Morphometric Estimates of Infiltrative Cellular Changes During the Development of Bleomycin-Induced Pulmonary Fibrosis in Hamsters

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The sequence of cellular infiltration into interstitial lung tissue subsequent to intratracheal administration of bleomycin was examined in hamsters. Quantitation of lesions following bleomycin treatment showed a transient increase in the percentage of lung involved, which peaked at 21 days and decreased thereafter. Associated with these lesions was a significant increase in interstitial cell profile density at 21 and 28 days. The total number of cell profiles decreased after 28 days. The cellular composition of the lesion was dominated by monocytes, neutrophils, and macrophages in the initial phase of the fibrosis. Subsequently, monocytes were significantly decreased at 21, 28, and 42 days, as compared with the 7-day value. Similarly, neutrophils were significantly decreased at 21 and 28 days, as compared with the 7-day value. In contrast, macrophages were significantly decreased in the initial phase (7 days) of the cellular infiltration and the later phase at 35 and 42 days, as compared with the value at 4 days after treatment. Lesion composition in the later phase exhibited significant increases in fibroblasts and eosinophils, accompanied by a general increase in lymphocytes. It is concluded that bleomycin-induced inflammatory sequela exhibits temporally based changes in cellular composition of the infiltrate and that the temporal changes in cellularity might be one of the determinants in the pathophysiology of pulmonary fibrosis induced by bleomycin. (Am J Pathol 1983, 112:170–177)

BLEOMYCIN is a chemotherapeutic agent used against several squamous cell carcinomas.[1] Unfortunately, a dose-related development of interstitial pulmonary fibrosis is associated with its use.[1,2] In order to study the pathologic mechanisms of pulmonary fibrosis an experimental animal model has been developed by using a single intratracheal injection of bleomycin in the hamster.[3–5] The histopathologic and biochemical evaluations of this model have shown it to be similar to the pulmonary fibrotic changes in humans produced by the use of bleomycin.[6–9]

The pathogenesis of pulmonary fibrosis is not fully understood, but a growing body of evidence suggests that the immune and inflammatory systems affect the development of pulmonary fibrosis.[10–14] Morphometric examination of the pulmonary lavage in bleomycin-induced pulmonary fibrosis shows an increased number of neutrophils and lymphocytes in the lavage.[15] Similarly, the lung interstitium exhibits an infiltration of neutrophils, lymphocytes, plasma cells, and macrophages.[8] These studies[7,8,16] have reported subjective changes in the cellular composition of the lung following intratracheal administration of bleomycin. However, there exists little information on the sequence of the infiltrating cells and the possible interactions of these cells in the overall sequelae of pulmonary fibrosis. We used morphometric methods to examine more precisely the tem-

This work represents a portion of David B. Chandler's dissertation in partial fulfillment of the requirements for PhD degree in pharmacology and toxicology from the University of California, Davis, California.

Supported by National Heart Lung and Blood Institute Grant 1RO1 HL 27354-02.

Accepted for publication March 15, 1983.

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0002-9440/83/0805-0170$01.20

poral changes in the cellular composition of the pulmonary lesion following intratracheal administration of bleomycin in this investigation. Our results show that the development of pulmonary fibrosis is characterized by sequential changes in infiltrating and resident connective tissue cell populations and suggest some form of interaction among the cells during the fibrogenic period.

Materials and Methods

Adult male Golden Syrian hamsters (90–100 g, Harlan, Madison, Wis) were used. Under pentobarbital anesthesia (60–70 mg/kg) the hamsters were treated with 1 unit of bleomycin intratracheally by the method described by Giri et al[7] and Zuckerman et al.[6] Controls were similarly treated intratracheally with an equal volume of the vehicle (0.9% saline). The bleomycin-treated hamsters were sacrificed at 4, 7, 21, 28, 35, and 42 days after treatment. The controls were sacrificed at 7 days after treatment. In addition, naive hamsters were sacrificed for comparison purposes. The lungs of the hamsters were prepared for fixation by opening the thoracic cavity under pentobarbital anesthesia (90 mg/kg) and tying off the heart to isolate the pulmonary vasculature. The trachea was cannulated with a blunt 18-gauge needle, and the lungs and heart were removed from the thoracic cavity. The lungs were floated in 0.9% saline and perfused with buffered formol via the trachea with the use of a constant pressure apparatus with a pressure of 30 cm of fixative for 2 hours. At the end of 2 hours the trachea was tied off, and the tissue was stored in buffered formol until preparation for sectioning.

Prior to preparation of the lung tissue for glycol methacrylate embedding, lung volume was determined by weight displacement.[17] The right caudal lobe of each lung was removed, and a representative section was removed by cutting longitudinally down the lobar bronchus. A second cut was made 4 mm lateral to the initial cut. The 1-sq cm blocks of tissue were cut from the slab, dehydrated in ethanol, and embedded in glycol methacrylate. Sections 2 μ thick were cut with a Sorvall JB4 microtome, mounted on glass slides, and stained with hematoxylin and eosin (H&E).

Morphometry

The volumetric ratio of lesions within the lung was estimated by point-counting techniques.[18] A lesion was defined as a cluster of 10 or more inflammatory cells within the interstitium in the earlier phase, whereas it also included regions of interstitial fibrosis in the latter phase. A Weibel projection device was used with a Zeiss projecting microscope and a double square lattice test system (D64) with 1024 points at a final magnification of 40×.[19] Every field on a slide—about 20 per slide—was evaluated.

For each slide a second cascade level of subsampling was used with systematic area weighted quadrate sampling for estimation of the volumetric ratio of components constituting the lesions at a final magnification of 400×.[20] In this latter method of sampling, we used a simple square lattice system of 100 points in which 1 point is designated as the test point. The slide was scanned in a systematic manner until the test point fell on a lesion. Subsequently, a random number generator was used for selection of an additional point of the 100 possible points, which, when selected, was superimposed on the location originally marked by the test point. This method ensures random location of the grid relative to the lesions. Ten fields were used per slide for this subsampling method. Volume ratios were estimated by the use of point-counting techniques.

Numerical profile densities of cells within interstitial lesions (number per square millimeter) were estimated with the use of the previously described sampling scheme and the counting rule of Gundersen.[21] Numerical profile densities were estimated from nuclear profiles of cell types within interstitial lesions per field. Cells that were involved in the inflammatory response within the interstitium included macrophages, monocytes, fibroblasts, eosinophils, neutrophils, and lymphocytes. Cell identification used classic definitions of cell shape, nuclear shape and heterochromatin, nuclear-to-cytoplasmic ratio, and cytoplasmic volume and staining characteristics. Differences between macrophages and monocytes were decided by the nuclear-to-cytoplasmic ratio. When the ratio was less than 1 to 2, the cell was determined to be a monocyte. Sampling was sufficient for an estimation of the mean of all morphometric parameters within ± 10% of the animal mean with the use of a 95% confidence interval. All morphometric values were analyzed with the use of analysis of variance (PIV of BMDP program) with $P \leqslant 0.05$ considered the level of significance.[22]

Results

Following intratracheal administration of saline, the lungs of the control hamsters showed no changes when compared with naive lungs. In contrast, the

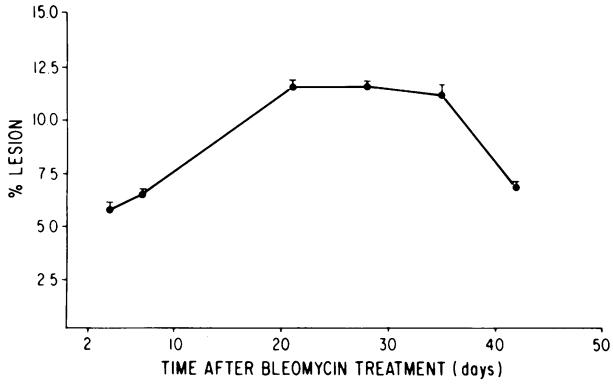

Figure 1—The effects of intratracheal administration of bleomycin on lesion development in the lung. Each point represents the mean ± SE of 5 hamsters at 4 days; 6 hamsters at 7 days; 4 hamsters at 21 days; 2 hamsters at 28 day; and 3 hamsters at 35 and 42 days. The number of animals at each time point is the same for all subsequent figures.

hamsters treated with bleomycin developed extensive fibrotic lesions. Quantitation of the lesions showed that there was a linear increase in the percentage of the lung involved, from 5.8% at 4 days to 11.6% at 21 days (Figure 1). The percentage of the lung demonstrating lesions then stabilized, showing 11.6% at 28 days and 11.2% at 35 days, followed by a decrease to 6.8% at 42 days. In addition, lung tissue showed a linear increase in the total number of cell profiles involved in the lesions from 7976 cells /sq mm at 4 days to 13521 cells/sq mm at 28 days, being significantly elevated at 21 days (11210 cells/sq mm) and 28 days after bleomycin treatment (Figure 2). Subsequent to 28 days, decreases in the number of cells/sq mm were seen at 35 and 42 days, but none were significant. At all time points subsequent to bleomycin administration, the number of cells was significantly elevated above that for hamsters that received only saline, which showed 2229 cell profiles/sq mm of interstitium.

The cellular composition of the lesion was observed to change over the course of this study following bleomycin treatment. As seen in Figure 3, in the early phase the lesions were composed of a significantly higher number of monocytes, 3094/sq mm at 7 days, as compared with the lesions seen in the later phase, which exhibited significant decreases in the number of monocytes to 1385, 1236, and 1611 monocytes/sq mm at 21, 28, and 42 days, respectively. In contrast to a marked reduction in the numbers of monocytes during the late phase of lesion development, the fibroblasts exhibited significant elevations at 21, 28, 35, and 42 days to 4514, 4789, 4116, and 3936 fibroblasts/sq mm, respectively, as compared with 203 fibroblasts/sq mm at 4 days (Figure 3).

The macrophages exhibited a complex response, as opposed to other cellular components (Figure 3). The macrophage population was seen to decrease significantly from 3860 macrophages/sq mm at 4 days to 2160, 1876, and 1652 macrophages at 7, 35, and 42 days after treatment, respectively. Although macrophages were generally increased at 21 and 28 days, neither increase was significant.

Similar to monocytes, the neutrophil composition of the lesion was significantly higher at 7 days (899 neutrophils/sq mm), as compared with 212 and 0 neutrophils at 21 and 28 days after treatment, respectively (Figure 4). The infiltration of eosinophils in response to bleomycin treatment exhibited a significant increase at 28 days to 1351 eosinophils/sq mm, as compared with 66 eosinophils/sq mm at 4

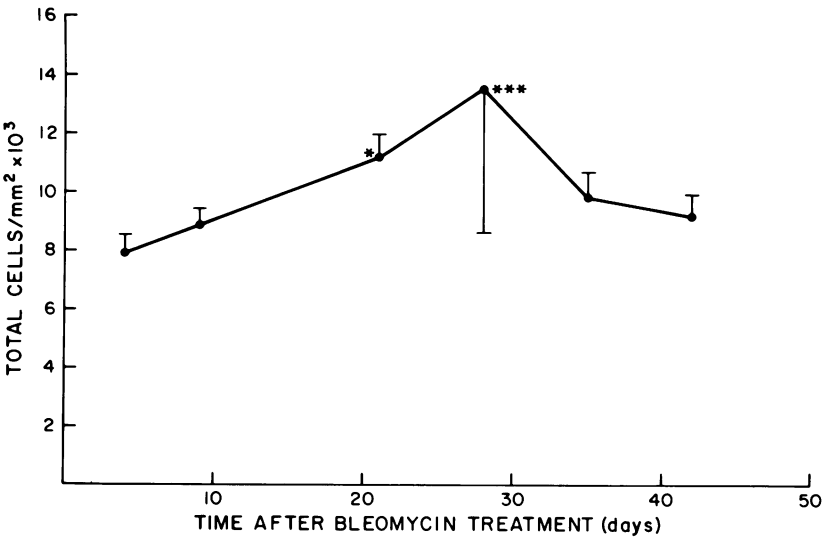

Figure 2—The effects of intratracheal administration of bleomycin on the number of cells per square millimeter. Each point represents the mean ± SE. For determination of significance, all comparisons in experimental animals were made against the 4-day value. $*P \leq 0.05$. $***P \leq 0.01$. The number of cells in saline-treated control animals was 2229/sq mm.

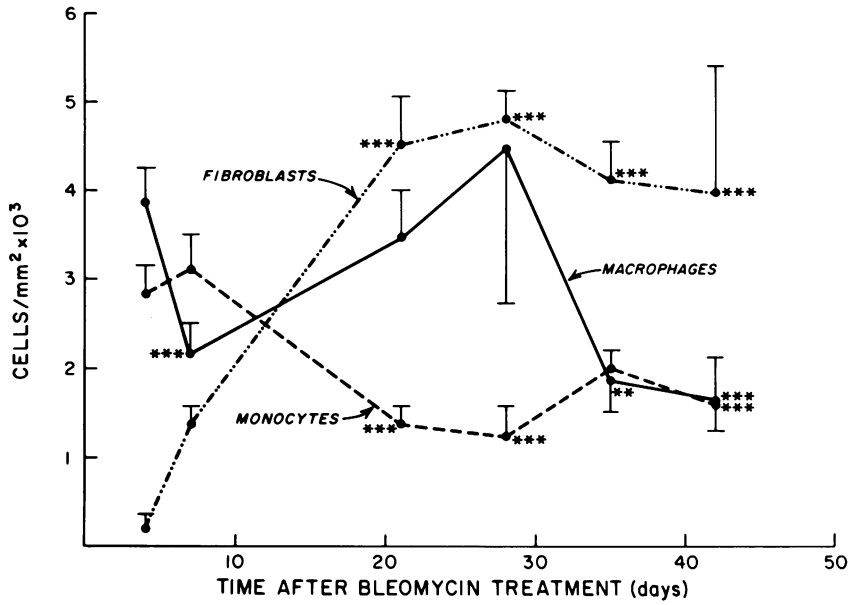

Figure 3—The effects of intratracheal administration of bleomycin on the number of monocytes, macrophages, and fibroblasts per square millimeter. Each point represents the mean ± SE. For determination of significance, the macrophage and fibroblast values in experimental animals were compared against the 4-day, and the monocyte values against the 7-day values. $^{**}P \leq 0.02$. $^{***}P \leq 0.01$. Cell profiles per square millimeter in saline-treated controls were macrophages, 339; monocytes, 244; fibroblasts, 1284.

days, decreasing to 396 eosinophils/sq mm at 42 days (Figure 4).

The infiltration of lymphocytes into the lung showed a significant elevation at 7 days to 1028 lymphocytes/sq mm, as compared with 399 lymphocytes/sq mm at 4 days following bleomycin treatment (Figure 5). Lymphocytes were generally increased at all other time points except 42 days after treatment, but the changes were not significant. All of the significant elevations of inflammatory cell profiles during lesion development were significantly greater than the values estimated in the controls. Cell profiles per square millimeter were 339 for macrophages, 244 for monocytes, 1284 for fibroblasts, 92 for neutrophils, and 270 for lymphocytes within the interstitium of hamsters administered saline alone. No eosinophils were observed in these control hamsters. Volumetric density changes of cells after bleomycin treatment are shown in Table 1. The volumetric changes generally paralleled the changes in cell profile density. In addi-

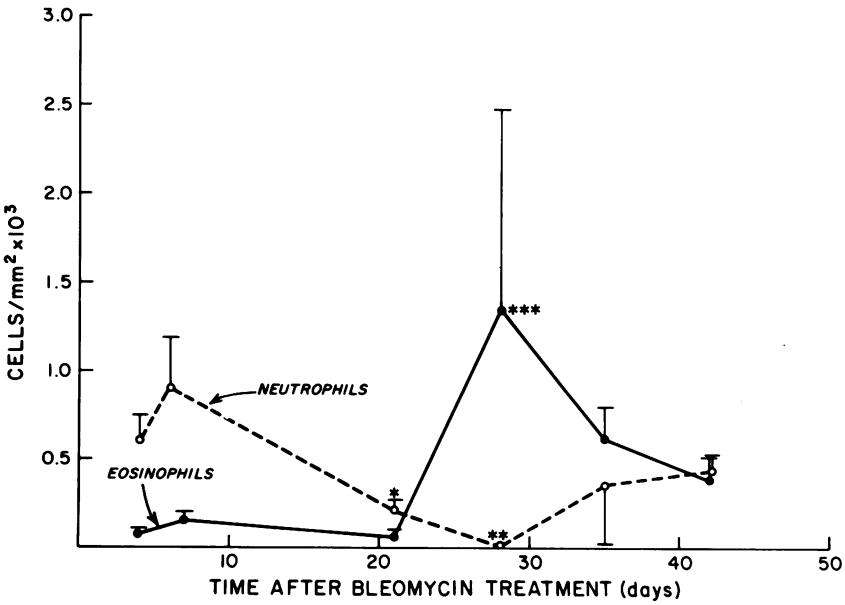

Figure 4—The effects of intratracheal administration of bleomycin on the number of neutrophils and eosinophils per square millimeter. Each point represents the mean ± SE. For determination of significance, the neutrophil values in experimental animals were made against the 7-day values and for eosinophils against the 4-day values. $^{*}P \leq 0.05$. $^{**}P \leq 0.02$. $^{***}P \leq 0.01$. Cell profiles per square millimeter in saline-treated controls were neutrophils, 92; eosinophils, 0.

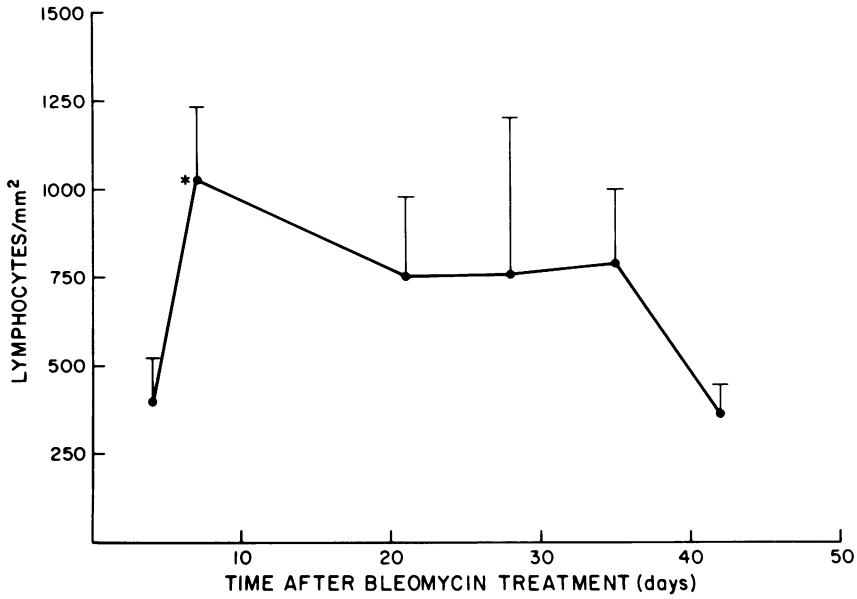

Figure 5—The effects of intratracheal administration of bleomycin on the number of lymphocytes per square millimeter. Each point represents the mean ± SE. For determination of significance, the lymphocyte values in experimental animals were compared against the 7-day value. $*P \leq 0.05$. The lymphocyte profile in the saline-treated control was 270/sq mm.

tion, the lung volume between control and treated hamsters showed no significant changes (data not shown).

Discussion

Bleomycin has been used for the development of an animal model of pulmonary fibrosis. A single bolus intratracheal injection of bleomycin in hamsters produces a disorder that has several of the physiologic, biochemical and histologic features of human pulmonary fibrosis.[3-5] The lesions that develop produce both functional and biochemical changes affecting lung volume, compliance, and collagen content.[4,5,7] Before 7 days the lesions are classified as diffuse hemorrhagic interstitial pneumonia. Thereafter, lung collagen is significantly increased; and the lesions have less hemorrhage and edema, more inflammatory cells, and thicker interalveolar septa.[7] In addition, histopathologic studies have reported a marked cellular hypertrophy and hyperplasia, accompanied by the infiltration of inflammatory cells.[3,5,8] The cellular infiltration of the lung in response to intratracheal administration of bleomycin is characterized by changes in the numbers of neutrophils, monocytes, and lymphocytes and accompanied by proliferating fibroblasts.[5,7,8,18] We attempted in the present study to delineate the temporal sequential changes in the cellular infiltrate, which may contribute to the pathogenesis of bleomycin-induced fibrosis.

The initial step in the sequelae appears to be an increase in macrophages. This is an interesting finding, because such predominance of macrophages is not a striking feature of lung insults caused by other chemicals.[23-27] The exact role of the macrophages in bleomycin-induced pulmonary fibrosis is unknown. However, it has been suggested that macrophages are chemotactic toward monocytes and neutrophils and stimulate fibroblast proliferation.[14,28,29,30] The exact chemotactic factors are not known, although hydroxyeicosatetraenoic acids (HETEs) and a factor of 5000 molecular weight have been implicated.[29,30]

In the present study the possibility that macrophages are chemotactic toward neutrophils and monocytes and stimulate the proliferation of fibroblasts as well is supported by several observations. First, macrophages tend to increase prior to the influx in neutrophils and monocytes. Second, macrophages appear earlier in the temporal sequence and precede the proliferation of fibroblasts. Thus, our results suggest that macrophages might be instrumental in initiating neutrophil and monocyte infiltration and the proliferation of fibroblasts.

The macrophage-mediated infiltration of monocytes may serve a dual function. The monocytes could aid in debridement of the damaged lung cells following insult[31,32] and aid in the stimulation of the fibroblasts.[33] However, Jimenez et al[11] have reported that the mononuclear cell supernatant inhibits collagen synthesis by fibroblasts from normal human skin *in vitro*. The role of the monocyte-macrophage system in stimulating the activity of fibroblasts appears to be controversial. There is, however, evidence that lymphocytes, another component of the cell infiltration, might be involved in proliferation of

Table 1—Effects of Intratracheal Administration of Bleomycin on the Volumetric Density of Inflammatory Cells

Cell type	Time after bleomycin treatment in days* 4 (5)†	7 (6)	21 (4)	28 (2)	35 (3)	42 (3)	P‡	Comparison time points
Mac	0.161 ± 0.025	0.120 ± 0.019	0.227 ± 0.046	0.207 ± 0.042	0.153 ± 0.26	0.124 ± 0.043	$P < 0.1$	4 days
Mono	0.121 ± 0.021	0.113 ± 0.022	0.039 ± 0.010 ($P \leq 0.02$)§,‖	0.024 ± 0.004 ($P \leq 0.02$)	0.083 ± 0.022	0.077 ± 0.028	$P < 0.05$	7 days
Fibro	0.006 ± 0.004	0.038 ± 0.009	0.209 ± 0.027 ($P \leq 0.01$)	0.125 ± 0.016	0.237 ± 0.076 ($P \leq 0.01$)	0.226 ± 0.092 ($P \leq 0.01$)	$P < 0.001$	4 days
Eos	0.003 ± 0.001	0.005 ± 0.002	0.001 ± 0.001	0.004 ± 0.001	0.026 ± 0.008 ($P \leq 0.01$)	0.010 ± 0.002	$P < 0.001$	4 days
Neut	0.020 ± 0.004	0.018 ± 0.007	0.004 ± 0.002 ($P \leq 0.05$)	0.00 ± 0.00	0.012 ± 0.005	0.013 ± 0.003	$P < 0.1$	7 days
Lym	0.008 ± 0.003	0.016 ± 0.005	0.009 ± 0.002	0.004 ± 0.004	0.010 ± 0.006	0.008 ± 0.004	$P < 0.1$	4 days

Estimation of volumetric density is described in detail in the Materials and Methods Section.
Mac, macrophage; mono, monocyte; fibro, fibroblast; eos, eosinophil; neut, neutrophil; lym, lymphocyte.
* Values expressed as mean ± SE.
† The number in parenthesis represents the number of animals at each time point.
‡ Results of one-way analysis of variance.
§ As compared with stated time point. There was no significant difference between 4 and 7 days for any one of the cell types.
‖ Results of Student *t* test.

fibroblasts, increased collagen synthesis, and/or its secretion from fibroblasts.[12,13,34,35] This is supported in the present study by the lymphocyte influx occurring simultaneously with the fibroblast proliferation and reported increases in collagen accumulation.[7,36] The stimulus that triggers the influx of lymphocytes at the site of the inflammatory lesion is not known. However, it has been suggested that macrophages and neutrophils play a role.[12,33] This is supported by our results, which show an increase in the neutrophils and macrophages preceding the lymphocyte infiltration. However, it is not known whether this stimulation by the neutrophils and/or macrophages is the sole stimulus responsible for the sustained elevation of the lymphocytes in the lung interstitial tissue as seen in this study. Thus, there appear to be a number of cell types that might stimulate fibroblast proliferation and collagen synthesis. However, the question of which cell type provides the primary stimulus for the proliferation of fibroblasts has not yet been resolved.

In contrast to the possible stimulation of collagen synthesis by macrophages, monocytes, and lymphocytes, neutrophils and eosinophils have been frequently associated with collagen degradation.[37] Neutrophils were seen to peak early in the temporal sequence following the macrophage influx and coincided with the influx of monocytes. Neutrophils also showed a secondary increase in the temporal sequence, which was accompanied by a significant increase in eosinophils.

These changes in neutrophil and eosinophil numbers with time are interesting in view of Bassett's interpretation of their differential roles.[38] Bassett et al suggest that neutrophils are breaking down newly synthesized collagen while eosinophils are "remodeling" this newly synthesized collagen. This suggestion is supported by two lines of evidence. First, Thrall et al[39] have shown that in rats that are made neutropenic, collagen buildup associated with bleomycin treatment is accelerated. Secondly, neutrophils have been shown to digest preferentially Type I collagen,[40] which appears to be predominantly synthesized by bleomycin-stimulated fibroblasts.[41] These findings complement earlier work in our laboratory[36] that showed a low amount of collagen accumulation at the time points corresponding to the early increases in neutrophils and a reduction in the amount of collagen with the concomitant increases in eosinophils and neutrophils. In addition, morphometric determinations of the lung show evidence of a trend toward a decrease in the amount of lesion at the corresponding time points (Figure 1). We were rather surprised to find a seeming paradox between our morphometric results dealing with the number of fibroblasts and our biochemical results reported earlier[7] for the amount of collagen in the lungs of bleomycin-treated hamsters. The peak number of fibroblasts occurred at a much later time (28 days) than the peak amount of collagen (14 days). At present we cannot explain this discrepancy with any degree of certainty. There are, however, at least two possibilities. First, the rate of collagen degradation exceeds the rate of collagen synthesis; second, the amount of collagen synthesized per fibroblast is drastically reduced after 14 days of bleomycin treatment. It

therefore appears that the resulting collagen deposition in the lung might be affected by the presence of the cellular elements due to their intrinsic activities.

In conclusion, it appears that bleomycin-induced lung damage is accompanied by an inflammatory response. It has been demonstrated that the presence of an intact cell-mediated immune system is not an absolute requirement for the development of this lesion.[42] However, several studies[8,43] have shown that modification of this inflammatory sequela has significant effects on the subsequent time course of collagen deposition. Furthermore, changes in the neutrophil and lymphocyte populations produce significant changes in the subsequent collagen accumulation.[35,39] This evidence suggests that the inflammatory response has different roles in the sequelae of pulmonary fibrosis induced by bleomycin. In this study it appears that the influx of neutrophils, in the early phase, might modulate collagen deposition as a result of their collagenase activity. In contrast, the later phase is characterized by an influx of lymphocytes resulting in collagen deposition. The entire sequence of events appears to be complex and interdependent. Manipulation of the inflammatory sequelae,[8,35,39,43] however, may be beneficial in the treatment of chemically induced or idiopathic pulmonary fibrotic disorders.

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