Role of Polymorphonuclear Leukocytes in Silica-Induced Pulmonary Fibrosis

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Silicosis is usually attributed to fibroblast stimulation by secretion of damaged alveolar macrophages (AMs), but the role of polymorponuclear leukocytes (PMNs) and of continuing cell injury in the pathogenesis has not been fully studied. Mice given intratracheal injections of 2 mg of silica received ^{3}H-thymidine 1 hour before death at intervals to 20 weeks. Cellular populations and lysosomal content of lavage fluids were correlated with morphology, DNA synthesis, and collagen content of the lung. The initial response involved rapid PMN and AM recruitment to the alveoli. Some free particles crossed Type 1 epithelial cells, and silica was found in interstitial macrophages. Focal Type 1 cell damage was rapidly repaired by Type 2 cell proliferation. Although PMN numbers dropped after a few days, they never reached control levels and rose again after 8 weeks; the number of AMs fell to control values from 2 to 8 weeks, then increased again. Glucosaminidase and glucuronidase levels in the lavage fluid were much higher than control levels throughout the study. Increased DNA synthesis by interstitial cells occurred from 2 days to 20 weeks; increased collagen synthesis was found from 4 weeks onward. The continuing inflammatory response of the lung to silica suggests that both AMs and PMNs and their secretory products may contribute to fibroblastic stimulation. (Am J Pathol 1984, 117:37–43)

THE DEPOSITION of silica in human and animal lungs results in a fibrotic reaction. Investigations into the pathogenesis of silicosis have concentrated largely on the early response of alveolar macrophages (AMs)[1-3] and the later fibroblastic stimulation.[4-6] The most accepted mechanism of injury involves the disruption of lysosomal membranes of alveolar macrophages by silica and the subsequent stimulation of fibroblasts by a secreted macrophagic product.[6-8]

The role of the polymorphonuclear leukocyte (PMN) in the pulmonary reaction to silica has not been studied. It is known that the initial response of the lung to silica involves an inflammatory process with PMN recruitment to the alveoli.[2,3,6] However, the relationship between PMNs and the longer term fibrotic process is not clear. Some reports mention the presence of these cells in the lung at specific stages of the reaction,[3,5] but in other studies an exposure-related PMN response was not found.[9] In addition, although there is some evidence that free silica particles cross the Type 1 epithelium to reach the interstitium,[10] it is not known whether this cell is injured in the process.

In the present study we have correlated the cellular population and lysosomal enzyme content of bronchoalveolar lavage fluids with morphologic, autoradiographic, and biochemical evidence of cellular injury and repair in the lung over a 20-week period following intratracheal instillation of silica in mice.

Materials and Methods

Two milligrams of silica (crystalline quartz particles, 0.3-μ diameter, Dowson and Dobson, South Africa) in 0.1 ml sterile water was instilled intratracheally to 70 male Swiss Webster mice under mild anesthesia. The animals, in groups of 4, were killed at Days 1, 2, 3, 5, 7, and 10 and at Weeks 2, 4, 6, 8, 10, 12, 16, and 20; each animal received 2 μCi/g tritiated thymidine 1 hour before death. Control animals were given 0.1-ml injections of sterile water and were sacrificed less frequently after the first week, because a previous study showed no changes in the lung at 1 week when the animals were compared with control animals that did not receive injections.[11]

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At sacrifice, a tracheotomy was performed, and the lungs were washed four times with 1.0 ml saline. The lavage fluid was pooled for each animal, and the total number of cells was counted on a hemocytometer. The cell suspension was centrifuged, and a smear was made and stained. Differential counts of PMNs and AMs were made on 500 cells per slide. The total number of cells of each type was calculated for each time studied, and the mean ± SE of the total cells per lung in the 4 mice per group was plotted against time. The remaining cells were centrifuged to a pellet, which was fixed in osmic acid and prepared for embedding in Spurr plastic. Both 0.5-μ and ultrathin sections were examined.

The supernatant of the lavage fluid from each mouse was used for determining the activity of the lysosomal enzymes glucuronidase[12] and glucosaminidase.[13] After lavage, the bronchus leading to the right lung was clamped; this lung was removed, weighed, and frozen for biochemical analysis. The left lung was inflated with 0.5 ml 2% buffered glutaraldehyde and removed, and most of the tissue was processed for embedding in glycol methacrylate. Sections (0.75 μ thick) from three random blocks per animal were prepared for autoradiography with the use of Kodak NTB2 emulsion.[14] We determined the percentages of ^{3}H-thymidine-labeled cells at each time point by counting 3000 lung cells per animal. The means and standard error (SE) were calculated for each group. These sections were thin enough to allow identification of pulmonary cell types, and differential counts of labeled cells were carried out on 300 labeled cells per animal. The product of the differential percentage and the total labeling percentage gave the labeling index for each cell type. The index for epithelial, interstitial, and endothelial cells was calculated at each time studied. The remaining tissue sample was postfixed in osmic acid and dehydrated through alcohols before embedding in Spurr plastic. Thin sections were examined by electron microscopy.

The right lung of each mouse was homogenized in water, and biochemical assays were carried out on duplicate samples. Determinations of DNA and total protein were carried out by conventional methods. As an index of collagen content, hydroxyproline levels were determined after hydrolysis with hydrochloric acid.[15]

Results

Bronchoalveolar Lavage

Injection of silica promoted a rapid efflux of PMNs to the lungs (Figure 1). The number rose from near zero in untreated animals to about 8 × 10^5 at 1 day. Although the acute phase was over by 5 days, PMNs never disappeared, and around 1 × 10^5 were found to 8 weeks,

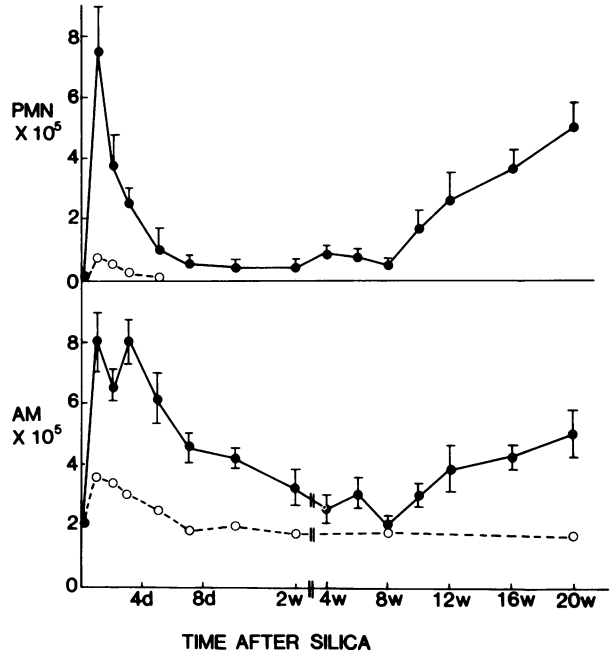

Figure 1 – Numbers of PMNs and AMs (± SE) recovered by lavage at intervals after silica (*closed circles*) and water (*open circles*).

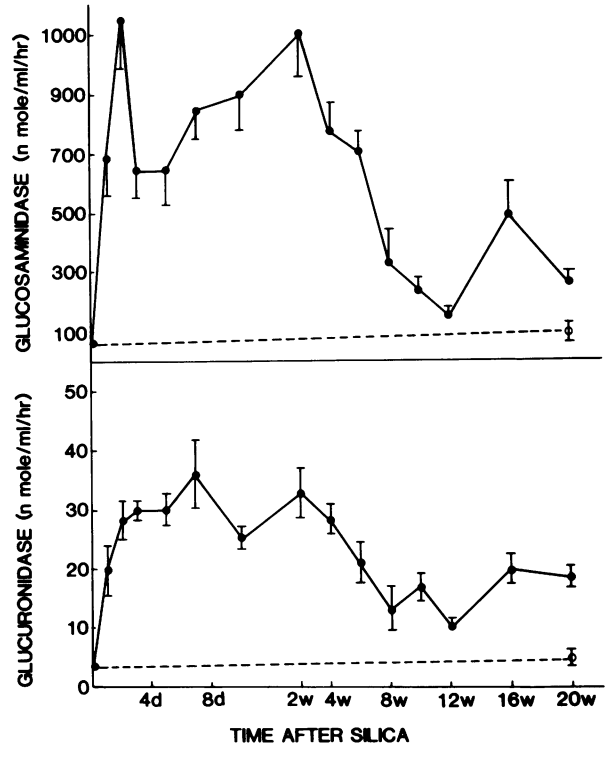

Figure 2 – Lysosomal enzyme activity (± SE) in lavage fluid recovered at intervals after silica. The control range is shown by the *broken lines*.

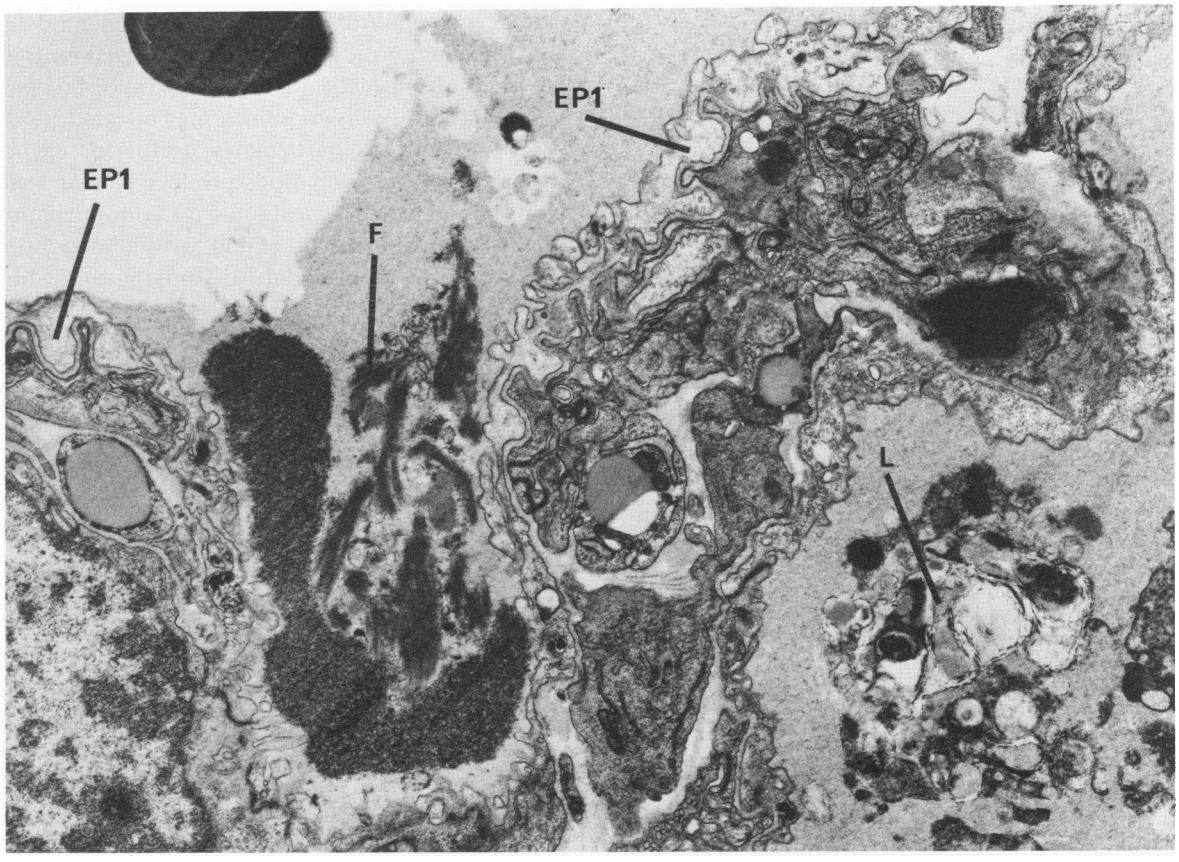

Figure 3—Electron micrograph of alveolar area 1 day after silica. Swelling and necrosis of type 1 epithelial cells (*EP1*) is accompanied by a proteinaceous exudate that includes fibrin (*F*) and lipid material (*L*). (×14,000)

when the number steadily increased again. A brief small increase in PMNs was seen after injecting water alone. A similar pattern occurred in AM numbers, which showed the rapid increase to about 4 times normal after silica, then slowly declined to the normal range at from 2 to 8 weeks before increasing again (Figure 1).

Examination of the pellets of lavaged cells confirm the mixed nature of the free lung cells. Silica particles were phagocytized by both AMs and PMNs. Cell injury, in particular lysosomal swelling, was observed in both cell types[16]; many dead PMNs and cellular debris were seen, particularly in the first 2 weeks. Levels of the lysosomal enzymes glucuronidase and glucosaminidase in the alveoli increased dramatically after silica injection and, though the levels fluctuated, each value was significantly above the control range throughout the study period (Figure 2).

Lung Morphology

The lesions observed were predominantly centrilobular in distribution. In the early stages, many PMNs and AMs were seen in the alveoli and in the pulmonary interstitium. Some particles were found free, some were phagocytized by the PMNs and AMs, and some at 1 day were seen in the cytoplasm of Type 1 epithelial cells. Later silica was observed in interstitial macrophages. At 1 day evidence of injury and necrosis of Type 1 alveolar epithelial cells was found accompanied by focal collections of intraalveolar fibrin and areas resembling alveolar proteinosis (Figure 3). At Days 2 and 3 an occasional mitotic figure was seen in Type 2 cells. Subsequently, the epithelium appeared normal, and no fibrin was observed. After a week the interstitium became thicker, there was an apparent increase in fibroblasts, and at later times it appeared that collagen content also increased. Over the 5-month period, focal nodules of fibrosis developed, particularly in peribronchiolar regions.

Autoradiography

The percentage of labeled lung cells rose almost immediately and peaked at 2 days (Figure 4). Though the number dropped subsequently, it never fell to control values and in fact increased again between 8 and 16

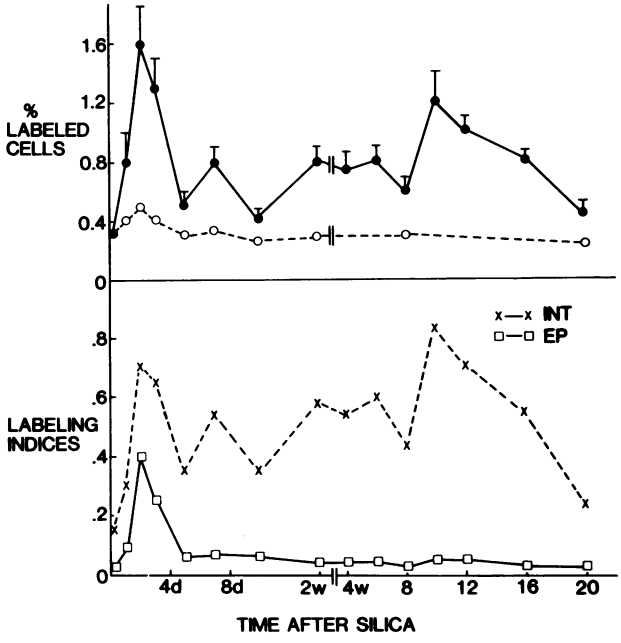

Figure 4—*Upper*, Percentage of thymidine-labeled lung cells after silica (*closed circles*) and water (*open circles*). *Lower*, Labeling index of interstitial cells (*crosses*) and Type 2 epithelial cells (*squares*) after silica. Control values are the time 0 numbers.

weeks. This percentage is a composite value for all lung cells and, after we made differential counts, it was found that the increase in labeling had two components (Figure 4). DNA synthesis in Type 2 epithelial cells was significantly above normal for a brief period at Days 2 and 3; these cells were recognized by their epithelial location in alveolar corners and by their round cytoplasmic inclusions typical of lamellar bodies (Figure 5). Thereafter, the predominant labeled cell population was interstitial, with thymidine incorporation consistently above normal throughout the study period (Figure 6). The animals that received water injections remained at the control time 0 values.

Biochemistry

Total DNA and protein rose over the course of the study, but the main biochemical changes related to collagen synthesis. Figure 7 shows that the total hydroxyproline (HYP) and the ratio of HYP to DNA were significantly greater than those of controls from 4 weeks onward. The ratio of HYP to protein was significantly increased as early as 10 days after silica injection.

Discussion

Deposition of silicon dioxide in the human lung results in the formation of nodular areas of fibrosis in which the major cellular components appear to be macrophages and fibroblasts.[6] Experimental models for study of the fibrotic reaction have involved both inhalation and intratracheal administration of fine particulate quartz, usually to guinea pigs or rats.[4,5,9,17] The use of aerosols is particularly useful for clearance studies,[10] and both methods produce similar lesions. However, the intratracheal route is a convenient means of delivering a precise load and gives a known time 0 for cytokinetic studies and for studying the sequence of pathologic changes.

Administration of silica to mouse lung produces a rapid inflammatory response. The numbers of PMNs and AMs recovered by lavage in the first week are similar to those observed after injecting inert particles such as carbon or latex.[18] It has been postulated that the interaction of particles with macrophages in the alveoli releases chemotaxins for both PMNs and AMs. This has been shown after exposure of AMs to silica *in vitro* and in studies of lavaged cells up to 2 weeks after silica injection.[1,2,19] The mechanism is probably nonspecific, because we have shown previously that lavage fluid, recovered after carbon or latex instillation to the lung and injected into normal mice, produced a chemotactic response.[11] The case of silica may be somewhat different, because there is morphologic evidence of cell injury, and other properties of macrophages such as

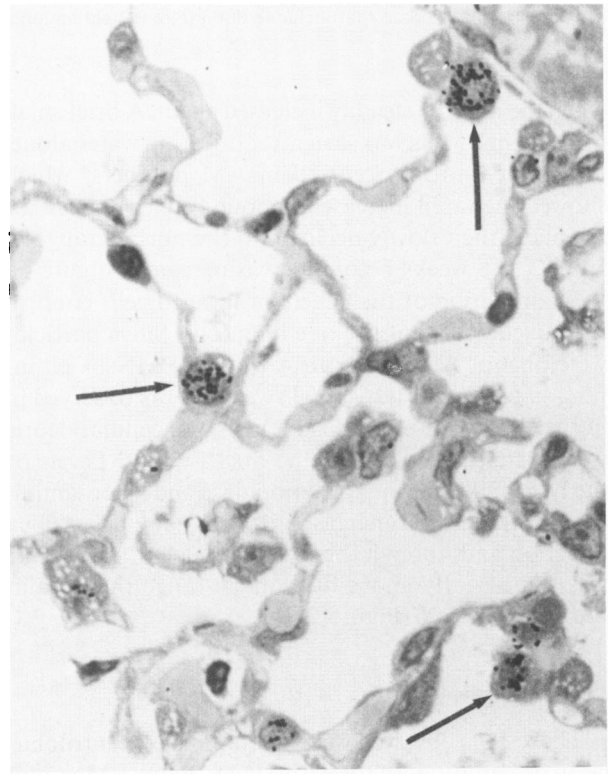

Figure 5—Autoradiograph, 2 days after silica, showing labeled cuboidal epithelial cells (*arrows*). (×900)

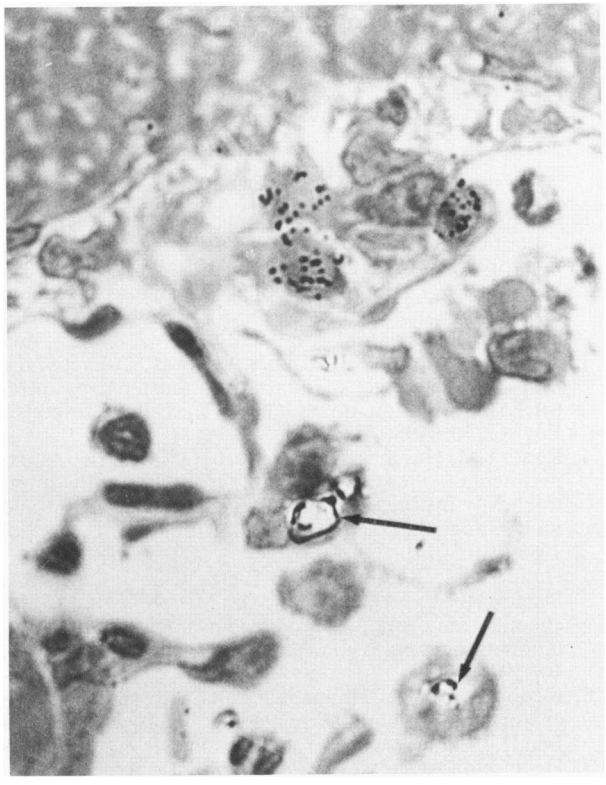

Figure 6—Autoradiograph, 4 weeks after silica, showing three labeled cells in the perivascular interstitium. Free macrophages containing silica can also be seen (*arrows*). (×900)

adherence and migration are altered by exposure to silica.[20]

Although the numbers of PMNs and AMs decline after the acute phase, the lavaged cell population never reaches control levels. By examining frequent time points in the present study, we have shown a continuing inflammatory response in the lung up to 20 weeks. PMN numbers are much greater than in normal lung, where very few such cells are seen in the alveoli. The finding of elevated numbers of AMs and PMNs, many of which are injured or die in the alveoli, correlates with the high levels of lysosomal enzymes in lavage fluids, particularly in the initial stages. Enzyme levels in the alveoli remain high in the 2–8-week period when the number of AMs is not significantly higher than normal but when the lavaged PMN number is 1×10^5, compared with near zero in controls. Lavage fluid also contains many dead cells and debris, most probably derived from disrupted short-lived PMNs. These findings suggest that the enzymes measured in this 2–8-week period arise mainly from PMNs. In a study of the rat lung, Sykes et al have shown that peptidase and collagenase recovered by bronchoalveolar lavage 100 days after silica were derived mainly from PMNs.[21,22] From 8 weeks to the end of the present experiment, enzyme levels correlated with both PMN and AM numbers. In previous studies, increased PMNs were either not detected[9] or were found in lavage fluids when examined at a single time point, 3 months[3,21] or 6 months[5] after administration of silica. Using asbestos, Kagan et al have shown that supernatants of recovered macrophages release chemotatic factors for up to 15 months.[23] The present experiments indicate that the pulmonary reaction to silica involves a continuing inflammatory response with discharge of lysosomal enzymes, an ongoing process that may be involved in fibroblastic stimulation.

The most accepted theory on the fibrotic process was first proposed by Heppleston and Styles, who showed that macrophagic products stimulated fibroblast growth and collagen production.[7] The key event is believed to be damage to lysosomal membranes and secretion of fibroblast-stimulating factors. Several studies have supported this hypothesis, and there is recent evidence of regulation of lung fibroblast stimulation by AMs.[8] The emphasis in these studies has been placed on the reaction of the AM, whereas the present investigation indicates that the PMN may also be involved. It ingests particles and either by lysosomal membrane disruption due to silica or by cell death releases its enzymes. It has been shown that lactate dehydrogenase levels, an index of

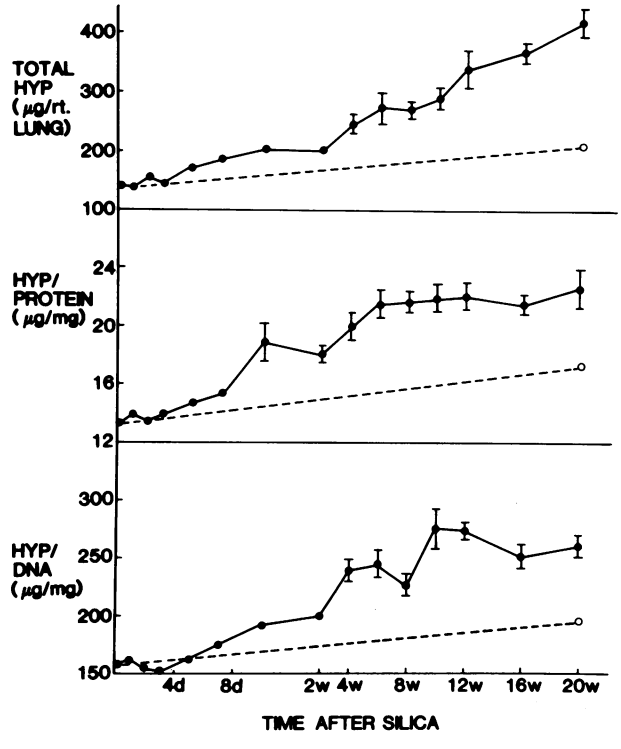

Figure 7—Levels of hydroxyproline (*HYP*) in the lung after silica (*solid circles*). Control values are given by the *broken lines*. SE shown when significantly greater than control ($P < 0.01$).

cell injury, are elevated in the lung after silica instillation.[24] The ongoing recruitment of PMNs to the alveoli and the high levels of lysosomal enzyme activity indicate that continuing tissue injury occurs in the pulmonary interstitium and alveoli. The finding of collagenase and peptidase derived from PMN in lavage fluid after silica also suggests a role for this cell in collagen turnover in the silicotic lung.[21,22] It is suggested that products of both AMs and PMNs contribute to the fibrotic process after silica.

The fibrotic reaction, as shown by morphologic and biochemical evidence, appears to begin as early as 1 week and continues for as long as 1 year.[2,4,17] Although late effects of silica have often been attributed to concurrent respiratory disease, we saw no evidence of infection. Others have found no differences in the response of normal and respiratory disease-free rats to intratracheal silica[4,17]; both developed silicotic nodules of similar collagen type distribution.[4] In the present study, fibroblast proliferation begins early and continues at higher than normal levels to 20 weeks. It is likely that some of the labeled interstitial cells seen in the first week are dividing macrophage precursors, because some of the increased number of AMs arise from dividing interstitial precursors.[18] After 1 week, when the number of AMs is lower, interstitial labeling is predominantly due to fibroblast proliferation, and collagen production is increased by 4 weeks. The enhanced fibroblastic activity may be due to factors released by phagocytes in the alveoli and in the pulmonary interstitium, because free silica crosses the Type 1 cell[10] and is taken up by interstitial macrophages. Factors released by these interstitial cells and by PMNs attracted to these sites may produce stimulatory factors in the immediate vicinity of the interstitial fibroblast.

An additional component in the reaction is the response of the alveolar epithelium. A few previous reports have mentioned epithelial hyperplasia and increased secretion by Type 2 cells in relation to alveolar lipoproteinosis.[6,17] We observed focal Type 1 epithelial cell swelling and necrosis, which would account for leakage of blood components, including fibrin, into the alveoli. Injury to Type 1 cells does not occur after normal lung lavage or following injection of carbon and latex suspensions.[11,18] Type 1 cell injury is normally repaired by Type 2 cell proliferation and differentiation,[14] and this probably occurs after silica, because labeled Type 2 cells were found in the autoradiographs. The pinocytic vesicle membrane of the Type 1 cell may be injured during the process of transporting silica. Products of this interaction may also stimulate the underlying fibroblast. Furthermore, focal necrosis of Type 1 cells would be expected to facilitate particulate passage to the interstitium and enhance macrophage–silica interactions at that site.

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