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SEQUENCE OF BRONCHOALVEOLAR LAVAGE AND HISTOPATHOLOGIC FINDINGS IN RAT LUNGS EARLY IN INHALATION ASBESTOS EXPOSURE

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To assess the early cellular inflammatory response of the lungs, 7 rats per group were exposed nose-only to 13 mg/m³ of chrysotile asbestos, 7 h/day for 2, 4, or 6 wk. Lung histopathology and bronchoalveolar lavage (BAL) were analyzed. In exposed animals, dose-related bronchiolitis and fibrosis were found that were not seen in control rats (p < 0.001). In exposed rats, total BAL cells were increased six- to sevenfold over matched controls, and more cells were retrieved with longer exposure (p < 0.001). In the BAL, counts of macrophages, lymphocytes, and polymorphonuclear cells (PMNs) were each elevated in the exposed rats (each p < 0.001). PMNs seen histologically and in the BAL may be related to the time period examined. PMNs and lymphocytes observed throughout this 6-wk study support the idea that these cells may have an important role in the early events of asbestos lung injury.

INTRODUCTION
Asbestos exposure and lung disease are known to be associated. However, the mechanisms involved in asbestos-initiated lung fibrosis remain enigmatic. This study was designed to quantitate the early events of asbestos lung injury.

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changes in cell populations and pulmonary histopathology during asbestos inhalation in rats.

A central unresolved issue is the stimulation of different lung cells by asbestos and their relative importance in fibrogenesis. In previous inhalation experiments, the time periods studied have been either very brief (1 h) (Brody et al., 1981) or very long after fibrosis is already evident (3 mo or longer) (Wagner et al., 1974, 1980; Holt et al., 1964; Tetley et al., 1976; Davis et al., 1978; Barry et al., 1983). In these time periods, increases only in the number of macrophages have been uniformly observed. However, numerous investigations in vitro have demonstrated alteration in the function of lymphocytes (Rola-Pleszcynski et al., 1981; Miller and Kagan, 1977, 1981; Miller et al., 1979; Lemaire and Dubois, 1983) and polymorphonuclear cells (PMNs).

Lung histopathologic changes have been assessed by several investigators. Brody et al. (1981) analyzed the cellular reaction after 1 h of inhaled chrysotile asbestos exposure to an airborne concentration of 9 mg/m$^3$. Among the early events were engulfment of fibers by type 1 epithelial cells, and macrophage migration to airway bifurcations where fibers deposited. These bifurcations became thickened by macrophage infiltration within 8 d after exposure. Barry et al. (1983) found that after 5 d of daily exposure to 9 mg/m$^3$ of chrysotile asbestos there was an increase in the number of alveolar macrophages, and the alveolar walls adjacent to terminal bronchioles appeared thickened. Wagner et al. (1974), Tetley et al. (1976), and Davis et al. (1978) each independently showed that rats that inhaled chrysotile asbestos at doses of 10–15 mg/m$^3$, 7 h/d, for 15 wk or longer developed fibrosis. This study used daily inhalation of asbestos, at a concentration known to cause fibrosis in rats, for periods of 2, 4, or 6 wk. These exposure durations fall between the chronic studies, in which fibrosis had already occurred, and the acute studies. This allowed examination, by bronchoalveolar lavage and histologic analysis, of the sequence of cellular changes during a presumed critical period of early fibrosis.

**METHODS**

**Animals**

Forty-eight Fischer-344 male rats, 175–199 g, 2–3 mo old (Hilltop Laboratories, Scottsdale, Pa.), were studied. Six were randomly selected, sacrificed, and examined for infectious diseases. *Mycoplasma pulmonis* titers were negative. Histologic examination by light microscopy indicated that lung and spleen were normal. The remaining 42 were acclimatized for 3 wk to nose-only exposure tubes before the asbestos inhalation period. Once the exposure began, the control and exposed animals were housed in separate chambers simultaneously.
breathing high-efficiency particulate absolute (HEPA) filtered, purified air. All procedures were conducted in accordance with the National Research Council's recommendations for the care and use of laboratory animals.

**Experimental Design**

Rats were randomly divided into 2 equal groups of 21 to serve either as controls or as asbestos-exposed animals. During the 6-wk exposure, 7 rats from the exposed group were randomly selected for termination of exposure at 2, 4, and 6 wk. Seven control rats were also removed from the study at these same time intervals. Each group was studied immediately after they were terminated from exposure.

**Exposures**

The rats were exposed in a modified nose-only exposure system after the design of Raabe et al. (1973) (Sandia Research and Development Corporation, Albuquerque, N. Mex.). The exposure system was housed within a Rochester-type exposure chamber at the University of California, Irvine, Air Pollution Health Effects Laboratory. Each rat was held in a plastic cylinder with its nose protruding through one end. The nose end of the cylinder was then inserted into an exposure port on the outer wall of the exposure unit, with the nose protruding into the port. The asbestos–air mixture inside the airtight apparatus flowed past each rat's nose through individual ports in a manifold distribution system.

Exposure was to chrysotile asbestos (Plastibest-20, Johns-Manville Jeffrey Mine, Asbestos, Quebec, Canada) of intermediate length (nominal 80% less than 10 μm in length and greater than 96% chrysotile asbestos) obtained from the National Institute of Environmental Health Sciences (NIEHS), Research Triangle Park, N.C. (Campbell et al., 1980). Airborne fibers were generated from the NIEHS material by a modified Timbrell generator (Timbrell, 1968), and passed through a radioactive 85Kr discharger used to reduce the charges on fibers acquired during aerosolization (Vincent et al., 1981). A mean concentration of 13 mg/m³ was maintained in the exposure system in a 10 l/min flow of pre-HEPA-filtered, humidified air. The animals were exposed to this atmosphere in the nose-only system for 7 h/d, 5 d/wk, for up to 6 wk.

The matched control animals were simultaneously placed in plastic tubes, breathing HEPA-filtered, purified air.

**Fiber Characteristics**

Three independent fiber characterizations were performed. The airborne asbestos mass concentration (expressed in milligrams per cubic meter) was measured gravimetrically with a Sartorius analytical balance (type 2434, Fabnr 199793, Sartorius-Werke GMBH, Gottingen,
Germany) from multiple daily samples collected on Metricel (PVC) membrane filters, 0.8 μm pore (Gelman Sciences, Inc., Ann Arbor, Mich.), using a Gelman filter holder. Samples were acquired from a nose exposure port of the exposure unit. Fibers were collected for an average of 35 min at an airflow regulated at 4 l/min.

The aerosol particle aerodynamic size distribution was measured daily by using a Cascade Impactor (model 220, Sierra Instruments, Inc., Carmel Valley, Calif.). One port of the animal exposure unit was used throughout the study for sampling with the Cascade Impactor. Collection was for 1 h with an airflow of 4 l/min through the impactor. The impactor filters were analyzed gravimetrically.

Nuclepore filters, 0.2 μm pore (Nuclepore Corp., Pleasanton, Calif.), were used to collect hourly samples (1.0 l/min for 1 min) for calculation of asbestos fiber concentration (expressed as number of fibers per cubic centimeter of air), and estimation of fiber length, diameter, and aspect ratio by scanning electron microscopy (SEM).

The nuclepore filters were examined in an ETEC SEM. At ×10,000 viewing-screen magnification, random fields of view were searched and at least 90 fibers were counted and measured. Elemental analysis of at least 50 fibers was also performed using energy-dispersive X-ray analysis (EDXA).

**Bronchoalveolar Lavage (BAL)**

At the end of the designated exposure period, the 7 animals in each control and exposed group were anesthetized with 0.1 ml im ketamine hydrochloride at 100 mg/ml (Parke-Davis, Morris Plains, N.J.) and 0.05 ml im Innovar Vet (Pittman-Moore, Washington Crossing, N.J.). Once anesthetized, the chest was opened and the animals were exsanguinated by cardiac puncture. The lungs were removed by tracheal excision (1 cm above the carina) and weighed after the heart and excess tissue were removed. The trachea was intubated with a modified, sterile, 19-gauge butterfly infusion set (Abbot Hospitals, Inc., North Chicago, Ill.). The tubing was placed into the right mainstem bronchus for lavage of the right lung only. Silk suture tied around the outside of the right mainstem bronchus assured stabilization of the tube and prevented leakage around the tube.

The right lung was lavaged with 37°C Hanks balanced salt solution. The lung was filled and emptied 3 times with 4 ml of fluid for a total volume of 12 ml/animal. The BAL was centrifuged at 200 × g for 10 min. The cell pellet was resuspended in 0.6 ml RPMI medium 1640 with 25 mM HEPES buffer (Gibco Laboratories, Grand Island, N.Y.). A hemocytometer was used to obtain the total leukocyte cell count. Leukocyte differential cell counts were obtained from 100 cells counted from a Wright's stained smear of cytocentrifuge prepared slides (Cytospin, Shandon Southern Products, Ltd., Cheshire, England).

After lavage, the tubing was retracted above the carina and both
lungs were fixed and inflated with 7 ml of 1% glutaraldehyde/2% formaldehyde solution (phosphate-buffered) instilled over 1 min.

Pathology

Tissue samples were coded to assure unbiased interpretation by the reader. Right and left lung sections of paraffin-embedded tissues were stained with hematoxylin and eosin (H&E), elastic van Gieson (EVG), and Perl's iron (Fe) stains for light microscopic analysis. The fibrosis was graded in each EVG section using the recently proposed grading system for asbestosis established by the Pneumoconiosis Committee of the College of American Pathologists and the National Institute for Occupational Safety and Health (NIOSH) (Craighead et al., 1982): 0 = no fibrosis associated with bronchioles; 1 = fibrosis involving wall of respiratory bronchiole with extension into no more than the immediately adjacent alveolus; 2 = fibrosis as in 1, plus alveolar ducts or two or more layers of adjacent alveoli, but not bridging adjacent bronchioles; 3 = fibrosis as in 2, but with coalescence of fibrosis between two adjacent bronchioles. As a modification, intermediate grades were given when variation within a slide was present. Additionally, the epithelial and inflammatory cell changes (bronchiolitis) were graded on a 0–4 scale as follows: 0 = normal, 1 = difficult to detect, 2 = easily seen but sparse, 3 = numerous, 4 = severe. The average grade for fibrosis and for bronchiolitis was determined for all the slides from each exposure period. Some tissues were also embedded in plastic for transmission electron microscopy, and some were saved for fiber burden and characterization studies.

Statistical Analysis

The BAL cell counts were analyzed by two-way analysis of variance using the BMD-2PV statistical analysis program (Dixon and Brown, 1979). The analyzed groups were controls (unexposed) and exposed animals, and the experimental time periods: 2, 4, and 6 wk. The log \(_{10}\) of the cell counts was taken as a variance-stabilizing transform. The number 1 was added to all counts prior to transformation so that counts of zero would not be excluded. All results are expressed as mean ± SEM.

The median pathologic scores were analyzed by chi-square analysis for the differences between control and exposed animals. The test of linear trend in proportions was used to evaluate the differences among the median scores of the exposed rats with longer exposure durations.

RESULTS

Aerosolized Fiber Characteristics

The mean total asbestos concentration gravimetrically determined over the 6-wk period was 12.9 ± 7.5 mg/m\(^3\) (time-weighted average of
233 filters collected during the 6-wk study). SEM analysis of fibers collected on 4 examined nuclo pore filters indicated that the length of fibers varied from 0.5 to 61 μm; 79% of the fibers were less than 5 μm in length, and 95% less than 10 μm. The mean length was 3.7 ± 0.6 μm. The mean aspect ratio (length to diameter) was 23 ± 8. The mean fiber concentration was 5.5 ± 3 × 10^9 fibers/m^3 air. No fibers other than chrysotile asbestos were observed in over 2000 fibers analyzed.

The aerosol particle aerodynamic size analysis revealed an average mass median aerodynamic diameter of 0.86 μm; the observed geometric standard deviation was 3.4.

**Bronchoalveolar Lavage**

Approximately 80% of the lavage fluid was recovered. There was no significant difference in volume collected between the control and exposed groups.

The results are presented in Table 1. Comparing the control animals to the exposed groups, the exposed rats demonstrated a significant nonlinear increase in the total number of cells retrieved at each time period studied (p < 0.001). The longer the rats were exposed to the asbestos, the more cells were retrieved in the lavage (p < 0.001). The cell number was not proportional to dose but showed a striking increase between 4 and 6 wk of exposure.

The differential percentages of leukocytes retrieved in the exposed groups also were significantly different from the controls at each time period. The percentage of leukocytes in the control groups did not vary significantly during the 6-wk study. In the exposed groups, the mean percentage of macrophages was consistently a smaller fraction of the total cells (p < 0.0001). The decrease in the percentage of macrophages was associated with a significantly higher percentage of PMNs and lymphocytes (both p < 0.0001). The ratios of the three leukocyte populations remained relatively constant for all three exposure durations.

While there was a decrease in the percentage of macrophages recovered from exposed rats, these were percentages of an increasing total number of cells recovered. When the total counts of individual cell populations were considered, significant differences existed between the control and exposed groups at the three study periods. Each leukocyte population in the exposed group had increased numbers of lymphocytes (p < 0.001), macrophages (p < 0.001), and PMNs (p < 0.001). Within the exposed group, macrophages were the predominant cell lavaged and their numbers increased with longer exposure, as did the lymphocytes (p < 0.001). The number of PMNs was elevated by two weeks, but it did not increase further with longer exposure.

**Pathology**

The control animals (Fig. 1a) had no fibrotic lesions (grade 0) or evidence of bronchiolitis (grade 0) at any time period.
### TABLE 1. Mean (±SEM) Bronchoalveolar Lavage Cell Counts and Differentials from Rats after Exposure to Asbestos or HEPA-Filtered Clean Air (Controls)

<table>
<thead>
<tr>
<th>Group (n = 7/group)</th>
<th>Exposure duration (wk)</th>
<th>Total cells* ($\times 10^5$)</th>
<th>Leukocyte differential (%)</th>
<th>Leukocyte cell count ($\times 10^6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lymphocytes</td>
<td>Macrophages</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>1.5 ± 0.8</td>
<td>4 ± 0.4</td>
<td>95 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.7 ± 0.5</td>
<td>5 ± 1.4</td>
<td>94 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.3 ± 0.8</td>
<td>5 ± 1.6</td>
<td>95 ± 1.7</td>
</tr>
<tr>
<td>Asbestos-exposed</td>
<td>2</td>
<td>8.7 ± 3.1</td>
<td>8 ± 2.3</td>
<td>73 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>11.6 ± 1.5</td>
<td>14 ± 2.1</td>
<td>68 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>22.9 ± 1.7</td>
<td>14 ± 1.2</td>
<td>76 ± 4.9</td>
</tr>
</tbody>
</table>

*aRight lung only was lavaged.
At 2 wk (Fig. 1b) several changes were evident in the exposed groups' animals. All groups had bronchiolitis (median grade 1.0) and mild fibrosis (median grade 0.5). At higher magnification (Fig. 2), PMNs and edema were noted in the interstitium of bronchioles and small vessels. Some lymphocytes were noted in the interstitium. The air spaces contained primarily macrophages and PMNs and, very rarely, lymphocytes. Iron-positive macrophages were seen rarely.

The animals exposed for 4 wk (Fig. 1c) clearly exhibited fibrosis

FIGURE 1. Representative lung sections of control and each exposed group. Results of grading of each section (fibrosis/bronchiolitis): (a) control from 6-wk group 0/0; (b) 2-wk exposed (0.5/1.0).
FIGURE 1. Representative lung sections of control and each exposed group. Results of grading of each section (fibrosis/bronchiolitis) (continued): (c) 4-wk exposed (1.0/1.5); (d) 6-wk exposed (1.5/2.0) EVG; x75.

(median grade 1.0) and bronchiolitis (median grade 1.5). Macrophages were more numerous than PMNs in the airspaces, and interstitial PMNs were less numerous than lymphocytes. Macrophages with iron staining were present in greater numbers than at 2 wk, but no coated fibers were noted.

After 6 wk of exposure (Fig. 1d) the total numbers of cells significantly increased, the median fibrosis grade was 1.5, and the median bronchiolitis grade was 2.0. Epithelial nodules at bronchiolar–alveolar
FIGURE 2. Lung section of 2-wk exposed rat. Interstitial edema and cellular infiltrate [PMNs (arrows) and mononuclear cells] are present in the peribronchiolar interstitium and in nearby airspaces. H&E, ×400.

junctions were noted. Macrophages were the predominant cell in airspaces, but some PMNs were seen. In the interstitium there were more lymphocytes than PMNs. Numerous iron-positive macrophages were now seen as well as several coated fibers.

The median grading scores of the rat lungs for severity of the bronchiolitis and the fibrosis were plotted against each exposure duration (Fig. 3). There was a significant difference between the control and exposed animals at all three exposure periods for both the bronchiolitis and fibrosis scores ($p < 0.0001$). With longer exposure there was a significant, apparently linear, increase in the scores for both bronchiolitis and fibrosis ($p < 0.001$).

DISCUSSION

The concentration of asbestos used in this experiment, when inhaled daily for 3 mo or longer, is known to cause fibrosis in rat lungs (Wagner et al., 1974; Holt et al., 1964; Tetley et al., 1976; Davis et al., 1978; Barry et al., 1983; Wagner et al., 1980). Using the same dose for shorter exposure periods of 2, 4, and 6 wk revealed (1) a demonstrable inflammatory and fibrotic process in the terminal airways after 2 wk of
exposure (Figs. 1b and 2); (2) that the cells infiltrating both the interstitium and the airways were primarily monocyte/macrophage cells but also included significant numbers of PMNs and lymphocytes (Fig. 2); and (3) that there was progression of the pathologic lesions with continued exposure: bronchiolitis associated with fibrosis (Fig. 1b–d and 3).

The asbestos fiber aerosol characteristics in this system were similar to those reported by Pinkerton et al. (1983). The high concentration (13 mg/m$^3$) was chosen so that these results could be compared with the inhalation studies cited.

The time period of this experiment was important, since it allowed examination of changes at times previously unexplored yet theoretically important to the subsequent development of fibrosis. This study demonstrated that the lower respiratory tract airways and surrounding interstitium are involved in an active inflammatory and reparative process soon after initiation of heavy asbestos exposure.

![Graph showing the median grading scores for severity of bronchiolitis and fibrosis compared to exposure duration. Asbestos-exposed rats: filled circles, bronchiolitis; open circles, fibrosis. Control rats: half-filled circles, combined bronchiolitis and fibrosis. Exposed rats at each exposure duration had significantly higher pathology scores than matched controls ($p < 0.001$); with longer duration of exposure there was worsening of the pathology grade for both bronchiolitis and fibrosis ($p < 0.001$).]
The striking total BAL cell increase in the 4- and 6-wk exposed animals did not change linearly with the dose or with the length of exposure. It is unclear from this study what mechanism caused the effects seen.

The histopathologic changes at terminal airway bifurcations were similar to the findings reported by Brody et al. (1981). In addition, at the time points studied, there was a firm correlation between exposure duration and response (fibrosis or bronchiolitis) (Fig. 3).

Both PMNs and macrophages appear to infiltrate the air spaces early after exposure to asbestos. Although the mechanisms of asbestos-initiated fibrogenesis are not completely understood, the macrophage apparently plays a key role in mediating the tissue injury (Barry et al., 1983; Miller, 1978; deShazo, 1982). In this experiment the macrophage was the predominant cell, but the presence of PMNs at sites where collagen deposition later occurs suggests the need for further investigation of their involvement in the development of early peribronchiolar fibrosis.

Variable findings have been previously reported regarding PMN involvement in the early cellular accumulation in the lung. A diffuse alveolitis consisting of PMNs was found in guinea pigs as the initial response secondary to instillation of asbestos fibers in saline intratracheally (Schoenberger et al., 1982). Yet exposure by the same method in sheep revealed only a mononuclear-cell accumulation during a similar time period (Begin et al., 1981). Using inhalation exposure methods, rats exposed for 1 h (Brody et al., 1981) or 5 d (Barry et al., 1983) revealed only a macrophage accumulation at terminal bronchioles.

This study examined the cellular response after a different exposure duration than those cited above. PMNs were increased in number in the BAL and tissue sections. The PMN appears to be an early-responding cell that persists with longer exposure as mononuclear cell accumulation increases. In Bozelka et al.'s (1983b) mouse model of inhalation, a hypercellular response seen in the peribronchiolar interstitium and alveolar spaces after 8 wk of daily exposure included increased numbers of PMNs (BAL was not performed). Mice examined 1 yr after 4 mo of exposure had increased numbers of PMNs in the BAL coincident with peribronchiolar fibrosis. This late BAL finding of increased PMNs in relation to already developed fibrosis is similar to human BAL results (Bignon et al., 1978; Gadek et al., 1981; Xaubet et al., 1984). An increased number of PMNs in the BAL of humans with asbestos exposure is found in the group of workers with fibrotic changes on chest radiograph. Observations made at this late stage of the disease when both PMNs and fibrosis are present cannot determine if the PMN initiated the fibrotic process. The time course examined in the study by Bozelka et al. (1983b) and Rola-Pleszczynski et al.'s (1984) sheep model revealed
the presence of PMNs prior to fibrosis development. The findings reported here agree with Bozelka et al.'s (1983b) findings that PMNs accumulate early during asbestos exposure prior to fibrosis development.

In the animals examined in this study, the increase in PMNs was seen most readily in the BAL analysis. The percentage and absolute increase in PMNs was seen in the BAL from 2-, 4-, and 6-wk exposed groups. The histologic samples only showed PMNs in large numbers in the 2-wk animals, perhaps the result of the relative number of PMNs in relation to other cell types (i.e., macrophages and lymphocytes) which continued to increase to much higher numbers as exposure time progressed. Differences between sampling by BAL and histologic examination of lung parenchyma is also a factor. For example, cells residing in the airways would be represented more strongly in BAL samples.

Immunologic aberrations are found in humans (Turner-Warwick and Parkes, 1970; Kagan et al., 1977a,b; Gaumer et al., 1981; deShazo et al., 1983; Miller et al., 1983; Bozelka et al., 1983a; Rola-Pleszczynski et al., 1982) and laboratory animals (Rola-Pleszczynski et al., 1981; Miller and Kagan, 1977, 1981; Miller et al., 1979; Bozelka et al., 1983b) exposed to asbestos. It is not known, though, if these abnormalities represent lymphocytic involvement in fibrogenesis. The finding of elevated and increasing numbers of lymphocytes in this study indicates that they are recruited to the lung early. The presence of lymphocytes during active collagen deposition may be evidence that they function as activated effector cells in early asbestosis lung injury.

Cell types seen in the BAL (Table 1) are similar to the types seen histologically in the airways and interstitium. The increasing bronchiolitis and fibrosis observed histologically were paralleled in the BAL by an increasing number of total cells retrieved and an increase in the total macrophage and lymphocyte number. The cellular differential and the total PMN number, which did not change over the exposure periods, did not parallel the worsening bronchiolitis and fibrosis. The apparent plateau in the PMN absolute number in the BAL may be related to either the short lifespan of PMNs compared to lymphocytes and macrophages and/or to the stimulus of a constant exposure to chrysotile fibers. For example, Wagner et al. (1974) showed that the chrysotile burden of the lung plateaued despite continuing exposure. If the recruitment of PMNs is dependent on fiber interaction with epithelial cells, macrophages, and/or extracellular inflammatory mediators, the limited fiber burden may be associated with the PMN findings in the BAL.

During the first 6 wk of heavy asbestos exposure, progressive pathologic changes were seen. The findings of increased number of macrophages, PMNs, and lymphocytes suggest that all these cells play a role in the early events of asbestosis.
REFERENCES


