American Journal of Pathology, Vol. 135, No. 4, Oct. 1989

Quantitative Immunohistologic Assessment of Lymphocyte Populations in the Pulmonary Inflammatory Response to Intratracheal Silica

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Immunogold-silver staining was used to identify T lymphocytes, T lymphocyte subsets, and B lymphocytes in lung tissue from mice injected intratracheally with silica, titanium dioxide, or saline alone. Morphometric quantitation revealed a marked influx of T lymphocytes in the silica-treated animals during the first 3 weeks after injection. The relative numerical density of these cells remained elevated when compared with saline-treated controls throughout the 12 weeks of the experiment. Cells expressing the CD4 and CD8 antigens were both increased in number, with the former accounting for approximately two-thirds of the T lymphocytes. An increased number of B lymphocytes was also apparent from 6 weeks after treatment with silica. The T lymphocyte response preceded the development of significant pulmonary fibrosis by several weeks. No lymphocyte response was observed in the lungs of mice injected with nonfibrogenic titanium dioxide. These observations are consistent with the hypothesis that lymphokines secreted by T lymphocytes play a role in the pathogenesis of silicotic inflammatory lesions and their progression to fibrosis. (Am J Pathol 1989, 135:605–614)

The pathogenetic mechanisms involved in the development of pulmonary fibrosis have not been completely defined. Histopathologically, there is usually evidence of interstitial and often intra-alveolar proliferation of fibroblasts, with deposition and rearrangement of collagen, an accompanying inflammatory response dominated by mononuclear cells, and hypertrophy and hyperplasia of type 2 pneumocytes.[1] Possible interactions between these cell populations and the role of various cytokines in mediating such interactions have been the focus of a number of recent studies. Much information has accumulated that supports the belief that cytokines secreted by cells that participate in the inflammatory response have the potential to stimulate fibroblast chemotaxis, proliferation, and collagen synthesis.[2-13] The biological role of such molecules *in vivo*, however, has not yet been elucidated.

Most studies of fibroblast-stimulating cytokines in pulmonary fibrosis have concentrated on the macrophage as the cellular source of such mediators. These have established that, in both experimental and clinical disease, macrophages are able to release factors that may promote fibroblast growth, collagen synthesis, or both.[14-18] However, studies of the induction of secretion of macrophage-derived growth factors *in vitro* suggest that these cells exhibit responses of comparable magnitude to both fibrogenic and nonfibrogenic stimuli.[2,19] Therefore, if macrophage-derived growth factors have relevance in the development of fibrosis *in vivo*, mechanisms must exist that regulate the secretion of growth factors, so that sustained secretion of biologically active cytokines occurs only in response to a fibrogenic stimulus.

Such regulatory signals may be provided by the secretion of lymphokines that could activate pulmonary macrophages to secrete growth factors. Lymphocytes are frequently present in the interstitial inflammation associated with the development of pulmonary fibrosis and may participate in an immunologic response to alterations in the structural components of lung tissue induced by the fibrogenic stimulus.[20,21] However, there have been few studies of the secretion of mediators by pulmonary lymphocytes. There is some evidence supporting both the hypothesis that lymphocytes secrete mediators that directly stimulate fibroblast proliferation[22] and the possibility that lymphocyte–macrophage interactions play a role in the induction of growth factor secretion by macrophages.[23]

Supported by grants from the National Health and Medical Research Council of Australia and from the Australian Tobacco Research Foundation.

Accepted for publication May 26, 1989

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Ongoing studies in this laboratory are directed towards evaluating the possible contribution of lymphokines to the development of particle-induced pulmonary fibrosis initiated by intratracheal injection of silica in mice. This experimental system is considered a model of acute or accelerated silicosis.[24] That lymphocytes participate in the inflammatory response in human silicosis has long been recognized, and accompanying alterations in immunologic responsiveness have also been documented.[25] However, there is little quantitative information available regarding the involvement of lymphocytes in pulmonary inflammation induced by silica and virtually no studies of their possible pathogenetic role have been performed.[25,26] Therefore, as a first step towards assessing the functional capacity of lymphocytes to influence the development of silicotic fibrosis, this study was undertaken to quantitate lymphocyte populations in the pulmonary inflammatory response induced by silica, compared with the response induced by nonfibrogenic titanium dioxide or by saline alone. T lymphocytes, T lymphocyte subsets, and B lymphocytes were identified by immunogold-silver staining, and their relative numerical densities were estimated by appropriate morphometric methods. The involvement of these cells was correlated with the development of histologically recognizable pulmonary fibrosis.

Materials and Methods

Animals

Twelve-week-old female specific pathogen-free BALB/c mice were obtained from the animal breeding facility at the Australian Nuclear Science and Technology Organisation, Sydney, Australia. Animals were housed in filter-top cages on autoclaved bedding in a barrier environment. Irradiated food and autoclaved water were allowed *ad libitum*. All experimental procedures complied with the requirements of the Animal Ethics Committee of the University of New South Wales (ref. no. 88/3).

Particulates

Silica with a particle size less than or equal to 5 μm (Min-U-Sil, Pennsylvania Glass Sand Co., Pittsburgh, PA) (a generous gift from Dr. G. E. R. Hook, NIEHS, Research Triangle Park, NC) was prepared as previously described by washing in hydrochloric acid.[27] For intratracheal injection, particles were suspended in 0.9% saline at 40 mg/ml and ultrasonicated for 60 seconds to break up aggregates. Titanium dioxide (Sigma, St. Louis, MO) was similarly dispersed at 40 mg/ml by ultrasonication. Particles were resuspended by vigorous syringing before injection.

Intratracheal Injection

Mice were anesthetized by intraperitoneal injection of a mixture of 100 mg/kg of ketamine (Ketalar, Parke... Sydney) and 3.3 mg/kg of xylazine (Rompun, Bayer, Sydney). The ventral aspect of the neck was shaved and the skin incised in the midline. A pediatric ocular speculum was inserted to expose the strap muscles, which were separated by blunt dissection to reveal the trachea. Using a 1 ml tuberculin syringe attached to an angled #26 needle, 0.05 ml of a suspension of particles or of vehicle alone were injected into the tracheal lumen. The incision was closed with interrupted silk sutures.

Experimental Design

Animals were divided into 13 groups of six each and received intratracheal injections of either 2 mg of silica, 2 mg of titanium dioxide, or saline alone. Silica-treated animals were killed 1, 2, 3, 4, 6, 8, and 12 weeks after injection. Animals receiving titanium dioxide or saline alone were killed 1, 4, and 12 weeks after treatment.

Preparation of Tissues

Animals were anesthetized with 75 mg/kg of pentobarbital (Nembutal, Ceva Chemicals, Sydney) and killed by transection of the aorta. The pulmonary trunk was cannulated with a #21 needle and the lungs perfused with 0.9% saline under a pressure of 60 cm H_2O for 60 seconds to remove blood from the pulmonary capillary bed. The trachea was then cannulated with a blunted #19 needle and the lungs were inflated to total lung capacity with 1 ml of frozen section embedding medium (Tissue-Tek, Miles Scientific, Naperville, IL). Three horizontal slices, each approximately 1-mm thick, were cut from the midzone of the single-lobed left lung. One slice was fixed in 10% phosphate-buffered formalin for 24 hours before embedding in paraffin. Four-μm-thick sections from the paraffin-embedded tissue were stained either with hematoxylin-eosin or with a modified Masson trichrome stain in which the nuclear counterstain was omitted to improve contrast. The other two slices of lung were frozen in isopentane precooled in liquid nitrogen and stored at −70 C. Six-μm-thick sections were cut on a Reichert Frigocut 2800E (Reichert-Jung, Nussloch, West Germany), fixed in acetone for 10 minutes, and stored at −20 C until use.

Immunostaining

Rat monoclonal antibodies to the mouse T lymphocyte surface membrane differentiation antigens Thy-1 (pan-T

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lymphocyte), L3T4 (CD4), and Ly-2 (CD8)[28] were obtained from SeraLab (Crawley Down, UK). Monoclonal anti-mouse IgM was obtained from Serotec (Oxford, UK). Affinity-purified goat anti-rat IgG conjugated to 5 nm colloidal gold particles (AuroProbe LM) and IntenSE II silver enhancement reagent were purchased from Janssen Life Sciences (Olen, Belgium).

Immunogold-silver staining (IGSS) of T lymphocytes and B lymphocytes was performed using a capillary action staining system as previously described.[29] Components for the manual Probe-On slide staining system were obtained from Fisher Scientific (Pittsburgh, PA). Briefly, frozen sections were mounted on Probe-On slides coated with 0.5% gelatin in 0.05% chromic potassium sulfate. Sections were blocked with 1:10 diluted normal goat serum, incubated with appropriately diluted monoclonal rat antibody for 45 minutes, washed, and incubated with gold-conjugated goat anti-rat antibody for 45 minutes. After washing and postfixing the sections in 1% ethanolic formalin, the gold label was visualized by precipitation of silver around the particles with IntenSE II reagent. Sections were counterstained with methyl green and eosin. Each batch of slides that was immunostained included sections of normal mouse spleen that served as controls for the IGSS procedure.

Morphometry

A systematic sampling procedure with a random start was employed as recommended by Weibel.[30] For each animal, the blocks and the block faces to be sectioned were chosen randomly. Stained sections were examined with a Zeiss microscope (Carl Zeiss, Oberkochen, FRG) to which a light video camera (Sony Corp., Tokyo, Japan) was attached, and images were projected on a color monitor (48 cm diagonal). The screen was overlaid with a coherent point-counting lattice of 7 × 5 points, spaced 5 cm apart, of which one point was designated as the test point. To examine each slide, the test quadrat was initially located at one corner of the section. A random start was achieved by using a computer-generated random number to select one of the 35 points and moving the stage so that the tissue underlying the selected point was superimposed on the location marked by the test point. Thereafter, fields were systematically sampled at 1-mm intervals using the vernier stage movement. Every field in each section was evaluated, yielding an average of approximately 15 fields per section.

Quantitation of the relative numerical density of T lymphocytes, B lymphocytes, and T lymphocyte subsets was performed using an approach similar to that of Chandler et al.[31] Sections were examined with a ×16 magnification objective at a final magnification of ×1667. Profiles of positively immunostained cells with a visible nucleus were counted in each field, applying the rules discussed by Weibel,[32] and the number of points falling on the section was recorded. The cell count was then expressed as number of cells/mm^2 of tissue section. The volume density of collagen in Masson trichrome-stained sections was determined by relative point-counting of positive-staining areas in parenchymal tissue using a ×40 objective.

Statistical Methods

Data from the cell counts were logarithmically transformed before analysis. Differences between control and particle-treated groups of animals were assessed by one-way analysis of variance. The effect of intratracheal injection of particles at each time point was compared with the combined means of the saline-treated control groups by the multiple contrasts procedure of Scheffe's S test.[33]

Results

Intratracheal injection was successful in all 78 animals, and none of the animals died during the course of the experiment.

Histopathologic Findings

Animals injected with silica exhibited a florid pulmonary inflammatory response. In sections stained with hematoxylin and eosin, lesions were most often found in the posteromedial portion of the lung. As early as 1 week after injection of silica, the most striking component of the response was the development of granulomas, consisting mostly of modified macrophages with the morphology of epithelioid cells. These were usually distributed in relation to respiratory bronchioles. Particles of silica were often identifiable within the granulomas by polarization microscopy. These particles were frequently surrounded by small clusters of neutrophils. A granulomatous response was also noted in hilar lymph nodes at this stage. Between the granulomas, many alveoli contained foamy, granular eosinophilic material. Particles of free silica were often identified in these alveoli, which also contained numerous free alveolar macrophages, many of which had highly vacuolated cytoplasm. Perivascular aggregates of

Figure 1. *Pulmonary inflammatory response induced 1 week after intratracheal injection of silica. Numerous epithelioid cell granulomas are present and many alveoli contain granular eosinophilic debris. Clusters of lymphocytes surrounding small blood vessels are also visible (arrows) (H&E, original magnification × 120).*

lymphocytes were frequently seen (Figure 1). In sections stained by IGSS, most of these cells were T-lymphocytes that stained positive for the Thy-1 antigen (Thy-1$^+$) (Figure 2), although a small proportion of B lymphocytes exhibiting surface membrane staining for IgM (sIgM$^+$) was also noted.

Polarizable silica particles continued to be visible in the granulomas for the duration of the experiment. In later lesions, the inflammatory response was sustained and was accompanied by obvious alveolar septal thickening, with large numbers of plump cuboidal epithelial cells covering the alveolar surfaces. The morphology of these cells suggested that they were probably hypertrophic type 2 pneumocytes. Nodular aggregates of lymphocytes around blood vessels and bronchioles were quite prominent by approximately 6 weeks after injection of silica. IGSS revealed that most of these cells were now sIgM$^+$ B lymphocytes (Figure 3).

Examination of sections stained with Masson's trichrome demonstrated deposition of collagen fibers in the lung parenchyma from 4 weeks onwards, although some animals exhibited fibrosis as early as 1 week after injection of silica. Fine bundles of collagen fibers were seen in peri-

Figure 2. *Frozen section of lung from an animal [illegible] silica 2 weeks previously, demonstrating perivascular aggregates of T lymphocytes, which exhibit membrane staining for the Thy-1 antigen (IGSS-methyl green counterstain, original magnification × 80).*

vascular and alveolar interstitial regions, whereas nodular intra-alveolar fibrosis occurred in relationship to the silicotic granulomas (Figure 4).

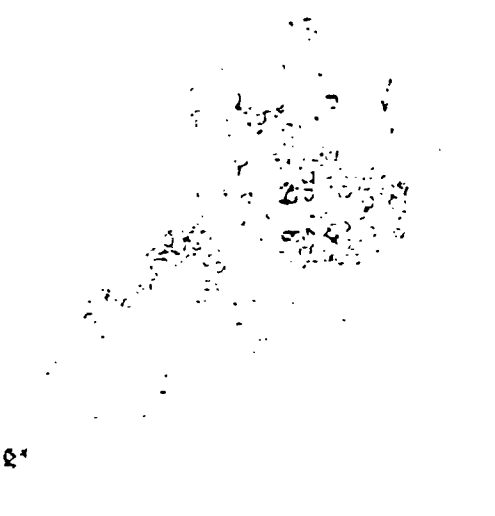

Figure 3. *Frozen section of lung from a silica-treated animal after 6 weeks, showing a nodular aggregate of lymphocytes adjacent to a blood vessel. Most of the cells are B lymphocytes that exhibit surface membrane staining for IgM (IGSS-methyl green counterstain, original magnification × 200).*

Figure 4. *Deposition of collagen fibers within silica-induced granulomas from an animal 4 weeks after intratracheal injection (Masson trichrome, original magnification ×200)*

In contrast to the response observed with silica, animals injected with titanium dioxide exhibited little if any inflammatory response to the particles. Much of the titanium dioxide was phagocytized by alveolar macrophages. In addition, free titanium dioxide was readily demonstrable in the alveolar lumina (Figure 5). These appearances persisted throughout the period of study. Particles of titanium dioxide were also found within macrophages in the hilar lymph nodes.

No inflammatory changes were observed in animals injected with saline alone.

Morphometry

The qualitative changes observed were validated by the results of the morphometric analysis. In the silica-treated animals, enumeration of Thy-1$^+$ T lymphocytes revealed a marked early influx of these cells during the first 3 weeks after injection. Thereafter, the number of Thy-1$^+$ cells in the lungs decreased somewhat, but remained significantly elevated relative to the saline-treated controls for most of the period of study (Figure 6). The numbers of CD4$^+$ and CD8$^+$ cells were both increased (Figure 7) and together accounted for the increase in the number of Thy-1$^+$ cells. Most T lymphocytes at each time point studied were CD4$^+$ cells. The ratio of the mean number of CD4$^+$:CD8$^+$ cells was similar to the ratio of 2.4:1 found in the lungs of control animals.

In contrast, sIgM$^+$ B lymphocytes were only modestly increased early in the inflammatory response, but were present in significantly increased numbers from 6 weeks onwards (Figure 8). Correspondingly, the ratio of the mean number of B:T lymphocytes rose from 0.4:1 in the first 2 weeks to over 1.6:1 in the period 6-to-12 weeks after injection, compared with a mean ratio of 0.7:1 in the control animals.

The volume fraction of stainable collagen increased during the early weeks of the experiment and reached statistical significance at 8 weeks after injection of silica (Figure 9).

No significant changes in either the relative numerical densities of pulmonary lymphocytes or the volume fraction of collagen were found in the animals injected with titanium dioxide (Figures 6, 8, and 9).

Discussion

The pulmonary response induced by intratracheal injection of silica has been previously described. Most studies

Figure 5. *Aggregated particles of titanium dioxide and particle-laden macrophages in the alveoli of an animal 4 weeks after intratracheal injection (H&E, original magnification ×200)*

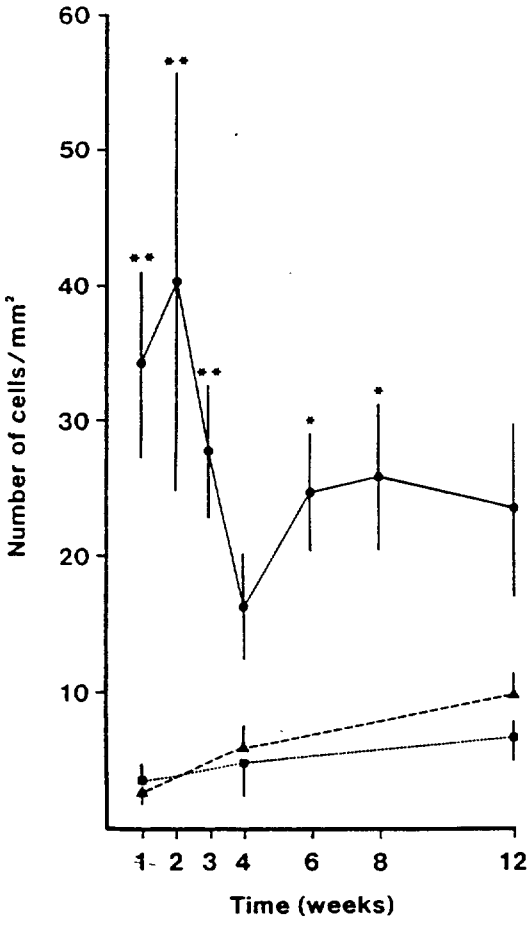

Figure 6. *Relative numerical density (mean ± SEM) of Thy-1⁺ cells in the lungs of animals at various times after treatment with silica (● —— ●), titanium dioxide (▲ - - - ▲), or saline alone (■···■). Significant differences from the combined means of the saline-treated control groups are shown as *, P < 0.05 and **, P < 0.01.*

have emphasized macrophage-dominated granulomatous inflammation,[34,35] the development of alveolar lipoproteinosis[24,35] and accompanying hypertrophy, and hyperplasia of type 2 pneumocytes.[35,36] In the experiments described here, the response was entirely consistent with that found in earlier studies. These reports also commented on the presence of lymphocytes in the inflammatory cell population, both in the interstitium surrounding the macrophage-dominated silicotic granulomas[34] and in peribronchiolar aggregates.[35] However, so far as the author is aware, quantitative immunohistologic analysis of the responding lymphocytes has not previously been undertaken.

The data from the present experiments establish that a significant influx of Thy-1⁺ T lymphocytes occurs early in pulmonary inflammation induced by intratracheal injection of silica. CD4⁺ and CD8⁺ cells are correspondingly increased with CD4⁺ cells accounting for approximately two thirds of the T lymphocytes participating in the response. Although the Thy-1 antigen is not unique to murine T lymphocytes, being expressed to a significant extent on NK cells and dendritic cells,[37] the CD4 and CD8 antigens are not markers for these populations,[38] and the observation that the increase in numbers of CD4⁺ and CD8⁺ cells accounted for the increase in Thy-1⁺ cells confirms that the Thy-1⁺ cells enumerated were T lymphocytes. The T lymphocyte response, which precedes the development of significant fibrosis by 3 to 6 weeks, is sustained through the evolution of the pulmonary lesions. In the later stages, there is also a marked increase in the number of sIgM⁺ B lymphocytes in the lung.

Statistical evaluation of the morphometric data probably underestimates the magnitude of the lymphocyte response within the silica-induced lesions. The coefficient of

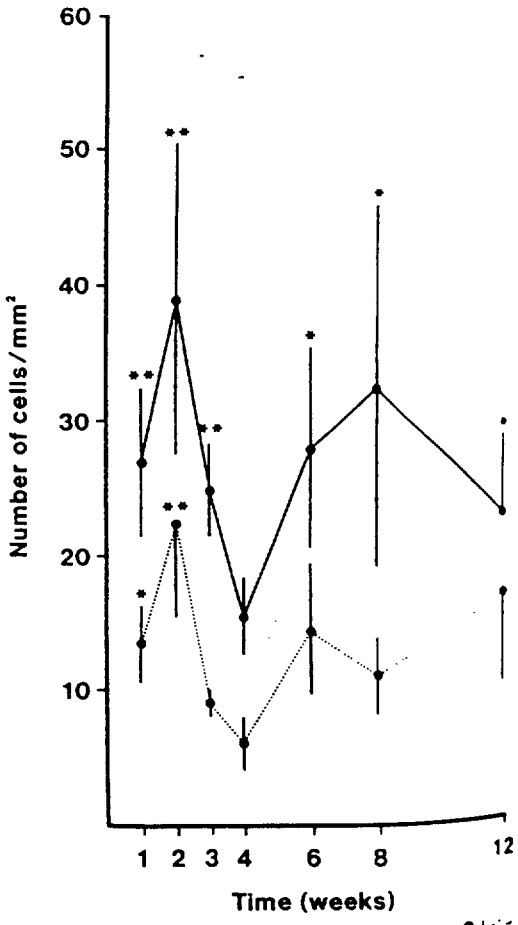

Figure 7. *Relative numerical density of CD4⁺ (● —— ●) and CD8⁺ (●···●) cells in the lungs of animals treated with silica. For the sake of clarity, data for other experimental groups are not shown. Significant differences from the combined means of the saline-treated control groups are shown as *, P < 0.05 and **, P < 0.01.*

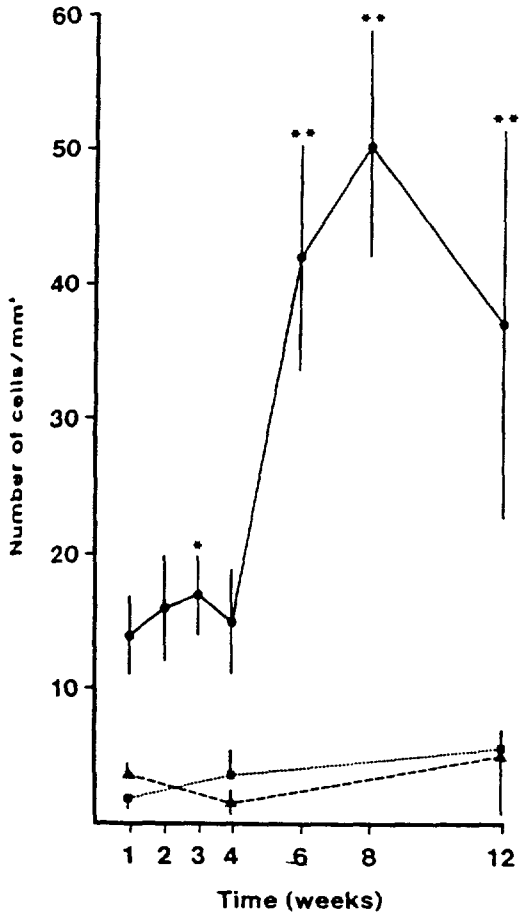

Figure 8. *Relative numerical density of sIgM⁺ cells in the lungs of animals treated with silica (● ——— ●), titanium dioxide (▲ - - - ▲), or saline alone (■ · · · ■). Significant differences from the combined means of the saline-treated control groups are shown as *, P < 0.05 and **, P < 0.01*

ariation for most groups was high; statistical significance sing the conservative procedure of Scheffe's *S* test, erefore, was only achieved when the mean differences n the number of cells were large. Quantitative variations n the alveolar delivery of silica and the inherent biological ariability of the inflammatory response undoubtedly con- buted to the high coefficient of variation. However, an mportant reason for the variability was the nonuniform stribution of the lesions, which is a well-recognized roblem associated with the delivery of particles by intra- acheal injection.[39] In some of the sections examined, a arge proportion of the microscopic fields evaluated con- sted of uninvolved lung tissue, when sections from an acent block from the same animal exhibited lesions olving a much greater proportion of the section, a more ond cellular response, or both. This appeared to be the ain reason for the low relative numerical densities of lymphocytes recorded for several animals of the group that had received silica 4 weeks previously. Nevertheless, to maintain the integrity of the random sampling procedure, no sections were excluded from evaluation on the grounds of inefficient sampling.

Recently, Struhar et al[40] demonstrated that, following administration of silica to rats by intratracheal injection, there was an early increase in the number of T-lymphocytes obtained by bronchoalveolar lavage or by dissociation of lung tissue with collagenase. In agreement with the results reported here, the lymphocytic response was dominated by $CD4^+$ cells and was maximal at 14–30 days postexposure. It remains to be established whether human pulmonary disease induced by inhalation exposure to silica is also associated with a sequential increase in the number of T lymphocytes and B lymphocytes in the lung parenchyma, as has been demonstrated in this experiment. However, it is noteworthy that perivascular and peribronchial aggregates of lymphocytes were described in mice exposed to silica dust by inhalation,[41] although no assessment of lymphocyte populations was carried out. Similarly, the presence of lymphocytes surrounding the

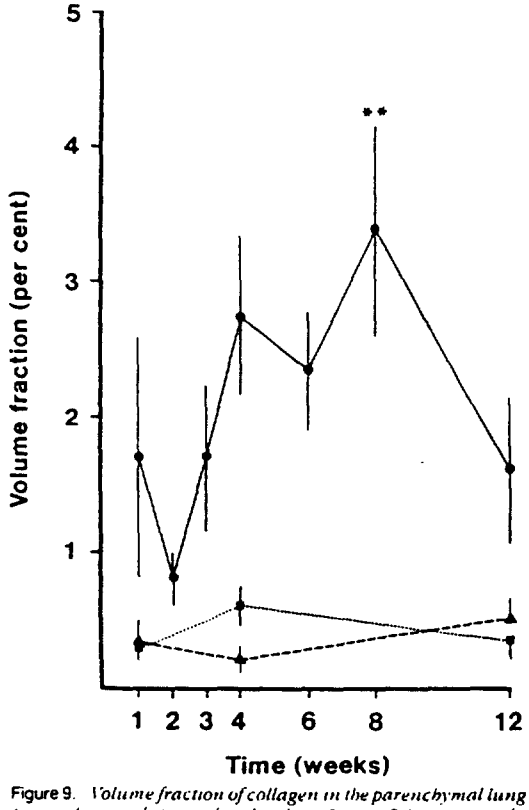

Figure 9. *Volume fraction of collagen in the parenchymal lung tissue of animals treated with silica (● - - - ●), titanium dioxide (▲ - - - ▲), or saline alone (■ · · · ■). Significant differences from the combined means of the saline-treated control groups are shown as **, P < 0.01.*

granulomatous lesions in human silicosis has long been recognized, but no information about the phenotype of the cells involved at different stages of the disease is available.[25]

The mechanisms underlying the demonstrated pattern of T lymphocyte and B lymphocyte responses are unknown. A plausible hypothesis might be that T lymphocytes are recruited as a result of the secretion of chemotactic factors by macrophages.[42] Macrophages that phagocytize silica are stimulated to secrete interleukin 1,[43] which has been shown to be chemotactic for T lymphocytes *in vitro*.[44] Disruption of the alveolar epithelial surface by the initiating injury may lead to the generation of immunogenic fragments from components of the interstitial matrix such as collagen and elastin,[45,46] which have been shown to be capable of evoking an immunologic response.[20,21] T lymphocytes participating in such a response might be activated to secrete lymphokines, such as interleukins 2, 4, 5, or 6, or interferon-γ, which can trigger the recruitment and/or proliferation of B lymphocytes.[47]

At this stage one can only speculate on what role, if any, these cells play in the progression of the silicotic lesion. However, the belief that T lymphocytes contribute to the development of pulmonary fibrosis is supported by studies of lung disease induced by bleomycin.[48,49] The observation that the influx of T lymphocytes in silica-induced inflammation precedes demonstrable fibrosis by 3 to 6 weeks is also consistent with such a hypothesis. Although experiments employing lung slices in organ culture suggest that the development of pulmonary fibrosis does not necessarily require the presence of an inflammatory response,[50,51] the possibility exists that lymphokines secreted by T lymphocytes stimulate fibroblast proliferation and collagen synthesis, either directly or by activation of macrophages to secrete growth factors for fibroblasts. A direct role for T lymphocyte-derived mediators is suggested by T cell-dependent models of fibrogenesis[52,53] and is consistent with evidence of reduced fibrosis after bleomycin-induced pulmonary injury in T lymphocyte-deficient nude mice.[54] The properties of a lymphocyte-derived fibroblast-activating factor are the subject of continuing investigation.[12,13,55] Mediators that might trigger growth factor secretion could include lymphokines such as interleukin-4 and interferon-γ, which are known to activate macrophages.[47] There is some experimental evidence for such a role for interferon-γ.[23]

T lymphocyte-derived lymphokines, which might cause a polyclonal proliferation of B lymphocytes as hypothesized above, could also stimulate immunoglobulin synthesis by these cells. This could provide an explanation for the disordered regulation of immunoglobulin synthesis observed in human silicosis, which is characterized by circulating antinuclear antibodies and rheumatoid factors and an increased incidence of autoimmune diseases.[56,57] Local synthesis of immunoglobulins might also explain the long-recognized presence of immunoglobulins within the hyaline material in a classical silicotic nodule.[58]

The results of the present study provide a foundation for future *in vitro* experiments focusing on the possible contribution of lymphokines to the progression of the silicotic inflammatory lesion and the ensuing fibrosis.

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Acknowledgment

I thank Mrs. Daphne Greening and Mrs. Mignon Wegner for assistance in the preparation of tissue sections, Mr. Alfred Dekolbe for caring for the experimental animals, [illegible] Department of Medical Illustration, University of New South Wales for preparation of the figures.

Immunoph[illegible] Characteri[illegible] Non-Hodgk[illegible]

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Diffuse, mixed small (DML) are a heterog[illegible]kin's lymphomas. Th[illegible]mmunophenotypic [illegible] DML, and they includ[illegible]s unclear whether [illegible]lonal, oligoclonal, o[illegible]ng monoclonal anti[illegible] LN1, LN2, and L26) [illegible]mbedded, B5-fixed t[illegible]udied. This method [illegible]ion between cell mor[illegible]ype compared with f[illegible]. In addition, Sou[illegible]ation was used to i[illegible]he neoplastic cell pop[illegible]ation of large B lymp[illegible]mmunohistology. In [illegible] immunoglobulin l[illegible]zen section immun[illegible]ocytology, or both, s[illegible]he B lymphocytes ar[illegible]he cases (12 of 13), t[illegible]ited predominantly [illegible]obulin gene rearra[illegible]ren cases, but no T [illegible]ents were detected. [illegible]re monoclonal lymp[illegible] non-neoplastic T ce[illegible]989, 135:615–621)

[illegible]se mixed small and l[illegible]es (DML), or diffuse mi[illegible]mas, were described [illegible] consisting of a prolifer[illegible]ed "reticulum cells" ("h[illegible]