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Mesothelial Cell Proliferation after Instillation of Long or Short Asbestos Fibers into Mouse Lung

Ian Y. R. Adamson, J. Bakowska, and D. H. Bowden

From the Department of Pathology, University of Manitoba, Winnipeg, Canada

The relationship of asbestos deposition in the lung to subsequent cell proliferation at the pleural surface is not clear. The present study examines DNA synthesis by various pulmonary cells, particularly those at the pleura after intratracheal injection of 0.1 mg crocidolite to mice using: 1) long fibers (>20 μ), which are deposited in bronchiolar regions and induce fibrosis; 2) short fibers (<1 μ), which reach alveoli but do not induce fibrosis. Mice also received 2 μCi/g tritiated thymidine 1 hour before death at intervals to 16 weeks. Short fibers induced only a small increase in labeling of bronchiolar epithelial and interstitial cells, which subsided by 5 days, when a small increase in labeled mesothelial and subpleural cells was seen. In contrast, long fibers damaged the bronchiolar epithelium and became incorporated into connective tissue. During regeneration, 12% of cells were labeled at 3 days and labeling was greater than controls to 4 weeks. Increased peribronchiolar labeling of fibroblasts and interstitial macrophages was seen around long fibers, and increased DNA synthesis by mesothelial and subpleural cells was found. Up to 2% of mesothelial cells were labeled 1 week after long fibers compared to near zero in controls. No long fibers were found at the pleura. Activation of interstitial macrophages in response to long crocidolite fibers is associated with fibroblast proliferation. It is now suggested that mesothelial cells may also be stimulated by cytokines from activated interstitial macrophages that diffuse across the interstitium, without requiring actual fiber translocation to the pleura. (Am J Pathol 1993, 142:1209–1216)

Deposition of asbestos fibers in the lungs of humans or experimental animals is associated with the production of pulmonary fibrosis, which involves the small airways.[1–3] To a lesser extent, cells at or near the pleural surface are also affected, leading to pleural fibrosis or mesotheliomas.[4] The precise mechanism for activation of cells in the pleural region following fiber deposition in the air spaces is not known. Direct injection of fibers to the pleural space is associated with tumor production,[5] and a lower incidence has been found after long-term inhalation or after intratracheal instillation.[6] Questions remain regarding how inhaled asbestos fibers can reach the pleura and whether the quantity is enough to promote a neoplastic response.

Although the mechanisms of neoplasia and repair may or may not be linked, the initial stages of any fibrotic or tumorigenic response at the pleural surface involve cell proliferation. In an earlier brief report, transient DNA synthesis by mesothelial cells was seen after one inhalation exposure of rats to chrysotile asbestos.[7] This was a mixed length sample, which allowed fiber deposition throughout the lung. We have now instilled via the trachea 1) long crocidolite asbestos fibers, which are deposited in the airways inducing a fibrotic response, and 2) short fibers of the same asbestos, which reach the alveoli, where they are phagocytized without inducing fibrosis.[8,9] The proliferative responses of various pulmonary cell types is now examined, in particular to determine whether either sample induces proliferation of mesothelial or subpleural cells and whether this is related to the location of deposited fibers and to the development of fibrosis.

Materials and Methods

Standard UICC crocidolite asbestos was separated into long and short fibers by sedimentation.[8,9] A sample of short fibers measured 0.6 μ ± 0.1 SE,

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Address reprint requests to Dr. I. Adamson, Department of Pathology, University of Manitoba, 236–770 Bannatyne Avenue, Winnipeg, Canada, R3E 0W3.

with more than 98% of fibers being shorter than 2.5 μ. Long fibers had a mean length of 24.4 μ ± 0.5, with 88% longer than 2.5 μ. The size distribution was as follows: 12% at <2.5 μ; 17% at 2.5 to 5 μ; 31% at 5 to 10 μ; 22% at 10 to 20 μ; and 18% at >20 μ. Each asbestos sample, at a dose of 0.1 mg suspended in 0.1 ml sterile water, was instilled intratracheally into 70 male Swiss Webster mice under mild anesthesia. At this dose level, approximately 10^8 long fibers or 5×10^9 short fibers were instilled. The animals, in groups of four, were killed at days 1, 2, 3, 5, and 7 and at weeks 2, 4, 8, 12, and 16; each animal received 2 μCi/g tritiated thymidine 1 hour before death. Control animals were injected with 0.1 ml sterile water and were killed at days 1, 5, and 7 and at weeks 4, 8, and 16. At sacrifice, a tracheotomy was performed, and the bronchus leading to the right lung was clamped. This lung was then removed, weighed, and frozen for biochemical analysis. The left lung was inflated with 0.5 ml of 2% buffered glutaraldehyde, removed, and processed for embedding in glycol methacrylate.

Methacrylate sections (0.75 μ thick) from three random blocks per animal were prepared for autoradiography. Slides were dipped in Kodak NTB 2 emulsion (diluted 1:1 with distilled water), exposed in darkness for 2 weeks, developed in D19, and stained with basic fuchsin. The percentage of [^{3}H]-thymidine-labeled cells was determined by counting 3000 lung cells per animal, excluding bronchial epithelial cells. Means and standard error were calculated for each group. The 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 total labeling percentage and the differential labeling percentage gave a radiographic index for each cell type.

In addition to the overall percentage of labeled lung cells, more specific counts were made. The labeling percentage of bronchial and bronchiolar epithelial cells was calculated after counting 500 of these cells per animal. The labeling index of mesothelial cells was calculated after counting the number labeled in all mesothelial cells identified in the three sections per lung. This gave a total of at least 1000 mesothelial cells per rat, with no apparent difference in total cell number seen in these random sections at various times after fiber injection. The labeling index for subpleural cells was calculated after counting cells lying between the mesothelial lining and the adjacent first alveolar space. One thousand cells per section were counted and a mean labeling index ± standard error was calculated for each group. Statistical difference from control at the same time interval was determined using Student's *t*-test.

The right lung of each mouse was homogenized in water, and biochemical assays were carried out on duplicate samples. Measurement of total protein was carried out[10] and, as an index of collagen content, hydroxyproline levels were determined after hydrolysis with hydrochloric acid.[11]

Results

Morphology

The sequential morphological changes that occur in the lung after instilling 0.1 mg long or short asbestos fibers have been described in detail previously[8,9] and will be summarized here. Deposition of short fibers induced a brief inflammatory response, almost all fibers were phagocytized by alveolar macrophages, and the number in the alveolar cells declined to normal after 2 weeks. Only a few fibers reached the interstitium but there was little evidence of cell injury, no granulomas formed, and no fibrosis was observed up to 16 weeks.

After instilling the same dose by weight of long fibers, early injury to bronchial and bronchiolar epithelial cells was found at areas of deposition. Fibers then became incorporated into underlying connective tissue and, by 1 week, the regenerating epithelium overgrew these areas. No long fibers were found in the alveoli or in alveolar macrophages. However, in the peribronchiolar interstitium where long fibers were exclusively located, giant cells formed by 2 weeks and later granulomas of mixed macrophages and fibroblasts were produced (Figure 1). A detailed description of fiber incorporation and granuloma formation has been reported previously.[9]

In some sections of the lung, the peribronchial or peribronchiolar interstitium could be traced directly to the pleural surface with no intervening alveolar spaces. In the long-fiber group, granulomas containing asbestos were seen in such anatomic locations, where the reactive interstitial cells are directly linked to cells in subpleural and mesothelial locations (Figure 2). In some sections of lung, small foci of lymphoid tissue were observed at the pleural surface, attached to underlying connective tissue. In places, a continuous interstitium linked a peribronchiolar granuloma to these pleural lymphoid cells (Figure 3). The granulomas and peribronchiolar interstitium became more fibrotic with time and, al-

though there were some focal areas of subpleural fibrosis, no plaques were observed and no mesotheliomas were found at 16 weeks. Although it was relatively easy to recognize long fibers of asbestos in these plastic sections, as illustrated by Figure 1, long fibers were never observed in the pleural wall or in contact with mesothelial cells at any time.

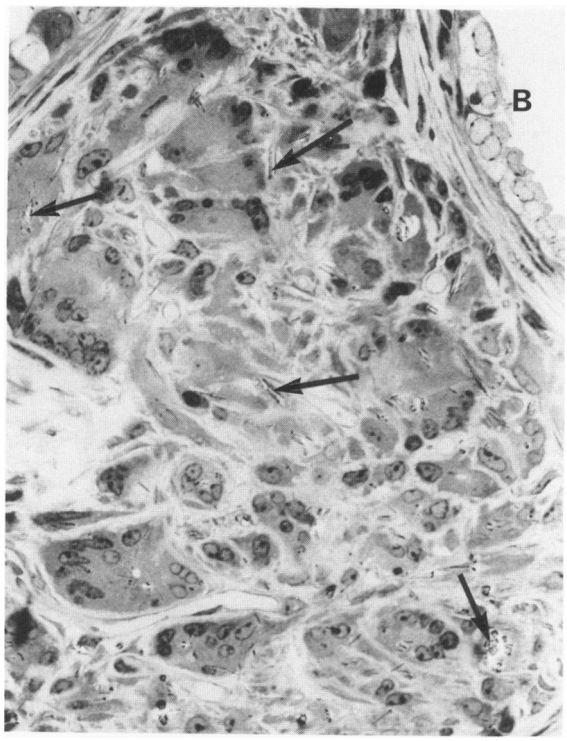

Figure 1. *Lung section 2 weeks after instillation of long asbestos fibers. Beneath the bronchiolar epithelium (B) is a granuloma, composed of macrophages, fibroblasts, and giant cells with many incorporated fibers (arrows). Magnification, ×950.*

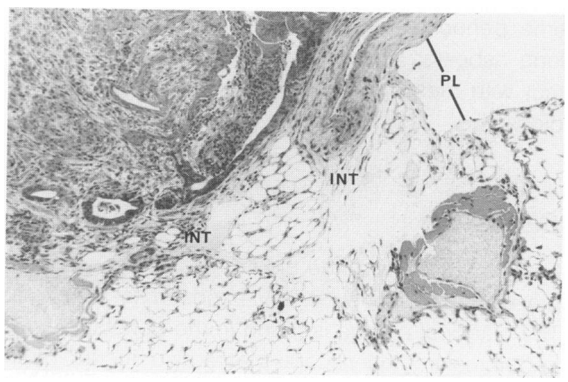

Figure 2. *Lung section 4 weeks after instillation of long asbestos fibers. The large peribronchiolar granuloma containing asbestos lies in the pulmonary interstitium (INT), which connects directly to the nearby pleural surface (PL). Magnification, ×200.*

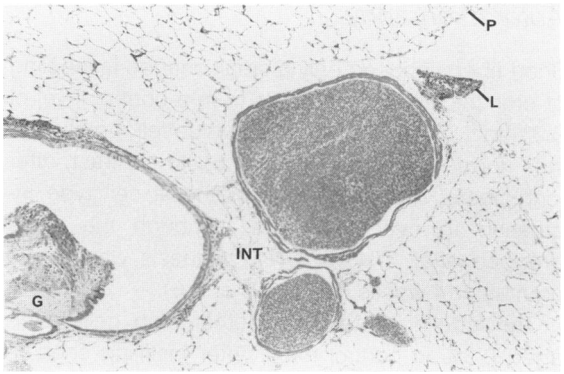

Figure 3. *Lung section 8 weeks after instillation of long asbestos fibers. The peribronchiolar granuloma (G) lies in an area of interstitium (INT), which connects to the pleura surface (P) and an attached lymphoid follicle (L). Magnification, ×200.*

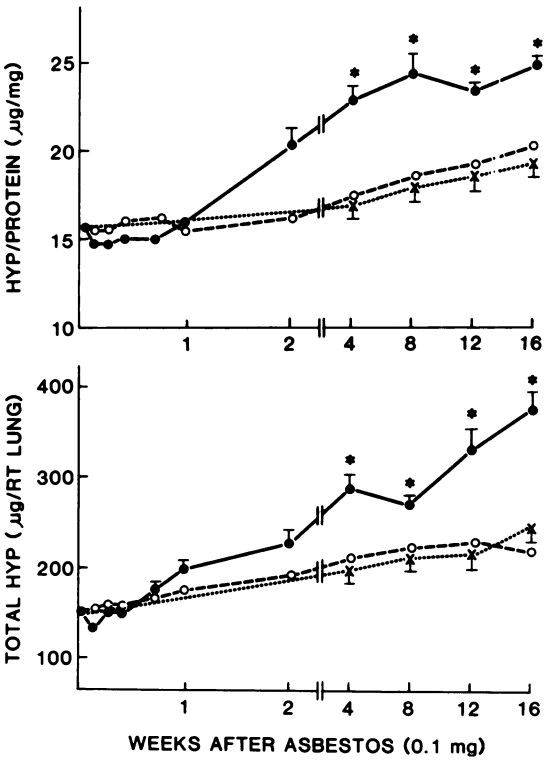

Figure 4. *Hydroxyproline (HYP) content expressed per protein and per dry weight of right lobe in controls (×), and after long (●) or short (○) asbestos fibers. *P other groups.*

Biochemistry

Total hydroxyproline (HYP) increased with the age of animal in controls and no difference was found following the instillation of 0.1 mg of short asbestos fibers (Figure 4). However, following long fibers, the ratio of HYP to total protein rose above control values by 2 weeks and, in the right lobe, total HYP increased significantly from 4 weeks to the end of the experiment.

Autoradiography

Short fibers induced only a small change in labeling of bronchiolar epithelial cells from a control level of 0.2% to 1.7% at 1 day, but the value fell rapidly and was normal by 5 days (Figure 5). In contrast, after long fibers, labeling of this epithelial cell type increased to over 12% and, although the value dropped after 3 days, DNA synthesis was above normal for 4 weeks (Figures 5 and 6).

The labeling index of all other lung cells averaged 0.3% in control mice. After instilling short fibers, the level rose briefly to about 0.7% at day 3 before falling to normal values by day 5 (Figure 7). Long fibers produced a more substantial and prolonged increase in lung cell labeling. Values peaked at over 3% of cells labeled at 1 week and did not return to the control range for 4 weeks (Figure 7). Following differential counts of labeled cells, a radiographic index for the various cell types was calculated. The major change occurred in thymidine uptake by the interstitial cell population (Figure 7). Labeling of these cells increased rapidly after long fiber instillation, and from the morphology, both interstitial macrophages and fibroblasts were labeled in the first week (Figure 6), whereas fibroblast labeling predominated subsequently.

The pattern and timing of lung cell labeling shown in Figure 7 was repeated when counts of labeled subpleural cells were made (Figure 8). There was only a brief, small increase in response to short fibers, whereas after long fibers, labeling increased sixfold by 1 week and did not return to normal until

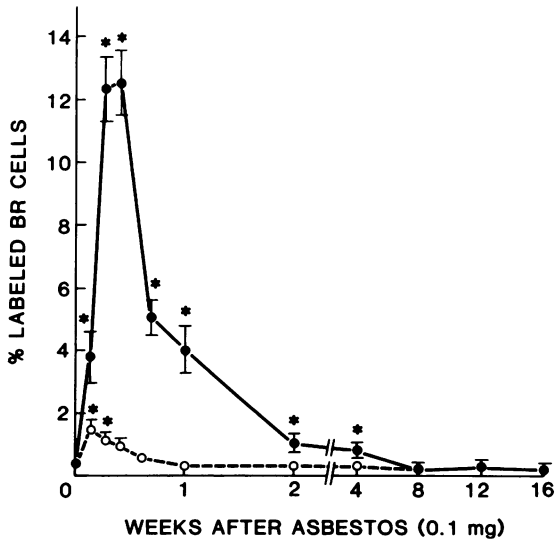

Figure 5. *Percentages of labeled bronchial and bronchiolar (BR) epithelial cells after long (●) or short (○) asbestos. Control values were equal to time 0.* *P < *0.01, greater than control.*

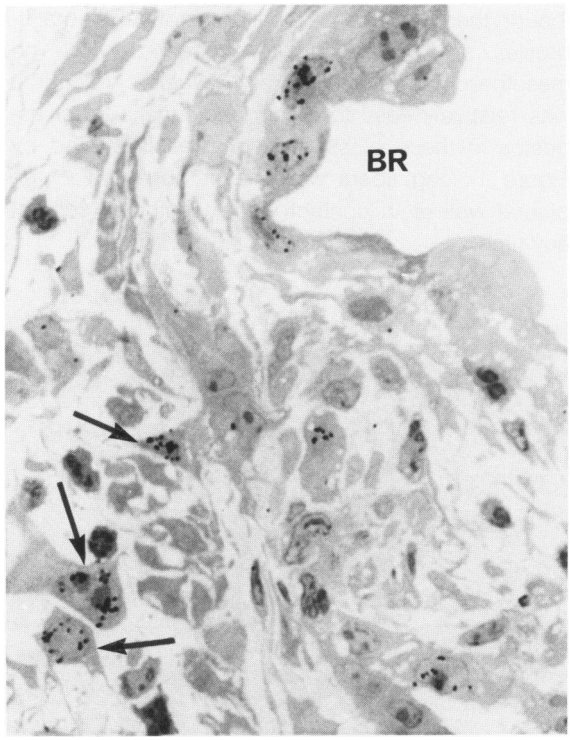

Figure 6. *Autoradiograph 5 days after long fibers. Labeled bronchiolar epithelial cells (BR) overlie the interstitium containing labeled cells (arrows). These appear to be mixed macrophages and fibroblasts. Magnification, ×950.*

after 4 weeks. Most of these labeled cells were fibroblasts located beneath the mesothelial lining (Figure 9). When mesothelial cells only were counted, only a small increase in labeling occurred at day 5 in the short fiber group (Figure 10). After long fibers, however, the labeling percentages of mesothelial cells increased from a near zero control value to over 2% at 1 week, and did not fall to control values until week 4 (Figures 10 and 11). In carrying out these cell counts, the total pleural surface in three random sections from each of four mice per time period was examined at high magnification; long asbestos fibers were never observed in contact with mesothelial cells or in the subpleural regions.

Discussion

Fibrotic reactions at the pleura are common in people who are occupationally exposed to asbestos. The classical plaque is found in the parietal pleura, and fibrosis of the visceral pleura also occurs, often in association with asbestos-related fibrosis of the lung.[1] A low incidence of mesothelioma is also associated with crocidolite exposure.[4] Although it is

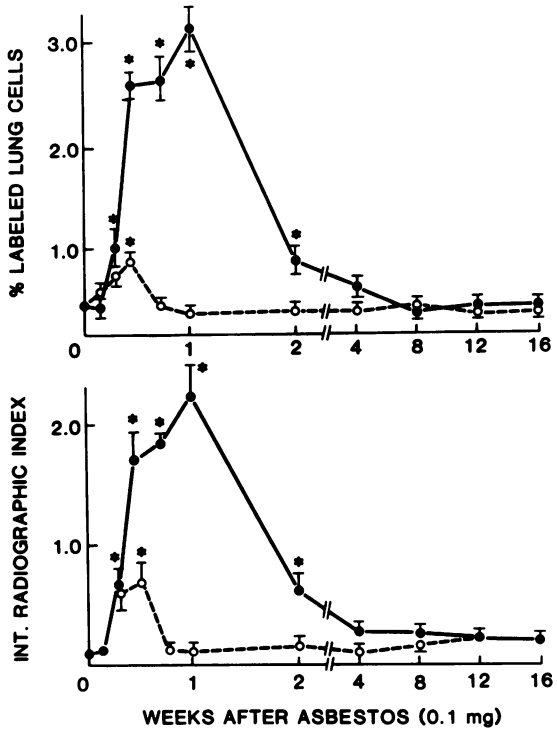

Figure 7. Top: *Percentages of labeled lung cells (excluding BR) after long (●) or short (○) asbestos fibers.* Bottom: *Radiographic index for the interstitial cell (INT) population. Total lung cell label percent multiplied by the percentage of labeled cells identified as interstitial.* *P < *0.01, greater than control (time 0).*

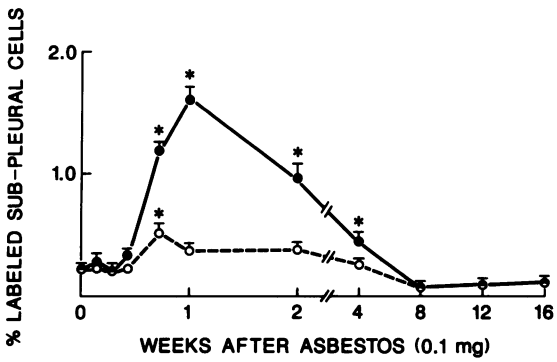

Figure 8. *Percentages of labeled cells in subpleural locations after long (●) or short (○) fibers.*

difficult to estimate asbestos content of pleural lesions using histological sections, recent tissue analysis has shown that fibers are present in plaques and mesotheliomas in humans in approximately the same concentration as in the lung, when expressed per gram of tissue.[12] In analyzing pleural plaques, Churg[13] has shown that the incidence correlates with the content of long fibers. This may be important, since long fibers are known to be more potent than short fibers in the induction of pulmonary fibrosis.[8,9,14,15] Similarly in carcinogenesis, the incidence of mesotheliomas in animals exposed to asbestos is much higher in long-fiber groups, and these animals also have more advanced pulmonary fibrosis.[6,15] It is generally assumed that fibers become incorporated into lung tissue and somehow are transported to the pleural surface. Davis and Jones conclude that a substantial number of fibers must reach the pleura to induce such a response[6] and suggest lymphatic transport as the most likely

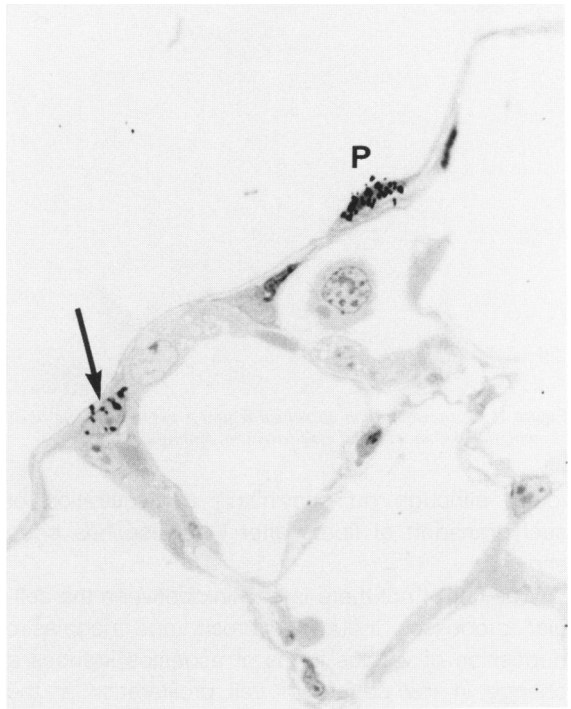

Figure 9. *Autoradiograph of pleural region 5 days after instilling long fibers. Thymidine-labeled cells are seen in the interstitium beneath the pleura (arrow) and at the surface (P). Magnification, ×950.*

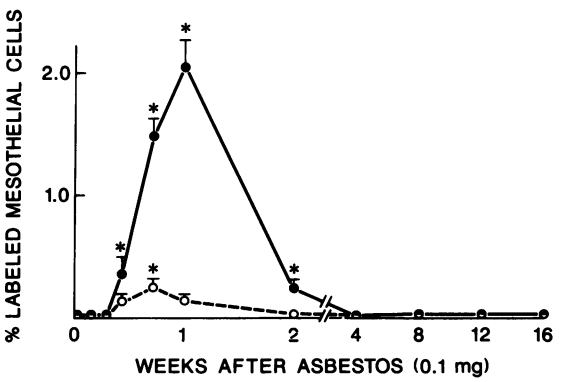

Figure 10. *Percentages of mesothelial cells labeled up to 16 weeks after long (●) or short (○) asbestos fibers.* *P < *0.01, greater than control.*

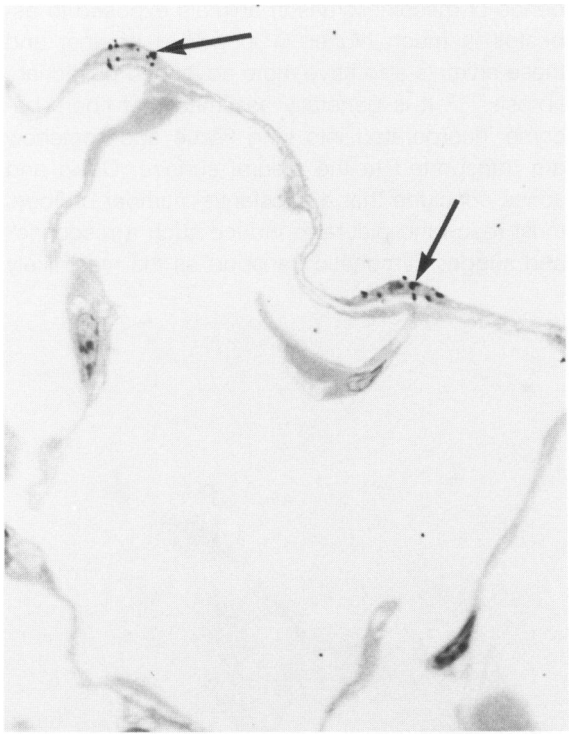

Figure 11. *Autoradiograph of pleural region 1 week after long fibers showing labeled mesothelial cells (arrows). Magnification, ×950.*

route, although no convincing demonstration of such transport of fibers after inhalation has been shown.

Whether or not there is any link between the cellular processes inducing fibrosis and neoplasia, deposition of various forms of asbestos induces a change in the pattern of cell proliferation at the pleura. In the present study, we have analyzed thymidine incorporation by various lung cell types after instilling the same dose of long or short crocidolite. Despite the very much larger number of short fibers administered, they induced minimal changes in all cell types examined. At this dose level, short fibers have been shown previously to be almost all phagocytized by alveolar macrophages, and only a few cross the epithelium.[8] In the present study, short fibers induced little change in lung cell proliferation and no fibrosis ensued. In earlier experiments, alveolar macrophages were found to secrete a growth factor for fibroblasts in response to short asbestos, but it was concluded that such a secretion into the alveolar space did not cross the epithelium to activate fibroblasts in the interstitium.[14]

Pulmonary fibrosis was induced by the instillation of long fibers; this was seen morphologically and confirmed biochemically. The single intratracheal injection allows a precise time zero for cytokinetic studies, while the deposition of fibers in bronchiolar and alveolar duct areas is similar to that found after inhalation.[7] The subsequent pathological sequence of peribronchiolar granulomas and fibrosis after intratracheal injection is similar to the small airway disease seen in humans.[2,9] In the present study, the reaction was mostly peribronchiolar where long fibers penetrated the injured epithelium. Regeneration was rapid as shown by the high labeling index of bronchiolar cells, and a similar burst of bronchiolar cell proliferation was demonstrated previously following an inhalation exposure to chrysotile asbestos.[16] The incorporation of long crocidolite fibers into the peribronchiolar connective tissue promoted a macrophagic response in the interstitium. Some of these cells proliferated and within a few days, giant cells had formed. Surrounding fibroblasts also showed increased proliferation as granulomas were produced.

The pattern of interstitial cell labeling in areas of long-fiber retention in the first 2 weeks is matched by the graphs of labeled subpleural cells (mostly fibroblasts) and of mesothelial cells. No long fibers were observed in these anatomic locations and no cell necrosis was seen, but the DNA synthesis was increased.

If asbestos fibers reached the mesothelium, they could cause injury and repair, since direct injection of crocidolite to the peritoneum induces a proliferative response of mesothelial cells on the peritoneal surface.[17] However, in the case of asbestos deposition in the airways, fiber translocation to the pleural surface may not be essential to provoke cell division. In a published abstract, a brief increase in thymidine-labeled mesothelial cells was found soon after an inhalation exposure to chrysotile of mixed length.[7] The present study, using intratracheal administration of crocidolite, contrasts the cellular responses at the pleural surface to fibers of different length in terms of cell proliferation and the development of fibrosis. Short fibers reached terminal alveoli close to the pleural surface, but virtually no change in pleural or subpleural labeling was seen. In contrast, long fibers, trapped in peribronchiolar interstitium, were associated with a rapid pleural proliferative response. The concomitant increase in proliferation of fibroblasts in both peribronchiolar and subpleural locations and in mesothelial cells may not be coincidental but may represent a multicellular response to cytokine secretion by interstitial macrophages in response to long asbestos fibers.

Pulmonary macrophages are believed to be key cells in the secretion of cytokines in the lung. After activation in various conditions, including asbestos exposure, macrophage-derived growth factors have

been identified and are usually associated with fibroblast growth.[18–21] In particular, interstitial macrophages isolated after instilling long fibers to rat lung secrete a factor(s) that stimulates lung fibroblast growth.[21] One of the possible macrophage-derived cytokines is platelet-derived growth factor (PDGF), which is known to stimulate fibroblast growth.[22] PDGF receptor has also been demonstrated in rat mesotheliomas induced by asbestos.[23] The interstitial macrophage population, activated by long fibers, may produce PDGF or other cytokines within the lung interstitium. Such a molecule could activate local fibroblasts but may also diffuse through the interstitium to reach subpleural and mesothelial cells. Transfer of a factor from alveolar macrophages to mesothelial cells is less likely, since 1) virtually no mesothelial cell reaction to short fibers was seen in this study; 2) alveolar macrophages secrete growth factors in response to short fibers yet no fibrosis is seen *in vivo*[14]; and 3) in a cell culture system, PDGF does not cross a normal pulmonary epithelial layer.[24] These findings suggest that an intra-alveolar secretion may not reach fibroblasts or mesothelial cells.

Changes in proliferation of mesothelial and subpleural cells were confined to the long-fiber group, yet no asbestos fibers were found in this location at any time. Although histological examination is not the ideal method to locate fibers, it should be noted that at least 1000 mesothelial cells and 3000 underlying connective tissue cells were examined at high power per rat, yet no long fibers were detected at the pleura, whereas they were readily detected elsewhere in the same lungs. In subsequent experiments, we have lavaged to pleural cavity and separated mesothelial cells by mild collagenase treatment after long-fiber instillation. We have not been able to find fibers in these cells or in recovered fluid up to 1 month later, when the proliferative response at the pleura is over (unpublished observations). In the present study, pathological changes were confined to the long-fiber group in which asbestos was readily recognized in peribronchiolar regions. In some anatomic locations, the peribronchiolar interstitium with a reactive granuloma was close to and continuous with the subpleural region. Cytokine transfer via peribronchiolar lymphatics to the pleura is also possible. In this way, cells at the pleura may respond to cytokines generated within the interstitium without requiring actual fiber translocation to that anatomic location. Such general activation by locally generated cytokines may account for cases of pleural fibrosis or mesotheliomas in which little or no asbestos exposure is indicated.

In conclusion, it has been shown that mesothelial and subpleural cell proliferation occurs in response to long crocidolite fibers, and it is suggested that this proliferative response results from cytokine secretion by interstitial macrophages in response to these fibers. The fact that extensive pleural fibrosis or mesotheliomas were not induced in the present study may be related to the single asbestos exposure, to the dose level, or to the limited observation period. Although these two different pathological end points to fiber exposure may involve different growth factors and molecular pathways, the demonstration that a cell proliferative response occurs at the pleura soon after long-fiber crocidolite deposition in the airways and its reaction with interstitial macrophages leads us to speculate that repeated or prolonged activation of these cells may be involved in the generation of subpleural fibrosis or mesotheliomas.

References

1. Craighead JE, Mossman BT: The pathogenesis of asbestos-associated diseases. N Engl J Med 1982, 306:1446–1455
2. Wright JL, Churg A: Morphology of small airway lesions in patients with asbestos exposure. Hum Pathol 1984, 15:68–74
3. Bowden DH, Adamson IYR: Bronchiolar and alveolar lesions in the pathogenesis of crocidolite-induced pulmonary fibrosis in mice. J Pathol 1985, 147:257–267
4. Mossman BT, Gee JBL: Asbestos-related diseases. N Engl J Med 1989, 320:1721–1730
5. Stanton MF, Wrench C: Mechanisms of mesothelioma induction with asbestos and fibrous glass. JNCI 1972, 48:797–821
6. Davis JMG, Jones AD: Comparisons of the pathogenicity of long and short fibers of chrysotile asbestos in rats. Br J Exp Pathol 1988, 69:717–737
7. Coin PG, Moore LB, Roggli V, Brody AR: Pleural incorporation of ^{3}H-TdR after inhalation of chrysotile asbestos in the mouse. Am Rev Resp Dis 1991; 143:A604
8. Adamson IYR, Bowden DH: Response of mouse lung to crocidolite asbestos. 1. Minimal fibrotic reaction to short fibers. J Pathol 1987, 152:99–107
9. Adamson IYR, Bowden DH: Response of mouse lung to crocidolite asbestos. 2. Pulmonary fibrosis after long fibers. J Pathol 1987, 152:109–117
10. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 1951, 193:265–269
11. Woessner JF: Determination of hydroxyproline in connective tissue. Hall DA (Ed): The Methodology of Connective Tissue Research. Oxford, Joynson and Bruvvers, 1976, pp 227–233
12. Kohyama N, Suzuki Y: Analysis of asbestos fibers in lung parenchyma, pleural plaques and mesothelioma

tissues of North American insulation workers. Ann N Y Acad Sci 1991, 643:27–52
13. Churg A: Asbestos fibers and pleural plaques in a general autopsy population. Am J Pathol 1982, 109: 88–96
14. Adamson IYR, Bowden DH: Pulmonary reaction to long and short asbestos fibers is independent of fibroblast growth factor production by alveolar macrophages. Am J Pathol 1990, 137:523–529
15. Davis JMG, Addison J, Bolton RE, Donaldson K, Jones AD, Smith T: The pathogenicity of long *versus* short fibre samples of amosite asbestos administered to rats by inhalation and intraperitoneal injection. Br J Exp Pathol 1986, 67:415–430
16. Brody AR, Overby LH: Incorporation of tritiated thymidine by epithelial and interstitial cells in bronchoalveolar regions of asbestos-exposed rats. Am J Pathol 1989, 134:133–140
17. Moalli PA, MacDonald JL, Goodglick LA, Kane AB: Acute injury and regeneration of the mesothelium in response to asbestos fibers. Am J Pathol 1987, 128: 426–445
18. Bitterman PB, Adelberg S, Crystal RG: Mechanisms of pulmonary fibrosis: spontaneous release of the alveolar macrophage-derived growth factor in the interstitial lung disorders. J Clin Invest 1983, 72:1801–1813
19. Bissonnette E, Rola-Pleszczynski M: Pulmonary inflammation and fibrosis in a murine model of asbestosis and silicosis: possible role of tumor necrosis factor. Inflammation 1989, 13:329–337
20. Lemaire I, Beaudoin H, Masse S, Grondin C: Alveolar macrophage stimulation of lung fibroblast growth in asbestos-induced pulmonary fibrosis. Am J Pathol 1986, 122:205–211
21. Adamson IYR, Letourneau HL, Bowden DH: Comparison of alveolar and interstitial macrophages in fibroblast stimulation after silica and long or short asbestos. Lab Invest 1991, 64:339–344
22. Schapira RM, Osornio-Vargas AR, Brody AR: Inorganic particles induce secretion of a macrophage homologue of platelet-derived growth factor in a density and time dependent manner *in vitro*. Exp Lung Res 1991, 17:1011–1024
23. Walker C, Bermudez E, Stewart W, Bonner J, Molloy CJ, Everitt J: Characterization of platelet-derived growth factor and platelet-derived growth factor receptor expression in asbestos-induced rat mesothelioma. Cancer Res 1992, 52:301–306
24. Mangum JB, Everitt JI, Bonner JC, Moore LR, Brody AR: Co-culture of primary pulmonary cells to model alveolar injury and transport of proteins. In Vitro Cell Dev Biol 1990, 26:1135–1143