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The objective of this study was to explore the use of alveolar macrophage culture to evaluate the cytotoxicity of two glass fiber materials, a building insulation fiberglass (a relatively long and thick fiber) and a glass microfiber (a short and thin fiber). Alveolar macrophages were obtained from male Sprague-Dawley rats by bronchoalveolar lavage and were cultured with varying fiber concentrations for up to 3 d. Fiber toxicity was assessed by assaying cell viability, membrane integrity, and phagocyte function. The microfibers exhibited a concentration-dependent cytotoxicity shown by the loss of cell viability and function. The building insulation fiberglass had little effect on cell viability and did not change macrophage function in this assay system. The results of this study show that short and thin glass fibers are more toxic than long and thick fibers in vitro, supporting a role of fiber dimension in toxicity.

The inhalation of asbestos by humans is associated with lung pathology including pulmonary fibrosis, lung cancer, and mesothelioma (Dement et al., 1986). There is a continuing debate over which properties of asbestos and other fibers are most critical in producing toxicity. An important theory, the Stanton hypothesis, proposed that fiber dimension is the major determinant of pathogenicity following intraperitoneal instillation (Stanton et al., 1981). In that study a correlation was found between carcinogenicity and the number of fibers with length >8 μm and diameter <1.5 μm. A link has also been found between fiber length and fibrogenic activity both in vivo (Adamson & Bowden, 1987a, 1987b; Wright & Kuschner, 1977) and in vitro (Brown et al., 1979).

Chemical factors related to asbestos fibers have also been implicated in their toxicity. The removal of magnesium from chrysotile asbestos decreases its cytotoxicity (Morgan et al., 1977; Harrington et al., 1971). Iron associated with asbestos fibers has been shown to generate reactive oxygen species via a Haber–Weiss reaction (Turver & Brown, 1987; Shatos et al., 1987; Garcia et al., 1988). This iron-catalyzed oxidant generation has been associated with asbestos-induced DNA damage in vitro; such DNA damage may lead to carcinogenesis (Moyer et al., 1994).

The debate concerning chemistry versus morphology has also been lively in the study of man-made vitreous fibers. Man-made vitreous fibers (MMVF), also known as man-made mineral fibers, gained popularity for use as asbestos substitutes after the dangers of asbestos inhalation became apparent. MMVF include fiberglass, rock and slag wools, and ceramic fibers. These substances are widely used in industry and construction. As with asbestos, there is evidence that fiber dimension may be an important determinant of MMVF toxicity (Stanton et al., 1981; Wright & Kuschner, 1977). Specifically, Brown et al. (1979) found in an in vitro system that glass fibers below 10 µm in length were inactive while longer fibers were cytotoxic. Data also exist indicating that durability and other chemical factors play an important role in the pathogenicity of MMVF; that is, fiberglass is less fibrogenic than ceramic fibers (Hesterberg et al., 1993).

In addition to the chemistry versus morphology debate, there are also questions about the relevance of in vitro testing of fibers to in vivo lung disease. For example, fiberglass has been shown to be cytotoxic to various cell types in vitro (Brown et al., 1979). However, chronic inhalation of fiberglass in rats resulted in neither pulmonary fibrosis nor cancer (Hesterberg et al., 1993). In contrast, an in vitro assay system has been able to verify epidemiological data suggesting that wollastonite is less pathogenic than asbestos (Pailes et al., 1984).

The development of an appropriate, accurate, in vitro test of the potential toxicity of fibers would have several advantages. In vitro screening of fibers for safety would be faster and less costly than chronic in vivo studies. Additionally, an appropriate in vitro study system would more easily allow the characterization of the cellular and molecular events, as well as the chemical and physical properties of fibers that are important in fiber toxicity. Such information would allow elucidation of mechanisms involved in the pathogenicity of fibers and assist in developing strategies for prevention and treatment.

The alveolar macrophage is a logical candidate for a viable in vitro screen of the fibrogenicity of fibers. As the major phagocytes of the lung, alveolar macrophages are important mediators of inflammation and fibrosis in response to agents such as silica and asbestos. When macrophages encounter inhaled particles, the cells become activated and/or damaged. As a result, macrophages release substances such as lysosomal enzymes and cytokines. These agents may directly damage the lung parenchyma, as well as recruit and activate other cells (e.g., neutrophils and fibroblasts) that are important in alveolitis and the development of fibrosis. In our laboratory, alveolar macrophage cultures have proven to be an accurate system to evaluate the toxicity of chrysotile asbestos and wollastonite (Pailes et al., 1984); chrysotile was determined to be toxic, while wollastonite was deemed far less toxic and latex beads were found to be nontoxic. These in vitro results correlate with the epidemiological finding of Shasby et al.

(1979) that wollastonite is nontoxic to exposed workers. This system has also proven useful in evaluating the relative importance of particle number and surface area as contributors to cytotoxicity of crocidolite and its nonasbestiform polymorph, riebeckite (Castranova et al., 1994).

The objective of the present study was to explore the use of alveolar macrophage cultures to evaluate the cytotoxicity, and presumably fibrogenicity, of two glass fiber materials, a building insulation fiberglass and a glass microfiber. The hypothesis of the study was that decreased macrophage viability and function after fiber exposure in vitro correlates with the potential of the fibers to cause lung inflammation and fibrosis in vivo. These fibers were selected in an effort to evaluate the contribution of physical characteristics of fiberglass to cytotoxicity, as the building insulation sample represented long and thick fibers while the microfibers were short and thin. Extreme-sized fiber populations (long and thick vs. short and thin) were selected for this study to maximize potential differences in responses. Toxicity of fibers was assessed by assaying cell viability (trypan blue exclusion), membrane integrity (lactate dehydrogenase and β-galactosidase release), and phagocyte function (zymosan-stimulated oxygen consumption and hydrogen peroxide release and release of β-galactosidase).

METHODS

Source and Characterization of Fiber Samples

Owens-Corning building insulation was obtained commercially from a retail building materials store, while Manville Code 100 microfibers were supplied by the manufacturer. To reduce fiber length, bulk samples were processed using a 20,000 rpm knife mill. Samples were examined using a combination of light, scanning electron microscopy, and transmission electron microscopy. Estimates were made of total particle count/mass. For elongated particles (aspect ratio >3 : 1), length, width, and aspect ratio were measured. Surface areas of the fiber samples were measured by the gas adsorption method.

Cell Culture

Alveolar macrophages were obtained by bronchoalveolar lavage of specific pathogen-free male Sprague-Dawley rats (200–250 g). Briefly, rats were anesthetized with sodium pentobarbital (200 mg/kg body weight). The trachea was cannulated and the lungs were lavaged 10 times with 8-ml aliquots of Ca^{2+},Mg^{2+}-free phosphate-buffered medium (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, 5.5 mM dextrose; pH = 7.4). Cells were centrifuged at 500 × g for 5 min and resuspended in fresh buffer for determination of cell number and purity using an electronic cell counter equipped with a cell sizing attachment. Cells were then centrifuged and resuspended in Medium 199 supplemented with glutamine (1 mM), penicillin (100

units/ml), streptomycin (100 µg/ml), kanamycin (100 µg/ml), and 10% fetal bovine serum. Alveolar macrophages (1×10^7 cells/4 ml) were transferred to 25-cm^2 Corning cell culture flasks and incubated for approximately 2 h at 37.5°C and 80% relative humidity to allow the macrophages to adhere to the flasks. After this preincubation period, 1 ml of Medium 199 or fibers (sterilized at 160°C for 90 min, then suspended in Medium 199) was added to the control or test culture flasks, respectively. Total culture volume (5 ml) and flask size (25 cm^2) did not vary from culture to culture. After 1, 2, or 3 d, macrophages were harvested by cooling the flasks to 2°C and shaking them vigorously. Cultures were done for up to 3 d in an effort to assess whether lower fiber numbers cause toxicity when cells are exposed to them for longer periods of time. The harvested cell suspensions were then centrifuged at $500 \times g$ for 5 min. The supernatant was saved for analysis of enzymes, and the cells were suspended in HEPES-buffered medium (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM CaCl$_2$, 5.5 mM dextrose; pH = 7.4).

Cell Viability

Cell counts and viability were determined microscopically (Phillips, 1973). An aliquot of cells was mixed with 0.04% (w/v) trypan blue for 4 min before the addition of buffered formalin (1%). Cells were counted under a light microscope using a hemacytometer, and viability was determined as the percent of cells excluding dye.

Oxygen Consumption

Oxygen consumption by alveolar macrophages was measured at rest and in the presence of zymosan (2 mg/ml) using an oxygraph equipped with a Clark electrode (Van Scott et al., 1987). The oxygraph was calibrated using media equilibrated with gases of known oxygen content.

Hydrogen Peroxide Production

Hydrogen peroxide secretion from alveolar macrophages was determined in the presence of zymosan (2 mg/ml) by measuring the oxidation of scopoletin. The fluorescence of scopoletin (1.2 µM) was measured in the presence of type IX horseradish peroxidase (6.6 units/ml) at an excitation wavelength of 350 nm and an emission wavelength of 460 nm. Fluorescence changes were converted to nanomoles of H$_2$O$_2$ using a standard curve (Van Scott et al., 1987).

Lactate Dehydrogenase

Lactate dehydrogenase activity of culture supernatants was determined using the method of Cabaud and Wroblewski (1958) as given in Sigma Technical Bulletin no. 500 (Sigma Chemical Company, St. Louis, MO). Specific activity was expressed as the amount of enzyme that would reduce 4.8×10^{-4} µmol pyruvate/min at 25°C.

Beta-Galactosidase

β-galactosidase activity of culture supernatants was determined by the method of Conchie et al. (1959). The substrate was 2.5 mM p-nitrophenyl-β-galactopyranoside in citrate-phosphate buffer (pH = 3.6). A 0.25-ml sample was used for the enzyme reaction, which was stopped by the addition of 0.4 M glycine–NaOH buffer (pH = 10.8). The specific activity was expressed as nanomoles of p-nitrophenyl released per hour per 10^6 cells as measured spectrophotometrically at 420 nm.

Data Analysis

Statistical significance was determined at $p < .05$ using analysis of variance. For the building insulation experiments, $n = 12–15$; for microfiber experiments, $n = 5–9$. Values for n represent independent experiments and not duplicate cultures from a single cell pool. Please note that scales differ for building insulation and microfiber results on most graphs.

RESULTS

This study examined the effects on cultured rat alveolar macrophages of two glass fibers of different physical dimensions. The fiber length and width distributions are reported in Figure 1. Clear distinction can be made between the two samples for both parameters. Table 1 lists median length and width along with other physical measurements made on the samples. Note that the microfiber sample contained 50 times as many fibers per unit mass and had 6.3 times the
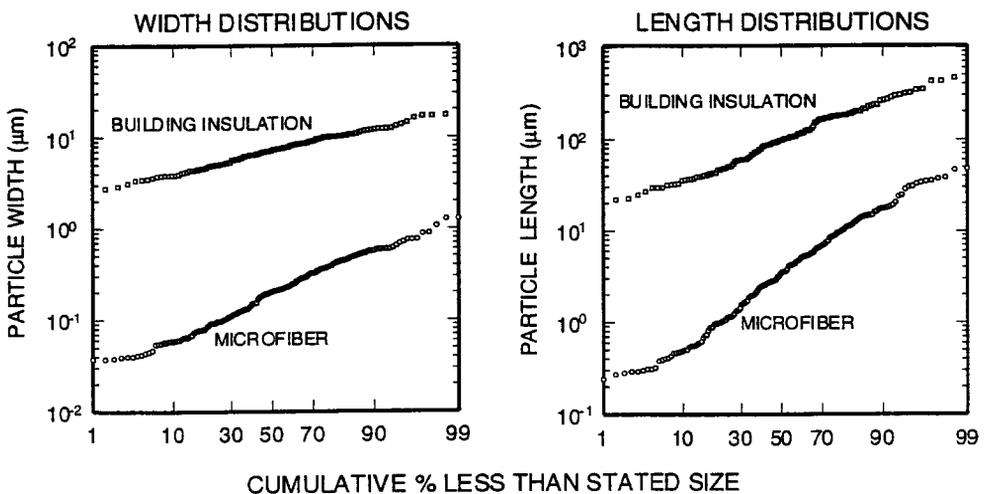

FIGURE 1. Log-probability plots of fiber length and width measurements of building insulation and microfiber samples.

TABLE 1. Physical features of glass fiber samples

Characteristic	Glass microfiber	Building insulation fiberglass
Count median length (μm)	3.4	98
Count median width (μm)	0.20	7.3
Count median aspect ratio	17 : 1	16 : 1
Particle count/mg	5.0×10^8	1.0×10^7
Surface area (m^2/g)	4.7	0.74

surface area as the building insulation sample and that the insulation sample had a median length 29 times greater and median width 36 times greater than the microfiber sample.

Figure 2 contains photomicrographs of the microfiber (Figure 2A) and building insulation sample (Figure 2B), which further illustrate the
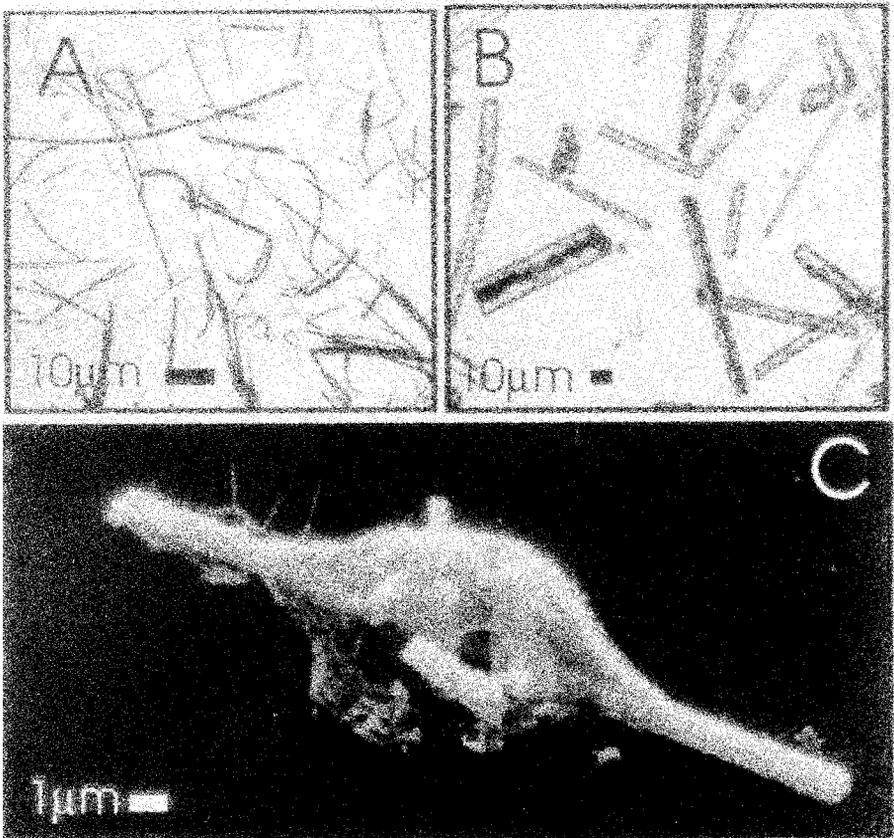

FIGURE 2. (A) Transmission electron photomicrograph of microfiber sample; (B) light photomicrograph of building insulation sample; (C) scanning electron photomicrograph of alveolar macrophages exposed to microfibers.

differences between the two fiber samples. Figure 2C is a scanning electron photomicrograph showing the interaction of microfibers with a rat alveolar macrophage. The interaction of the phagocyte with a particle too large to be ingested is particularly interesting.

The effects of these fibers on the cell viability and function of alveolar macrophages in vitro were assessed. Figure 3 shows the effect of fibers on cell viability measured by trypan blue exclusion. The glass microfibers caused a clear concentration-dependent decrease in cell viability. The effect of the larger building insulation fiberglass was much less dramatic; in fact at 72 h none of the concentrations used caused a significant loss of cell viability.

Similar results were obtained from the measurement of lactate dehydrogenase (Figure 4), a cytosolic enzyme the release of which is used as an indicator of loss of membrane integrity. The building insulation fiberglass did not increase lactate dehydrogenase release. The smaller glass microfibers, on the other hand, significantly increased lactate dehydrogenase release in a concentration-dependent manner, indicating cytotoxicity or cell membrane damage.

The release of a lysosomal enzyme, β-galactosidase, was also measured following incubation of macrophages with fibers (Figure 5). The release of this enzyme indicates phagocyte damage or activation. Glass microfibers, but not the larger building insulation fibers, significantly increased β-galactosidase release. Again the microfiber effect increased with concentration.

Two assays were performed to evaluate macrophage function. Figure 6 shows the results of measuring zymosan-stimulated oxygen consumption. The building insulation glass fibers did not affect macrophage oxygen consumption, but smaller glass microfibers decreased oxygen consumption at all concentrations and time points studied. Zymosan-stimulated hydrogen peroxide release was measured as another functional assessment. Microfibers, but not the thicker and longer building insulation fibers, significantly decreased the release of hydrogen peroxide from zymosan-stimulated macrophages (Figure 7). For both functional assays, the baseline values among the groups did not differ; that is, the values for oxygen consumption and hydrogen peroxide release in the absence of zymosan stimulation were not elevated in the fiber-treated cells (data not shown). As with the cytotoxicity assays, the decrease in cell function was related to concentration of microfibers used.

DISCUSSION

The demonstration of the serious pulmonary pathology resulting from asbestos inhalation led to a demand for safe asbestos substitutes. Man-made vitreous fibers are widely used as asbestos substitutes. Understandably, there is a great concern to demonstrate the safety of
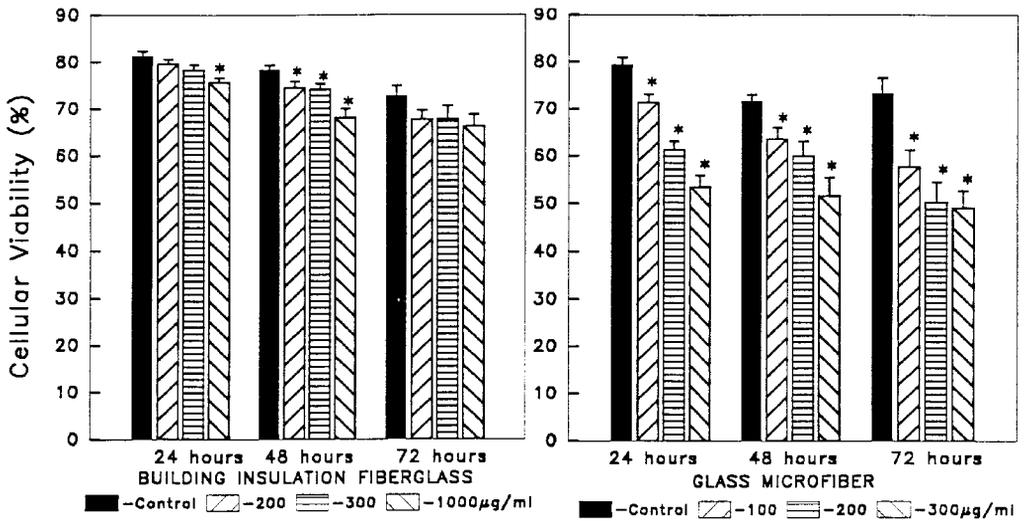
FIGURE 3. Trypan blue exclusion of macrophages incubated in vitro for 1–3 d with or without (control) various concentrations of building insulation fiberglass or glass microfibers. Asterisk indicates a significant decrease from control ($p < .05$).

such fibers. At the same time, there is much interest in uncovering the mechanism of fiber toxicity. There is evidence that both physical and chemical properties of fibers may be important in toxicity.

In vitro test systems of fiber toxicity offer several advantages. They are faster, less costly, and much easier to set up than inhalation studies.
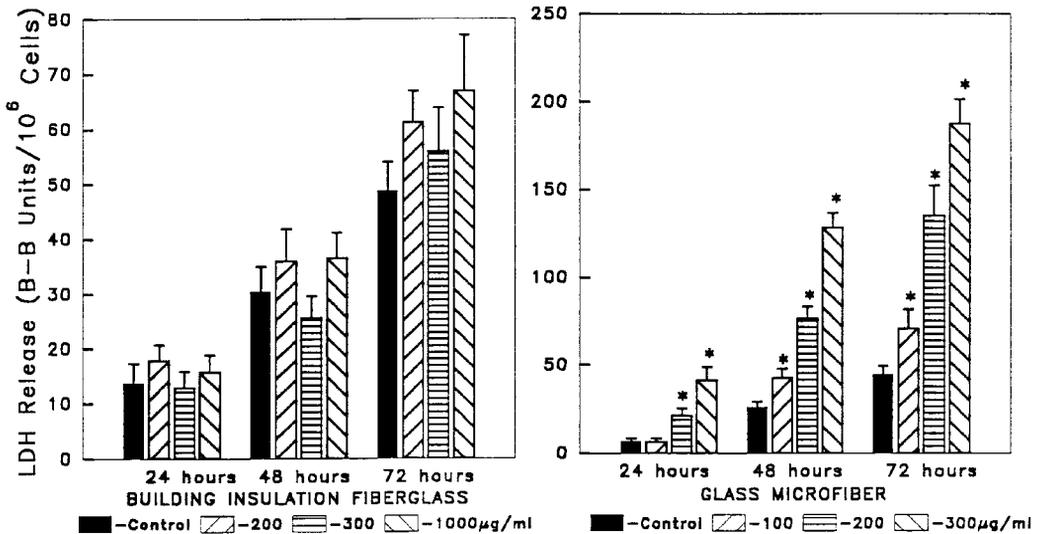

FIGURE 4. Lactate dehydrogenase (LDH) release from macrophages treated in vitro for 1–3 d with different concentrations of building insulation fiberglass or glass microfibers (control = no fibers). Asterisk indicates a significant increase from control ($p < .05$). Note that the y-axis scales differ.
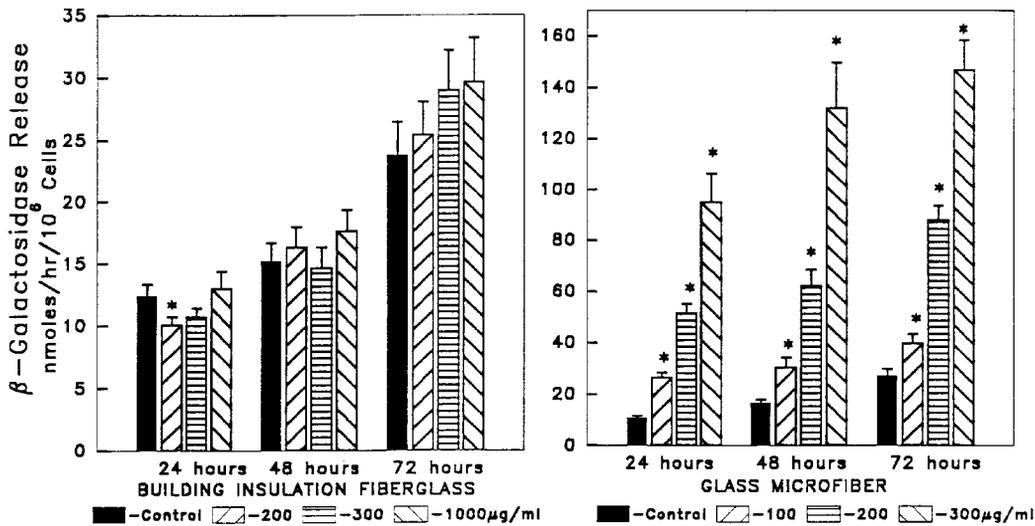
FIGURE 5. β-Galactosidase release from macrophages treated in vitro for 1–3 d with or without (control) various concentrations of building insulation fiberglass or glass microfibers. Asterisk indicates a significant increase from control ($p < .05$). Note the different scales of the y-axes.

In vitro screening also more easily allows the study of the cellular and molecular mechanism(s) of fiber toxicity and the role of chemical and physical characteristics of fibers in toxicity. There remains a question, however, as to the relevance of in vitro testing versus in vivo inhalation exposures.
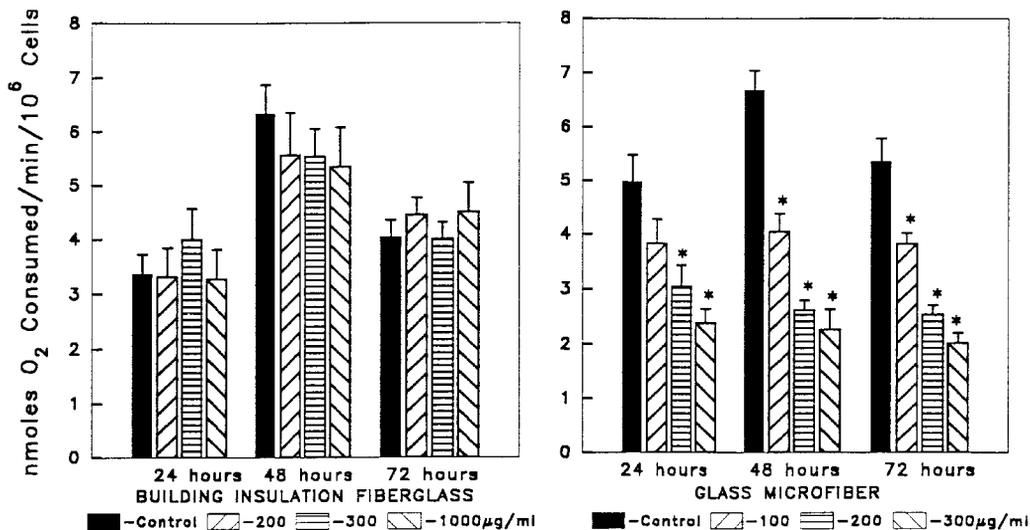

FIGURE 6. Zymosan-stimulated oxygen consumption by alveolar macrophages treated in vitro for 1–3 d with or without (control) different concentrations of building insulation fiberglass or glass microfibers. Asterisk indicates a significant decrease from control ($p < .05$).
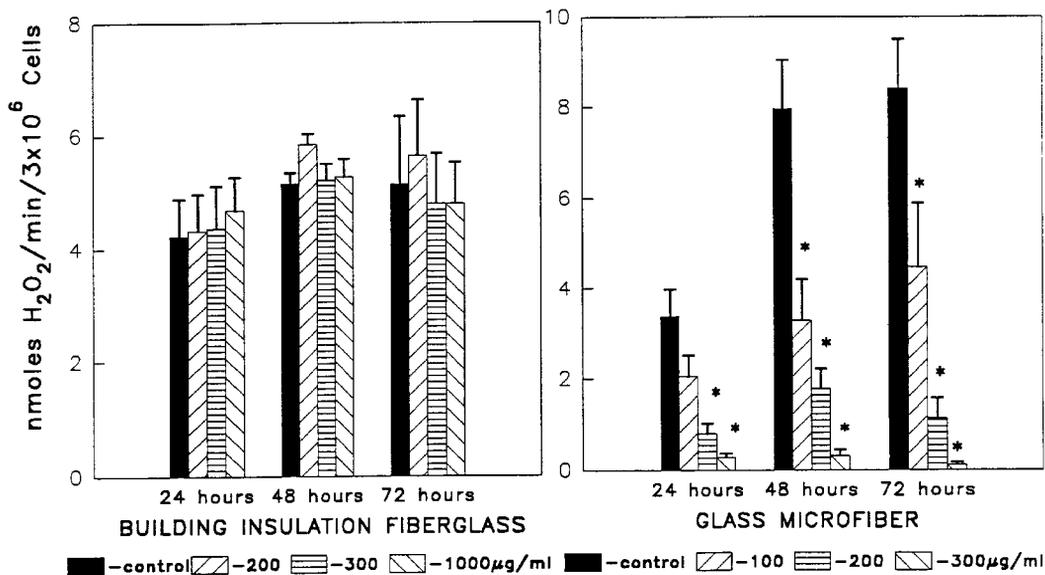
FIGURE 7. Zymosan-stimulated hydrogen peroxide release from macrophages treated with building insulation fiberglass or glass microfibers in vitro (control = no fibers). Asterisk indicates a significant decrease from control ($p < .05$). Note the different scales of the y-axes.

As reviewed by Brochard et al. (1994), a small excess of lung cancer has been noted in groups of workers exposed to some types of MMVF. The epidemiological study of human MMVF exposures is still very difficult, however. The latency of cancer and fibrosis development can be very long, as with asbestos; it may, therefore, be impossible at this time to accurately detect an increased risk from MMVF exposure in humans. Animal studies, therefore, have been used to assess the carcinogenicity and fibrogenicity of MMVF. Warheit and colleagues (1995) summarized the results of several rat and hamster inhalation studies with various classes of MMVF. Different fibers were associated with lung inflammation, fibrosis, and cancer. There is evidence that fiber dose, size, and physicochemical characteristics such as biodurability may be important in fiber pathogenicity.

A recent study by Hart et al. (1994) attempted to correlate in vitro results with those of the various inhalation studies. This group used cultures of Chinese hamster ovary cells and found that the in vitro effects did not correlate well with the in vivo studies.

The incubation of alveolar macrophages with fibers has been shown to be an accurate system to assess the toxicity of chrysotile asbestos and wollastonite (Pailes et al., 1984). The comparison of crocidolite asbestos and riebeckite, its nonasbestiform polymorph, in an in vitro system demonstrated that physical characteristics of particles may not be the sole determinant of toxic potential (Castranova et al.,

1994). Therefore the use of a potential target cell, the alveolar macrophage, may be a more appropriate assay system for the in vitro study of fiber toxicity.

The goal of the present study was to extend the use of a macrophage in vitro culture system to examine the effects of glass fibers. The focus of this study was on cell viability and function rather than genotoxicity. Alveolar macrophages were used since they are the primary phagocytes of the lung and would therefore be important in the initial response of the lung to respired fibers and directing later events in the inflammatory/fibrotic cascade. To evaluate the importance of physical characteristics in glass fiber toxicity, a thick and long fiber was compared to a short and thin fiber.

Since the goal of this study was to test the validity of this in vitro culture system, extreme-sized fibers were used to maximize potential differential responses. While the respirability of fibers is not a critical issue in vitro, if these fibers were tested in vivo size would be a consideration and fibers of respirable dimension would be neces-sary. Another consideration in choosing such large insulation fibers for this study was to examine the hypothesis of frustrated phagocytosis. Archer (1979) proposed that oxidants and enzymes leaking from alveo-lar macrophages unable to completely engulf fibers contribute to the damage that follows fiber exposure. The large insulation fibers, then, were expected to elicit this frustrated phagocytosis as they were, for the most part, too large to be engulfed by a single macrophage.

The thin microfibers were highly cytotoxic as exhibited by the loss of cell viability and membrane integrity. Glass microfibers also had a significant detrimental impact on macrophage function. All of the effects of glass microfibers were concentration dependent, with larger concentrations causing more damage. The larger building insulation fiberglass sample, on the other hand, exhibited little effect on cell via-bility and did not change macrophage function in this assay system. This study, then, supports a role of fiber dimension in toxicity.

When comparing the toxicity of fibers of different dimensions, it is important to consider fiber number and surface area as well as mass. If surface properties of fibers are important determinants of toxicity, surface area consideration is especially needed. The results of the pre-sent study are presented in terms of effects of given fiber masses. However, the glass microfibers were much thinner than the building insulation fiberglass; therefore the microfiber samples contained more fiber particles and had a greater surface area per mass than the building insulation fibers. So in comparing the relative toxicity of the two fiber types, consideration of fiber number or surface area is appropriate. The microfibers had over six times the surface area of the thick fibers. Comparing the effect of 100 μg/ml microfibers to 1000 μg/ml building insulation, a greater than sixfold difference, demon-

strates that the glass microfibers are much more toxic than the larger insulation fiberglass on an equivalent surface area basis, as well as an equivalent mass basis.

As mentioned earlier, the microfiber samples contained a greater number of fibers per mass. Even at the concentration extremes (1000 µg/ml building insulation vs. 100 µg/ml microfibers), the microfiber samples contained around five times as many fibers as the insulation samples. This fivefold difference is not that great in light of the fact that microscopic counts, by nature, are not exact. Furthermore technical considerations made it impractical to increase the insulation concentration beyond 1000 µg/ml. This is one reason cultures were extended to 3 d. For example, a comparison of d 1 microfiber results to d 3 insulation results allows the assessment of whether lower fiber numbers cause toxicity when cells are exposed to them for prolonged periods.

The results of this study, then, suggest that short and thin glass fibers are more toxic than long and thick fibers in vitro. While the current investigation did not assess factors such as biodurability, it does implicate fiber dimension as an important determinant of toxicity. Future studies will attempt to explore further the role of fiber dimension by independently assessing fiber length and diameter. This will be done, for example, by comparing the effects of fibers of identical length but differing diameters. Additionally, future study using this system could be directed at studying cytokines, which are mediators of inflammation and fibrosis. Similar in vitro systems have demonstrated that macrophages release cytokines and eicosanoids when incubated with refractory ceramic fibers (Leikauf et al., 1995). Such study of cytokine release following fiber exposure and the investigation of how cytokines direct inflammation, fibrosis, and possibly carcinogenesis may help finally elucidate the mechanism of fiber toxicity.

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