In Vitro Assessment of Biopersistence Using Mammalian Cell Systems

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Biopersistence of fibers in the respiratory airways is a concept including both the physical durability of the fibers and their chemical stability. Physical durability results from several events of diverse origins: fiber etopurization by the lung clearance mechanisms, internalization by scavenger cells and fiber splitting. Fibers residing in the lung milieu will be attacked and modified chemically, structurally, and physically (size and shape). Fiber toxicity, which is very likely to be dependent on physical fiber characteristics, will also be dependent on the duration of the fiber's stay in the tissue. Biopersistence, therefore, will be a key issue in determining fiber toxicity. So far, few in vitro systems have been used to study parameters involved in biopersistence. However, examples exist of investigations of fiber phagocytosis by mammalian cells in culture, either by macrophages, or epithelial or mesothelial cells, and studies have also been reported of the fate of internalized fibers in relation to fiber dimensions and chemical stability, especially within macrophages and mesothelial cells. The methods will be presented and discussed to determine to what extent the development of in vitro biophysical models could help in determining those parameters, known or thought to be relevant to fiber persistence. — Environ Health Perspect 102(Suppl 5):55-59 (1994)

Key words: biopersistence, epithelial cells, durability, in vitro cell systems, macrophages, man-made vitreous fibers, mesothelial cells, mineral fibers, particle internalization, solubility

Introduction

The concept of biopersistence is relatively new in research on the effects of respirable synthetic fibers and minerals. Its importance in solid particle toxicity has been developed mainly from biological studies carried out with man-made vitreous fibers (MMVF). But even in the early experiments on the effects of asbestos fibers on cells and animals, the notions involved in biopersistence were considered important in accounting for the effects of this kind of particulate matter.

Biopersistence of a particle can be defined as a parameter resulting from two factors, its chemical instability and its clearance. Some examples of the instability of asbestos fibers include the reported release of metal constituents of chrysotile administered in experimental animals (1) and the solubility in vivo of other fibers as demonstrated by a loss of constituents of amphiboles and MMVF during retention in the lung (2-4). Particle clearance also has been studied as a measure of physical persistence—the actual amount of fibers remaining in the tissue (5-8).

The concept of biopersistence currently is a key concept in the toxicity of mineral or synthetic fibers, although its meaning is not always well defined. The term durabil- ity used by some authors (9) is more related to chemical instability. Durability is generally considered a necessary attribute of a fiber presenting a health hazard, but how it is measured is not always clear (10). Clearance, and solubility, also are considered but they refer, variably, to short- or midterm clearance (5,8), half-time (7), or long-term persistence assessed by lung burden (11,12). Clearly, therefore, the notion of biopersistence cannot be said to be well defined; neither are the methods of assessment sufficiently standardized to establish to what extent toxicity and carcinogenicity depend on biopersistence. However, this does not mean that clearance and solubility are unimportant events in dust-related diseases.

Because of the chemical instability of many particle types in mammalian tissues, the first studies on the biopersistence of particles investigated their solubility characteristics. In vitro acellular systems have been developed to study the release of chemicals from fibers in conditions close to the physiological status (13-16) found in an organism. The conclusions are often difficult to extrapolate because many compartments of different compositions exist in an organism.

The clearance of particles can only be investigated in in vivo studies. The assays may, however, be difficult to interpret, especially with fibers, since it may be expected that while clearance would decrease the number of intrapulmonary fibers and thus reduce the risk, solubilization might actually increase the number of critical fibers. Complex phenomena may occur, since some fibers would be both split as short as shortened in vivo (17). Between in vivo studies and studies in in vitro acellular systems, in vitro cell systems offer a bridge that can provide a close assessment of biological likelihood, and an indication of the mechanisms of action of the particles in different parts of the organism. So far, few investigations of biopersistence have been made in in vitro cell systems. This article will summarize the methods that have been applied and will suggest further research to resolve the issues considered here.

Assessment of Biopersistence in In Vitro Cell Systems

Physical Biopersistence

In vitro cell systems should make it possible to investigate particle clearance. Many studies have indeed been carried out to investigate particle internalization, but few of them have been quantitative. Morphological studies have been performed mainly by scanning and transmission electron microscopy, with macrophages, epithelial, and mesothelial cells (18-23). No systematic study has compared the ability of different cell types

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to ingest a particle of a given type. It has been found generally that both epithelial cells and macrophages may ingest particulate matter. Less clear-cut results have been obtained with nonembryonic fibroblasts; both an absence of internalization (24) and an easy uptake have been reported (25,26). Fibers longer than the cell and extruding fibers often are noted with different cell types (19,21–23). Ex vivo studies have confirmed that particle internalization occurs in vivo (27–29). Quantitative determination of internalized particles is difficult to achieve, but it has been reported with Syrian hamster embryo cells treated with MMVF (30) and rat tracheal epithelial cells treated with chrysotile and crocidolite (31).

While internalization of particles has been widely studied, phagocytosis has not always been looked for where, within the phagocytic vacuole of the lysosome, degradation of the fiber occurs. Chrysotile fibers are readily phagocytized by rat pleural mesothelial cells involving the lysosomal enzyme degradation (20). This is important since in the internalization process, particles are exposed to different environments—extracellular, intraphagosomal or cytoplasmic (32–34), each differing in its chemical attack (Figure 1). Because of these differences in the chemical nature of the different parts of the cell, studies of particle internalization and phagocytosis are an essential preliminary to the study of the biopersistence of a particle.

Because of the relationship of particle dimensions to fiber carcinogenicity as demonstrated by Stanton et al. (35) and confirmed by others (36–38), the influence of dimensions on the fate of intracellular fibers following internalization also has been investigated. In several studies a perinuclear localization of the fibers has been reported in certain cell systems (30,31,39,40). Short crocidolite fibers have been found to concentrate in the perinuclear region (41), a finding also observed with chrysotile (42). Moreover, studies carried out with Syrian hamster embryo cells have indicated that long fibers are internalized preferentially to short fibers (30). All these observations are important, since they suggest that when cells are exposed to fibers of variable size distribution, long fibers would be ingested preferentially. The longest fibers might then interact with the chromosomes and be involved in cell transformation while the smallest fibers, if internalized, would follow saltatory cell movements (41).

These studies all indicate that particle physical biopersistence may be assessed in in vitro cell systems, in which the amount and size of the particles taken up are determined, both for macrophages and for epithelial cells, and possibly also for fibroblasts. This would give a measure of the ability of the macrophages to clear the particles, and of the likelihood that the epithelial cells would suffer chromosome damage, leading to cell transformation. In addition, if fibroblasts were used, the interstitial migration could be assessed.

These studies can be performed by classical morphological electron microscopy, but they will be significant only if fibers of relevant dimensions are used and if the experimental conditions are well defined. These conditions include the nature of the culture media and the addition of certain biological macromolecules (surfactants, mucus components; see below).

### Chemical Biopersistence

The chemical stability of particles in in vitro cell systems has been investigated in several studies. Physical studies, in which macrophages, epithelial, or mesothelial cells have been used are summarized in Table 1.

In all experiments both fibers and nonfibrous material displayed chemical instability. When the same particle has been studied in different cell systems, a difference has been observed between the efficiency of macrophages and epithelial cells (43,47), possibly due to a pH effect, among others (see below). These studies emphasize the difference between the phagosome compartment of macrophages and epithelial cells, in particular their pH.

Certain in vitro studies (45,46) have shown a good correlation with in vivo studies. However, in one study, using an acellular dissolution system (45), there was no correlation neither with the in vivo study nor with the in vitro cell system.

A wide range of methods of analysis of particle solubility in cell systems has been used including radioactive tracing, microprobe analysis, atomic adsorption and size calculation. It would be of interest to use standard particles to compare the limits of the different methods of analysis applied to such a standard.

In summary, these results indicate that chemical biopersistence can be assessed with in vitro cell systems. Different cell types and methods of investigation could be used and would merit development. At present, insufficient data are available to compare the results obtained in in vitro acellular systems with those in cellular systems. It would be useful to make the comparison using fibers that had been tested in animals.

### The Further Development of in Vitro Cell Systems

Certain factors have to be considered for the development of in vitro cell systems—the particle dimensions, the nature of culture media, and the cell viability. (These factors also may need to be considered in the development of in vitro acellular systems.)

#### Particle Dimension

Investigation of the potential effects on the respiratory airways is significant only if particles of "respirable" dimensions are used. This is also true in the study of biopersistence, where the solubility of a particle is dependent on its dimensions (49,50). If very long fibers encounter a macrophage or an epithelial cell, a combination of both intracellular and extracellular solubility may come into play; in macrophages the dissolution rate is higher for smaller particles (Table 1). Both the dimensions of fibers and the method of analysis must be carefully chosen. The medium used to cul-

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**Figure 1.** (A) Schematic representation of the different cell components. (B) Schematic representation of particle fate after deposition in the respiratory airways. A particle can be exposed to different environments, and may encounter different extracellular media. If free particles are internalized, they are first in contact with the cell membrane, then phagocytosis may occur and the particles become enclosed in a phagocytic vacuole, and so may be cleared. If particles are toxic, cell death may occur exposing the internalized particles to cytoplasmic and extracellular medium, possibly modified by the release of necrotic cell factors.
ture the cells is important and contains proteins and lipids; therefore it is necessary to determine fiber dimensions in the plain medium used to culture the cells.

Nature of the Culture

Many additives are present in culture media to preserve cell homeostasis. These include proteins, which are liable to be adsorbed at the surface of the particles, modifying the susceptibility of the particle to attack (51,56). The addition of proteins, as either albumin or serum, is not recommended, since proteins are already present in the interstitial fluid and in the cellular fluid, where a concentration of 300 mg/ml may be found (57).

Mucus is present at the bronchus surface, and surfactant, which contains phospholipids, at the alveolar surface. In vivo, particles may interact with these macromolecules, which will cover partly or totally the particle surface (55). This could alter the parameters governing biopersistence, and may affect deposition (58) or biological effects (59).

Cell Viability

When solubility is being determined by chemical measurement of elements released in the cell, the effect of cell death is critical, since it is associated with a release into the extracellular medium of intracellular components including metals. If fiber dissolution releases toxic elements in the cell—iron or silicon for instance—this may cause cell death (60–64), making it difficult to decide if the elements detected in the filtrate are due to an action of the culture medium on the fiber or arise from the intracellular component.

In conclusion, biopersistence can be studied in in vivo cell systems, which, if they are correctly standardized, should make very efficient assays. Human and animal bodies are “black boxes” where many different environments coexist, each involved in different aspects of biopersistence. The data obtained so far show that in vitro experiments can be designed to investigate all these aspects, provided the necessary basic research is done to support their development.

Table 1. In vitro solubility with cell systems.

| Cell types | Particle | Solubility in medium | Solubility in cells | Method | Reference |
|------------|----------|----------------------|---------------------|--------|-----------|
| Rabbit alveolar macrophages (AM) | UICC Rhodesian chrysotile, 2–4 μg/cm² | Not tested | Yes | AM > PMC | Microprobe analysis Si/mg S/mg |
| Rat pleural mesothelial cells (PMC) | MnO₂, 0.1–0.5 μm; 10 μg/ml | Yes | Yes | Human = rabbit | Mn in filtrate atomic absorption |
| Human AM | Be metal powder | Yes | Yes | Hot-pressed > powder | Be in filtrate atomic absorption |
| Dog AM | 57Cr²⁺, < 2 μm | Small > large | Yes | Human < dog Small > large | 57Cr in filtrate atomic absorption |
| Rat AM | Glass fibers, 35 μg/ml | Not tested | Yes | AM > NEC | Particle size |
| Rat nasal epithelial cells (NEC) | Glass fibers, 200 μg/ml | Yes | Yes | Si > Fe, Al | Elements in filtrate atomic absorption |

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