

SILICA-INDUCED PULMONARY FIBROSIS INVOLVES THE REACTION OF PARTICLES WITH INTERSTITIAL RATHER THAN ALVEOLAR MACROPHAGES

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SUMMARY

Macrophage-derived products have been implicated in fibroblast stimulation following particle deposition in the lung. To assess the role of macrophages in the alveolus versus those in the interstitium in the induction of pulmonary fibrosis, we compared the pulmonary response to silica when phagocytosis occurred predominantly in each of these compartments. One group of mice received intratracheal silica which was phagocytosed largely by alveolar macrophages (AM). A second group was exposed to whole body irradiation prior to receiving the same dose of silica. This prevented the usual efflux of PMN and monocytes into the air sacs, allowing passage of silica particles across the alveolar epithelium to reach the interstitial macrophages (IM). In the irradiation plus silica group, many large interstitial granulomas were formed at 2-4 weeks, and collagen levels were significantly greater than in all other groups at 16 weeks. More silica was found in a lung tissue residue and in lymph nodes of these animals. Pulmonary fibrosis was limited to interstitial areas where there was a high level of retained silica, whereas peripheral regions of the lung, where free AM containing silica were found, did not show fibrosis of the alveolar walls. The results suggest that factors secreted by IM in response to silica are more effective in stimulating fibrogenesis than secretions made by the AM into the alveolar space.

KEY WORDS—Pulmonary fibrosis, silica, alveolar macrophages, interstitial macrophages, granulomas, experimental animals.

INTRODUCTION

The induction of pulmonary fibrosis in response to inhaled particles is believed to result from products of activated macrophages.¹⁻³ Although most attention has been paid to the secretory activity of the alveolar macrophage, there is a large population of macrophages in the pulmonary interstitium that is potentially more important because of its proximity to the fibroblast. In earlier studies after asbestos instillation, we have shown that a dose of 0.1 mg of short fibres reached the alveoli, was retained in alveolar macrophages over a long period, and yet did not induce pulmonary fibrosis.⁴ In contrast, the same dose of long fibres of the same asbestos type

reached the peribronchiolar interstitium where particle-macrophage interaction stimulated the induction of fibrotic granulomas.⁵ This suggests that products of the interstitial macrophage may be more effective in stimulating fibroblasts than factors released by free alveolar cells. This implies that translocation of particles across the epithelium to the interstitium is a critical step in the development of pulmonary fibrosis.

To study this hypothesis further, we have examined the development and extent of fibrosis in lungs exposed to silica in a situation where particulate clearance was depressed, thereby allowing relatively free passage of particles to the interstitium. We have previously shown that irradiation prior to the instillation of inert carbon to the lung reduces the inflammatory cell response and inhibits particle clearance, so that the particulate content of the

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interstitium is subsequently increased.⁶ In the present study we have used this experimental model to compare the pulmonary reactions to a fibrogenic dust, silica, when the predominant site of particle-macrophage interaction is either interstitial or alveolar.

MATERIALS AND METHODS

Two groups of Swiss-Webster mice (25 g males) were exposed to 650 Rad whole body irradiation. The animals were housed in individual sections of a plastic box during exposure.^{6,7} Three days later, one of these groups received an intratracheal injection of silica (Dowson and Dobson, South Africa) in 0.1 ml sterile water while under mild nembutal anaesthesia. A third group of mice was instilled with silica only, while a fourth group received no treatment and served as control. Animals in sets of 4 were killed at intervals to 16 weeks.

When the chest was opened, a blood sample was taken by cardiac puncture and the white blood cell (WBC) count was made. Then a tracheotomy was performed and the lungs were washed four times with 1 ml of saline. The bronchoalveolar lavage fluid (BAL) was pooled for each animal and the total cell count determined using a haemocytometer. The remaining cells were centrifuged to a pellet, which was fixed in osmic acid and prepared for embedding in Spurr plastic for electron microscopy. After the lavage procedure, the lungs were inflated with 2 per cent buffered glutaraldehyde and removed. After fixing for 2 h, most of the tissue was processed for embedding in glycol methacrylate. The remaining lung samples were post-fixed in osmic acid and processed for electron microscopy.

Extra animals from each group were killed at 16 weeks to assess fibrosis and the silica content of the lungs. From one group of four mice, the left lungs were processed uninflated for microscopy, while the right lungs were removed and frozen immediately for the measurement of collagen by the hydroxyproline assay method.⁸ In addition, hilar lymph nodes were taken from each animal for embedding in Spurr resin and the morphology was assessed in 0.5 μ m sections. From a separate set of four mice, the lungs were removed immediately, chopped up, and incubated in 40 per cent KOH overnight in an 80°C waterbath to digest the tissue. After cooling, the solution was centrifuged at 1500 rpm for 15 min and a residue was obtained. This was washed twice

in distilled water, then 1 drop was placed on a coated grid for examination by electron microscopy. The remainder was dried and the weight of the residue was determined.

RESULTS

Blood and lavaged cells

The WBC count from animals that received silica alone was not significantly changed from the control non-injected mice. However, both groups that received irradiation showed a marked reduction in WBC in the following 2 weeks (Fig. 1). At the time when irradiated mice received silica, the WBC level was very low, about 25 per cent of the control. The leukocyte count fell further, then recovered slowly and returned to the normal range by 4 weeks.

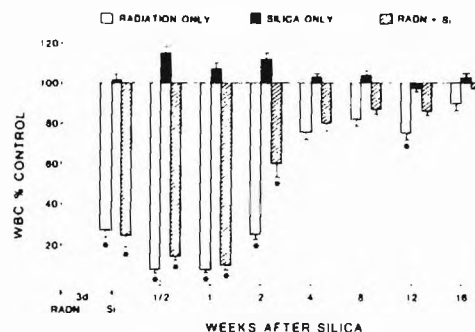


Fig. 1—Percentage change from controls in white blood cell (WBC) numbers after irradiation and or silica. Values are means + SE. * $P < 0.01$ less than controls

The total cell count recovered by BAL is shown in Fig. 2. About 2×10^5 cells, all macrophages, were recovered from control animals and the numbers were not altered by irradiation alone. Instillation of silica to otherwise normal mice induced a rapid large increase in total cells which appeared on the haemocytometer to be predominantly polymorphonuclear leukocytes (PMN) at early times, and mostly alveolar macrophages (AM) after 1 week. When irradiated mice received silica, there was a delay in mounting the usual inflammatory response. There was only a small increase in cells recovered by BAL before 2 weeks, then the numbers increased greatly, and though they fell later, the cell number was always above normal and above the silica only group (Fig. 2).

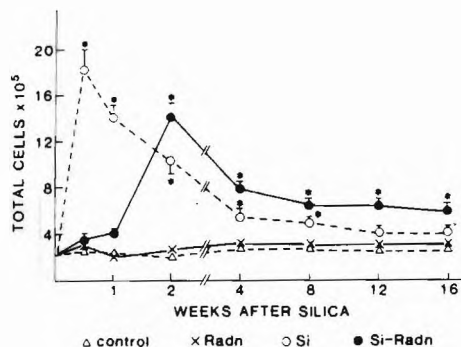


Fig. 2—Total cells (mean + SE) recovered by BAL in the various groups. * $P < 0.01$ greater than controls

Pellets of lavaged cells from the irradiation only group contained an almost pure population of macrophages at all times. Mice that received silica only showed mixed PMN and AM early with both cell types containing silica as previously described.^{8,9} Later, AM became the predominant cell. Cell pellets prepared in the first 2 weeks from the silica plus irradiation group contained so much free silica that sections were of very poor quality. After 2 weeks, less free silica was found and as the cell number increased, particles were seen in phagosomes of PMN and AM (Fig. 3). Subsequently, the proportion of AM increased, though at 16 weeks some PMN were still present in the lavaged cell population. A few silica particles were found in the AM at this time.

Lung morphology

(a) *Irradiation alone*—The sequence of lung injury and repair induced by this dose of irradiation resembled that reported previously.⁷ Briefly, there was ultrastructural evidence of endothelial injury in the first 2 weeks but subsequently these cells appeared normal. By 4–8 weeks, focal thickening of some regions of the air–blood barrier by fibroblasts and collagen was seen and linear scarring of alveolar walls was evident after that time (Fig. 4).

(b) *Silica alone*—Instillation of silica resulted in a rapid inflammatory response and many PMN and AM were seen in the alveoli, particularly in alveolar duct regions (Fig. 5). By electron microscopy, silica particles were seen in both of these cell types and in addition a few particles were observed at 3 days in type I alveolar epithelial cells and later in interstitial macrophages. As described previously,⁹ there was some necrosis of type I cells at this time, followed by

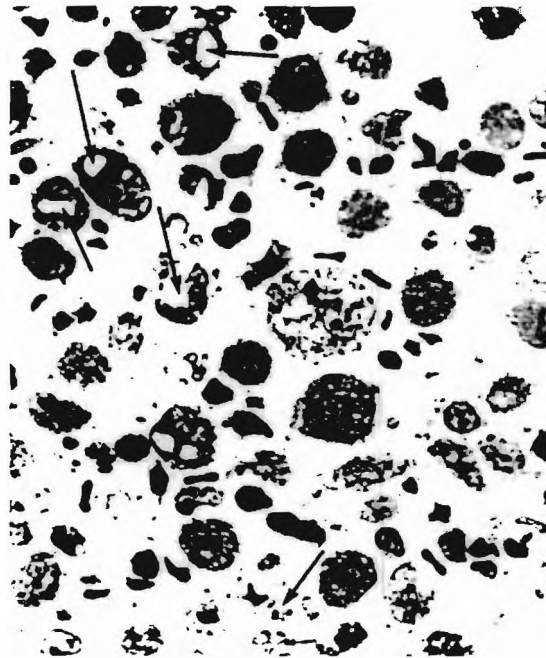


Fig. 3—0.5 μ m section of cell pellet from BAL 2 weeks after silica instillation to irradiated mice. A mixed population of PMN and AM is seen, both containing ingested silica (arrows). Toluidine blue

type 2 cell proliferation during repair. After 4 weeks, some small interstitial granulomas of macrophages and fibroblasts were seen and these became more fibrotic up to 16 weeks (Fig. 6).

(c) *Silica plus irradiation*—Up to 2 weeks after silica, very few inflammatory cells were seen in the lung; the alveoli contained macrophages, some cell debris, and free silica (Fig. 7). In contrast to the silica alone group, particles were seen frequently within the type I cell cytoplasm, and by 2 weeks many interstitial macrophages contained phagocytosed silica particles (Figs 8A and 8B). At this time, large granulomas of mixed macrophages and fibroblasts were evident in areas where there was a heavy load of interstitial silica (Fig. 9). These interstitial granulomas became more fibrotic with time but still contained macrophages and silica (Fig. 10). In contrast, in many peripheral alveolar regions there were large free macrophages containing silica often appearing trapped in the alveoli. These cells were seen up to 16 weeks in both lavaged and non-lavaged lungs, and it was observed that the neighbouring alveolar walls were thin with no signs of fibrosis (Fig. 11).

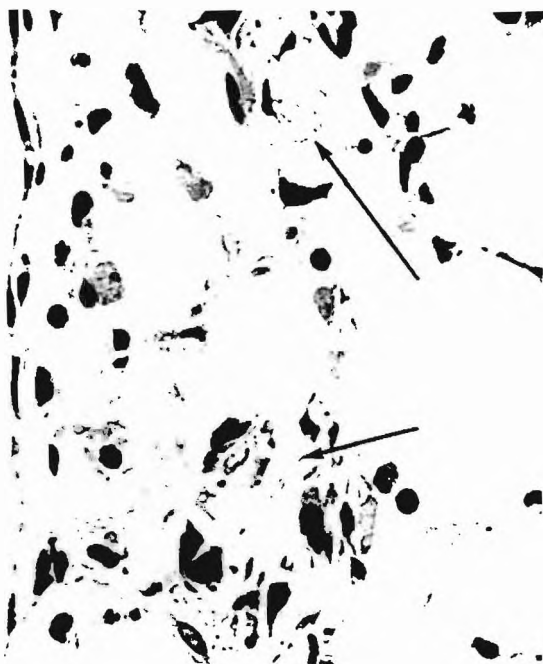


Fig. 4—Lung section 8 weeks after irradiation only. Focal thickening of alveolar walls with collagen (arrows) is seen in some peripheral areas. Basic fuchsin



Fig. 5—Section of an alveolar duct region 3 days after silica only. An inflammatory exudate with a large number of PMN is seen in the air sacs. Basic fuchsin

Lymph nodes

Hilar lymph nodes were compared at 16 weeks. Nodes from mice that received silica only appeared normal on gross examination but by light microscopy were found to contain a few silica particles. The lymph nodes from irradiated mice that received silica 16 weeks earlier were enlarged and contained a considerable amount of silica (Fig. 12).

Biochemistry

The hydroxyproline (HYP) content of the right lungs at 16 weeks is shown in Fig. 13. Both silica and irradiation alone produced pulmonary fibrosis with an approximate 70 per cent increase in HYP. Administration of silica to irradiated animals, however, resulted in a 130 per cent increase over age-matched controls, and the collagen level was also significantly higher than after irradiation or silica alone.

Lung residue

An amorphous, non-particulate residue was obtained from normal lung and from the fibrotic



Fig. 6—Lung section 8 weeks after silica only showing a small interstitial granuloma. No fibrosis is seen in alveolar walls



Fig. 7—Lung section 3 days after silica with prior irradiation. An inflammatory response is seen in the alveoli, which contain a few cells and free silica particles

lung after irradiation alone (Fig. 13). The residue from mouse lung after instillation of silica was whitish in appearance, contained particles as seen by electron microscopy, and weighed twice as much as that from controls. After digesting tissue from the irradiation plus silica group, a white residue, rich in silica, was obtained and this weighed significantly more than that prepared from all the other groups.

DISCUSSION

Deposition of particles in the lung, whether by instillation or by inhalation results in an immediate inflammatory response with an initial efflux of PMN followed by an increase in AM in the alveoli. This has been shown for a number of particles including silica, which, because of its small diameter, readily reaches the alveoli.⁹ In the first week, most particles are cleared from the lung, either in the free state or else in phagosomes of these inflammatory cells. Some particles may also be retained in the lung, either in free AM or in the connective tissue as a result of transverse the alveolar epithelium before phagocytosis by interstitial macrophages.⁹ It is the subsequent interstitial cell response with the

formation of granulomas that later become fibrotic that constitutes the predominant pulmonary response to silica.

In the present experiment, the combination of irradiation and silica administration to mice clearly accelerates and exaggerates the pulmonary reaction to silica. Irradiation alone produces an increase in collagen but, by microscopy, this is in the form of linear scarring of alveolar walls. Silica alone induces little of this type of fibrosis, even in focal regions of the lung where AM containing silica are trapped. The distribution of fibrosis after silica is nodular, often at alveolar ducts where there appears to be a higher proportion of deposited particles, and a greater interstitial content of silica. It is this nodular granulomatous type of fibrosis that predominates when irradiated mice receive silica. Both silica groups received the same dose of particles delivered to the alveoli, but the major difference in animals that had prior irradiation is the great increase in phagocytosed particles within interstitial macrophages. It is likely that the increased activation of these cells promotes the formation of large granulomas and later the fibrotic process in these localized areas of the lung.

The highest levels of collagen were found in the lungs of the silica-irradiation group, which also had the highest level of retained silica. Irradiation caused a great reduction in circulating WBC at the time when silica was given and over the following 2 weeks. This resulted in a considerable delay in mounting the usual inflammatory response that follows particle instillation. By reducing the influx of PMN and monocytes-macrophages to the airways, particles remained free in the alveoli for a longer time and the number crossing the epithelium was increased. Many particles were then phagocytosed by interstitial macrophages. The subsequent increase in cellularity was due to both macrophages and fibroblasts as large granulomas formed as soon as 2-4 weeks. With time, fibroblasts predominated but large numbers of macrophages containing silica appeared trapped in these interstitial fibrotic areas. This was in direct contrast to the appearance of some peripheral areas where there were free AM containing silica surrounded by alveolar walls in which there was no evidence of fibrosis. Previously a similar picture was found when small asbestos fibres were instilled into mouse lung; fibres were present in alveolar macrophages up to 20 weeks with no evidence of pulmonary fibrosis.⁴

The heavy particulate burden within the interstitium was not cleared over the 16-week period. It is

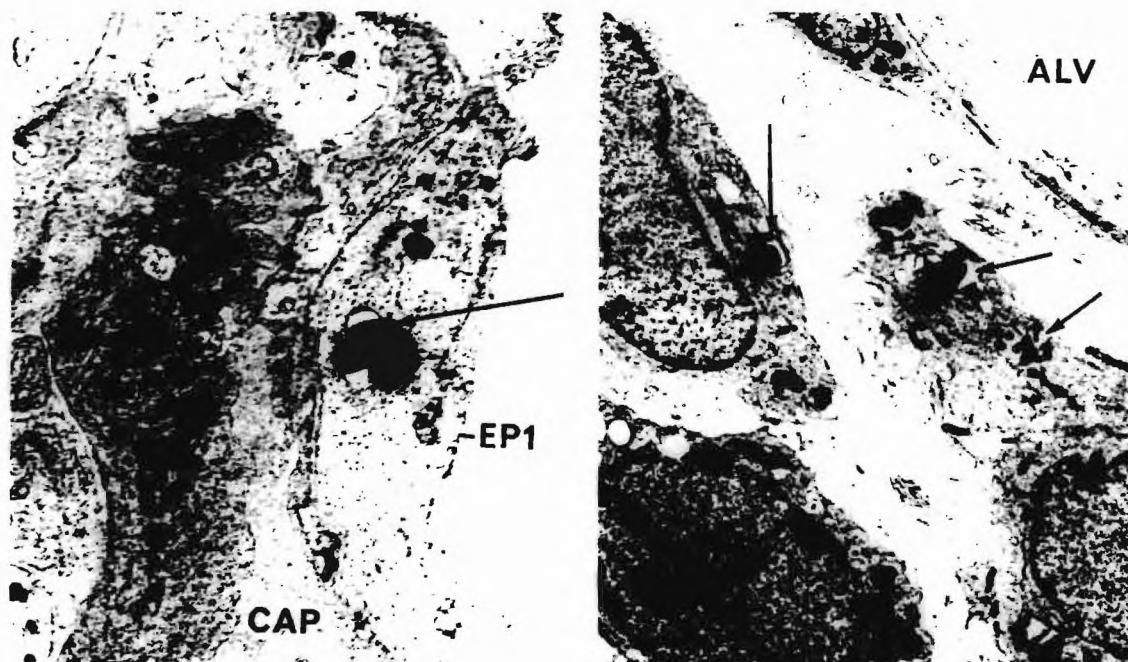


Fig. 8—Electron micrographs of lung 1 week after silica with prior irradiation. (A) Silica particles (arrows) are seen in type I epithelial cell (EP1). CAP = Capillary. $\times 9000$. (B) In the interstitium, particles are located in macrophages (arrows). ALV = Alveolus. $\times 7500$



Fig. 9—Lung 2 weeks after silica with prior irradiation. A large granuloma shows mixed fibroblasts and macrophages containing silica (arrows)



Fig. 10—Lung 12 weeks after silica with prior irradiation. Granulomas become more fibrotic but still contain much silica (arrows)



Fig. 11—Peripheral alveolar region of non-lavaged lung, 12 weeks after silica with prior irradiation. Many macrophages containing silica are seen free in the alveoli. Adjacent alveolar walls are thin with no evidence of fibrosis

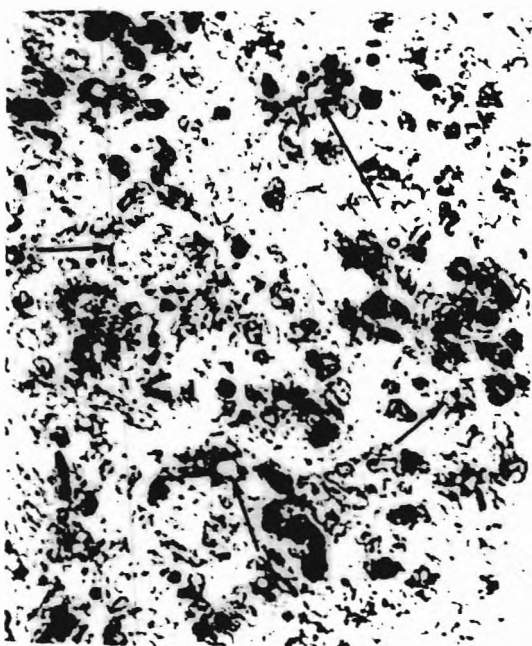


Fig. 12—0.5 μ m section of hilar lymph node 16 weeks after silica with prior irradiation. Some areas of the node are heavily laden with silica particles (arrows)

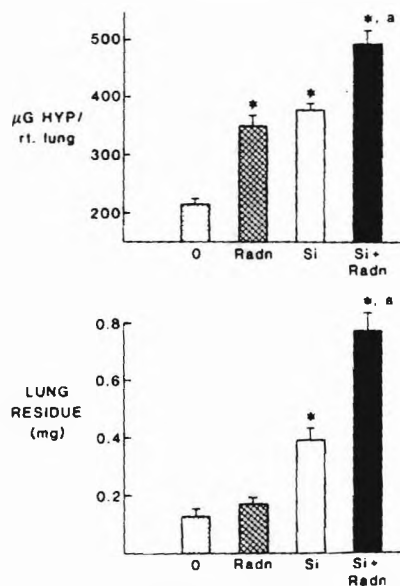


Fig. 13—Top: Hydroxyproline (HYP) content of right lungs at 16 weeks in the various groups. Bottom: Weight of lung residue recovered at 16 weeks. Radn = irradiation only; Si = silica only; Si + Radn = silica 3 days after irradiation; 0 = age-matched control. * $P < 0.01$ greater than control; ^a $P < 0.01$ greater than all other groups

possible that some interstitial macrophages could have migrated to the alveoli and there was also evidence of some clearance to lymphatics. The particle content of hilar lymph nodes in the irradiation plus silica group was much higher than that of the silica only group and probably reflects the greater interstitial particulate content of the former group of animals. It is possible that, with time, a fibrotic reaction may occur in these lymph nodes as a consequence of macrophage activation and subsequent fibroblast stimulation.

The role of macrophage secretions in fibrogenesis is well established.¹⁻³ In the lung, the populations of alveolar and interstitial macrophages are different^{10,11} and may not contribute equally to the fibrogenic process. The results of this study indicate that activation of interstitial macrophages by silica induces a greater fibrotic response than a macrophage-particle interaction confined to the alveolus. Alveolar macrophages lavaged from fibrotic lungs after silica have been shown to secrete fibroblast growth factors in serum-free conditions.¹² This may reflect a general consequence of chronic inflammation since a variety of growth factors have been identified in alveolar macrophage secretions in various examples of fibrotic lung disease.^{1,13-15} *In vivo*, however, there is an epithelial cell barrier

between the macrophage in the alveolus and the fibroblast in the interstitium. It is also possible that various inactivating factors, perhaps in serum, could diminish the effects of an alveolar secretion on the interstitial fibroblast. In contrast, the high level of particle phagocytosis by the interstitial macrophage and its subsequent activation would allow direct transfer of any growth factors to adjacent fibroblasts. Thus, it may be inferred that any pulmonary condition which results in diminished production of an inflammatory response or in decreased phagocytosis in the airways will increase the likelihood of transepithelial passage of particulates to reach the interstitial macrophage. A macrophage-fibroblast interaction at this location is more likely to result in the induction of pulmonary fibrosis.

ACKNOWLEDGEMENT

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