



## MORPHOLOGICAL AND CHEMICAL MECHANISMS OF ELONGATED MINERAL PARTICLE TOXICITIES

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**Much of our understanding regarding the mechanisms for induction of disease following inhalation of respirable elongated mineral particles (REMP) is based on studies involving the biological effects of asbestos fibers. The factors governing the disease potential of an exposure include duration and frequency of exposures; tissue-specific dose over time; impacts on dose persistence from in vivo REMP dissolution, comminution, and clearance; individual susceptibility; and the mineral type and surface characteristics. The mechanisms associated with asbestos particle toxicity involve two facets for each particle's contribution: (1) the physical features of the inhaled REMP, which include width, length, aspect ratio, and effective surface area available for cell contact; and (2) the surface chemical composition and reactivity of the individual fiber/elongated particle. Studies in cell-free systems and with cultured cells suggest an important way in which REMP from asbestos damage cellular molecules or influence cellular processes. This may involve an unfortunate combination of the ability of REMP to chemically generate potentially damaging reactive oxygen species, through surface iron, and the interaction of the unique surfaces with cell membranes to trigger membrane receptor activation. Together these events appear to lead to a cascade of cellular events, including the production of damaging reactive nitrogen species, which may contribute to the disease process. Thus, there is a need to be more cognizant of the potential impact that the total surface area of REMP contributes to the generation of events resulting in pathological changes in biological systems. The information presented has applicability to inhaled dusts, in general, and specifically to respirable elongated mineral particles.**

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<sup>1</sup>The term "REMP" as used in this article is not restricted to the six types of fibers currently defined under federal asbestos regulations. The term "REMP" is specifically chosen here to avoid confusion associated with interpretations of scientific studies where there is a wide range of particle origins including mineral habits ranging from asbestiform to nonasbestiform in the original rock formations. Historically the term "fiber" has been used to define elongated (length/width ratios  $\geq 3.0$ ) particles per various counting schemes (Millette, 2006). Any of these entities that meet the specific counting definition could be considered morphologically as a "REMP" if sufficiently small to be inhaled. The definition of "respirable" as used in this term (REMP) is based on the theoretical dimensions considered as defining "respirable" particles for humans (Lee, 1985). The upper limit of diameter for a rounded structure has been suggested as  $>10 \mu\text{m}$  and for an elongated particle as  $<3.5 \mu\text{m}$  (Lee, 1985). In reality, one of the authors (RFD) has evaluated thousands of REMPs from human lung tissue and found that these are rarely  $>1 \mu\text{m}$  in diameter.

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An overview of the health effects/toxicities of respirable elongated mineral particles (REMP)<sup>1</sup> freed from minerals occurring with habits ranging from asbestiform to nonasbestiform, as well as combinations thereof, requires a basic understanding of known biomolecular, cellular, and tissue responses to these entities regardless of the source of exposure. These components of toxicology, following measures of exposure and dose establishment in relevant tissues, are well recognized as critical for the establishment of predictive models for the effects of chemicals and have been incorporated in the NRC report "Toxicity Testing in the 21st Century: A Vision and Strategy" (Krewski et al., 2010). The resulting framework is equally applicable to inhaled particles, including REMP and those from asbestos, as is considered in this report. The obvious commonality in environmental exposures to "naturally occurring asbestos" (NOA), occupational exposure to REMP from asbestos mining, manufacturing, uses, and para-occupational or domestic exposures is that inhalation of REMP, given sufficient quality, quantity and individual susceptibility, induce disease in humans (Craighead et al., 1982; Dodson, 2006; *Lancet*, 2008). Unlike most other dusts, REMP from asbestos also are recognized as carcinogens in that they may induce lung cancer and mesothelioma (Craighead et al., 1982). A recent review by Samet et al. (2006) also considered there was sufficient evidence for a suggestive, if not causal, link for inducing cancer in other sites in the body. Subsequently, the International Agency for Research on Cancer (IARC) Monograph Working Group, comprised of 27 scientists from 8 countries, determined that REMP from asbestos also are causal agents for laryngeal and ovarian cancer in humans (Straif et al., 2009).

Epidemiological observations are in turn supported by mammalian toxicology studies that also indicate toxicities for a broader range of REMP than just those from truly mineral as well as nonmineral origins (Stanton et al., 1981; Cook et al., 1982; Pott et al., 1987; Davis et al., 1991b; Wagner et al., 1985; Coffin et al., 1992; Poland et al., 2008; Sakamoto et al., 2009). Exposures to REMP that are not

identified as one of the six forms of fibrous minerals defined in the regulations are of increasing concern for risk to human health. High numbers of mesothelioma and pleural disease cases have been reported in a Turkish village (Artvinli & Baris, 1982; Dogan et al., 2006). The causal agent has been strongly suggested as a naturally occurring fibrous form of the zeolite mineral erionite. In addition, exposure to mined vermiculite from Libby, MT, has been associated with occurrence of diseases similar to those that are "asbestos induced" (Whitehouse et al., 2008; Horton et al., 2008; Rohs et al., 2008). REMP source material sampled from the vermiculite ore body consists of three amphiboles (84% winchite, 11% richterite, and 6% tremolite by particle number) as defined by Meeker et al. (2003).

#### **MORPHOLOGICAL AND MINERALOGICAL DEFINITION AND IDENTIFICATION OF REMP CATEGORIZED AS CLEAVAGE FRAGMENTS**

A subject of considerable interest in discussions involving REMP is the hypothetical lack of potency for those REMP termed "cleavage fragments." OSHA defines cleavage fragments as "mineral particles formed by comminution of minerals, especially those characterized by parallel sides with a moderate aspect ratio (usually less than 20:1)" (OSHA, 1994a). OSHA further concluded, "Most cleavage fragments of the minerals are easily distinguishable from true asbestos fibers. This is because cleavage fragments usually have larger diameters than 1  $\mu\text{m}$ . Internal structures of elongated particles with diameters larger than this usually have no internal fibrillar structure. In addition, cleavage fragments of the monoclinic amphiboles show inclined extinction under crossed polars with no compensator. Asbestos fibers usually show extinction at zero degrees or ambiguous extinction if any at all. Morphologically, the larger cleavage fragments are obvious by their blunt or stepped ends showing prismatic habit. Further, they also tend to be acicular rather than filiform" (59 FR 40964, p. 343). In practice, "cleavage fragment" relative potency issues are largely relevant to

assessment of hazards associated with amphibole REMP since amphiboles have a common double-chain silicate structure and comprise all but one of the regulated asbestos minerals.

When dust particles are less than 1  $\mu\text{m}$  in diameter and have an aspect ratio greater than or equal to 3:1, it is recommended that the sample be analyzed by SEM or TEM if there is any question whether the fibers are cleavage fragments or particles from asbestiform minerals. Care needs to be taken when analyzing by electron microscopy because the interferences are different from those in light microscopy and may structurally be similar to asbestos. The classic interference is between anthophyllite and biopyribole or intermediate fiber. The same morphology clues are used for electron microscopy as are used for light microscopy, e.g. fibril splitting, internal longitudinal striation, fraying, curvature, etc. (OSHA, 1994b, 59 FR 40964, p. 343)

In essence, such physical distinctions between asbestiform fibers and cleavage fragments noted by OSHA are generally applied to a population of particles rather than single particles. Furthermore, identification criteria such as inclined extinction were reported as unreliable, particularly in environmental applications (Meeker et al., 2006).

The simplest differentiation between asbestiform fibers and elongated particles created by cleavage is the original mineral growth texture in parent rocks (asbestiform, in contrast to nonasbestiform or nonfibrous). A cleavage fragment is defined as a particle created by breakage along specific crystallographic planes from a mineral that did not originally grow along its long axis with a fibrous habit (for additional subject review see companion publication by Case et al., this issue). While not meeting a mineralogical definition of a fibrous or asbestiform structure, a cleavage fragment may appear in some morphological comparisons as similar or even identical to an asbestiform structure of the same mineral. Making a distinction between these particle types during routine microscopic particle analysis is subjective and has become a major point of discussion in the scientific/regulatory community (Case, 1991). The federal regulations governing workplace exposures and general protection of public health from

asbestos exposures currently are governed under the asbestos standards as applicable to the asbestiform varieties of these minerals.

There are unique settings such as the metamorphic transition between fibrous anthophyllite and talc. This results in evolution of a fiber that has features in part of the structure that are more consistent with anthophyllite asbestos (as would be governed as to exposure levels under the asbestos regulatory documents) and other portions of the elongated mineral particle being definable with features consistent with fibrous talc. Such complex structures have been defined structurally as "transition fibers." The issues of distinguishing such morphological details to interpret the form of a REMP have appreciable connections to legal issues, since the discussions of exposure levels are referenced back to definitions in regulatory guidance documents. If the dust is determined to contain "asbestiform structures" of a regulated asbestos mineral, then an entire set of federal/state/local regulations applies in that human exposures to dusts from asbestos are recognized to induce diseases primarily on the basis of data accumulated from studies of past occupationally exposed cohorts.

#### **ENVIRONMENTAL HEALTH RISK CONSIDERATIONS COMPLICATE HAZARDOUS REMP DEFINITION AND TOXICITY RISK ASSESSMENT**

Acquisition of the fullest understanding of modes of action associated with exposures to all REMP is desirable in order to ensure the strongest basis for all health risk assessments. This principle already has been extended to assessment of new nanomaterials (Oberdorster et al., 2005a) that have the potential to create new inhalation exposures to elongated synthetic particles. In this regard, note recent reports of mesothelioma induced by intraperitoneal (ip) exposures of mice to multiwalled carbon nanotubes (Takagi et al., 2008) and inhaled carbon nanotube translocation to subpleural tissue in mice (Ryman-Rassmusin et al., 2009). Poland and colleagues (2008) evaluated the response of the mesothelial lining of the body cavity when

exposed to multiwalled carbon nanotubes. The conclusions were that “asbestos-like” responses were length-dependent and pathological reactions, including inflammation and formation of granulomas. Muller et al. (2008) contributed several studies in the evaluation of multiwall carbon nanotubes (MWCNT) and induction of pathological responses in tissue. In one study the investigators assessed the exposure via the ip model as a 2-yr bioassay (Muller et al., 2008). The reasons for a lack of carcinogenic response in their findings were suggested as being “the length of the MWCNT tested,” “the absence of a sustained inflammatory reaction to MWCNT,” and “the capacity of these MWCNT to quench free radicals.” In contrast, Sakamoto and associates (2009) administered MWCNT to rats by a single intrascrotal injection and found that some animals “at 37–40 weeks died or became moribund due to ip disseminated mesothelioma.” Sakamoto et al. (2009) concluded that “MWCNT possesses carcinogenicity causing mesothelioma at high rate in intact rats under experimental conditions.” In evaluation of these findings one needs to recognize the observations are scientifically important in evaluation of the potential pathogenicity of REMP in situ but the exposure routes are nonphysiological.

A statement from Acting Surgeon General Steven K. Galson in recognition of National Asbestos Week (Galson, 2009) contained some applicable thoughts regarding the breadth of exposure levels of concern: “Low levels of asbestos are commonly in the air as fibers enter the environment from natural rock outcroppings, products that contain asbestos, former asbestos mining and milling operations, and from disturbance of asbestos-containing material.” While it is evident that exposure to greater levels of REMP from asbestos carries the most concern for induction of disease, Galson (2009) further stated, “There is no level of asbestos exposure that is known to be safe and minimizing your exposure will minimize your risk of developing asbestos-related disease.” An editorial in the journal *Lancet* (2008) states that “all countries should listen to WHO’s advice: the only way to eliminate asbestos-related disease

is to stop the use of all types of asbestos, all over the world.” The same logic would apply to other inhaled toxic particulates, including biopersistent REMP. However, there are some viewpoints that suggest this is only the case for certain types of or subsets of elongated mineral particulates based on more specific and limiting physical/chemical features. Some of those factors are discussed in detail in the companion papers in this issue.

It should be recognized that for diverse environmental exposures there are concerns with respect to evaluating a broad range of REMP types, origins, size and shape distributions, and exposure levels of potential concern. Understanding such diverse relationships can provide a maximum knowledge basis for defining and applying fundamental mechanism of action models. The focus of this review includes discussion of the physical features of REMP as factors inducing pathological events, as well as a discussion of specific chemical and biological interactions that lead to pathological changes. The emphasis of the latter feature will focus on the chemical compositions that generate reactive oxygen species (ROS) and induce production of reactive nitrogen species (RNS), which may contribute to the resultant pathology in cells and tissue.

### **PATHOLOGICAL POTENTIAL OF REMP**

This report emphasizes the need to identify and assess toxicological mechanisms for REMPs under a fundamental assumption that the risks of irreversible changes in cells/tissues are correlated with increasing levels of the particles reaching various target tissues. Unlike most dust/poorly soluble particles (PSP), these elongated or fiber-like structures also are recognized as carcinogens in that they may induce lung cancer and mesothelioma (Craighead et al., 1982). A recent review concluded specifically that there was evidence following exposure to asbestos “to infer causal relationship for laryngeal cancer; to be suggestive for pharyngeal, stomach and colorectal cancers; and to be inadequate for esophageal cancer.” (Samet et al., 2006). Consistent

with established extrapulmonary transport of nanoparticles, other sites should be of continuing concern for pathological reactions following exposure to inhaled dusts. Further, as shown by Cook and Olson (1979), amphibole elongated particles ingested with drinking water are subsequently observed in human urine. Drinking filtered water greatly reduced or eliminated the amount of amphiboles found in urine. The same amphibole particles were also found in kidney tissue from fish caught from the drinking-water source and were attributed to ingestion of food and associated water (Batterman & Cook, 1981). The elongated particles that passed through the various barriers of the gastrointestinal tract (GIT) and urinary systems of humans or other vertebrates were predominantly less than 3  $\mu\text{m}$  in length.

Another example of the translocation of asbestos particles in the body was recently reported by Hasanoglu et al. (2008). In this study, chrysotile ingested by rats was reported to result in asbestos bodies in spleen, lungs, and pleura in association with mesothelial proliferation (Hasanoglu et al., 2008). At the end of the 1-yr period of observation, interstitial fibrosis in the lungs of exposed rats was also observed. Chrysotile is typically inhaled as a shorter, thinner particle form of asbestos and is much less likely to be found as the core of ferruginous bodies in human tissue than amphibole REMP (Dodson, 2006). Unfortunately, electron microscopy of tissue samples was not done in the study by Hasanoglu et al. (2008) and thus the tissue burdens of uncoated chrysotile bundles/fibrils relative to observed asbestos bodies are unknown. As will be further discussed, the shorter REMP from asbestos are also the ones more likely to reach extrapulmonary sites in the body following deposition in the respiratory tract.

Asbestos-particle-induced diseases and especially the rare asbestos-particle-induced tumor of the serosal linings, mesothelioma, occur decades after first exposures (Friedman, 2006; Hammar, 2006). Goldberg and Luce (2009) recently reviewed the impact of nonoccupational exposure to asbestos and concluded that "studies concerning natural asbestos in

the environment show that the exposure that begins at birth does not seem to affect the duration of the latency period, but the studies do not show whether early exposure increases susceptibility (to asbestos induced cancer); they do not suggest that susceptibility differs according to gender. Solid evidence shows an increased risk of mesothelioma among subjects whose exposure comes from a paraoccupational or domestic source. The risk of mesothelioma associated with exposure as result of living near an industrial asbestos source (mines, mills, asbestos processing plants) is clearly confirmed" (p. 421). Furthermore, they stated, "Non-occupational exposure to asbestos may explain approximately 20% of the mesotheliomas in industrialized countries."

The potential for inhaled REMP to induce irreversible cytopathological changes is the result of complex inherent features of the fibrous dusts. From a simplistic standpoint the bulk material can break down into smaller respirable components when subjected to processing or physical trauma. Similarly, inhaled REMP were suggested as further subject to alteration over time through dissolution and shape/size alteration in vivo (for additional reviews on the subject see companion articles in this issue). Extreme in vivo exposure dose changes are possible through these mechanisms. For example, the extensive longitudinal splitting of relatively short, wide REMP of ferroactinolite (amphibole) in rat lungs following intratracheal instillation exposures was associated with high incidences of both lung cancer and mesothelioma (Cook et al., 1982). The increased potency was attributable not only to greatly elevated numbers of thinner REMP but, more importantly, to a rise in the total REMP surface area available for cellular contact. Lippmann (1988) concluded, for example, "that asbestosis is most closely related to the surface area of retained fibers." The presently known potential toxicity determinants of fibrous dusts are divided into physical and chemical characteristics of the dusts and molecular features that include inherent surface characteristics including effective or

bioavailable surface areas, charges, chemically reactive sites, variations of elemental composition, and an array of changes induced by contributions/alterations of these features in biosystems.

### **INHALATION OF REMP AND LUNG CLEARANCE**

As with exposures to most dusts, REMP are initially deposited in the body via the portal of entry through the respiratory system (Robertson, 1980). The clearance of dust from the respiratory system is a critical process that attempts to keep the surface of the airways clean of foreign debris. Under ideal conditions, the clearance process was noted by Gross and Detreville (1972) to eliminate "98 to 99%" of inhaled particulates, even in individuals who are heavily exposed to dust. A review of the issues regarding clearance in mammalian species has been presented by Snipes (1989). In this review, retention is described as the time-dependent distribution pattern of particles or their reaction products that have not yet been cleared/translocated from the lung. As previously noted, REMP released from asbestos, asbestiform or nonasbestiform habits of minerals historically used as asbestos, or other minerals that simply produce elongated particles when crushed may accumulate in lymph nodes, which serve as loci for translocation from the lung to other sites in the body (Netter, 1979; Dodson et al., 2007).

Thus, particulate burdens are a function of both deposition and clearance. Clearance is discussed in terms of physical processes that transport particles from one place to another and the dissolution-absorption process, as is discussed in other companion articles in this issue. Snipes (1989) further states that "some particles appear to be retained for extended periods in phagocytic cells in the alveoli. Other particles are found in cellular constituents of the interstitium. Most, or all, of the particles in the interstitium appear to be in mobile cells that have the options of remaining in the interstitium, moving to the mucociliary escalator, moving to

lung-associated lymph nodes, or moving into the lymphatic or circulatory systems" (p. 175).

Davis and associates (1991a) explored an additional impact of translocation of REMP from asbestos that seems to affect potency. The translocation/clearance was examined in rat model following 1 yr of exposure and 2-yr follow-up. Davis et al. (1991a) acknowledged that the rat pleural lining differs from the human in thickness and morphological complexity. Thus, mechanisms of translocation though the visceral pleura may be appreciably different between rats and humans. However, when combining the exposure of chrysotile or amosite with nontoxic levels of titanium dioxide or quartz, observed differing levels of reactions and endpoints (i.e., tumor formation) were noted. Quartz "greatly increased fibrosis above that produced by the asbestos types alone." The occurrences of pulmonary and mesothelioma tumors in animals receiving fibers from asbestos and the other dusts were greatly increased. Davis et al. (1991a) also observed that the "presence of particulate dusts made little difference in the amounts of amosite fibre retained in the lung tissue, but, [according to their interpretation] with chrysotile, titanium dioxide appeared to increase retention while quartz reduced it." Although the subject of mixed exposures to nonfibrous and fibrous dusts may seem beyond the scope of the present publication, it is useful to remember that most human exposures to REMP, or specifically fibers from asbestos, involve mixed dusts and this may amplify effects. It is also useful to recognize that while the molecular mechanisms discussed in the various sections may emphasize characteristics of biopersistent elongated particles, many of the same mechanisms may have applicability to respired PSP that are of the same elemental composition and crystal structure but not in an elongated form.

### **TRANSLOCATION OF REMP FROM THE LUNG**

Dust overloading of the upper respiratory tract can reduce mucociliary/macrophage

clearance-associated elimination of inhaled small particulates, resulting in the enhanced accumulation of dust particles in the lower respiratory system over time, thus increasing elongated particle dose. Factors considered in resultant induction in pathological responses include levels of dose, intensity of dose, frequency of exposure, durability of the dust in a biological system, toxicity of a given dust, and individual susceptibility to these variables. Dust overloading and the impact on lung function have been discussed in several extensive reviews. When the lung cannot eliminate the inhaled dusts, the potential for damage to the lung tissue and the potential for relocation of fibers to extrapulmonary sites are increased (Pritchard, 1989; Oberdorster, 1995). This is parallel to the well-accepted increase in lung cancer risk associated with combined smoke and elongated particle inhalation (Lippmann et al., 1980; McFadden et al., 1986; Churg et al., 1987).

Lymphatic accumulation of dust including asbestos was reported in the loci (lymph nodes), including those where initial translocation may occur from the lung in route to other extrapulmonary sites within the body (Oberdorster et al., 1988; Dodson et al., 2007). Oberdorster and associates (1988) studied in a dog model the transfer to the lymphatics of neutron-activated amosite fibers from the lower respiratory system. The animals were subjected to intratracheal instillation of the labeled fibers and samples of thoracic lymph nodes and postnodal lymph were examined by scanning electron microscopy (SEM) at 24 h post exposure. Data showed that there was a fast translocation of fibers from the airspaces of the lung to the lymph nodes and even into postnodal lymph. The findings indicated that the "structures of the peripheral lung and lymph node itself act like size selective filters, permitting only the fine fiber fraction to penetrate." This conclusion is consistent with the findings of the actual fiber burden in the lung and lymph nodes from exposed humans (Dodson et al., 2007). Oberdorster and colleagues (1988) further concluded that "fibers below about 9  $\mu\text{m}$  in length and below about

0.5  $\mu\text{m}$  in diameter can be cleared into the postnodal lymph and thus can reach any organ of the body."

The drainage of lymphatics and associated dusts from the lung to the various levels of lymph nodes within the chest cavity has been anatomically defined (Netter, 1979). The comparison of the asbestos burden in lung tissue with that of lymph nodes within the chest cavity was the subject of a study by Dodson and associates (2007). The evaluation used analytical transmission electron microscopy (ATEM) and a sampling scheme based on a Naruke node map for classification of the levels within the thoracic cavity for the sampled nodes. The findings confirmed the similarities with regard to the types of fibers from asbestos found in each of the sites, as well differences in the physical features between the types of fibers in the sites. The fibers detected in the lymph nodes were appreciably shorter than those of the same type found in the lung tissue.

A hypothesis as to how REMP reach the extrapulmonary sites from the lung (site of original deposition) was offered by Miserocchi et al. (2008). Data indicated that "fibers can pass the alveolar barrier and reach the lung interstitium via the paracellular route down a mass water flow due to combined osmotic (active  $\text{Na}^+$  absorption) and hydraulic (interstitial pressure is subatmospheric) pressure gradient" (p. 1). Specifically, it was noted "fibers may be dragged from the lung interstitium by pulmonary lymph flow (primary translocation) wherefrom they reach the blood stream with subsequent distribution to the whole body (secondary translocation). Primary translocation across the visceral pleura and towards pulmonary capillaries may also occur if fiber/elongated particle-induced lung inflammation increases pulmonary interstitial pressure which can reverse the trans-mesothelial and trans-endothelial pressure gradients" (Miserocchi et al., 2008, p. 1). It was further stated that "secondary translocation to the pleural space may occur via the physiological route of pleural fluid formation across the parietal pleura; fibers accumulate in parietal pleura stromata (black spots)

reflect the role of parietal lymphatics in draining pleural fluid” (p. 1). Evidence indicated that “ultrafine fibers (length  $<5 \mu\text{m}$ , diameter  $<0.25 \mu\text{m}$ ) can travel larger distances due to low steric hindrance (in mesothelioma about 90% of the fibers are ultrafine).”

Another study indicated that asbestos fibers accumulate not only in the lung tissue but also in the retroperitoneal and mesenteric lymph nodes (Uibu et al., 2009). Results demonstrated that “even low-level occupational exposures result in the presence of crocidolite, amosite, anthophyllite, tremolite, or chrysotile in these abdominal lymph nodes.” Furthermore, it was found that the results “support the hypothesis of lymph drainage as an important translocation mechanism for asbestos in the human body.”

Monchaux and associates (1982) evaluated the translocation of REMP via the respiratory system following intrapleural injection of either UICC chrysotile A, UICC crocidolite, or JM 104 glass fibers into the pleural cavity of rats. “Ninety days after their intrapleural injection, fibres were found in all organs analyzed: mediastinal lymph nodes, lung parenchyma, spleen, liver, kidneys and brain. The resultant fibre concentrations were in the same range for all organs except in thoracic lymph nodes where they were considerably higher (10–100 times)” (p. 311). Data indicated that given time the average length of the fibers in the lung increased as would be expected due to the potential for shorter fibers to clear more readily from the airway side of the lung. The diameter of each individual fiber was estimated and used to create 8 classes with nominal width dimensions of  $10^{n/4} \mu\text{m}$ , where  $n$  is an integer ranging from 16 to +1. The measurement model compensated for clumps when encountered. Monchaux et al. (1982) indicated that if the “hypothesis formulated by Lauweryns and Baert (1977) that particles are removed from the pulmonary interstitium by blood capillaries and lymphatics is correct, it may be assumed that only short fibers are cleared in this way, whereas long fibers are entrapped within the alveolar walls” (p. 316). However Monchaux et al. (1982) found that after the short fibers

have migrated rapidly, fibers of all lengths are found in lung parenchyma, with a marked rise in the mean length whatever the fiber type. The fact of short fibers being preferentially translocated from the lung parenchyma to the thoracic lymph nodes is certainly what has been found when such tissues from asbestos-exposed individuals have been evaluated (Dodson et al., 1990, 2007).

The importance of the lymphatics as a pathway for relocation of asbestos fibers within the body is further illustrated by the findings of Kanazawa et al. (1970). The crocidolite fibers in their study were injected subcutaneously (sc) into one or both flanks of mice. Results showed that the fibers were relocated within the body via the lymphatic pathways and “accumulate in lymphatic tissue, particularly in the regional/axillary lymph nodes, [and] smaller amounts were found in inguinal, mediastinal and lumbar nodes.” It was emphasized that there was a need for analytical transmission electron microscopy (ATEM) evaluation since many of the uncoated asbestos fibers are of a size requiring the detection/identification capability of this instrument. When the reported lengths and widths of fibers that are found in the extrapulmonary sites are reviewed, it is evident that the latter point raised in their study is critical if the asbestos fibers are to even be detected since most are  $<5 \mu\text{m}$  in length and are individual “fibrils,” which are much below the detection limit (based on diameter) for detection by light microscopy or even at low magnification by electron microscopy.

#### **PHYSICAL FACTORS OF REMP RELATED TO DOSE AND PATHOGENIC POTENTIAL**

Size, shape, and biodurability of REMP in inhaled dust exposures are considered important determinants of the relative carcinogenicity for dusts once deposited in a mammalian respiratory tract and in extrapulmonary tissues through translocation over time. The weighted roles that these factors play in

inducing pathologically irreversible responses are still the subject of research. The conflicting opinions with regard to the contribution that physical diameters and lengths play in contributing to pathological responses are also discussed in companion publications in this special issue. The reports most often cited for the physical dimensions of fibers related to risks for inducing tumors are the studies of Stanton and Wrench (1972) and Stanton et al. (1981) and those of Pott et al. (1974, 1987). These important references for initial discussions regarding the influence of the physical characteristics of elongated dust particles share the conclusion that long thin fibers have higher potencies for inducing tumors than short thicker fibers of the same type material. The findings in both studies are similar despite the use of nonphysiological exposure models and different mesothelial tissue sites. Stanton et al. (1981) used gelatin implants into the pleura for assessment of tissue reaction, while Pott et al. (1974, 1987) chose an ip injection placement of the fibers of interest. Pott et al. (1974, 1987) clearly defined the peritoneal doses given as being heavy with the intent of eliciting a response, and the Stanton et al. (1981) pleural doses would be considered heavy for samples with great potencies but helpful for investigation of samples with lower potencies/mass dose. The investigators used preparations that in some cases had been subjected to milling and other preparative procedures. These alterations, while useful in producing altered particle size distributions, may have also produced alterations of the surface/chemical makeup of the materials used in the exposures when compared to the exposures to these particles in a less unaltered (more natural) state.

The study of Stanton et al. (1981) has often been cited as the basis for near exclusive potency of long, thin fibers, often defined as "Stanton fibers" or "Index fibers," implying that fiber carcinogenicity is based on these specific physical dimensions alone. The actual statement of Stanton et al. (1981) was, "The probability of pleural sarcoma correlated best with the number of fibers measured 0.25  $\mu\text{m}$  or less in diameter and more than 8  $\mu\text{m}$  in

length, but relatively high correlations were also noted with fibers in other size categories having diameters up to 1.5  $\mu\text{m}$  and lengths greater than 4  $\mu\text{m}$ " (p. 965). It is important to recognize that many alternative size fraction fiber concentrations will correlate with tumor incidence in these studies because of the inherent covariation of the data. The ideal dose metric would predict tumor probability on the basis of the collective relative potencies of all elongated particles placed in the mesothelial tissues. The Stanton et al. (1981) data would seem to indicate that "long thin" structures carry more potential risk for inducing tumors if they reach the pleura (as per the nonphysiological exposure method used). However, statements interpreting these findings often ignore their caution that fiber concentration "data on the amphibole asbestoses: amosite, tremolite, and crocidolite though estimates of the dimensions of the asbestoses are especially liable to error. Chrysotile, although as carcinogenic as the amphiboles at comparable dimensions, could not be included since it has proved difficult to be measured with any degree of precision" (p. 965). The obvious, but often overlooked, reality in understanding fiber dose-response relationships is that their interpretation and use can only be as good as the relevance, accuracy, and applicability of the analytical data that are used to define them.

Pott et al. (1974) pointed out that "It is suggested that the fibrous shape (of dust particles) leads to a high multiplication rate of cells and predisposes to tumor formation. Fibrosis, in the other hand, does not so predispose." Pott and colleagues (1974) reached conclusions that were in contrast to the emphasis in the later findings of Stanton et al. (1981) and others that long/thin particles had appreciably more carcinogenic potential. The observation from the study of Pott et al. (1974) was that they "are inclined to believe that even short fibers less than 10  $\mu\text{m}$  in length can induce tumors. The milled chrysotile A contained only few fibers exceeding 10  $\mu\text{m}$  in length, and 99.8% of the fibers were shorter than 5  $\mu\text{m}$ . Data do not confirm that the carcinogenic effect can be limited to fibers with a diameter less than 0.5  $\mu\text{m}$ .

it is presumed that the maximum diameter of an effective fiber is limited by the length of the fiber that still induces damage to the membrane of a cell" (p. 315). Pott et al. (1974) reported that 30–40% of the animals in the milled (short) chrysotile group developed tumors.

Care needs to be taken when statistically deriving relative potency values for REMP from toxicology studies involving comparisons of equal masses of samples containing highly variable fiber size distributions. One problem associated with these efforts is the unavoidable covariance of alternative dose measures being evaluated. In contrast to frequent attempts to determine relative potencies based on correlations of concentrations of fibers in discrete size categories, fiber doses can better be described as continuous functions of the sum of individual fiber potencies based on physical characteristics like length and width and potentially modified for chemical factors like bio-persistence/dissolution rate and surface chemistry. In this regard, Pott (1978) proposed a relative carcinogenic potency conceptual model for fibers based on potency variation as a continuous function of length and width. This conceptual model appeared to incorporate both particokinetic effects on durable fiber size distribution and tissue concentrations in lung after inhalation exposure and consequent cellular effects leading to cancer. The shape of the Pott (1978) fiber potency model in graded length by width space appears close to what one might expect for potency related to surface areas of fibers ultimately residing in tissues involved in carcinogenesis. One consequence of the model is that, for fibers of a given length, greater potency is associated with greater width (within the respirable range) rather than thinness.

As discussed later in this report, effects of REMP on cells depend on chemical reactions at the particle–cell interface. It should be clear that potencies of particles with a common surface chemistry vary in proportion to their effective surface areas (Oberdorster et al., 2005a). While this has not been documented for fibers from asbestos and REMP in general, it is well established for non-elongated particles.

Oberdorster and associates (2005b) provided a clear demonstration of the importance of surface area with a comparison of pulmonary-inflammatory neutrophil responses determined by lung lavage 24 h after intratracheal instillation of graded mass doses of 0.02- $\mu\text{m}$  and 0.25- $\mu\text{m}$  diameter titanium dioxide ( $\text{TiO}_2$ ) particle samples in both rats and mice. While responses to the two particle sizes were markedly different on an equal mass (0.02- $\mu\text{m}$  diameter  $\text{TiO}_2$  appears most potent) or equal number (0.25- $\mu\text{m}$  diameter  $\text{TiO}_2$  appears most potent) dose basis, the fibers displayed equal potency on a particle surface area dose basis for both rats and mice. While the same principle applies to REMP or fibers from asbestos specifically, it is more difficult to directly provide such an absolute test of the surface area dose metric because of the complexity of the heterogeneous particle size distributions involved. However, it seems unreasonable to expect differences in elongated particle shapes and sizes alone to negate the fundamental effective surface area dose-response relationships observed for more spherical particles in lungs.

Potency as a function of REMP surface area is very consistent with the concept of greater potency as a function of length (potential reactivity of an individual long fiber versus that of a shorter fiber of the same type), but is inconsistent with the concept of thinness related to greater potency. In toxicology studies the appearance of greater potency for thin fibers from asbestos is the often-overlooked consequence of gauging potency on an equal mass dose basis. As with the  $\text{TiO}_2$  example earlier, differences in REMP widths exert large impacts on number of particles or total particle effective surface areas per unit mass of test samples. For example, REMP of the same length can have particle number or surface area concentrations per unit mass of sample that vary more than 100-fold as a function of particle diameters associated with different samples. The consequence is that many REMP require testing of greater sample masses to directly compare potencies, on either a fiber number or surface area concentration basis, to the thinnest fibers from asbestos.

Fiber/elongated particle number and surface area to mass relationships do not appear to have been considered for amphibole REMP samples composed of "cleavage fragments," as further discussed later. However, one study involving 10 g ip injections of 6 tremolite samples in rats (Davis et al., 1991b) reported peritoneal mesotheliomas for 3 samples that appeared to be largely composed of tremolite amphibole cleavage fragments yet produced tumors. More importantly, the reported particle number and size data allow calculation of surface areas for the REMP with aspect (length/width) ratios  $\geq 3$ . The total surface area concentrations calculated for each tremolite sample are highly correlated to the tumor incidence values reported. Adding surface areas for all particles with aspect ratios  $< 3$  (sample surface area concentration based on all particles) destroys the correlation, implying low potency for low-aspect-ratio (less elongated) particles.

The findings of asbestos fiber toxicity studies using animal models for direct site exposure, inhalation, or intratracheal modes support greater cancer risk per exposure to a long fiber in comparison to a short fiber of the same type. To the extent that particle surface area increases with length and long particles, once deposited in the lung, are more persistent, a long fiber should be more potent than a short fiber for inducing lung diseases. However, far greater numbers of shorter fibers are inhaled in most exposure settings than longer fibers and thus accumulate in the lung despite faster clearance rates for short fibers. This is especially so for continuing, chronic exposures that allow steady-state concentrations to be approached in the lungs and ultimately in extrapulmonary tissues. Thus, larger numbers of short fibers should reasonably offer greater overall biochemical reaction potential since their collective surface area in the lung is appreciably greater than that for longer REMP at any post-exposure point of time. The consideration of the potential for short fibers ( $< 5 \mu\text{m}$ ) to carry a risk for inducing tumors is reasonable in that they constitute the majority of fibers in the lung despite their potential for preferential clearance associated with "fitting" inside

of the mobile pulmonary macrophages as well as interstitial and epithelial cells (Brody et al., 1981; Pinkerton et al., 1984; Warheit et al., 1984; Dodson, 2006).

Short fibers predominate in extrapulmonary sites, as reported in the published literature (LeBouffant, 1980; Sebastien et al., 1980; Dodson et al., 1990, 2007; Kohyama & Suzuki, 1991; Boutin et al., 1996). This includes those sites within the body lined by serosal membranes where the rare tumor mesothelioma originates (LeBouffant, 1980; Sebastien et al., 1980; Dodson et al., 1990; Kohyama & Suzuki, 1991; Boutin et al., 1996). Logic would therefore suggest that since fibers  $< 5 \mu\text{m}$  are the particle fraction more likely to be in extrapulmonary sites where asbestos related changes/tumors occur, these short fibers contribute to the pathogenicity/tumorigenicity at these sites. Contrasting opinions exist as to the potential contribution of short fibers to development of tumors; however, there are no published electron microscopy data that contradict their being the majority fiber size in extrapulmonary sites.

### REMP IN EXTRAPULMONARY SITES

There have been limited numbers of studies regarding the asbestos content of human pleural tissue, as discussed in the following and in sections of companion papers in this issue. Boutin and associates (1996) evaluated parietal pleural samples with an emphasis in assessing asbestos content of areas considered as representing "pleural lymphatic vessels," referred to in the article as "black spots." The logic of the study was to characterize the tissue burden in these sites to question, "can these findings explain why the parietal pleura is the target organ for mesothelioma and plaques?" The first consideration of these data needs to begin with the recognition that the predominant REMP found in lung tissue were amphiboles. Thus, these should constitute the fiber type available for relocation to extrapulmonary sites. However, it is noteworthy that Boutin et al. (1996) stated that "from a technical point of view, short and thin chrysotile

fibers could be less easily detected among a 'background' of particles in anthracotic samples." The conclusions from the study indicated that "22.5% of the fibers were 5  $\mu\text{m}$  or longer and 10% were 8  $\mu\text{m}$  or longer." The diameter of fibers recovered from the black spots was "greater than 0.1  $\mu\text{m}$ ." It was further stated that "our observation is thus contrary to the conclusion by Lippman (1988) that only fibers with diameters  $\leq 0.1 \mu\text{m}$  reach the pleural space." Boutin et al. (1996) indicated that the finding of some longer fibers had been reported in previous fiber burden studies. "LeBoufant (1980) reported 7% of the amosite and 5% of the chrysotile in the parietal pleura were 5  $\mu\text{m}$  or longer. Sebastien and coworkers (1980) noted that 24% of amphiboles and 14% of chrysotile fibers in the parietal pleura exceeded 4  $\mu\text{m}$ . Dodson et al (1990) found that 10% of amphiboles and 3.1% of the chrysotile fibers were longer than 5  $\mu\text{m}$ " (p. 448). Comparison across these data generated from previous studies as well as that of Boutin et al. (1996) suggested that the majority of asbestos fibers that reach the parietal pleura are shorter than 5  $\mu\text{m}$  in length. The suggestion by Boutin et al. (1996) is that the black spots accumulate coal dust (i.e., black spots) and asbestos dust in the same location. Thus, the question was raised of whether the black spots, which contain asbestos fibers, may be important sites for initiation of pathological events within the parietal pleura and thus as a site of origin for pleural mesothelioma as well as development of pleural plaques.

A subsequent study of tissue collected from 150 consecutive necropsies of urban dwellers in several studies further examined the significance of "black spots" as sites of origin for pathological changes in pleural tissue (Mitchev et al., 2002). Results demonstrated that "there was no relationship between the predominant locations of black spots and hyaline pleural plaques." An additional study by Muller et al. (2002) was conducted with the aim "to understand the formal pathogenesis of 'black spots' and their possible role in the development of mesothelioma." The pleural tissue containing "black spots" was obtained from 12 individuals from a region (German Ruhr region)

where such structures are commonly found in the parietal pleura. Data showed that "our results do not support the hypothesis whereby mesotheliomas develop preferentially in the regions of pre-existing black spots. In our collective studies of former miners of the Ruhr area no asbestos fibers were found especially amphibole fibers directly located in black spots, but silicates, quartz and silicone as well as aluminum-rich minerals such as muscovite was detected. There were no hints that black spots play a possible role in the development of mesothelioma" (p. 266).

#### **AN EXAMPLE OF REMP IDENTIFICATION AND RISK ASSESSMENT COMPLEXITY: AMPHIBOLE CLEAVAGE FRAGMENTS WITH CHRYSOTILE ASBESTOS**

As previously noted, REMP from asbestos, asbestiform, or nonasbestiform habits of minerals historically used as asbestos, or other minerals that simply produce elongated particles when crushed, all accumulate in lymph nodes, which serve as loci from which translocation from the lung to other sites in the body occurs (Netter, 1979; Dodson et al., 2007). A subject of considerable interest in discussions involving REMP is the hypothetical lack of potency for those REMP termed "cleavage fragments." OSHA defines cleavage fragments as "mineral particles formed by comminution of minerals, especially those characterized by parallel sides with a moderate aspect ratio (usually less than 20:1)" (OSHA, 1994a). As a point of further clarification, OSHA further concluded, "Most cleavage fragments of the asbestos minerals are easily distinguishable from true asbestos fibers. This is because true cleavage fragments usually have larger diameters than 1  $\mu\text{m}$ . Internal structures of particles larger than this usually have no internal fibrillar structure. In addition, cleavage fragments of the monoclinic amphiboles show inclined extinction under crossed polars with no compensator. Asbestos fibers usually show extinction at zero degrees or ambiguous extinction if any at all. Morphologically,

the larger cleavage fragments are obvious by their blunt or stepped ends showing prismatic habit. In addition, these fragments tend to be acicular rather than filiform" (59 FR 40964, p. 343). In practice, "cleavage fragment" relative potency issues are largely relevant to assessment of potential hazards associated with amphibole REMP, since amphiboles have a common double-chain silicate structure and comprise all but one of the regulated asbestos minerals.

When the particles are less than 1  $\mu\text{m}$  in diameter and have an aspect ratio greater than or equal to 3:1, OSHA (1994a) recommends "that the sample be analyzed by SEM or TEM if there is any question whether the fibers are cleavage fragments or asbestiform particles." The regulation further states that "care must be taken when analyzing by electron microscopy because the interferences are different from those in light microscopy and may structurally be very similar to asbestos. The classic interference is between anthophyllite and biopyribole or intermediate fiber. The same morphology clues used for light microscopy, e.g. fibril splitting, internal longitudinal striation, fraying, curvature, etc." (OSHA, 1994b, 59 FR 40964, p. 343) may be used for electron microscopy. Such physical distinctions between asbestiform fibers and cleavage fragments noted by OSHA are generally applied to a population of particles rather than single particles. Furthermore, identification criteria such as inclined extinction were reported as unreliable, particularly in environmental applications (Meeker et al., 2006).

However, the simple differentiation is that a "fibrous habit" (asbestiform, in contrast to nonasbestiform or nonfibrous) is a growth texture. Conversely, a cleavage fragment is defined as a particle created by breakage along specific crystallographic planes from a mineral that did not originally grow along its long axis with a fibrous habit. While not meeting a mineralogical definition of a fibrous or asbestiform structure, a cleavage fragment may appear in some morphological comparisons as similar or even identical to an asbestiform structure of the same mineral. Making a distinction between

these particle types during routine analysis is subjective and has become a major point of discussion in the scientific/regulatory community (Case, 1991). The federal regulations governing workplace exposures and general protection of public health from asbestos exposures currently are governed under the asbestos standards as applicable to the asbestiform varieties of these minerals.

Particle characteristics are affected by unique mineral crystal growth impacts, such as the metamorphic transition between fibrous anthophyllite and talc. In this example, particles possess features in part of the structure that are more consistent with anthophyllite asbestos (exposure levels subject to the asbestos regulatory documents), with other portions of the elongated mineral particle having features consistent with fibrous talc. Such structures have been defined structurally as "transition fibers." The issues of distinction by morphological interpretation as to the detailed form of an elongated mineral particle have appreciable relevance to legal issues, since discussions of exposure levels are referenced back to those in regulatory guidance documents. If the dust contains "asbestiform structures" of a given mineral, then an entire set of federal/state/local regulations applies in that asbestos is recognized to induce the aforementioned diseases based primarily on data accumulated from assessment of occupational exposed cohorts.

The historical/scientific basis for determining what structure is counted in a NIOSH 7400 method by light microscopy is discussed in a publication by Langer et al. (1991). The article discusses the mineralogical distinction between asbestiform and nonasbestiform structures of the same mineral, as well as acknowledging that the establishment of the definition of a fiber under the light microscopy count scheme ( $\geq 5 \mu\text{m}$  with an aspect ratio of  $> 3:1$ ) was made with full awareness that "short fiber,  $< 5 \mu\text{m}$  in length, was the predominant component in air, [and] it constituted a small component of the total dust assayed by light microscopy at 100x magnification" (p. 254). The latter point needs to be taken into account when

interpretation of the health-related value of the permissible exposure limit (PEL) is made following certain exposures and further relates to the consideration of short asbestos fibers (elongated mineral particles) in potential inhalation and induction of disease. An example of the problems associated in distinguishing the morphological definition of an elongated mineral particle as being in the asbestiform habit or nonasbestiform habit is the mineral tremolite. McDonald et al. (1989) concluded, following a lung tissue evaluation from 78 cases from autopsy in Canada, that "fibrous tremolite, contaminant of many industrial minerals including chrysotile, probably explained most cases [of mesothelioma] in the Quebec mining region and perhaps 20% elsewhere" (p. 1544).

In an additional study, Roggli et al. (2002) concluded that "tremolite in lung tissue samples from mesothelioma victims derives from both talc and chrysotile" and that tremolite accounts for a considerable fraction of the excess fiber burden in end-users of asbestos products. It was further stated that tremolite was a "contaminate" of fibrous talc and vermiculite. However, from a morphological standpoint the question is: Which form of tremolite is the biologically active entity in producing disease? Possibly reasonable consideration should be given to a report from one of the more active chrysotile mines in Canada and a point of reference in the report by McDonald et al. (1989).

A mineralogical assessment of the amphibole content of the Jeffrey Mine in Asbestos, Quebec, was conducted by a team led by Williams-Jones et al. (2001). Data showed that the Jeffrey Mine contained the presence of the "following amphibole-group minerals: anthophyllite, cummingtonite, hornblende and tremolite-actinolite." The "bulk of the amphibole, however, was in the form of tremolite and actinolite, and found mainly in serpentinite adjacent to or included within felsic dykes. Appreciable quantities of amphibole also are present in pyroxenite (tremolite) and slate (actinolite) in contact with serpentinite distal to the ore zones. Significantly, the chrysotile ores are essentially amphibole-free. Most of the

amphibole is fibrous, but a small proportion is asbestiform according to criteria established by the U.S. Occupational Safety and Health Administration" (p. 89). This is the quandary that may not be unique for only the tremolite form of REMP. The mineralogical study found that tremolite associated with a given mined mineral (chrysotile) is not asbestiform and thus could be argued to not be a regulated fiber. Yet distinguished Canadian researchers feel that "most or all" of the asbestos-related diseases, especially mesotheliomas that occur in the Canadian miners and millers and potentially in other exposed groups, are induced by tremolite (McDonald & McDonald, 1995; McDonald et al., 1989). The implications from their conclusion raise questions regarding potential risks to human health from exposure to "cleavage fragments" in the form of REMP as associated with other minerals.

Dr. Bruce Case, who has authored or co-authored several of the Canadian studies, reasonably concluded regarding the cleavage fragment/asbestiform particle origin debate that "The major flaw in the substitution of mineralogical definitions for microscopical characteristics is a reliance of the first on gross morphology" (Case, 1991). Case (1991) further stated, "For regulatory and health assessment purposes, it is microscopical morphology that counts: there is no evidence that potentially affected cells can distinguish between 'asbestiform' and 'nonasbestiform' fibres having the equivalent dimensions" (p. 357). This would seem to be a worthy hypothesis for further testing with regard to molecular/surface reactions, as discussed in the following section of this paper and in companion papers in this issue.

### ENTRY OF ASBESTOS INTO CELLS

Asbestos fibers are known to produce diverse effects in a variety of different cell types. The mechanisms by which the fibers produce these effects are still not well understood. Minerals have crystal structures with specific arrangements of elements in repeating, periodic arrays. The surfaces of mineral particles will display characteristic surface charge and

topography at the atomic scale, as a result of this inherent internal structure and the “life history” of the particular surface exposed to the environment. With asbestos particles, these characteristics vary with the mineral type. In experiments *in vitro*, crocidolite and amosite were observed to be negatively charged and to bind different proteins than the positively charged chrysotile (Desai & Richards, 1978; Valerio et al., 1986; Scheule & Holian, 1990). This may result in attraction and binding of specific biological molecules, such as proteins, *in vitro* and *in vivo*.

Internalization of the fibers into the cells is likely to play an important role in this process. First, how the fibers actually enter the cells may be instrumental in some of the mechanisms underlying toxicity. If their surface chemical properties enable them to serendipitously utilize preexisting mechanisms of endocytosis, then signaling pathways could be triggered, which would contribute significantly to their effects. Second, the fact that they are inside the cells changes the chemical environment for the fibers. They become exposed to high concentrations of chemicals, such as ADP, citrate, and other organic acids (potential chelators) and ascorbate or cysteine (reductants) at concentrations in the millimolar range. The chelators may mobilize the iron (Fe) away from the fibers, allowing it to move throughout the cell, making it bioavailable, while the reductants may produce redox cycling of Fe on the fibers or bound to chelators, resulting in production of deleterious reactive oxygen species (ROS) or reactive nitrogen species (RNS) that may damage biomolecules. Third, being taken inside the cell places the fibers in much closer proximity to the ultimate potential targets, such as lipids, proteins, and DNA. Fourth, if endocytized, the fiber is surrounded by a membrane that controls conditions, such as pH, making the environment more acidic, thus impacting subsequent events.

### Vitronectin Binding and Endocytosis

If fibers are capable of binding high levels of specific proteins, this may result in disguising

them as a protein familiar to the cells, which might trigger programmed biological responses. This is precisely what Boylan et al. (1995) found when they investigated the effects of various proteins on the uptake of NIEHS crocidolite asbestos (amphibole with a nominal composition  $(\text{Na}_2\text{Fe}_3^{+2}\text{Fe}_2^{+3}\text{Si}_8\text{O}_{22}(\text{OH})_2)$ ) by rabbit pleural mesothelial cells. Coating the fibers with serum or vitronectin, a major adhesive protein in serum, tissues, and fluids, including lung lining fluid, enhanced the uptake of the fibers. Further it was found that the internalization of the vitronectin-coated fibers was mediated by integrin  $\alpha v\beta 5$ . Bronchoalveolar lavage fluid also coated crocidolite with vitronectin. The lung lining fluid in the lavage fluid is known to contain abundant levels of vitronectin (Memmo & McKeown-Longo, 1998). Another mineral, wollastonite (pyroxenoid with a nominal composition  $\text{CaSiO}_3$ ), Nyglos I, milled to a geometry mimicking that of crocidolite, also bound vitronectin, but was not internalized by vitronectin receptors on the mesothelial cells (Boylan et al., 1995). Evidence indicated that the wollastonite-bound vitronectin might assume a different conformation, due to a difference in surface interaction, preventing the binding to receptors, such as  $\alpha v\beta 5$  integrin.

Pande et al. (2006) expanded these studies and found that serum-treated NIEHS crocidolite fibers were internalized into a human lung epithelial cell line (A549) and human primary small airway epithelial cells by  $\alpha v\beta 5$  integrin. The internalization, not just binding to the  $\alpha v\beta 5$  integrin, was required for efflux of reduced glutathione from these cells. This is an important observation and is discussed in more depth in a subsequent section, since this influences the intracellular redox status substantially and contributes to ultimate effects of the fibers on the cells.

### Summary

The binding of vitronectin by crocidolite and the subsequent uptake of the fibers by  $\alpha v\beta 5$  integrin appear to mediate the endocytosis by more than one cell type and may play

a critical role in important changes in the intracellular redox environment by mechanisms not well understood. These changes in intracellular redox state may contribute greatly to the ultimate effects the fibers have on the cell and will be discussed at more length in sections to come.

## CHEMICAL CHANGES IN ASBESTOS

### Structural and Physical Properties of Asbestos Important for Reactions

Before considering the chemical changes that asbestos may undergo, it is important to have a brief review of the chemical structure of these crystalline minerals. The amphibole minerals, crocidolite and amosite, are composed of octahedrally coordinated cations, including Fe, sandwiched between two double silicate chains (Hardy & Aust, 1995). The oxygen atoms coordinate both the Si and other cations, predominantly Fe and magnesium (Mg). Chrysotile has a cylindrical structure resulting from a misfit between the  $\text{Mg}(\text{OH})_2$  octahedral sheet and the  $\text{SiO}_4$  tetrahedral sheet. The Mg ions may be substituted with Fe in modest amounts, which accounts for trace to minor amounts of Fe in the structure. The intrinsic Fe content of asbestos is highest in the amphiboles, crocidolite and amosite, up to 27% by weight. Also important to note is the valence of Fe in the idealized structure of these two amphiboles. In amosite the iron is primarily Fe(II), while in crocidolite both Fe(II) and Fe(III) are present.

Erionite is an aluminosilicate of the zeolite group, composed of silicon–oxygen tetrahedra, which associate into six-membered rings to form channels and cages. Systematic substitution of Al atoms for Si gives rise to an overall negative charge on the framework tetrahedra. This negative charge is balanced by monovalent and divalent cations binding within the channel and cage structure. Various cations and anions might migrate into the 4.3-Å openings of the network of channels and replace existing ions in reactions that are dependent upon temperature and the chemical environment. This open, cage-channel structure can,

under the right conditions, give erionite a significantly higher “surface” area for certain reactions than the surface areas of comparably sized amphibole asbestos fibers. Thus, while there may be significant differences in surface area between amosite and crocidolite samples, rather large differences exist between the amphiboles chrysotile and erionite, even when considering fibers of comparable dimension. Many of the reactions that these fibers may undergo will be influenced by the surface area and surface topography.

Exposed silicate mineral surfaces can form silanols ( $\text{Si}(\text{OH})_x$ ). With asbestos minerals, the number of surface silanol groups varies largely for the various fiber types, the environment, and the “life history” of the particle. These silanol groups may also contribute heavily to the reactions these minerals undergo. The surface silanols influence the interaction of a given fiber with cations, such as Fe, that were not originally part of the structure. This is referred to as “iron binding” or “iron acquisition,” which are discussed in detail in sections that follow.

**Summary** The two amphibole forms of asbestos, crocidolite and amosite, contain relatively high levels of intrinsic Fe (up to 27% by weight), which is coordinated in the structure by the oxygen from the silica tetrahedra. The idealized structure of other silicates, such as erionite, does not contain Fe. The oxygen in the surface silanol groups of asbestos or erionite may also coordinate cations, such as Fe, thus increasing Fe content on the surface. Thus, the surface of the fibers becomes important.

### Reactions Catalyzed by Asbestos

**Fiber Surface** In considering the determining factors that control reactivity of asbestos, one must first take into serious consideration the surface of the fibers. This is where the fiber interacts with its environment and may be affected by milling, suspension in aqueous solution, or changes in temperature or pressure.

*Active sites* Mineral surfaces are complex not only in atomic structure and composition, but also in terms of microtopography

(Hochella, 1993). "Active sites" often occur where surfaces are rough or the microtopography is uneven. These areas may possess dangling bonds, silanol groups, and unoccupied cation coordination sites. Milling might create "active sites" making the fibers more reactive (Hochella, 1993).

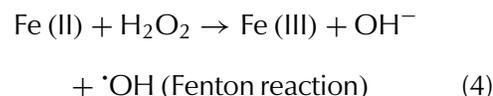
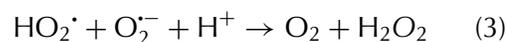
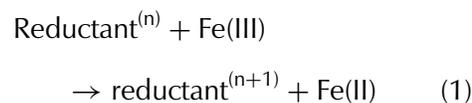
*Milling effects* Fubini et al. (1990) observed homolytic and heterolytic cleavage of chemical bonds on the surface of silicates after grinding, which produces distorted siloxane bridges, peroxide bridges, silica radicals,  $\text{Si}^+$  or  $\text{Si}-\text{O}^-$  surface charges,  $\text{Si}-\text{O}_2^\cdot$  peroxyradical, and  $\text{Si}^+\text{O}_2^{\cdot-}$ . These reactive groups would be rapidly dissipated shortly after inhalation. While reactive groups may be involved in acute biological effects, their contribution to chronic disease development, such as carcinogenicity, is less likely unless the exposures were continuous over chronic durations.

*Surface, redox-active iron* The amount of Fe on the surface of the mineral fibers is more likely to control the long-term reactivity, since it has the capability of redox cycling, generating ROS, superoxide, hydrogen peroxide, and hydroxyl radical over long periods of time.

Shen et al. (1995) found that the total amount of redox active Fe on the surface of NIEHS crocidolite or NIEHS amosite remained constant even after repeated oxidation and reduction cycles. This may be very important in the reducing environment of the cell where  $\text{O}_2$  is also present. While many investigators seem mostly concerned with how much Fe(II) is present on the fibers, this seems irrelevant compared with the total amount of Fe that might redox cycle. If asbestos-generated hydroxyl radical or a similar ROS is responsible for the DNA damage that produces asbestos-induced cancer, then it is unlikely that surface-reactive Fe is responsible, since ROS as reactive as those would not travel more than 3 nm from the fiber, thus requiring the fiber to be present in the nucleus for this damage. While this was observed, it is infrequent and would diminish the possibility that this is a plausible mechanism for introduction of oxidative DNA damage.

**Hydroxyl Radical Generation by Asbestos**  
Oxygen cannot react with biological molecules

directly but requires reduction by a transition metal, such as Fe(II), to produce ROS that are capable of reacting with biological molecules, as in the reactions that follow. These reactions, the modified, Fe-catalyzed Haber-Weiss reactions, lead to the formation of the hydroxyl radical  $\cdot\text{OH}$  (Lund & Aust, 1990):



The ferryl iron species,  $\text{Fe}^{\text{IV}} = \text{O}$ , and  $\text{Fe}^{\text{II}}-\text{Fe}^{\text{III}}-\text{O}_2$  complex have also been proposed to be involved in reactions catalyzed by Fe, but methods for studying them are not readily available. Therefore, there exists more evidence for the generation of hydroxyl radical or a species of similar reactivity. Common biological reductants, such as cysteine or ascorbate, are excellent reductants of Fe. It is important to note that the intermediate oxygen species, superoxide  $\text{O}_2^{\cdot-}$  and hydrogen peroxide  $\text{H}_2\text{O}_2$ , do not react directly with biological molecules, it is the  $\cdot\text{OH}$ , or a similarly reactive species, that is of importance.

It is extremely reactive with biological molecules and only diffuses about 3 nm, the average diameter of a typical protein, before reacting (Hutchinson, 1957). Of great importance to consider in understanding the mechanism by which Fe from asbestos might catalyze damage to biological molecules is that the  $\cdot\text{OH}$  does not cross the cell membrane. Thus, damage occurs where it is generated and requires the presence of catalytically active Fe.

Early studies with asbestos were focused on determining whether Fe associated with the fibers was capable of undergoing these types of reactions. If so, then the hydroxyl radical, or a similarly reactive species, generated by

reactions with Fe(II) might contribute to the mechanisms by which asbestos induced damage in biological systems. Subsequent studies asked questions about whether the ROS were generated by surface-bound Fe or Fe mobilized away from fibers. Indicators for the generation of ROS used in these studies included electron paramagnetic resonance spectroscopy (EPR) spin trapping, salicylate hydroxylation, lipid peroxidation, DNA oxidation (strand breaks or oxidation of deoxyguanine), and oxygen consumption. Perhaps the most sensitive method to detect the generation of  $\cdot\text{OH}$  was the induction of DNA single-strand breaks (SSB), using closed circular, plasmid  $\phi\text{X-174}$  RFI DNA.

Weitzman and Graceffa (1984) were the first to study the radical generation by asbestos fibers, UICC crocidolite, UICC Canadian chrysotile, and UICC amosite. Using electron paramagnetic resonance (EPR) spin trapping, it was noted that chrysotile, crocidolite, or amosite generated  $\cdot\text{OH}$  when suspended in  $\text{H}_2\text{O}_2$ -containing, aqueous solutions. The addition of the Fe chelator desferrioxamine B inhibited the formation of  $\cdot\text{OH}$ , indicating that the reactivity was due to Fe. Pezerat and coworkers (1989) also conducted extensive studies with electron spin resonance (ESR) to examine the role of Fe in the chemical reactivity of UICC asbestos samples. It was proposed that there are two types of ROS generated by Fe, which are responsible for the production of  $\cdot\text{OH}$  or similarly reactive species that attack DNA and another strongly oxidizing species capable of initiating lipid peroxidation.

A comparison of the ability of a fiber to generate  $\cdot\text{OH}$  and the formation of 8-OHdG yielded a strong correlation (Nejjari et al., 1993). Grinding was used to increase the oxidizing potential of the minerals. After grinding, UICC amosite or UICC crocidolite had a 145- or 125-fold rise in ability to generate DMPO adducts and a 53- or 22-fold elevation in ability to catalyze the formation of 8-OHdG, respectively. Erionite showed little ability to generate EPR spin adducts before or after grinding, with a fivefold increase in 8-OHdG. Grinding the amphiboles likely produced the radical species, discussed earlier under "Milling Effects," and

exposed new surfaces with Fe(II). The limited amount or lack of Fe in native erionite may explain the lack of activity compared with the Fe-containing minerals. Further studies by Ghio et al. (1992b) showed a relationship between total surface Fe and the ability to generate  $\cdot\text{OH}$ .

To determine whether Fe on the surface of asbestos or Fe mobilized into solution by chelators was the most significant contributor to the ROS generated by asbestos, Aust and Lund (1991) and Lund and Aust (1991) compared the oxygen consumed and EPR spin adducts produced by NIEHS crocidolite in the absence or presence of chelators, e.g., citrate, ADP, and ethylenediamine tetraacetic acid (EDTA). Results indicated that mobilization of Fe from the crocidolite fibers with chelators greatly increased its reactivity with oxygen to produce  $\cdot\text{OH}$ . A more extensive discussion of Fe mobilization follows.

### Iron-Catalyzed Damage to Biomolecules

Iron-catalyzed generation of ROS produces damage to DNA similar to what was seen with x-rays or  $\gamma$ -rays. One of the major products that was quantified is 8-OHdG. This damage leads to mutations from the misincorporation of bases during replication of the damaged DNA (Loeb et al., 1988; Shibutani et al., 1991) if the damage is not repaired by enzymes synthesized by cells for that purpose. These and mutations induced by other ROS-produced lesions may be involved in the initiation of cancer.

Iron-catalyzed reactions also damage lipids. The role of Fe in lipid peroxidation was extensively studied, and the identity of the oxygen species responsible remains controversial. However, molecular oxygen is required for Fe-catalyzed lipid peroxidation. Products of lipid peroxidation, such as 4-hydroxynonenal, 4-hydroxyhexenal, and malonaldehyde, were extensively studied and are considered dangerous reactants because of their long lifetimes and ability to traverse the cell (Halliwell & Gutteridge, 1999). These products react with DNA and protein and may be involved in initiation of cancer.

Iron-catalyzed ROS also damages proteins. The ROS responsible is not known, but, once again, oxygen is required (Halliwell & Gutteridge, 1999). What role these damaged proteins may play in development of cancer is not understood, but modification of function of proteins, such as glutathione (GSH) or glutathione peroxidase (GSPx), or DNA repair enzymes may compromise the cell's ability to defend itself against an assault by ROS.

In summary, the studies discussed here broadly support that asbestos fibers are capable of generating ROS and that this activity is due to Fe, either on the fibers or more likely mobilized from the fibers. Studies specifically designed to compare reactivity of Fe on the fibers, compared with that from Fe mobilized by chelators, found that the mobilized Fe was much more reactive and capable of generating ROS. Iron-catalyzed generation of ROS produces damage to cellular DNA, lipids, and proteins.

#### **Iron Mobilization From Mineral Fibers**

Under normal conditions, Fe is absorbed from the diet and transported in the blood by the protein transferrin. Specific receptors for transferrin on the cell membrane enable Fe to be transported into the cell only when the cell requires Fe. Iron can then be used in the synthesis of essential proteins or stored in the protein ferritin. When bound by these proteins, the reactions of Fe are controlled. However, if Fe enters the body via routes other than dietary, it may produce serious adverse effects, because the body has not developed methods for efficiently sequestering Fe to preferentially facilitate insertion into functional or storage proteins. Thus, if Fe-containing fibers are inhaled into the lungs and endocytized into cells where Fe might be released intracellularly, bypassing the normal transport mechanisms and control of reactivity by proteins, the metal may potentially interact with reductants, such as ascorbate and cysteine, becoming reduced and subsequently passing electrons to molecular oxygen in a series of reactions that produce damaging ROS.

Thus, endocytosis of asbestos fibers constitutes an uncontrolled entry of Fe into the cell, because this bypasses control by the protein

transferrin. As just discussed, there is redox-active Fe on the surface of the fibers, which in the presence of cellular reductants initiates redox cycling. However, also within the cell are low-molecular-weight (LMW) chelators, such as citrate, which are present at millimolar concentrations. If these chelators have the ability to mobilize Fe from the fibers, the redox activity might be altered, producing more ROS, and the chelate complex may diffuse throughout the cell and have the potential of catalyzing the formation of the hydroxyl radical or a similarly reactive species within the nucleus, thus inflicting oxidative damage to DNA.

The Fe(II) chelator ferrozine has often been used to quantify the amount of Fe(II) Fe in solutions because it forms a colored complex of high extinction coefficient with Fe(II) (Stookey, 1970). The amount of Fe mobilized by LMW chelators, such as citrate, ADP, EDTA, and nitrilotriacetic acid (NTA), is determined by incubating asbestos with millimolar concentrations of each chelator for a prescribed time, then quantifying the amount of Fe mobilized into the solution by doing a total Fe assay with ferrozine. Chelators, such as ADP, EDTA, NTA, and citrate, increase the redox activity of Fe, while ferrozine or desferrioxamine B inhibit redox activity. That is why desferrioxamine B is frequently used to determine whether Fe is involved in chemical or biological activities.

*Factors affecting mobilization* Fiber characteristics that become important for mobilization of Fe from different mineral fibers are crystalline structure, surface area, surface structure, and Fe content. Lund and Aust (1990, 1991, 1992) studied mobilization of Fe from NIEHS crocidolite, amosite, chrysotile, tremolite, and erionite (from Rome, OR) in vitro. Results from these studies revealed that Fe mobilization required the presence of a chelator at physiological pH (Lund & Aust, 1990). This suggests that "leaching" of Fe from asbestos, which was observed in vivo (Holmes & Morgan, 1967; Morgan et al., 1971; Jaurand et al., 1977; Spurny, 1983; Parry, 1985), was the result of chelation of Fe and mobilization from the fibers. Lund and Aust (1990) also found that the rate of mobilization depended

upon the chelator being used. Since the stability constants for the chelators with Fe do not correlate well with the rate of mobilization of Fe from the fibers, other factors, such as fiber surface geometry, size and charge of the chelator, and complementarity of the coordination of Fe with that of the fiber, are important (Hardy & Aust, 1995).

The Fe content in NIEHS crocidolite and that in NIEHS amosite are similar, but when Fe mobilization rates were compared, the rate of mobilization from crocidolite was higher than amosite on a weight basis (Lund & Aust, 1990). However, when corrected for differences in surface area, the rates were comparable (Lund & Aust, 1990). Since the vast majority of comparative studies use weight as the standard for comparison, no reliable information on relative reactivities is available. Little Fe was mobilized from NIEHS long or short fiber chrysotile, none from tremolite (Lund & Aust, 1990), and no Fe was mobilized from erionite from Rome, OR (Eborn & Aust, 1995). Some forms of asbestos, such as the amphiboles, resist dissolution in the lung and reside for decades. Thus, Fe may be removed from these fibers for prolonged periods of time. Studies on long-term removal of Fe from the amphiboles, NIEHS crocidolite and amosite, revealed that Fe could still be mobilized, albeit at slower rates, even after incubation of fibers with desferrioxamine B for up to 90 d. After incubations for varying periods of time with desferrioxamine B, the fibers produced fewer oxygen radicals (Ghio et al., 1992a) and produced fewer DNA SSB (Chao & Aust, 1994). If Fe is involved in the biological effects of asbestos, desferrioxamine B treatment should reduce, but not eliminate, the effects of the fibers on cultured cells. Observations in cultured cells of several different types reported in the literature are consistent with this conclusion (Goodglick & Kane, 1986, 1990; Mossman et al., 1987; Shatos et al., 1987; Kennedy et al., 1989; Kamp et al., 1990; Lund & Aust, 1992). Iron mobilization does impact the structure of the outer layer of the fibers, but the silicate structure, somewhat amorphous but intact, subsequently binds cations, such as Fe, from solution.

Other factors that impact mobilization of Fe from asbestos in vitro and that may impact the effects of asbestos inhaled from environmental exposures are temperature, pH, and time in aqueous suspension. Iron mobilization rates decrease as NIEHS crocidolite is incubated in aqueous suspension. Increased temperature during those incubations, as with autoclaving, results in an even more significant reduction (Mossop, 1992). The rate of Fe mobilization from crocidolite or amosite increased as the pH was lowered from 7.5 to 5, the pH observed in phagosomes of cells (Lund & Aust, 1990; Chao & Aust, 1994), suggesting that uptake of fibers by cells may enhance Fe mobilization.

Theoretically, the oxidation state of Fe on the surface of the fibers might impact the rate of mobilization. However, organic acids, such as citrate or ADP, which are the likely candidates for mobilization of Fe in vivo, were found to mobilize both Fe(II) and Fe(III).

*Synergistic effects of cigarette smoke* It is known that there is a synergistic effect between cigarette smoking and asbestos exposure on the development of bronchial carcinoma (Selikoff et al., 1968; Kamp et al., 1992). There are many known carcinogens in cigarette smoke. A discussion of the association of those carcinogens with the asbestos fibers and how this may potentiate a greater risk of cancer is provided in Huang et al (2010). A different possible mechanism for this synergism was proposed by Qian and Eaton (1989, 1991) when it was found that organic acids, stearic and palmitic acid, present in cigarette smoke, were able to chelate, mobilize, and translocate Fe from crocidolite and amosite into intact red blood cells in vitro, where it remained. Thus, bypassing protein control of Fe uptake with transferrin may result in elevated LMW pools of Fe inside the cells, resulting in generation of ROS which may damage DNA.

*Summary* Mobilization of Fe from minerals is dependent upon crystalline structure, surface area, surface structure, and Fe content of the mineral. Mobilization at physiological pH requires the presence of a chelator to coordinate Fe and move it into solution. The rate of mobilization is (1) dependent upon the

chelator being used, (2) inversely proportional to pH, and (3) directly proportional to temperature. Exposure of the mineral to aqueous environments before treatment *in vitro* or *in vivo* may slow the rate of mobilization. These effects increase with temperature, pH, and time of exposure. Thus, how samples are handled before treatment is critical for consistency of outcome in experimental results.

**Intracellular Iron Mobilization** Leaching of Fe and other ions from chrysotile *in vivo* was observed (Holmes & Morgan, 1967; Morgan et al., 1971; Jaurand et al., 1977; Spurny, 1983; Parry, 1985), but no quantification was available. Using neutron-activated NIEHS crocidolite, containing  $^{55}\text{Fe}$ , Chao et al. (1994) were able to quantify the amount of Fe mobilized from the fibers in human lung epithelial (A549) cells. After 24 h of treatment of cells with  $4.5 \mu\text{g}/\text{cm}^2$  crocidolite, the majority of Fe mobilized within the cells (1.4 mM) was found associated with proteins, but a significant amount,  $22 \mu\text{M}$ , appeared in the <10-kD, or LMW, pool. There was a linear relationship between the cytotoxicity of the crocidolite to the cells and the amount of Fe in the LMW pool, but not total Fe mobilized. The rate of Fe mobilized in A549 cells was comparable to the rate of Fe mobilized by citrate *in vitro* at pH 7.5 (Chao et al., 1994). Since no Fe was removed from the fibers by incubation in the cell culture medium, only when cultured cells were present, and crocidolite was internalized by the cells, these findings suggest that Fe was being mobilized by intracellular chelators similar to citrate. This is consistent with the observations that the concentrations of organic acids, such as citrate, are at millimolar levels in the cell, but not in the culture medium or extracellular fluids *in vivo*.

Normally, ferritin uptake of Fe is postulated to protect cells from the damaging effects of Fe. Using the same neutron-activated crocidolite, as described earlier, ferritin synthesis and the movement of  $^{55}\text{Fe}$ , after mobilization from crocidolite, were examined in human lung epithelial (A549) cells (Fang & Aust, 1997). Iron mobilization began immediately after treatment of the cells, but ferritin synthesis did not

begin until about 4 h after treatment. Despite the incorporation of Fe into ferritin and non-ferritin proteins, the amount of Fe in the LMW fraction increased immediately and remained relatively constant throughout the treatment. This suggests that ferritin synthesis may not have fully protected the cells. In studies to be discussed in a later section, crocidolite treatment of A549 cells, under the same conditions as described earlier, resulted in the formation of 8-OHdG, which required the presence of Fe from the fibers, indicating that Fe in the LMW fraction was redox active and generating ROS or inducing the production of peroxynitrite  $\text{ONOO}^-$ , to be discussed in more detail in a later section of this report (Chao et al., 1996).

### Biological Significance of LMW Iron

The presence of an intracellular, LMW pool of potentially redox-active Fe after crocidolite treatment is of significant concern, since this is observed *in vivo* only in disease states, such as Fe overload disease hemochromatosis. Analysis of blood from hemochromatosis patients contains citrate-chelated Fe (Grootveld et al., 1989), and it is well known that citrate-chelated Fe produces damage to biomolecules in the presence of a reductant, such as ascorbate (Aruoma et al., 1989; Lund & Aust, 1990). The LMW Fe mobilized from crocidolite was directly related to the cytotoxicity of the fibers and may be similar to Fe in other pathological conditions, such as hemochromatosis.

Iron has been implicated as a cancer-causing agent in a number of different exposure conditions. Iron ore miners were reported to have an increased incidence of lung cancer (Boyd et al., 1970; Kinlen and Willows, 1988). There is an elevated risk of liver cancer among victims of Fe overload diseases, hereditary hemochromatosis (Bradbear et al., 1985; Akarasereenont et al., 1995) and porphyria cutanea tarda (Salata et al., 1985; Okuda, 1986). There is also a direct correlation between increased body Fe stores and an elevated risk of cancer of all organs and tissues in individuals not suffering from Fe overload diseases (Stevens et al., 1986, 1988). Thus,

increasing LMW Fe in lung or mesothelial cells after inhalation of asbestos may pose a risk for cancer.

*Summary* Iron is mobilized from asbestos into an LMW (<10,000 MW) fraction in cultured cells and after being inhaled by humans. In cultured cells, the amount of Fe in the LMW fraction was directly proportional to the toxicity of the asbestos. The LMW Fe from asbestos is likely to pose an increased risk for cancer, as it does for other disease conditions where it is found.

### Iron Binding

An important observation made from studies of silicate mineral fibers *in vivo* is that the fibers acquire Fe from the biological system, as well as liberating the metal. The formation of ferruginous bodies in individuals exposed to silicate fibers demonstrates that this does occur *in vivo*. This additional Fe may potentiate the ability of the fibers to produce pathological damage. Until recent observations, discussed next, the most generally accepted theory was that most humans coat fibers with Fe and that this process of coating affords protection to the tissue from the physical threat of the needlelike fibers (Davis, 1970; Pooley, 1972). However, recent studies to be discussed, investigating the reactivity of the Fe deposited on asbestos, would lead one to question that conclusion.

### Ferruginous Bodies

Fibers are known to acquire Fe after inhalation into the lungs. These coated fibers may become so large that they are visible via light microscopy and are designated as ferruginous bodies (Dodson et al., 1991), or asbestos bodies if the core is asbestos. The first report of ferruginous bodies was in 1906 (Marchand, 1906). The presence of asbestos bodies in the lungs of patients was used as an indication of previous exposure to asbestos (Barclay, 1984). Factors that seem important in determining whether fibers are coated are length, physical characteristics (Dodson et al., 1982, 1983), and number of fibers inhaled (Churg & Warnock,

1981; Morgan & Holmes, 1985). It is known that crocidolite, amosite, chrysotile, and erionite all form ferruginous bodies after long-term exposure *in vivo* (Hammar & Dodson, 1994; Hammar, 1994).

Macrophages were proposed to be the source of Fe for the formation of ferruginous bodies. Koerten et al. (1986) found that when crocidolite was added to cultures of macrophages, only fibers too large to be completely phagocytized were coated to form asbestos bodies. After 4 wk in culture, the large fibers were beginning to be coated, but the thickness and the segmentation of the added material became more prominent after longer durations of exposure.

Inflammatory macrophages, which appear after inhalation of mineral fibers, have a greater uptake of Fe than resident macrophages from sources, such as effete erythrocytes (Birgegard & Caro, 1984). The factor that seems the most important for Fe-coating of mineral fibers seems to be the coating ability of the macrophages. Thus, phagocytosis of mineral fibers with a high affinity for Fe by these macrophages may lead to Fe deposition and ultimately formation of ferruginous bodies. The time required for formation of ferruginous bodies is not known, but individuals vary in their ability to deposit Fe on fibers. This is likely related to the variation in body burden of Fe.

### Intracellular Source of Iron

Ferritin or hemosiderin, oxidized ferritin from lysosomes, was proposed as the intracellular source of Fe (Hammar & Dodson, 1994) The Fe coating may be segmented into spherical or rectangular units spaced along the fiber, like beads on a necklace (Churg & Warnock, 1981). Gloyne (1931) and more recently Botham and Holt (1971) described in an animal model that the earliest form of asbestos body has a sheathlike coating of Fe that with time becomes segmented. Investigations by Pooley (1972), using transmission electron microscopy and x-ray diffraction, suggest that asbestos bodies are coated with a crystalline Fe oxide material with a composition similar to the Fe oxide core

of ferritin, the Fe storage protein. Subsequent to that, the Fe core of ferritin was determined to be ferrihydrite (Treffry et al., 1987).

In studies *in vitro*, where NIEHS crocidolite or amosite was incubated in FeCl<sub>3</sub> solutions for 14 days, Fe was found to be removed from the solutions, using a ferrozine total Fe assay, and to be deposited on the fibers, using x-ray photoelectron spectroscopy (XPS) (Shen et al., 2000). Analysis of the surfaces of the fibers, using atomic force microscopy, revealed that the microtopography of the amosite was rough compared with that of the untreated fibers, further evidence of Fe(III) accumulation on the fiber surfaces, but in specific locations formed three-dimensional structures on the surface. X-ray diffraction (XRD) analysis of the Fe(III)-loaded crocidolite or amosite suggested that ferrihydrite, a poorly crystallized hydrous ferric iron oxide, had formed. This was in the absence of any ferritin. Thus, although *in vivo* ferritin may be involved, it is not required to contribute the ferrihydrite form of Fe. That forms naturally with Fe(III). XRD analysis of amosite-core asbestos bodies taken from human lungs also showed that ferrihydrite was present. When Fe(II) iron solutions were exposed to crocidolite, a limited amount of Fe(II) was bound, but quickly stopped. It is postulated that the Fe(II) was undergoing ion exchange with the Na<sup>+</sup> sites. When these sites were saturated, the binding stopped. Thus, fibers loaded with Fe(III) may be useful models for the study of chemical characteristics and biological potential associated with asbestos bodies.

#### **Chemical and Biological Activity of Asbestos Bodies or Iron-Loaded Fibers**

Asbestos bodies with amosite cores, isolated from the lung tissue of a deceased 68-year-old insulator, were examined for their ability to induce DNA SSBs in  $\phi$ X174 RFI DNA, a sensitive method for detection of ROS. The asbestos bodies induced SSB, while an equal number of amosite fibers of similar length did not (Lund et al., 1994). This activity appeared to be due to Fe coating on the asbestos bodies,

since EDTA, an Fe chelator that mobilizes Fe from fibers and potentiates redox activity of Fe, increased SSB, while desferrioxamine B, an Fe chelator that inhibits redox activity of Fe, blocked the formation of SSB. This suggests that Fe deposited on the fibers *in vivo* is bioavailable and may potentiate damage to biological molecules, such as DNA.

There have been a variety of studies to investigate the impact that Fe binding to crocidolite, amosite, or chrysotile asbestos, as well as a few other silicates, has on chemical and biological activities. Ghio et al. (1992a) investigated the effect of acquisition of Fe(III) exerted on the reactivity of crocidolite, silica, kaolinite, and talc. All of these minerals bound Fe, resulting in an increased formation of thiobarbituric acid-reactive products over untreated minerals, but crocidolite showed the greatest rise in reactivity. It was also found that rat alveolar macrophages exposed to Fe-treated fibers released more leukotriene B<sub>4</sub>. When crocidolite, silica, kaolinite, or talc was injected into the pleural cavities of rats and left for 4 d, the recovered minerals demonstrated increased amounts of chelatable Fe on their surfaces. Thus, Fe bound from inorganic or biological sources enhanced the abilities of the minerals to generate oxidants and was proposed to be responsible for the biological activities observed. In similar studies, Ghio et al. (1994) compared the abilities of Fe(III)-loaded crocidolite, amosite, or chrysotile to induce DNA SSB and thiobarbituric acid-reactive products. The reactivities of the Fe(III)-loaded fibers correlated with the amount of Fe(III) bound, with crocidolite > amosite > chrysotile.

In investigating the ability of erionite to catalyze the formation of hydroxyl radical, Zalma et al. (1987) found that the native fibers were unable to produce the hydroxyl radical spin adduct. Using a more sensitive technique for detecting hydroxyl radical, induction of SSB in  $\phi$ X174 RFI DNA, Eborn and Aust (1995) also concluded that erionite was not capable of catalyzing the production of hydroxyl radical. However, when they exposed erionite to solutions of Fe(II) or Fe(III), data showed that the fibers readily bound the iron, Fe(III) >

Fe(II). Ferrous iron appeared to bind via ion exchange, while Fe(III) likely had a mechanism of binding active sites and then forming three-dimensional structures on the erionite in a precipitation or crystallization process (Mann et al., 1993). After Fe binding, the fibers were able to catalyze the formation of DNA SSB in vitro in the presence of a reductant and/or chelator. The amount of SSBs was proportional to the amount of Fe mobilized when a chelator was present. This may explain why erionite may induce cancer but appears nonreactive in vitro.

*Summary* Mineral fibers have been found to accumulate Fe to form ferruginous bodies after inhalation. It is postulated that these deposits come from inflammatory macrophages. The Fe that is deposited to form large asbestos bodies in the lung may be mobilized in vitro and does redox cycle to generate ROS. Whether this happens in vivo is not known. In vitro experiments to model formation of asbestos bodies using Fe(III) solutions showed continuous uptake of Fe with time, forming small three-dimensional structures over the surface of the fibers. Thus, it is likely that the formation of asbestos bodies happens in stages, perhaps starting with more uniform coating and eventually developing the thicker plaques in some areas. This seems consistent with what has been observed in animals after inhalation of fibers. Smaller fibers may never form the thicker plaques, but only have the more uniform coating of iron. The potential for release of deleterious Fe is there, but whether this actually happens in vivo is not well understood.

### **INTRACELLULAR REDOX CHANGE AND PRODUCTION OF REACTIVE NITROGEN SPECIES**

#### **Efflux of Reduced Glutathione**

Reduced glutathione (GSH) participates in conjugation and reduction reactions. Some of the conjugation reactions are enzyme catalyzed and others are spontaneous chemical reactions. These reactions detoxify foreign compounds and carcinogens, both organic and

inorganic. Some of the reduction reactions serve to maintain reduced cysteine in functional proteins/enzymes, while others also serve the function of detoxification, but in this case by donating electrons to ROS. In these detoxification reactions, GSH is converted to the oxidized form GSSG. Normally at least 90% of the GSH is in the reduced form, and the remainder in the oxidized form. An increased ratio of GSSG to GSH indicates oxidative stress in the cells (Halliwell & Gutteridge, 1999).

Decreases in cellular reduced glutathione (GSH) were reported by Janssen et al. (1995) in rat pleural mesothelial cells exposed to crocidolite, by Boehme et al. (1992) in rat alveolar macrophages exposed to crocidolite, and by Israbian et al. (1994) in human pulmonary epithelial-like cells (WI-26) exposed to UICC amosite asbestos. All of these investigators attributed the decrease in GSH to formation of oxygen radicals.

However, Boehme et al. (1992) further reported release of GSH into the extracellular medium after crocidolite exposure. Golladay et al. (1997) also observed a release of 50% of the intracellular GSH into the extracellular medium after treatment of human lung epithelial (A549) cells with NIEHS crocidolite. This did not appear to be the result of membrane damage, since lactate dehydrogenase (LDH) activity was not observed in the medium in either case. It also did not appear to be apoptosis related, since removal of Fe with desferrioxamine B pretreatment of the NIEHS crocidolite fibers had absolutely no marked effect on the intracellular levels of GSH when compared with untreated crocidolite, but increased the survival of the treated cells. Golladay et al. (1997) also noted a 47% decrease in  $\gamma$ -glutamylcysteine synthetase activity without changes in the activities of GSH reductase, GSH peroxidase, GSH S-transferase, or glucose-6-phosphate dehydrogenase. Since  $\gamma$ -glutamylcysteine synthetase is involved in the GSH synthesis pathway, the decrease in activity would likely lead to less synthesis of GSH and may have contributed to marked intracellular fall in GSH. As discussed in the section on endocytosis of asbestos, the endocytosis

of the fibers, not just binding to  $\alpha v\beta 5$  integrin, was required for the observed decrease in intracellular GSH (Pande et al., 2006). Thus, the significant fall in GSH was independent of Fe associated with the fiber, but dependent upon endocytosis of the fiber. This suggested that the decrease in GSH had nothing to do with detoxification of ROS. Since GSSG did not increase with any of the treatments described, this further suggested that ROS had not overwhelmed the ability of the cell to cope with the asbestos challenge. The significant reduction in GSH resulted in an overall rise in the oxidizing environment for protein thiols in the cells. Although the role that this change may play in the overall effects of crocidolite on the cell are not completely understood, the change in the oxidizing environment might serve a regulatory function that activates or inactivates proteins via thiol oxidation leading to control of signaling pathways and the function of the cell.

### Induction of Inducible Nitric Oxide Synthase

Treatment of human lung epithelial (A549) cells with NIEHS crocidolite resulted in the formation of 8-OHdG in the DNA (Chao et al., 1996). Formation of this potentially mutagenic and carcinogenic base in the DNA is the result of oxidation by a highly oxidizing species, such as hydroxyl radical  $\cdot\text{OH}$  (Loeb et al., 1988; Huang et al., 2011). Previous discussions of the reactivities of Fe from crocidolite suggest that  $\cdot\text{OH}$  may be produced after the asbestos treatment (reactions 1–4 described earlier).

However, there is another chemical candidate that might produce this base modification, and that is peroxynitrite. The formation of this highly oxidizing species would require the production of nitric oxide by the inducible enzyme nitric oxide synthase (iNOS). In the same A549 cells where 8-OHdG was observed, synthesis of mRNA for the inducible form of iNOS (Park & Aust, 1998) and increased intracellular nitrite, a stable oxidation product of nitric oxide ( $\cdot\text{NO}$ ) (Chao et al., 1996), were detected after NIEHS crocidolite treatment. Thus, it is possible

that peroxynitrite may be formed in these cells, resulting in oxidation of DNA.

Further experiments in A549 cells showed that Fe from the crocidolite was required for both nitrite formation and 8-OHdG formation (Chao et al., 1996). The formation of 8-OHdG required the presence of Fe from crocidolite and nitrite formation. Taken together, these results suggested that DNA oxidation was the result of peroxynitrite  $\text{ONOO}^-$  resulting from reaction of Fe-dependent  $\text{O}_2^{\cdot-}$  (reaction 2 shown earlier) with iNOS-produced  $\cdot\text{NO}$ .



Both  $\cdot\text{OH}$  and  $\text{ONOO}^-$  produce an array of DNA base oxidation products, including 8-OHdG. But the representative array for each differs, much like a fingerprint. Thus, more conclusive evidence as to the identity of the oxidizing species responsible awaits complete analysis of the DNA oxidation profile produced after crocidolite treatment.

To better understand how induction of iNOS was occurring, Park and Aust (1998) investigated whether the decrease in intracellular GSH in A549 cells was involved in the induction process. The results indicated that the fall in the thiol reducing capability within the cells, a GSH decrease after crocidolite treatment, was required for induction of iNOS.

The induction of iNOS was also observed in other cell types after asbestos exposure. Choe et al. (1998) noted induction of iNOS protein expression in asbestos-treated mesothelial cells. Inducible NOS was also activated in macrophages after asbestos exposure (Quinlan et al., 1998). Thus, the induction of iNOS by asbestos is not isolated to one cell type.

### Peroxynitrite

Peroxynitrite is a unique oxidant formed from the reaction of the radical species  $\cdot\text{NO}$  and  $\text{O}_2^{\cdot-}$ , as shown above in reaction (5). The rate of this chemical reaction, which does not require an enzyme, is extremely fast, near diffusion-controlled (Kissner et al., 1997), making it an order of magnitude faster than the

superoxide dismutase (SOD)-catalyzed dismutation of  $O_2^{\cdot-}$ . Thus, if  $O_2^{\cdot-}$  and  $\cdot NO$  are present,  $O_2^{\cdot-}$  is more likely to react with  $\cdot NO$ . In biological systems, the  $\cdot NO$  is produced enzymatically by the inducible form of nitric oxide synthase (iNOS). Macrophages produce high levels of  $\cdot NO$  as a cytotoxic agent in the immune or inflammatory response (Hibbs et al., 1988; Marletta et al., 1988). Under these conditions,  $\cdot NO$  is released along with other species, including  $O_2^{\cdot-}$ . This may set the stage for high levels of formation of  $ONOO^-$ . This may explain the apparent association between inflammation and cancer. Thus, when macrophages are activated by asbestos, there is likely  $ONOO^-$  produced, which would be extracellular to target cells, such as lung epithelial cells and pleural mesothelial cells. Peroxynitrite is a potent and relatively long-lived oxidant with a half-life of approximately 1 s under physiological conditions (Beckman et al., 1990). It is estimated to travel up to 9  $\mu m$  in the cell, where it reacts with bicarbonate and thiols, limiting its further migration (Beckman, 1996). It is 400-fold more likely to penetrate cell membranes than  $O_2^{\cdot-}$  (Marla et al., 1997). Thus, it is possible for it to move from extracellular synthesis through the membrane of a target cell, like the epithelial or mesothelial cell, and react. However, the concentration of  $ONOO^-$  produced within a target cell would likely be higher.

It is clear that the stability of  $ONOO^-$  in biological systems is higher than  $\cdot OH$ , since the  $\cdot OH$  will react within 3 nm. This provides much more flexibility regarding where  $ONOO^-$  might be produced and still allow for damage to a biomolecule, such as DNA.

*Summary* Exposure of various cell types to asbestos results in decreased intracellular GSH. This does not appear to be the result of detoxification of ROS produced by Fe associated with the fibers, but the result of decreased synthesis of GSH combined with efflux of GSH into the extracellular space with no apparent increase in GSSG. In separate experiments, the induction of iNOS with synthesis of  $\cdot NO$  was observed. Studies focusing on the mechanism for these events in human lung epithelial cells (A549) showed that exposure to crocidolite resulted in

intracellular Fe mobilization from the fibers and a fall in intracellular GSH from the cells (independent of Fe, but requiring endocytosis). The unexpected, simultaneous occurrence of these two events appeared to be required for the induction of iNOS mRNA and production of  $\cdot NO$  (possibly  $ONOO^-$ ) and oxidation of DNA (Chao et al., 1996). When iNOS is induced in macrophages by asbestos, it is also possible that  $ONOO^-$  was produced and moved into other cells to potentiate DNA damage, but the concentration would be decreased as a result of moving through fluids that contain compounds with which it reacts.

## CONCLUSIONS

The mechanisms by which an inhaled particulate induces pathological changes include physical features, such as width and length. These two-dimensional features are important when considering the respirable fraction of a given dust within the air, as well as the depth to which the components are inhaled. There is a need for a generalized, comprehensive term for describing the wide range of particles studied in vivo and in vitro for effects and mechanisms of action. The term should be relevant to health risks historically associated with exposure to respirable fibers in dusts associated with asbestiform minerals. For this purpose, the acronym "REMP" to connote "respirable elongated mineral particles" was utilized, while recognizing that some elongated synthetic particles also exhibited related behaviors. The sizes of REMPs are important, as they impact on the clearance/translocation functions of dust particles reaching within the lung. The larger the particle (including REMPs), the less likely is it to be inhaled. Likewise the larger the inhaled particle (including REMPs), the less efficiently it is cleared. However, the smaller the particle, the more likely it is to be inhaled more deeply into the lung. Each of these factors needs to be considered when assessing the durability/longevity for particulates to remain in the lung.

Total dust exposures as well as cumulative impact of multiple exposures also are important considerations with regard to risk

for irreversible dust-induced alterations. The smaller particulates, including REMP, are not only subjected to more rapid clearance from the lung, but also constitute the fraction of the dust burden (for REMP, majority  $<5 \mu\text{m}$  in length) within the lung that more readily become translocated to extrapulmonary sites. Thus, better definition of relative potencies of all sizes and shapes of biodurable REMP, ideally within mechanism of action constructs, remains a critical risk assessment need. This is especially so for assessing increasingly diverse and complex environmental exposures and supporting prospective evaluations of new nanomaterials.

The simple impact of physical features of REMP is more complex than the two-dimensional definition often used. Elements on the surface of the REMP may impart surface charges, unique physical/chemical features, and topography. These surfaces are influenced by intentional processing of the materials for commercial applications, as well as environmental alterations (including *in vivo*) occurring in aqueous solutions, for varying periods of time. Because the surface potential for reactivity is so important in all interactions with biosystems, comparison of *in vitro* reactivities or *in vivo* biological effects may best be done using dose based on surface areas, rather than weights.

In considering the mechanism(s) by which inhaled particles exert their toxicological effects once they reach their targets, it is important to consider first that the particles, in and of themselves, are chemically active. Observations discussed here support the conclusion that intrinsic or acquired Fe that is mobilized from the particles intracellularly is responsible for most of the chemical and some of the biochemical properties of the most carcinogenic forms of asbestos. This appears to be the result of Fe-catalyzed generation of ROS, such as hydroxyl radical or a similarly reactive species, which is generated in cell-free systems and appears to be generated in cultured cells and possibly whole animals. Second, it is important to consider how particles interact with biological systems. The surface charge, physical/chemical features, and topography determine how biological systems "see" the particles and whether proteins bind

their surfaces, enabling the particles to be endocytized by the cells, potentially initiating a cascade of intracellular biochemical pathways and reactions, which may exert a dramatic impact on the pathology of the particles. The induction of iNOS and production of RNS may be the result, in part, of just such a process. More investigation is required before firm conclusions can be made regarding this. However, it is the generation of ROS, such as hydroxyl radical, and/or RNS, such as peroxyxynitrite, that might lead to damage of DNA and other biological molecules.

Macrophages are known to produce the ROS and RNS mentioned earlier. It may be possible that this cell type is one of the sources of these damaging species responsible for the pathological effects of particles. However, there is accumulating evidence that the target cells for the pathology in the lung and mesothelial tissue are capable of generating these same reactive species after exposure to the particles. The concentrations of the ROS and RNS within the target cell are likely to be higher when these species are generated inside the cell.

In summary, the effective surface area of REMP *in vivo*; particokinetic impacts on particle persistence and alteration in affected tissues, including the roles of lymphatic transport routes and impacts of nonelongated particles on REMP clearance or translocation; and particle tissue residence time to affect relationships all contribute to the effective exposure of the REMP to the target cells. The chemical reactions driven by reactive surface species or intrinsic, or acquired, Fe to produce ROS, and the potential biochemical reactions of the cells in response to the particles to produce RNS ultimately damage DNA and other biological molecules. All of these factors are necessary considerations for interpreting *in vivo* toxicity data.

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