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Minerals, Fibrosis, and the Lung

by Alfred G. Heppleston*

Determinants of pulmonary fibrosis induced by inhaled mineral dusts include quantity retained, particle size, and surface area, together with their physical form and the reactive surface groups presented to alveolar cells. The outstanding problem is to ascertain how these factors exert their deleterious effects. Both compact and fibrous minerals inflict membrane damage, for which chemical mechanisms still leave uncertainty. A major weakness of cytotoxicity studies, even when lipid peroxidation and reactive oxygen species are considered, lies in tacitly assuming that membrane damage suffices to account for fibrogenesis, whereas the parallel occurrence of such manifestations does not necessarily imply causation. The two-phase procedure established that particles, both compact and fibrous, induce release of a macrophage factor that provokes fibroblasts into collagen synthesis. The amino acid composition of the macrophage fibrogenic factor was characterized and its intracellular action explained.

Fibrous particles introduce complexities respecting type, durability, and dimensions. Asbestotic fibrosis is believed to depend on long fibers, but scrutiny of the evidence from experimental and human sources reveals that a role for short fibers needs to be entertained. Using the two-phase system, short fibers proved fibrogenic.

Other mechanisms, agonistic and antagonistic, may participate. Growth factors may affect the fibroblast population and collagen production, with cytokines such as interleukin-1 and tumor necrosis factor exerting control. Immune involvement is best regarded as an epiphenomenon. Downregulation of fibrogenesis may follow collagenase release from macrophages and fibroblasts, while augmented type II cell secretion of lipid can interfere with the macrophage-particle reaction.

Introduction

Elucidation of the steps by which pulmonary fibrosis is engendered as a result of retention of the commoner types of inhaled particle constitutes not only a scientific exercise but also a means to explore the possibility of intervention, should preventive measures prove impracticable. Findings derived from the operation of relatively simple mineral compounds may apply to more complex ones as well as to diverse biological stimuli and thus be germane to the fibrotic process in general. Experimental observations, both *in vivo* and *in vitro*, have figured prominently, though their relevance needs to be assessed in the light of evidence derived from humans.

As the principal cell type responsible for collagen formation, the fibroblast becomes the prime focus of attention, and its participation involves functional augmentation in existing cells, with which an increase in population may be associated. Being a simple and ubiquitous compound as well as a powerful stimulant of fibrosis, silicon dioxide in the crystalline form of quartz has been widely employed. The impact of quartz on fibrogenesis eventually proved to be indirect and to involve a sequence of events, among which more than one cellular phase may be identified, though other facets are thought to modulate the interaction. It is informative to consider the principal minerals separately.

Silica

Determinants of Fibrogenesis

Quantitative. Of the particle properties recognized to influence the reaction, the quantitative aspect assumed obvious importance. To minimize the elimination that occurs during and after inhalation exposure, intratracheal injection established a dose-response relationship between quartz or tridymite and the severity of fibrosis (*1,2*). However, inhalation of dust mixtures by rats led to fibrosis only when airborne and lung dusts contained 20% or more of quartz (*3*). Similarly, in humans, typical silicotic change with massive fibrosis was observed when the level of quartz in lung dust generally exceeded 18% (*4*).

Particle Size and Area. Severity of fibrosis and its rate of development proved maximal for injected flint (a mixture of quartz and cristobalite) particles in the 1 to 2 μm range when based on constant surface area, with smaller ones being less active and larger ones least so, even though all size fractions had equal solubilities. At constant weight, the intensity of pulmonary fibrosis, as judged visually, was directly related to diminishing particle size (*5*), with those $< 1\ \mu$m being maximally fibrogenic (*6*). Although these observations implicated both size and surface area, others considered the latter to be irrelevant and the former to operate in conjunction with the degree of silica retention, so that particles $< 1\ \mu$m caused less fibrosis than those measuring 1 to 3 μm or 2 to 5 μm (*7*). However, recent evidence suggested that, at constant surface area, particles with an average diameter of 5 to 11.2 μm were more fibrogenic than those of 1 μm after ensuring corresponding deposition and rate of clearance (*8*). The issue might be settled by determination of the precise anatomical sites of deposition in the bronchial tree for a range of inhaled compact particles in terms of size, shape, density, and surface area.

Structure. The most striking determinant of silicotic fibrogenesis to be identified experimentally was the orientation of the Si-O tetrahedra on which physical form depends. With intratracheal dose and particle size standardized as accurately as

*Institute of Occupational Medicine, Edinburgh, UK.
Address reprint requests to A. G. Heppleston, Bridgeford Gate, Bellingham, Hexham, Northumberland, NE48 2HU, England.

possible, the severity of the fibrotic reaction was least for amorphous silica, but much greater for the crystalline forms, increasing through quartz, then cristobalite to a maximum for tridymite, yet all varieties again had similiar solubilities (*9*). Still greater divergencies were revealed by the high-pressure, high-temperature forms of silica administered by injection. Much less pulmonary fibrosis was produced by coesite as compared with quartz (both tetrahedrally coordinated) of similar size range (*10*), while with pure samples of similar surface area, the rate of fibrosis by coesite was only one-tenth that of quartz, and stishovite (octahedrally coordinated) behaved as inert dust (*11*).

Collagen Production

Silica Solution. Of the earlier hypotheses, silica solubility achieved prominence, originating with the observations of Gye and Purdy (*12*) on the toxic properties of colloidal silica. However, this concept fell into disrepute when disparities emerged between solubility and fibrogenicity, in respect of amorphous and different crystalline modifications of silica and of differently sized particles (*5,9*), as well as when silica particles of various forms were etched with hydrofluoric acid or sodium hydroxide (*13*). An extended solubility theory envisaged that precursors of collagen adsorbed silicic acid, which induced them to polymerize into mature forms (*14*), but this idea too did not overcome the objections. Diffusion chamber experiments also offered no support to simple solution as the means by which silica led to fibrosis. Composed of membranes of known pore size, chambers retained particulate silica but allowed escape of the colloidal form; implanted intraperitoneally or subcutaneously, connective tissue failed to develop around the chambers, although all particulate forms were fibrogenic on direct contact with tissues (*15*).

As a logical consequence of the theory, solubility depression was attempted, principally by the use of aluminum or its compounds, which achieved some success experimentally though not in humans. Rabbits inhaling quartz developed silicotic lesions, which were largely suppressed when metallic aluminum was added to the quartz (*16,17*). Later studies concurred with this result partially or completely in various species and by different routes of administration (*18*). Soluble aluminum compounds, as the hydroxide, hydroxychloride, or chlorhydroxyallantoinate, administered in the form of aerosols, succeeded against quartz prophylactically, but the therapeutic effect was both partial and temporary (*18,19*). Aluminum lactate, a recently available inert and soluble compound, given to sheep intrabronchially, proved of value in prevention, possibly through more rapid clearance of quartz particles, but treatment of the established condition by injection or inhalation had less effect (*20,21*). Quartz coated with aluminum lactate elicited a much reduced inflammatory response in the lung after injection (*22*). The inhibitory effect of metallic aluminum is considered to reside in the slow release of a soluble form that reacts with and covers the silica surface but substitution of Al ions for Si ions in the SiO_2 lattice seems more likely.

A controlled trial of aluminum therapy in silicotic pottery workers and coniotic miners revealed no clinical or radiological evidence of improvement (*23*). Long-term prophylaxis by inhalation of metallic aluminum apparently eliminated silicosis in Canadian gold miners, but dust suppression measures applied contemporaneously were probably responsible for the benefit (*24*).

Membrane Damage. Disturbances of cellular membranes, both plasma and lysosomal, are readily recognized by changes in permeability, revealed through dye penetration and enzyme escape. Cell types employed are mainly macrophages from alveoli and peritoneum, but also permanent phagocytic lines in an attempt to standardize the target. The latter, however, are unreal substitutes, being derived from a mouse macrophagelike tumor ($P388D_1$), Chinese hamster lung fibroblasts (V79-4), or a human type II alveolar epithelial tumor (A549), none of which corresponds closely with the alveolar macrophage.

Erythrocytes permit the reaction with a membrane to be isolated. These cells carry a net negative surface charge, as does quartz, thereby excluding simple electrostatic attraction. The presence of positively charged trimethylammonium groups on the surface of red blood cells may nevertheless exert a high affinity for quartz particles (*25*). The formation of H-bonded complexes, notably with phospholipids of cell membranes, through hydrogen donation by polymeric silicic acid, was considered to mediate silica toxicity (*26*). Hemolysis was much reduced by the polymer polyvinylpyridine-*N*-oxide (PNO) (*27*), which was known to inhibit both cell damage by quartz in culture and experimental silicosis. PNO might thus establish H-bonds preferentially with silicic acid and so preserve cell membranes, but the polymer may also accumulate on the surface of quartz particles to present a simple barrier (*28*). Hydrogen bonding carried undertones of the solubility hypothesis. Moreover, modification of the surface charge on quartz particles by means of organosilanes suggested that damage to cell membranes and subsequent pulmonary fibrosis depended on electrostatic interactions rather than H-bonding (*29*). Aluminum and iron cations inhibited quartz reactivity *in vitro* by binding to its negative surface centers, a phenomenon that may be concerned when individuals are exposed to contaminated silica or to coal dusts. Even among quartz samples from different geological sites, activity against red cell membranes varied 50-fold (*30*). An alternative view attributed membrane damage by silica to abstraction of protein components (*31*) rather than to lipid binding. Uncertainty about the mechanism of erythrolysis is further illustrated by the proposal that quartz particles adsorb cell constituents such as red cell ghosts or synthetic liposomes of dipalmitoyl lecithin (*32*). However damage is inflicted, it results in increased permeability followed by alterations of intracellular electrolyte balance (especially Na and K) with osmotic changes leading to rupture (*28*). It should be emphasized that, for the same crystal sample, hemolytic rate did not always parallel the fibrotic response *in vivo* (*8*).

Hydration of quartz leads to the formation of surface hydroxyl (silanol) groups that are thought to be the adsorption sites for cell membranes, possibly via their phospholipids, since conversion of silanol into siloxane (Si-O-Si) groups by high temperature rendered the particles much less active biologically (*33*). The electron theory of catalysis has also been applied to gain information about the electron trap structure of silica dusts and the configuration of surface silanol groups along with indications of surface contamination (*34*). Difficulties nevertheless remain in correlating these physical parameters with biological behavior *in vitro* and *in vivo* as well as with epidemiological findings, as for instance in connection with coal workers' pneumoconiosis

(see below). The piezoelectric mechanism of fibrogenesis, thought to be induced by deformation of quartz crystals *in vivo* (*35*), failed to account for the lack of such response from tridymite, despite its powerful fibrogenicity. Surface molecular topology could well determine the relative biological activities of different crystalline forms of silica and of TiO_2, depending on atomic density in combination with surface irregularities from which active oxygen atoms protrude (*36*). The pathological permeability of membranes induced by quartz, as revealed by entry of eosin or trypan blue, gives a conclusive end point, and light microscopy permits a quantitative estimate of cell death.

Enzyme release from phagolysosomes and cytosol affords an *in vitro* means of grading cytotoxicity by measurement of acid hydrolases or reduction of triphenyltetrazolium chloride (TTC). However, enzyme assays do not always exhibit consistency, and quartz cannot be assumed to be a standard compound, since cytotoxicity and *in vivo* fibrogenicity are affected by source (*37–39*). These differences may depend on surface contamination with amorphous silica or on incorporation of foreign ions such as aluminum. Lecithin, a major constituent of surfactant, when adsorbed onto particles of quartz suppressed its cytotoxicity, which was restored by subsequent digestion with phospholipase (*40*). Accordingly, the toxicity of quartz to alveolar macrophages *in vivo* is likely to depend on the effectiveness of surfactant removal by enzyme activity in phagolysosomes. Human macrophages are now known to be much more resistant to silica toxicity than cells of animal origin (*41*), thereby casting further doubt on *in vitro* assessments employing standardized cell lines. Whether the nonspecific inhibition by glutamate of quartz cytotoxicity and of the development of silicosis can be translated from the experimental (*42,43*) to the human scene remains doubtful.

Implication of the Macrophage. None of the mechanisms mentioned takes account of fibrogenesis as a two-stage process, in which phagocytosis of quartz precedes formation of connective tissue by fibroblasts. The intimate connection between them, obscured in organ culture and *in vivo* where the cellular components coexist, became apparent when cell culture techniques allowed phagocytosis and fibrogenesis to proceed independently (*44,45*). The discovery of the macrophage fibrogenic factor (MFF) relied on several control procedures. Application of the cell-free extract, derived from the interaction of quartz and macrophages (obtained without elicitation), to independently grown fibroblasts consistently led to a highly significant elevation of hydroxyproline (HOP) production, but no such effect was obtained when dissolved or particulate quartz was applied directly. Furthermore, extracts from untreated macrophages had no effect on collagen formation, and disintegrated cells failed to react with quartz. The stimulatory action of quartz could not therefore be explained solely by damage to plasma or other membranes. Pretreatment of macrophages with PNO did, however, abolish the quartz effect, which may thus be attributed to an initial attack on cell membranes followed by an intracellular reaction leading to the formation or release of the MFF. Throughout, the DNA levels in fibroblasts were unchanged from control values, suggesting that the response represented augmented functional activity rather than cellular proliferation. Being a nonfibrogenic dust, TiO_2 was used as an *in vitro* control and proved to be inactive. The initial observations relied on peritoneal macrophages, which exhibit functional differences from cells of alveolar origin, but the latter tested against quartz likewise produced the MFF. The technique excluded immune mechanisms, humoral or cellular, from participation.

By the same dual culture procedure, confirmatory observations relied mainly on homologous systems and isotope estimation of collagen (*46–52*), the most important contributions emanating from Kulonen's laboratory, where the MFF was characterized and its mode of action illuminated. Relying on experimental granuloma slices for their content of fibroblasts, Kulonen et al. reaffirmed that *in vitro* a soluble macrophage factor augmented collagen synthesis without affecting the level of DNA and without reliance on release of lysosomal enzymes (*49*). The silica-liberated factor, which proved to be a homogeneous protein having a molecular weight of about 14,300 (*53*), was regarded as being released from, rather than synthesized in, macrophage phagolysosomes (*54*). It was found to act on fibroblast polysomes (*53*), the yield of which was increased and whose RNA was preserved from degradation with corresponding augmentation of their stability (*55*). Synthesis of collagen having occurred in rough endoplasmic reticulum, whose RNA was elevated (*56*), the protein was transferred in secretory vesicles to the extracellular fibrillary state (*57*). Collagen formation was not linked to transcription, and collagenase was not involved (*54*); instead, a huge increase in RNA (particularly nuclear) translational capacity was detected in rat lung affected by experimental silicosis and was directed mainly to collagen synthesis (*58*). Antiserum against the purified and concentrated factor from silica-treated rat macrophages neutralized or inhibited to high titer its fibrogenic activity both in culture medium and in granulation tissue growing *in vivo* (*59,60*). Since human as opposed to animal macrophages proved far less susceptible to the toxic effects of silica (*41*), better survival may facilitate generation of the MFF and hence the development of silicosis. Treated with quartz, human monocytes/macrophages preserved their lysosomal membranes but developed a vacuolar network, in which cellular products and particles lay and which opened onto surface pits (*61*). Channels thus became available for discharge into the extracellular environment of secretions, among which mediators such as the MFF could well be included.

Further indications of the relevance of *in vitro* findings to the response in intact animals were soon forthcoming. Silica-treated macrophage extract enhanced collagen synthesis under *in vivo* conditions (*62*) and, reversing the arrangement, extract from silicotic rat lung stimulated proline incorporation by granulation tissue fibroblasts (*60*). Human rheumatoid synovium or fluid contained an extractable agent that was able to release a fibrogenic factor from macrophages (*49,62*). Furthermore, silica-treated monocytes and macrophages from humans, along with a line of human histiocytic lymphoma cells and transformed mouse macrophages, released a collagen-stimulating factor active against both rat granuloma fibroblasts and human synovial cells (*63*). The fibrogenic activity of homogenized silicotic lung was shown to originate in alveolar macrophages, and the protein nature of the agent was confirmed, though its molecular weight of 16,000 was slightly higher than earlier thought (*64*). Purification of this acidic protein demonstrated its effectiveness on fibroblasts at a concentration of 10^{-10} M in a dose-dependent manner, its purity permitting the amino acid composition to be

determined. Total lung RNA in experimental silicosis was greatly increased, and the maintained high level of type I procollagen mRNAs explained the continuous accumulation of collagen; it also transpired that the fibrogenic factor may exist in interconvertible forms (*65*). Silica inhalation by rats led to increased lung levels of type III procollagen mRNA followed by type I procollagen mRNA (*66*). Investigations such as these encourage further exploration of the effector mechanism. Formation of the MFF is thus an intracellular and not a surface event and its target under natural conditions must be regarded as interstitially located fibroblasts, known to be exposed *in vivo* by concomitant silica-induced damage to type I epithelium (*67*).

A parallel *in vivo* situation arose when extracts from CCl_4-poisoned mouse liver were applied to murine fibroblasts and led to elevation of collagen synthesis without affecting the rate of proliferation (*68,69*), the hepatic cell responsible having the ultrastructure of the macrophage (*70,71*). Enhanced rates of collagen mRNA transcription were thought to explain the accumulation of type I collagen mRNA in fibroblasts treated with the fibrogenic factor derived from thioacetamide-poisoned rat liver (*72*). The SiO_2-macrophage extract was also able to elevate collagen synthesis in liver slices (*62*). Biological as well as mineral agents thus provoke generation of the MFF, whose operation evidently has a relevance beyond the immediate concern.

An important role was identified for macrophage alkaline ribonuclease (RNAse), which silica rapidly released from subcellular constituents and then adsorbed; in consequence the level of RNAse in the medium become very low as fibroblast capacity for protein synthesis was enhanced (*53,73–75*). The macrophage RNAse rapidly entered fibroblasts, its target being mainly their nuclear RNA (*76*). One of the RNAses recovered from macrophages, irrespective of treatment with silica, inhibited proline incorporation into proteins of cultured fibroblasts as a result of RNA degradation (*77*); correspondingly, *in vivo*, alkaline RNAse activity was inversely related to proline incorporation into collagen (*64*). Antiserum to macrophage RNAse, isolated from culture and then purified, was inhibitory to the enzyme not only *in vitro* but also in alveolar macrophages lavaged from silicotic rat lung, though there was no cross-reaction with antiserum against the fibrogenic factor when tested by ELISA (*78*). Anti-RNAse serum also depressed hydroxyproline formation by silica in the rat lung, presumably because mineral particles were so heavily coated with adsorbed immune complexes as to leave little surface free to set in train the process of MFF generation. These results reinforced the conclusion that a separate, directly operating biological agent was involved in fibrogenesis by silica. The response to the mineral may therefore be interpreted as a dual mechanism, one leading to preservation of fibroblast RNA from degradation by binding of macrophage RNAse to quartz particles, and the other to release of a fibrogenic agent, which may be referred to as fibrosin, having a distinct identity and composing another of the cytokines liberated by macrophages.

Coal Mine Dust

Determinants of Disease

Under colliery conditions, the problem of fibrogenesis assumes greater complexity, on account of multiple components in the airborne dust, though most attention has focused on the role of quartz. However, the simple dust lesion of coal workers and the silicotic nodule possess distinct structural features, especially in respect to connective tissue content, and differences in pathogenesis may be anticipated.

Human Evidence. The long-term survey of coal workers, carried out under the Pneumoconiosis Field Research program of British Coal, established that progression of simple pneumoconiosis among face workers correlated directly with the colliery mean mass concentration of respirable dust (*79*) but subsequently detected disparities between the prevalence of pneumoconiosis and the mineral content, notably quartz, of the airborne dust (*80*). High progression was sometimes associated with low dust concentration or low progression with high dust concentration (*81*). Wide and unexplained colliery-associated variations occurred and were not explicable in terms of the quartz content of the respirable dust, averaging 5% and rarely exceeding 10%. Case-control analysis revealed the lack of an overall effect of quartz and showed that only with higher levels of quartz exposure were unusual radiological changes observed (*82,83*).

When lung dust was related to morphological changes, wide variation was found between dust content and particular forms of lesion (*84*), while for different pathological grades, percentage composition was similar in respect of coal belonging to the same rank (*85*). Coal workers have developed simple pneumoconiosis when little or possibly no quartz was present in the lungs, and a similar situation arose in hematite workers. Lesions comparable to those in coal workers have also been encountered in individuals exposed to carbon and graphite dusts as well as to nepheline, yet the quartz content was minute or absent.

Experimental Evidence. *In vivo* observations failed to incriminate quartz as the prime determinant of pulmonary fibrosis following inhalation of coal mine dusts. PNO, effective against experimental silicosis, proved of little prophylactic or therapeutic value in rats and monkeys inhaling coal-quartz mixtures or on the reaction to coal mine dusts possessing low levels of quartz, results which have already been outlined (*86*). The integral clay minerals were believed to inhibit the deleterious action of quartz on the human lung (*80*), a view for which experimental evidence existed (*87*). However, the inhibitory action of clays was not always permanent and might also differ according to source. This variability may reflect the content of aluminum in or its release from clays, the minerals of which (muscovite, illite, and kaolin) possessed collagen-forming ability independently of quartz (*39*). Various combinations of quartz, clays, or carbonaceous elements in mine dusts may thus be expected to exert different overall fibrogenic effects and emphasize the difficulty of elucidating pathogenesis in terms of particular components. Surfactant adsorption may even augment the cytotoxicity of kaolin (*40*).

Fibrogenesis

Membrane Damage. Cytotoxicity studies gave little indication of the fibrogenic capacity of different coal mine dusts and were therefore unable to implicate disturbance of membranes as a prime mechanism. In relation to coal rank, hemolysis exhibited disparities with clinical findings, and a role for quartz was not detected. Applied to the unnatural cell line $P388D_1$, toxicity was not defined solely by quartz content, and some mine dusts proved

less deleterious than control titanium dioxide (*88*). A role for quartz also failed to emerge from a toxicity study of European mine dusts applied to the same tumor cell line (*89*). It is now known that P388D$_1$ cells do not behave consistently and are therefore unreliable substitutes for freshly harvested alveolar macrophages. Using the TTC reaction, toxicity of respirable coal mine dusts to peritoneal or alveolar macrophages could not be correlated with the quartz or mineral content (*90,91*). An extensive interlaboratory comparison was unable to correlate dust composition with toxicity or epidemiology and *in vivo* procedures afforded little encouragement (*92*). Gauging cytotoxicity now demands reliance on human monocytes/macrophages because of their particular resistance to particle-induced damage (*41,93*).

Role of the Macrophage. Tests of toxicity concern only the first step of the process that leads to fibrogenesis, to judge which it is necessary to employ both phases by applying to fibroblasts the products of the macrophage reaction with different mine dusts; in addition, the technique required a quantitative basis. Using coal-quartz mixtures and natural mine dusts of respirable size from European sources, dust concentration emerged as more important than composition, the quartz and ash contents bearing no apparent relationship to fibrogenicity (*94*).

On the combined evidence, a specific role for the minor quartz component of coal mine dusts may therefore be discounted. Elimination of the characteristic quartz effect is explicable as simple dilution by the heavily predominant nonquartz components, as loss of Si ions in exchange for those of Al existing in silicate constituents, or as interference with the surface silanol groups. That these coal mine dusts operate nonspecifically through generation of the MFF in relatively low concentration nevertheless seems a justifiable conclusion, although the intracellular interplay of the different components has yet to be clarified. In any event, macrophages are shielded from the intimate contact with native quartz particles required to release fibrosin in sufficiently high concentration.

Fibrous Particles

Determinants of Fibrosis

Fiber Burden. The prevalence of parenchymal changes as detected radiologically depended largely on duration of exposure and atmospheric fiber concentration as, for instance, in amosite/crocidolite miners and textile employees (*95–97*), though environmental data do not always explain individual variations.

Estimation of the uncoated fiber content of the lung entails technical problems (*98*), and correlation with the grade (extent × severity) of asbestotic fibrosis introduces further complications. In particular, expression of fiber concentration in terms of dry weight is influenced by the density of affected tissue, higher grades of fibrosis giving lower estimates of fiber content than lighter tissue containing similar numbers of fibers. Added complexity in matching exposure to disease may arise by virtue of changes in fiber concentration and type within the lung during exposure and subsequent survival as a consequence of eliminatory mechanisms. Despite these reservations and the wide range of reported counts, an overall relationship between dose and disease is believed to exist in both human (*99–101*) and experimental (*102,103*) material. Per gram of dried lung, uncoated fiber contents by phase-contrast microscopy are commonly given as 5×10^3 to 10^5 for control material, 10^6 to $n \times 10^7$ for mild asbestosis and 10^6 to 5×10^8 for severe asbestosis. The fiber content of lung with complicating bronchogenic carcinoma parallels that of the associated asbestosis, but for mesothelioma extends from the control range up to $n \times 10^8$; in the latter case fiber concentration at the pleural surface may well be more important than estimates made on tissue from deeper locations.

Nevertheless, the particular form of the pathological reaction is not apparently determined solely by fiber concentration, since aerated lung sometimes revealed a figure similar to that for advanced disease elsewhere in the same lung (*99*). Experimentally, deposition of chrysotile was affected by the length and number of divisions in conducting air passages, as well as by gravity, all of which influence air flow (*104,105*). Size, mass, and surface area may also influence the distribution of fibers in humans (*106*). Such factors may explain in part the uneven manner in which asbestotic fibrosis affects the human lung, though they hardly account for high fiber concentrations in adjacent nonfibrosed areas. Furthermore, it should not be overlooked that poor correlations emerged between fiber counts performed in seven different laboratories, which relied on similar human material and preparative techniques and employed light and electron microscopy (*107*).

Fiber Type. All the main forms of asbestos have been regarded as fibrogenic to humans (*108*), though not necessarily to the same degree. In rats exposed by inhalation and sacrificed at scheduled times, Canadian chrysotile and anthophyllite were the most fibrogenic, Rhodesian chrysotile and crocidolite less so, and amosite gave the least fibrosis (*102*). As in humans, progression of asbestosis occurred after exposure was terminated. Although chrysotile proved more harmful to rats, it transpired that amphiboles played a greater role in human disease. The distinction arose because amphibole predominated in human lung even when exposures were mainly to chrysotile (*101,109–111*). Citing findings from diverse human sources, Pooley and Wagner (*112*) reaffirmed the excess of amphibole over chrysotile content of the lung both in respect of asbestosis grade and of mesothelioma. They also made the important point that nonfibrous minerals could greatly outweigh fibrous ones in lung dust and possibly contribute to disease, while attention was drawn to admixture of nonasbestos fibers (*113*). Clearance of fibers after comparable inhalation exposures of rats disclosed a much greater loss of chrysotile as compared with crocidolite, which broke down into shorter, thinner fibers (*114*). To account for the disparity, it may be suggested that alveolar deposition is reduced because curled chrysotile fibers offer a larger collision area than straight amphiboles, or aggregate into boli as a result of their hygroscopic property and so alight on conducting airways from which removal is easier, though short chrysotile fibers may also be straight and penetrate to alveoli. Alternatively, the susceptibility of chrysotile to chemical dissolution, especially if the rate remains constant, may imply more effective clearance over the much longer life span of humans, in whom inception of disease could in consequence be both delayed and less pronounced. On the other hand, because chrysotile persists long enough over the comparatively short life span of animals, its fibrogenic and neoplastic potential becomes more evident. A further

complication depends on the tendency of chrysotile fibers to separate *in vivo* into constituent fibrils, thereby exposing a greater surface area and possibly initiating a more vigorous reaction, at least initially, relative to amphiboles. The fibrogenicity to humans of the commoner types of amphibole proved to be of a similar order when surface area was taken into account (*115*).

Further suspicion about the role of chrysotile in human disease arose because so few cases of mesothelioma occurred in a large cohort of miners and millers (*116*). Correspondingly, the lung content of crocidolite or amosite increased in parallel with the severity of fibrosis, but the level of chrysotile remained unchanged or diminished (*117,118*). It transpired that a much higher proportion of tremolite to chrysotile was present in the lungs of miners and millers than existed in the ore, suggesting that during life chrysotile was removed but tremolite retained (*101,119*). Substantiation came from cases of mesothelioma following exposure to chrysotile mine dust, where the pulmonary concentration ratio of tremolite compared with controls was much higher than the corresponding chrysotile ratio (*120*), though lower ratios were found in workers exposed to processed chrysotile (*121*). Hence a much higher lung burden for chrysotile than for amosite or crocidolite is implied in the induction of mesothelioma (*122*). Tremolite may be blamed for nonmalignant pleural disease and pulmonary fibrosis (*123*) as well as for neoplasia (*124*). Inhaled by rats, tremolite led to pulmonary fibrosis and lung carcinoma with an occasional mesothelioma (*125*). The altered amphibole/chrysotile ratio in lung tissue compared with the ore has, on the basis of animal experiment, been attributed to preferential clearance of chrysotile by fiber fracture rather than by dissolution (*126*). The latest source of contention arises from claims that a high relative risk of mesothelioma occurred among railroad machinists exposed to chrysotile (*127*) and that Japanese cases were caused solely by it (*128*).

Fiber Dimensions. Diameter rather than length predominantly determines the free falling speed of fibers, especially when the aspect ratio is high, and an upper limit of about 3 μm enables such particles to negotiate conducting passages and reach respiratory parenchyma.

Early experimental observations (*129,130*) suggested that fibrosis was dependent on long rather than short fibers, a belief receiving impetus from Stanton and Wrench (*131*), who concluded from pleural implantations that the crucial dimensions for mesothelioma induction were $\geq$ 8 μm length and $\leq$ 1.5 μm diameter (optimum $<$ 0.25 μm). Such figures have also been taken to apply to fibrosis, for which the operative sizes by inhalation are usually given as $\geq$ 10 μm long and $<$ 0.3 μm diameter with mesothelioma being attributed to fibers $>$ 5 μm long and $<$ 0.25 μm across. Recent investigations have been designed to establish conclusively the critical fiber length. Employing long (11% $>$ 10 μm) and short (almost all $<$ 5 μm) amosite fibers by inhalation and injection in rats, it was found (*132*) that, in contradistinction to long fibers, short ones failed to excite fibrosis despite greater retention and aggregation of dust-laden macrophages in relation to respiratory air passages. This study contended that the ball-milled sample of short amosite retained its crystalline structure and elemental composition, but fiber comminution by milling has been shown to affect structural and surface characteristics of fibers as well as their reactions with cell membranes (*133,134*). Moreover, the ball-milled sample of amosite used (*132*) contained only a minority of particles which could properly be classed as fibers and, as with coal or hematite, fibrosis in animals would be less likely than in humans. When inhaled by rats, short fibers of chrysotile induced much less severe fibrosis and fewer pulmonary neoplasms than did long fibers (*135*), though the length distributions were not widely dissimilar. However, on this evidence it cannot be assumed with confidence that short fibers are necessarily less pathogenic than long ones, since greater retention of the former during exposure was followed by much more rapid loss from the lungs. The observed differences could equally be attributed to more effective clearance or dissolution of short fibers, which nevertheless retained some fibrogenic and neoplastic potential. The failure of short fiber chrysotile to induce pulmonary fibrosis in exposed rats and monkeys (*136*) is explicable by the low dose which would facilitate clearance, and by the altered structure caused during preparation of the sample by ball-milling. The inability of Vorwald et al. (*130*) to obtain a significant reaction to short (ball-milled) chrysotile occasions no surprise, since 98.6% of their dust was nonfibrous. It should also be recollected that pulmonary fibrosis was induced in rats and guinea pigs with ball-milled serpentine or amphibole fibers (*137,138*), which by electron microscopy were $\leq$ 5 μm in length including many of the order of 1 μm (*139*). Confirmatory evidence was derived from rats, exposed very briefly to chrysotile or crocidolite, after which the great majority of fibers retained in the lung measured less than about 7 μm even though there was a tendency for length to increase with survival time (*140,141*).

Injected into mice crocidolite (78% of whose fibers were $<$ 5 μm) caused multifocal mecroses of bronchial and bronchiolar epithelium and a brisk exudative reaction, which rapidly spread through the walls and left focal granulomas (*142*). Long crocidolite fibers (85% $\leq$ 20 μm), similarly administered, again induced an acute inflammatory reaction with focal necroses in larger air passages of mice, but short ones (98.8% $<$ 2.5 μm, separated by sedimentation) elicited only a macrophage response in alveoli, a distinction held to incriminate long but not short fibers in fibrogenesis (*143,144*). Intratracheal injection of particles in fluid suspension interferes with the natural mechanisms of deposition, permitting long fibers to impact as a bolus in conducting airways, whereas short fibers, being easier to disperse, almost exclusively reach the alveolar region. Granulomatous lesions thus produced in air passages do not correspond in character or location with the changes seen in animals or humans after prolonged inhalation of low concentrations. Such a comparison of length in relation to fibrogenicity will also have been affected detrimentally by the ready clearance of short fibers. Fewer mesotheliomas developed from ball-milled short than long fibers of crocidolite following intrapleural injection, but the lesions suffered a selective loss of short fibers (*145*); had the latter been retained they could well have contributed to mesothelioma induction by the long fiber sample.

Attribution of pathogenicity to long fibers was also made on the basis of cytotoxicity studies (*146,147*), but short fibers were often prepared by ball-milling and unnatural cell lines again employed. When human alveolar macrophages were used in culture, short chrysotile fibers proved more toxic than long ones (*148*) and short fibers can exhibit high toxicity to cultured cells (*149*). Recent evidence suggests that both long and short crocidolite fibers are

cytotoxic to macrophages *in vitro* through oxidant and surface iron-dependent mechanisms and also that *in vivo* short fibers are cytotoxic when their clearance from the peritoneum is prevented (*150*). Furthermore, a role for short fibers emerged from *in vitro* assessment of fibrogenicity, as opposed to cytotoxicity, by means of the macrophage fibrogenic factor (*151*).

Protrusion of long fibers from the surface of macrophages may enable inflammatory mediators to be released, but short fibers, even though completely ingested, may do so too; as laden macrophages die the particles will be transferred to fresh cells and mediators, including the MFF, could simultaneously be released and the process would be continuous. A defect of toxicity tests lies in the absence of macrophage recruitment, a feature that may well account for some of the emphasis laid on long fibers. The term "recruitment" is best confined to the marrow response caused by a stimulus acting from a distance (*152*), though it is loosely applied to the composition of an exudate where vascular permeability and chemoattractants cooperate locally. Under the latter circumstances, short-fibered amosite induced in the mouse peritoneum a much less intense inflammatory reaction than long fibers (*153*). Both dust samples were, however, derived from the same sources as those used earlier (*132*), the double ball-milled amosite containing over 60% of nonfibrous particles, which being compact would be unlikely to excite an inflammatory reaction as readily in animals as in humans.

The pronounced neoplastic potential of erionite provided another approach to the question of fiber length. A peculiarity of zeolites, to which erionite belongs, is the occurrence within their internal structure of spaces which communicate through minute pores with the exterior (*154*). The greatly increased surface area in comparison with crocidolite or chrysotile may well account for the exceptional activity of erionite. Airborne fibers from a region in Turkey where mesothelioma was unusually prevalent were mostly $\leq 10\ \mu m$ in length (*155*). Induction of the tumor in mouse peritoneum was effected by fibers $< 8\ \mu m$ long with some being much shorter (*156*). Inhaled erionite was carcinogenic to rats when 56% of airborne fibers were $\leq 5\ \mu m$ and 92.6% $< 10\ \mu m$ long (*157*). In a similar experiment, particles $\leq 5\ \mu m$ failed to induce mesotheliomas, but those $> 3\ \mu m$ (of which 15.3% were 3–6 μm and 7% $> 6\ \mu m$) were highly effective (*158*). However, the final number of long fibers per gram of dried lung was 2.4 times greater than short ones, and a comparable exposure to crocidolite gave an even higher ratio of 6.1 despite the virtual failure in pathogenicity by long fibers. Both types of fiber contained many compact particles in the disc-milled short form, and the degrees of pulmonary fibrosis caused by different fiber lengths varied by only a single grade on an eight-point scale. Definitive information is still needed to establish whether after inhalation similar numbers of retained short and long fibers, sized within narrow limits and indisputably separated, differ in pathogenicity; to compensate for lack of length, an excess of short fibers would be required.

With so many reservations, the experimental findings fall short of certainty on the issue of fiber length in asbestos pathogenicity. Human lung analyses cast further doubt on the overriding importance of long fibers. According to Sebastien et al. (*159*) and Pooley and Clark (*160*), all fiber types were $< 0.5\ \mu m$ diameter and 70 to 90% had lengths $< 5\ \mu m$, a proportion being $< 1\ \mu m$ long. Lung parenchyma contained a preponderance of short fibers (mean 4.9 μm), notably in alveoli (mean 3.3 μm) where amphibole exceeded chysotile, and also in lymph nodes (mean 2.5 μm) which concentrated amphibole (*110*). A pronounced excess of fibers $< 8\ \mu m$ with mean geometric lengths of 3.2 to 4.2 μm occurred in the lungs of mesothelioma cases (*161*), as had been found for both amphiboles and serpentine (*120,121,162*). Analysis of material from cases of asbestosis or mesothelioma confirmed that chrysotile and tremolite possessed fiber lengths with a geometric mean of $< 3\ \mu m$ and $< 5\ \mu m$ allowing for geometric SDs (*122*). Peripheral sites of human lung affected by mesothelioma retained fibers the great majority of which were $< 8\ \mu m$ long and $< 0.25\ \mu m$ in diameter (*113*). Brochoalveolar lavage recovered from occupationally exposed subjects fibers which were very largely $< 5\ \mu m$ in length and the biological risk from fibers longer than 5 μm was considered to be nonproven (*163*). Similarly, the lungs of nonoccupationally exposed subjects revealed chrysotile and amphibole fibers with mean lengths $< 2\ \mu m$ (*164*). Unlike animal studies, fiber length of amphibole and chrysotile in human lungs did not increase with time since last exposure; for both types of fiber mean geometric lengths remained $< 3\ \mu m$ and were still $< 5\ \mu m$ after paying regard to SDs (*165*). The same situation obtained in individuals residing near a chrysotile mining town and who developed pleural plaques (*166*).

Relating fiber size and degree of fibrosis in miners and millers, a positive correlation emerged for tremolite and a lesser one for chrysotile concentrations; no relationship to grade of fibrosis was found for chrysotile fiber size, mass or surface area, but in the case of tremolite these parameters were negatively correlated (*167*). Similarly in amosite-induced asbestosis the longer the fiber the lower the grade of fibrosis (*168*). It was admitted that short fibers may be more important in pulmonary fibrogenesis than is commonly believed. The relative risk of mesothelioma was related to the pulmonary concentration of amphibole fibers $\geq 8\ \mu m$ long with no additional information being provided by shorter fibers (*169*). The attributable risk for short fibers was, however, little less than for long ones, and a role for the former is not excluded especially as they probably represent the size operative in the pleura. It is worth recalling that in rats inhaling tremolite the cloud consisted predominantly of short fibers (80% $< 6\ \mu m$) (*125*), while fibers ultimately retained after injection comprised over 80% $< 10\ \mu m$ when differential clearance between chrysotile and amosite was studied in guinea pigs (*126*). Lung fibers in Finnish anthophyllite miners were usually $< 7\ \mu m$ in length and a threshold length of 10 μm regarded as suspect (*170*), but their diameters up to the limit of respirability probably accounted for the occurrence of fibrosis and carcinoma and the absence of mesothelioma. However, Timbrell (*171*) recently reemphasized the need to reconsider surface area and fiber diameter as important determinants of fibrosis and neoplasia.

It thus seems unjustifiable to relegate short fibers to an insignificant role in human pathology. Mean fiber lengths $< 5\ \mu m$ may conceal a small proportion of longer fibers, but emphasis should surely be placed on the size range which greatly predominates. With increasing retention, short fibers may be expected to operate in a manner similar to compact particles of coal and to initiate a relatively low grade but progressive fibrotic

response with the added potential for neoplastic change. Greater abundance of short fibers with a comparatively low aspect ratio may well compensate for lack of length, as appeared to be the case experimentally (*172*) and after human exposure (*120,161*). Retention of short fibers in alveolar cells was considered to be an essential component of the reaction to inhaled asbestos (*173*). Sufficient ground thus exists to challenge an exclusive role for long fibers in fibrogenesis and also in carcinogenesis.

Mechanisms of Fibrogenesis

Membrane Damage. Amphiboles and chrysotile differ in their ability to impair the integrity of isolated membranes, the former being much less hemolytic than the latter, whose magnesium but not silicon content bore a linear relationship with the degree of lysis (*27*). The activity of chrysotile evidently depended at least in part on its outer layer of magnesium hydroxide, since removal by acid leaching exerted a depressive effect (*174*), as did nonspecific chelation of metal ions by sodium ethylenediaminetetraacetate (*27*), though silica does not require magnesium for lysis. Because it lacks a hydrogen bond, PNO afforded minimal protection. Extraction of membrane lipids, followed by increased permeability rather than rupture, provided one explanation for chrysotile hemolysis, which could be inhibited by prior adsorption of dipalmityl lecithin (DPL) liposomes or red cell ghosts onto the fibers (*175,176*). Possessing a negative surface charge by virtue of their sialic acid component, membranes attract the positively charged (apparently by Mg^{2+}) chrysotile fiber, the binding being thought to distort glycoprotein complexes. In consequence clustering of surface proteins occurred with the formation of ionic-sized gaps (as with silica), which led to disturbance of Na and K balance and osmotic rupture (*177*). Removal of red cell sialic acid residues by neuraminidase reduced the lytic effect of chrysotile (*27*), but though crocidolite also bound to red cells its distorting effect was not thereby abolished and the fiber's negative charge could instead become attached to oppositely charged groups on phospholipids or proteins (*177*).

Electrostatic attachment to alveolar macrophages, such as is available to chrysotile, is not open to negatively charged crocidolite. Nullification of the electrostatic charge reduced the fibrotic and neoplastic reaction to inhaled chrysotile, presumably by diminishing retention (*178*). As with red cells, sialic acid residues on macrophage membranes serve to bind chrysotile but not oppositely charged crocidolite (*179*). How amphiboles attach to membranes is unknown, though it may be effected through protein or phospholipid components or via cationic receptors (*180*), but, unlike anionic receptors, they are difficult to demonstrate (*181*). A trypsin-sensitive receptor on alveolar macrophage membranes may cooperate with extracellular calcium to promote particle-cell binding (*182*), but such ions penetrating the plasma membrane led to cell death (*183*). The surface charges of amphibole and serpentine asbestos may *in vivo* be altered by surfactant, although *in vitro* dipalmitoyl lecithin had little effect on the mortality of alveolar macrophages from chrysotile (*184*). Release of lysosomal enzymes from macrophages varied with fiber type, amphiboles and TiO_2 in the rutile phase being ineffective but chrysotile, like silica, proving active (*185*). The idea that chrysotile, in contrast to silica, permitted selective release of lysosomal enzymes as compared with those of the cytosol (*186*) has not proved to be a sharp distinction. Magnesium removal from chrysotile led to inconsistent biochemical effects, and *in vivo* as well as in culture the fiber showed a nonhomogeneous loss of magnesium (*187*). However effected, binding of chrysotile by macrophages led to nonspecific production of arachidonic acid metabolites, which were capable of mediating an inflammatory response (*188*). A similar situation arose when cattle alveolar macrophages were stimulated *in vitro* by crystalline silica; a dose-dependent shift occurred from primarily cyclooxygenase to lipoxygenase products (notably leukotriene B_4), a change that preceded the toxic effect (*189*).

Lipid peroxidation constitutes a means for membrane damage by minerals, the products of the reaction being elevated in the plasma of workers exposed to asbestos or silica (*190*). Peroxidation affecting rat alveolar macrophages has been attributed to chrysotile from a very early stage of the particle-cell interaction and suggested as a factor in asbestos-induced cell damage, which metallic elements may facilitate (*191*). Enhanced peroxidation was also detected in microsomal and lysosomal membranes of rat lung cells after treatment with crocidolite whether *in vivo* or *in vitro* (*192,193*), and the effect was inhibited by antioxidants (*194*), but the mechanism did not prove to be directly responsible for irreversible toxicity to macrophages by this type of fiber (*195*). Injection of silica led to stimulation of lipid peroxidation in lung tissue along with lysosomal enzyme release before the development of fibrosis (*196*), while after inhalation of exceptionally high doses of quartz, peroxidase activity in lining fluid was elevated (*197*). Lipid peroxidation had earlier been recognized in silicotic lung (*198,199*), but it appeared that in membranes this phenomenon was not the primary mechanism for the fibrogenic activity of quartz (*200,201*).

Reactive oxygen species have also been ascribed a role in cell toxicity by mineral particles acting as primers; damage may be inflicted on proteins and the hydroxyl radical may indeed operate through lipid peroxidation (*202*). Alveolar macrophages derived from humans with pneumoconiosis released the superoxide anion and hydrogen peroxide spontaneously and they were considered capable of damaging parenchymal cells (*203*). Generation of this anion by alveolar macrophages became particularly evident in coal workers affected by progressive massive fibrosis, though cases of simple pneumoconionsis also revealed the change but to a lesser degree (*204*). Free radicals detected on freshly fractured coal dusts and also in coniotic lung tissue raised the question of their significance in pathogenesis (*205*). Chrysotile was able to elicit reactive oxygen intermediates from macrophages (*206*), and damage from crocidolite could be prevented by scavengers of superoxide anion and hydrogen peroxide, though antioxidants failed to prevent injury by silicates or cristobalite (*207,208*). Augmented release of superoxide from alveolar macrophages of the rat or hamster proved to be a feature of fibrous rather than compact dusts (*209*). *In vitro*, superoxide stimulated rat lung fibroblasts to produce collagenous and noncollagenous proteins, but inhalation of crocidolite also caused a compensatory increase of superoxide dismutase (*210*). *In vivo*, therefore, the effect of superoxide may be nullified and other evidence casts further doubt on its participation. Spontaneous release of superoxide was not elevated in alveolar macrophages derived from sheep injected intratracheally with chrysotile or quartz, though these cells could be primed to do so (*211*). Reactive oxygen intermediates were considered not to be

a major factor in toxicity to broncho-alveolar leukocytes from the rat after treatment *in vitro* with quartz or asbestos (*212*), though they may facilitate detachment of type II cells (*213*). Implication of these intermediates as an initiatory event in dust-induced damage to parenchymal cells of the lung cannot yet be regarded as convincing. Moreover, to imply that the balance between production of oxygen radicals and antioxidants can explain nearly all aspects of the development of simple and complicated pneumoconiosis in coal workers (*214*) seems extravagant. Indeed, red cell and plasma antioxidant enzyme levels in these men probably constitute effects of the disease and not predictive features.

Anchorage Dependence. As with other mineral particles, membrane damage by asbestos, however mediated, and enzyme liberation from macrophages do not on their own suffice to account for fibrogenesis and attention needs to be redirected to the subsequent fibroblast phase. In suspension culture, fibroblasts became attached to glass fibers and growth proved maximal when the latter were long and narrow (*215*). It was proposed that linear extension stimulated cell division, but collagen production was not measured and phagocytosis was omitted.

Macrophage Fibrogenic Factor. Evidence from humans and animals indicates that, from an early stage, exposure to fibers is followed by persistent macrophage accumulation not only in alveoli but also in the interstitium (*173,216*). Brief inhalation of chrysotile (77% < 5 μm long) by rats soon sufficed for this purpose, leading to interstitial fibrosis and transient increase of epithelial cells (more evident in type I than type II) with the whole reaction confined to the vicinity of divisions of respiratory air passages (*217*). Interstitial macrophages have also been accorded a prominent role in silicotic fibrogenesis (*218*). The augmented proliferative activity of epithelial and interstitial cells in these regions, though not in larger conducting airways of chrysotile-exposed animals (*219,220*), did not distinguish the steps leading to fibrosis. Employing both phases, the MFF was activated in macrophages treated with chrysotile or amphiboles, but there was no increase in collagen formation when the macrophage step was omitted or fibers were applied directly to fibroblasts (*54,151*). Epithelial separation in combination with protease degradation of the pulmonary framework under the influence of dusts (*221*) may facilitate access of the MFF to interstitial fibroblasts. Enclosed with macrophages in diffusion chambers, chrysotile led to surrounding fibrosis in the peritoneal cavity, though the response subsided with time (*222*). Silica was also active under these conditions, but only with the smallest dose, which would be less destructive to cells; hematite similarly tested lacked fibrogenic capacity and exclusion of macrophages abolished the response. This procedure reinforces the dual nature of the process leading to fibrosis, but by restricting the supply of cells it again emphasizes the importance of macrophage recruitment to sustain the reaction. Distinguishing circulating monocytes from mature alveolar macrophages by means of monoclonal antibodies, enhanced recruitment and *in situ* replication were both implicated in the accumulation of mononuclear phagocytes in the lower respiratory tract of asbestos-exposed subjects (*223*). Using the dual *in vitro* system, fibrous glass stimulated production of hydroxyproline (*151*) and, inhaled by baboons, it led to fibrosis (*224*), despite the belief that it is an unlikely cause of disorder in humans. From the evidence suggesting an increased risk of lung cancer in the past from exposure to fibrous glass (rock or slag wool) (*225*), some degree of preceding pulmonary fibrosis may be deduced, though not necessarily recognizable radiologically.

Collagen formation by fibroblasts can also be provoked by extracts of mesothelial cells cultured with quartz and these same cells are able to serve as the target for connective tissue formation (*226*). Mesothelium may thus operate as initiator and effector in fibrogenesis and, assuming a corresponding reaction with asbestos, suggests a mechanism for pleural fibrosis and possibly the mesenchymal component of mesothelioma.

Cooperative and Opposing Mechanisms

In parallel with the means outlined, others may come into play either to potentiate or to oppose mineral fibrogenesis and direct attention to the fibroblast population.

Macrophage/Monocyte Participation

Cytokine-mediated interactions are known to regulate the proliferation of fibroblasts under *in vitro* conditions and the monocyte/macrophage figures prominently as an initiator. Increase in the fibroblast population may then contribute to connective tissue formation. Enhanced growth of fibroblasts and collagen production were promoted by blood monocytes via mediators (*227*) or by a factor released from human alveolar macrophages after stimulation by nonmineral particles (*228*). However, this macrophage-derived growth factor (MDGF) was unable by itself to stimulate fibroblast replication, for which initiating factors provided by fibroblasts or platelets were required to establish competence. Regulation of fibroblast growth by this means has exposed complexities. Supernatants from cultures of density-defined human alveolar macrophages, whether stimulated or not, inhibited human lung fibroblast proliferation in a dose-dependent manner and in direct relation to their ability to activate prostaglandin (PG) production by the fibroblasts (*229*). The conditioned medium (CM) of alveolar macrophages lavaged from hamsters affected by bleomycin-induced pulmonary fibrosis led to bidirectional effects on collagen formation (*230*). The latter was suppressed by higher concentrations of CM, a response attributed to macrophage secretion of PGE_2. Moreover, pretreatment of fibroblasts with indomethacin, which inhibits PG formation, enabled CM in suitable concentration to increase collagen production, a situation that indicated the presence of stimulatory factor(s) originating in macrophages. Bronchoalveolar lavage fluid from individuals with idiopathic pulmonary fibrosis stimulated proliferation of human lung fibroblasts *in vitro* (*231*), and fibroblasts from fibrotic human lungs possessed a higher growth rate than control cells and could come to dominate the process of repair (*232*). It may also be noted that supernates from human blood monocytes suppressed the growth of dermal fibroblasts through powerful stimulation of PGE_2 synthesis (*233*) and that by a similar mechanism such monocytes possessed a factor capable of inhibiting collagen formation by chondrocytes (*234*). Fibronectin, a glycoprotein component of the extracellular matrix derived from human plasma or alveolar macrophages, represents another growth promoter for fibroblasts; acting in the early part of the G_1 phase of the cell cycle, it facilitated replication of fibroblasts but only in the

presence of other factors (*235*). PGE_2 counteracted the proliferative response of human lung fibroblasts to MDGF and fibronectin (*236*). Stathmokinesis by colchicine led to suppression of fibronectin and MDGF release by cultured alveolar macrophages that had been lavaged from cases of interstitial pulmonary fibrosis, raising the possibility of therapeutic application (*237*). Overall, fibroblast proliferation under the influence of monocyte/macrophage products appears to be restrained through the agency of PGE_2.

Fibroblast growth factor (FGF), whose activity as a mitogen and as a modifier of other functions extends to diverse cell types, has been well characterized and its amino acids sequenced (*238*). It originates from a multiplicity of cells including fibroblasts and macrophages. Collagenase release comes with the range of FGF activities, as does collagen and fibronectin synthesis, and it also serves as a chemoattractant. Platelet-derived growth factor (PDGF) from rat alveolar macrophages is homologous with that from human cells, and its target is believed to be the interstitial fibroblast (*239*).

Modulation of MDGF release from murine peritoneal macrophages may be effected by arachidonic acid metabolites, cyclooxygenase products being antifibrogenic and lipoxygenase products being proliferative (*240*). Disturbance of the equilibrium between these opposing actions seems likely to determine whether fibrosis proceeds or recedes, in combination with control of endogenous fibroblast PGE_2 production. One cytokine may indeed account for multiple biological activities and vice versa, while one factor may arise from a variety of cell types, and considerable overlap may exist between the effects of separately described mediators. Although the chemical structure of MDGF is not fully known, it exhibits properties resembling those of PDGF, which macrophages secrete along with FGF and tumor necrosis factor. The activities of MDGF may therefore be attributed to known cytokines originating in macrophages rather than to a separate entity (*238*). By positive or negative feedback in the cell of origin, cytokines are able to enhance or inhibit the response of other cells and the system becomes particularly involved when attempting to apply *in vitro* findings to the intact animal, a problem that is not alleviated when dust participation is considered.

Mineral Involvement

Implication of mineral particles in macrophage regulation of fibroblast proliferation stems from observations on both compact and fibrous dusts. Having reacted with quartz instilled *in vivo*, guinea pig alveolar macrophages inhibited or enhanced growth of fibroblasts in culture according to whether treatment was applied for a short or long period (*241*). Lung lavage fluid from quartz-instilled rats stimulated both DNA synthesis and collagen formation in cultured lung fibroblasts (*242*). Under the influence of quartz or coal mine dusts, human macrophages released a growth factor for fibroblasts (*243*) and alveolar macrophages from coniotic subjects exposed to silica, coal or asbestos behaved similarly (*203*). The claim that silica-exposed human alveolar macrophages provided a powerful stimulus to fibroblast proliferation *in vitro* without release of PGE_2 (*244*) suggests the use of too high a dose of quartz which killed macrophages before PG secretion could occur. Aalto et al. (*49*) were, however, unable to detect a macrophage mediated effect on collagen synthesis by PGs or bradykinin. As in silicosis, the primary cellular reaction in asbestosis is dominated by macrophages, and treated *in vitro* with chrysotile, they produced a growth factor for fibroblasts (*245*). A similar effect was exerted by alveolar macrophages lavaged from rats injected with chrysotile and release of fibroblast growth inhibition factor by blood monocytes was depressed as pulmonary fibrosis developed (*246,247*). However, conditioned medium (CM) from monocytes of control human subjects exerted a greater stimulus to fibroblast proliferation than did monocyte CM from asbestotic subjects, as measured by mitogenic activity and mRNA levels for the B chain of PDGF (*248*). Whether this response reflects immaturity of circulating monocytes from asbestos-exposed individuals remains undecided.

Pleural mesothelial cells possessed in culture the ability to synthesize collagen (*249*), an activity that was augmented by short fiber amosite (*250*). The two-phase procedure had earlier demonstrated the release of a fibrogenic factor when mesothelial cells reacted with quartz (*226*).

The growth factors formed by macrophages, in particular PDGF, appear to be distinct from the MFF released after ingestion of mineral particles, since the former typically stimulate fibroblast proliferation *in vitro* while activity of the latter emphasizes collagen formation that occurs under both *in vitro* and *in vivo* conditions (*64*).

Additional Regulators

Accumulating evidence suggests that other cytokines play a part in communication between macrophages and fibroblasts and that mineral particles may intervene, though doubts about the *in vivo* relevance persist.

Interleukin-1. Interleukin-1 (IL-1) represents two closely related polypeptides, derived from mammalian phagocytes among other cells, whose functions include activation of T-lymphocytes and fibroblasts and also mediation of acute inflammatory responses (*251,252*). Recombinant IL-1 stimulated dermal fibroblast growth and synthesis of type I procollagen along with collagenase and PGE_2 (*253*), thus appearing to be distinct from and less specific than the MFF. However, IL-1 alone did not prove a potent mitogen for a fibroblast cell line, though PDGF made cells more responsive (*254*), and proliferation of human lung fibroblasts, mediated by human blood monocytes or alveolar macrophages, was inhibited through intervention of IL-1, an effect which indomethacin blocked but which PGE_2 restored (*255*). Cells of dermal origin or a standard line evidently do not respond in a manner similar to those derived from adult humans and reinforce the finding that dermal or tendon as contrasted with granuloma fibroblasts do not release the MFF (*49,256*).

Inhalation of amphibole or serpentine asbestos by rats produced alveolar macrophages which, cocultured with lymphocytes, led to an elevated level of IL-1 and IL-2 and to increased DNA synthesis by human dermal fibroblasts (*257*), as well as to macrophage activation (*258*). Provocation of fibroblast proliferation via an IL-1-like factor released from monocytes or macrophages treated with quartz or asbestos (crocidolite or chrysotile) has also been reported, but, again fibroblasts derived from skin or cell lines were used (*259,260*), oil elicitation of macrophages (*261*) is to be deprecated (*49*), while in none of these studies was collagen formation measured and reservations

have been expressed on the *in vivo* applicability of such findings (*262*).

Tumor Necrosis Factor. Tumor necrosis factor (TNF) constitutes another cytokine derived from macrophages and implicated in inflammatory states. The molecular structure of TNF has been determined (*263*), and it is believed to interact synergistically with IL-1 or interferon (IFN) to inhibit proliferation of human lung fibroblasts (*264,265*). The action of TNF combined with IFN was largely independent of PG production, but that of IL-1 plus TNF appeared to be partly mediated by fibroblast PG. When activated by lipopolysaccharide, alveolar macrophages synthesized and released TNF (*266*). Cultured in the presence of chrysotile or quartz, rat alveolar macrophages produced TNF and the lipoxygenase metabolite leukotriene B_4 which amplifies TNF production (*267*). Restraint in proliferative activity of fibroblasts may in this way be expected when mineral particles react with alveolar macrophages, but instead enhanced growth of fibroblasts was claimed (*268*). Elevated release of TNF, along with IL-1, was detected in alveolar macrophages from cases of coal workers' pneumoconiosis (*269*); the significance of these cytokines in the response to coal mine dust was undetermined, though, in parallel with quartz and asbestos, restriction of the fibroblast population may be anticipated. At present it appears inappropriate to regard the decline in TNF levels (as assessed in monocytes) during progression of coal workers' pneunoconiosis as preventing the development of massive fibrosis (*214*).

Chemotaxis. Apart from circumscribed proliferation in response to a localized stimulus, fibroblasts are believed to be sensitive to chemoattractants. Released by macrophages, fibronectin proved chemotactic for dermal fibroblasts in culture (*270,271*). Alveolar macrophages from patients with idiopathic pulmonary fibrosis produced fibronectin at a rate 20 times higher than did normal cells, and it was chemotactic for human lung fibroblasts (*272*). PDGF possessed a similar chemotactic capacity as well as inducing mitosis in dermal fibroblasts (*273*), as did human collagens of types I, II, and III along with collagen-derived peptides (*274*), thereby suggesting that products of collagen degradation may be instrumental in recruiting to sites of inflammation cells capable of replacing lost connective tissue. Moreover, fragmented fibronectin was a potent chemoattractant for monocytes but not for neutrophils or lymphocytes (*275*), and it may thus concentrate together the two cell types principally required for fibrogenesis. A role for fibronectin in pneumoconiosis remains uncertain, since in lavage fluid from asbestotic humans and sheep the level was elevated (*276*) but depressed after rats inhaled coal mine dust (*277*). Furthermore, impaired chemotaxis of macrophages by inhaled particles was confined to those that were fibrogenic and proved to be independent of dust burden (*278*), but the agent responsible was not identified.

In Vivo Relevance

Acute versus Chronic States. Expansion of the fibroblast population and operation of chemoattractants are readily comprehended in acute-phase inflammatory reactions, such as an organizing pneumonic exudate or granulomatous conditions of the lung where tissue destruction is a feature. Inflammatory mediators secreted by alveolar macrophages from individuals affected by asbestosis include leukotriene B_4, a lipoxygenase metabolite of arachidonic acid (*279*), and plasminogen activator which is particularly evident at an early stage of the disorder (*280*), but how they contribute to fibrogenesis remains speculative. A role for plasminogen activator appears somewhat dubious, since its secretion by alveolar macrophages *in vitro* disclosed no correlation with the pathogenicity of fibrous or compact dusts (*281*).

The requirement for fibroblasts is rather less obvious in disorders which are chronic *ab initio* and silicotic nodules not only remain strictly circumscribed but ultimately present as almost acellular lesions whose collagen is hyalinized and disposed concentrically. Findings on the proliferative behavior of fibroblasts toward macrophage products depend largely on *in vitro* procedures, which are unlikely to be reproduced *in vivo*, where constraints imposed by closely apposed structures are liable to limit population increase or restrain movement of connective tissue cells. Considered in relation to phenotypic variability of proliferative capacity within human lung fibroblast lines (*282*), these kinetic studies expose a series of potential interactions whose complexity renders precise outcomes difficult to anticipate. The culmination is more predictable in terms of the activity of the MFF, which is produced *in vivo* (*64*) and whose operation could readily proceed from indigenous interstitial lung fibroblasts with only restricted replication and no apparent need for migration. A decisive influence on lung collagen formation is likely to be the size and turnover of the macrophage population as well as its secretory rate, as provoked by mineral dusts. On this basis, fibrogenesis by coal mine dusts would not be expected to be as pronounced as by quartz.

Mast Cells. These cells accumulate in relation to fibrotic lesions of diverse origin and are considered to provide mediators, such as serotonin and histamine, for events composing the inflammatory reaction. To this pattern experimental asbestosis, whether induced by amphiboles or serpentine, conformed (*283,284*). A similar occurrence typified human and experimental silicosis (*285*). Although the exact part played by mast cells in the chronic events of fibrosis provoked by dust remains obscure, interaction with macrophages and fibroblasts may contribute, the latter by proliferation.

Involvement of the Immune System

Primary

The immunological theory as applied to mineral-induced lung disease can hardly be considered as an initiatory mechanism. Immunological phenomena, humoral or cellular, affect only a minority of humans and occur as a consequence of fibrosis whether the result of exposure to quartz, coal, or asbestos. The presence of humoral components in silicotic lesions, on which emphasis was originally laid, does not necessarily imply production of an autoantigen or an adjuvant effect, since sequestration of serum proteins in the dust lesions could alter their configuration nonimmunologically so they first stimulated formation of rheumatoid factor, then reacted with it and finally bound complement. In the light of *in vitro* observations on mitogen-induced T-cell proliferation and IL-1 release by alveolar macrophages under the influence of silica or asbestos (*260,286,287*) an accessory cell function for these macrophages, encouraging a cell-mediated immune response, may be envisaged as an

epiphenomenon occurring simultaneously with or following fibrogenesis. The heterogeneity in immunological function of alveolar macrophages may explain the inconstant humoral and cellular responses found in coniotic subjects.

Secondary

A consequential role for immune reactions may, however, be admitted, especially if particulate irritants alter the chemical structure of tissue components, among which denaturation of collagen may play a part. Antiserum to lung connective tissue incubated with macrophage supernatants and then applied to fibroblasts led to increased levels of collagen, but antibodies alone had no such effect (*288*). In this system antibodies had first to be stimulated by connective tissue. Denaturation of newly formed collagen *in vivo* might, with macrophage cooperation, continue the process. Release of IL-1 by macrophages exposed to quartz or chrysotile was considered to be consistent with nonspecific stimulation of the immune system, such as could occur in asbestotic or silicotic subjects (*289*). Interleukin-2 may act via TNF as a mediator of interactions between alveolar macrophages and lymphocytes in conditions of cell-mediated immunity, so serving to maintain the inflammatory process (*290*) and perhaps aggravate complicated pneumoconiosis. Abolition by chrysotile of the inhibitory effect of artificially-activated lymphocytes on fibroblast growth *in vitro* (*246*) may conceivably imply a similar role. Estimation of splenic T-lymphocyte function and humoral immune response induced by pathogenic dusts exposed inconsistencies; either depression occurred after injection of quartz or chrysotile (*291*) or elevation by quartz and depression by chrysotile followed inhalation (*292*), with TiO_2 showing no effect via both routes. Moreover, antibody-forming splenic lymphocytes were depressed similarly by quartz and control TiO_2, but more so by chrysotile, given intraperitoneally (*293*). Even if these changes were consistent and could be shown to persist beyond the acute phase, they too could fall into the category of epiphenomena unrelated to fibrogensis. Chronicity does not, however, necessarily rely on immune intervention, since dust alone and especially quartz instigates a self-propagating state by the local reingestion cycle and by systemic recruitment of monocytic cells to maintain *in situ* production of the MFF.

Cell-mediated immune features may nevertheless be mounted in connection with asbestos-induced malignancy. Natural killer cell activity of human blood lymphocytes from normal and exposed subjects was suppressed in culture by amphibole or serpentine asbestos, an effect which may assist in the development of pulmonary malignancy (*294*). It should be remembered that asbestosis is now regarded as the presursor for development of carcinoma of the lung (*117,295,296*). Human mesothelioma cells, although resistant to lysis by natural killer cells, proved to be susceptible to lymphokine-activated killer cells and afforded a therapeutic possibility (*297*).

Implication of humoral or cellular mechanisms in the genesis of human pneumoconiosis thus remains subordinate. Furthermore, individual susceptibility, as revealed by histocompatibility antigens, has been shown to have no bearing on the prevalence of pneumoconiosis. Although histocompatibility antigens HLA-A29 and HLA-B44 occurred in excess among silicotic subjects, no clinically useful parameters (including PMF) correlated with the presence of either antigen (*298*). To this general position rheumatoid pneumoconiosis constitutes an exception. It is well known that, on epidemiological grounds, Welsh coal workers affected by rheumatoid arthritis had a much increased prevalence of progressive massive fibrosis and about a quarter showed discrete radiological opacities, subsequently identified pathologically as rheumatoid nodules. Arthritis and lung lesions usually appeared together, though either may antedate the other, and their respective severity sometimes differed. The rheumatoid diathesis may therefore be seen as a determinant of the peculiar reaction to inhaled particles, which include silica and asbestos as well as coal mine dust.

Downregulation of immune reactions within the pulmonary parenchyma may be effected by surfactant lipids, which suppress the proliferative response of peripheral blood lymphocytes to mitogens (*299*). Through its phospholipids, surfactant may also modulate the toxicity of human alveolar macrophages and monocytes toward tumor cells, with enhancement by the main components probably exceeding depression by a minor one (*300*).

Connective Tissue Destruction

Macrophages and fibroblasts not only combine in fibrogenesis but individually are able to reverse the process by production of collagenases. Macrophage populations from various sources differed widely in their capacity to degrade collagen and proteoglycans, but fibroblasts possessed greater collagen degrading ability and could be stimulated to do so by a cytokine secreted by macrophages (*301,302*). Moreover, human alveolar macrophages proved capable of producing in culture not only a procollagenase but also a collagenase inhibitor, both of which were indistinguishable from analogous products of human fibroblasts (*303,304*). Degradation of procollagen probably occurs intracellularly and soon after synthesis, but degradation of extracellular collagen, that is where cross links are established, is a slower process to avoid destabilization of structure (*305*). It therefore becomes important to investigate the conditions that might determine whether collagen anabolism or catabolism predominates at a particular time. PGE_2 constitutes a known signal for collagenase synthesis by macrophages (*306*). Similar control mechanisms may conceivably operate in mineral fibrogenesis, but Aalto et al. (*54*) found no evidence of collagenase participation. Moreover, extracellular collagen in dust lesions is mature, sometimes hyalinized and presumably stable.

Collagenolytic metalloproteinase secretion by monocytes could play an important role in their migration through vascular basement membrane, while invasion of blood vessels by malignant tumor cells may be effected similarly (*307*) and be relevant to the neoplastic complications of asbestos exposure.

Lipid Intervention

Alveolar lipo-proteinosis is a recognized consequence of intense exposure to quartz, but lipid participation also deserves attention in other coniotic states, especially when present to a minor degree. Inhaled particles provoke activity not only by alveolar macrophages but also by type II alveolar epithelium which is responsible for secretion of surfactant with its prominent phospholipid component. Continued contact of epithelium with particles is assured by newly deposited atmospheric dust

as well as by dust liberated from laden macrophages as they perish and before it is reingested by newly arrived cells. However, stimulation of type II cells may depend on macrophage cooperation rather than on direct contact of particles with epithelium. Macrophage products and bronchoalveolar lavage fluid augmented DNA synthesis by type II cells *in vitro* (*308–310*), and *in vivo* these cells underwent division following accumulation of dust-laden macrophages in alveoli (*311*).

Coal mine dust or carbon inhaled by rats sometimes leads to a mild excess of lung phospholipid and the degree may vary according to the rank of coal (*312*). Pulmonary phospholipidosis constitutes a nonspecific reaction since experimentally it occurs after administration of TiO_2, diesel particulates, volcanic ash, metals, asbestos, and alpha particles. Chrysotile inhalation led to pronounced elevation of surfactant level, hyperplasia of type II cells, and alterations in surface properties of the lung (*313,314*). Macrophages exposed *in vitro* to chrysotile or silica markedly stimulated secretion of arachidonic acid metabolites, but again the effect appeared to be nonspecific (*188,189*).

The lipid component of the response to inhaled particles tends to the counteract fibrogenesis. Rapid retention of larger amounts of quartz emphasizes lipidosis, while slower accession of a smaller dose permits silicotic nodules to form (*315*). Pronounced accumulation of lipid, as in alveolar lipo-proteinosis, discloses a paucity or even an absence of macrophages and quartz particles become isolated from cellular contact. Consequently, lipidosis stabilizes, local production of the MFF is largely prevented, and such fibrosis as occurs is atypical both in form and distribution. Subtler changes may ensue when lipid secretion is only mildly increased, as by a low dose of a toxic mineral or by a higher dose of one that is less deleterious to cells. A limited degree of lipidosis may then determine the induction of diffuse interstitial fibrosis instead of the focal or circumscribed forms of dust lesions characteristic of simple silicosis, coal workers' pneumoconiosis and asbestosis (*316*). Human pulmonary interstitial fibrosis affords a comparable situation, since the more severe fibrotic features were associated with a reduction of phospholipid in bronchoalveolar lavage fluid (*317*). The proposal that surface radicals exposed on freshly fractured particles of crystalline silica are implicated in the genesis of accelerated silicosis (*318*) or that such radicals account for pulmonary injury by fresh coal mine dust (*205*) fails to take account either of the lipid component or of the macrophage-fibroblast interaction.

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