ADSORPTION ON SILICA SURFACES

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Modulation of Silica Pathogenicity by Surface Processes

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I. INTRODUCTION

Lung disease associated with occupational mineral dust exposure has been recognized in the written record for over two millennia, at least since the time of Hippocrates. Crystalline silica is probably the first material to have been recognized as particulate toxicant, being responsible for the development of long-term disease in people exposed to respirable-sized silica dusts. However, its mechanism of action at the molecular level is still obscure. This is due in great part to the extreme

variability in surface properties among quartz dusts arising from different sources, so that any classification of crystalline silica dust as single substance is somehow cumbersome. This is reflected in the conclusions of most present literature in the field, such as the IARC monograph on silica carcinogenicity (IARC, 1997) and a few publications which have followed (Donaldson and Borm, 1998; Fubini, 1998b) describing crystalline silica dusts as a variable entity. Much of this variability resides in surface processes taking place when the dust is generated, stored, airborne, and inhaled. In this respect, adsorption processes on silica dusts are of paramount importance to understanding the pathogenic mechanisms.

II. BIOLOGICAL RESPONSES TO SILICA

A. Silica-Related Health Effects

Exposure to some kinds of silica dusts adversely affects the lungs, causing silicosis and, as recently established by the International Agency for Research on Cancer (IARC), bronchogenic carcinoma (IARC, 1997). Recently evidence was also reported of the association between several autoimmune diseases and exposure to silica (Steenland and Goldsmith, 1995).

Chronic silicosis (nodular pulmonary fibrosis) has been recognized since ancient times as an occupational disease which afflicts people chronically exposed to dusts containing crystalline silica. *Acute silicosis* (alveolar proteinosis) usually occurs in occupations where silica is fractured or ground into fine powders by mechanical processes (drilling, sandblasting, etc.). In contrast to chronic silicosis, acute silicosis becomes clinically apparent within a few years of exposure and is a serious, often fatal disease, resulting from acute injury to alveolar lining cells. *Bronchogenic carcinoma* is a lung cancer which can occur in experimental animals exposed to silica dusts and is suspected to occur preferentially with patients with silicosis (IARC, 1997). It also is associated with smoking, which may act synergistically with silica or which may confound epidemiological evidence. Silica-related autoimmune diseases are typically *systemic sclerosis, rheumatoid arthritis, lupus*, and *chronic renal disease*.

When discussing chemical models it has to be stressed that different mechanisms and thus different surface functionalities may be involved in the various diseases provoked by silica inhalation, although some interplay may occur between these events. In all cases the primary event is inflammation and recruitment of defence cells in the alveoli (macrophages, neutrophils, etc.).

B. Fate of an Inhaled Particle in the Lung

Respirable particles which penetrate deep into the lung and settle onto the respiratory bronchioles or the pulmonary alveoli will first contact a surfactant coating on the thin fluid coating of the epithelium. That hypophase contains surfactants consisting largely of lipids and proteins synthesized and recycled by the alveolar type II epithelial cells. Those surfactants exist as a surface film on the air–liquid interface and as micellar dispersions within the aqueous lining layer. A primary function of this surfactant coating is the decrease and regulation of the pulmonary surface

tension (Clements et al., 1970; Bourbon, 1991). It appears that another function is the prompt suppression of otherwise direct membranolytic action of some mineral dusts on the alveolar epithelium. Lavaged pulmonary surfactant or primary components of that surfactant have been shown to rapidly adsorb to the silica surface and to transiently suppress cytotoxic activity (Emerson and Davis, 1983; Wallace et al., 1985).

The subsequent fate of the particle and nature of the disease process depends at least in part on interactions of the so-conditioned particle with pulmonary alveolar macrophages free on the alveolar surface. Macrophages can phagocytose a surfactant-coated quartz particle and subsequent cellular digestive processes may modify or remove the prophylactic surfactant coating with consequent restoration of cytotoxic activity. A particle may be cleared to the ciliated airways and out of the lung (Fig. 1, path a). Conversely the macrophage may die and the particle, perhaps associated with cellular residue, will be available again in the alveolar space for possible recoating and possible reingestion by other recruited macrophages, establishing a continuous ingestion–reingestion cycle with accumulation of the free particles in the lung (Fig. 1, path b). During these cycles the activated macrophage will secrete a large amount of substances such as cytokines, reactive oxygen species (ROS), reactive nitrogen intermediates (RNI), arachidonic acid metabolites and growth factors, which cause persistent inflammation and may severely damage epithelial target cells (Fig. 1, path c).

In the case of chronic silicosis and some other mixed-dust pneumoconioses, the evidence of animal model studies and of histopathology examination of human pulmonary tissue is that some particles do enter the interalveolar septa; this may involve transport by the alveolar macrophage or other mechanisms of penetration

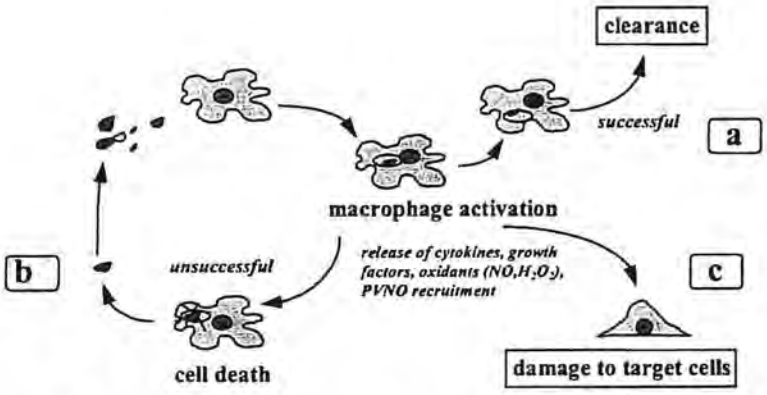

FIG. 1 Events following phagocytosis by alveolar macrophages: (a) clearance to the ciliated airways and out of the lung; (b) ingestion–reingestion cycle with accumulation of the free particles in the lung; (c) damage to epithelial target cells following persistent inflammation caused by macrophage activation.

of the alveolar epithelium into the interstitium. Interaction is then possible with the pulmonary macrophage, fibroblasts, or other cells within the septa. *In vivo* studies of pulmonary response of mice to intratracheal instillation of silica dust using irradiation to suppress the initial inflammatory influx of alveolar macrophages and polymorphonuclear leukocytes indicated translocation of silica particles through the pulmonary alveolar epithelium with subsequent formation of granulomas and fibrosis there (Bowden et al., 1989). It is not clear as to which disease processes result from interactions of particles with the free surface alveolar macrophages prior to this distribution of the particles, or which follow processes begun within the septa adjacent to the pulmonary fibroblasts located there.

Research has indicated that the path followed by a deposited particle to benign clearance or to pathogenic interaction with alveolar macrophages or with cells behind the alveolar epithelium, may be dependent on the state of the particle surface. In particular, the path taken may be determined by endogenous biochemical constituents of the lung or exogenous organic or mineral materials adsorbed onto the particle surface.

C. Mechanisms of Silica Toxicity and Chemical Functionalities Involved

A proposed general mechanism of toxicity of silica causing silicosis and lung cancer (Kane, 1996; IARC, 1997; Donaldson and Borm, 1998) is schematized in Fig. 2. On the basis of literature data, surface functionalities have been associated with the biological steps in which such functionalities appear involved (Fubini, 1998b). Cytotoxity may be related to the distribution and abundance of silanols (SiOH) groups at the surface (Pandurangi et al., 1990; Hemenway et al., 1993; Fubini et al., submitted) and/or to silanol groups dissociated in water (Nolan et al., 1981). It has been long hypothesized that cytotoxicity was originated by strong adsorption of membrane components onto the silica particle (Nash et al., 1966). A role for silanol activity in the membranolytic action of quartz was supported by the more recent observation of the diminution of hemolytic activity by quartz dust with dehydroxylation of the quartz particle surface by calcination (Razzaboni et al., 1990). It has been suggested that toxic membranolytic activity is based upon hydrogen-bonding of silica surface silanol groups with nitrogen or oxygen moieties of macromolecules of biological membranes or upon interaction of dissociated anionic surface silanols with ionic lipids or proteins of biological membranes (Nolan et al., 1981); or radicals on the surface of freshly fractured silica causing damage of membrane lipids by direct peroxidation or by Fenton reaction generation of hydroxyl radicals (Castranova et al., 1997). One or more of these reactive particle surface sites might then interact with a pulmonary macrophage, triggering toxic events in the cell and pathogenic effects in the pulmonary alveolus. An activated macrophage cycle may be established, and particle-derived ROS (Fubini et al., 1989; Giamello et al., 1990) and cell-derived ROS (Vallyathan et al., 1992) will both contribute to a state of oxidative stress, persisting as long as the inflammation persists. Cells will also release nitric oxide, which contributes to the oxidative stress and in the presence of the superoxide ion forms the dangerous compound peroxonitrite. Particle-derived ROS, such as free

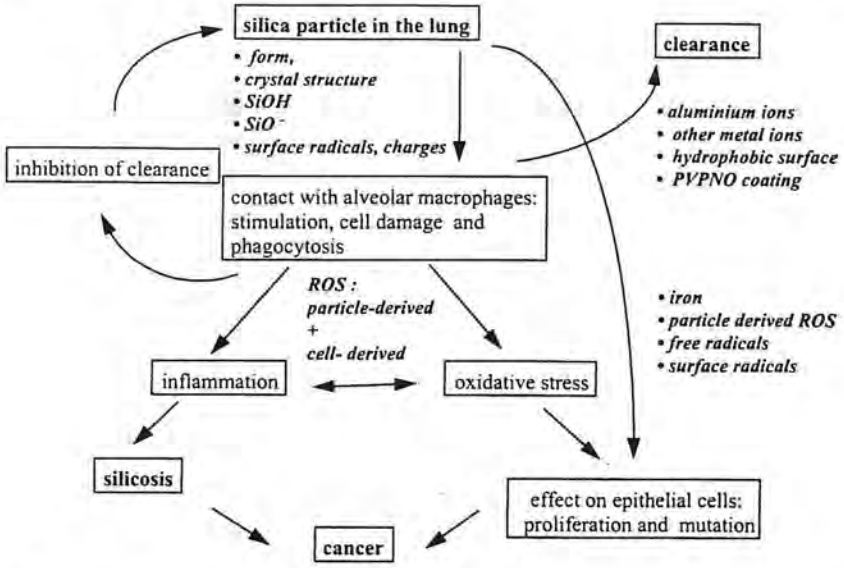

FIG. 2 The possible role of physico-chemical factors in the sequence of events leading to the pathologies associated with inhalation of crystalline silica reported by Fubini (1998a).

radicals or peroxides, may also be implied in direct damage to the epithelial cells. Several particle-derived ROS have been reported, such as hydroxyl radical, superoxide anion, and peroxides (Shi et al., 1995). The production of silica-originated free radicals is much higher on freshly ground materials, where surface peroxide or hydroperoxides are formed (Fubini et al., 1990; Giamello et al., 1990), therefore such a step is more relevant in the case of freshly ground than of aged silicas. If some iron, even in trace amounts, is present or has been adsorbed at the silica surface—which is a very common situation with minerals—Fenton chemistry may be activated, with consequent prolonged release of radicals, which may cause DNA damage and transformation in target cells. Free radical generation does not usually relate to the actual amount of iron but to small fractions of iron with a particular redox and co-ordination state (Fubini et al., 1995b; Gilmour et al., 1995). As a consequence of the above effects, mutations and proliferation in epithelial cells may initiate a neoplastic transformation. It appears therefore that the potential of the inhaled particles to catalyze ROS release and to persistently activate macrophages would determine the pathogenicity of a given dust. Surface processes act to inactivate these processes, e.g., removal of radical generating sites such as adsorbed iron or others, should also inhibit the pathogenic response. Any surface property, moreover, favoring path a instead of b, by lowering the extent of accumulation of the dust in the lungs and consequent inflammation, will also lower the pathogenic potential.

However, theories of particle surface functional groups and interactions responsible for the disease-inducing potentials of silica must consider the possible modulation of silica pathogenicity by endogenous or exogenous processes of particle surface modification.

Several studies of exogenous pretreatment of dust surfaces have been performed. If a respirable dust is covered by polymers (Mao et al., 1995), has been chemically modified (Wiessner et al., 1990), is hydrophobic (Hemenway et al., 1993; Fubini et al., submitted), or has been treated with aluminum salts (Brown and Donaldson, 1996), the effect of silanols is much reduced or even eliminated. Under these circumstances the particle may follow path a in Fig. 1, i.e., will be cleared out from the lung in the upper airways or to lymph nodes by macrophages. Alternatively, following path b, clearance will be inhibited and phagocytosis will eventually cause cell death following disruption of the phagolysosome membrane.

There are two broad concerns bearing on the role of endogenous modification of silica surface in the determination of pathogenic activity *in vivo*. One is the question of the prompt modulation of silica particle surface activity from conditioning by the mucus and surfactant protective layers on the lung surface, e.g., the prompt masking or neutralization by surfactant adsorption of particle surface silanol groups or surface free radical species (Marks, 1957; Emerson and Davis, 1983). The other is the question posed by the comparable *in vitro* cytotoxic activities of silica and some aluminosilicate dusts, despite the relatively weak disease-inducing potentials of the aluminosilicate dusts *in vivo* (Brown et al., 1980; Daniel and LeBouffant, 1980). That is, what differences in surface properties prevent clays but not quartz from expressing comparable toxicities *in vivo*? Tests of postulated mechanisms of silicosis typically do not test such comparably cytotoxic but relatively weakly pathogenic mineral silicate clay dusts as a negative control. However, measures of the *in vitro* cytotoxicity of quartz and aluminosilicate clay respirable dusts have found that they are comparably cytotoxic to lavaged macrophages (Vallyathan et al., 1988). This suggests that the outstanding hazard of quartz dust is not due to the presence of unique surface toxic functionalities and interactions, but rather is due to a lack on the quartz surface of surface functionalities and interactions which are responsible for prophylactic effects on other silicate mineral surfaces.

III. ADSORPTION OF ENDOGENOUS MATERIALS

A. Particle Adsorption of Proteins

Proteins are large amphipatic molecules which, being intrinsically surface active, tend to adsorb to all surfaces. The enthalpy of adsorption varies over a wide range and depends upon the chemical nature of both surface and proteins. In some cases the enthalpy of adsorption may even be positive and the process of adsorption is entropically driven (Brash and Horbett, 1995). Entropic factors are always relevant because of the displacement of water molecules from both surface and protein as well as limited unfolding of the protein at the surface.

The physicochemical nature of the solid surface determines the kind of protein which preferentially adsorbs, as well as the strength of the bond. As a general rule

the more hydrophobic the surface, the greater the extent of adsorption (Brash and Horbett, 1995). This rule has several exceptions. Low-density lipoproteins are adsorbed preferentially on hydrophilic silica surfaces (Ho and Hlady, 1995); often adsorption on hydrophilic surfaces is underestimated because the adsorbate is more easily removed (Brash and Horbett, 1995). Serum albumin is strongly adsorbed at silica or glass surfaces; heterogeneity in adsorption sites, namely the presence of calcium ions acting as electron acceptors, attracts the electron donor moieties of the protein (Van Oss, 1994). EDTA in fact decreases adsorption.

Silica does adsorb proteins, likely via hydrogen bonding of their polar parts onto silanols (Van Oss, 1994). One of the hypotheses for the lytic action of silica on some cell membranes was in fact a strong adsorption of the external part of membrane protein onto silanols (Summerton et al., 1977).

The adsorption of several proteins was thoroughly investigated by Kozin et al. (1982) in the course of an investigation of the membranolytic potential of several form of silicas. The following order of affinity for adsorption by quartz was found when results were expressed as percent of protein bound: fibrinogen > lysozyme > IgG > ribonuclease > ovalbumin > β-lactoglobulin > bovine serum albumin. Differences were also noted in the affinity of various silicas for proteins. Too much speculation on these differences could be pointless because the authors used "synthetic" and mineral samples. The mineral cristobalite adsorbed two orders of magnitude more than the synthetic one, probably because the synthetic one had experienced a high-temperature treatment which inactivates surface functionalities (Fubini, 1998b). The hemolytic potential of the silica particles correlated with their capacity to adsorb IgG or lysozyme (LYS), both positively charged, but not bovine serum albumin (BSA), negatively charged. Expressed as a molar ratio of adsorption LYS/BSA, the results indicated that only particles with such a ratio of 5.0 or below were membranolytic, suggesting a requirement of an optimum negative surface charge for membranolysis. Adsorbed proteins partly inhibited hemolysis.

Adsorption may also stem from hydrophobic interactions. The adsorption behavior of human plasma fibronectin was investigated on silica substrates of different surface energy, one hydrophilic and one hydrophobic (Jönsson et al., 1982). Fibronectins are high-molecular-weight glycoproteins present on many cell surface, in connective tissues, and in extracellular fluids. They are involved in many cell adhesion phenomena. Similarly to what happens with human fibrinogen, there was an increased amount of protein adsorbed at the plateau on a hydrophobic surface as compared to a hydrophilic one. Furthermore, adsorption was nearly irreversible on a hydrophobic surface but partially reversible on the hydrophilic one. Interaction of antibodies with preadsorbed fibronectin suggests that fibronectin adsorbs in different conformations and/or arrangements on the two types of surfaces.

The surface of crystalline silica exhibits hydrophilic and hydrophobic areas (Bolis et al., 1992; Fubini et al., 1992, 1993). Proteins will therefore be adsorbed at different sites depending on their characteristics. The possibility that particular arrangements of hydrophilic and hydrophobic sites on a crystalline surface may strongly fix and modify a protein is one of the hypotheses put forward years ago to explain the peculiar biological properties of most crystalline silica polymorphs

(Langer, 1978) and has never been fully discarded. Irreversible modifications of biomolecules at the surface may activate the immune system which will regard them as nonself, hence the autoimmune silica-related diseases and a possible "immune" pathogenesis of silicosis as postulated by Pernis and Vigliani (1982). It has to be pointed out that plasma proteins adsorbed on silica dusts acquired antigenic properties as reported long ago (Scheel et al., 1954).

B. Silica Adsorption of Pulmonary Surfactant and Inhibition of Toxicity

Pulmonary surfactant is a multicomponent mixture of lipids, proteins, and carbohydrates (Clements et al., 1970; King and Clements, 1972; King et al., 1973; Haagsman and van Golde, 1985; Bourbon, 1991). Lipids are the major component of pulmonary surfactant; the major fraction is phospholipids, principally diacyl-(palmitoyl)phosphatidylcholine (DPPC) (Gilfillan et al., 1983). DPPC in physiological saline can reproduce almost all of the surface-tension-altering behavior of pulmonary surfactant on the air–liquid interface of the alveolar surface. Therefore DPPC in saline frequently is used as a model for pulmonary surfactant. Adsorption of complete (lavaged) pulmonary surfactant or of DPPC can suppress the *in vitro* cytotoxicity of quartz (Marks, 1957). This suggests a surfactant role in preconditioning mineral particle surfaces to prevent such interaction from damaging alveolar epithelial cell membranes.

Decrease in hemolytic activity versus surface-normalized amounts of adsorbed DPPC has been measured for quartz and kaolin respirable dusts (Keane et al., 1990; Wallace et al., 1985). Isotherms at 37°C for adsorption of DPPC from dispersion in physiological saline were measured in two ways. The DPPC remaining in dispersion indicated that approximately 15 mg DPPC adsorbed per square meter quartz surface area (the surface area was measured by BET nitrogen gas adsorption) (Wallace et al., 1988). However, multiple saline rinsing of the quartz dust removed all but about 4 mg DPPC/m^2 as determined by organic solvent elution and wet phosphate assay quantification (Wallace et al., 1992). The kinetics of enzymatic removal of this tightly adherent DPPC suggest that this coating may be a bilayer with ionic phosphatidylcholine headgroups of the DPPC oriented toward the quartz surface for the inner molecular layer and oriented toward the outer aqueous media on the outer molecular layer, with the fatty acids tails between (Fig. 3). Such DPPC pretreatment and rinsing of quartz dust fully inhibits the hemolytic activity of quartz against erythrocytes *in vitro* and against lavaged rat pulmonary macrophages as measured *in vitro* by lactate dehydrogenase, beta glucuronidase, and beta *N*-acetyl glucosaminidase release assays (Wallace et al., 1985).

Prompt and total suppression of quartz membranolysis by DPPC phospholipid surfactant adsorption has been observed for freshly fractured quartz dust as well as for standard quartz dusts. It is not clear that surface free-radical or highly reactive sites would survive a surfactant adsorption–digestion cycle of events. Perhaps the question to be asked of a role for free-radical sites is: Under what conditions could highly reactive silica surface sites produce significant levels of toxic intermediates by reaction with lung-lining organics? And, are such highly reactive surface sites also

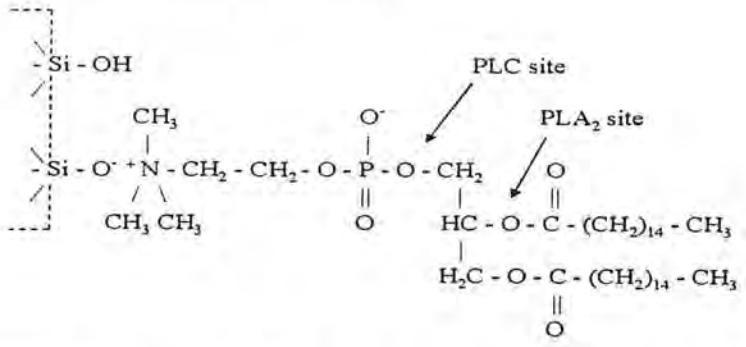

FIG. 3 Adsorption of DPPC surfactant on a quartz surface silanol. The cationic trimethylammonium end of the DPPC interacts with an acidic silanol surface site. Silicate basic aluminol surface site interactions with the acidic phosphate may alter this DPPC conformation. Sites of phospholipase A2 and C hydrolysis are shown. This molecule would be backed by an outer oppositely directed DPPC molecule.

present on freshly broken mineral dusts which are not strongly pathogenic? With such information one could then reasonably speculate about involvement of such interactions in acute or accelerated silicosis.

C. Surfactant Removal and Restoration of Toxicity

The lack of persistence of prophylactic effects of surfactant on quartz and the consequent restoration of particle toxicity have been measured in cell-free system models of enzymatic digestion processes, for cellular processes *in vitro*, and in limited *in vivo* animal model studies. In cell-free enzymatic digestion experiments, phospholipase A2 (PLA2) (Fig. 3) digestion of DPPC-treated quartz dust indicated that enzyme could hydrolyze the fatty acids from the adsorbed DPPC, with subsequent loss of lysolecithin digestion product from the particles. Analysis of the digestion rate data used a kinetics model for enzymatic digestion of an adsorbed bilayer considering steric hindrance both of the outer molecular layer to the inner particle-surface-associated DPPC layer, and of hindrance by the enzyme–substrate complex to enzyme access to adjacent DPPC molecules. The analyses suggested that DPPC is adsorbed as a tightly held bilayer, with the outer part of the bilayer rapidly digested by PLA2, and the inner part of the bilayer digested much more slowly, and with mineral specific rates. Hemolytic activities of parallel samples showed a correlation of restoration of membranolytic activity with removal of the particle-surface-adsorbed layer of surfactant (Wallace et al., 1992). These cell-free analyses indicated that phospholipase could digestively remove DPPC from silica and consequently restore particle cytotoxicity; but cell-free experiments cannot predict absolute values of rates of such restoration for cellular or *in vivo* processes (Wallace, et al., 1994).

In vitro rates of cellular digestive removal of radiolabeled DPPC from respirable quartz dust using the macrophage-like P388D1 cell line found that two-thirds of the mineral surface-adsorbed DPPC was digested in a one-week period. Tests used radiolabeled DPPC adsorbed to quartz to challenge a cell line *in vitro*. Over the one-week period after dust challenge, column chromatography and subsequent liquid scintillation counting were used at one- to three-day intervals to determine the radioactivity which remained with the DPPC fraction of lipid extracted from particles after cell disruption; that is, to determine the rates of digestion of particle-adsorbed DPPC. This found that approximately two-thirds of the adsorbed DPPC was digested in a six- to nine-day period by these cells *in vitro*, with no significant mineral specificity. Lysosomes of these cells were acidic. In addition, extracellular phospholipase digestive activity was seen (Hill et al., 1995). Lavaged rat pulmonary macrophage viability and DNA damage induced *in vitro* by DPPC-treated dust challenge was measured in parallel with loss of fluorescent-labeled phospholipid surfactant from phagocytosed particles over a one-week period. Rates of digestion of a commercial organoboron fluorescent-labeled DPPC from quartz after *in vitro* challenge of lavaged macrophages was measured by fluorescence confocal microscopy. Parallel *in vitro* systems measured DNA damage by the single-cell gel "comet" assay, and viability by trypan blue dye exclusion and by a dual fluorescence commercial "live–dead" assay. Surfactant adsorption fully suppressed quartz toxicity at one day after *in vitro* challenge; approximately 60% of the labeled-phospholipid fluorescence was lost over a seven-day period, and viability was restored to three-quarters to full activity of native quartz over that period. DNA damage activity also was initially suppressed; but activity began to appear again after several days (Liu et al., 1997, 1998). Using Chinese hamster lung fibroblast-derived V79 cells, *in vitro* challenge with silica dusts was found to induce micronucleus formation at high dust doses. DPPC pretreatment of the dusts suppressed this activity over a five-day post silica challenge time period (Liu et al., 1996).

A commercial bovine-based pulmonary surfactant treatment of silica was used to challenge lavaged rat alveolar macrophage *in vitro*. Cell viability as measured by trypan blue dye exclusion was approximately 10% of controls for 1 h challenge with native silica, and 90% for surfactant-treated silica. After 24 h of challenge by surfactant-treated silica, viability dropped to about 70% of the control value. This bovine surfactant also was used *in vivo* by tracheal instillation in the rat, with subsequent assay of bronchiolar lavage assays at 1 and 14 days for total protein, beta glucuronidase activity, and neutrophil influx. Significant differences indicative of a protective function were seen between the native and surfactant-treated challenges at one day for protein and enzyme assays, but not for neutrophil infiltration. The protective effects had diminished to nonsignificant levels at 14 days (Antonini and Reasor, 1994). Another set of experiments suggested that the loss of surfactant prophylaxis is due to phospholipase enzymatic effects. Prior to and following intratracheal instillation of silica, rats were treated orally with amiodarone, a drug known to induce phospholipidosis in the lung, presumably by inhibiting phospholipase activity. At 60 days after silica instillation, lung weight, hydroxyproline content as an indicator of fibrosis, neutrophil

percentage of lavaged cells, and lavage fluid content of albumin, β-glucuronidase, and lactate dehydrogenase were measured. In all the assays the values for silica-challenged but non-drug-treated animals were significantly increased over the negative control non-silica-challenged animal values; for the silica-challenged and amiodarone-treated animals the values were significantly greater than the negative controls, but were significantly lower than the values for silica-challenged but non-drug-treated animals (Blake et al., 1996).

D. Surface–Surfactant Interactions and the Unique Toxicity of Silica

In order to attempt to find the mineral-specific surface properties and interactions responsible for the uniquely strong pathogenic potential of quartz dust, explicit direct comparisons of quartz dust with kaolinite aluminosilicate clay respirable dusts have been made for some of the surfactant interactions discussed above. This has shown the two dusts to have comparable cytotoxicity *in vitro* as measured by hemolysis or by macrophage damage, to have equivalent adsorption capacities per unit specific surface area for the primary phospholipid component of pulmonary surfactant, and to have their cytotoxicities fully suppressed by surface coverage with DPPC surfactant. It appears to be firmly established that silanol groups are membranolytic, and although transiently suppressed by adsorption of surfactant, the activity can be restored through a cycle of prophylactic surfactant adsorption and removal; that is, the potential membranolytic activity survives the initial defensive interaction in the lung. A corollary to this is that it is not a lack of toxic silanol groups which makes aluminosilicate clays relatively nonpathogenic; rather it is the presence of adjacent protective surface functional groups, e.g., aluminol groups or heteroatoms on clay which counter the silanol toxicity (Wallace et al., 1989; Keane et al., 1990). The differing pathogenic potentials appear not to be due to differences in initial surfactant interactions as both kaolin and quartz adsorb surfactant and are promptly inhibited in their direct membranolytic action. The question arises: Are there significant differences in restoration of toxicity due to prophylactic surfactant being removed more fully or more quickly from quartz, such that quartz pathogenicity is expressed and other silicate activity remains inhibited for a period that is long compared to particle clearance or retention in the lungs?

Cell-free phospholipase digestion removed DPPC more rapidly from quartz than from kaolin: half the tightly adherent DPPC was digested from both dusts at a comparable rapid rate, while the remaining half, postulated to be the molecules in contact with mineral surfaces, showed a fourfold decrease in the kinetic rate constant for removal from kaolin compared to that for removal from quartz (Wallace et al., 1992). Those cell-free system studies used a porcine pancreatic exudate phospholipase A2, which was optimally active at neutral pH. However, the P388D1 cell *in vitro* studies did not see a mineral specificity for intracellular surfactant digestion or restoration of cytotoxicity. Preliminary data do not show mineral-specific differences in the rate or restoration of cytotoxicty of DPPC-treated quartz and kaolin to lavaged rat macrophage *in vitro* (Gao et al., 1999). The P-

388D1 cell-line studies observed an extracellular phospholipase released by the cells *in vitro*; this phospholipase was a pH-neutral-acting enzyme which did show a mineral specificity for DPPC removal from quartz and kaolin. Thus extracellular phospholipase appears to be more strongly active against quartz-adsorbed DPPC, while the intracellular phospholipase of phagocytic cells with acidic lysosomes and low-pH-active phospholipase do not appear to show a mineral specificity in restoration of dust toxicity.

A hypothesis emerges from these considerations on the effects of endogenous surfactant in determining the exceptionally strong pathogenicity of silica. Silica or silicate particles depositing in the deep lung adsorb components of pulmonary surfactant and are thereby promptly inhibited in their cytoxicity. Such inhibited particles are phagocytosed by alveolar macrophages or make their way into the alveolar epithelium. The surfactant coating on the particles is subject to attack by lysosomal enzyme in the alveolar macrophage phagolysosome or in the cellular lysosomes of interstitial cells, or attack by extracellular enzyme in the interstitium. The rate of success of this enzymatic digestion depends on the detailed structure of the particle surface, different surface functional groups having different affinities for surfactant functional groups, affecting the strength of adsorption and the conformation of the adsorbed surfactant molecule and thereby affecting the rate or degree to which the digestive enzymes can remove the prophylactic surfactant. A molecular model is that the conformation of kaolin-adsorbed DPPC provides greater hindrance to phospholipase enzyme access to the phosphate and carbonyl ester bonds which the enzymes hydrolyze along the glycerol moiety of the phosphopholipid molecule. Both quartz and kaolin have acidic silanol surface sites which can interact with the positively charged choline trimethylammonium group at the head of the DPPC molecule; however, clay and not quartz would have basic or amphoteric aluminol surface sites to interact with the acidic phosphate or carbonyl groups. Thus the clay surface interactions with the hydrolysis-susceptible region of the molecule might result in an adsorbed DPPC conformation which retards enzyme access and activity there. Thus both minerals would have potentially toxic surface functions, but quartz would lack adjacent "protective" functional groups. The particular type of cell interacting with the particle may also be critical in this process, as alveolar macrophages have acidic phagolysosomal processes, while epithelial and mesothelial cells have neutral pH lysosomes (Johnson and Maples, 1994). Thus molecular conformations and susceptibility to enzymatic action may vary between alveolar and interstitial locations of the particle. In the case of DPPC surfactant, the available data suggest a nonmineral specific restoration of particle toxicity by alveolar macrophages and the possibility of more rapid restoration of silica particle toxicity compared to clay for particles subject to neutral pH phospholipase activity, e.g., sequestered in the interstitium behind the alveolar epithelial surface. However, the question is open as to the effects and kinetics of other components of pulmonary surfactant.

Research is needed on the comparative rates of loss of pulmonary surfactants and restoration of toxicity for quartz and clay particles within the alveolar interstitium to determine if intracellular or extracellular enzymatic processes these

could distinguish the longer term *in vivo* response for different respired mineral dusts.

IV. MODIFICATION OF SILICA SURFACE PROPERTIES AND TOXICITIES BY EXOGENOUS MATERIALS

A. Modulation of Toxicity by Adsorption of Organics

1. Organosilanes

The effect of surface chemical modifications of the surface of quartz on the development of lung disease was investigated by treating quartz with various organosilanes (Wiessner et al., 1990). The samples have been tested *in vivo* on a mouse model measuring parameters linked to lung inflammation and fibrosis. Red-blood-cell lysis was also measured as a means to compare the reactivity towards the cell membrane with the pathogenic response. The samples studied were well characterized and they were administered to mice by intratracheal injection with each crystalline material at constant surface area, which implies that it is the surface of the particles which elicits the pathogenic response investigated. Several parameters related to the inflammatory and fibrotic response were investigated, such as lung index, cell number, lavage protein concentration, and hydroxyproline level. The functional groups attached to the quartz surfaces were (—CN), ($-CH_3$), ($-NH_2$), and ($-N(CH_3)_3$). The crystals showing the highest degree of biological activity were native quartz, and $-N(CH_3)_3-$ and —CN-modified quartz. Conversely, the $-NH_2$-modified one was as unreactive as the crystal preparation modified with the hydrophobic group $-CH_3$. The authors conclude that electrostatic interactions may be more important in determining effective biological activities than are hydrogen-bonding interactions, as the $-NH_2$ group, which can give hydrogen bonds, was as unreactive as the hydrophobic $-CH_3$ terminal group. This very nice set of data deserves probably further interpretation as it contains information still to be drawn on the effect of the chemical modifications in the various steps of the pathogenic process. For instance, the possibility that the terminal groups of the chain might be involved in interaction with some other surface sites has not been considered. All silane-modified surfaces were less hemolytic than pure quartz. This is likely due to the elimination of surface hydroxyls in the silanization and to the hydrophobicity brought about by the hydrocarbon chain. Dispersion of the quartz dusts in the pulmonary surfactant DPPC inhibited hemolysis but had little or no effect in inflammation and fibrosis. Inflammation and fibrosis were higher for pure quartz than for the modified ones, but the effect of the $-N(CH_3)_3$ and —CN ones was higher than those of the $-CH_3$ and $-NH_2$ ones.

The effect of a commercial organosilane reagent for treatment of laboratory glassware, Prosil®, was tested for its effect on silica toxicity *in vitro*. The organosilane was applied to silica dust by incubation at 100°C for 10 min. This treatment reduced the hemolytic activity of silica by 78% from the untreated silica-induced value. *In vitro* challenge of lavaged rat macrophage showed a decrease in damage to cellular membrane integrity as measured by a fluorescent propidium uptake assay

at 5 h after cell challenge for organosilane-treated quartz compared to native quartz. Organosilane treatment of silica also lowered by 83% the oxidant release from challenged lavaged macrophages *in vitro* as measured by chemiluminescence with a luminol indicator. Intratracheal instillation of Prosil®-treated silica in the rat resulted in no apparent significant difference in the viability of cells lavaged one day after dust challenge between native and coated silica dusts, and no apparent significant difference in amounts of protein in the lavage fluid. A decrease, not tested for statistical significance, is seen for the induction of beta glucuronidase in lavage fluid for the treated dust (Castronova et al., 1997).

It has been observed that some preparatory conditions for *in vitro* toxicity studies of silica dusts can inadvertently alter the measurements, apparently by modification of the quartz particle surface. In the course of sterilizing dusts for long-term *in vitro* testing it was observed that boiling quartz dust in glass test tubes suppressed the cytotoxic and membranolytic activity of the quartz (Wallace et al., 1990b). It is not clear if this is due to contamination of the quartz dust by organosilane or other laboratory glassware treatment materials partially released by the boiling conditions, or due to contamination by a low-solubility silicon or aluminum or boron or other compound released by the glassware glass surface itself under boiling conditions. The suppression did not occur when quartz was boiled in polycarbonate tubes. However, it did occur when quartz was boiled in polycarbonate tubes containing glass beads or ground up glass cover slips. The latter result suggested that the prophylactic effect was not due to release of some organic coating on the test tubes. An alternative hypothesis is that some slightly soluble form of silica in aqueous media has a greater solubility from glass than from crystalline quartz surface in boiling water, resulting in silicic acid or some polymerized derivative being adsorbed on the quartz surface.

2. Polyvinyl-pyridine-*N*-oxide (PVPNO)

One of the most used silicosis inhibitors is polyvinyl-pyridine-*N*-oxide (PVPNO), which is much more effective against quartz-induced fibrosis than against asbestosis. The —NO groups of PVPNO provide a periodic point for strong attachment to silanols via H-bonding.

The mechanism of action is still partially unclear. It is likely due to the coating of the surface of the dust by the polymer (Schlipköter and Brockhaus, 1960) but it has also been found to act as a scavenger of free radicals (Gulumian and van Wyk, 1987). Binding to the surface is certainly important and may explain the differences found between the effect of PVPNO on quartz and asbestos on the basis of their different H-bonding potential. Investigating the effects of surface-modifying agents on the production of reactive oxygen metabolites by polymorphonuclear leucocytes, Klockars et al. (1990) found remarkable differences between quartz and various asbestos types. PVPNO inhibited oxygen metabolite production by quartz but had little effect on asbestos. Conversely, carboxymethylcellulose only reduced chrysotile activity, but was ineffective with other particulates, including quartz. There is clearly a sort of surface-inhibitor specificity in polymeric inhibitors which is determined by the surface properties of the particle surface (H-bonding, charges, etc.).

The toxicity of quartz to fetal rat lung epithelial cell line was investigated by Mao et al. (1995) on Min-U-Sil as received and precovered by two forms of PVPNO (2-vinyl and 4-vinyl), one of which (2-vinyl) only was active in blocking hemolysis (Nolan et al., 1981). The toxicity was evaluated as colony-forming efficiency (proportion of cells that survived to form colonies).

Binding of both forms of PVPNO effectively inhibited the toxicity of quartz to the cell line investigated, mostly at the same level. The authors suggest that quartz toxicity to epithelial cells may be caused by hydrogen-bonding interaction of the silanol group with cellular components (different from those involved in hemolysis) and/or formation of free radicals at the silica surface.

B. Modulation of Toxicity by Surface Inorganic Materials

1. Aluminum Salts

Soluble aluminum salts, administered during the exposure to silica dusts, blunt the adverse effect of silica. Long ago Haldane (1917) hypothesized that the nonfibrogenicity of some quartz-containing dusts could be related to the presence of some components—mainly clay silicates—capable of blunting silica toxicity. Some 20 years later it was reported that the inhalation of aluminum dusts inhibited in experimental animals the pathogenic response to crystalline silica dust. Considering that most probably the inhibition is carried out by soluble aluminum salts, Le Bouffant and associates experimented with different salts and administration protocols (Le Bouffant et al., 1977). They reported that among various aluminum salts, aluminum lactate was the most appropriate, because of its high stability in aqueous solutions. Inhalation of aerosols prevented the development of pulmonary fibrosis in rats exposed to pure crystalline silica dusts or silica-containing mineral dusts (Le Bouffant et al., 1987). Several studies then followed by Bégin and associates with sheep as experimental animals, confirming that aluminum lactate inhibits the development of silicosis (Bégin et al., 1987). They demonstrated that surface chemistry modification of quartz by aluminum lactate significantly reduced the biological activity of quartz and increased its clearance with no detectable particle retention in the lung 10 months after exposure. Further studies demonstrated that the inflammatory response to quartz in the lung could be ameliorated even when aluminum was administered post exposure (Dubois et al., 1988).

The mechanism of action is not fully clarified, but it is noteworthy that aluminum is active if deposited on the silica particle and if administered either with silica or few days after administration of silica to experimental animals. Once the pathogenic mechanism had proceeded, aluminum was ineffective. This points to a direct action of aluminum ion on some surface functionalities of silica implied in the pathogenic mechanism.

In order to detect surface modifications brought about by aluminum lactate, the adsorption of NH_3 was measured on a pure quartz dust before and after a treatment with aluminum lactate, following the protocol used by Bégin and associates (1987) to prevent silicosis (Fubini et al., 1995a). The most remarkable difference between the two samples was found in the irreversible reaction with ammonia on

the partially dehydrated samples, which evidences a much higher Lewis acidity on the aluminum-treated sample. Aluminum decreases silica solubility. Aluminum in a silica framework substitutes for silicon in a tetrahedral position, and acts as a Lewis acid (electron acceptor), but also enhances Brønsted acidity (proton donor), by facilitating H^+ donation from nearby silanols. A higher acidity may affect surface affinity for biomolecules, membranolytic potential and the overall reactivity of the surface. The presence of aluminum at the silica surface likely favors path a (clearance) in Fig. 1 relative to path b (macrophage activation and death).

Epidemiological studies of fibrogenic pulmonary lung disease in some occupations with exposure to quartz in mixed mineral dusts have failed to find correlations of disease risk with the quartz component of cumulative respirable dust exposure. This is a well-known phenomenon in epidemiological studies of pneumoconiosis or progressive massive fibrosis in coal workers (Walton et al., 1971; Hurley et al., 1982; Robock and Bauer, 1990; Attfield and Morring, 1992). Innate aluminosilicate surface contamination of respirable quartz particles in the mixed dusts may explain the seeming anomaly. Submicroscopic layers, e.g., a few atomic layers to tenths of a micrometer thick, of aluminosilicate clay or other minerals of low fibrogenicity covering a respirable-sized quartz particle would be detected only as low-level bulk contaminant of the exposure by conventional industrial hygiene exposure characterization methods, i.e., infrared spectroscopy or x-ray diffraction of collected dust samples. But if such clay coatings of the quartz substrate particles exist and remain adherent following deposition in the lung, then they would represent a qualitative difference in the nature of the exposure: the particle would have the toxicological properties of the occluding mineral coating rather than that of the underlying mineral particle identified by the conventional exposure analysis.

Auger spectroscopic analysis and thermoluminescence analysis of silica particles from German coalmine dusts found evidence of aluminum contamination on all silica particles studied (Kriegseis and Scharmann, 1982). *In vivo* toxicity studies challenging the rat with silica added to coalmine dust of low silica content versus a coalmine dust of equal inherent silica content indicated that the silica found inherent in the coal dust did not possess the strong pathogenic activity of pure silica (Le Bouffant et al., 1982). Laser microprobe mass spectrometric (LAMMS) study of quartz particles in German coalmine dusts found evidence of admixtures of quartz with clay minerals as a surface contamination of quartz particles. From this it was postulated that most quartz particles are neutralized by such coatings in the coalmine dusts, and proposed that the *in vivo* toxicities of the dusts are functions of non-quartz-related factors (Tourmann and Kaufmann, 1994). The use of scanning electron microscopy with x-ray spectroscopic analysis was modified to detect submicrometer-thick coatings of aluminosilicate on respirable-sized quartz particles by performing the analyses at successively lowered electron beam accelerating voltages, thus generating elemental x-ray spectra from decreasing depths into a particle (Wallace et al., 1990a). This revealed the presence of aluminosilicate occlusion of some fraction of quartz particles in dusts from clay works and from coalmines. This analysis of dusts from a limited number of U.S. coalmines suggested that the fraction of quartz particles surface occluded increases with decreasing coal rank, coincident with the epidemiological observation that disease risk per

unit cumulative respirable coalmine dust exposure decreases with decreasing coal rank (Harrison et al., 1997).

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