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# In Vivo Evaluation of Chemical Biopersistence of Nonfibrous Inorganic Particles

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The lung's response to deposited particles may depend upon the physical-chemical properties of the particles, the amount initially deposited, and the persistence of the particles. Clearance involves mucociliary transport as well as the action of phagocytic cells in nonciliated regions of the lung. Depending on the animal species studied, particle type, and particle load, inorganic materials are ingested by macrophages on alveolar surfaces with half-times of 0.6 to 7 hr. Particle-laden macrophages may migrate to airways, but we believe that an important mechanism of clearance is the dissolution of particles within alveolar macrophages and the subsequent translocation of dissolved materials to the blood. Particle dissolution *in situ* has long been recognized but was often thought to be carried out extracellularly in the alveolar lining layer, airway mucus, or interstitial fluid. However, many particles such as cobalt oxide or iron oxide which dissolve very little in simulated lung fluid, are solubilized more rapidly within alveolar macrophages. Clearance of particles from the lungs can be followed by a number of techniques, both invasive and noninvasive. The approaches vary in expense and resolution, and can be directed toward quantifying mechanical removal of particles versus their intracellular dissolution. Noninvasive methods permit repeated measurements of particle retention in the lungs of the same animal or human and thus allow replications and serial measurements. Greater precision with respect to the sites of retention and redistribution is achieved with quantitative morphometric methods that utilize fixation followed by physically dividing the respiratory tract into individual pieces. Microwave drying or slam-freezing can eliminate the possibility of significant particle redistribution or loss of particles and dissolved elements during tissue processing. Detection of particles and therefore evidence of clearance can rely upon any distinctive property of the aerosol. Particles may be radioactive, fluorescent, or magnetic, or may have a characteristic visual appearance. Detection techniques include radiography, analyses of radioactivity, magnetometry, and microscopic approaches such as fluorescence and confocal microscopy, X-ray emission analysis, and electron energy loss spectrometry (EELS). Using these approaches, considerable evidence has been accumulated to conclude that particle dissolution *in situ* within alveolar macrophages and subsequent absorption by the circulation, rather than bulk transport, is the dominant mechanism for the long-term clearance of many insoluble minerals from the lungs. — Environ Health Perspect 102(Suppl 5):119–125 (1994)

Key words: clearance mechanisms, dissolution, magnetopneumography, microwave drying, slam-freezing, electron energy loss spectrometry, EELS

## Introduction

The lung's response to aerosols depends not only on the amount of particles deposited but also on the amount retained over time. Retention is the amount of material present in the lungs at any time and equals deposited mass minus cleared mass. An equilibrium concentration of retention is reached during continuous exposure to aerosols when the rate of deposition equals the rate of clearance. Clearance may represent either bulk, mechanical transport from the airways and parenchyma, or solubilization and the removal of individual ions or molecules by body fluids. Both the amount of particles retained within a specific lung region over

time and those properties of the retained particles determine the magnitude of the response.

Deposition influences subsequent fate since where particles deposit in the lungs determines the mechanisms used to clear them, how fast they are cleared, and the amount retained over time. Examples of the implications of particle characteristics on integrated retention have been described (1) based on a model developed by the Task Group on Lung Dynamics (2). Calculations show that the total amount as well as the distribution of retained dose between nose and pharynx, trachea and bronchi, and the pulmonary and lymphatic compartments are dramatically altered by particle size and solubility.

This article begins with a brief review of the fate of particles deposited either in the conducting airways or on alveolar surfaces. The mechanisms responsible for particle clearance are summarized with particular emphasis on the fate of nonfibrous inorganic particles. We then discuss a number of new methods that are particularly useful in describing the fate of particles deposited in the respiratory tract.

## Airways: Mucociliary Transport

Many inhaled particles will deposit on the liquid-covered conducting airways that serve as conduits for inspired air. Such airways are well populated with cells containing cilia. These ciliated epithelial cells of the airways and nasal passages are covered by a complex multilayered mucus blanket. Early models describe a top, high-viscosity gel layer and a bottom, low-viscosity serous layer. The gel mucus blanket along with any less-soluble particles that deposit on it are moved toward the pharynx by the cilia. Also present in this moving mucus carpet are cells and particles that have been deposited there or transported from the nonciliated alveoli to the ciliated airways. Mucus, cells, and debris from the nasal cavities and lungs meet at the pharynx, mix with salivary secretions, and enter the gastrointestinal tract after being swallowed. In humans the ciliated epithelium extends from the trachea to the terminal bronchioles. The particles are removed with half-times of minutes to hours but may be retained much longer. The speed of the mucus blanket is faster in the trachea than

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in the small airways and is affected by factors influencing either the cilia or the amount and quality of mucus. In the airways there is less time for solubilization of slowly dissolving materials because of mucociliary transport. In contrast particles deposited in the nonciliated compartments may have longer residence times.

Ciliary action may be affected by the number of strokes per minute, the amplitude of each stroke, the time course and form of each stroke, the length of the cilia, the ratio of ciliated to nonciliated area, and the susceptibility of the cilia to intrinsic and extrinsic agents that modify their rate and quality of motion. The characteristics of the mucus layer are critically important. The thickness of the mucus layer and its rheological properties may vary widely. In the peripheral bronchioles, for example, the gel layer is less evident. Many of the factors that influence mucociliary clearance have been reviewed (2,3).

Many investigators have estimated mucociliary transport from whole lung clearance curves. These curves are generated by monitoring the amount of radioactivity in the lungs over time (hours to days) following the inhalation of a radiolabeled aerosol. This method, when first used, indicated that the clearance curve could be divided into a fast phase and a slow phase (5). The fast phase is complete within 24 to 48 hr and generally has been attributed to tracheobronchial clearance; the slow phase has been attributed to alveolar clearance (6). However, later evidence indicates that clearance from the airways is incomplete in the first 24 hr (7); this may be even more pronounced in patients with lung disease. Later, it was noted that particles instilled into the trachea could be sequestered in epithelial cells (8). Latex particles were found trapped in the periciliary sol fluid below the gel mucus blanket (9). The particles may have been displaced there by surface tension forces created by surfactant (10,11). Both fast and slow phases of clearance were noted even in humans given a bolus of aerosol particles delivered only 45 ml beyond the larynx (12). When radiolabeled particles were delivered with a bronchoscope to airway generations 6 to 10 in dogs, it was found that about 20% of the particles were cleared slowly (12).

Cough also operates to remove particles trapped in mucus when mucociliary transport is compromised or overwhelmed. After a series of coughs one often notices an accumulation of mucus in or just below the pharynx. How did the mucus get there?

Was it pushed along as a film or carried suspended in the airstream? There is detailed information concerning the flow of gas during cough (13), but we also need to consider the flow of mucus. The importance of mucus rheology to mucus transport is appreciated, but for cough we also need to consider coupling between gas and liquid and how these are affected by mucus viscosity, surface tension, elasticity, and thickness (15). Yet, we do not know precisely which rheological properties are appropriate. What characteristics of mucus enhance mucus transport? The problem is complicated by the fact that mucus is a highly non-Newtonian material. The viscosity of mucus varies over three orders of magnitude as the shear rate varies, so one needs to know what shear rates exist *in situ* during cough to evaluate these strategies properly.

### Bronchial Circulation and the Redistribution of Deposited Dose

Ions and molecules from soluble particles that land on the airway surfaces and dissolve can pass through the mucus layer and enter the circulation. So may solubilized components from particles that enter airway cells and are dissolved intracellularly. These materials then can leave the lungs, but some portion also may be carried via the circulation to other lung regions. Although the extent of this redistribution by the lung's circulation is still unclear, it could deliver solubilized materials beyond initial deposition sites and lead to accumulation elsewhere, such as sites in the parenchyma or airway cartilage.

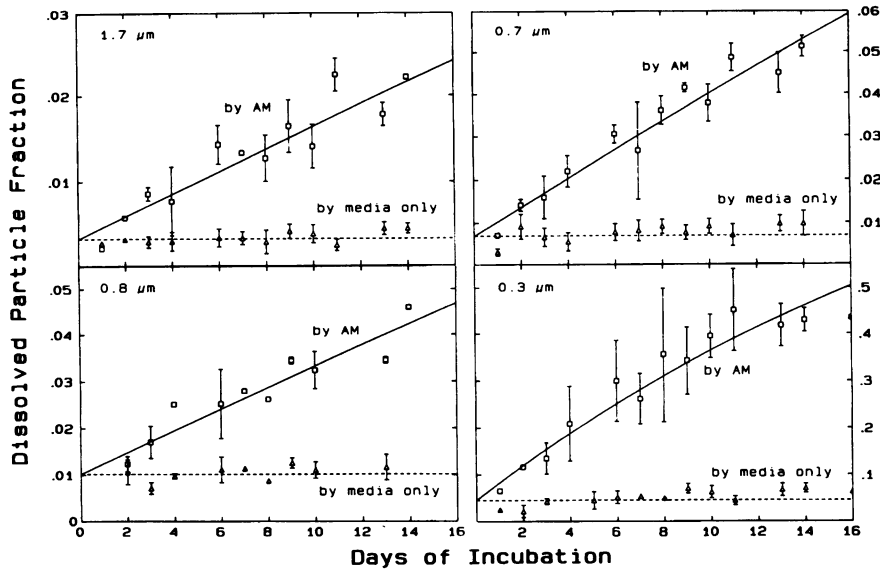
The lung has two blood supplies: the pulmonary circulation, which nourishes the parenchymal tissues, and the smaller bronchial circulation, which nourishes the airways from the trachea to the terminal bronchioles as well as the associated nerves, lymph nodes, and supporting tissues including the esophagus (16). As the main bronchial arteries enter the lungs they follow the branching pattern of the airways. From the arteries arise a series of arterioles and then capillaries that form an extensive vascular bed in the mucosa under the airway epithelium. The capillary bed drains into numerous postcapillary venules in the mucosa and then to underlying collecting venules and veins (17,18). In sheep, rabbits, and possibly humans, the collecting venules in the submucosa exist as long sinuses that run longitudinally along the airways (18,19). Thus soluble materials need only travel across the mucus and

epithelial cell layers to reach the subepithelial capillary network and have access to the bronchial circulation. Once in the mucosal and submucosal circulation, materials can diffuse back into the interstitium and lumen in response to local concentration gradients. Transport of materials toward peripheral airways and acini is favored since in the bronchi both the subepithelial capillaries and venule sinuses tend to be oriented longitudinally; this favors drainage toward the pulmonary vasculature (18).

The fate of dissolved materials also depends upon where inhaled particles land in the airways (20). The veins draining the extrapulmonary airways join the systemic venous return to the right heart; most veins from the intrapulmonary airways join the pulmonary circulation via bronchopulmonary anastomoses and return to the left heart (16). As a result, material that dissolves in the extrathoracic airways could either enter the venous sinus network or be cleared from the lungs but then reappear, although in a more diluted state, in the lung parenchyma within the pulmonary circulation. Materials that enter the intrapulmonary circulation have the best chance to be redistributed to both airway and the parenchymal tissues. However, materials that enter the circulation beyond the terminal bronchioles are not redistributed to the airways. In the normal lung the bronchial circulation is only 1 to 2% of the total cardiac output. Yet blood flow increases with general lung injury and in the presence of inflammatory mediators such as histamine, platelet activating factor, and prostaglandin F<sub>2</sub><sup>α</sup>; in some chronic disease conditions bronchial blood flow may be as much as one third of the total cardiac output (16,18).

### Lung Parenchyma

Particles that deposit in the nonciliated portion of the lungs are cleared mechanically, by dissolution, or by the combined action of these processes. During mechanical clearance most particles move toward the ciliated region within alveolar macrophages. Some particles also may move as a result of a surface tension gradient due to surfactant that extends from the alveoli to the airways (21). Other particles may enter the interstitial tissues (7), particularly when deposited levels are high (22). Macrophages are credited with keeping the alveolar surfaces clean and sterile. These cells rest on the continuous epithelial layer of the lungs. It is their phagocytic and lytic potentials that provide most of the microbicidal properties of the lungs. Rapid endo-



**Figure 1.** Mean dissolved  $^{57}\text{Co}$  fractions of the initial  $^{57}\text{Co}_3\text{O}_4$  particle mass averaged (with standard deviations) for each day of incubation. Data are presented for four different-sized porous particles phagocytized in canine AM or in cell-free media. Note that the vertical scales differ for the four particle sizes. From Kreyling et al. (37); reprinted by permission.

cytosis prevents particle penetration through the alveolar epithelia and facilitates alveolar-bronchiolar transport. The biology of lung macrophages has been summarized (23–26) and a review has emphasized that many kinds of lung macrophages exist (27). These include alveolar, airway, connective tissue, pleural, and intravascular macrophages.

Particles can enter the lung connective tissue by translocation through type I pneumocytes (7,28). There are also limited data that indicate that a few alveolar macrophages can carry particles into the interstitium as well (29). Once in the interstitium most particles are phagocytized by interstitial macrophages; some particles may remain free temporarily. Interstitial particles are cleared eventually either through dissolution or transportation to regional lymph nodes through lymphatic pathways. In pulmonary lymph nodes, particles are cleared with half-lives ranging from a few days to thousands of days depending on their solubility.

### Intracellular Particle Dissolution

There is abundant evidence that inorganic particles such as metal oxides are ingested by alveolar macrophages (AM). However, the subsequent fate of these particles is controversial. Particle dissolution in the respiratory tract has been recognized as an important clearance pathway (30,31), but many authors have suggested that dissolu-

tion occurred extracellularly. For example, attention has been focused on the properties of the alveolar lining layer, airway mucus, or interstitial fluid. This has led to experimental models estimating dissolution rates of particles in serum or simulated lung fluids (32). There is growing evidence that such models are inappropriate. Studies in dogs showed that various forms of inhaled homogeneous cobalt oxide particles present after several weeks cleared primarily by dissolution of the particles in the lungs, followed by translocation of the dissolved Co to the blood (33,34). The rate of dissolution of these cobalt oxide particles depended on the physical chemical properties of the particles such as size, density, specific surface area, and chemical composition. If the particles were cleared by reproducible mechanical transport systems, one would have expected their clearance rates to be more similar. These particles dissolve negligibly in serum lung fluid simulants, and in saline solutions (35,36).

Studies have been carried out on the dissolution of a family of uniform cobalt oxide particles within monolayers of human and canine alveolar macrophages that were maintained alive and functional *in vitro* for more than 2 weeks (37). Complete phagocytosis of moderately soluble, monodisperse  $^{57}\text{Co}_3\text{O}_4$  test particles of four sizes was obtained by optimizing the cell density of the monolayer and the particle-to-cell ratio. The soluble fraction of the initial particle mass that was soluble

increased over time when the particles were ingested by AM but remained constant at a very small level when in culture medium alone (Figure 1). Smaller particle sizes had a faster characteristic intracellular dissolution rate constant than did larger particles. The dissolution rates differed between AM obtained from two human volunteers and those obtained from six mongrel dogs (38). These *in vitro* dissolution rates were very similar to *in vivo* translocation rates previously obtained from human and canine lung clearance studies after inhalation of the same or similar monodisperse, homogeneous cobalt oxide test particles as part of an interspecies comparison of lung clearance including seven species (39). We believe the data show convincingly that an important clearance mechanism for inhaled aerosol particles deposited in the lungs is *in vitro* dissolution. This is particularly true for acid soluble particles and at later time points when the efficiency of mechanical clearance is diminishing.

### Measuring Particle Retention

Once particles have been introduced into the respiratory tract, the challenge must be faced of quantifying the amount and distribution of retained particles and solubilized derivatives. Not only are the technological solutions frequently complex, but the nature of the problem is frequently obscure. How should the dose to the respiratory tract be calculated? Should it be averaged over the whole lung, or should the local airway or alveolar epithelial dose be estimated?

An extensive menu of measurement techniques is available. The approaches vary in expense, resolution, and the extent to which the subject must be disturbed. There are advantages in using nondestructive, noninvasive methods of detection. These methods allow repeated measurements on the same animal or person and thus permit replications and serial measurements, and thus clearance kinetics. For human experimentation such detection methods are essential. However, noninvasive measurements of retention in whole animals may provide inadequate detail about the distribution of dose to structures of interest. The greatest precision is achieved by killing and dissecting animals. By freezing or drying the lungs, they can be made rigid and then sliced. It is then possible physically to divide the respiratory tract into individual pieces or specific lung compartments and analyze the particle content of each piece. Depending on one's patience and the sensitivity of the detection method,

the distribution of particle retention can be described with increasing detail. These approaches permit an identification of anatomical location of retained particles. A recent review details many of the available methods (40).

Actual detection of particles in the lung pieces can rely upon any distinctive property of the particles. They may be radioactive, fluorescent, radio-opaque, or magnetic. They may have a characteristic visual appearance, which can be identified with light and electron microscopy. Their elemental or molecular nature may be identified by a repertoire of techniques including colorimetry, atomic absorption, neutron activation, nuclear magnetic resonance, or electron energy loss spectrometry. The type of question suggests which properties of the particle can be exploited for ease and specificity of detection. Various approaches have been reviewed in greater detail (41).

Radioactivity has been used frequently for studies of particle deposition because of its potential for noninvasive measurement and its sensitivity and resolution. Hundreds of radioisotopes are produced, and labeled particles can be synthesized with a wide spectrum of energies, type of

radiation ( $\alpha$ ,  $\beta$ ,  $\gamma$ , positrons), and half-lives.  $\gamma$ -emitters penetrate through tissue and therefore are suitable for making measurements externally.  $\alpha$ - or  $\beta$ -emitters are better suited for producing autoradiographs; their short path-lengths, which make them unsuitable for external detection, produce high resolution images on film in contact with particle-laden tissue.

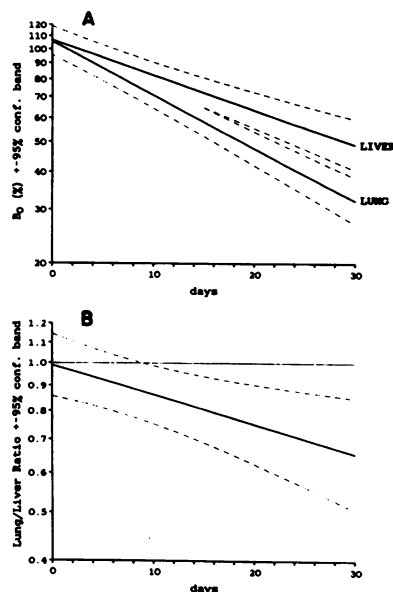
### Magnetometric Measurements of Particle Dissolution

Some aerosol particles are magnetizable, and sensitive magnetopneumography can measure their concentration and distribution in the lungs (42,43). The technique consists of applying a magnetic field to the whole thorax or to localized areas and detecting the resultant alignment of ferromagnetic domains in retained lung particles. Accumulations of magnetic particles have been measured in foundry workers, arc welders, coal miners, and asbestos miners (44). The greatest advantage of this technique is that the duration of measurement is not limited by radioactive decay. Measurements can be made as long as sufficient dust remains in the lungs. Thus one can noninvasively describe clearance kinetics over years (42). Two magnetic dusts suitable for studying retention in the lung are  $Fe_3O_4$  (magnetite) and  $\gamma-Fe_2O_3$  (a magnetic form of hematite) (47). Both are inert, relatively insoluble at physiological pH's, and can be aerosolized. The magnitude of the remnant field quantifies the amount of dust remaining in the lungs.

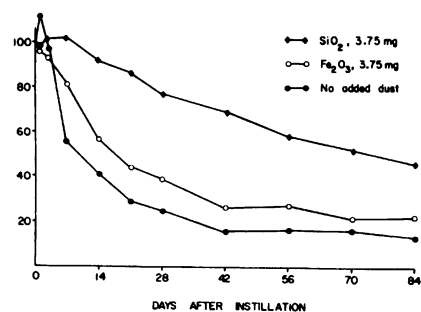
Particle clearance has been compared using magnetometry in two different organs, the lungs and liver (45). In the lungs, the retained particles are almost exclusively located in the alveolar macrophage; in the liver, the particles are

in the hepatic macrophage or Kupffer cell. These sister macrophages differ in several important ways. The alveolar macrophages are not fixed; rather, they move about on the alveolar surface seeking particles and pathogens. Some have asserted that these macrophages can migrate to the airways carrying insoluble particles with them. In contrast, hepatic macrophages are attached to the endothelial lining of the liver sinusoids. No net cell motion for phagocytosis is needed since the particles are presented to these cells by the circulating blood. The rates of particle clearance from each organ have been compared (45). Pulmonary clearance includes not only *in situ* solubilization of iron oxide particles, but may also represent bulk, mechanical transport from the respiratory tract by mucociliary transport or macrophage migration. In the liver, however, there is no evidence for either transport mechanism. For instance, 30 years after injection of thorium oxide (Thorotrast) in patients, particles were still found in Kupffer cells (46). It would seem then that particles ingested by Kupffer cells would disappear primarily by solubilization.

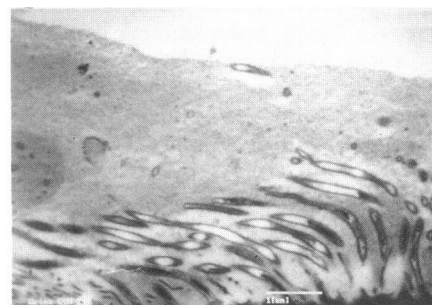
Sprague-Dawley rats were given submicrometric  $\gamma-Fe_2O_3$  particles suspended in saline (45). The particles initially were prepared by a combustion of iron pentacarbonyl and collected on a filter (46). A suspension of these particles was injected intravenously (1.5 mg/kg) or instilled intratracheally (1 mg/kg) into the lungs. Individual  $\gamma-Fe_2O_3$  particles were 0.1 to 0.2  $\mu m$  but tended to agglomerate into chains that were almost always  $<1 \mu m$ . Magnetometric measurements were then made (46) and the clearance of particles from both organs was measured (Figure 2). Clearance of  $\gamma-Fe_2O_3$  particles from the liver and lungs of rats was followed for 30 days post-injection; over the entire 30-day



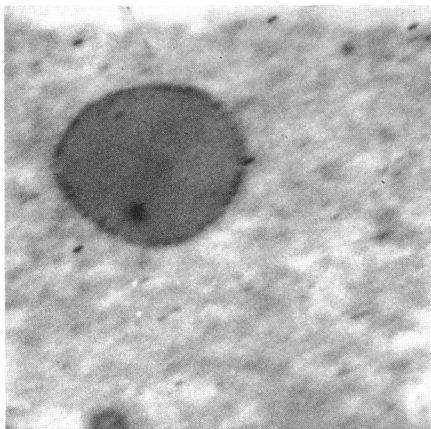
**Figure 2.** (A): Clearance of  $\gamma-Fe_2O_3$  from lungs and liver. Clearance is measured by following field strength at time 0 (B) with time and analyzing with random-effects regression technique to produce an average curve for lung clearance and an average curve for liver clearance. (B): Difference between lung and liver clearance over time is expressed by lung-clearance-to-liver-clearance ratio. Ratio departed from 1 with statistical significance ( $p < 0.05$ ) after 9.5 days. From Weinstein and Brain (45). Reprinted by permission.



**Figure 3.** Clearance of  $Fe_3O_4$  from hamster lungs ( $n = 6$ ) using magnetometry. Each point is the mean of the field strengths immediately after magnetization on the day shown.  $SiO_2$  significantly slows the clearance of  $Fe_3O_4$ , nonmagnetic iron oxide ( $Fe_2O_3$ ) has a much smaller retardation effect (49).



**Figure 4.** Hamster bronchial mucosa with overlying mucus layer (arrows). The tissue was prepared by slam freezing, followed by molecular distillation. After infiltration with Spurr's resin, it was sectioned and examined in a Zeiss CEM 902 electron microscope. There is a 1- $\mu m$  bar in the bottom right.



**Figure 5.** Latex particle *in situ* in hamster covered with an osmiophilic layer. The tissue was fixed, prepared, and examined as described in Figure 4.

experiment, clearance from the lungs was more rapid than from the liver as shown by the decline of the lung-to-liver ratio from just under 1.0 at 0 days to 0.65 at 30 days. The ratio departed from 1.0 with statistical significance ( $p < 0.05$ ) after about 9.5 days, as shown in the lower part of the figure.

We also have used magnetometry to describe the long-term clearance of iron oxide from human lungs. Three heavy cigarette smokers and nine nonsmokers inhaled a trace amount of magnetic dust,  $\text{Fe}_3\text{O}_4$  (42). From periodic measurements with a sensitive magnetic detector of the amount of this dust remaining in the lungs, a clearance curve was described for each subject. We found that the dust clearance in smokers was considerably slower than that seen in the nonsmokers. For example, after about 1 year, 50% of the dust originally deposited remained in the lungs of the smokers, whereas only 10% persisted in the lungs of the nonsmokers.

We also used magnetometry in animals to look at the impact of toxic particles such as  $\alpha$ -quartz ( $\text{SiO}_2$ ) to affect the clearance of magnetic iron oxide ( $\text{Fe}_3\text{O}_4$ ). Syrian golden hamsters were intratracheally instilled with 0.2 mg  $\text{Fe}_3\text{O}_4$  alone or in combination with one of the following: 3.75 mg  $\text{SiO}_2$  or 3.75 mg  $\text{Fe}_2\text{O}_3$  (49).  $\text{Fe}_3\text{O}_4$  alone had a clearance half-life ( $T_{1/2}$ ) of  $8.8 \pm 1.0$  days (Figure 3). However, in combination with the 3.75 mg doses of  $\text{Fe}_2\text{O}_3$  or  $\text{SiO}_2$ , clearance kinetics were slowed. The 3.75 mg dose of nonmagnetic  $\text{Fe}_2\text{O}_3$  (a nontoxic dust) caused the ( $T_{1/2}$ ) to rise to  $18.4 \pm 2.4$  days. The 3.75 mg dose of  $\text{SiO}_2$  slowed clearance even more dramatically; ( $T_{1/2}$ )

increased to  $74.1 \pm 9.0$  days. We conclude that  $\text{Fe}_3\text{O}_4$  in magnetometry can be used to evaluate the effects of various mineral fibers and other particulates on pulmonary clearance.

Magnetometry has some advantages for studies of particle dissolution. In the case of radioactive particles, even when the particles are dissolved the atoms or ions remain radioactive. Thus dissolution may have occurred, but if the components have been retained in an organ or if they have been resynthesized in other forms the radioactivity may persist. For example, iron oxide can be solubilized, then iron can be resynthesized in the form of ferritin and hemosiderin (7). But when the particles are magnetic, they must remain as macroscopic magnetic domains to be detectable by magnetometry. After dissolution, even if they persist in a cell or organ, they are no longer detectable.

### Improved Preservation Methods for Airway and Alveolar Lining, Layers, Particles, and Cells

To understand the fate of inhaled particles, we must preserve precisely anatomic relationships among particles, cells, and the fluids that line the respiratory tract. Unfortunately, most routine methods for tissue fixation involve filling the lungs with liquid or at least multiple liquid steps. Such methods make it very likely that particles and cells will be displaced to new locations or lost altogether.

Two methods eliminate this possibility. The first is microwave drying of inflated lungs, followed by slicing and examination by fluorescence microscopy or confocal microscopy. Such dried lungs are never exposed to any liquid and thus the possibility of particle displacement or loss is eliminated. Naturally, there is loss of detail at the microscopic or ultrastructural level, but the essential features of airways and alveoli are well preserved. In fact, one can rehydrate specimens and obtain reasonable quality light microscopic sections (50).

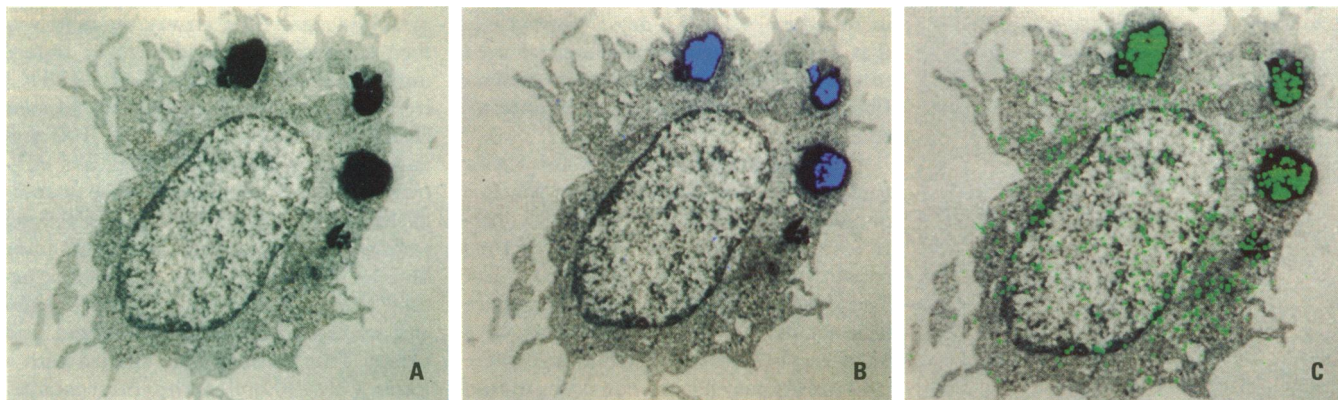
More recently, we used a technique commonly known as slam freezing to preserve airways, mucus, and particles and to prepare them for electron microscopy. Airways were cut into 3-mm segments while minimizing any disturbance to the mucus layer. The segments were opened longitudinally and mounted so that the mucus-covered surface could be slam frozen *en face* with a LifeCell CF100 cry-

ofixation unit. The airway segment was then transferred to liquid nitrogen storage until processed in the LifeCell (MDDC) freeze drying unit. Dehydration, gaseous fixations with  $\text{OsO}_4$  (10 mg or 100 mg) and paraformaldehyde (100 mg), and infiltration with Spurr's resin were accomplished in the MDDC unit. The airways were sectioned and stained with toluidine blue for light microscopy. For ultrastructural studies, sections were cut at 60 nm and stained with uranyl acetate and lead citrate. Thin sections were studied with a Zeiss CEM902 electron microscope. Electron spectroscopic imaging enhanced contrast.

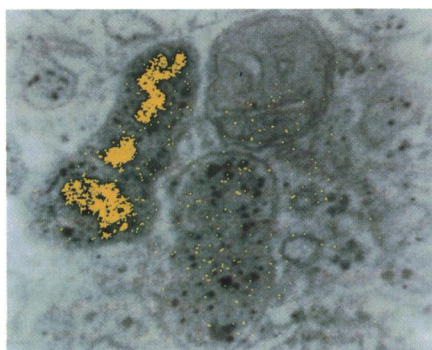
Figure 4 illustrates the preserved mucus layer of airways, cilia, and epithelial cells from the bronchus of a hamster. A latex bead surrounded by an osmiophilic layer is illustrated in Figure 5. We have consistently found that the mucus layer appears thicker and more uniform than in previously published studies. We sometimes observe two layers as previously described, but frequently the distinction is less pronounced compared to other fixation and dehydration methods. We think this is because the hydrophilic and hydrophobic components of mucus are far better preserved.

### Electron Energy Loss Spectroscopy (EELS)

As particles gradually dissolve within macrophages, soluble ions may become dispersed throughout the cell and throughout tissue. Identifying these materials and quantifying their distribution is a difficult task. Electron energy loss spectroscopy (EELS) can enable us to map the distribution of elements in relation to the ultrastructural morphology of macrophages and other lung tissue. This is demonstrated for cobalt oxide particles within alveolar macrophages (Figure 6). Moreover, the anatomic distribution of cobalt and oxygen throughout the cell can be described (Figure 6B,C). Soluble metallic ions also can be imaged (Figure 7). In this case hamster lung macrophages were incubated with 1 mM solutions of  $\text{VCl}_3$ . After a 3-hr incubation, the cells were fixed, embedded in araldite, sectioned at 30 nm, and studied unstained with the Zeiss CEM902. The cells incubated with vanadium had large, irregularly shaped phagolysosomes which had accumulated and concentrated vanadium. Lesser amounts of vanadium were found in other structures such as mito-



**Figure 6.** (A) Cobalt oxide particles are shown within a dog alveolar macrophage recovered by lung lavage. The spatial distribution of cobalt (B) and oxygen (C) throughout the cell can be seen using EELS on a Zeiss CEM 902.



**Figure 7.** A portion of an alveolar macrophage from a hamster is shown with numerous irregularly shaped phagolysosomes. Cells obtained by bronchoalveolar lavage were cultured with soluble vanadium ions. The cells were embedded in araldite, sectioned at 30 nm, and studied unstained with the Zeiss CEM 902. The vanadium (yellow) has become localized in a phagolysosome-like organelle.

chondria. We believe that EELS will be a powerful tool in describing the anatomic location of metal oxide particles and in following the fate of dissolved elements.

### Conclusion

Quantitative assessment of the health risks resulting from inhaled toxic particles inevitably depends on understanding the quantitative and mechanistic aspects of clearance. In both airways and in alveoli, we believe that macrophages play an important role. They ingest insoluble particles of every type; the disappearance of these particles then depends on the migratory behavior of macrophages, and especially on their ability to dissolve particles within acidic phagolysosomes. New tools

are becoming available to supplement classic approaches to analyzing macrophage function. We believe that improved methods of fixation such as microwave radiation and slam freezing will preserve particle-macrophage relationships. We also believe that new technologies such as magnetometry and EELS can be used to study particle fate. Finally, we believe that dissolution of particles within macrophages is a more important determinant of long-term clearance kinetics for many mineral dusts, than is mucociliary transport and the migratory potential of lung macrophages. In turn, biopersistence may be correlated with increased risk of chronic injury.

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