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In Vitro Studies on the Biologic Effects of Fibers: Correlation with *In Vivo* Bioassays

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In vitro **studies employing organ cultures, primary cell cultures, cell lines, and bacterial systems have been used to assess the toxicity, mutagenicity, and carcinogenic potential of asbestos and nonasbestos fibers. These experiments have been useful in defining mechanisms contributing to the causation of fiber-associated lung diseases. Long (> 8 μm), thin asbestos fibers are more active *in vitro* than short (≤ 2 μm) fibers or nonfibrous particles, an observation supporting the importance of fiber dimension in disease. Although *in vitro* bioassays cannot evaluate characteristics such as clearance and/or durability of fibers which may be critical determinants of fiber toxicity in lung, they can be used both to address dosimetry at the cellular level (i.e., number of fibers per cell that elicit a measurable biologic end point) and to evaluate preventive approaches to fiber-induced cell injury. Development of *in vitro* models employing target cells of the lung, i.e., mesothelial cells, tracheobronchial epithelial cells, and lung fibroblasts, as well as carefully characterized preparations of fibers and particles, will be necessary to evaluate whether *in vitro* bioassays are amenable to predicting the pathogenic potential of synthetic and naturally occurring fibers comparatively.**

Introduction

The interactions between fibers and cells *in vitro* have been studied over the past 10 years in an effort to understand the mechanisms of cell injury and lung disease (*1–3*). Most of these experiments have employed various types of asbestos, fibers associated with the development of mesothelioma, bronchogenic carcinoma, and asbestosis in asbestos workers (*4*). Because asbestos is a documented carcinogenic and fibrogenic agent in the workplace and because a ban on usage of asbestos in the United States has been advocated by the Environmental Protection Agency, a number of naturally occurring and synthetic fibers have been proposed as substitutes for asbestos in industry. Thus, it would be useful, in view of the expense of long-term toxicologic studies using rodents, to determine if *in vitro* bioassays could provide an indication of the biologic activity and mechanisms of action of nonasbestos fibers.

Work with asbestos *in vitro* has elucidated physicochemical features of asbestos important in induction of toxicity, proliferation, and morphologic transformation of cells *in vitro*. In this paper, these data are summarized and compared with the results of whole animal studies.

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Results and Discussion

Studies on Cytotoxicity of Asbestos and Nonasbestos Fibers

The mechanisms of asbestos-induced cytotoxicity were first explored in red blood cells (*5*) and later in cell cultures and tissues from the respiratory tract maintained in culture. A recent volume addresses toxicity of asbestos in detail (*6*). Several points are important to this discussion. First, the geometry and dimensions of fibers are important in eliciting cytotoxic responses. In studies reported thus far, longer, thinner fibers (i.e., those with lengths ≥ 8–10 μm and diameters ≤ 0.25 μm) of both asbestos and glass are more toxic to cells than short, blunt fibers or nonfibrous particles (*7,8*). Cytotoxicity of both attapulgite and sepiolite fibers is directly related to fiber length in cultures of human lung carcinoma and Chinese hamster lung fibroblasts (V79-4 cells) (*9*), and inhibition of growth or release of cytoplasmic enzymes is not observed in a macrophagelike cell line (*10*) or hepatocytes (*11*) after exposure to short fibers (≤ 1.0 μm length).

A difficulty in the interpretation of *in vitro* studies is the introduction of fibers on a mass (milligram of fibers per dish) rather than a numerical (numbers of fibers per dish) basis. Recently, the cytotoxic effects of chrysotile, crocidolite (both U.I.C.C. reference samples), and two

samples of erionite fibers were compared in V79 fibroblasts using both these approaches (*12*). No differences in cytotoxicity were observed between groups when fibers were evaluated on a mass basis, but both samples of erionite were several orders of magnitude more potent in causing cell death than crocidolite, a fiber of intermediate potency, or chrysotile, the least toxic fiber, when equal numbers of fibers were evaluated. These results correlate with the higher tumorigenic potential of erionite in comparison to both types of asbestos in rodent inhalation experiments (*13*).

The role of active oxygen species (AOS) in fiber-induced cell damage is a burgeoning area of exploration in several laboratories. These reactive species may be second messengers of asbestos-elicited cell damage, as they can cause changes in membrane fluidity, lipid peroxidation, and breakage of DNA. Whereas short fibers and particles are incorporated into phagolysosomes, longer fibers are phagocytosed unsuccessfully by cells *in vitro*, a process liberating AOS (*14*). The importance of AOS in contributing to asbestos-associated toxicity *in vitro* has been confirmed by studies in which asbestos and antioxidants have been added simultaneously to a number of cell types (*15*). Under these circumstances, asbestos-induced cell death is ameliorated. Extracellular mechanisms may also be important in the generation of AOS by asbestos as asbestos fibers in cell-free solutions of H_2O_2 or physiological saline generate superoxide ($O_2^{\bar{}}$ and hydroxyl ($OH^{\cdot}$) radicals by redox reactions occurring on the fiber surface (*16–18*). Recently, the generation of AOS has been reported by rockwool and glass fibers (*19*). However, their contribution to toxicity of these fibers is unclear, as scavengers of AOS fail to prevent cytotoxicity of glass fibers in tracheobronchial epithelial cells (*20*).

Studies on Cytogenetic Effects and Morphologic Transformation of Asbestos and Nonasbestos Fibers *In Vitro*

Whether *in vitro* models can be used to predict the carcinogenic potential of fibers is questionable because results with asbestos in bacterial and cell transformation systems are inconsistent. In comparison to the other 21 agents designated as group 1 carcinogens by the International Agency for Research on Cancer, asbestos (the type was not specified) and conjugated estrogens were defined as nongenotoxic in both the Ames test and rodent bone marrow bioassay, a test detecting chromosomal aberrations and micronucleated erythrocytes (*21*). Unlike most chemical carcinogens, asbestos tests negatively in bacterial mutation assays (*22,23*) and is not mutagenic in liver epithelial cells (*24*) or Syrian hamster embryo fibroblasts (SHE) (*25*). However, morphologic transformation is achieved in SHE cells after addition of crocidolite and chrysotile, glass fibers, and nonfibrous silica (albeit at lower concentrations than either asbestos or fiberglass). The transformation frequencies of asbestos and glass fibers vary appreciably from laboratory to laboratory (*25–28*). In the SHE assay, longer, thinner fibers of both asbestos and glass are more potent in the induction of morphologic transformation (*26*), an observation consistent with the results of cytotoxicity studies in a variety of cell types. Nonasbestos fibers have, for the most part, not been evaluated in bacterial mutation or cell transformation systems (*29*). However, glass, erionite and potassium octatitanate (Fybex) fibers cause morphologic transformation of rodent fibroblast cell lines.

Studies on Proliferation of Cells

Asbestos appears to resemble a tumor promoter in the development of bronchogenic carcinoma, both *in vitro* (*30*) and *in vivo* (*31*). The induction of cell proliferation by various types of asbestos and nonasbestos fibers in cell and organ cultures of rodent tracheobronchial epithelial cells (*8,30,32*) and the appearance of squamous metaplasia in response to fibers in the latter bioassay (*30,34*) have been useful in defining the characteristics of fibers important in causing altered proliferation of epithelial cells, the progenitors of bronchogenic carcinoma. Results are comparable with the *in vitro* toxicity and transformation assays. When assayed at comparable concentrations (on a mass basis), long ($\geqslant$ 8 μm) fibers (both asbestos and nonasbestos) cause enhanced incorporation of tritiated thymidine, increased biosynthesis of polyamines (growth regulatory molecules necessary for cell division to occur) and increased amounts of squamous metaplasia and keratinization in organ cultures of hamster trachea. In contrast, nonfibrous particles do not cause these changes, whereas short ($\leqslant$ 2 μm) fibers must be introduced at severalfold higher amounts than long fibers to achieve these effects.

Correlations with *In Vivo* Studies

The importance of fiber size in the induction of mesotheliomas and pleural sarcomas in experimental animals has been known for several years (*35,36*). Both intraperitoneal and intrapleural injection studies have been useful in indicating fibers with marked carcinogenic potential, i.e., those fibers longer than 8 μm with diameters of less than 0.25 μm, or Stanton fibers. The Stanton hypothesis also is supported by the results of recent inhalation studies in which long and short ($\leqslant$ 5 μm length) preparations of chrysotile and amosite asbestos have been administered to rats (*37*). In these experiments, long fibers of chrysotile and amosite caused both asbestosis and pulmonary tumors. Conversely, short fibers of amosite produced no lung disease, whereas short chrysotile invoked a small proportion of asbestosis and malignancies attributed by the author to contamination of the short chrysotile preparation by some longer chrysotile fibers. Long amosite fibers, in comparison to short amosite and titanium dioxide, were associated with a more pronounced inflam-

matory response and increased activation of macrophages after injection intraperitoneally into mice (*38*).

Future Directions

As emphasized previously, *in vitro* models have been valuable in defining mechanisms of interaction between fibers and cells and dimensions of fibers important in eliciting these biologic responses. However, the crude dosimetry and poorly defined characteristics of fibers used in these studies have not allowed precise comparison of individual fiber types over a range of concentrations. Recent studies by Palekar and colleagues (*12*) suggest that cytotoxicity of fibers, if compared on a numerical basis, can be used to predict the pathogenicity of fibers. Including positive (i.e., erionite) or negative controls (i.e., inert or nuisance dusts) for evaluation will be essential to testing this hypothesis further.

The choice of cell type to be used in bioassays with fibers also will be important. Thus far, rodent fibroblast lines have been used in most experiments to evaluate toxicity and morphologic transformation after exposure to fibers. These cells may be less sensitive to the *in vitro* effects of asbestos and nonasbestos fibers than mesothelial cells or tracheobronchial cells, the target cells of asbestos-induced tumors in lung. Indeed, studies by Lechner and colleagues (*39*) have shown that human mesothelial cells *in vitro* are 100- and 1000-fold more sensitive to the cytotoxic effects of asbestos than human tracheobronchial epithelial cells or lung fibroblasts, the most insensitive cell type. The development of a human mesothelial cell model that can be passaged continuously (*40*) may be a promising tool for future work in this area.

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