Asbestos Exposure and Retention as Determinants of Airway Disease and Asbestos Alveolitis[1-3]

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SUMMARY To evaluate the relationships of asbestos exposure, retention, airway response, and the asbestos alveolitis, we exposed 2 groups of sheep every 2 wk for 3 yr to either 100 ml phosphate-buffered saline (PBS) or 100 mg UICC chrysotile fibers in 100 ml PBS. The sheep were evaluated periodically by pulmonary function tests (PFT), chest radiograph (CR), bronchoalveolar lavage (BAL), and transbronchial lung biopsy (TLB). At Month 24 of the study, all asbestos-exposed sheep had significant increases in lung resistance and upstream resistance. However, only 9 of the 16 asbestos-exposed sheep had significant changes in TLB, CR, Cst, and VC, which clearly separated them from the other 6 sheep in these parameters. The 2 groups, however, had similar air-flow limitation. At lung biopsy, all asbestos-exposed sheep had significant peribronchiolar fibrosis, with significant alveolitis only in the group of 9 sheep with radiographic and functional changes of early asbestosis. The 9 sheep also had significant changes in BAL cellularity and biochemical profile, which differentiated them from the other 6 asbestos-exposed sheep. Analysis of BAL fiber content at that point revealed that despite identical exposure, the group with interstitial lung disease had significantly more fiber retention ($p < 0.01$). The data demonstrate that whereas asbestos airway disease appears to be primarily an exposure-dose-related response, the lung response appears to be more closely related to alveolar retention of the dust.

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Introduction

Recently, it has been well documented that inhalation of asbestos dust (or other mineral dust) can produce a small airways disease in exposed workers that is distinct from airway disease associated with cigarette smoking (1-5). This asbestos airway disease has been thought to cause significant air-flow limitation whether the workers were cigarette smokers (1, 6-13) or lifetime nonsmokers (14). Furthermore, in humans and in animal models with either asbestos alveolitis (early asbestosis) or well-established asbestosis, similar airway dysfunction has been reported (15-18). This suggests that air-flow limitation in asbestos workers could be a marker of early parenchymal pulmonary damage and should preclude further exposure.

In a previous study (15) of a sheep model of early asbestos peribronchiolar alveolitis with restricted lung volumes, we found a concomitant small airways disease that significantly limited air flow. Morphologic features and function of the small airways were studied in detail only after the initial alveolitis, which did not permit a study of the relationship of asbestos airway disease and parenchymal pulmonary damage.

To provide additional information on the relationship of asbestos airway disease and early asbestosis, we obtained repeated bronchopulmonary function and morphologic data in 15 sheep exposed to asbestos dust for 3 yr. The data clearly document that air-flow limitation occurred in all exposed sheep, whereas only 9 of 15 developed asbestos alveolitis. Further analyses of BAL fiber content suggested that whereas airway disease is related to exposure dose, the asbestos alveolitis is primarily related to alveolar retention of the fibers.

Methods

Animals

Twenty-six sheep weighing 25 to 40 kg were used in this study. They were prepared and accustomed to the pulmonary techniques as previously reported (19-21).

Experimental Design

The flock was divided into a group of 11 sheep exposed to phosphate-buffered saline (PBS) only and a group of 15 sheep exposed to 100 mg UICC Canadian chrysotile asbestos fibers in 100 ml PBS every 2 wk. These fibers were relatively uniform and well characterized (22), 92% being less than 0.25 μm in diameter and 20 μm in length. Exposures were carried out after nasotracheal intubation via repeated slow infusions of the suspension in the trachea at 2-wk intervals. The animals were studied prior to exposure and at 3- to 6-month intervals after exposure by chest radiographs (CR), pulmonary function tests (PFT), bronchoalveolar lavage (BAL) analyses, and transbronchial lung biopsies (TLB).

Chest Radiograph

Each sheep was positioned on a mobile cart with a wooden board and a grid cassette under the thorax. The X-ray source was placed at a 30-degree caudal angle 2 feet from the cassette. The intubated animal was held at TLC using a giant syringe, and radiographs were taken at exposure factors 80 kV, 20 mAs, and 0.02 s. Each radiograph was scored according to the International Labor Organization classification of radiographic profusion of parenchymal opacities (23).

Pulmonary Function Tests

The methods used in PFT assessment of the sheep have been published (15, 19-21). Briefly, transpulmonary pressure was monitored with a nasoesophageal 7-ml balloon catheter and an airway catheter connected to a Hewlett-Packard 270 differential pressure transducer (Hewlett-Packard, Waltham, MA). Gas flow at the airway opening was measured by connecting the cuffed endotracheal tube to a Fleisch no. 2 pneumotachygraph (Dynasciences, Blue Bell, PA) attached to a flow integrator recorder system and a Mink data processing system (Digital Equipment, Montréal, Quebec), for on-line analysis and storing of the data. Each PFT measurement was obtained after 3 inspiratory capacity measurements at a constant volume; TLC was defined as the lung volume at a transpulmonary

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pressure of +35 ± 5 cm H_2O, and RV was defined as the lung volume at a transpulmonary pressure of −35 ± 5 cm H_2O. The static expiratory lung compliance (Cst) was determined by multiple-step syringe deflation between TLC and FRC. For the measurement of maximal expiratory flow rates, the animal was anesthetized with pentobarbital (initial dose, 50 mg/kg) and curarized with succinylcholine (initial dose, 1 mg/kg), placed in a fast integrated-flow plethysmograph, and ventilated with a Harvard pump (Harvard Apparatus Co., South Natick, MA) between procedures. Total maximal expiratory flow volume (MEFV) curves were obtained by suddenly connecting the airway opening to a large vacuum source pressurized at −200 mm Hg after the animal lungs were inflated to a transpulmonary pressure of 35 cm H_2O (TLC). The flow-volume curves were replotted in relation to absolute lung volume and transpulmonary pressure, the effective driving pressure producing Vmax at any lung volume, and the upstream resistance (Rus) was plotted as the ratio of transpulmonary pressure over Vmax at lung volumes between near TLC and FRC. The above were expressed in relation to absolute lung volume. Diffusion capacity (D_{LCO}) was obtained by a passive rebreathing method using a gas mixture of 10% helium, 0.30% carbon monoxide, and 21% oxygen in nitrogen, a Collins catherometer (Warren E. Collins, Braintree, MA), and a Beckman infrared carbon monoxide analyzer (Beckman Instruments, Fullerton, CA). For the D_{LCO} test, the animals were passively ventilated with a 2.5-L syringe at a rate of 30 breaths/min with a 1-L tidal volume.

Transbronchial Lung Biopsy

Under topical local anesthesia and without fluoroscopic guidance, TLB were obtained following the technique previously published (13). Subsegmental bronchi were randomly selected, and the TLB sample was immediately placed in 10% buffered formaldehyde fixative and processed as routinely done for human lung tissues. All sections were stained with hematoxylin-eosin, and selected biopsies were also stained with Masson-trichrome.

Bronchoalveolar Lavage and Fluid Analysis

Most of the techniques in BAL procedures and analyses have been previously described (19-21). The BAL effluent was passed through 4 layers of cheesecloth to remove mucus, and the cells were pelletized by centrifugation. Cells were counted in a hemocytometer, and cell viability was determined by the trypan blue exclusion technique. Cytocentrifuge smears served to identify the cellular populations recovered with the Wright-Giemsa and naphthyl acetate esterase stains. In the supernatant, albumin and IgG were measured by the immunochemical methods of Killingsworth and Savory (24), using the Behring laser nephelometer instrument (Behring LN modular system; Hoechst Behring, Frankfurt, WG). For sheep albumin and IgG, specific antiserum raised in rabbits was obtained commercially (Cappell Laboratories, Downingtown, PA). Lactate dehydrogenase, alkaline phosphatase, and β-glucuronidase were measured by standard methods (25-27). All results were expressed per milliliter of BAL fluid.

Electron Microscopy

The analytical transmission electron microscope was used to look for the presence of chrysotile fibers in lung lavages. All samples were prepared in a "clean room," using filtered chemicals and carefully cleaned glassware. Thirteen milliliters were isolated from the third effluent of lavage and allowed to react at room temperature with sodium hypochlorite, in order to digest the biologic material. An aliquot of the suspension, corresponding to 5 ml of BAL, was filtered on a polycarbonate membrane filter 25 mm in diameter with a pore size of 0.2 μm pore size (Nucleopore Corp., Pleasanton, CA). Particles collected at the upper surface of the membrane were embedded in a carbon film and subsequently transferred onto 200-mesh grids (28). Several grid openings were observed in the transmission mode at a screen magnification × 10,000. Chrysotile fibers were identified by their morphologic features and their elemental composition as determined by energy-dispersive X-ray spectrometry (PGT system IVR; Princeton Gamma Tech, Princeton, NJ). The length of each individual fiber encountered was measured directly on the screen to the nearest 0.2 μm: diameter was measured using an eyepiece graticule.

Statistical Analysis

In the presentation of the results, values of the data for each group of sheep are followed by the standard error as an index of dispersion. The data were evaluated by analysis of variance for experiments having repeated measurements on the same subjects. When a significant effect was detected, a Kruskal-Wallis test was used to determine which group means were significantly different (29). Differences with $p < 0.05$ were considered significant.

Results

Subsets of Asbestos-exposed Sheep

At Month 24 and after, the group of 15 sheep exposed to asbestos had significantly higher pathology scores, higher radiographic scores, lower VC, lower static lung compliance, and lower arterial P_{O_2} (data not shown), and these changes were accentuated in the year after. Within the group, these changes were the effect of disease limited to some of the sheep. We then separated the group of asbestos-exposed sheep into 2 subsets: a subset of 6 sheep who had minimal or no alveolitis shown by TLB and CR scores of 0/0 or 0/1, which were within the 95% tolerance limit of PFT of saline-exposed sheep; this subset is reported as without asbestos alveolitis hereafter. The second subset of asbestos-exposed sheep was composed of 9 sheep with changes shown by TLB, CR, and PFT consistent with those of the initial asbestos alveolitis previously reported in this model (15, 20); this subset is reported as with asbestos alveolitis hereafter.

Pathologic Findings

In the saline-exposed sheep, the lung tissue remained normal. In the asbestos-

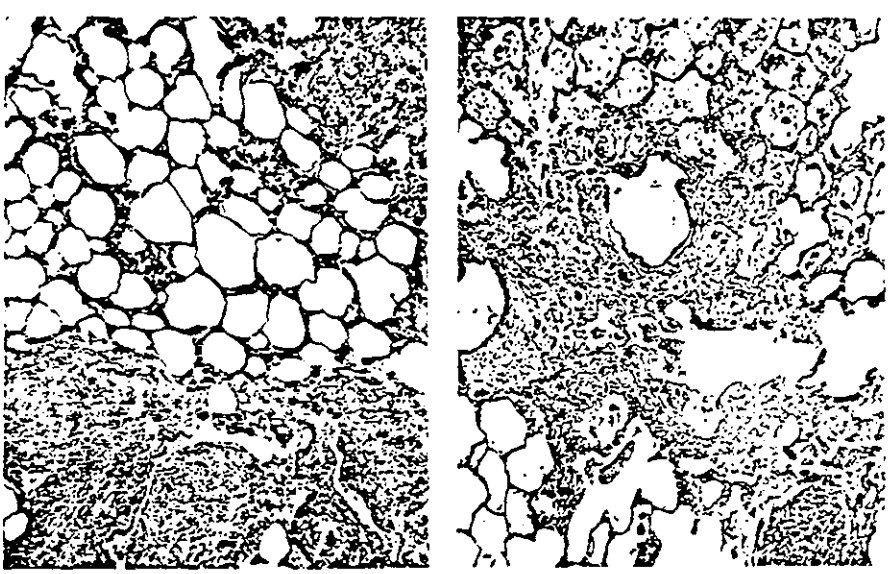

Fig. 1. Representative histopathologic features of the airway lesions and surrounding lung tissue. The left panel is from an asbestos-exposed sheep with severe airway disease characterized by peribronchiolar leukocyte accumulation, fibroblastic proliferation, severe airway distortion, but minimal alveolitis. The right panel is from a sheep with less severe airway disease associated with diffuse leukocyte infiltration of the surrounding alveoli and interstitium (hematoxylin-eosin stain; magnification: × 63).

exposed sheep without alveolitis, there was a diffuse peribronchiolar fibrotic process with minimal alveolitis (figure 1, *left panel*). In the sheep with alveolitis, there was the same peribronchiolar process and in addition extensive accumulation of macrophages and leukocytes in the alveoli and interstitium adjacent to the airway lesions (figure 1, *right panel*).

Chest Radiographs

All CR of control animals were without infiltrates or pleural lesions throughout the experiment. In the asbestos-exposed sheep without alveolitis, the same observations (scores of 0/0 or 0/1) were recorded until Month 24 (figure 2, *left panel*) where 1 of the 6 sheep had a 1/0 infiltrate in 2 lung fields, which appeared in 2 other sheep at Month 36. In the asbestos-exposed sheep with alveolitis, scores of parenchymal opacities of 1/0 or greater were present in each of the 9 sheep (figure 2, *right panel*), and the average score went from 2.7 ± 1.7 at Month 24 to 7.3 ± 2.7 at Month 36 ($p < 0.05$) (figure 2).

Lung Volumes

Between the 3 groups of sheep, the only significant lung volume difference was for VC, which was significantly lower in the asbestos-exposed sheep with alveolitis from Month 24. For control sheep at Month 24 VC was 3.05 ± 0.05 L, for the group without alveolitis VC was 2.99 ± 0.05 (NS), and for the group with alveolitis, VC was 2.80 ± 0.06 ($p < 0.05$).

Diffusion Capacity

For control sheep at Month 24 D_{LCO} was 23 ± 0.6 ml/min/mm Hg, for the group without alveolitis D_{LCO} was 23 ± 1.4 (NS), and for the group with alveolitis D_{LCO} was 19 ± 0.8 ($p < 0.05$).

Pulmonary Mechanics

In the upper panels of figure 3, the time course of static lung compliance (C_L), lung resistance (R_L), and upstream lung resistance (Rus) at low lung volume are presented. In the lower panels of the same figure, the full upstream resistance curves at selected times are presented. It can be seen that both groups of asbestos-exposed sheep had lower C_L than did control sheep, with values for the sheep with alveolitis showing a significantly steeper rate of fall of C_L ($p < 0.05$). The air-flow resistance increased significantly and similarly for both groups of asbestos-exposed sheep in the large (R_L) and small airways (Rus, 40%), which was confirmed on the full Rus/lung volume curves (*lower panels*).

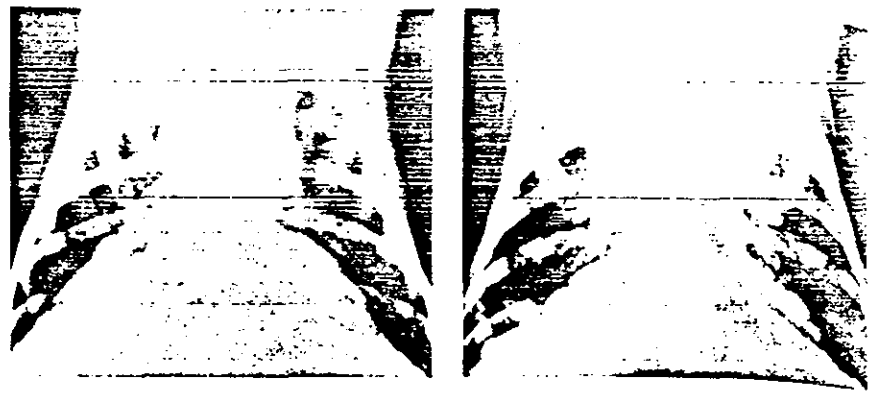

Fig. 2. Representative chest radiographs of exposed sheep with airway disease (*left panel*) and a sheep with airway and interstitial lung disease (*right panel*). The left panel radiograph is essentially normal, whereas the right panel radiograph shows diffuse reticulonodular infiltrates of the lung parenchyma.

Alveolitis

In contol sheep, there was no significant change in BAL cells and biochemical analyses over time in any of the parameters. In the 6 asbestos-exposed sheep without alveolitis, the only significant changes were slight increases in BAL albumin from Month 30 and IgG from Month 24. In the 9 asbestos-exposed sheep with alveolitis, there were significant changes in all BAL parameters which clearly differentiated those sheep from the sheep exposed to the same cumulative asbestos exposure but without alveolitis ($p < 0.05$). These changes were seen from Month 12 for lactate dehydrogenase, from Month 24 for neutrophils, and from Month 30 for total BAL cells, macrophages, and lymphocytes (the time

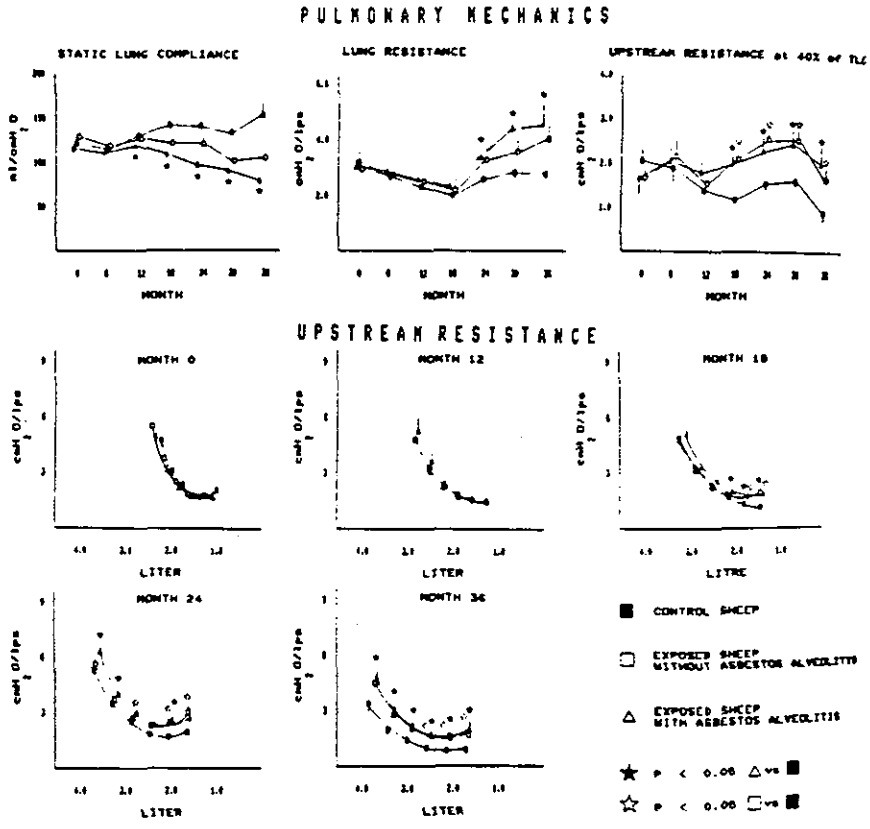

Fig. 3. Results of pulmonary mechanics test presented for the 3 groups of sheep in the upper panels, with selected upstream resistance/lung volume curves in the lower panels.

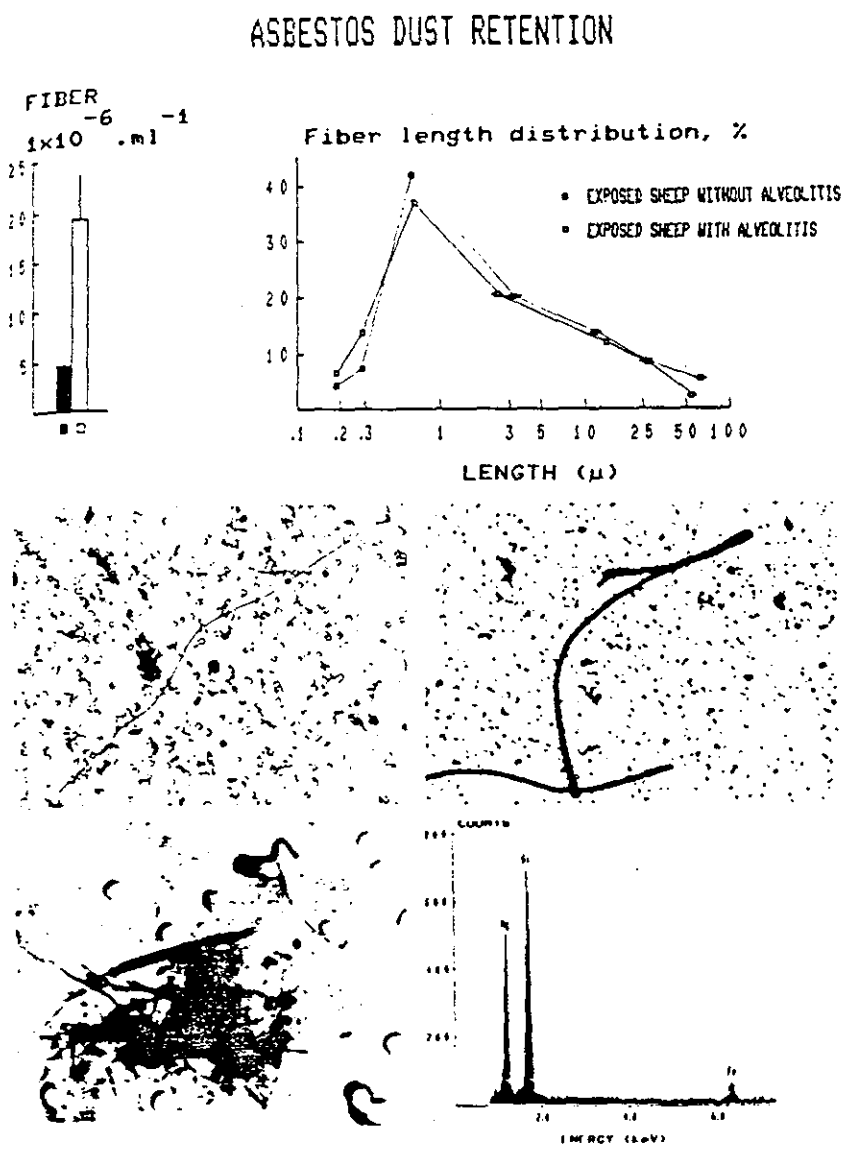

Fig. 4. Results of analysis of fiber content of lung lavage at Month 24. The left upper panel is a bar diagram of fiber counts in million per milliliter of BAL effluent. The right upper panel is a fiber length-distribution diagram of the fiber analysis of BAL showing similar length distribution in the 2 groups of exposed sheep. The middle left panel shows a fiber 15 μ long, the middle right panel an asbestos body 32 μ long by 0.75 μ wide, and the left lower panel shows bundles of fibers 1 μ long, all on a background of a 0.2-μ Nucleopore® membrane. The right lower panel shows the characteristic X-ray energy spectrometric pattern of a chrysotile fiber used to ascertain the nature of the fibrous materials observed on BAL.

course of these have been reported separately, in relation to our analysis of gallium-67 lung uptake in the process [30]).

BAL Fiber Analyses

In figure 4, the results of mineralogic analyses of BAL fluid obtained at Month 24 as an index of asbestos dust alveolar retention are presented. In control sheep, fiber counts averaged $1.8 \pm 1 \times 10^6$/ml, and all were shorter than 1 μ in length. In the sheep with airway disease only, we found $4.56 \pm 0.55 \times 10^6$ fibers/ml, and in the sheep with airway disease and alveolitis, we found $19.2 \pm 4.4 \times 10^6$ fibers/ml ($p < 0.01$). Analysis of size distribution did not differentiate the exposed groups; a few asbestos bodies were observed in the 2 asbestos-exposed groups.

Discussion

This study showed that all sheep exposed to a cumulative exposure dose of 5 g of asbestos dust developed diffuse airway lesions that caused significant air-flow limitation. Furthermore, this study demonstrated an individual heterogeneity of lung tissue response to the same exposure dose, with 40% of the sheep having disease limited to the airways and 60% also having a concomitant alveolitis. Analysis of BAL fiber content in the 2 groups of exposed sheep showed a fourfold difference between the 2 groups without significant difference in the type, size distribution, width, or aspect ratios of the fibers.

Although the relationship of asbestos exposure and airway disease has been a controversial subject during the last decade, recent reports have contributed substantially to clarifying the issue. In the animal model, it has been shown that asbestos fibers are primarily deposited and retained at the bifurcation sites of the peripheral airways where the initial airway lesion is rapidly established (31). In asbestos workers, these airway lesions have been observed in the absence of asbestosis (interstitial lung disease) and have been associated with higher asbestos content of the lungs (32). However, because similar pathologic changes of the small airways have been observed in association with a variety of mineral dust exposures (4), the specificity of the lesions appears to be related to mineral dust exposures, including asbestos. Our data on 15 sheep exposed chronically to asbestos dust confirmed the existence in all of these animals of airway lesions that caused significant air-flow limitation independent of the development of interstitial lung disease. In this animal model, it is clear that the peripheral airway lesions were more severe than those reported in asbestos workers in terms of intensity of the bronchiolar disease and its effects on lung function. The airway lesions in this multiple intratracheal asbestos instillation model differ from the human disease associated with asbestos inhalation, as the animals were subjected to more intense and less frequent exposures. This exposure produces a more intensely inflammatory airway lesion in sheep and more severe airway dysfunction than that reported in lifetime nonsmoking asbestos workers (14). Indeed, in a recent study of 34 lifetime nonsmoking, long-term miners and millers of the Quebec asbestos industry, we found a 30% reduction in air-flow conductance at low lung volumes, which was consistent with the concept of an asbestos airway lesion. However, this peripheral airway dysfunction did not reduce the expiratory flow rates on the flow volume in the lifetime nonsmoking, chrysotile miners and millers (33).

Is this airway lesion a precursor of interstitial lung disease? In humans, this airway lesion was observed at total lung burden substantially lower (50%) than that of patients with asbestosis and may be linked to pleural changes (32). Its relationship to the development of interstitial lung disease has not been reported. In our sheep with alveolitis at Month 24, significant changes in several parameters of interstitial lung damage preceded the airway disease documented at Month 18: lung compliance was reduced from Month 9 (figure 3), ^{67}Ga lung uptake and lung lavage fibronectin increased from Month 6 (30), and lung lavage LDH increased from Month 12. In the sheep without alveolitis at Month 24, only 3 of 6 developed interstitial lung disease despite continuous exposure for the following year. Thus, on the basis of these data, the asbestos-induced airway disease cannot be seen as a precursor of interstitial lung disease but should be considered as a relatively independent process.

The asbestos-induced alveolitis, which in our view constitutes the initial lung tissue reaction leading to asbestosis (15, 20, 21), has been further characterized in this study. In a recent report on the same groups of sheep, we have reported serial measurements by BAL and scan of ^{67}Ga lung uptake during the induction of the disease process and have found that the uptake of the marker occurred before the disease process could be detected by other methods (30). The enhanced ^{67}Ga lung uptake correlated best with the excessive accumulation of BAL fibronectin. These observations confirmed our previous reports on ^{67}Ga lung uptake as a useful indicator of early lung damage in asbestos workers (34) and further suggested that macrophage activity producing excessive amounts of fibronectin was primarily responsible for the enhanced uptake of the marker.

The present study further investigated the heterogeneity of lung tissue response in our sheep with similar dust exposures, 60% of them developing the alveolitis at Month 24. This observation in the animal model is similar to the clinical observation of individual susceptibility of humans to develop the disease (35). To investigate the individual susceptibility factor, immunologic background and clearance capacity have been considered. Reports on human immunologic histocompatibility have failed to document definite markers identifying the more susceptible workers (36), and our own work in progress with some 200 asbestos workers tested appears to agree with these earlier reports. In the sheep, histocompatibility testing is not currently feasible.

Alveolar dust clearance capacity in humans has not been directly studied in asbestos-exposed workers as a determinant factor for individual susceptibility to the disease. Lung tissue burden of fibers have been found to be increased in asbestos workers compared with that in the general population (37). In the exposed workers, it has been documented that workers with isolated airway disease have twice the fiber content of the lungs of workers without airway disease but only 50% the fiber content of patients with asbestosis (31). In the lung tissue of patients with asbestosis, the lung fiber burden appears to correlate with severity of the disease (38–41). Analysis of BAL fiber content in workers with asbestosis have yielded results in the same range as that of our sheep with asbestos alveolitis, but lower values were found in exposed workers without asbestosis (28, 42). Because in human studies, the amount of exposure is difficult to determine with precision, it has not been possible to compare groups of workers with similar exposures but different disease activity.

Given that all our exposed sheep had similar exposures, BAL fiber content can be seen as an index of alveolar dust retention (43) and hence assesses the individual alveolar clearance capacity. In the sheep with alveolitis, we found a fourfold increase in the number of fibers retained in the alveolar space without difference in type, size distribution, width, or aspect ratio of the fibers. These data in the sheep model clearly link the individual susceptibility to develop the asbestos alveolitis to alveolar retention of the dust and thereby to the individual dust clearance capacity. To verify the relevance of these findings to the human condition, we have initiated alveolar clearance studies in asbestos workers.

In conclusion, this study on the sheep model of asbestos-induced lung injury has shown that chronic asbestos dust exposure can induce airway disease with significant air-flow limitation in the absence of interstitial lung disease. Within the exposed population, there is a heterogeneity of lung tissue response, and the susceptibility to develop interstitial lung disease appears to be related to the individual capacity of alveolar dust clearance.

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