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Biochemical and Cellular Mechanisms of Dust-Induced Lung Fibrosis

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The sequence of cellular and biochemical events in response to the deposition of dust particles in lung tissue is described. Primary reactions at the lung surface include changes in the free cell population, the alveolar surface protein and in the quantity of pulmonary surfactant, a lipoprotein-rich material secreted by Type II cells. The relationship between these changes and lung fibrogenesis is discussed. It is suggested that such primary changes are protective mechanisms which may assist in the prevention of fibrogenesis rather than lead to an increase in collagen formation and deposition. If these primary defenses are overcome, then the interstitial fibroblastlike cell may have a prominent role in fibrogenesis. Therefore detailed observations of the interaction between lung fibroblasts and mineral dusts *in vitro* are described. As fibrogenesis may be arrested *in vivo,* or possibly reversed, and does not always progress to fibrosis, final consideration is given to the step from fibrogenesis to fibrosis. It is suggested that this step may involve other tissue proteins apart from collagen and that the irreversible nature of fibrosis can be explained by the formation of strong intermolecular crosslinks between different proteins. The types of crosslinks that may be involved are discussed. Emphasis is placed on the role of calcium-dependent transglutaminases in fibrosis, as these enzymes have hitherto received little attention.

For many centuries it has been known that inhalation of certain dusts can cause human lung disease. For example, historical evidence of silicosis has been documented in Egyptian mummies. The Romans were apparently aware of certain health hazards associated with asbestos, because Pliny in AD 50 mentioned that the weavers producing wicks for the lamps of the vestal virgins wore masks to avoid inhaling the dust. Silicotic lungs commonly show fibrous adhesions in the pleural cavity, with plaques visible over the pleural surfaces. In section, fibrotic nodules vary in size from a few millimeters to larger conglomerate areas of massive fibrosis. Each nodule consists of hyalinized collagen fibers in a central region and reticulin fibers on the periphery, the whole having a concentric arrangement and showing some fibroblastic activity at the edges of early lesions (*1*). The pathological features of asbestosis have been described by numerous investigators, and it is suggested that asbestos fibers excite a phagocytic and giant cell reaction, followed by interstitial fibrosis leading to obliteration of alveoli, alveolar ducts and respiratory bronchioles (*2*). It is generally agreed that both human asbestosis and silicosis develop many years after the first exposure and are charaterized by a slow, progressive fibrosis often associated with the excessive deposition of collagen.

There are a number of whole animal and cell models involving inhalation, instillation, injection or addition of such compounds as dusts, bleomycin, paraquat, butylated hydroxytoluene (± oxygen) and cadmium which purport to study experimentally induced "fibrosis." However, different experimenters have used quite different criteria to recognize the endpoint of "fibrosis." For example, some pathologists equate hypercellular areas of sections stained with hematoxylin and eosin with the sites at which fibrosis will develop. Alternatively, some consider an increase in reticulin (silver stain) as evidence of fibrosis, while others use connective tissue stains such as aniline blue or van Gieson which are said to identify collagen specifically.

Apart from the difficulties of quantitative interpretation of stained sections, the histopathologist is faced with other problems. Is it certain that the increased uptake of any of these stains presents conclusive evidence of irreversible fibrosis? Is it not possible that an increase in reticulin stain simply provides evidence for fibrogenesis which may be quite reversible? In attempting to quantify fibrosis the biochemists are faced with equally formidable problems. Fibrosis may often be confined to localized areas of lung tissue, and therefore biochemical analysis of biopsy specimens can be misleading. Alternatively, analysis of whole lung homogenates may prove insensitive unless the fibrotic reaction is widespread. Another problem is whether elevated levels of prolyl hydroxylase activity or an increase in the incorporation of radiolabeled proline into hydroxyproline-containing moieties is sufficient evidence of fibrosis. Whereas such studies may well show that more collagen (as procollagen or tropocollagen) is made,

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and it is likely therefore that more fibrous collagen will be deposited, (i.e., fibrogenesis), it should not then be assumed that this process is irreversible (i.e., fibrosis).

In this review we consider the ordered sequence of cellular and biochemical events in response to the deposition of dust particles in the lung to be: (1) the primary reaction at the lung surface; (2) reactions of dusts with lung interstitial cells; (3) the step from fibrogenesis to fibrosis. One of the central themes is the important distinction between fibrogenesis and fibrosis. However, several novel concepts are put forward which hitherto have received little attention. The first of these is that one of the primary effects of dusts which involves damage to the alveolar epithelium and accumulation of pulmonary surfactant may assist in the prevention of fibrogenesis rather than lead to an increase in collagen formation and deposition. Second, if these primary defenses are overcome, then the interstitial fibroblast has a prominent role in fibrogenesis. However, fibrogenesis may be arrested, or possibly even reversed, and does not always progress to fibrosis. Final consideration is therefore given to the step from fibrogenesis to fibrosis, which we suggest involves other connective tissue proteins apart from collagen. The irreversible nature of fibrosis may depend on the formation of strong intermolecular crosslinks catalyzed by calcium-dependent transglutaminases.

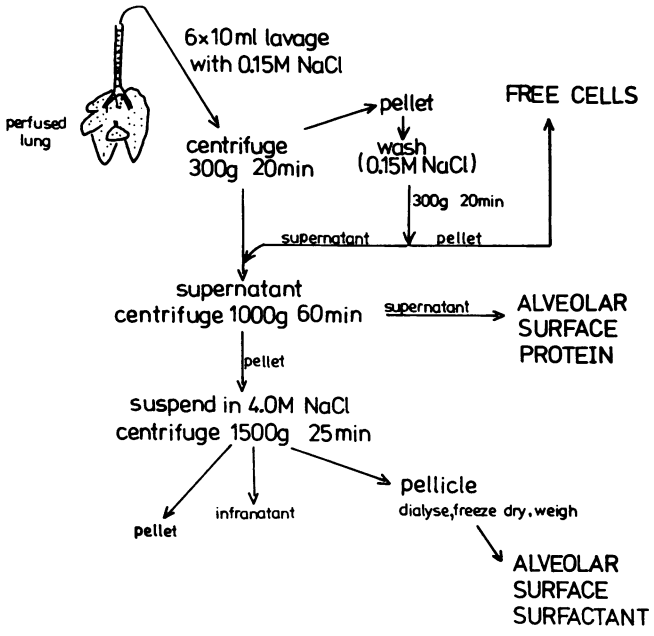

FIGURE 1. Procedure for lung lavage and isolation of alveolar surface components. This is a procedure modified from Abrams (*5*) to obtain pulmonary surfactant. It should be noted that the 1000*g* supernatant fraction, designated as alveolar surface protein fraction, also contains up to 50% of the total lipid on the lung surface.

Primary Reactions of Dust Particles at the Lung Surface

These reactions include alterations in the epithelial lining layer (Type I and Type II cells) and in pulmonary lipoprotein (surfactant), the free cell population and the alveolar surface proteins.

The three components can be isolated from control and dust-treated animals by centrifugation of alveolar lavage (Fig. 1). In control animals, the free cell population is mostly macrophages, but following deposition of "cytotoxic" dusts the population is more variable. In control animals, the alveolar surface protein is mainly albumin and immunoglobulin G with varying amounts of secretory immunoglobulin A (*3*). Analysis of pulmonary surfactant from a number of species show it to be approximately 10% protein and 90% lipid, the major lipid moiety being phosphatidylcholine. Fatty acid analysis of this fraction shows that it contains a very high proportion of palmitic acid (hexadecanoic acid, 16.0), indicating that much of the phosphatidylcholine fraction is dipalmitoylphosphatidylcholine (*4*). Thus, alveolar surface surfactant isolated by a modification of the method of Abrams (*5*) is relatively pure, being minimally contaminated with membrane lipids and is of very similar composition to that found in Type II cell lamellar bodies.

Changes in Alveolar Surface Surfactant Levels in Rats Exposed to Dust by Inhalation or Instillation

Alveolar surface surfactant accumulates in the lungs of rats exposed to chrysotile (white) asbestos (a mineral considered to produce fibrosis in humans) over a 15-week period. At this time, mineral-treated lungs contain 6 to 12 times the amount of surfactant found in normal animals (*6,7*). Removal of the animals from the inhalation chamber produces some reduction in the surfactant levels after 30 weeks (15 weeks exposure to dust followed by a 15-week clearance period), but the levels do not return to those found in control animals (*6*). This accumulation of surfactant is probably dose-dependent, and exposure to low doses of chrysotile does not promote surfactant levels (*8*). The isolated surfactant is of similar chemical composition in control and chrysotile-treated rats (*7*), and thus it seems probable that the fibrogenic chrysotile promotes lipoprotein accumulation by increasing Type II cell proliferation (*9*) without a corresponding change in degradation (*7*). This hypothesis has received support from investigations with other dusts, some of which produce fibrosis in humans and others which may have relatively low fibrogenic potential. Rats inhaling fiberglass (low fibrogenic potential) show only a transient, small accumulation of surfactant which returns to control levels following a clearance period (*8*). Similar studies with a paste polymer polyvinyl chloride,

at "nuisance dust" exposures, produced small granulomatous areas in the lung but little or no accumulation of surfactant and no evidence of fibrogenesis or fibrosis over the time period studied (*10*). By contrast, inhaled amosite asbestos (which produces fibrosis) induces surfactant accumulation in rats, and these levels do not return to normal following a clearance period (*8*). Further inhalation experiments with α-quartz (fibrotic in humans) showed that rats exposed briefly to high concentrations of the mineral over 8 weeks followed by a clearance period of 16 weeks had 16 times the level of surfactant found in control animals (Hardy and Richards, unpublished data; *11*).

Studies on rats given small doses of dusts by instillation show a similar trend (Table 1). Most dusts of weak fibrogenic potential promote very little or no accumulation of surfactant at 4 or 15 weeks after instillation. However, the highly active α-quartz produces a dramatic response with surfactant levels between 15 and 20 times higher than those found in normal animals. Chrysotile did not provoke the same extensive increase in surfactant levels in instillation experiments (Table 1) as found previously in inhalation studies. The reason for this is not yet clear, but one important difference is in the size distribution of the minerals used in the two studies. For the instillation studies, the dust was prepared by sedimentation of UICC chrysotile in water, and consequently the fiber size of the dust fraction was very small (< 2 μm length). It is conceivable that these small particles could be cleared relatively rapidly, whereas in inhalation studies the clearance may be slower due to the combined effects of the deposition of larger particles and continuous exposure.

Collectively the inhalation and instillation studies show that certain dusts can promote the accumulation of alveolar surface (and probably intracellular) pulmonary surfactant (*12*) and that the degree of response is dependent on such factors as dose, particle/fiber size distribution and the surface chemistry of the minerals. The greatest and most persistent response is obtained with dusts which produce fibrosis in human lung. Why is the synthetic/degradative balance of pulmonary surfactant disturbed by some dusts and what cellular mechanisms are involved? Does the "wounded" lung have more need of pulmonary surfactant? What are the consequences of surfactant accumulation in relation to fibrogenesis and fibrosis? The answers to these questions are unknown but some positive suggestions can be made. For example, certain quantities of a cytotoxic dust probably damage the thin Type I cell barrier and Type II cells replicate quickly to plug the epithelial gap. The number of Type II cells would thus increase, resulting in the production of more surfactant, which if not degraded by the wounded lung would lead to surfactant accumulation. This accumulation may be important for lung protection in that surfactant is known to coat cytotoxic dusts, reducing their ability to damage cell membranes (*13*). In addition, the increase in the amount of lipid products may also increase the influx of macrophages (*14*) which could then assist in the clearance of the offending dust. Indeed, preliminary experiments (Table 2) have shown that increasing the dose of instilled surfactant elevates the number of free cells recoverable from rat lung lavage. Moreover, the metabolism of these cells appears to be changed in that the specific activity of two lysosomal enzymes is often

Table 1. Pulmonary surfactant levels (mg/rat) in experimental animals exposed to 5 mg quantities of instilled dust.

Dust	Probable fibrogenic potential	Surfactant levels at various times after exposure, mg/rat[a]	
		4 weeks	15 weeks
None (Control)	—	0.87 ± 0.24	1.35 ± 0.32
A380 silica	Weak	2.22	0.92
R972 silica	Weak	—	1.60
Cabosil	Weak	1.50	—
DQ12 α Quartz	Strong	15.35	22.72
Anatase	Weak or none	0.85	—
Rutile	Weak or none	0.92	—
Rutile + Ni	Weak or none	0.85	—
Rutile + Cr	Weak or none	0.63	—
Carbon fiber (small)	Weak	2.20	1.87
Carbon fiber (ground)	Weak	2.06	—
Activated charcoal	Weak	1.83	2.10
Calcium silicates			
A	Weak	1.02	—
B	Weak	0.63	—
C	Weak	0.97	—
Prepared chrysotile asbestos	Strong?	2.50	—

[a] Control surfactant levels (SD) are from five separate experiments on pooled samples from four rat lavages (20 animals total). The weight of surfactant in dust-treated rats represents the mean value/animal from a pooled sample of 4 to 8 rats for each determination.

Table 2. Effects of instilling different doses of pulmonary surfactant in the rat.[a]

	Time after instillation, (days)	Amount of surfactant instilled, mg/rat				
		0	2.3	4.5	9.0	18.0
Free cell number	6	9.8	25.4	26.5	26.5	42.8
× 10^{-6}	21	11.3	15.2	19.2	21.4	23.4
	42	14.3	18.4	18.9	18.1	24.9
Acid RNase, units/	6	0.83	0.95	1.01	1.38	1.49
10^6 cells	21	0.81	1.32	0.78	0.68	0.44
	42	0.67	1.12	1.39	0.99	0.87
Acid protease,	6	0.31	0.58	0.60	0.70	0.75
μmole tyrosine	21	0.37	0.42	0.42	0.38	0.56
equiv./10^6 cells	42	0.25	0.35	0.47	0.42	0.41
Alveolar surface	6	8.8	5.0	7.5	4.5	7.6
protein, mg/rat	21	4.7	6.1	4.0	4.6	6.8
	42	4.9	7.2	4.6	4.5	2.7

[a] Freeze-dried rat surfactant was collected from numerous experiments and stored frozen. When required it was suspended to the instilled concentration and sonicated in 0.1 M NaCl, after which it was instilled in a volume of 0.5 mL/rat.

increased (Tetley and Richards, unpublished data). By contrast, the amount of alveolar surface protein is rarely altered after surfactant instillation (Table 2). It has been proposed that the macrophage has an important role in surfactant degradation (*15*), but the simultaneous accumulation of both surfactant and macrophages does not necessarily support this suggestion. Investigations indicate that macrophages are not particularly efficient in degrading the intact lipoprotein complex but nevertheless may metabolize the partially degraded products of surfactant (*16*). Another consequence of the extensive accumulation of surfactant, particularly in experimental silicosis, is that it may affect surface tension and therefore alter physiological measurements of lung function. However, it is difficult to determine whether some of these changes are directly related to surfactant accumulation or whether they arise from the effects of dust in the interstitium.

A further attractive hypothesis is that surfactant accumulation could act as a "trigger" mechanism in interstitial connective tissue initiating changes in fibroblast cell growth or alterations in collagen synthesis/deposition. Such a proposal is similar to that suggested by Fallon (*17*), who considered that phospholipids, derived from macrophages, could stimulate fibrogenesis. The possibility that surfactant might influence the behavior of interstitial cells was tested experimentally by using stationary phase lung fibroblast cells. These cells were exposed to increasing concentrations of pulmonary surfactant or its major component, dipalmitoyl-phosphatidylcholine (DPPC) in the presence of [^{3}H]-thymidine and [^{14}C]-proline. While little or no change was detected in the incorporation of ^{3}H into DNA over 24 hr, pulmonary surfactant depressed

Table 3. Effects of rabbit alveolar surfactant and dipalmitoylphosphatidylcholine on stationary phase rabbit lung fibroblasts incubated with [^{3}H]-thymidine and [^{14}C]-proline.[a]

Description of culture	[^{3}H]-Thymidine incorporated into DNA, dpm/μg	[^{14}C]-Proline incorporated into cell mat protein, dpm/mg × 10^{-3}	[^{14}C]-Hydroxyproline labeled moieties in culture medium, dpm/μg	[^{14}C]-Hydroxyproline labeled moieties cell mat, dpm/μg
Control 1 (containing serum)	1708 ± 220	107 ± 13	308 ± 61	221 ± 27
Control 2 (serum-free)	1591 ± 312	98 ± 9	328 ± 36	173 ± 31
Surfactant added, mg/mL				
0.1	1642 ± 472	68 ± 7*	224 ± 14*	146 ± 62
0.25	1449 ± 367	63 ± 16*	235 ± 41*	184 ± 20
0.50	1520 ± 314	50 ± 12*	225 ± 11*	114 ± 24*
1.00	1710 ± 334	35 ± 22*	111 ± 79*	68 ± 44*
Dipalmitoyl phosphatidylcholine, mg/mL				
0.075	1970 ± 551	83 ± 19	220 ± 34*	179 ± 19
0.180	1681 ± 777	83 ± 13	236 ± 24*	203 ± 41
0.370	1815 ± 692	91 ± 12	347 ± 69	190 ± 11
0.750	1541 ± 248	78 ± 28	352 ± 131	211 ± 48

[a] Cells were incubated in the presence of 0.3 μCi/mL of [^{3}H]-thymidine and 0.2 μCi/mL of [^{14}C]-proline for 24 hr (*3*).
* Significant difference (*t*-test) from control 2.

Table 4. Preliminary studies on the effects of dusts (5 mg single instillation) on protein metabolism by lung slices taken from animals 15 weeks after first exposure.[a]

Dust sample	Total lung wet weight, g[b]	Total lung dry weight, g[b]	Surfactant, mg/rat[c]	Lung protein (dry weight) mg/g[c]	Hypro, (dry weight), mg/g[c]	Bound [^{3}H] / Total[^{3}H]	Free [^{3}H] / Total [^{3}H]	Bound [^{3}H] / Lung dry weight dpm/hr/g × 10^{-3}	Bound [^{3}H] / Lung protein dpm/hr/mg × 10^{-3}
Control	2.08	0.29	1.35	751	7.06	0.48	0.52	2220	2.95
Ground carbon fiber	2.26	0.35	ND	645	5.26	0.46	0.54	1792	2.78
Activated charcoal	2.42	0.36	2.10	700	5.42	0.43	0.57	1523	2.18
Anatase	2.07	0.28	1.15	746	8.12	0.47	0.53	1720	2.30
Calcium silicate A	1.88	0.26	1.25	838	8.23	0.49	0.51	2350	2.10
Calcium silicate B	2.21	0.31	1.15	768	7.61	0.53	0.47	1845	2.40
A380 silica	2.21	0.31	0.92	855	10.03	0.40	0.60	1755	2.05
R972 silica	2.05	0.27	1.05	896	10.48	0.51	0.49	1994	2.22
DQ12 quartz	4.36	0.76	22.72	551	4.69	0.25	0.75	529	0.96

[a] Lung tissue slices (250 mg wet weight) were preincubated in serum-free medium which was then replaced with medium containing [^{3}H]-proline. Free and bound radiolabel were separated by washing and perchloric acid treatment and synthetic rates (final two columns) calculated from samples processed at hourly intervals up to a period of 4 hr. The methods are slightly modified from Bradley et al. (*18*).
ND = not determined.
[b] Data derived from four individual animals.
[c] Data from pooled samples from four rats.

the incorporation of [^{14}C]-proline into protein, the formation of [^{14}C]-hydroxyproline containing moieties in the culture medium (the "soluble" collagen) and [^{14}C] hydroxyproline containing moieties in the cell mat (deposited collagen) in a dose-dependent manner (Table 3). Interestingly, the DPPC itself had little effect. These results from cell culture suggest that a component(s) of pulmonary surfactant, when present in sufficient amounts, could interfere with lung connective tissue metabolism in such a way as to reduce protein (including collagen) synthesis. It is also possible that dusts which elevate surfactant levels *in vivo* will interfere with protein metabolism in the lung. Evidence that fibrogenic minerals, such as quartz, alter lung protein metabolism is shown in Table 4. Radiolabeling studies with [^{3}H]-proline were carried out on lung slices which had been obtained from rats exposed for 15 weeks to 5 mg quantities of different dusts given by instillation. Despite the fact that the quartz-treated tissue accumulates more total radiolabel, much of this remains unbound and the actual incorporation of [^{3}H] into protein in terms of dry weight or total lung protein is less than that found in control animals or in rats which have been exposed to other dusts. These results imply that although exposure to quartz increases the total protein content of the lung, there is no evidence of a proportional increase of proteins containing proline and these would include collagen. Lung protein and hydroxyproline expressed as a proportion of the lung dried weight are lower in quartz-treated animals compared with control rats probably because the mineral induces an extensive lung lipidosis, which contributes significantly to lung dry weight.

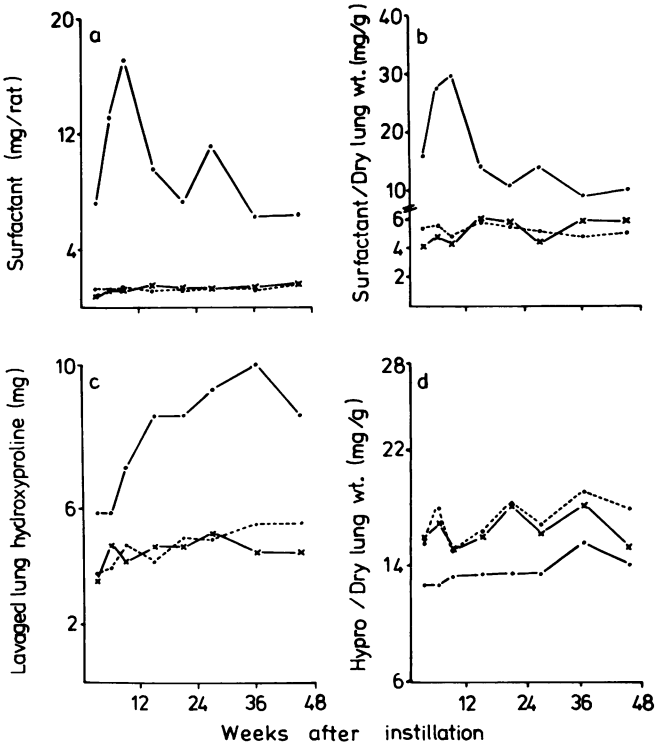

FIGURE 2. Pulmonary surfactant and hydroxyproline levels in lavaged lungs of rats exposed to 5 mg quantities of (●——●) DQ12 quartz and (●--●) titanium dioxide (anatase) compared with control animals (×——×). The dusts were given at zero time as a single instillation in 0.5 mL of 0.15 M NaCl. Surfactant values represent the mean level from pooling samples from at least four rats, and hydroxyproline data are given as the mean level from individual determinations on four animals (ranges not shown).

In order to determine whether lipidosis and, in particular, if surfactant levels remain elevated with progression of lung disease, a further experiment was conducted to assess the long-term effects of the instillation of 5 mg quantities of anatase or quartz on the rat lung. In rats treated with the anatase, a nonfibrogenic mineral, surfactant and lung hydroxyproline levels are similar to those found in control rats (Fig. 2), and histopathological examination indicates that no additional reticulin or collagen fibers are deposited over a 45-week period. In contrast, fibrogenic DQ12 quartz causes a rapid increase in surfactant, particularly in the first 9 weeks, and although this effect is gradually reduced, the amount of alveolar surface lipoprotein is still 5 times higher in mineral-treated rats

Table 5. Hydroxyproline and protein levels in rats exposed to mineral dusts (5 mg, one instillation) at different time intervals after first exposure.

Parameter	Dust treatment	Time after first exposure							
		3 weeks	6 weeks	9 weeks	15 weeks	21 weeks	27 weeks	36 weeks	45 weeks
Hydroxyproline, mg/lung	Control	3.5 ± 0.4	4.8 ± 0.2	4.2 ± 0.3	4.8 ± 0.2	4.8 ± 0.9	5.3 ± 0.9	4.4 ± 0.4	4.5 ± 1.2
	Anatase	3.8 ± 0.3	4.1 ± 0.7	4.8 ± 0.7	4.3 ± 1.0	5.0 ± 0.5	4.9 ± 1.0	5.6 ± 0.5*	5.6 ± 0.7
	R972	5.0 ± 0.6*	4.8 ± 1.0	5.6 ± 1.3	4.5 ± 0.6	5.8 ± 0.7	6.1 ± 1.3	6.1 ± 1.2	5.1 ± 0.6
	Quartz	5.9 ± 0.8*	5.9 ± 0.3*	7.6 ± 2.3*	9.4 ± 2.2*	9.2 ± 1.8*	10.3 ± 1.3*	11.1 ± 3.8*	9.3 ± 1.1*
Protein, mg/lung	Control	127 ± 18	146 ± 17	139 ± 10	191 ± 29	207 ± 16	223 ± 29	194 ± 21	233 ± 49
	Anatase	138 ± 16	125 ± 7	158 ± 19	181 ± 27	226 ± 34	199 ± 39	234 ± 47	275 ± 31
	R972	192 ± 30*	136 ± 11	173 ± 22	173 ± 25	247 ± 34	224 ± 30	247 ± 18*	229 ± 17
	Quartz	224 ± 38*	207 ± 36*	252 ± 43*	415 ± 96*	466 ± 91*	420 ± 74*	474 ± 73*	436 ± 44*
Hydroxyproline/protein, μg/mg	Control	27.7 ± 1.2	33.1 ± 3.3	30.5 ± 3.3	25.5 ± 4.0	23.3 ± 2.4	23.7 ± 2.2	22.7 ± 1.7	19.3 ± 2.9
	Anatase	27.8 ± 2.0	33.1 ± 6.8	30.3 ± 3.3	23.8 ± 2.6	22.3 ± 2.5	24.8 ± 2.2	23.5 ± 2.9	20.3 ± 0.6
	R972	26.4 ± 3.3	34.9 ± 5.8	32.2 ± 3.3	25.9 ± 2.6	23.5 ± 1.3	27.1 ± 2.4	24.7 ± 3.5	22.1 ± 1.2
	Quartz	26.4 ± 1.9	29.2 ± 5.1	29.8 ± 4.9	22.6 ± 1.1	19.9 ± 0.5	24.6 ± 1.6	23.2 ± 6.0	21.3 ± 0.4

* Significantly different from control (*t*-test).

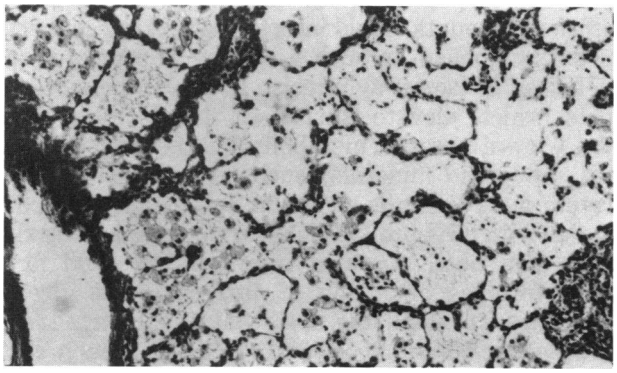

FIGURE 3. Rat lung after 15 weeks exposure to DQ12 quartz. Note extentive alveolar surface reaction. Formalin-fixed sections; hematoxylin and eosin (HE). ×132.

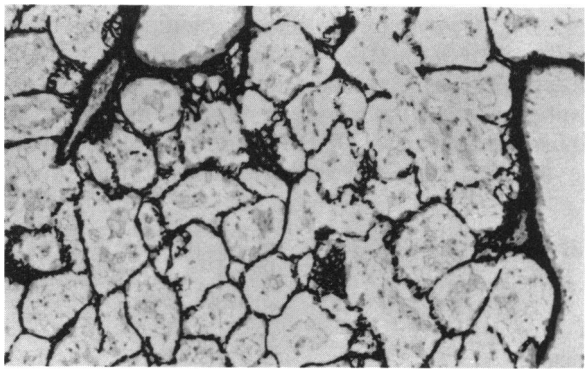

FIGURE 4. Rat lung after 15 weeks exposure to DQ12 quartz. Formalin fixed section, silver stain for reticulin (R). ×132.

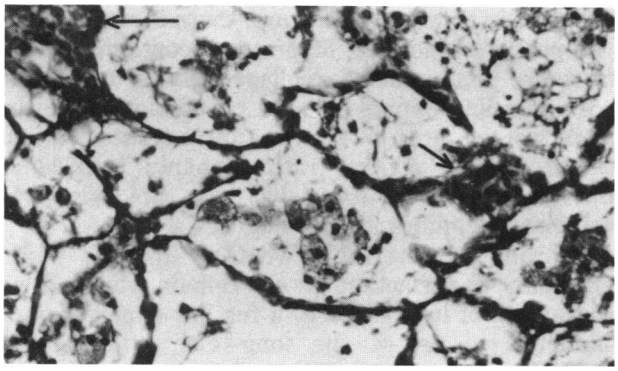

FIGURE 5. Rat lung after 15 weeks exposure to DQ12 quartz. Extensive surface reaction and cellular thickening in some areas (arrows). Formalin fixed section, Azan/aniline blue stain for collagen (AA). ×330.

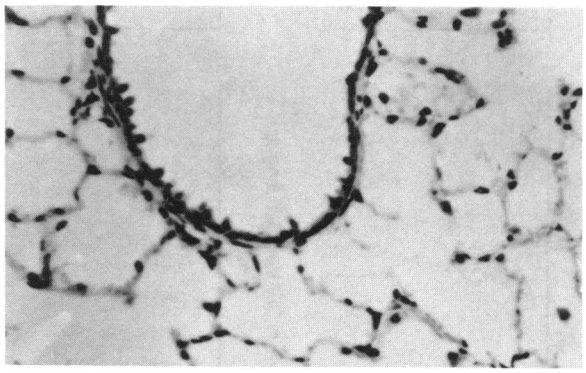

FIGURE 6. Normal rat lung. Control for 45 week exposure. Formalin fixed section, HE stain. ×330.

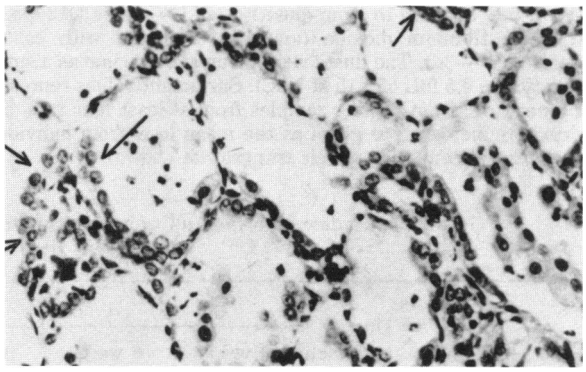

FIGURE 7. Rat lung after 45 weeks exposure to DQ12 quartz. Thickened alveolar walls prominent with large numbers of epithelial-like cells lining the alveolar surfaces (arrows). HE stain. ×330.

than that found in control animals at 45 weeks after exposure (Fig. 2*a*). The result is similar if surfactant levels are expressed relative to lung dry weight (Fig. 2*b*). Total lung hydroxyproline levels are higher in quartz-treated rats, particularly after 9 weeks, and remain high up to 45 weeks after first exposure (Fig. 2*c*). This would seem to provide biochemical evidence for fibrogenesis because additional collagenlike material is present in the whole lung. However, hydroxyproline content expressed as a proportion of the dried weight is lower in quartz-treated lungs than that found in control animals (Fig. 2*d*). Similarly, while total lung protein increases with time after exposure to quartz, the proportion of hydroxyproline-containing proteins is not increased until at least 27 weeks after exposure. Even then hydroxyproline/protein ratios are not significantly different in quartz, anatase-treated and control rats (Table 5). Histological examination of rat lungs treated with quartz (5 mg, instillation) in an identical manner to those described above was also carried out. After quartz exposure for 15 weeks the major reaction is seen at the alveolar surface (Figs. 3–5) with possibly some additional reticulin in areas of alveolar wall thickening (Fig. 4), although such regions may not stain prominently with aniline blue (Fig. 5). Similar though more extensive effects are found after 28 weeks exposure to quartz (data not shown). At 45 weeks much of the normal lung architecture (Fig. 6) is disturbed with evidence of numerous areas of epithelial-like cell

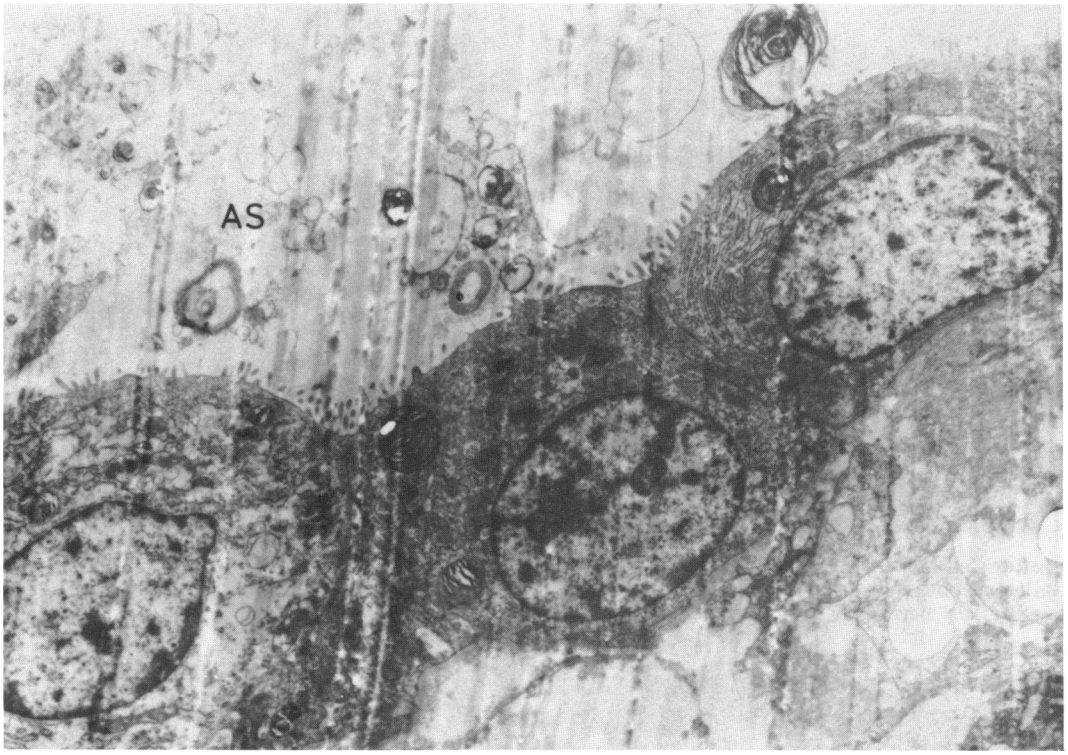

FIGURE 8. Alveolar surface of a section prepared for electron microscopy from formalin fixed material taken from an area of the lung where epithelial-like cells are prominent (Fig. 7). The micrograph is not of high quality because the glass knife drags quartz particles across the section surface. Nevertheless the micrograph shows a highly active row of Type II cells lining the alveolar space (AS). ×28,000.

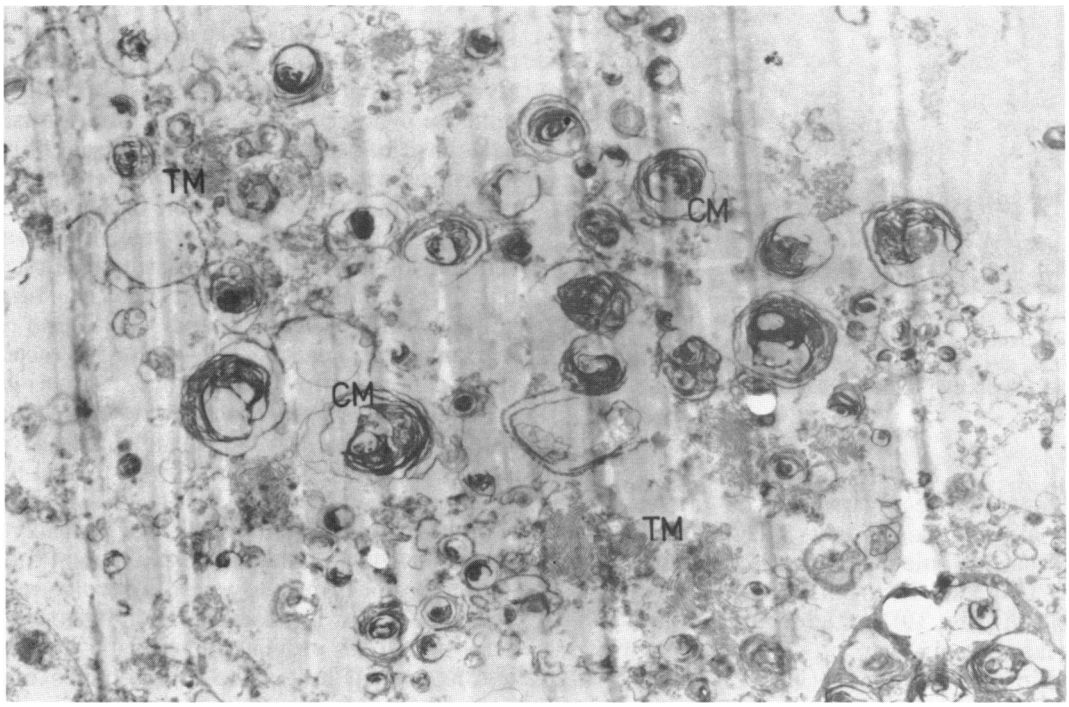

FIGURE 9. Material found in the alveolar space after 45 weeks exposure to DQ12 quartz. Details as for Fig. 8 Note the presence of both common (CM) and tubular myelin (TM). ×28,000.

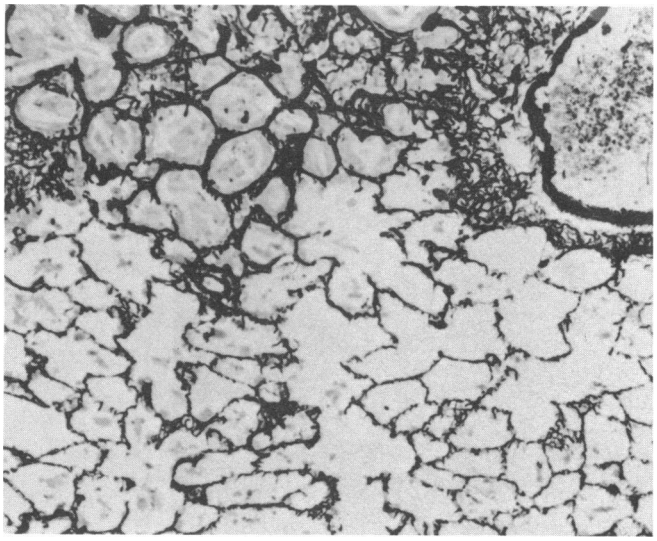

FIGURE 10. Lung after 45 weeks exposure to DQ12 quartz showing extensive deposition of reticulin in some areas of the lung, while other areas are essentially normal. R stain. ×129.

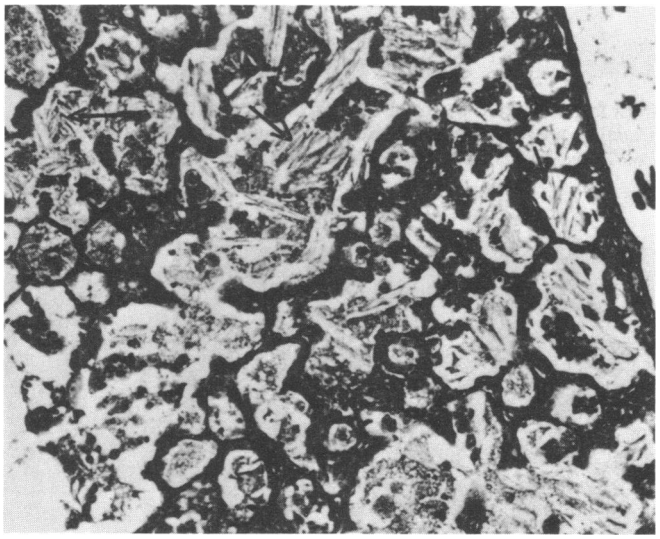

FIGURE 11. Rat lung after 45 weeks exposure to DQ12 quartz demonstrating alveolar wall thickening and heavy collagen deposition. Note also the prominent consolidation of alveolar spaces with the presence of cholesterol clefts (arrows). AA stain. ×129.

proliferation (Fig. 7). Further examination of such areas by electron microscopy reveals the presence of rows of hyperactive Type II cells (Fig. 8) and the accumulation of both tubular and common myelin in the alveolar spaces (Fig. 9). In localized areas, excessive reticulin deposition (Fig. 10) and additional aniline blue staining material (Fig. 11) can be seen in comparison with that found in control animals (Fig. 12). Therefore, the instillation of quartz promotes extensive changes at the alveolar surface, a progressive increase in Type II cells and interstitial cells with some excessive formation of reticulin and aniline blue-positive material in localized

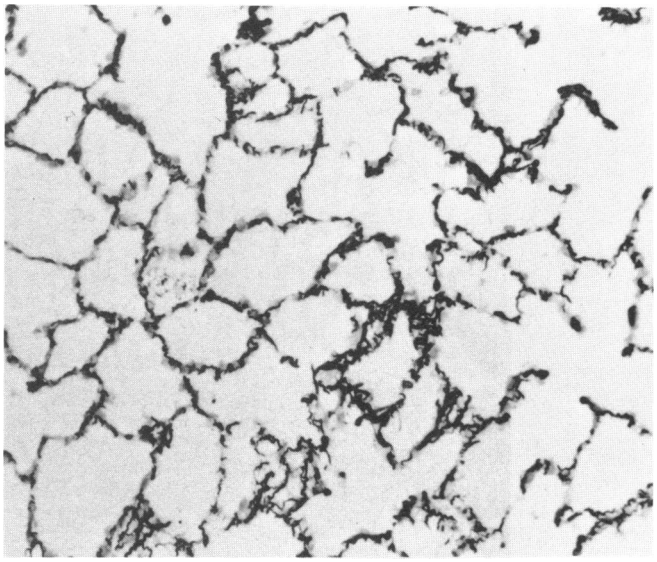

FIGURE 12. Normal lung, control for 45 week exposures. R stain. ×323.

areas. However, at 45 weeks exposure to this dose (5 mg) of quartz, there is no evidence of nodular fibrosis. This raises the possibility that the instilled dose of 5 mg of quartz is not sufficient to produce fibrosis but that the primary reactions at the lung surface are adequate in preventing any further progression of the localized fibrogenesis observed. Alternatively, the time scale of the experiment is too short for fibrosis to be observed. It would seem pertinent to compare these findings with those of Heppleston et al. (*12*), who found a widespread alveolar lipoproteinosis in rats inhaling quartz but minimal evidence of fibrogenesis or fibrosis.

Deposition of small amounts of fibrogenic dust interferes with the metabolism of epithelial lining cells resulting in the accumulation of alveolar surface surfactant. This primary effect may be advantageous in reducing dust toxicity and assisting in particle clearance, but if continually provoked may be responsible for trapping free particles and dust-laden cells in the intra-alveolar spaces. The prolonged accumulation of surfactant may provide additional protection by reducing collagen formation. The importance of surfactant accumulation and the re-epithelialization of the lung surface in preventing extensive mineral-induced fibrogenesis is probably very similar to that reported for the combined effects of butylated hydroxytoluene and oxygen (*19*). In quartz-treated lungs the re-epithelialization process would seem to involve mostly Type II cells and there is no evidence for the reversal to the correct relative proportions of Type I to Type II found in the normal lung. The maintenance of an effective epithelial barrier by Type II cells and/or intact alveolar basement membrane would reduce the risk of particle entry into the interstitium. Such a protective mechanism may be vital in the prevention of fibrogenesis.

The Free Cell Population

In investigations of mineral-induced diseases, the macrophage, which constitutes a major proportion of the free cell population, can lay claim to being the most studied cell. Attention has been focused on this cell because of its extensive secretory and phagocytic activities, e.g., macrophages, when "activated," have been shown to secrete complement products, a number of lysosomal hydrolases, plasminogen activator, prostaglandins, lymphokines and two enzymes (collagenase and elastase) of possible significance in connective tissue turnover. It is not always clear, however, how these processes relate to fibrogenesis or fibrosis. A number of studies *in vitro* support the view that phagocytosis of silica by macrophages and the subsequent death of the cells could lead to fibrogenesis. The cytotoxicity of silica is intimately linked with alterations in the lysosomal system (*20*), and some dusts, for example, asbestos, stimulate selective early release of lysosomal enzymes from macrophages (*21*). Thus, the idea that hydrolases released from macrophages/or other cell types provide a basis for the remodeling of connective tissue seems attractive. However, the connection between the release of lysosomal enzymes and fibrogenesis has not been adequately explained. Would these hydrolases which show maximum activity in the pH range 3–5 also be active *in vivo* against connective tissue with a pH of approximately 7? It may be argued that areas of damage in the lung have a relatively low pH. However, other important features of dust-treated lungs must also be considered. For example, how would the activity of released lysosomal enzymes be affected by large amounts of pulmonary surfactant or alveolar surface protein, which also accumulates in dust-treated lungs? Would this accumulation reduce access of macrophage hydrolytic enzymes to the interstitial connective tissue of the lung? Of equal importance is the question of whether the release of hydrolases from macrophages is specific only to fibrogenic dusts, or does the release simply reflect changes in the metabolism of a cell under stress. In other words, while there may be some specific release of hydrolases from alveolar macrophages *in vitro* (depending on the external stimulant), this may not be abnormal but part of the aging process (*22*). Nevertheless, it is still difficult to envisage how released acid proteases (if active) would aid in increasing lung protein and collagen which is still regarded as a necessary first step in fibrogenesis. In much the same way, proteolytic enzymes that are active at neutral pH such as collagenase, elastase and neutral proteinases, if released from "activated" free cells (macrophages/neutrophils), would presumably only help to reduce the lung extracellular connective tissue components. In a review by Cohen (*23*) on the potential adverse effects of lung macrophages and neutrophils (the latter cells also accumulating as a primary reaction to many inhaled toxic compounds), there is no such contradiction. He suggests that superoxide radicals and enzymes (particularly elastase) released from macrophages and neutrophils may well be linked to lung emphysema, where there is a considerable loss of connective tissue elements. Cohen's theory may be extended by suggesting that elastase released from free cells can damage the epithelial barrier. Indeed this enzyme is successfully added to pulmonary lavage media to obtain an enriched Type II cell population. As indicated in a previous section, the penetration or destruction of the alveolar epithelial barrier can lead to direct interaction of any invading organism or toxin with the interstitial cells such as fibroblasts and this step may be equally, if not more important, in the progression of emphysema.

Another theory which popularizes the role of the macrophage in fibrogenesis results from the work of Heppleston and Styles (*24*). Under the fibrogenic action of silica (*14*), a factor which is released from silica-treated macrophages, but not from titanium dioxide-treated cells, is suggested to promote hydroxyproline production in fibroblast culture. Other investigators, using various techniques, have claimed to be successful (*25*), partially successful (*26*) or unsuccessful (*27*) in reproducing these studies. Indeed, in some instances a sufficient number of intact macrophages alone (without any stimulant) appear capable of inducing fibroblast cultures to deposit more collagen (*26*). Other workers (*28*) have fractionated a factor released from silica-treated peritoneal macrophages which enhances the incorporation of labeled proline into medium and cell fractions of granulation tissue slices (derived by sponge implantation) and into isolated polysomes from the same granulation tissue. The same, or a similar factor, apparently increases [^{3}H]-thymidine uptake (not necessarily into DNA) by cultured granuloma cells. Interpretation of these experiments is somewhat complicated because of the unknown cell composition of the granuloma culture. Nevertheless, if it is accepted that the macrophage has a stimulating factor capable of promoting growth and/or collagen synthesis in fibroblasts, then is it so highly active that it overcomes the activity of the released degradative enzymes such as collagenase from the same (or different) macrophages so that fibrogenesis may take place? In many of the experiments referred to above, because they are often short-term and some consider only the formation of soluble as opposed to deposited collagen, there are difficulties in establishing whether or not fibrogenesis is taking place. Further carefully controlled experiments using a number of fibrogenic and nonfibrogenic agents would seem necessary. For example, if the macrophage factor has an important role in fibrogenesis, then should not the same factor be released from bleomycin, paraquat, asbestos and cadmium-treated macrophages? Even if there is a macrophage factor which increases collagen deposition in the lung (fibrogenesis), without the toxin having any direct effect on the interstitium, then, unless the collagen or any other protein is retained in the lung, the definition of fibrosis has not been satisfied.

The activities of macrophages and other free cells

represent only part of the primary wound healing process in the lung. They are directed towards the promotion of clearance of toxic (and nontoxic) agents and removing and digesting the debris which results from edema and cell damage. Clearance of offending particles may take place via lymphatic drainage and some dust-laden macrophages may enter the interstitial tissue. During the course of such activities the macrophages can secrete a number of products, some of which, if present in sufficient amounts and in the absence of sufficient quantities of specific inhibitors, may affect the growth and/or synthetic activity of neighboring cells. In the absence of further experimental evidence, particularly from *in vivo* studies, aimed at establishing how macrophage secretory activity may cause an imbalance in the lung in favor of excessive degradation (emphysema?) or excessive connective tissue synthesis and retention (fibrogenesis?), it is difficult to ascribe a central role for the macrophage cell in these disease processes. Similarly, the neutrophils may have a transient role only.

Alveolar Surface Protein

Gel filtration (using Sephadex G-150) of the 1000*g* supernatant from rabbit lung lavage fluid gives three major protein peaks corresponding to albumin, immunoglobulin G (IgG) and secretory immunoglobulin A (sigA) (*29*). Albumin and IgG, both of which are derived from serum, have been identified in the rat alveolus by histological techniques (*30*) and in human lung washings (*31*). Scarpelli et al. (*32*) indicated that one protein in rabbit lung lavage was not derived from serum, and this protein was identified as sigA (*33*), a finding repeated for rabbit (*3*) and man (*31*).

One of the early responses of the lung to a toxic mineral is the formation of protein-rich edematous fluid at the alveolar surface (*2*). Similarly, an 11-fold increase in protein occurs in the lung lavage fluid taken from dogs during the development of radiation-induced fibrosis and no major changes are observed in the qualitative composition of the proteins (*34*). Similarly, in human alveolar proteinosis there is also an increase in lung wash protein with only minor changes in qualitative composition (*35*). These observations raise a number of questions about the effects of different minerals on the lung surface protein fraction. Would different minerals produce qualitative as well as quantitative changes in lung surface protein(s) and would the accumulation of proteins have a role to play in fibrogenesis or fibrosis? Early experiments by George (*3*) showed that a rapid and extensive accumulation of alveolar surface protein occurred in rats instilled with quartz and chrysotile asbestos (99% of fibers <2 μm). By contrast, the response was far less marked in rats given titanium dioxide (Table 6). Similar results were obtained in a comparative study of the effects of fibrogenic and weakly fibrogenic minerals in animals after 15 weeks exposure (unpublished data, Table 7) and in longer term

Table 6. Alveolar surface protein levels at 1 and 4 weeks after a single instillation of 5 mg quantities of different dusts.[a]

	Alveolar surface protein, mg/rat	
	1 week	4 weeks
Control	5.9–8.3	5.2–5.3
Anatase	6.5	5.1
Chrysotile	11.8	9.3
DQ12 quartz	21.4	29.7

[a] Range for control animals given. Values for dust-treated animals are from single pooled sample (four rats).

Table 7. Alveolar surface protein levels at 15 weeks after a single instillation of 5 mg quantities of different dusts.[a]

Dust	Alveolar surface protein, mg/rat
Control	6.73
Ground carbon fiber	9.09
Activated charcoal	8.10
Anatase	7.10
Calcium silicate A	8.50
Calcium silicate B	7.90
R972 silica	7.50
DQ12 quartz	49.00

[a] Fibrogenic potential of dusts given in Table 1. Data from pooled sample of four rats.

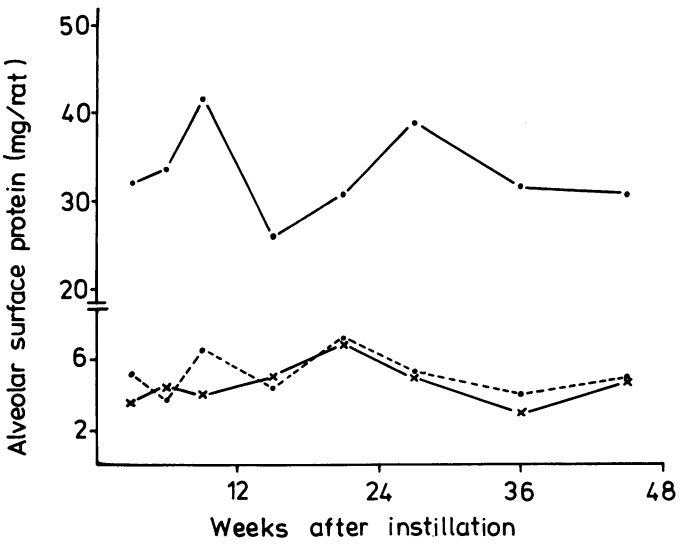

FIGURE 13. Alveolar surface protein levels in the lungs of rats exposed to 5 mg quantities of DQ12 quartz (●——●) and anatase (●--●) compared with control animals (×——×). Data as for Fig. 2.

studies up to 45 weeks after exposure (Fig. 13). No significant changes in protein composition are found in the 1000*g* lavage fraction at 4 weeks exposure to quartz or titanium dioxide (doses up to 5 mg) when analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (*3*). While these studies have not been carried out for the long-term investigations, it can be tentatively concluded that a fibrogenic dust such as quartz will

induce an accumulation of alveolar surface protein of similar composition to that found in control animals.

What is the significance of protein accumulation? Jones et al. (*36*), have shown that a number of dusts adsorb serum proteins. Desai and Richards (*37*) suggest that this adsorption is a selective process dependent on the surface chemistry of the dust. The adsorption of protein(s) (and pulmonary surfactant) would presumably reduce the cytotoxicity of dusts. This theory is supported by a number of studies *in vitro* which show that minerals coated with organic materials no longer induce membrane damage (*13*). Thus, it can be envisaged that a rapid increase in alveolar surface protein along with pulmonary surfactant (the second, perhaps a more "desperate" measure by the lung) in response to quartz deposition would enable the neutralization of any immediate toxic effect of the dust on alveolar macrophages and particularly on epithelial cell membranes. If the dust, for example, titanium dioxide, is relatively inert, then such a protective mechanism may be unnecessary. In addition, the "candy coating" of a dust particle, particularly for a mineral which is cytotoxic, will presumably mask its true identity to the surface receptors of macrophages and other cell types. A coated particle may therefore attach to the macrophage surface or be endocytosed, and, providing there is no exchange of components of the mineral coat which result in cell death or macrophage immobility, the offending dust may then be rapidly cleared. Alternatively, removal of the coat, for example, by hydrolases, may expose a "naked" particle which, once released from the lysosomal system, is free to carry out exchange/adsorption reactions in the cell cytoplasm. This process could lead to cell death and reduce the rate of dust clearance.

Does the accumulation of alveolar surface protein have any direct role in fibrogenesis? The answer is not known but when stationary phase lung fibroblast cultures are exposed to purified (lipid-free) alveolar surface protein, the incorporation of [^{14}C]-proline into radiolabeled protein and the synthesis of [^{14}C]-hydroxyproline-containing moieties is suppressed (*3*) (Table 8). Fibroblast cultures treated with albumin (the major component of alveolar surface protein) in concentrations equal to that of the total protein fraction show no significant changes in protein metabolism (Table 8). The effect of the alveolar surface protein on fibroblasts is similar, but not as extensive as that found with pulmonary surfactant (Table 3).

The mechanism by which some dusts increase the levels of alveolar surface protein is unknown but may be linked to changes in the permeability of the capillary endothelium (*3*) or result from damage to the alveolar and capillary basement membranes. Dusts which are fibrogenic in humans stimulate the greatest accumulation of alveolar surface protein and this additional protein may provide some protection to epithelial and macrophage cell surfaces. One or more components of the alveolar surface protein fraction may inhibit to some extent the formation of proteins which contain proline and/or hydroxyproline.

The primary reactions at the lung surface to deposited minerals described above, involving macrophages, neutrophils, alveolar surface proteins, epithelial cells and surfactant seem to be early protective mechanisms following insult. Collectively, these changes aid clearance and reduce dust toxicity and minimize the amount of mineral penetrating the alveolar wall. Presumably, unless these protective mechanisms are overloaded, then fibrogenesis and fibrosis are unlikely.

Reactions of Dust with Lung Interstitial Cells

Is there any evidence to suggest that particles or toxic compounds can penetrate the lung epithelium and react with interstitial cells? In a study of the pathology of silicosis found in gold miners, Webster (*38*) states, "It

Table 8. Effects of rabbit alveolar surface protein and bovine serum albumin (BSA) on stationary phase rabbit lung fibroblasts incubated with [^{3}H]-thymidine and [^{14}C]-proline.[a]

Description of culture	[^{3}H]-Thymidine incorporated into DNA, dpm/μg	[^{14}C]-Proline incorporated into cell mat protein, dpm/mg × 10^{-3}	[^{14}C]-Hydroxyproline labeled moieties in the culture medium, dpm/μg	[^{14}C]-Hydroxyproline labeled moieties in the cell mat, dpm/μg
Control 1 (containing serum)	1708 ± 220	107 ± 13	308 ± 61	221 ± 27
Control 2 (serum-free)	1591 ± 312	98 ± 9	328 ± 36	173 ± 31
Alveolar surface protein				
0.1 mg/mL	1459 ± 309	54 ± 5*	253 ± 54	191 ± 25
0.25 mg/mL	1488 ± 426	50 ± 7*	226 ± 8*	176 ± 23
0.50 mg/mL	1565 ± 287	53 ± 4*	221 ± 37*	141 ± 29
1.00 mg/mL	1719 ± 255	60 ± 2*	239 ± 17*	132 ± 17
Bovine serum albumin				
0.1 mg/mL	1821 ± 385	96 ± 14	266 ± 34	171 ± 27
0.25 mg/mL	1513 ± 474	114 ± 16	359 ± 43	173 ± 4
0.50 mg/mL	1621 ± 219	97 ± 23	269 ± 30	195 ± 12
1.00 mg/mL	1698 ± 306	96 ± 22	367 ± 26	177 ± 16

[a] Details as for Table 3.
* Significant difference (*t*-test) from control 2.

is concluded that the dust is removed from the alveoli not only by the alveolar phagocytes but also by direct penetration of the alveolar walls. This has also been indicated by Watt et al. (1916) and Gross and Westrick (1954)."* Some recent electron microscopy studies by Brody and co-workers (*39,40*) have provided experimental support for the ideas proposed by Webster. Thus, they have shown that inhaled silica and asbestos may be found in interstitial cells at alveolar duct bifurcations in rat lungs. They have also demonstrated that microcalcifications occur in the interstitium just 4 weeks after exposure to chrysotile (the importance of which is further discussed below). It would seem therefore that dust particles gain access to the pulmonary interstitium but what cells are likely to be affected? Suzuki (*41*) argues for the presence of asbestos in "stromal macrophages," and presumably numerous interstitial cells would be potential targets. However, the most likely target cell when considering lung fibrosis or emphysema is the fibroblastlike cell. Electron micrographs of lung interstitium prepared following asbestos inhalation in rats show mineral fibers in fibroblastlike cells (*39*). Do the particles change the metabolism of the interstitial fibroblast? No data are available on the effects of dust on fibroblasts *in vivo*, but extensive studies have been carried out *in vitro* (*42–44*). Before discussing these effects of dusts, it is necessary to briefly review the rather complicated metabolism of lung fibroblast cells maintained *in vitro*.

Normal, primary-derived lung fibroblastlike cells, when maintained in tissue culture over 24 days, show two or three main stages of differentiation. These are summarized in Figure 14, the data for which are taken from a number of different, primary-derived rabbit lung strains (subcultures normally between 4 and 10). Although all the results are not strictly comparable because of unknown differences between strains, nevertheless there are a number of consistent features. During logarithmic growth (up to day 10) there is a rapid increase in cell number, evidence of mitotic activity and elevated DNA levels and sometimes very low but gradually increasing amounts of hydrolase enzyme activity. These "young" actively dividing cells produce little collagen, most of which is in a soluble form and not deposited as fibers in the cell mat. The relative amounts of hyaluronic acid (HA) with respect to dermatan sulfate (DS) are high in the culture medium of fibroblasts in logarithmic growth (Fig. 14). These young cells have an extensive glycocalyx (fuzz, amorphous cell coat) some surface areas of which apparently lack a typical "tramline" membrane structure but instead, numerous microfilaments (actin/myosin complexes) are found in close proximity to the fuzz (*43*). These surface areas equate to regions of undulating membrane as seen by using time-lapse microscopy (*42*). Once a cell enters stationary phase undulating membrane activity ceases, much of the surface fuzz seems to disappear and plasma membrane extensions or microvilli become prominent. Changes are also seen in the golgi, endoplasmic reticulum and in nuclear conformation (*45*). All of these features are consistent with a cell preparing for active secretion and indeed, mitotic activity is arrested, DNA levels remain constant and collagen deposition is increased, concomitant with a lowering of the HA/sulfated glycosaminoglycan ratio both at the cell surface and in the culture medium (Fig. 14). The change in the balance of glycosaminoglycans in fibroblast cultures and their relationship to the deposition of collagen accord well with the proposed model for fibrogenesis summarized in Figure 15. Towards the end of stationary phase there is some turnover of DNA or possibly cell loss (cell death?), and while there is an increase in the release of low molecular weight hydroxyproline-containing moieties into the medium (suggesting greater collagen turnover), collagen deposition continues to increase until at least day 24. It is important to state that the hydroxyproline-containing material laid down as fibers in the cell mat of rabbit lung fibroblast cultures is reticulin, i.e., the fibers stain prominently with silver stain but do not stain (or stain only lightly) with the collagen stains aniline blue or van Gieson (*46,47*). Other workers (*48*) have suggested that the small filaments and fibers without periodicity, produced by myofibroblasts derived from granulation tissue, may be analogous to Type III collagen and the material designated reticulin by the histochemists. The normal, rabbit lung fibroblasts deposit mainly reticulin (is this Type III collagen?) and present a good model for the study of fibrogenesis over a 24-day period.

What happens to cultured lung fibroblasts when they are exposed to minerals of different fibrogenic potency? When we posed this question in 1969 it was assumed that in a dust-damaged lung the fibroblast cells could rapidly divide to "thicken" the interstitium. Thus, many of the cells exposed to the dust *in situ* would be "young" (in logarithmic phase) fibroblasts. Therefore, it was decided to add minerals to "young" cells at day 2 or 3 in culture and the effects were examined by light and electron microscopy and using biochemical techniques. The first results were encouraging because the response of the cells to each mineral appeared to be specific. Extensive studies were carried out with the fibrogenic mineral, chrysotile asbestos, which was particularly toxic (*46*), but after 21 days exposure produced an increase in cell mat hydroxyproline (reticulin) levels (*49*). Reticulin deposition in chrysotile-treated cultures was "abnormal" (*47*), and other studies revealed that this mineral induced changes in secreted glycosaminoglycans (*44*). It was suggested that asbestos provokes the excessive accumulation of hydroxyproline-containing moieties in the cell mat by rapidly "aging" the fibroblasts (*43*). However, after further experiments these conclusions were shown to be too simplistic, because other forms of asbestos—less toxic to fibroblasts (*50*)—and the chrysotile asbestos itself (*42*) were found to produce a dual effect on the cultures. Although the same dose of

*Original articles not seen.

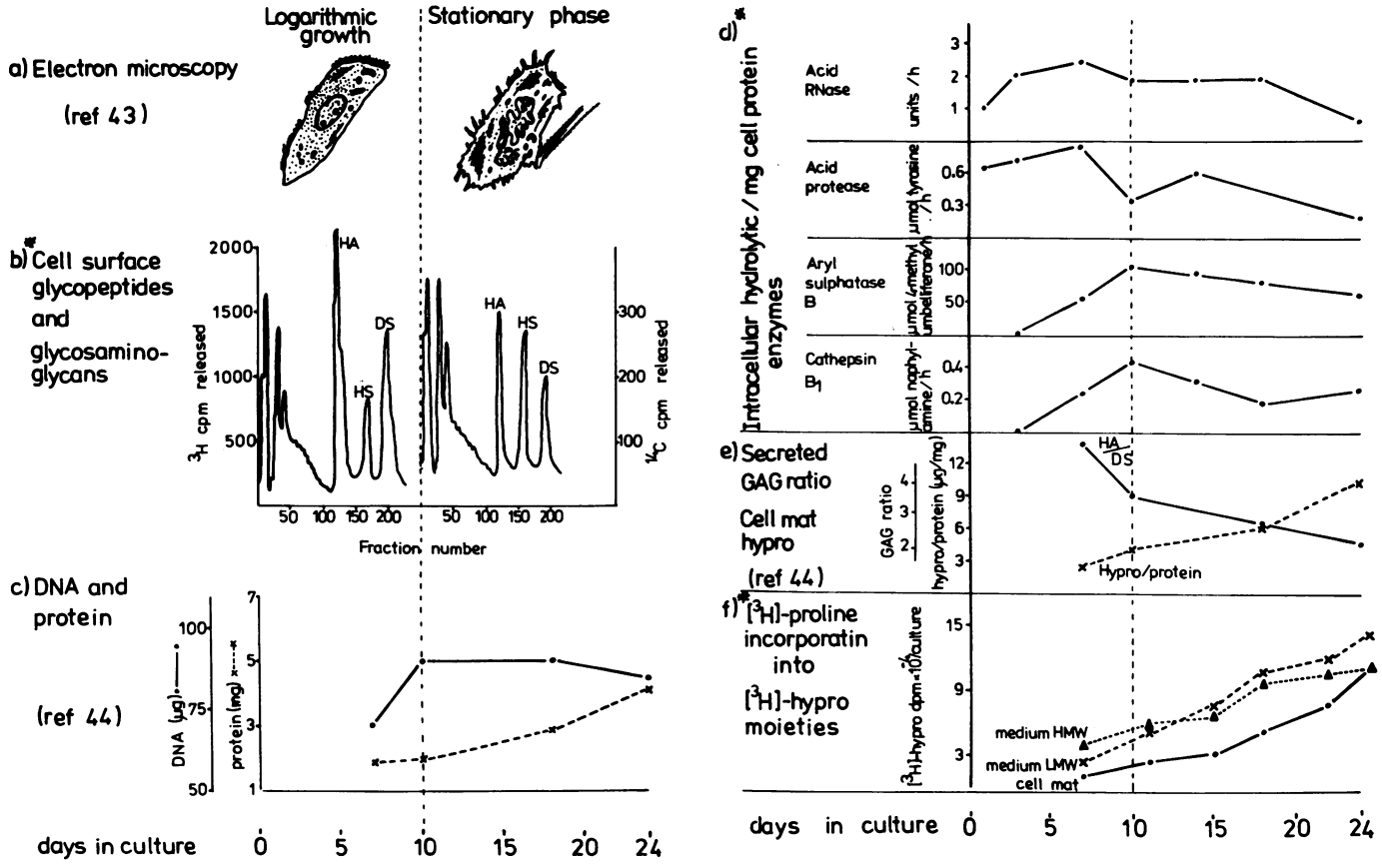

FIGURE 14. Differentiation of rabbit lung fibroblasts over 24 days in culture: (*a*) schematic of EM results; (*b*) DEAE-cellulose chromatography profile of trypsin released glycopeptides and glycosaminoglycans from normal rabbit lung fibroblasts incubated with [^{3}H]-glucosamine (log phase) or [^{14}C]-glucosamine (stationary phase) (unpublished data of Hext and Richards). The 5 ml fractions were collected from the column (15 × 1 cm) at a flow rate of 0.51 mL/min. The first 15 fractions were eluted with 5.0 mM Tris buffer pH 7.2 followed by linear gradient 0–0.3 M NaCl in the same buffer up to fraction 130, after which a second linear gradient 0.3–0.7M NaCl in Tris buffer was used; here HA = hyaluronic acid, DS = dermatan sulfate and HS = heparan sulfate. (*c*) Results refer to fibroblast cells in 6th passage maintained in 20% fetal bovine serum and Waymouth's medium with additional ascorbic acid. The cells were continuously labeled with [^{3}H]-proline. HMW = hydroxyproline containing material in the pellet precipitated from the culture medium with four volumes of ethanol and LMW is the hydroxyproline-containing moieties in the supernatant fraction from this treatment.

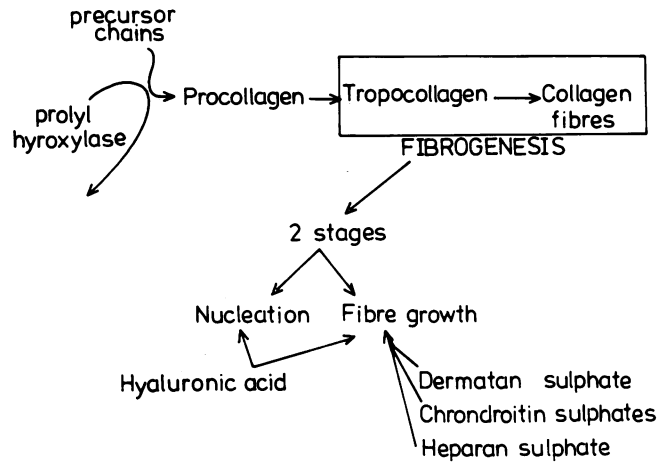

FIGURE 15. Basic, simplified model of fibrogenesis incorporating the role of glycosaminoglycans.

mineral was used sometimes the cell mat hydroxyproline levels were higher than that found in normal cultures whereas in different cell strains the levels were lowered (*42,50*).

Apart from differences between primary strains of fibroblasts, no logical interpretation for the data could be offered, although it appeared that the higher the dose, the more toxic the mineral and thus the greater likelihood of a depression in the amount of hydroxyproline-containing moieties. In retrospect, the doses of asbestos employed in all these experiments were probably far too high because there is a fine balance between mineral-treated cultures which deposit more collagen than control cultures and cultures in which cells were killed by asbestos which resulted in less collagen deposition. In spite of the toxic effects, persistent exposure to chrysotile asbestos can produce a substrain of cells which seem capable of depositing more reticulin (Fig. 16). A major difference in hydroxyproline levels

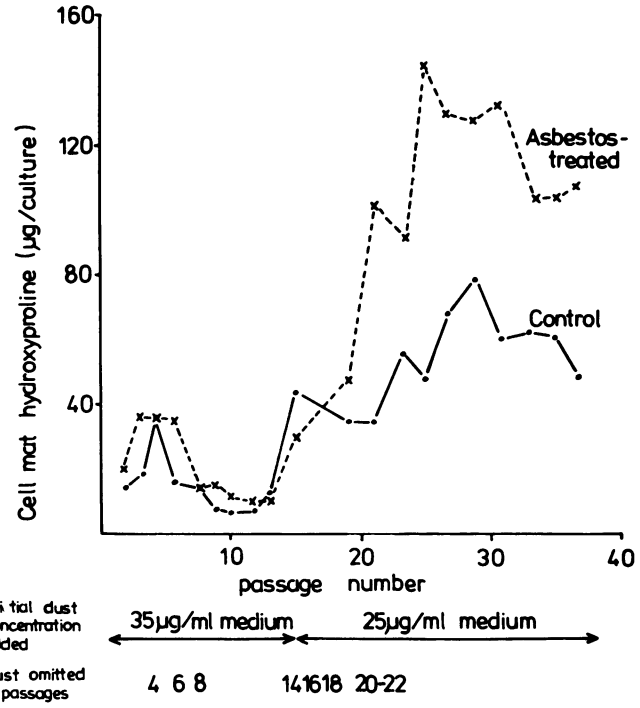

FIGURE 16. Effect of long-term exposure of chrysotile asbestos on a rabbit lung fibroblast cell strain (*51*).

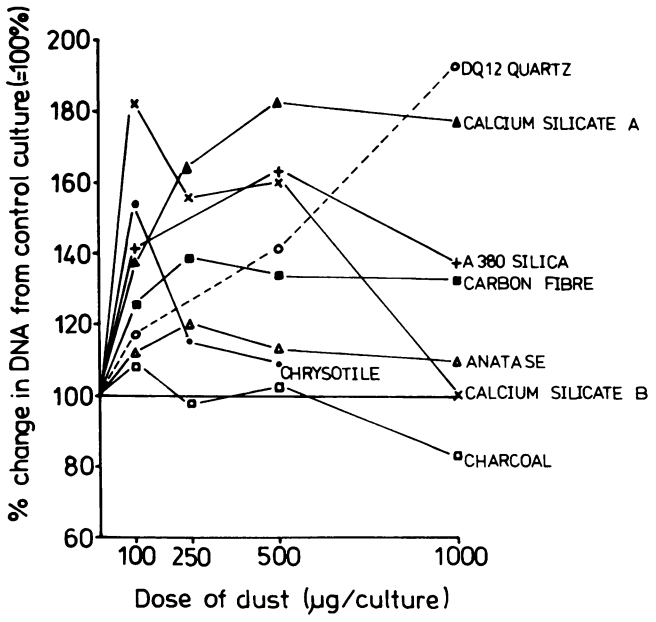

FIGURE 17. Changes in DNA levels in lung fibroblast cultures (at 24 days) 3 weeks after the addition of varying doses of different natural and man-made mineral particles (*52*).

between the control and asbestos-treated cultures is noted when the mineral exposure is reduced after passage number 13 and omitted on passages 16, 18, 20, 21 and 22 (Fig. 16) (*51*). This suggests that subtoxic amounts of chrysotile enhance reticulin deposition in fibroblast cultures, a result confirmed in other studies (*52*).

A number of other dusts apart from asbestos have also been shown to react differentially with fibroblast cultures. The effects of different concentrations of minerals added to 3-day-old rabbit lung fibroblast cultures, which are then analyzed for DNA and cell mat hydroxyproline on day 24, are shown (Figs. 17 and 18). Each dust may have a specific effect dependent on the dose. Most mineral-treated cultures have higher levels of DNA than those found in normal cultures, especially when treated with low doses of dust (Fig. 17). With one mineral (DQ12 quartz) there is a direct relationship between increase in DNA levels and dose. Min-u-sil quartz has also been shown to raise DNA levels in fibroblast cultures (*49*), and other investigators have shown that quartz will enhance the proliferation of human lung fibroblasts *in vitro* (*53*). Therefore if it is assumed that more cells are present in quartz-treated cultures, then they should ultimately deposit more collagen; indeed, this seems to be the case (Fig. 18). However, for the experiments carried out over 24 days *in vitro* it cannot be assumed that an increase in DNA levels equates directly with an increase in cell numbers. Individual cells treated with quartz may have higher

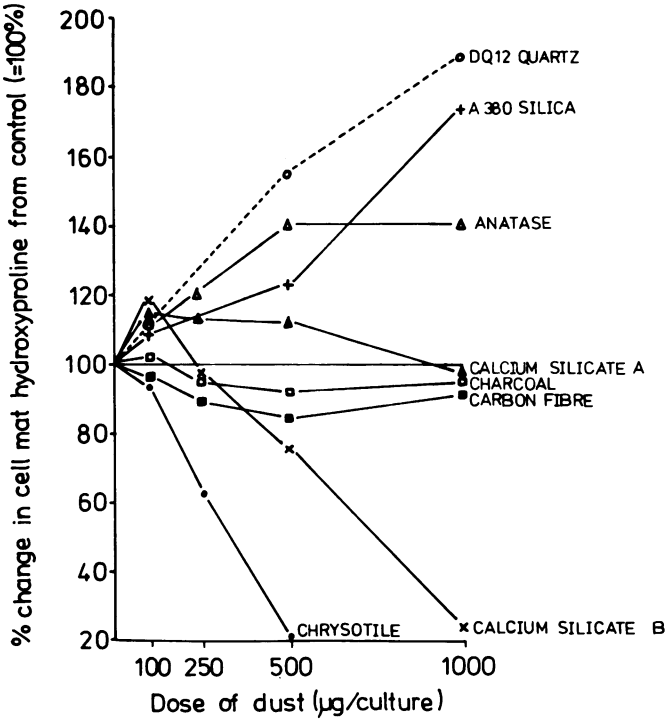

FIGURE 18. Changes in cell mat hydroxyproline levels in lung fibroblast cultures (at 24 days) 3 weeks after the addition of varying doses of different natural and man-made mineral particles (*52*).

Table 9. Mean DNA, protein and cell mat hydroxyproline levels in fibroblast cultures (day 24) following treatment with different doses of quartz (DQ12) for 21 days.[a]

Dose of quartz, μg	Total DNA, μg	Total protein, mg	Total hydroxyproline, μg	DNA / protein	Hypro / DNA	Hypro / protein
0	20.2 (± 2.2)	4.01 (± 0.20)	15.0 (± 0.6)	5.03	0.74	3.74
100	24.0 (± 1.5)	4.46 (± 0.26)	17.4 (± 0)	5.38	0.72	3.90
500	28.5 (± 0)	3.86 (± 0.11)	23.1 (± 2.1)	7.38	0.81	5.98
1000	39.0 (± 4.5)	3.64 (± 0.09)	28.5 (± 0.9)	10.71	0.73	7.82

[a] Range values given in parentheses are from duplicate cultures.

levels of DNA due to incorrect division (e.g., amitosis?) being binucleate (from cell/cell fusion) or because they "turn over" DNA at a slower rate than normal cells. Evidence for this suggestion can be deduced from the fact that the ratio of DNA to cell protein (which will include collagen) increases with increasing doses of quartz (Table 9). Indeed, cultures treated with the two highest doses of quartz have less "cell" protein than normal cultures, although the proportion of hydroxyproline-containing protein(s) increases with increasing amounts of added mineral (Table 9). Thus quartz may not only influence fibroblast growth or DNA levels in the culture but may have some direct action on collagen synthesis/deposition.

It is interesting that the amorphous silica (A380) behaves somewhat similarly (although not identically) to quartz (Figs. 17 and 18). However, other dusts produce different effects on fibroblast cultures. Calcium silicate A and carbon fiber increase DNA levels but cause little change in the amount of reticulin deposited. In contrast, anatase causes little change in DNA levels but promotes reticulin deposition. Chrysotile increases DNA levels but suppresses collagen deposition, and, in doses greater than 500 μg/culture, the mineral is highly cytotoxic and most cells do not recover. A similar result is found with calcium silicate B, although this mineral is far less toxic than chrysotile. A summary of the effects produced by different minerals is shown in Table 10, in which comparisons are made using a simple coding procedure.

Table 10. Summary of effects of minerals added to 3-day-old fibroblast (rabbit lung) cultures analyzed on day 24 (3 weeks exposure to a single dose).

Mineral	Amount required to effect a ± 40% change in the cultures, mg[a]: Toxicity	Total DNA	Total cell mat hydroxyproline	Coding [b]
Chrysotile	> 0.5	< 0.1	0.25 (−)	TDC
Calcium silicate B	> 1.0	< 0.1	< 1.0 (−)	TDC
Quartz	X	0.5	0.5 (+)	XDC
A380 silica	X	0.1	< 1.0 (+)	XDC
Calcium silicate A	X	0.1	X	XDX
Carbon fiber	X	0.25	X	XDX
Charcoal	X	X	X	XXX
Anatase	X	X	0.5 (+)	XXC

[a] The ± 40% change is chosen arbitrarily, and the results apply for cultures of ~ 0.5×10^6 cells in 10 mL of culture medium to which a maximum dust concentration of 1.0 mg has been added.

[b] T = toxic; D = effect on DNA; C = effect on collagen; X = no effect.

The Relationship between Studies *in Vitro* with Lung Fibroblasts and the Development of Fibrogenesis and Fibrosis *in Vivo*

It may well be unrealistic to attempt to equate *in vitro* and *in vivo* findings but nevertheless some logical extrapolations can be made with respect to the initiation of fibrogenesis. If a large amount of dust penetrates the epithelium in one area, if that dust is highly toxic to interstitial cells and if the process is maintained over a period of time, then presumably interstitial cells, including fibroblasts, will be continually killed and replaced. While no direct proof has been offered from *in vivo* studies that fibroblast cells are killed by dust, it has been shown that necrotic changes (lucent cytoplasm, dilated endoplasmic reticulum and vesiculation) occur in interstitial cells containing asbestos (*40*). If the "killing" effect overcomes the "replacement," then would not the interstitial tissue fall apart, leaving holes, a feature, characteristic of emphysema? Alternatively, if "replacement" defeats "killing," if the dose of dust penetrating the alveolar barrier is not too great or if the dust entering the interstitium is not highly toxic, then either cell growth may be promoted or cellular DNA turnover reduced. The net result might be the accumulation of mature fibroblastlike cells (equivalent to those in stationary phase of culture) which form a hypercellular area capable of producing more collagenlike (probably reticulin) material. This suggestion emphasizes the point that fibrogenesis may occur, before the condition of emphysema becomes manifest. This theory could well account for the variable response of experimental animals exposed to cadmium. Snider et al. (*54*) showed that rats exposed to cadmium chloride aerosol developed lung lesions which resembled human centrilobular emphysema, whereas other workers (*55*) produced diffuse fibrosis in hamsters by intratracheal injection of cadmium chloride. In this second study (*55*), when cadmium was given together with β-aminoproprionitrile (one property of which is the inhibition of lysyl oxidase),

bulbous emphysema was produced in the lungs. There are a number of interpretations to explain such data, but the first study (*54*) may simply represent an example of the "killing" effect predominating (the reversibility of the effect was not investigated). Similarly, in the second study (*55*), the bulbous emphysema may result because the killing effect predominates due to the combined exposure to two toxic agents, cadmium and β-aminoproprionitrile. In the second study, exposure to cadmium alone could result in some "killing," followed by replacement of connective tissue and consequently a diffuse fibrogenesis (reversibility, again not studied).

One of the marked effects of minerals on fibroblasts *in vitro* is the alteration in DNA metabolism. Is there any evidence that DNA metabolism is altered in lung tissue following treatment with minerals? DNA synthesis, as measured by [^{3}H]-thymidine incorporation into DNA, is approximately constant over 36 weeks following rat lung exposure to quartz (5 mg, single instillation) and is three times greater than that found in control animals (Fig. 19). Exposure to anatase (nonfibrogenic) also causes an increase in DNA synthesis at 6 and 9 weeks, but this effect is transient and not maintained over the 36-week period. Anatase has little effect on total lavaged lung DNA or DNA relative to lavaged lung dry weight (Fig. 19). By contrast, the fibrogenic quartz induces an increase in total DNA levels over 21 weeks, after which time the level drops. This suggests that the turnover of DNA is accelerated in the quartz-treated lungs after 21 weeks because the synthesis of DNA remains consistently high (three times control). It has not been determined which of the lung cell types (Type I, Type II, endothelial, fibroblastic) are involved in DNA synthesis/turnover following quartz exposure. Nevertheless, it is tempting to suggest that extensive proliferation of Type II epithelial cells is likely following quartz exposure to account for the high levels of pulmonary surfactant produced (Fig. 2). Histological examination of the tissue following quartz exposure supports this view but also indicates an increase in lung interstitial cells, perivascular cellularity and a thickening of the terminal bronchioles.

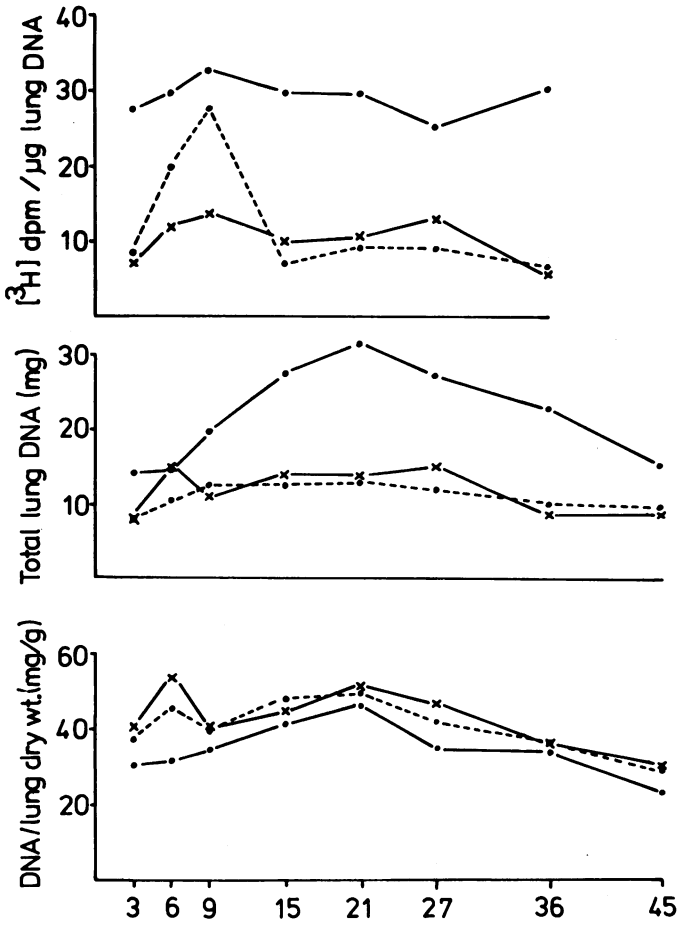

FIGURE 19. DNA levels and synthesis in the lungs of rats exposed to 5 mg quantities of (●——●) DQ12 quartz and (●--●) anatase compared with control animals (×——×). DNA synthesis was measured following a penal vein injection of [^{3}H]-thymidine and after 1 hr the lungs were removed and lavaged. Free label was removed from the lung sample and the [^{3}H] incorporated into DNA assessed. The data are from Richards and Hunt (unpublished) and given as the means from individual determinations on four animals (range values not shown). The synthesis experiments were concluded after 36 weeks.

In addition there is a large increase in free cell numbers at the alveolar surface (control rats, 10–20 $\times 10^6$ cells; quartz-treated 5 mg, 50–100 $\times 10^6$ cells) although these cells only contribute a small proportion to the total lung DNA (< 10%).

In summary, it would seem that the direct stimulation of fibroblast (and other interstitial cell) growth by minerals such as quartz would form the basis for initiating fibrogenesis, a critical factor being the amount and type of dust penetrating the epithelial cell barrier. With some dusts this penetration is unlikely; for example, anatase remains mainly at the alveolar surface following instillation and is gradually cleared. Presumably this could explain why this mineral is not fibrogenic *in vivo*, despite the fact that when it is administered to fibroblast cultures it induces an increase in collagen deposition.

Irrespective of how fibrogenesis is initiated by a mineral, i.e., by stimulating cell growth, altering DNA metabolism or directly affecting protein synthesis, the net result is an accumulation of connective tissue proteins. Reticulin and collagen may not be the only proteins involved; indeed, a fibrotic lesion may contain other components, such as fibrin or fibronectin. At what stage can we consider that fibrogenesis becomes nonreversible? Can we classify an agent which induces alveolar wall thickening and additional reticulin deposition, i.e., fibrogenesis, as an agent that will eventually produce fibrosis? The question, which has been discussed in an excellent review by Pickrell and Mauderley (*56*), is not easily answered. However, studies on rats with an amorphous silica (R972) suggest that the primary deposition of reticulin in granulomatous foci

does not progress further and indeed may be partially reversible. This mineral, which is not thought to cause fibrosis in humans, when given to rats (5 mg, single instillation) can produce a slight increase in lavaged lung hydroxyproline at 15 weeks after exposure (Table 4) although this result was not confirmed in a second longer term study (Table 5). Histological examination indicates that R972-treated lungs at 15 weeks exposure are very similar to normal lungs with no persistent alveolar surface reaction comparable to that detected in quartz-treated lungs, (Figs. 3 and 5). However, dense foci of cells are detected in parts of the lung (Fig. 20) and such foci have extensive reticulin deposits (Fig. 21) with surrounding areas showing a slight increase in deposition of reticulin in the alveolar wall. By 28 weeks the foci may be reduced in number, an effect also noted at 45 weeks after exposure (Fig. 22), although there may be some slight increase in reticulin in the alveolar walls (Fig. 23) compared with that found in control animals (Fig. 12). The particles of R972 silica are minute (even the largest aggregates reach only 1 μm maximum diameter) and may therefore be expected to gain access to the interstitium with relative ease. The mineral can promote growth and collagen deposition in fibroblast cultures and these effects may also occur in the lung. However, fibrogenesis does not progress, and, although the evidence is somewhat tenous, it may be that some reticulin deposition is partially reversible. A

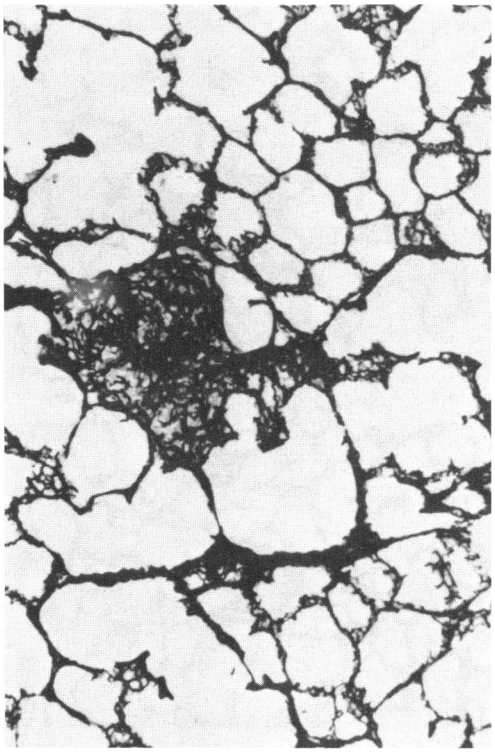

FIGURE 21. Rat lung showing deposition of reticulin in dense focal area after 15 weeks exposure to R972 silica. R stain. ×142.

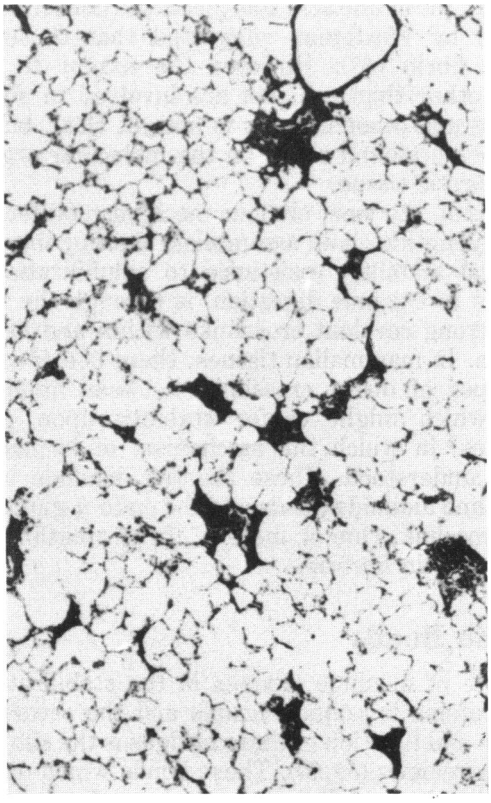

FIGURE 20. Rat lung after 15 weeks exposure to R972 silica showing patchy distribution of dense foci of cells. (AA stain.) ×56.

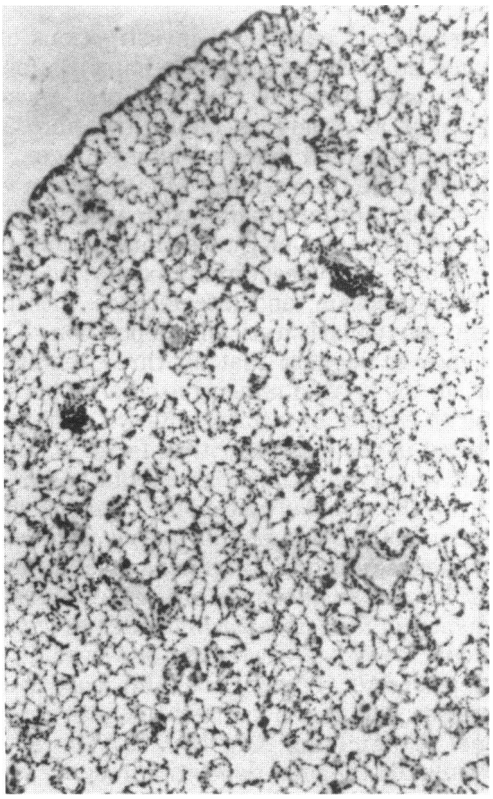

FIGURE 22. Rat lung after 45 weeks exposure to R972 silica. HE stain ×56.

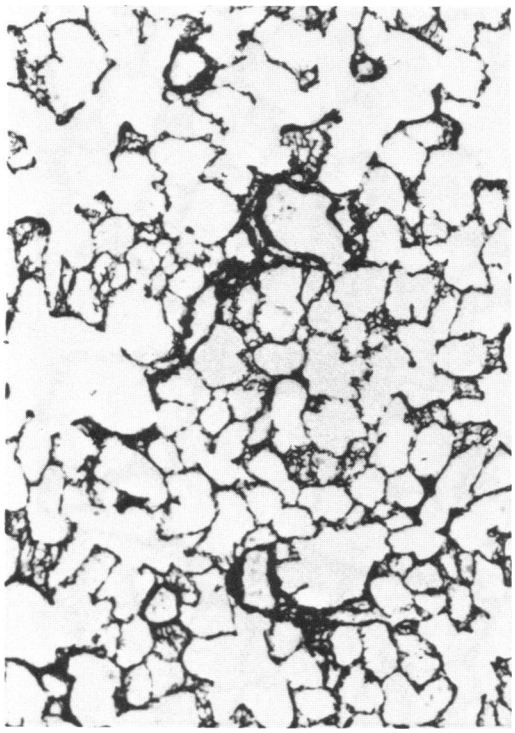

FIGURE 23. Rat lung after 45 weeks exposure to R972 silica R stain. ×142.

similar conclusion is reached in investigations of radiation-induced pulmonary damage in hamsters by other workers (*56*) who state "...the lung may repair and reverse damage as severe as interstitial fibrosis with reduced compliance and elevated lung collagen."

There is evidence from studies *in vitro* that a variety of minerals react differentially with interstitial cells with fibroblastlike morphology. The interactions may be all of, or one of three types: direct toxicity; interference with DNA metabolism; and interference with protein metabolism, particularly reticulin deposition. There is good evidence to suggest that all three reactions are dependent on the dose of mineral used and therefore in attempting to equate *in vitro* results with the reaction of the mineral in the lung, a critical factor in initiating fibrogenesis would seem to be the amount of dust which can penetrate the alveolar epithelium. This penetration may be very limited for nonfibrogenic minerals, but dusts which cause fibrosis in humans such as asbestos and quartz have been shown to enter the interstitium.

The response of lung fibroblast cultures to minerals is a useful model to study fibrogenesis. However, fibroblast cultures deposit only reticulin fibers (at least to day 24) and fibronectin and therefore may not provide a complete model for studying lung fibrosis. The extent to which reticulin or collagen deposition may be reversed remains unclear. Similarly, the exact reason for the progression of the disease process (although clearly related to dose of material) and the point at which fibrogenesis merges into an irreversible fibrotic reaction have not been elucidated. Can it be that there are further stages between fibrogenesis and fibrosis in which degradation of accumulated lung proteins is reduced or halted completely?

The Step from Fibrogenesis to Fibrosis

Fibrosis is considered to be the irreversible deposition of fibrous proteins, particularly collagen, but is this the only protein of any consequence in fibrotic nodules? Interestingly, analysis of fibrous tissue masses from cases of complicated coal workers' pneumoconiosis (PMF) showed that if the hydroxyproline content of the lesions could be taken as a measure of collagen present, there was no significant increase in the collagen when the PMF lesions were compared with the surrounding lung tissue (*57*). Moreover, biochemical, pathological, ultrastructural and immunological studies of the massive lesions in coal workers' lungs suggested that collagen is present in the capsule of these lesions, but in the center, other insoluble protein or proteins are present (*58*). This protein complex accounts for one-third of the weight of the lesions, the remaining two-thirds consisting of approximately equal amounts of mineral dusts and calcium phosphate. Of the material extracted with 8 M urea–0.05% dithiothreitol (13–40% of the lesion), the amino acid analysis of the noncollagenous protein(s) by Wusteman suggested that these most resemble fibrin (*58*). However the extent to which proteins other than collagen are involved in silicosis, asbestosis and other fibroses is not yet clear, but they may have a profound effect on the molecular organization of fibrotic tissue.

In general, the most obvious mechanism(s) for stabilizing polypeptide chain assemblies, thereby increasing mechanical stability, resistance to solubilization and resistence to enzymic digestion, is through the formation of strong covalent crosslinks within and between molecules. In mammalian tissues, there is evidence for three types of direct crosslinks between amino acid chains (which might confer stability upon protein aggregates) in which the mechanism of formation is partially understood. These are the disulfide bridge, the aldol and aldimide bonds and the endo-γ-glutaminyl-ε-lysyl crosslink. One or more of these crosslinks may be important in fibrosis.

Disulfide Bonds

The role of disulfide bridges in the stabilization of native proteins is unquestionable and the occurrence, formation and function of these bridges is the subject of numerous reviews (*59,60*). These bonds which occur in diverse globular and fibrous proteins are formed by the oxidation (either enzymic or nonenzymic) of thiols. The restriction of rotation around S-S bonds gives the

proteins that contain them an added rigidity in their microstructure, but do these bonds stabilize the fibrous deposits in fibrotic lesions? Such bonds are unlikely to be important because collagen, fibrinogen and even fibrin are typical of many extracellular proteins in that they do not contain free thiol groups and are therefore unlikely to undergo extensive polymerization through disulfide formation. By contrast, native fibronectin appears to contain between one and two free SH groups per monomer which are accessible to [^{14}C]-iodoacetamide titration. Disulfide bonding at the cell surface appears to contribute to fibronectin multimer formation (*61*) and possibly its binding to other proteins (*62,63*). For this reason, stabilization of fibrotic deposits by disulfide bonds cannot be ruled out completely, although there is no evidence to suggest that they make a significant contribution.

Aldol and Aldimide Crosslinks

Although these crosslinks are apparently confined to collagen and elastin, they are widely distributed in the sense that collagen in its various modified forms is the major protein component of many tissues. Extraction of bovine pleura and parenchyma with saline and organic solvents leaves an insoluble residue with an elastin content of 28% and 13.5% (w/w), respectively. By comparison, measurements of hydroxyproline content indicate that both pulmonary tissues contain about 70% collagen (*64*). In view of this, it seems likely that the changes in shape which normal lungs undergo is due to an alteration of the mesh-like structure of the collagen fibrils. It has been suggested that the elastic fibers play a secondary role of a restraining nature by preventing over-deformation of the collagen fibrils (*65*). Consequently studies concerned with the loss of function in lungs during ageing, fibrosis and other disease conditions have been preoccupied with changes in collagen structure. The tropocollagen molecules are held together in the fiber by relatively weak electrostatic forces so that the aggregate can be readily dissociated and the tropocollagen becomes soluble. However, the bulk of fibrous collagen in the tissues of adult animals is insoluble. To account for this and the considerable strength of collagen fibers, it has been accepted that during the maturation of collagen, covalent bonds form between the side groups of certain amino acid residues in adjacent polypeptide chains, thus crosslinking the chains. The nature of these crosslinks has been the subject of much research (*66,67*) and the discovery of the lysine-derived crosslinks in elastin (*68*) started the search for similar crosslinks in collagen. Although there were no desmosine and isodesmosine residues in collagen there was evidence for the presence of simpler lysine and hydroxylysine-derived crosslinks. These linkages depend on the deamination of the ε-amino group of lysine and hydroxylysine residues to α-amino adipic-δ-semialdehyde (allysine) and α-amino-δ-hydroxy adipic semialdehyde (hydroxyallysine), respectively. The crosslinks in collagen are produced when two of these aldehyde residues form aldol condensation products and also by the formation of Schiff-bases between one of the aldehyde residues and the free ε-amino group of lysine or hydroxylysine (*69–72*). A number of such bifunctional residues have been isolated (Fig. 24) and because they are reducible aldehyde and aldimine bonds they are referred to as reducible crosslinks. Surprisingly, these reducible crosslinks are present in greatest amounts in young (fetal) collagen that is still soluble, whereas with increasing age these particular linkages in pulmonary collagen progressively decrease (Table 11) so that simple aldol and aldimine linkages alone do not explain the insoluble nature of mature collagen. It is presumed that these are converted to more stable crosslinks of unknown structure. If this is correct then clearly the formation of such linkages in the collagen component might also contribute to the stabilization of fibrotic lesions.

Endo-γ-glutamyl-ε-lysyl Crosslinks

Neither γ-glutamyl-ε-lysyl nor γ-glutamyl-ε-hydroxylysyl crosslinks make any significant contribution to the stabilization of normal mature pulmonary collagen (*65*). However, it has already been pointed out in some fibrotic lesions a considerable proportion of the insoluble aggregates is composed of proteins other than collagen. Some of these (e.g. fibrinogen/fibrin) are substrates for transamidases of the transglutaminase type which catalyse the formation of endo-γ-glutamyl-ε-lysyl bridges. Consequently, there is the possibility that these isopeptide bonds play a part in the formation of relatively stable fibrous aggregates in the diseased lung.

Historically, the stabilization of fibrin clots was the first example of endo-γ-glutamyl-ε-lysyl bridges between polypeptide chains (*72*). The introduction of a few covalent crossbridges into the gel network [approximately 1 per 500 amino acid residues (*73,74*)] contrib-

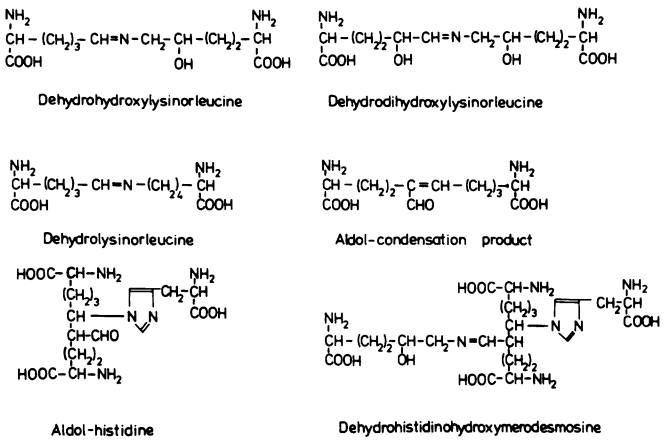

FIGURE 24. Crosslinks derived from lysine and hydroxlysine which have been shown to occur in collagen.

Table 11. Reducible crosslinks in pulmonary collagen fractions.[a]

	Elastase-insoluble				Collagenase-insoluble			
	DHLN		HLN		DHLN		HLN	
Age of animals	PL	PR	PL	PR	PL	PR	PL	PR
1 week	0.19	0.38	0.15	0.29	0.29	0.36	0.21	0.24
3 years	0.07	0.05	0.13	0.10	< 0.05	< 0.05	0.19	0.16
16 years	< 0.05	< 0.05	0.10	0.09	< 0.05	< 0.05	0.14	0.15

[a] Pulmonary collagen fractions ("elastase-insoluble" and "collagenase-insoluble") were prepared from reduced, salt-extracted and defatted pleura (PL) and parenchyma (PR) of bovine lungs. Concentrations of dihydroxylysinonorleucin (DHLN) and hydroxylysinonorleucine (HLN) are expressed as residues/300,000 g collagen. Data from Francis (*65*).

utes greatly to the physical stiffening of the gel network as expressed by an increase in the elastic storage modulus (*75,76*) and to its resistance to lytic enzymes (*77*). Fibrin crosslinking itself is catalysed by extracellular activated plasma Factor XIII (or FXIII[a] or fibrin-stabilizing factor) but there is a whole group of intracellular and extracellular transamidating enzymes with the specialized function of bringing about the post-translational modification of some native proteins enabling them to form covalently linked assemblies. Characteristically, these enzymes contain cysteine-thiol active centers and require calcium ions in order to catalyze the acylation/deacylation reaction depicted in Figure 25. In as much as the protein substrate is bifunctional carrying both the acceptor (i.e. γ-glutamine carboxyl) and the electron donor (i.e. ε-lysine amino) groups required for the reaction, there is the possibility for extended polymerization. The type of crosslinked product will depend on the nature of the protein substrates; in some physiological situations it may result in the direct formation of a specific precipitate (for example in the clotting of lobster fibrinogen or the formation of the copulation plug from vesicle secretion protein) or in the covalent fusion of individual molecules within an already existing ordered protein aggregate, as for example in the conversion of vertebrate fibrin gel to crosslinked or ligated gel. In the guinea pig copulation plug the average crosslink density may reach values as high as 1 per 23 amino acid residues (*78*). This high frequency of crosslinking has also been observed in the citrulline-containing protein obtained from the hair medulla of certain species (*79*).

In general, these crosslinks introduce into protein assemblies the property of resistance, that is, to deformation (rigidity), to mechanical breakage (strength), to solubilization and to chemical attack (molecular stability) (*80*). If these crosslinks are involved in the formation of dust-induced pulmonary fibrosis then the minimum requirements in the pulmonary interstitium would be: A source of transamidase activity, the appropriate protein substrates (both glutamine acceptors and amine donors) and calcium ions.

Pulmonary Transamidases

Although the specific protein substrates of only a few endo-γ-glutaminyl-ε-lysine transamidases are known,

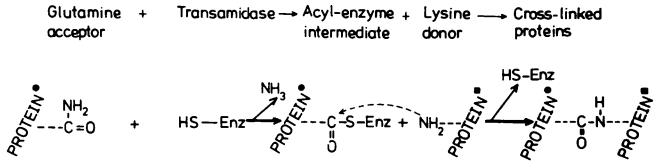

FIGURE 25. Crosslinking of proteins by transamidases (endo-γ-glutamyl transferases). The γ-carboxyamide groups of peptide-bound glutamine form an acylenzyme intermediate with a reactive thiol in the enzyme (Enz-SH), releasing free ammonia. Nucleophilic displacement by the ε-amino group of protein-bound lysine residues releases the enzyme and forms a strong covalent bond.

the ability to incorporate radiolabeled or fluorescent primary amine into proteins in a nonspecific manner (*81*) is a characteristic of enzymes derived from many tissues (*82*). In the guinea pig, the highest activities were found in liver, spleen, kidney, adrenal gland, heart and lung (*83,84*) and prostate (*85*). By contrast, and of particular interest to this discussion, in humans the lung and uterus are the richest sources of the enzyme (*84*). Classical subcellular fractionation studies of the rabbit lung shows that most of the enzyme activity is associated with the nuclear and high-speed supernatant fractions. The activity in the nuclear fraction is not tightly bound and can be washed out with dilute salt. The distribution of enzyme activity in the different lung cells has not been fully investigated, except that alveolar macrophages appear to have very low levels whereas fibroblasts in culture contain appreciable amounts of the enzyme activity at different stages of development (Curtis, unpublished work).

The potential transamidase activities in the blood may also be made available in the lung interstitium as a result of increased permeability caused by dust deposition. Human blood contains at least three distinct transamidases. The plasma enzyme (Factor XIII) is a thrombin-sensitive zymogen composed of two heterologous subunits (a_2b_2), the platelets yield a thrombin-sensitive zymogen with a postulated a_2 structure, while the red cell enzyme is a single subunit which does not require thrombin for activation.

Substrates for Transamidases in the Lung

Native proteins (serving as substrates for transamidase catalyzed incorporation of amines) that may be

relevant to fibrosis include fibrinogen, fibrin, fibronectin, myosin, tropomyosin and collagen. A common feature of these proteins is that they tend to form polymeric (fibrous) complexes. Although a number of these proteins are known to be present in the extracellular spaces of lung, the question arises as to whether these proteins would be crosslinked like-with-like and/or one protein to another. The crosslinking of fibrinogen and fibrin is well documented but other protein–protein associations are also possible. Nyman and Duchert (*86*) incubated different combinations of plasma and collagen with ^{125}I-fibrinogen and thrombin. When Factor XIII and Ca^{2+} were added, the amount of ^{125}I bound to collagen was 2.1 to 4.3 times that observed when either Factor XIII or Ca^{2+} was omitted. Moreover, when plasma, with a normal level of Factor XIII was clotted in the presence of collagen and Ca^{2+}, γ-dimer was absent, but when the lysine residues of collagen were blocked by acetylation, the γ-dimer was observed. Collectively, these Factor XIIIa-catalyzed interactions between collagen and fibrin suggest that collagen crosslinks to the γ-chain(s) of fibrin, although the nature of the crosslinks has not been positively identified. Nevertheless, such intermolecular associations might explain the electron micrographs of large stellate nodules in PMF, in the centers of which, the pattern of fibers appears to be interrupted by an amorphous material that spreads between the collagen fibers and separates them (*58*). The intermolecular associations in fibrosis may be even more complicated, because, apart from the well-known crosslinking of fibrinogen and fibrin, Mosher (*87*) has obtained convincing evidence for the crosslinking of fibronectin. Fibronectin, a β-globulin of 450,000 molecular weight is present in human plasma at a level of 0.33 mg/mL and is a major component of the surface of normal fibroblasts. One of its characteristics is its tendency to bind to fibrinogen from which it is difficult to separate. Indeed, at one time fibronectin was thought to be a modified form of fibrinogen (*88*). In the presence of Factor XIII, Ca^{2+} and a sulfhydryl-containing reagent, fibronectin molecules become crosslinked to each other, whereas in the presence of fibrin they preferentially crosslink to the α-chains of fibrin. Unlike Factor XIIIa, tissue transglutaminases bring about the nonspecific crosslinking of the α-chains of fibrin which makes it more likely that lung transglutaminase would crosslink fibronectin with the Aα-chains of fibrin. Other plasma proteins which may act as substrates for transglutaminases include α_2-macroglobulin and another unidentified 110,000 molecular weight protein (*87*). These proteins were recognized by their capacity to incorporate fluorescent amines, although there is no evidence for the crosslinking of α_2-macroglobulin to fibrin, to itself or to other proteins.

In silicosis and asbestosis, much emphasis has been directed towards the deposition of collagen. Although collagen itself is a substrate for transamidase, the interaction of reticulin or collagen with fibrinogen, fibrin or fibronectin cannot be ruled out. In 1975, Soria et al., (*89*) using phase-contrast microscopy, observed that the structure of collagen differed depending on whether polymerization occurred in the presence or absence of Factor XIIIa. In the absence of the enzyme, collagen assembled in fibers whereas in its presence the arrangement was more random. This supports the finding that Factor XIIIa lowered the amount of collagen polymerized but produced higher molecular weight subunits in the collagen which did polymerize. One possible interpretation of this data is that the γ-glutamyl-ε-lysine crosslinking occurred in such a way as to block sites on collagen normally use for noncovalent bonding (*90*). These data highlight the disadvantages of investigating disease processes using a single cell type in culture. The apparent inability to produce a fibrotic lesion in lung fibroblast cultures after dust treatment may be due to a lack of sufficient extracellular transamidase activity, a lack of noncollagenous crosslinking substrate, or that reticulin (Type III collagen?) is a poor substrate for crosslinking.

Conclusions

The central theme of this hypothesis is that the penetration of certain dusts into the pulmonary interstitium acts as a focal point for the nonspecific crosslinking of fibrous proteins in the extracellular spaces of lung tissue. Although the fibrotic tissue may involve several types of crosslinks, several factors favor the involvement of γ-glutamyl-ε-lysine bridges catalyzed by calcium-dependent transglutaminases. The increased permeability of the pulmonary vasculature of lungs exposed to dust and the influx of plasma into the interstitium provide suitable protein substrates and potential transglutaminase activity at the same site. The substrates in plasma include fibrinogen, fibronectin as well as other uncharacterized proteins. Moreover, the increase in pulmonary fibroblasts may well result in a net accumulation of fibronectin and collagen which are also substrates for transglutaminases. The major sources of transglutaminase activity would be Factor XIII in the plasma and the release of the more nonspecific tissue transglutaminase from damaged lung cells and hemolyzed red blood cells. Whereas under normal blood-clotting conditions plasma Factor XIII undergoes limited proteolytic cleavage by thrombin prior to calcium activation, even this enzymic step might not be necessary in the areas of interstitial microcalcification (*40*) or when high calcium concentrations (*58*) are attained in the late stages of fibrosis. Bearing in mind the number of potential fibrous protein substrates and the number of alternative sources of transamidase activity, the physical appearance of the fibrosis and its rate of deposition might depend on the relative concentrations and activities of these heterogeneous components. If this is the case, then the current view of fibrosis as the overproduction, deposition and retention of collagen may be oversimplified.

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