Biphasic Cellular and Tissue Response of Rat Lungs After Eight-Day Aerosol Exposure to the Silicon Dioxide Cristobalite

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Cristobalite is a crystalline silicon dioxide that elicits pulmonary inflammation and fibrosis in humans and experimental animals. Exposure of rats to aerosols of respirable cristobalite for 8 days led to a rapid influx of neutrophils and macrophages into alveolar and tissue compartments of the lung followed by a more gradual accumulation of T lymphocytes. This inflammatory response persisted throughout 52 weeks after the end of the exposure. For some variables studied there appeared to be a cyclical nature to the response. Statistical analysis of alveolar cell populations and lung tissue weight, protein, and hydroxyproline showed significant time-dependent fluctuations. Histologic analysis revealed a progressive deposition of collagen and type II cell hyperplasia centered on airways, however, there appeared to be some correlation between fluctuations in alveolar cell populations and overall tissue pathology. The observed cellular and biochemical fluctuations and the persistence of the inflammatory response may be due to the presence of silica in the lung, which serves as a source of repetitive stimulation of lung cells. (Am J Pathol 1989, 134:1243–1251)

The crystalline silicon dioxide, alpha-cristobalite (CRS), elicits intense pulmonary injury in experimental animals and has been specifically associated with human pulmonary disease in diatomaceous earth workers.[1-3] Rats exposed for short periods (ie, up to 8 days) to aerosols of respirable CRS (1 μ aerodynamic diameter) respond with a rapid influx of inflammatory cells into alveolar and tissue

compartments of the lung.[4,5] Pulmonary inflammation persists for many months after cessation of exposure and fibrosis ensues within 2 to 4 months after exposure with increased collagen deposition continuing for several months. It appeared from previous studies that, although the inflammatory response to CRS begins within a few hours of exposure and persists at a high level, there were two peaks of inflammatory cell influx into the alveolar spaces.[5] From the end of the 8-day exposure through approximately 8 weeks, neutrophil, macrophage, and lymphocyte influx into the alveolar spaces steadily increased and then declined over the next 8 to 10 weeks. A second peak of influx of inflammatory cells occurred approximately 24 weeks after the end of the exposure. A biphasic alveolar inflammatory response has also been documented in mice exposed to silica.[6]

The present study was undertaken to determine if the overall pulmonary inflammatory response to CRS is actually multiphasic or if the observed fluctuations are simply due to random variation. Cristobalite-exposed rats were studied at 2-week intervals for 1 year after exposure. Variables monitored included cell yield and cell type from bronchoalveolar lavage fluid, lymphocyte subsets, lung weight, lung protein and lung hydroxyproline content, and lung tissue morphology. The response for each variable was analyzed statistically to determine the significance of the time-dependent fluctuations.

Materials and Methods

Aerosol Exposures

Alpha-cristobalite (CRS) was obtained from C & E Mineral, Corp., King of Prussia, PA. Male Fischer 344 rats weighing approximately 175 g were exposed to aerosols of respira-

ble CRS (mass median aerodynamic diameter of 1.14 ± 2.5 μ geometric standard deviation) for 5 hr/day for a period of 8 days. Aerosols were generated from a Wright dust feeder and animals were exposed in horizontal flow chambers.[7] The mean exposure dose was 27.5 ± 10.0 mg/m^3 SD. The mean dose was calculated from samples taken from 4 levels of 3 chambers each day for 8 days. To minimize the effects of variation in exposure concentrations in the 4 levels of the chambers, animals were rotated systematically throughout the chambers at daily intervals as described previously.[8] When the total silica present in the rat lungs 1 day after the end of the exposure is determined, the variability among rats is found to be approximately 10 to 15%.

Three control and three exposed rats were killed 18 hours after the end of the exposure period and thereafter at 2-week intervals for 50 weeks.

Bronchoalveolar Lavage

All salt solutions and culture medium components were obtained from GIBCO (Grand Island, NY). Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (15 mg/100 g body weight). An 18-gauge needle fitted to polyethylene tubing was inserted into the trachea and the thoracic cavity was opened. Blood was collected via cardiac puncture and the lung vasculature was then perfused with 30 ml Dulbecco's phosphate-buffered saline (DPBS). The lungs were excised and lavaged with 10 equal instillations of DPBS without calcium and magnesium (CMF-PBS), using a total of 33 ml/100 g body weight. The installation volume of PBS based on body weight (rather than using a constant volume) was chosen because lung growth occurs with increasing weight of the animal. During the 50 weeks of observation, control lung weight increased 1.5-fold and exposed lung weight increased 2-fold, whereas body weight for both control and exposed increased 2-fold.

Bronchoalveolar lavage (BAL) fluid collected from the 10 instillations was pooled, centrifuged at 500g, and resuspended in Hank's balanced salt solution (HBSS) for counting and cytocentrifuge preparation. Cytocentrifuged cells were stained with May-Grunwald and Giemsa solutions and differential counts were made.

Lymphocyte Identification Using Monoclonal Antibodies

Lymphocytes were separated from BAL cell suspensions by incubating on glass wool columns; 80 × 10^6 BAL cells were suspended in 5.0 ml HBSS containing 20% fetal bo-

vine serum (FBS) and layered over a 2.0 × 8.0 cm column of glass wool and incubated for 30 minutes at 37 C and eluted with 50 ml warm HBSS. Total BAL cell suspensions and glass wool column eluted lymphocyte suspensions were fixed for 2 hours at 4 C in 1% paraformaldehyde in pH 7.2 cacodylate buffer. Fixed samples were air-dried on microscope slides and incubated for 15 minutes in 0.3% H_2O_2 in 0.05 M TRIS buffer, pH 7.6, rinsed in DPBS and air dried. Monoclonal antibodies OX-8 and W3/25[9] were obtained from Pel-Freez Biologicals, Rogers, AR. Antibody samples were diluted in DPBS and added to the fixed cells and the slides were incubated for 1 hour at ambient temperature in a humidified chamber, then rinsed in DPBS. Secondary antibody (goat anti-mouse IgG conjugated to peroxidase) (Cooper Biomedical, Malvern, PA) was added to the slides for 1 hour. After rinsing in TRIS buffer, the slides were stained with DAB (3,3-diamino-benzidine-tetrahydrochloride) for 10 minutes, rinsed in H_2O, and air dried. The slides were counter stained with 1% methyl green in 1% acetic acid.

Lung Tissue Biochemistry

Lobes were cut away from the trachea and large bronchi and homogenized with a Polytron tissue homogenizer. To determine dry weight, two 1.0 ml aliquots of the homogenized lung sample were evaporated for 24 hours at 80 C in preweighed glass tubes; this time and temperature has been determined previously to be sufficient for drying the sample. For protein determinations, lung homogenates were incubated for 24 hours in 0.2 M NaOH, then sonicated. Protein content was measured by the method of Lowry et al.[10] To measure hydroxyproline content, the lung homogenate was precipitated in 5% TCA, and centrifuged at 10,000g. The precipitates were hydrolyzed in 6.0 N HCl for 48 hours at 110 C, dried, and resuspended in 0.2M sodium pyrophosphate buffer, pH 8.0, for assay. Hydroxyproline was determined by a modified method of Prockop and Udenfriend.[11]

Histology

Before excision the lungs were vascularly perfused with DPBS. The lungs were fixed via the airways with Zenker's formol while physiologic pressure was maintained. Five-micron sections were cut from paraffin blocks and stained with hematoxylin and eosin (H & E) and with the Gordon and Sweets silver impregnation method for reticulin fibers.[12]

Statistical Analysis

A one-way analysis of variance (with time as the grouping variable) was performed to demonstrate variation in outcome (eg, lung hydroxyproline, BAL cell yield, etc.) over time. Further examination of the location and nature of this variation was made using fitted models. For lung weight, protein, and hydroxyproline, the mean values of exposed lungs minus the mean values of control lungs were the biochemical data used for statistical modeling. BAL data were analyzed using mean cell counts or mean percentages at each time point. Each of the outcome variables was modeled as a truncated Fourier series (through the fourth-order term). That is, models of the form

$$\beta_1 + \sum_{\kappa=1}^{4} [\beta_{2\kappa} \sin(\kappa\beta_0 t) + \beta_{2\kappa+1} \cos(\kappa\beta_0 t)]$$

were fit (t = 0, 2, . . . , 50 weeks and β_0 = 0.1). The coefficients $\beta_1 \cdots \beta_9$ were estimated by multiple regression using SAS PROC REG (SAS Institute, 1985). The value of β_0 was chosen by performing the regression analysis for a grid of possible values of β_0. A single value of β_0 was chosen to maximize uniformly the coefficient of determination for all variables.

Using the models, estimates of mean outcome at time t, $\hat{y}(t)$, and mean outcome across all 26 time points, $\bar{y}$, were calculated for each outcome measure. Statistical tests for equality of mean outcome at time t and mean outcome across all time points were made using the test statistic

$$T(t) = \frac{\hat{y}(t) - \bar{y}}{\sqrt{\text{Var}[\hat{y}(t) - \bar{y}]}}$$

with testing done at significance level 0.05. The variance of $[\hat{y}(t) - \bar{y}]$ was obtained using the identity

$$y(t) - \bar{y} = [y(t) - \hat{y}(t)] + [\hat{y}(t) - y]$$

and the regression result that

$$\text{var}[y(t) - \bar{y}] = \text{var}[y(t) - \hat{y}(t)] + \text{var}[\hat{y}(t) - \bar{y}],$$

where y(t) is the mean of the observed values at time t.

The variance of y(t) $- \bar{y}$ can be estimated using the mean squared error from the regression analysis. The variance of the residuals, y(t) $- \hat{y}(t)$, which is a function of the mean squared error, can similarly be determined from the regression analysis. However, the mean squared error from the regression analysis (MSREG) estimated lack of fit of the model in addition to pure error at a given time point. Therefore, the mean squared error from the one-way analysis of variance described above (MSAOV) was used to adjust the variances to reflect only pure error and

not lack of fit. Consequently, the variance of $\hat{y}(t) - \bar{y}$ was calculated as

$$\left[\frac{25}{26}\text{MSREG} - \text{VAR}[y(t) - \hat{y}(t)]\right]\frac{\text{MSAOV}/3}{\text{MSREG}}$$

The fit of the models was summarized in terms of the percentage of nonerror variability explained by the modeled relationship between response and time, ie, variability explained by the model divided by the sum: variability explained by the model plus variability explained by its lack of fit to the data. This was calculated as

$$\frac{\text{sum squares due regression} - 8\,\text{MSAOV}/3}{\text{sum squares total} - 25\,\text{MSAOV}/3}$$

where the regression and total sums of squares were derived from the regression analysis. The coefficients of determination, R^2, were determined from the regression analysis, based on the means.

Results

Eight-day aerosol exposure of rats to cristobalite elicited an influx of inflammatory cells into tissue and alveolar spaces that were present from the end of the exposure and throughout the observation period of 50 weeks. The fibrotic response, characterized by both increased tissue hydroxyproline content and increased histochemical staining of collagen in the lung interstitium, progressed from a few weeks after the end of the exposure period through 48 weeks of postexposure observation.

Previous studies have suggested a biphasic alveolar inflammatory response over a period of 6 months after an 8-day cristobalite exposure.[5] The data reported in the present study include tissue and cellular responses in lungs of 8-day cristobalite-exposed rats at 2-week intervals for a period of 1 year after exposure. The statistical methods described above were used to determine if the observed time-dependent fluctuations in the measured variables were random or nonrandom.

For all the variables, analysis of variance revealed highly significant differences in mean outcome across 26 time points ($P < 0.01$ for lymphocytes, and $P = 0.0000$ for all others).

The observed means and predicted means for selected variables are shown in Figures 1 to 3.

Bronchoalveolar Lavage Cells

BAL recovery of neutrophils, macrophages, and lymphocytes is shown in Figure 1. Using the model, biphasic in-
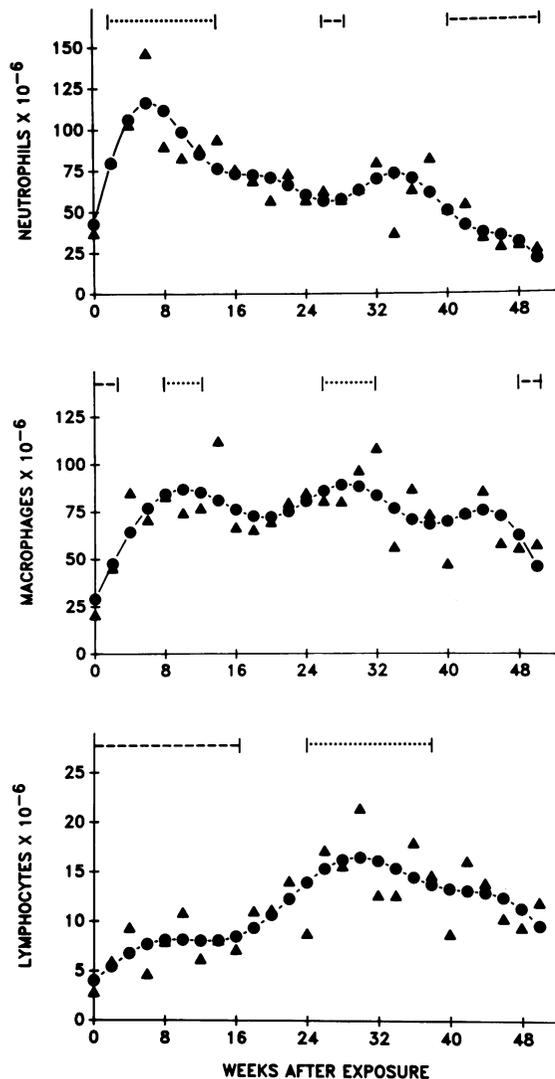
Figure 1. *Observed (▲) and predicted values (●) of macrophages, lymphocytes, and neutrophils recovered by bronchoalveolar lung lavage of cristobalite-exposed rats. Data are expressed as mean values of three rats at each observation time. (·····) denotes weeks when the predicted means were significantly above or (-----) significantly below the overall mean for each of the 26 time points (0, 2, ..., 50 weeks).*

creases and decreases were observed for neutrophils and macrophages. In contrast, lymphocytes showed a gradual rise, followed by a gradual decline. The peak in the lymphocytes coincided with the second peak in macrophages. Observed and predicted means for lymphocyte subsets (detected by monoclonal antibodies; W3/25, T-helper and OX8, T-suppressor) are shown in Figure 2. The data are expressed as percent of total lymphocytes and indicate statistically significant biphasic increases and decreases in the T-helper cells but not in the T-suppressor lymphocytes.

Lung Tissue Biochemistry

Means of observed and predicted values for lung dry weight, protein, and hydroxyproline content are shown in Figure 3. The patterns for each of these variables differs. Significant biphasic increases and decreases were found for lung weight, the first peak occurring at approximately 8 weeks and the second at about 34 weeks after exposure. The major peak for protein occurred at 32 weeks after exposure. Hydroxyproline levels generally increased over time, although some increases and decreases were observed.

Figures 1 to 3 indicate those weeks when the predicted means were significantly above (dotted lines) or significantly below (dashed) the overall mean. For each of the variables, the models were able to account for variability in the data other than error; the percentage varied, however, for the different variables. The percentages of nonerror variability accounted for by the models are given in Table 1. These ranged from 100% for excess lung weight to 30% for the T-suppressor lymphocytes.
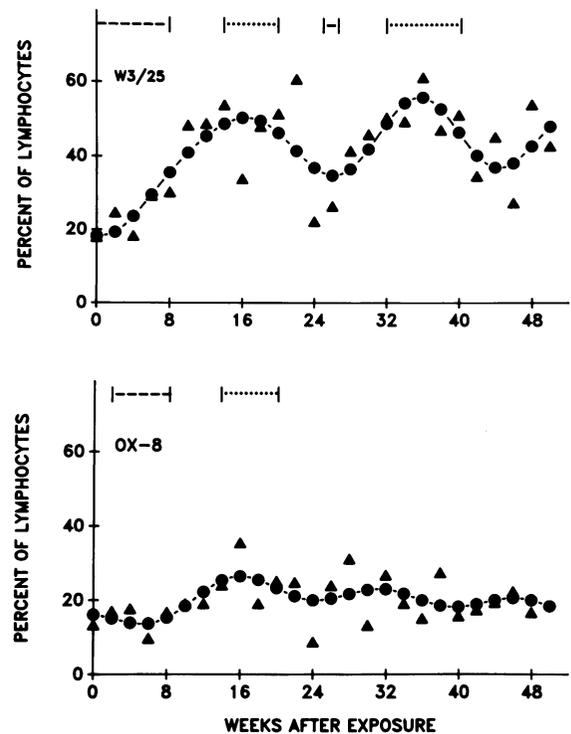
Figure 2. *Observed (▲) and predicted values (●) of lymphocyte subsets recovered by bronchoalveolar lung lavage of cristobalite-exposed rats and purified by glass wool filtration. Monoclonal antibodies, W3/25 identified T-helper subset and OX8 identified T-suppressor subset of lymphocytes. Data are expressed as percent of total lymphocytes and represent mean values of three rats at each observation time. (·····) denotes weeks when the predicted means were significantly above or (-----) significantly below the overall mean for each of the 26 time points (0, 2, ..., 50 weeks).*

Histopathology

Figures 4 and 5 show lung tissue sections from cristobalite exposed and unexposed rats at 8, 24, and 48 weeks after exposure. Tissue sections shown in Figure 5 were stained with ammoniacal silver for the demonstration of reticulin collagen fibers.

At 8 weeks after exposure the parenchyma contained multiple nodular inflammatory lesions centered on alveolar ducts and respiratory bronchioles. Within the lesions the

Table 1. *Percentage of Nonerror Variability Attributable to Model*

Variable	Percentage	R^2
Excess lung weight	100	0.80
Excess protein	72	0.58
Excess hydroxyproline	96	0.84
Neutrophils	84	0.79
Macrophages	58	0.53
Lymphocytes	78	0.65
T-helper lymphocytes	65	0.60
T-suppressor lymphocytes	30	0.30

Nonerror variability is the sum of variability explained by the truncated Fourier model and variability explained by the lack of fit of the model. Coefficients of determination, R^2, were determined from the regression analysis, based on the means.

Figure 3. *Observed (▲) and predicted values (●) of lung tissue dry weight, protein and hydroxyproline from cristobalite-exposed rats. Data shown are mean values of exposed lungs minus mean values of control lungs for lung weight, protein and hydroxyproline content for three rats at each observation time. (· · · · ·) denotes weeks when the predicted means were significantly above or (- - - - -) significantly below the overall mean for each of the 26 time points (0, 2, . . . , 50 weeks).*

alveolar spaces contained macrophages, neutrophils, nuclear dust, fibrin, and granular debris. Refractile CRS particles were distributed throughout the lung but appeared concentrated within these foci predominantly in macrophages. Also, within the lesions there was mild thickening of alveolar septa and discontinuous hyperplasia of Type II alveolar pneumocytes. The interstitium in these areas showed an occasional lymphocyte, plasma cell, and spindled interstitial cell. At low magnification, there was an overall periodicity to these lesions within the parenchyma, resulting from their airway association. A few peribronchial lymphocyte aggregates were seen, but were only slightly increased in number from those seen in unexposed lungs. No granulomas were identified at this time. There was an appreciable increase in interstitial collagen identifiable by silver impregnation. Interestingly, not only were the normally delicate reticulin fibers of the interstitium thickened in the inflammatory lesion, but they also were reduplicated and apparently more complex in their cross-fiber connections.

At 24 weeks after exposure, there was a definite increase in the size of the inflammatory nodules, many of which extending from the parenchyma to the pleura, the surface of which was diffusely thickened. Large peribronchial lymphoid aggregates containing noncaseating granulomas were observed. The interstitium was thickened in the areas of parenchymal inflammatory nodules. Prominent Type II cells formed a discontinuous epithelium along the surface of many peribronchiolar alveoli. Macrophages in nodules and alveolar spaces were foamy and many appeared to have degenerating cytoplasm, with refractile particulate material. The reticulin stain confirmed the presence of increased collagen within the inflammatory lesions and throughout the pleural surface. Reticulin density and complexity continued to increase progressively.

At 48 weeks after exposure, the parenchymal inflammatory lesions were less well defined and there was considerable overlap between lesions. Within the lesions there were fewer intra-alveolar macrophages and neutro-
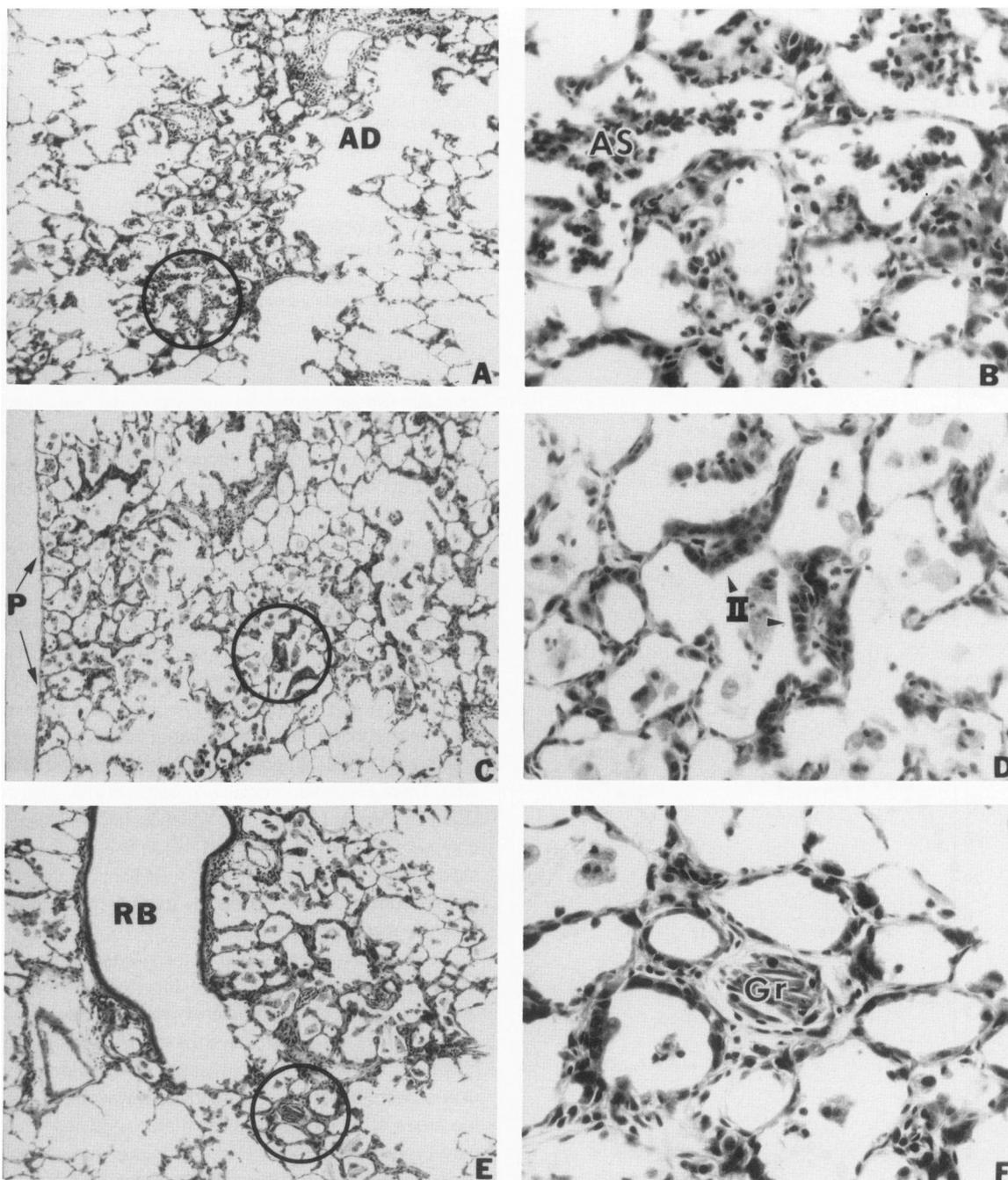
Figure 4. A *(X25)* and B *(X100)* demonstrate rat lungs 8 weeks after cristobalite exposure. Lesions are centered on alveolar ducts *(AD)*. Alveolar spaces *(AS)* contain aggregates of macrophages and neutrophilic leukocytes. By 24 weeks after exposure *(C [X25]* and D *[X100]) lesions are more frequently pleural (P) based. Enlarged airspaces are notable for strips of hyperplastic type II cells. E *(X25)* and F *(X100)* are representative photographs of rat lungs 48 weeks after exposure. Note the discrete centering of lesions on terminal airways *(RB)*. Continuous strips of hyperplastic type II cells are associated with septal fibrosis. Macrophages and neutrophils persist in airspaces. An intra-alveolar granuloma is present *(GR)*. Hematoxylin and eosin, all original magnifications.

phils. Type II cell hyperplasia was prominent and there was generalized thickening of the alveolar interstitium. Respiratory bronchioles were cuffed by lymphocytic aggregates in an irregular fashion. Within the inflammatory nodules, there were significantly more granulomas; these also were present in the submesothelial connective tissue. Coalesced macrophages in the parenchymal granuloma were frequently admixed with spindled cells resem-
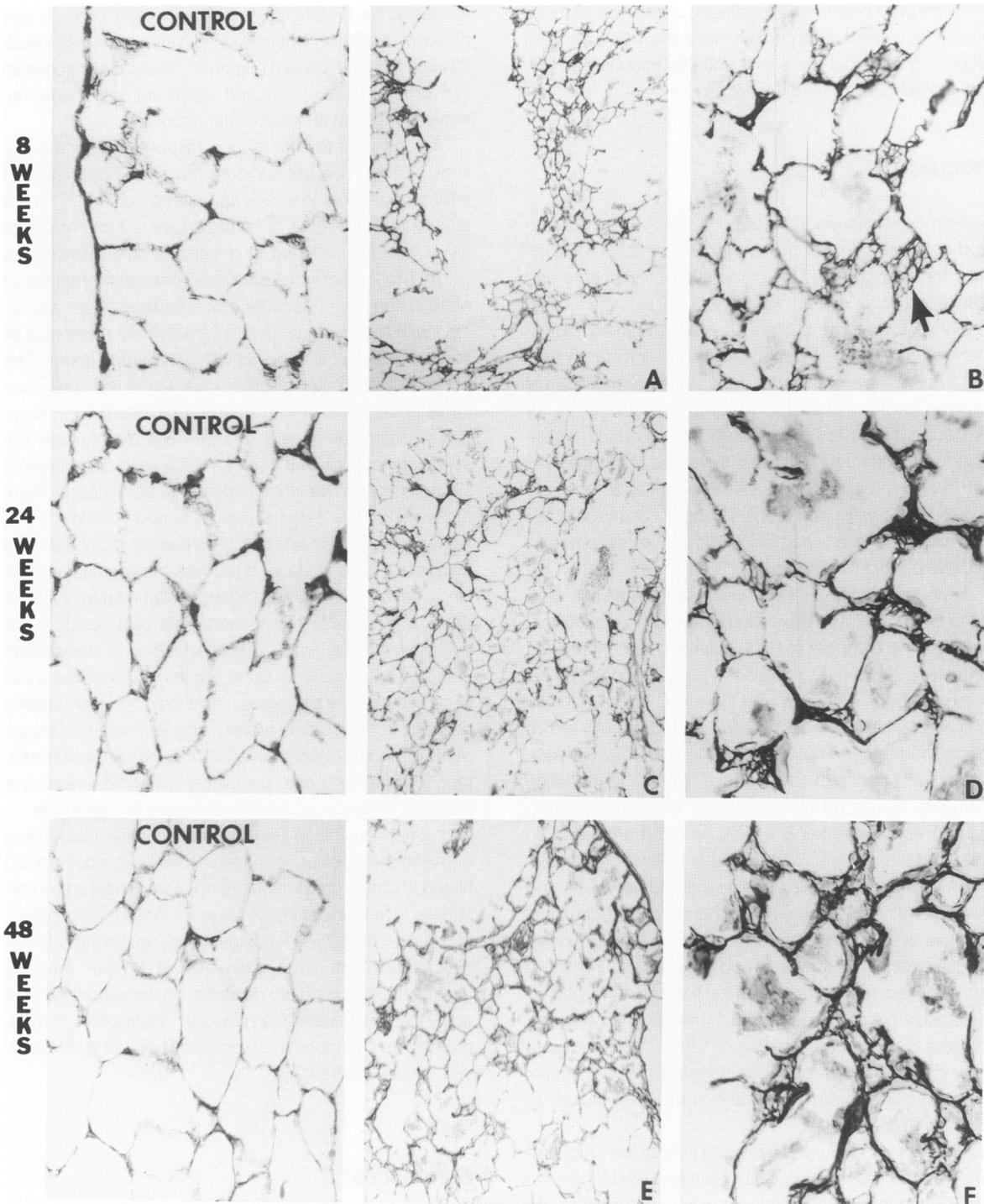
Figure 5. *Silver impregnated lung sections from 8 (A, ×25; B, ×100, 24 (C, ×25; D, ×100), and 48 (E, ×25; F, ×100) weeks after exposure to cristobalite. Note the progressive thickening and disorganization of interstitial reticulin collagen fibers compared with age-matched controls (×100).*

bling fibroblasts. In areas overlying the pleural-based nodules, mesothelial hyperplasia was now observed. The silver impregnation showed an impressive increase in parenchymal collagen centered predominantly on airways, blood vessels of all sizes, and the inflammatory lesions. Of particular note, parenchyma not involved directly by the inflammatory lesions also showed increased collagen fibers in a diffuse pattern. Furthermore, pleural-based le-

sions showed considerable collagen deposition focused on the subjacent inflammatory nodules but extending diffusely throughout the submesothelial interstitium in areas devoid of inflammatory cells.

Discussion

Experimental silicosis in laboratory animals is characterized by acute onset of pulmonary inflammation that persists for many months after cessation of exposure and results in development of fibrotic lesions. During the early response to crystalline silica, there is a marked recruitment of neutrophils and macrophages into lung alveoli.[4-6] Adamson and Bowden[6] found increased lysosomal enzyme activity in bronchoalveolar fluid within 1 day after exposure of mice to Dowson and Dobson quartz. Reiser et al[13] demonstrated significant increases in lung protein and hydroxyproline content within 1 week after exposure of rats to Dowson and Dobson quartz. Many of these early cellular and biochemical responses to silica persist for several weeks or months.[4,6,14]

In those studies where observation intervals were close and the total postexposure period was of sufficient duration, there appear to be at least two distinct phases of the lung inflammatory response to silica exposure.[5,6,14] In the study of Adamson and Bowden,[6] mice exposed to quartz exhibited an early and marked recruitment of macrophages and neutrophils to the alveoli, which subsided over a period of 1 to 2 weeks. Without further silica exposure, a second rise in alveolar macrophages and neutrophils began after 8 weeks, reaching a peak at 20 weeks after exposure. Early and late peaks of proliferative activity of interstitial cells were also noted, whereas lung hydroxyproline content showed a gradual and continual increase over a period of 20 weeks. In a study reported by Reiser et al,[14] lung weight and lung protein content of rats exposed to intratracheally instilled quartz appeared to increase over a period of 5 to 6 months, then subside, followed by a second increase at 12 months after exposure. In a preliminary report, we showed a biphasic influx of inflammatory cells in lung alveoli of rats exposed to aerosols of cristobalite.[5]

The present study was designed to evaluate long-term lung cell and tissue responses in rats exposed for 8 days to CRS and to use statistical methods to determine if observed time-dependent fluctuations in these responses are actually multiphasic or due to random variation.

The analysis indicated biphasic increases and decreases for neutrophils and macrophages (Figure 1). Total lymphocytes (Figure 1) showed only one major peak at about 30 weeks after exposure, however, the W3/25 positive lymphocyte subset did exhibit a significant bipha-

sic response (Figure 2). Lung dry weight showed two peaks of response, whereas protein showed a major peak 32 weeks after exposure (Figure 3). Fluctuations occurred in hydroxyproline content and significant peaks were observed at 32 and 48 weeks after exposure.

A consistent feature of lung response to silica exposure, whether quartz or cristobalite, is the persistence of inflammatory cells in alveoli and interstitium.[4-6,14,15] In the studies of Reiser et al,[13,14] a high dose (50 mg) of quartz given intratracheally led to development of granulomas within 1 to 2 weeks that continued through 12 months of observation after exposure. Silica particles were associated with the granulomas and intra-alveolar material at all times and at later time points within the lymph nodes. The continued presence of silica particles in the lung may serve as a repetitive stimulus for recruitment and activation of inflammatory cells. Our previous morphologic observations confirm that there is widespread distribution of silica particles in rat lungs exposed to aerosolized cristobalite during the 8-day exposure period.[4] While the observable pathologic effects of this particle distribution are diffuse at this early stage, a process of progressive particle concentration with associated inflammation is apparent by day 5 after exposure, principally centered on terminal airways. The lung content of silica in cristobalite-dusted rats persists at approximately 50% of the initial level (at the end of the exposure period).[16] Further, preliminary results from other studies[16] suggest continual movement of silica between alveolar and interstitial compartments of the lung and associated lymphoid tissue over period of several months after exposure to cristobalite.

It is not possible to establish a quantitative relationship between the alveolar cell populations and the developing histopathology; however, there appeared to be some correlation in response to CRS between these lung compartments over the 52 week postexposure observation. Histologic specimens were examined at 8-week intervals (weeks 8, 24, and 48 are described in this report). In general, the fluctuations in alveolar neutrophils, macrophages, and lymphocytes were corroborated by the analysis of tissue pathology.

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