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Incorporation of Tritiated Thymidine by Epithelial and Interstitial Cells in Bronchiolar–Alveolar Regions of Asbestos-Exposed Rats

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Inhaled asbestos causes progressive interstitial lung disease. The authors have performed a series of studies to elucidate early pathogenetic events at sites of fiber deposition in asbestos-exposed rats. This study reports that a single 5-hour exposure to chrysotile asbestos induces significant increases in incorporation of tritiated thymidine (^{3}HTdR) into nuclei of epithelial and interstitial cells of bronchiolar–alveolar regions. No cell populations in air-exposed or carbonyl iron-exposed control animals exhibited more than 1% labeling at any point in time. Immediately after the 5-hour asbestos exposure, incorporation was normal. By 19 hours after exposure there was a significant increase in incorporation of ^{3}HTdR, particularly by Type II epithelial cells of the first alveolar duct bifurcations. The greatest increase in degree of incorporation (up to 18-fold) was observed 24 hours after exposure, and increased percentages of ^{3}HTdR-labeled cells were maintained through the 48 hours postexposure period. Normal labeling was present by 8 days after exposure, and this level remained through the 1-month period studied. This apparent mitogenic response correlates with increased numbers of bronchiolar–alveolar epithelial and interstitial cells demonstrated by ultrastructural morphometry in correlative studies. The authors speculate that the incorporation of ^{3}HTdR could be induced by the direct effects of inhaled fibers or by mitogenic factors released from macrophages attracted to the inhaled asbestos. (Am J Pathol 1989, 134:133–140)

Inhalation of asbestos fibers results in progressive interstitial pulmonary fibrosis in humans[1] and experimental animals.[1-3] To establish the pathogenesis of this disease at the cellular level, we have developed an animal model wherein rats exhibit epithelial and interstitial alterations within 48 hours after a 1-hour exposure to chrysotile asbestos.[4,5] Anatomically, the initial lesion is confined to the alveolar duct bifurcations where the inhaled fibers were deposited originally[5,6] and where alveolar macrophages accumulated through the first 48 hours after exposure.[5,7-9] The lesion is characterized (48 hours after exposure) by increased numbers of alveolar and interstitial macrophages, increased volume and numbers of Type I and Type II epithelial cells, and an increased volume of the whole bifurcation region.[5] Through the 1-month period after exposure, the epithelial components appear to return to normal, but the interstitial compartment remains significantly enlarged in volume due to increased numbers of fibroblasts and a prominent non-cellular (mostly collagenous) matrix.[5]

This model of asbestos-induced fibrogenesis allows identification of the target cells on which inhaled fibers are deposited as well as the cells that respond initially by migration and phagocytosis. Alveolar and interstitial macrophages are migrating cells that actively phagocytize inhaled particles.[3-5] In addition, the alveolar epithelium[3,5,10,11] and interstitial fibroblasts[12,13] actively take up the inhaled particles. The nature and progression of the interstitial lesion is very likely due to the initial asbestos-induced cellular responses that occurred during the early 48 hours postexposure period. Chang et al,[5] as described briefly above, documented the morphometric alterations that occur, but there are no data indicating which of the cell types incorporate thymidine into DNA during this period of complex cellular responses. This information is essential in establishing a more complete understanding of the pathogenesis of the lesion and in making decisions about which cells to separate from the lung for further study *in vitro*. For example, if one postulates that a macrophage-derived factor is playing a mechanistic role in interstitial cell proliferation and matrix production during the fibrogenic response,[14-17] it will be necessary to learn as much as possible about the responses of the various cell types *in vivo*. Thus, air-exposed, iron-exposed, and as-

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bestos-exposed rats were injected with tritiated thymidine before they were killed at a wide range of times after exposure. Here we report that in the lungs of rats exposed to asbestos for 5 hours there is a striking increase in tritiated thymidine incorporation into nuclei of bronchiolar–alveolar duct epithelial and interstitial cells through the initial 19–48 hours after exposure.

Materials and Methods

Male CD (SD)BR rats (Charles River) 8 weeks old were exposed (5 rats per group) to room air (sham), to an aerosol of carbonyl iron spheres (30 mg/sq m) or to an aerosol of chrysotile asbestos (10 mg/sq m respirable mass) for 5 hours in open cages as previously described.[4,7,9] After recovery periods of 0, 19, 24, 33, and 48 hours and 1, 2, and 4 weeks after inhalation, tritiated thymidine (^{3}HTdR) (2 μCi/g) was administered intraperitoneally to the particle and sham-exposed rats. The animals were killed 4 hours after administration of the ^{3}HTdR by injection of 1 ml Na pentabarbitol (50 mg/ml) intraperitoneally. Animals then were perfused through the vasculature (by the pulmonary artery) with fixative (1% glutaraldehyde, 1% paraformaldehyde in 0.1 M Na cacodylate containing 5 mM $CaCl_2$) at 23 cm H_2O pressure for 5 minutes. The whole lungs were removed from the chest and immersed in fixative overnight as described previously.[4,7]

After tissues were fixed at least overnight, slices (2 × 5 × 10 mm) were taken at right angles to each mainstem bronchus from both right and left lungs and postfixed with 0.5% OsO_4 in veronal acetate buffer. The slices were embedded in soft Epox 812[4] and polymerized at 60 C for 15 hours. These plastic blocks were softened on a warming tray at 40 C, and slices 0.5 mm thick were cut parallel to the large tissue face with a double-edged razor blade. Bright light reflected from the surface of the cut block face clearly revealed the orientation of the lung tissue. A terminal bronchiole with its attached alveolar ducts, including the bifurcation between the ducts, was selected under a dissecting microscope and cut out of the warmed plastic block. Two terminal bronchiole–alveolar duct regions per animal were selected for a total of 10 anatomic units per treatment group. The selected tissue was glued onto a BEEM blank with epoxy glue as described previously.[4] For light microscopic autoradiography, 0.6 μ sections were cut with a diamond knife on a MT5000 microtome (DuPont, Wilmington, DE), and from the first two blocks sectioned at each time point, a thin section for electron microscopy was cut adjacent to the thick section taken for autoradiography. The thin sections were mounted on slotted grids (0.2 × 1 mm), stained with uranyl acetate and lead citrate, and photographed with a JEOL 100CX electron microscope. The autoradiography thick sections were placed on glass slides, coated with Ilford L4 emulsion, exposed for 3 weeks, developed with D-19 (Kodak, Rochester, NY), and stained with toluidine blue. For each treatment group, labeled nuclei (6 or more grains over a nucleus) were counted by light microscopy at ×100 magnification in the epithelium and interstitium of 4 anatomic units of the terminal bronchiole–alveolar duct regions: 1) terminal bronchiole, 2) alveolar duct walls between the terminal bronchiole and first alveolar duct bifurcation, 3) first alveolar duct bifurcation, and 4) ducts distal to the bifurcation (Figures 1 and 2).

Data were analyzed by Wilcoxon's Rank Sum test[18] for analysis of variance of the ranks of nonparametric values. Statistical significance was reached at a level of $P < 0.05$.

Results

Unexposed and Iron-Exposed Animals

Rats exposed only to room air (sham control) had occasional pulmonary cells which incorporated tritiated thymidine (^{3}HTdR, Figure 3). At no point in time during the course of the experiment did tissues from control animals show more than 1% labeled cells in any anatomic compartment (Figures 1–3). The same findings have been reported in other studies.[19,20]

The iron-exposed animals inhaled large numbers of small spheres, many of which were deposited on epithelial cells of the alveolar duct bifurcations (Figure 4). Previous studies have shown that this dose of particles is sufficient to cause macrophages to accumulate on the bifurcation surfaces.[9] To establish whether or not uptake of ^{3}HTdR was stimulated by nonfibrogenic iron spheres, we counted over 2000 epithelial cells and almost 1000 interstitial cells in the four designated anatomic regions (see Methods, Figures 1 and 2) between 18 hours and 2 weeks after exposure. In no animal, at any anatomic location or point in time, did more than 1% of the cells exhibit labeled nuclei. Furthermore, studies ongoing in our laboratory have shown that macrophages accumulated by 48 hours after exposure, but 1 month after exposure to the carbonyl iron spheres, no alterations could be detected by ultrastructural morphometry at alveolar duct bifurcations (unpublished observation).

Asbestos-Exposed Animals

Increased incorporation of ^{3}HTdR was first observed 19 hours after exposure and continued through the 2-day period (Figure 3). By 24 hours after exposure, and through the 33 and 48 hour time periods, 100% of the terminal bronchioles and 75–80% of the duct bifurcations exhibited at least one labeled epithelial or interstitial cell (data not shown). Ten anatomic units were analyzed at each

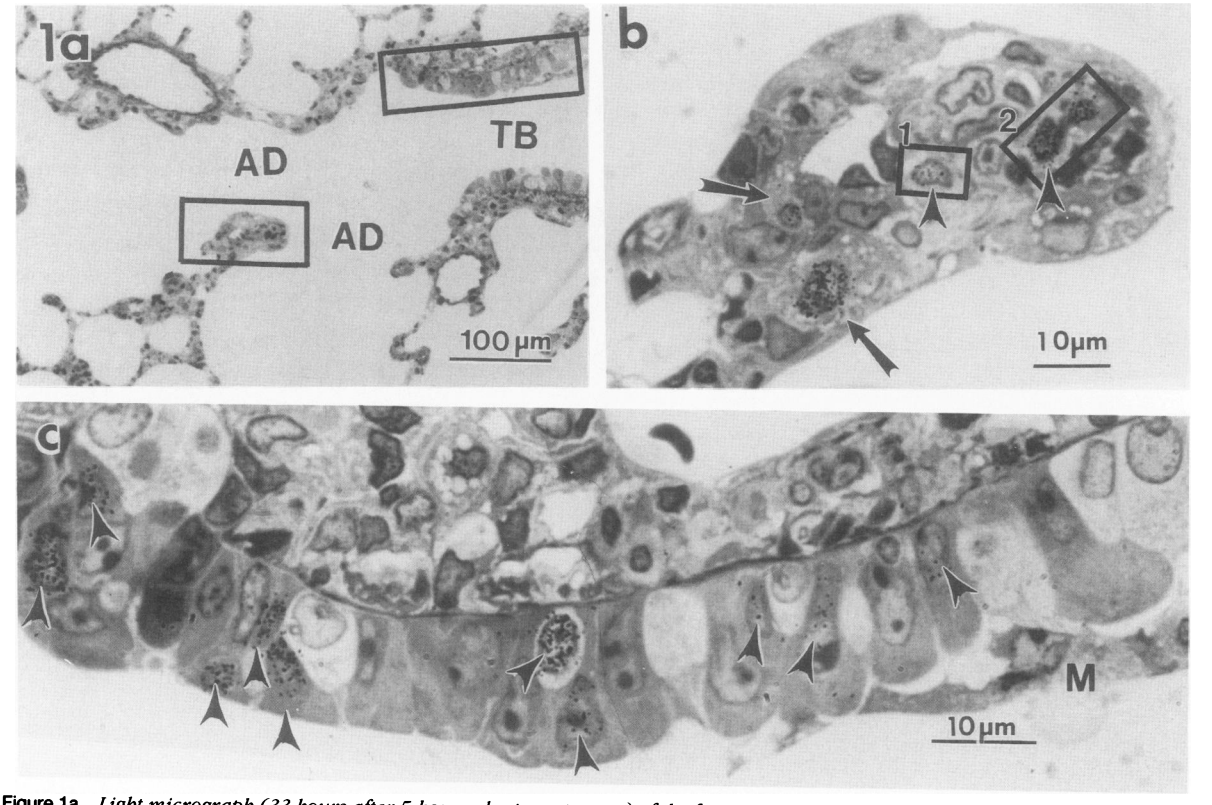

Figure 1a. *Light micrograph (33 hours after 5-hour asbestos exposure) of the four anatomic regions in which cells were counted for autoradiography. A terminal bronchiole (TB) leads into the alveolar duct region (AD) which splits at the first alveolar duct bifurcation (rectangle). This bifurcation is enlarged in (b) to show labeled cells. A portion of the TB (rectangle) is enlarged in c.* b: *First alveolar duct bifurcation enlarged from Figure 1a. Two interstitial cells (arrowheads) and two Type II epithelial cells (arrows) have been labeled with ^{3}HTdR. The two interstitial cells (boxes 1 and 2) are enlarged by electron microscopy in Figure 2.* c: *Portion of the TB demarcated in Figure 1a. At least 10 cells (arrowheads) in this segment of the TB have incorporated ^{3}HTdR. A cluster of macrophages (M) being cleared from the airway lumen is observed.*

time point to establish the percentages of the various cells labeled (Figure 3). There was no evidence of increased incorporation of ^{3}HTdR by any cell type immediately after the 5-hour exposure to asbestos (Figure 3). The epithelial cells of the first alveolar duct bifurcations were the first to show a clear response, and by 1 day after exposure, these cells exhibited an 18-fold increase over control levels (Figure 3). By 2 days after exposure, both the epithelial and interstitial compartments were returning to normal percentages of labeling, and normal levels were maintained through the remainder of the 30-day period studied (Figure 3).

The largest increase in ^{3}HTdR uptake was observed in the alveolar epithelial cells of first alveolar duct bifurcations (Figures 1 and 3). Here, and in the proximal alveolar ducts, the predominant epithelial cell type that incorporated ^{3}HTdR was the Type II cell. Figure 1 illustrates several cell types that obviously exhibit nuclei labeled with ^{3}HTdR. At least one of these clearly is a type II cell that is recessed in the alveolar wall and contains numerous vesicles. The electron micrograph (Figure 2b) of the tip of this bifurcation shows that numerous asbestos fibers remain within and on the alveolar epithelium at this period 33 hours after exposure. By this time, fibers have been translocated to the interstitium as well (see below).[5,10]

Significant percentages of interstitial cells also incorporated ^{3}HTdR. Because it was difficult to be sure that the labeled cells were indeed interstitial, 22 cells identified by light microscopy as ^{3}HTdR-labeled interstitial cells were thin-sectioned for further study by electron microscopy. All of these cells proved to be interstitial, but they apparently were undifferentiated. Thus, it was not possible to determine the precise identity of each cell type. Two of the labeled interstitial cells are illustrated by light microscopy in Figure 1b and by electron microscopy in Figure 2. They appear to be monocyte or macrophagelike, and one contains asbestos fibers in a phagolysosomallike structure (Figure 2a).

Epithelial cells of the terminal bronchioles also exhibited significant increases in incorporation of ^{3}HTdR (Figures 1 and 3). Nonciliated Claralike cells appeared to be the only epithelial cell type labeled (Figure 1a). Inasmuch as labeling of airway cells in normal animals is so low (<1%), the high percentages of labeled Clara cells in the asbestos-exposed animals were striking. In addition, interstitial fibroblastlike cells of the airway submucosa clearly

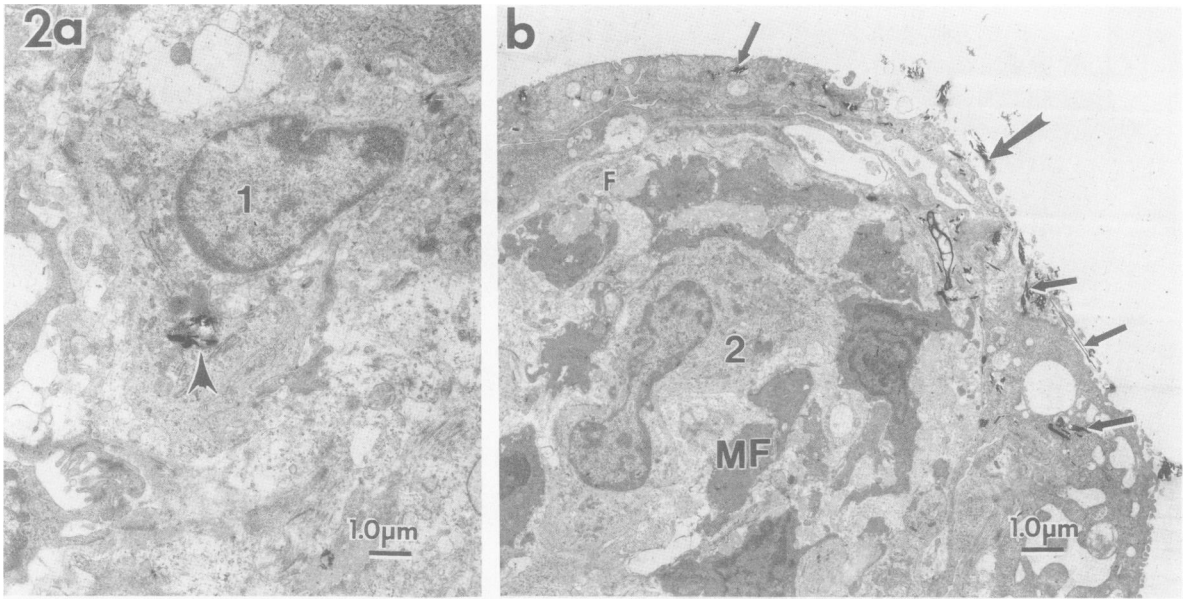

Figure 2a, b. *Electron micrographs of portions of the first alveolar duct bifurcation illustrated in Figures 1a and b. The labeled interstitial cells (#1 and #2) are macrophagelike and are surrounded by fibroblasts (F) and myofibroblasts (MF). One of the labeled cells (#1) has phagocytized asbestos fibrils (arrowhead). Note the abundance of asbestos fibers (arrows) on and within the alveolar epithelium (b) (see reference 10 for further details on epithelial uptake of fibers).*

were labeled (Figure 5), but the precise nature of this cell type was not ascertained.

Asbestos-induced accumulations of macrophages in alveolar spaces and airways were identified (Figure 1 and 2) at 19, 24, 33, and 48 hours after exposure.[7,8] No incorporation of ^{3}HTdR into nuclei was detected in 1500 of these macrophages observed in 20 animals.

Alveolar ducts distal to the first bifurcation (Figure 1) also were studied, but there were no significant increases in uptake of ^{3}HTdR by any cells of the distal ducts (data not shown).

Discussion

In previous studies, we have shown that a brief exposure to chrysotile asbestos induces cellular alterations and a progressive interstitial lesion at the first alveolar duct bifurcations where the fibers were deposited originally.[4,5] In attempting to understand the pathogenesis of this lesion, the present study demonstrates that a single 5-hour asbestos exposure causes increased percentages of both epithelial and interstitial cells of the bronchiolar–alveolar regions to incorporate tritiated thymidine by 19 hours after

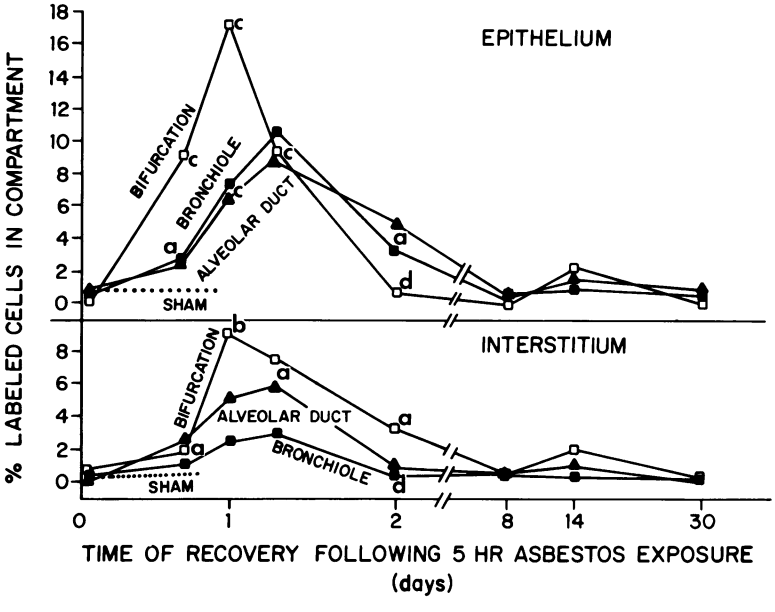

Figure 3. *The percentages of each cell type labeled with ^{3}HTdR. Note the dramatic increase in labeled epithelial and interstitial cells between 19 and 33 hours after exposure. Statistical comparisons* vs. *sham-exposed animals; Wilcoxon's Rank Sum Test[18]:* **a:** $P < 0.05$ **b:** $P < 0.005$ **c:** $P < 0.0005$ **d:** *NS.*

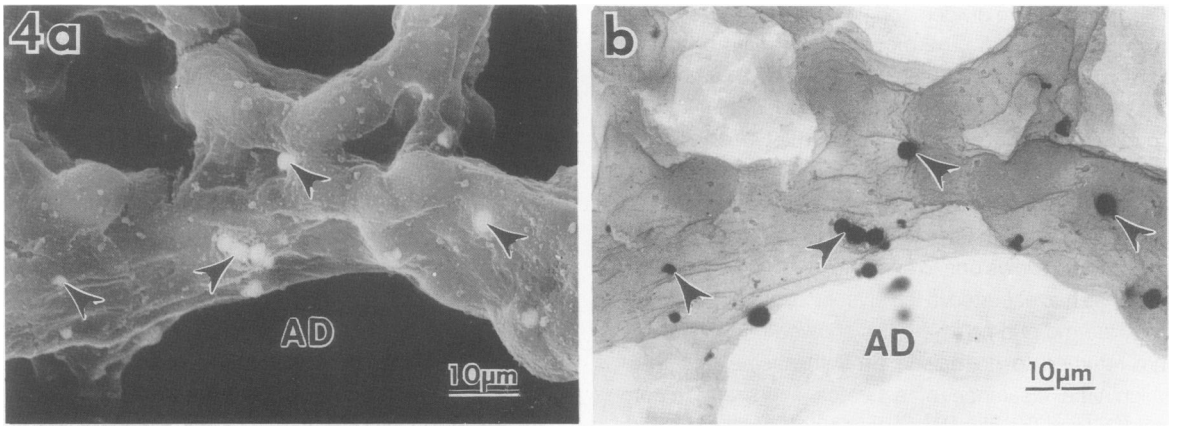

Figure 4a. *Scanning electron micrograph of the surface of a first alveolar duct bifurcation in the lung of a rat exposed to carbonyl iron spheres (arrowheads) for 5 hours. Immediately after exposure, numerous spheres are observed on the Type I epithelium. The alveolar duct (AD) and adjacent alveolar spaces are evident. The lungs of iron-exposed animals exhibited no increase in ^{3}HTdR incorporation by any cell types.* **b:** *Backscattered electron image of the same field shown in a. The distribution and number of iron spheres is highlighted by this technique (see references 4 and 9).*

exposure (Figures 1–5). This finding is in accordance with our recent morphometric studies on asbestos-exposed rats.[5] In that study, we showed that 48 hours after a single 1-hour asbestos exposure, epithelial cells of the first alveolar duct bifurcations were significantly increased in volume and number. One month after the exposure, the epithelial alterations had largely returned to normal, but there was an increased volume and number of myofibroblasts, fibroblasts, and interstitial macrophages as well as an increase in noncellular interstitial matrix, mostly collagen.[5] In addition, the entire bifurcation region was increased in volume. Interestingly enough, lung tissue sectioned randomly from distal regions of alveolar ducts showed no alterations in any anatomic compartment.[5] This coincides with our finding here of no increases in uptake of thymidine in the distal lung parenchyma. Because the earliest asbestos-associated lesion in the bronchiolar–alveolar region of the rat lung is characterized by increased numbers of epithelial and interstitial cells,[5] it seems likely that the lesion is initiated through a fiber-induced mitogenic effect. This effect appears to correlate well with the time-related events of fiber translocation and macrophage accumulation. Inhaled asbestos fibers can be found in type I epithelial cytoplasm 4 minutes after a 1 hour exposure.[10] Numerous fibers are located in or on this epithelium over the ensuing 4 days (Figure 2),[10] and this is precisely the period when increased numbers of Type I and Type II cells are apparent by morphometry,[5] presumably due to cell proliferation and transition from Type II to Type I alveolar cell phenotype. Furthermore, asbestos fibers reach the lung interstitium by 48 hours after exposure.[5,10] As reported here (Figure 3), increased ^{3}HTdR incorporation was noted in the alveolar epithelial compartment by 19 hours after exposure, but this response was initiated several hours earlier than our first observation was made. Thymidine incorporation by interstitial cells of the bifurcation appears to lag by 4–5 hours (Figure 3), and this is consistent with the timing of fiber deposition and subsequent translocation as just discussed. Complement-dependent macrophage accumulation also occurs in this same time frame,[8] and this potentially important phenomenon will be discussed below.

The mechanism through which an asbestos-induced mitogenic response might be mediated is unknown. Two possibilities seem to be reasonable based on what we know about the pathogenesis of the lesion. First, it is clear that inhaled asbestos fibers are deposited at all levels of the respiratory tract.[21] It is conceivable that the fibers that are distributed initially on epithelial membranes (Figure 2)[6] and are then taken into the cytoplasm of Type I alveolar epithelial cells[10] could directly provoke a mitogenic response in this epithelial cell population. We have presented no direct evidence in this paper that the epithelial

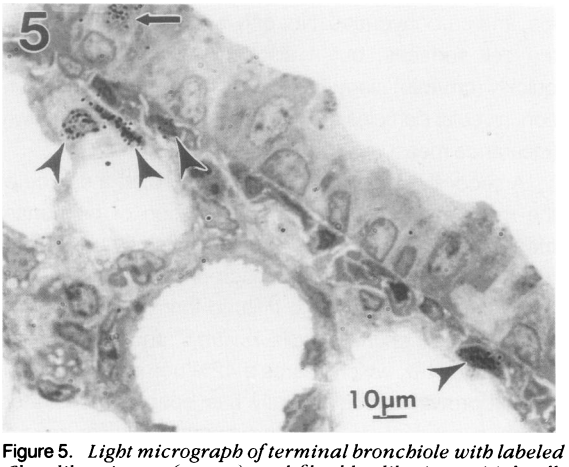

Figure 5. *Light micrograph of terminal bronchiole with labeled Claralike airway (arrow) and fibroblastlike interstitial cells (arrowheads).*

cells actually have divided and have not just incorporated thymidine into DNA. The morphometric data on this lesion, however,[5] clearly showed increased numbers of Type I and Type II cell at the alveolar duct bifurcations. Compared with unexposed controls, the type I cells were smaller in average volume while the Type II cells were enlarged. This suggests that there were increased numbers of cells in stages of transition from Type II to Type I morphology and allows one to speculate that a true mitogenic response has occurred in the alveolar epithelium. Interestingly, the morphometric studies showed that this epithelium returned to normal by 1-month after exposure, at which time clearance of asbestos fibers and macrophages from the alveolar surfaces had occurred.[5] Even though Type II cells very rarely take up fibers, it is the response of the Type I cells that can dictate a proliferative response in Type II cells. This has been shown clearly in inhalation studies where injury to Type I cells by oxygen[22,23] or NO_2[23] results in a mitogenic response by Type II cells. The fact that the Type I cells take up asbestos fibers and rapidly become thicker,[5] along with the development of a small but significant leak of serum proteins into the lung,[8] support the notion that the inhaled asbestos could induce a mitogenic effect through direct (perhaps membrane) interactions with alveolar epithelial cells. Marsh and Mossman[24] showed that uptake of fibrous minerals (including asbestos) by airway epithelial cells *in vitro* induced ornithine decarboxylase (ODC) activity and increased incorporation of ^{3}HTdR. ODC is a tightly regulated enzyme that is increased when cells respond to growth promoting stimuli. The investigators concluded that asbestiform minerals have direct effects on airway epithelial cells, causing, among other responses,[24] ODC production and cell proliferation. Direct effects on the terminal bronchiolar epithelium seem to be less likely to occur *in vivo,* yet these cells exhibited a highly significant increase in incorporation of ^{3}HTdR. Bronchiolar cells have not been shown to phagocytize asbestos fibers *in vivo,* and they are covered by a layer of secreted proteins, lipids, and carbohydrates. Not only would this layer protect the cell surfaces, but it also carries inhaled particles quickly (cm/min) along the airway surfaces.[25] In comparison, alveolar particles are known to move relatively slowly (clearance measured in hours and days).[25]

A second possibility to be considered as a source of the mitogenic stimulus is the population of pulmonary macrophages that responds to the inhaled fibers.[4,5,7,8] An as-yet unidentified alveolar macrophage-derived factor has been shown to induce tritiated thymidine incorporation by Type II epithelial cells *in vitro,*[26] and transforming growth factor-α, which shares a 42% homology with epidermal growth factor, recently has been demonstrated to be a product of activated human alveolar macrophages.[27] Furthermore, macrophage-derived growth factors for mesenchymal cells have been clearly demonstrated.[14,15,28] Because increased numbers of pulmonary macrophages accumulate in the bronchiolar–alveolar regions and phagocytize the inhaled fibers during the 24–48 hour postexposure period,[7,8] it is conceivable that these cells secrete mitogenic factors that induce the epithelial and interstitial cells to incorporate tritiated thymidine during proliferation. Although this hypothesis remains to be proven, the anatomic distribution of alveolar and interstitial macrophages appears to be appropriate to satisfy the relationships required of the various cell types.[5] In addition, we have shown in ongoing studies using alveolar[15,16] and interstitial[17] macrophages that asbestos and other particles stimulate the production of a pulmonary macrophage-derived homologue of platelet-derived growth factor (PDGF) that is mitogenic for rat lung fibroblasts *in vitro.* The data presented in the present paper suggest that we must begin our search for growth factor production *in vivo* within the first few hours after initiation of the exposure regimen. At this juncture in our studies, it is not possible to know just when the macrophages begin to produce the putative mitogenic factors, how long they might continue to secrete them, and whether or not fibroblasts and epithelial cells respond *in vivo* as they have been shown to do *in vitro.*

Interestingly enough, Adamson and Bowden[11] showed that instilled asbestos induced fibrosis in mice and was associated with increased incorporation of ^{3}HTdR by macrophages as well as by epithelial and interstitial cells of the bronchiolar and alveolar walls. It is clear that asbestos fibers cause an early significant incorporation of ^{3}HTdR by a variety of pulmonary cells. The fact that a lesion persists at the alveolar duct bifurcations for at least 1 month after exposure[5] suggests that the asbestos fibers that have been translocated to the interstitium continue to exert some influence upon the fibroblasts and/or macrophages which inhabit this compartment. As discussed above, the mechanism is not at all clear, but the timing of the apparent mitogenic response soon after exposure, followed by production of collagen, suggests that a "competence" and/or "progression" signal[14,15,17] could induce the initial response presented as ^{3}HTdR incorporation in this paper (Figure 3). The continued increase in cell number and extracellular matrix production could be stimulated by additional macrophage-derived growth factors such as transforming growth factor-β (TGF_β)[29] and interleukin-1 (IL-1).[30] It also is interesting to note that several of the macrophage-derived growth factors, including PDGF,[28] TGF_β, and IL-1, are chemotactic for macrophages and fibroblasts. This could account at least in part for the increase in cell numbers observed at sites of asbestos deposition and macrophage accumulation.

The epithelial cell types labeled in our study were relatively easy to identify, ie, Clara cells of the terminal bronchioles and Type II cells at the alveolar level. This is consistent with an extensive body of literature showing that

these two cell types provide the stem cell populations for their respective anatomic locations.[31-33] But the nature of dividing interstitial cells is more difficult to ascertain. It has been shown clearly that 2–3% of resident alveolar macrophages have the capacity to divide in the lung under normal circumstances.[34,35] The numbers of dividing macrophages appear to increase in animals treated with oxidant gas[36] and instilled carbon particles.[37] A segment of this dividing population of macrophages is found in the lung interstitium as well.[11] Because interstitial fibroblasts also are incorporating ^{3}HTdR, it is difficult to be sure, even by electron microscopy, which cell type is labeled and has been counted. Indeed, the original studies of Evans and Bils[38] showed that 13% of ^{3}HTdR-labeled cells in normal alveolar walls were unidentified interstitial cells. Our data suggest that both macrophagelike and fibroblastlike interstitial cells have incorporated ^{3}HTdR, and this is consistent with the morphometric data.[5] It is interesting to note that both cell types phagocytize inhaled asbestos fibers that have been translocated to the interstitium and remain for months after exposure.[12,13] Further studies using immunohistochemistry and a radiolabel over an extended period will be necessary to trace the dividing interstitial population as the cell types differentiate and thus can be identified. It is conceivable that there is a population of undifferentiated interstitial cells that responds rapidly to lung injury and provides a resident stem cell population.

In conclusion, we have shown that a single brief exposure to chrysotile asbestos causes a significant increase in incorporation of ^{3}HTdR by epithelial and interstitial cells of bronchiolar–alveolar regions in the lungs of rats exposed to asbestos fibers, but not to air or iron spheres. The time-course of ^{3}HTdR incorporation coincides with the progression of epithelial and interstitial lesions at sites of fiber deposition.[5] The mechanisms controlling the development of the lesion are not clear, although it seems conceivable that direct fiber-induced cell injury and/or mitogenic factors secreted by populations of pulmonary macrophages could cause cell proliferation in the appropriate anatomic locations. Further studies to test these hypotheses are ongoing.

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