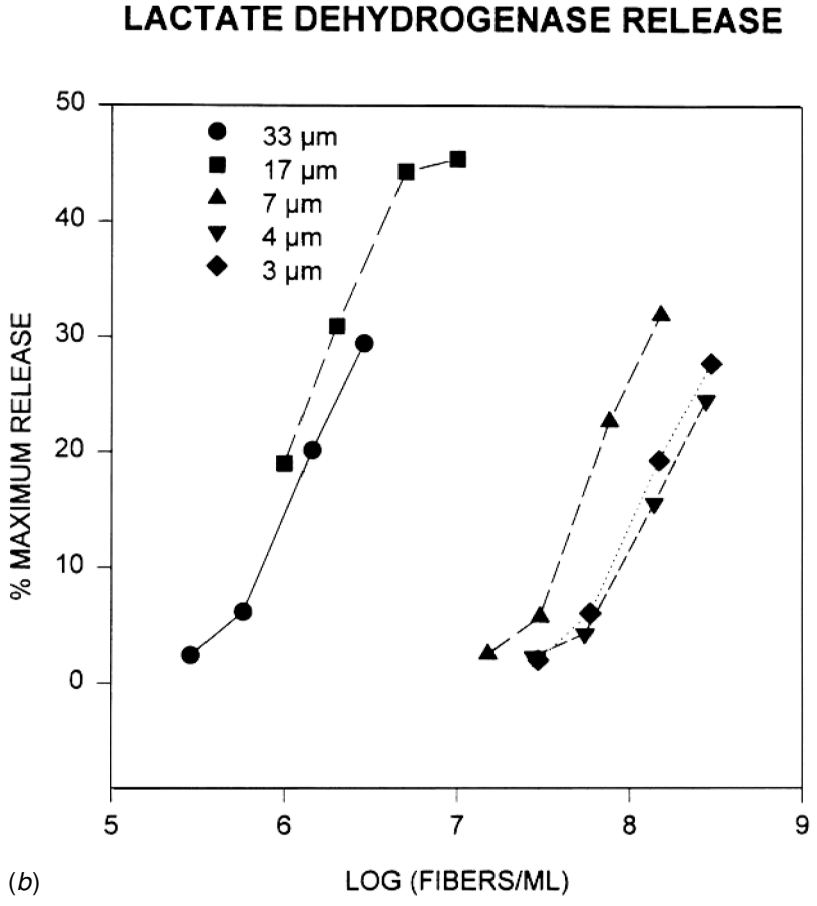
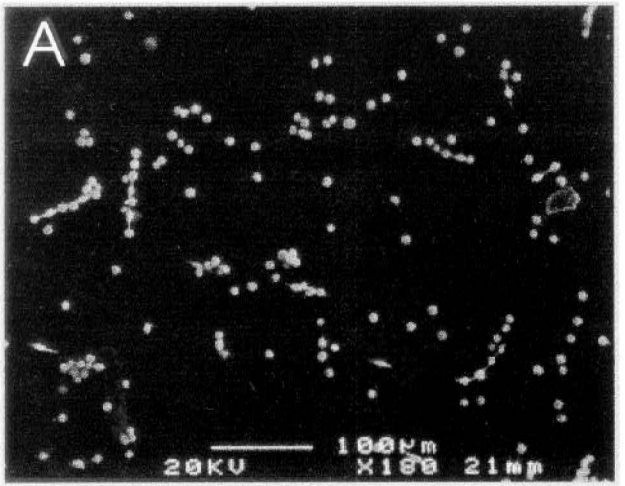
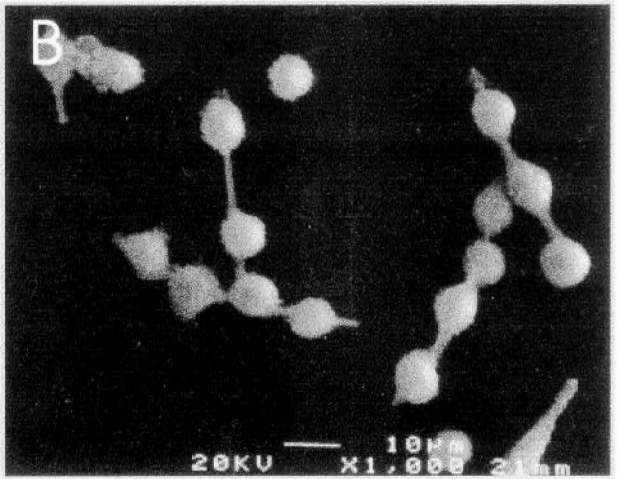
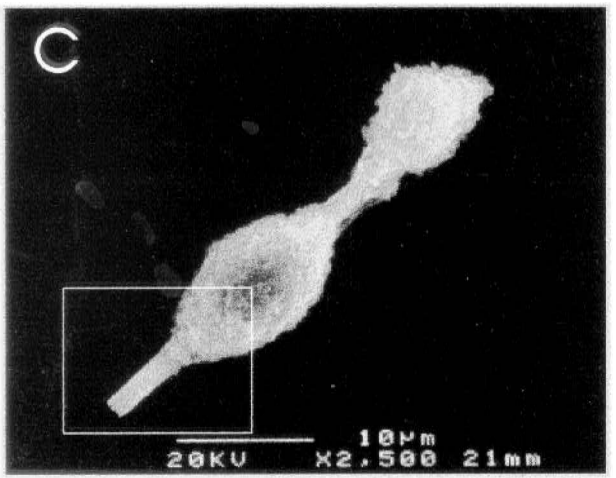
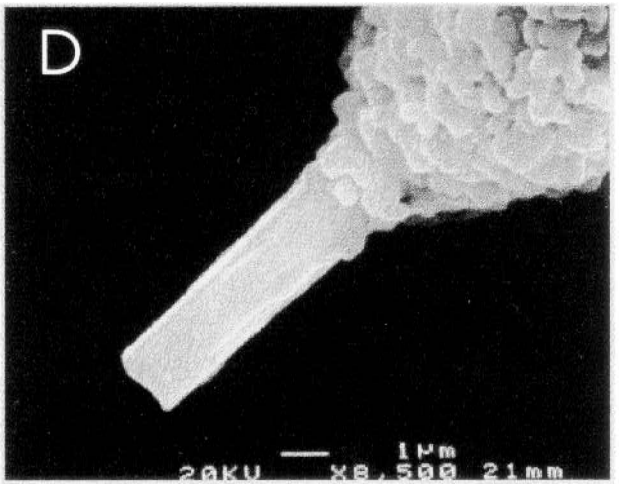


FIGURE 4. (*Continued*) Lactate dehydrogenase release from alveolar macrophages cultured with different samples of JM-100 fibers. Data are presented as log fibers/ml (B). The μg/ml data are means ± SD, while points represent calculated means for the fibers/ml data (n = 3–4). The μg/ml data were compared by two-way ANOVA and Student–Newman–Keuls method. There is a significant effect of both length and concentration and a statistically significant interaction between the two. For a given fiber sample, different letter denotations indicate groups that are significantly different from one another. The log fibers/ml data were not subjected to statistical analysis.


A
100µm
20KV X100 21mm


B
10µm
20KV X1,000


C
10µm
20KV X2,500 21mm


D
1µm
20KV X8,500 21mm

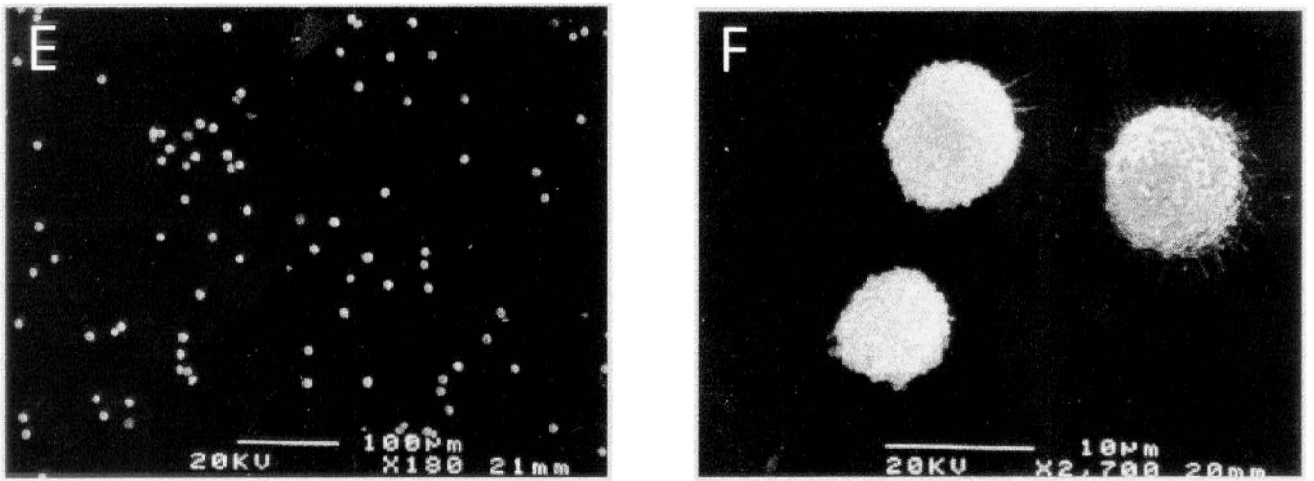


FIGURE 5. Scanning electron microscope images showing the interaction between alveolar macrophages and the 33-µm glass fiber sample (A, B, C, and D) and the 3-µm sample (E and F). Photograph D is the boxed portion of photograph C.

agglomerates would tend to approach the design fiber lengths and thus be hidden in the main distribution. In any event, due to the requirement of hydrosols for these experiments, it was not possible to take full advantage of the fiber classifier capability. Possible improvements might be achieved by breaking up more of the agglomerates prior to size classification.

These data, overall, show that fiber length is an important determinant in fiber toxicity. The present study also demonstrates a valuable biologic application of the dielectrophoresis classifier and the usefulness of the microculture assay system. These methods allow testing of very small (<5 mg) samples; small-scale cultures also reduce the consumption of resources and generation of chemical wastes.

Future goals for this application are to evaluate different types of fibers, such as other man-made fibers and asbestos. As the dielectrophoresis classifier is further refined, output is expected to increase. This may provide sufficient quantities of size-separated fibers to study the time course of fiber-induced changes, as well as additional parameters of AM activation and function, such as cytokine release, to further define the role of fiber length in toxicity. In addition, improved yields should allow for in vivo evaluation of size-separated fibers.

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