Characterization of the Bronchoalveolar Cellular Response in Experimental Asbestosis

Different Reactions Depending on the Fibrogenic Potential[1-3]

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SUMMARY Analysis of bronchoalveolar cell types and structure was performed during the development of asbestos-induced lung injury in the rat. Animals received single intratracheal injections of one of the following: saline (control), UICC chrysotile B asbestos (5 mg), or very short 4T30 chrysotile fibers (5 mg). Bronchoalveolar lavage (BAL) was performed at various intervals after instillation. Analysis of BAL fluid showed significant increase in inflammatory cells in response to asbestos, which persisted longer in animals treated with chrysotile B. Presence of numerous mitotic figures in BAL fluid of treated animals suggests that macrophage replication may contribute in part to this response. Differential cellular analysis indicated that after injection of long chrysotile fibers, which causes fibrotic lesions within 7 days, polymorphonuclear leukocytes (PMN) appear as early as Day 1 in significant concentration (40%) in the bronchoalveolar compartment and persist through Day 7 after treatment. From Day 7 to Day 21, multinucleated cells (MGC) were found in lavage fluid (5 to 8%). Most of these cells were binucleated, and none had more than 3 nuclei. By contrast, exposure to very short chrysotile fibers caused only a very transient influx of PMN on Day 1. By Day 7, there was a significant increase in MGC, which persisted through Day 21, at which time no fibrosis was apparent. Although most of these cells were binucleated, many cells had 3 or more nuclei. The giant cells were predominantly of the foreign body type, with MGC of the Langhans type also present. Our data demonstrate that in this rat model, lung alveolitis and fibrosis are associated with different bronchoalveolar cellular reactions and raise the question of a possible role for PMN and MGC in modulating macrophage function and lung response to injury.

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Introduction

Pulmonary fibrosis results from the inability of the lung to repair its altered structure. The mechanisms underlying the onset of fibrosis are unknown, but valuable insights into the pathogenesis of pulmonary fibrosis may be gained by studying animal models of the disease. Asbestos has been shown to cause pulmonary fibrosis in humans (1, 2) and in laboratory animals (3–11). Using inhalation or intratracheal instillation as the method of exposure, various investigators have demonstrated the histologic and physiologic similarities of asbestos-induced fibrosis in animals and asbestosis in humans (1–11). Inhalation exposure provides a physiologic model system that allows for the natural clearance of inhaled minerals, but it often requires months of experimentation and necessitates elaborate and expensive facilities. On the other hand, intratracheal instillation has some advantages because it is rapid, easily performed, and satisfactory in reproducing the histopathologic features of fibrosis in various animal models (8–11). Depending on their concentration, length, and aerodynamic diameter (12), asbestos fibers may reach the alveoli and eventually the pulmonary interstitium (13, 14). Cells of the bronchoalveolar compartment are among the first to react to this insult, and it is assumed that an orderly sequence of changes in their state and function must take place in order to protect the lung integrity. During these responses cells may interact with others or with humoral components, leading to nonspecific reactions as well as to more specific events involved in the fibrotic process.

Recently, in establishing a rat model of the disease, we have shown that asbestos fibers with similar chemical composition but different fiber length caused lung lesions that differed considerably both in nature and localization (10). In the present study, we compared the progressive bronchoalveolar cellular response to these 2 asbestos samples with different fibrogenic potential. Our data demonstrate that bronchoalveolar cell alterations correlate with differences in lung injury, and further document some aspects of alveolar cell response.

Methods

Animals

Male Wistar rats weighing 250 to 300 g were purchased from Charles River Canada Inc. (St-Constant, Québec). These animals were derived from a pathogen-free colony, shipped behind filter barriers, and housed in isolated temperature-controlled quarters. They were given Purina Chow and water ad libitum and were used 1 wk later. No control animals or animals injected with asbestos fibers showed evidence of infection when evaluated by histochemical staining.

Asbestos Fibers

The UICC standard sample of chrysotile B was obtained from the National Research Institute for Occupational Diseases, Johannesburg, South Africa. None of the fibers present in this sample was smaller than 2 μm, and 21% were longer than 10 μm (15). Very short 4T30 chrysotile fibers were isolated from Quebec 4T30 chrysotile, as described by Jolicoeur and coworkers (16). More than 98% of these fibers were smaller than 3 μm, with 50% smaller than 0.5 μm and 100% smaller than 8 μm. Except for differences in mean length and in specific surface area (short 4T30),

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m^2/g; UICC, 26.8 m^2/g) these 2 chrysotile asbestos samples had similar chemical and structural properties (10). Each sample was autoclaved for 45 min and suspended in sterile phosphate-buffered saline (PBS) (pH, 7.4) with a Dounce glass homogenizer prior to instillation into the animals.

Induction of Pulmonary Fibrosis

Rats were lightly anesthetized with a mixture of ketamine-xylazine (85%-15%, 100 mg/kg). The trachea was exposed surgically, and the asbestos suspension or saline was injected in a final volume of 0.5 ml. Three groups of 6 rats each received, respectively, a single intratracheal injection of saline (control), UICC chrysotile B (5 mg), and very short 4T30 chrysotile (5 mg). Animals were killed periodically (1, 7, 14, and 21 days) after instillation. Fifteen animals were killed at each time interval and evaluated for histologic changes, as described previously, and by bronchoalveolar analyses.

Bronchoalveolar Lavage

Animals were injected with sodium pentobarbital (30 mg/rat). The abdominal aorta was severed, and the trachea was cannulated. The lungs were lavaged with a total volume of 50 ml PBS in 5-ml aliquots. After recovery, lavage fluid was centrifuged at 500 *g* at 4° C for 10 min. The cell pellet was washed twice in PBS and finally resuspended in Dulbecco's modified Eagle medium (DMEM) (Grand Island Biological Co., Grand Island, NY). Cells were counted in a hemocytometer chamber, and viability was determined by trypan blue exclusion.

Differential Cellular Analysis

Differential counts of lavage cells were made from cytocentrifuge smears prepared with 2.5 × 10^4 cells and stained with Wright-Giemsa stain.

Multinucleated Giant Cells

The occurrence of multinucleated giant cells (MGC) in bronchoalveolar cells was quantitated by counting 10^3 cells and calculating the proportion of cells that contained 2 or more nuclei. In addition, the proportion of MGC was evaluated by the macrophage-fusion index, calculated by dividing the number of nuclei within MGC by the total number of nuclei in at least 10^3 cells, as described by Crawford and coworkers (17).

Statistical Analysis

Results are expressed as mean values ± SEM. The significance of differences between asbestos-treated and control groups was determined using Student's *t* test ($p < 0.05$).

Results

Asbestos-induced Lung Injury

Histologic evidence of lung injury was observed within 7 days after asbestos exposure. The histopathologic changes in the lung were essentially the same as we described previously (10). Treatment with UICC chrysotile B asbestos, which contains a high proportion of long fibers (21% smaller than 10 μm), caused typical fibrotic lesions consisting of a focal fibroblastic proliferation in peribronchiolar tissues, which distorted and obstructed small airways. The predominant lesions were located in and around terminal bronchioles, with some lesions located at bifurcations of the distal airways. By contrast, animals treated with the same dose of very short 4T30 chrysotile fibers (98% smaller than 3 μm) had lung lesions confined to the alveolar structures. It was characterized by multifocal septal thickening and alveolar distortions caused by interstitial mononuclear cell infiltration. The small airways were normal, and no evidence of fibrosis was found 60 days after exposure to this type of asbestos fibers.

Bronchoalveolar Cell Recovery at Various Stages after Asbestos Exposure

Lavage fluid recovery was 45 ml for control animals and, respectively, 44 and 40 ml for rats treated with very short 4T30 chrysotile and chrysotile B. For comparison, values of total cell counts in lavage fluid of asbestos-exposed groups were corrected for lavage fluid recovery, and all values were expressed as cell counts per 45 ml lavage fluid recovered. The BAL fluid of saline-injected animals contained an average of 5.9 × 10^6 cells 1 day after instillation, and no significant change was seen in the number of BAL cells of these animals on subsequent days after instillation. However, there was a significant increase in the total number of cells recovered from the lavage fluid of animals exposed to very short 4T30 fibers (12.5 × 10^6) and chrysotile B (9 × 10^6 cells) on Day 1 after treatment (figure 1). Higher cell counts were observed on Days 7 and 14 in these 2 groups of animals and returned to control values by Days 21 and 60, respectively, after treatment. Thus, the accumulation of inflammatory cells was more persistent in rats treated with long chrysotile fibers.

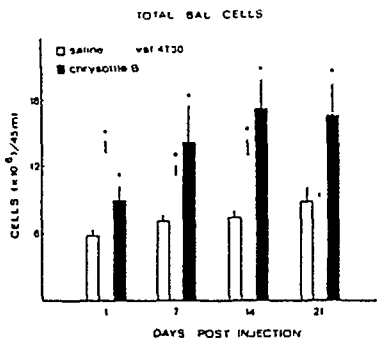


Fig 1. Total cell counts of lavage fluid at various times after intratracheal injection of saline, very short 4T30 chrysotile and UICC chrysotile B. Values are mean ± SEM in 5 animals. Significantly different from control (* = $p < 0.05$).

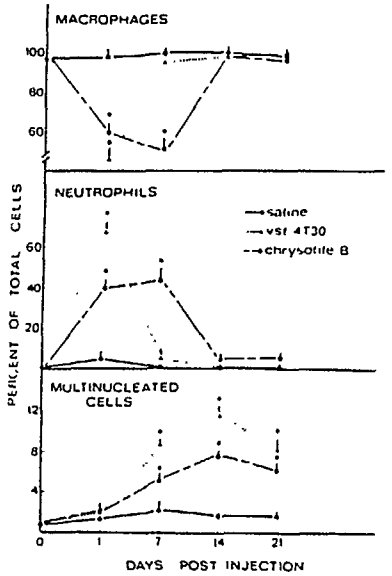


Fig. 2. Differential analysis of BAL cell populations at various times after asbestos exposure. Values are mean ± SEM in 5 animals. Significantly different from control (* = $p < 0.05$).

Differential Cellular Analysis and Kinetics of Cell Populations of Lavage Fluid

Lavage fluid obtained from saline-injected animals consisted of 97% macrophages with no eosinophils and very few polymorphonuclear leukocytes (PMN) (1%) and lymphocytes (1%). No change in the cell populations of these animals occurred throughout the study. Intratracheal injection of UICC chrysotile B asbestos caused a significant increase in PMN, which began to appear in the lavage fluid on Day 1 after treatment. The infiltration of PMN (44% of the total cells) was still present on Day 7 and returned to normal by Day 14 (figure 2). By contrast, a very transient increase in PMN seen on Day 1 only occurred in response to very short 4T30 chrysotile fibers (figure 2). The absolute number of macrophages (total cells times differential percentage of macrophages) increased significantly in response to both asbestos samples (from 5.9 × 10^6 in the control group to 15.5 × 10^6 and 11.2 × 10^6, respectively, in the chrysotile B and 4T30 chrysotile groups on Day 14).

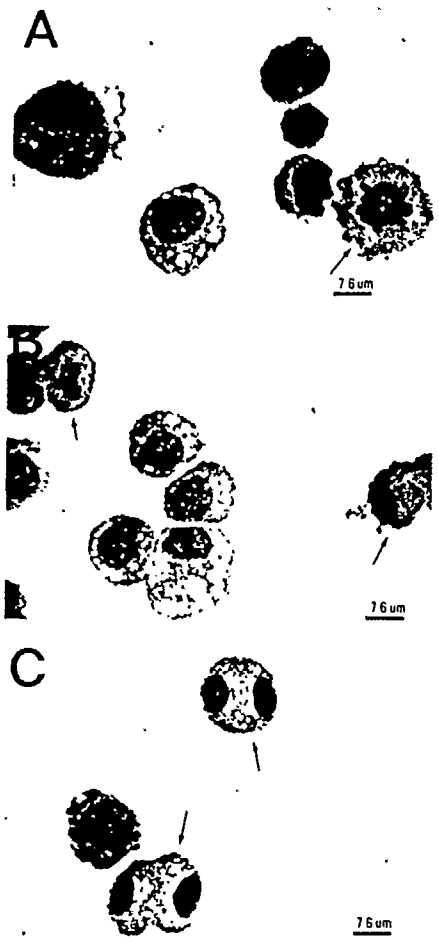
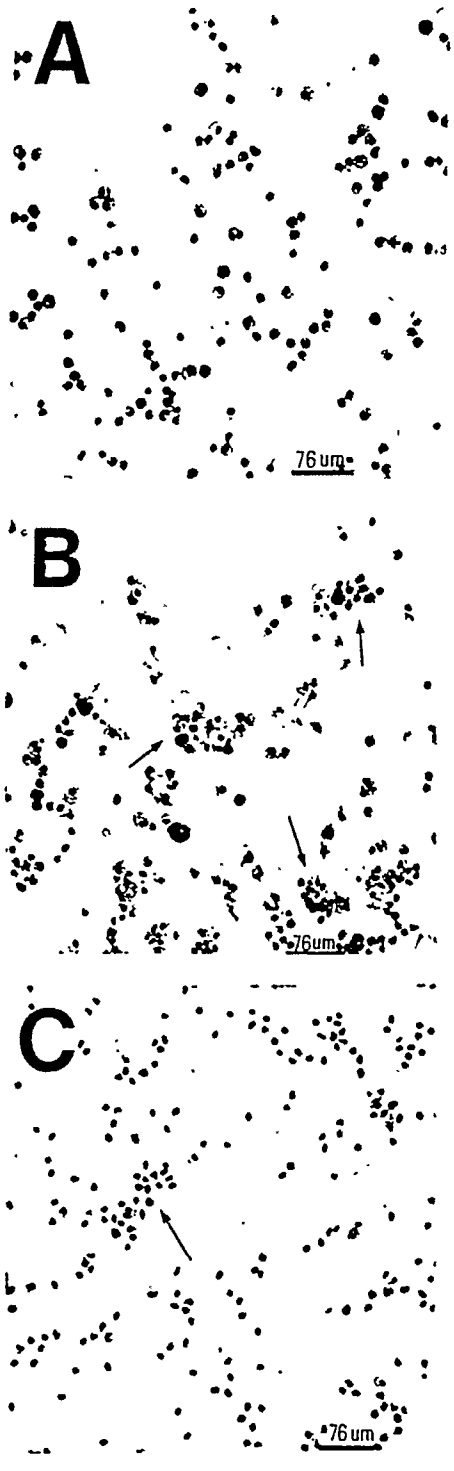


Fig. 3. Presence of mitotic figures in lavage fluid of asbestos-exposed rats. Cytocentrifuge smears were prepared and stained with Wright-Giemsa. A. Metaphase-anaphase (*arrow*). B and C. Telophase (*arrows*); original magnification: x 1,310.



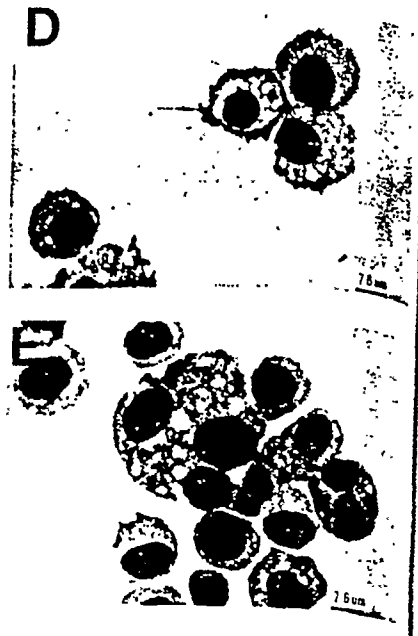


Fig. 4. Microscopic evidence of cell aggregates and cell contact in cytocentrifuge smears of lavage fluid rats exposed to asbestos. Cell preparations stained with Wright-Giemsa. A. Control. B and C Presence of cell aggregates in BAL fluid of rats tre ed with very short 4T30 fibers (B, *arrows*) and UIC chrysotile B fibers (C, *arrow*); original magnificat x 130 D and E. Evidence of cell contact betwee and more bronchoalveolar cells in lavage fluid of a bestos exposed rats; original magnification: x 1,1

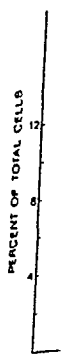


Fig 5. D s cells (3 or asbestos-e 10^5 cells Values are represent dex). Sig 0 05).

As illustrated in figure 3, microscopic evidence of cell replication was found in lung lavage of rats exposed to asbestos. The occurrence of mitotic figures, including anaphase and telophase in lavage fluid of these animals, was 3 to 5 times higher than in control animals, and suggests that local macrophage replication may contribute in part to this response. From Day 7 to Day 21, changes in the morphology of lung free cells were seen, and other cell populations consisting of MGC appeared in the bronchoalveolar milieu of rats treated with both asbestos samples (figure 2). Peak accumulation of MGC was seen 14 days after asbestos exposure and made up 7 and 11% of total cells, respectively, after treatment with UICC and 4T30 chrysotile compared with 1% in control animals. No significant changes in lymphocyte and eosinophil concentrations were observed at any given interval after asbestos exposure.

Analysis of Multinucleated Cells Present in Lavage Fluid

Microscopic analysis of cell preparations revealed the presence of numerous cell aggregates in lavage fluid of animals exposed to very short chrysotile fibers (figure 4B) compared with that seen in control animals (figure 4A). Aggregates were also present in rats treated with chrysotile B asbestos, although to a lesser extent (figure 4C). With respect to cell size, shape, and surface membrane characteristics, considerable heterogeneity was apparent in these preparations, and many cells showed vacuolate cytoplasm and long cytoplasmic extensions. In addition, evidence of cellular contact between two or more bronchoalveolar cells was commonly seen in BAL fluid of animals treated with short 4T30 fibers (figures 4D and

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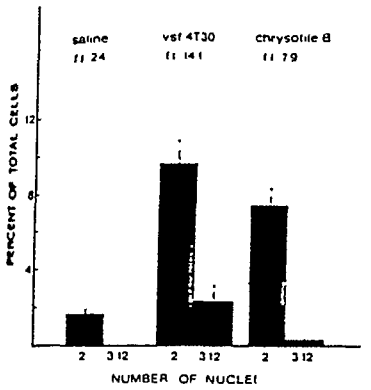

Fig. 5. Distribution of binucleated and multinucleated cells (3 or more nuclei) in lavage fluid of control and asbestos-exposed rats 14 days after instillation. The 10^3 cells were counted by 2 different observers. Values are expressed as percent of total cells and represent mean ± SEM of 5 animals (f.i. = fusion index). Significantly different from control (* = $p < 0.05$).

4E), which lends support to the concept that the formation of MGC probably arises from cell fusion. Some cells had as many as 16 nuclei, but cells with 3 to 7 nuclei were more commonly observed. The distribution of MGC in relation to the number of nuclei was determined in control and asbestos-treated animals 14 days after instillation. The number of binucleated cells was significantly higher in both groups of asbestos-treated animals (9.6 and 7.4% of total cells, respectively, for short 4T30 and UICC chrysotile B fibers) than in the control group (1.7% of total cells). However, giant cells with 3 or more nuclei were almost exclusively present in lavage fluid of rats exposed to very short asbestos fibers, where they represented 2.5% of total cells (figure 5). Similarly, the fusion index, a measure of cellular multinuclearity, was significantly higher in animals treated with very short fibers (14.1) than in animals treated with chrysotile B (7.9) and in control animals (2.4) (figure 5). The MGC seen in lung lavage displayed 2 types of nuclear arrangements: the majority had large nuclei spread in the whole cytoplasm (figure 6A) typical of the foreign body type giant cells, but a few had nuclei arranged at the periphery of the cell in an orderly fashion (figures 6B and 6C) characteristic of the Langhans type giant cell.

Discussion

Using a rat model we have compared the early and sequential bronchoalveolar cellular alterations after exposure to chrysotile asbestos fibers with different fibrogenic potential (10). A common finding in our study was the significant increase in total bronchoalveolar cells in both groups of asbestos-exposed rats. However, the nature and the duration of the inflammatory cell accumulation differed after exposure to these 2 chrysotile preparations. Even though an early and significant influx of PMN in the bronchoalveolar compartment occurred in response to both asbestos samples, the BAL fluid infiltration of PMN persisted longer in rats with fibrotic lesions and was only very transient in animals without fibrosis. This phenomenon preceded and paralleled the appearance of the fibrotic lesions and disappeared at a later stage of the disease. Similar increases in BAL neutrophils have been reported in experimental asbestosis (18, 19) and silicosis (20), and these cells have been implicated in the pathogenesis of pulmonary fibrosis (21). However, the role of PMN in fibrogenesis remains controversial. Whereas some investigators have demonstrated that wound healing occurs normally in the absence of PMN (22), others have presented data suggestive of a restraining role for PMN in the fibrotic process (23, 24). In this regard, it is worth mentioning that neutrophils have the potential to counteract collagen deposition by producing collagenase (25) and to inhibit fibroblast proliferation by releasing toxic mediators for fibroblasts (26). It is possible that PMN accumulation in BAL fluid of asbestos-treated animals may be secondary to the initial lung injury. Their presence may represent an index of the extent of lung injury rather than a determinant factor in the development of fibrosis. Alternatively, PMN accumulation in BAL fluid, although transient, may modulate adjacent cell functions and interfere in the initial stage of the disease process.

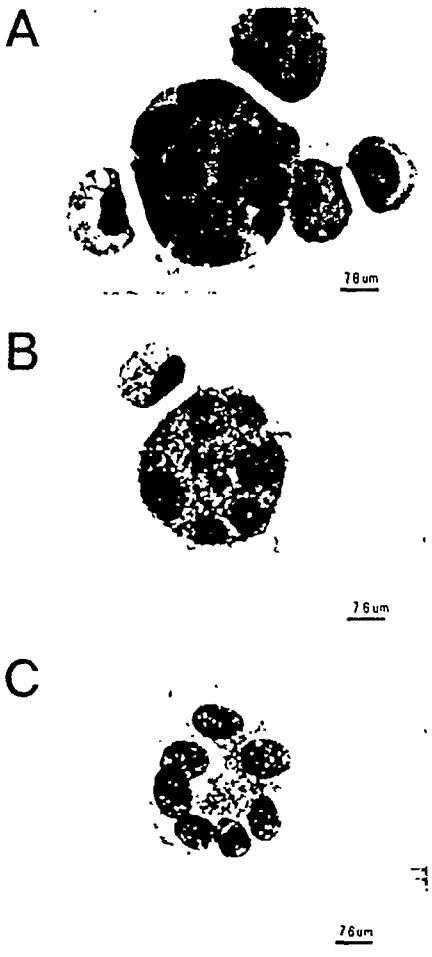

Fig. 6 Morphology of multinucleated cells present in lavage fluid of rats exposed to very short 4T30 chrysotile fibers. Cytocentrifuge smears were stained with Wright-Giemsa. A. Nuclei spread in the whole cytoplasm. B and C. Nuclei forming a ring at the periphery of the cytoplasm; original magnification: × 1,310

Our results also demonstrate that chrysotile B asbestos caused a more sustained increase in bronchoalveolar cells than did very short 4T30 chrysotile fibers. This increase was due largely to macrophages, and there may be a relationship between the persistence of macrophage accumulation and the severity of the lesions observed. Similar increases in macrophages have been reported after asbestos inhalation in rats (18) or intratracheal injection in sheep (27) and guinea pigs (28). It correlates well with the histopathologic finding of a macrophagic accumulation in lung lesions of experimental asbestosis (10, 11, 29). Although higher macrophage number in the bronchoalveolar milieu may be attributed to recruitment of blood monocytes, our finding of mitotic figures in BAL fluid of animals exposed to asbestos supports the assumption that macrophage replication may also contribute to this phenomenon. The proliferative capacity of the alveolar macrophage has already been demonstrated (30), and macrophage division was observed after exposure to NO_2 (31) and in some chronic inflammatory lung disorders (32). Whether mitogenic factor(s) for alveolar macrophages are present in BAL fluid of asbestos-exposed rats, as has been reported in other inflammatory exudates (33), is not known and will require further investigation.

A striking observation in our study was the morphologic heterogeneity of macrophages in the bronchoalveolar milieu of asbestos-exposed animals. The cells showed considerable range in size and shape, many with vacuolate cytoplasm

and long cytoplasmic extensions. In addition, a significant proportion of the cells were multinucleated. These cells have been previously found in histologic sections of lungs from animals inhaling chrysotile asbestos (5). More recently, 2 separate reports described briefly their presence in lung lavage of rats after inhalation of chrysotile asbestos (34, 35). Our study confirms these findings and further documents some aspects of giant cell formation after asbestos exposure. Although it is generally believed that MGC formation occurs by fusion of macrophages (36, 37), the mechanisms responsible for this process are not elucidated. There is evidence to suggest that increases in relative amounts of phospholipids, especially in lysolecithin, may favor cell fusion (38). However, chrysotile preparations that contain 15 to 21% of fibers longer than 10 μm (15) and that reportedly caused significant increase in pulmonary surfactant after intratracheal injection in rats (34) did not promote formation of cells with 3 or more nuclei in our study. Possibly, giant cells formation may be the result of macrophages simultaneously attached to the same ingestible material (39). In this case, variations in the degree of fusion caused by endocytosis of UICC chrysotile B and very short 4T30 chrysotile fibers may be related to differences in cytotoxicity (40, unpublished observations) or specific surface area (10). Alternatively, MGC formation may result from the *in vivo* release of lymphokines, such as macrophage fusion factor (41) selectively by sublethal concentration of very short 4T30 chrysotile fibers.

Our results also demonstrate that both foreign body and Langhans giant cells were present in BAL fluid of animals treated with very short chrysotile fibers. As suggested by Mariano and Spector (36), these 2 morphologic variants probably represent different phases in the life of the same cell rather than distinct cell types. Study of the kinetics of MGC formation revealed that although these cells were absent 1 day after asbestos instillation, they were found in significant numbers on Day 7. The reason for the initial delay before the appearance of MGC in BAL fluid of animals treated with very short chrysotile fibers is not known. Possibly the presence of great numbers of PMN 1 day after instillation may inhibit macrophage fusion. Similarly, the absence of giant cell formation after instillation of chrysotile B fibers may be related to a more persistent infiltration of PMN in BAL fluid of these animals. Another possible explanation for the delay in MGC formation may be that relatively "aged" macrophages are necessary for the formation of such cells (36).

The exact role of MGC in chronic inflammatory reactions is unknown. Previous data have demonstrated that these cells have a relatively short life span and a higher content of lysosomal hydrolases (42). On the basis of those characteristics, it was suggested that MGC may represent an efficient disposal system for unwanted macrophages that might possess injurious potential (36). Interestingly, the presence of MGC in BAL fluid is associated with a less persistent increase in inflammatory cells and the absence of fibrosis. Another possible role for MGC may be related to their lower contact inhibition of locomotion (42). This property may encourage and facilitate surface exploration of adjacent cells and enable them to modulate macrophage activity and involvement in inflammatory reactions.

In conclusion, our results demonstrate that bronchoalveolar cell populations are different, depending on the nature of lung injury induced by asbestos. The inflammatory cell response associated with lung fibrosis in our rat model is characterized by a more sustained increase in PMN and alveolar macrophages and the absence of giant cells with 3 or more nuclei seen in the alveolitis produced by very short fibers. The role of PMN and MGC in such lung lesions remains to be clarified. In light of the important role of cell cooperation in many physiologic processes, one must question the significance of the presence of PMN and MGC on macrophage function. This model will hopefully be useful in studying the interactions between these various cell populations and may prove valuable in providing new insights into the mechanisms of pulmonary fibrosis.

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