The Role of Soluble Factors in Bleomycin-Induced Pulmonary Fibrosis

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Endotracheal administration of bleomycin causes pulmonary fibrosis characterized by increased collagen synthesis and deposition. Incubation of normal lung mince with neutral salt soluble extracts of lungs from normal and bleomycin-treated rats caused a dose-dependent inhibition of collagen and noncollagenous protein synthesis. Bleomycin-treated lung extracts, however, were significantly less effective in such inhibition when compared with normal lung extracts. This inhibitory activity was not diminished by dialysis in tubing with nominal molecular weight cutoff of 10,000 but was destroyed by heat (70 C) and trypsin digestion. This inhibitory activity could not be ascribed to residual serum or bleomycin in the lung extracts. Fractionation on Sephacryl S-200 (Pharmacia, Piscataway, NJ) showed inhibitory activity to be heterogeneous with Mr (apparent molecular weight) > 100,000. Extracts from spleen showed similar inhibitory activity but showed no difference in intensity between normal and bleomycin-treated spleen. These data suggest that loss or decrease in production of lung inhibitory regulatory factors is partly responsible for the noted increase in collagen production and deposition in bleomycin-induced pulmonary fibrosis. (Am J Pathol 1981, 106:156–164)

BLEOMYCIN is an antitumor antibiotic that has good activity in squamous cell carcinomas but, when given in high (total accumulated) doses, causes fatal pulmonary toxicity.[1-3] An early interstitial pneumonitis terminates in diffuse interstitial fibrosis.[1-3] These changes have also been reported in various animal species when given this drug either endotracheally[4,5] or otherwise.[6-8] Endotracheal administration results in a prompt interstitial pneumonitis characterized by the presence of macrophage, plasma cells, lymphocytes, and scattered neutrophils and eosinophils. Early fibrosis is detectable beginning 10 days after treatment, both biochemically and histologically.[4] Lung collagen synthesis is increased starting at 4 days and returns to normal in 4–6 weeks.[9] This sequence of events following a single dose of bleomycin resembles other forms of injury with a consequent fibrotic response. The actual sequence of events resulting in the initiation of the fibrotic response remains unclear, although a great deal of suggestive evidence seems to indicate regulation of fibroblast proliferation and metabolism by mononuclear cells.[10-14] In this paper

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we have attempted to assay for soluble regulatory factors (affecting lung collagen synthesis) in homogenates of lungs and spleens of normal rats and to determine what changes these factors undergo upon bleomycin treatment.

Materials and Methods

Animals

All experiments were performed with male Fisher 344 rats weighing 150–200 g. These animals arrived in filtered cages from Charles River, Portage, Michigan, and were determined to be free of interstitial pulmonary disease. When indicated bleomycin (Blenoxane, Bristol Laboratories, Syracuse, NY), 1.5 units in 0.3 ml, was injected via tracheostomy under ketamine (Ketalar, Parke-Davis, Detroit, Mich) anesthesia, as previously described.[4,9] Normal controls underwent sham tracheostomy and were instilled with 0.3 ml sterile saline.

Soluble Extracts

The lungs from bleomycin-treated animals (7 days prior to sacrifice) and control animals were dissected from rats following ketamine anesthesia, transection of the inferior vena cava, and perfusion of the pulmonary artery with 10 ml cold phosphate-buffered saline (PBS). The lungs from 2 animals were combined and then minced with scissors, placed in 2 ml of cold PBS, and homogenized with the use of Polytron (Brinkman Instruments, Westbury, NY) with an ST10 generator in three 15-second bursts at a setting of 7. The volume was then adjusted to 4 ml with PBS and centrifuged at 30,000*g* for 60 minutes. The supernatant was then stored frozen at −20 C until ready for use. Prior to use, these extracts were sterilized and further clarified by filtration through an 0.45-μ filter (Millipore Corp., Bedford, Mass). Thus, "bleomycin lung extract" refers to that derived from pooling the lungs of 2 animals treated with bleomycin 7 days prior to sacrifice. "Control" or "normal lung extract" refers to the same method of preparation but derived by pooling of the lungs of 2 control animals that had undergone sham tracheostomies without bleomycin.

When indicated, these extracts were dialyzed prior to sterilization against 2 changes of 2 l cold PBS overnight. Dialysis tubing with a nominal molecular weight cutoff of 10,000 was used. Heating of extracts was accomplished by placement in a 70 C water bath for 15 minutes prior to sterilization. Trypsinization of extracts was performed by incubation with 10mg/ml of trypsin insolubilized on sepharose beads (Sigma Chemical Co., St. Louis, Mo), for 15 minutes at 37 C. Termination was accomplished by centrifugation and separation of the enzyme-coated beads. The trypsinized extract was then filtered as described above.

Lung soluble extracts were fractionated on a 2.5 × 90 cm column of Sephacryl S-200 (Pharmacia, Piscataway, NJ) and eluted with PBS. Chromatography was performed at 4 C, and 2 ml fractions were collected. Fractions were pooled as indicated in Figure 6, to give four fractions labelled V_1, V_2, V_3, and V_4.

Soluble extracts from spleen dissected free of surrounding adipose tissue, were prepared as described for the lung.

Collagen Synthesis Assay

This was performed essentially according to Peterkovsky and Diegelmann[15] with the lung mince method of Bradley et al,[16] as modified previously.[9] The bacterial collagenase was purified by gel filtration on Sephacryl S-200 (2.5 × 90 cm), eluted with 0.05 M Tris HCl, pH 7.6, and 5 mM $CaCl_2$ essentially as described previously.[15] It was free of detectable proteolytic activity against *Escherichia coli* protein. Lungs from normal, untreated Fisher 344 rats were removed and freed of trachea and bronchi. They were then pooled and minced with scissors to give 1–2 cu mm pieces. The mince was then placed in 35-mm Petri dishes in aliquots of approximately 200 mg. Usually six 200-mg portions of lung mince could be obtained from 1 animal. Two milliliters of 50% (volume % in PBS) Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% antibiotics (Streptomycin-penicillin, Grand Island Biologicals Co., Grand Island, NY), 100 μg/ml β-aminopropionitrile, 80 μg/ml sodium ascorbate, and 5 μCi/ml of ^{3}H-proline (L-[2,3-^{3}H]-proline, 20–40 Ci/mmol, New England Nuclear, Boston, Mass), were added into each dish and incubated at 37 C with 5% CO_2 for 3 hours. The minces were then washed with cold PBS, homogenized in 10% trichloroacetic acid (TCA). The samples were then processed essentially as described by Peterkovsky and Diegelmann.[15] Briefly, the homogenized, washed pellet was digested with the purified collagenase for 2 hours at 37 C and precipitated with 10% and 0.25% tannic acid; the supernatant (representing collagenous counts per minute (cpm) and the pellet (representing noncollagenous cpm) were then counted in a scintillation counter (24% efficiency). Substances (see below) to be tested for their ef-

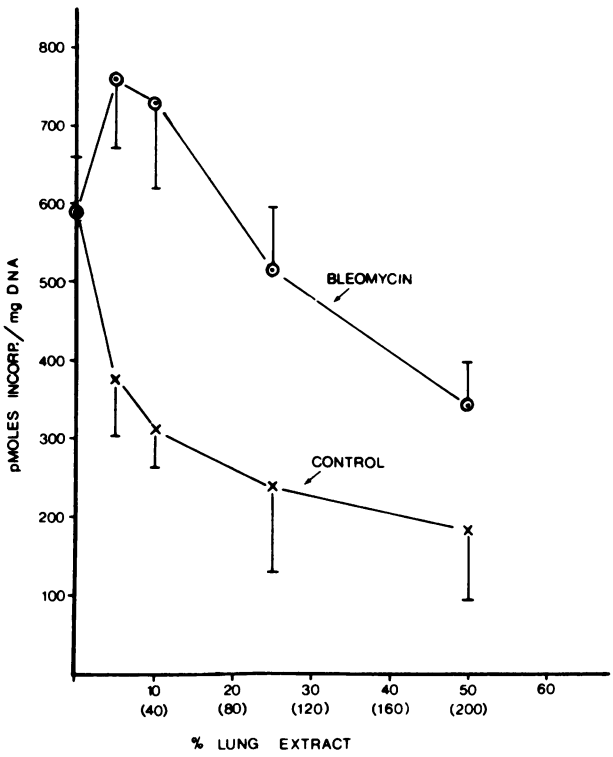

Figure 1—Effect of lung extracts on collagen synthesis. Normal lung mince was incubated in the presence of the indicated doses of lung extracts as indicated on the X axis. The dose is expressed as volume in milliliters of extract added per 100 ml total final volume of incubation mixture. The dose in brackets is the estimated ratio of lung mass from whence the extract came to the lung mass present in the incubation mixture (or lung mass equivalence). For example, 100% would indicate that the amount of extract added was obtained by extraction of an amount of lung tissue equal to that present in the assay. Each data point is the mean ± 1 SD, with $N = 6$. "Bleomycin" and "control" refer to extracts obtained from bleomycin-treated and untreated normal animals, respectively. At each dose, the bleomycin data were compared with the control data by the use of the Student t test. The results show them to be significantly different ($P < 0.05$) at every dose examined. With the same statistical analysis, the inhibition (comparing the data point at each dose versus that at a dose of 0%) by control lung extracts was significant ($P < 0.05$ for doses of 5% and 10%, and $P < 0.01$ for doses of 25% and 50%) at every dose examined. It is only significant ($P < 0.05$) for the bleomycin-treated lung extract at a dose of 50%).

fects on lung collagen synthesis were added directly into DMEM prior to addition to the lung mince. In each case, however, the final incubation volume was 2.0 ml with the same concentrations of all supplements. Synthetic rates were expressed as picomoles proline incorporated per milligrams deoxyribonucleic acid (DNA) during 3 hours of incubation.

When soluble extracts were tested for their effects on collagen synthesis, they were added in concentrations of 5%, 10%, 25%, and 50% (volume %) or as indicated in the figures, and the final volume was adjusted with sterile PBS. To examine the *in vitro* effects of bleomycin, we added it to the assay system in doses of 0.06, 0.12, 0.25, and 0.5 U/ml following dissolution in sterile PBS. Sera, when used, were obtained from normal and bleomycin-treated animals (treated 1 week prior to sacrifice). They were added directly into the assay system at concentrations of 5%, 10%, and 20% (volume %). An analog of prostaglandin E_1 (PGE_1) (Upjohn Co., Kalmazoo, Mich) was tested, since it has known collagen synthetic inhibitory activity.[17,18] The analog is more resistant to prostaglandin-15-dehydrogenase inactivation by substitution of a methyl group at the 15 position. The analog (15-M-PGE_1) was dissolved at a concentration of 10 mg/ml in absolute ethanol and diluted a hundredfold in PBS prior to addition to the assay medium at doses of 0.5, 1.0, 2.0, and 5.0 μg/ml.

Statistical Analysis

To establish any statistical significance in differences between sets of data points, the Student t test was used. Significance of inhibition or stimulation by the additions was established by comparing data points obtained at each dose of addition and the data points obtained in the absence of such additions (ie, at the 0% dose).

Results

Effects of Lung Soluble Extracts

The effect of lung soluble extracts on normal lung collagen synthesis is shown in Figure 1. Addition of extracts of normal untreated lungs *in vitro* caused inhibition of collagen synthesis by normal lung mince in a dose-dependent manner, reaching a maximum of 67% inhibition at a dose of 50% (v/v) (or 200% when expressed as lung mass equivalence; see legend to Figure 1). This curve resulted in an ED_{50} of 4% (ie, a dose of 4% [v/v] causes 50% of maximal inhibition or 35% inhibition below the rate obtained when no additions were present in the incubation mixture). Addition of extracts of bleomycin-treated lungs were significantly less effective in causing inhibition of lung collagen synthesis. Following a slight, statistically insignificant stimulation (over the rate obtained without any additions, ie, at the 0% dose) at doses < 20%, there was also a dose-dependent inhibition of collagen synthesis (below the rate obtained without any additions, ie, at the 0% dose), reaching a maximum of only 25% inhibition at a dose of 50% (v/v), resulting in an ED_{50} of greater than 35%. In-

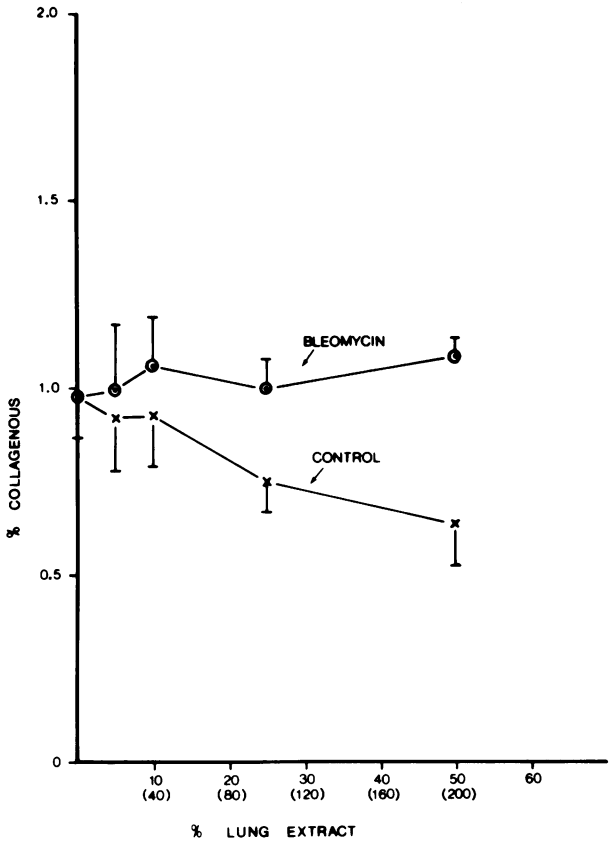

Figure 2—Selectivity of inhibition of lung collagen synthesis by lung extracts. "% collagenous" refers to the percentage of protein synthesis by lung mince devoted to production of collagen and was calculated as described in the text. Each data point is the mean ± 1 SD, with $N = 6$. Inhibition by control lung extract was statistically (t test) significant ($P < 0.05$) at doses of 25% and 50% only, while bleomycin-treated lung extract showed no significant effects at any dose examined (the data point at each dose was compared to the data point at a dose of 0%).

hibition was only statistically significant at the maximal dose tested (50%). At each dose, the inhibition by normal lung extract was significantly different from that by bleomycin-treated lung extract, at $P < 0.05$ (Student t test). This difference cannot be accounted for by the high protein concentrations in bleomycin-treated lung extracts (usually approximately 1.5 times greater than normal lung extracts) since the inhibitory dose response curve difference was opposite of what would be expected if the amount of inhibitory factor(s) was present at similar concentrations in both extracts on a per milligram total protein basis.

This inhibition of collagen synthesis was relatively specific, as shown in Figure 2. Increasing doses of normal lung extract caused increasingly lower amounts of collagen synthesized, relative to noncollagenous protein synthesis. Normal lung mince usually devotes approximately 1% of total protein synthesis to collagen synthesis (this number is obtained by division of the rate of radiolabeled proline incorporation into collagenous protein by the total amount incorporated into trichloroacetic-acid (TCA)-precipitable material, after correction for the lower content of proline in noncollagenous protein by multiplication with a factor of 5.04, ie, $C/(C+5.04[NC]) \times 100\%$, with C representing collagenous incorporation and NC representing noncollagenous counts). Upon addition of normal lung extracts, there was a dose-dependent inhibition of relative collagen synthesis, up to a maximum of 40% (below the relative rate obtained in the absence of any additions) at a dose of 50% (v/v) (Figure 2). Bleomycin-treated lung extracts exhibited no significant inhibition at all doses

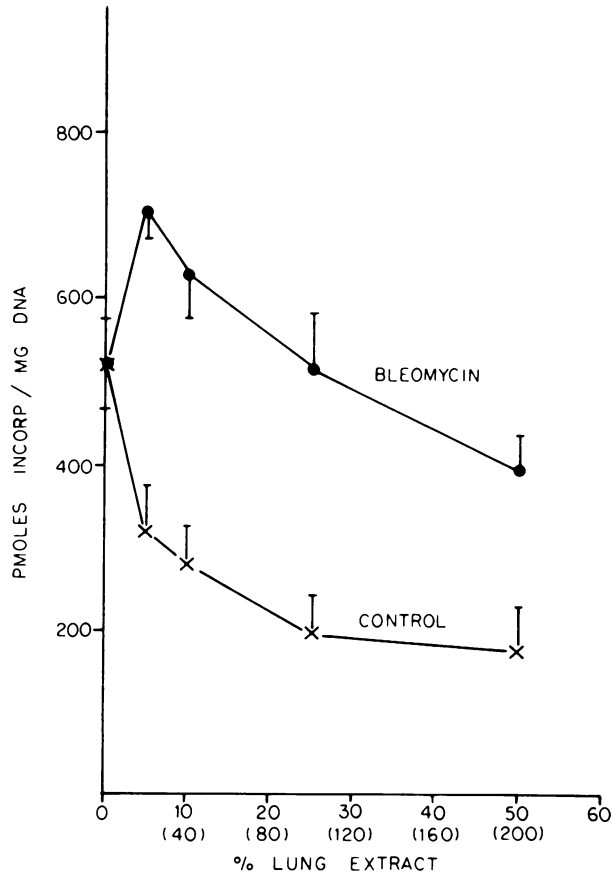

Figure 3—Effect of dialysis. The same protocol was used as described in Figure 1, except the extracts were dialyzed as described in Materials and Methods prior to their use in the assay. Each data point is the mean ± 1 SD, with $N = 4$. Statistical analysis shows results similar to those in Figure 1.

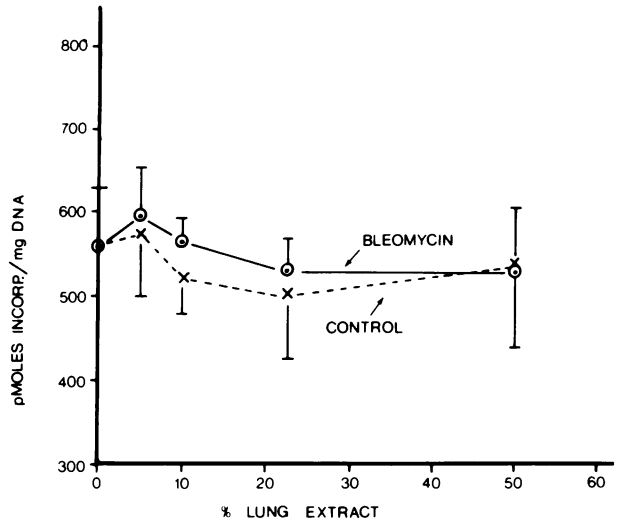

Figure 4—Effect of heat. The same procedure was used as for Figure 1, except the extracts were heated (70 C, 15 minutes) prior to assay. Each data point represents the mean ± 1 SD, with $N = 4$. Comparison of data points (t test) at each dose with the one at 0% dose revealed no statistically significant differences.

examined, suggesting the presence of inhibitory factor(s) in the normal lung extracts that are relatively specific for collagen synthesis versus other noncollagenous protein synthesis. These factors are present in significantly lower amounts in bleomycin-treated lung extracts.

Partial Characterization of Lung Extracts

Exhaustive dialysis in tubing with a nominal molecular weight cutoff of 10,000 caused no significant change in the dose-dependent inhibition of collagen synthesis by these lung extracts (Figure 3). This indicates 1) that the dose dependent inhibition observed was not due to dilution of radiolabeled proline precursor by cold free proline present in the extracts and 2) that the inhibitory factor(s) has Mr > 10,000 or is associated with molecules with Mr > 10,000 and would not undergo dissociation under the condition of dialysis.

To determine the nature of the inhibitory factor(s), the lung extracts were assessed for sensitivity to heat (70 C for 15 minutes) and trypsinization. Figure 4 shows complete abolition of inhibitory activity by heating. Trypsinization caused complete suppression of inhibition by normal lung extract at doses < 10% and partial suppression at doses > 10%. The inhibitory activity of bleomycin-treated lung extract was completely absent upon trypsinization (Figure 5). These findings suggest the proteinaceous nature of these inhibitory factor(s).

In an attempt to further characterize the molecular weight of this factor(s), the extracts were analyzed by gel filtration on Sephacryl S-200. Three major peaks and one minor peak were apparent in the chromatogram shown in Figure 6. Much less protein was apparent in the normal lung extracts and contained relatively much less protein in fractions V_1 and V_2 when compared with those in the bleomycin-treated lung extracts. The fractions were pooled as indicated in the figure and assayed for inhibitory activity. The data in Table 1 shows significant inhibitory activities only in pooled fractions V_1 and V_2, with apparent molecular weights > 150,000, in extracts from normal lungs. Fraction V_1 was more potent on a per milligram protein basis than V_2, but had less of a specific effect on collagen synthesis, since V_2 had no inhibitory activity against noncollagenous protein synthesis while inhibiting collagen synthesis by 28% at a dose of 2.1 mg/ml of total protein added. No significant inhibition was seen with the various bleomycin-treated lung extract fractions, except for V_1. The latter pooled fraction caused 10% inhibition at comparable doses (Table 1). No stimulatory effect was observed with any of the fractions.

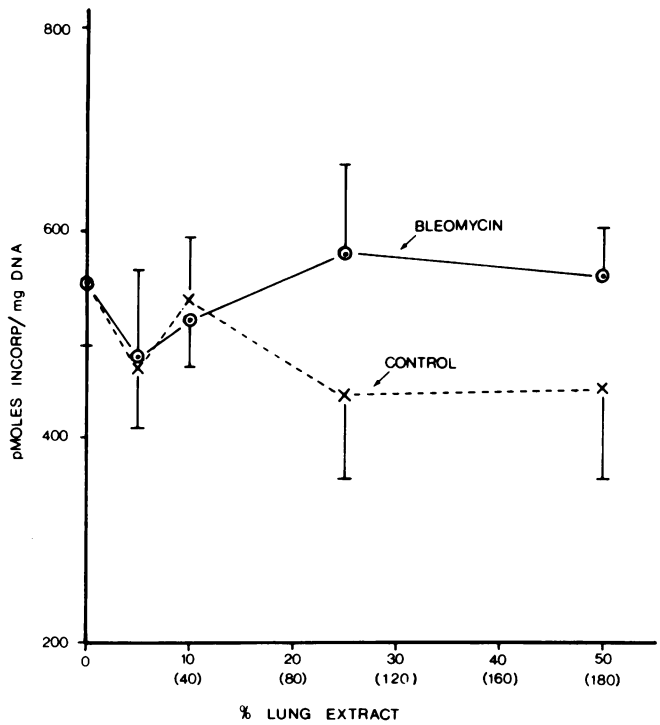

Figure 5—Effect of trypsinization. Lung extracts were trypsinized prior to the addition into the collagen synthesis assay medium as described in Materials and Methods. Each data point represents the mean ± 1 SD, with $N = 4$. All data points were not significantly different (t test) from the data point at a dose of 0%.

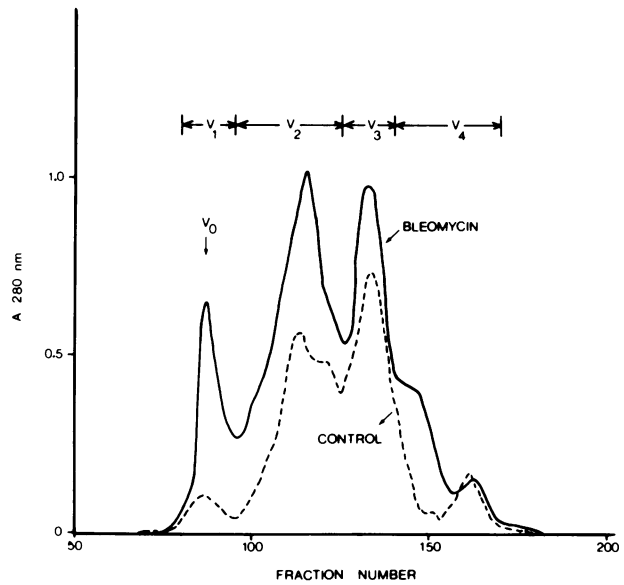

Figure 6—Chromatogram of lung extracts. Lung extracts were subjected to gel filtration on Sephacryl S-200 (2.5 × 90 cm). Fractions of 2 ml were collected, monitored at 280 nm, and pooled to give four major fractions (V_{1-4}) as indicated "V_0" refers to the void volume. The solid line represents bleomycin-treated lung extract, while the broken line represents normal lung extract.

Effect of Serum

Serum contains various regulatory factors with effects on fibroblast growth and metabolism. To determine whether the different inhibitory activities were due to residual serum in the lung extracts, we investigated the effect of normal rat serum as well as serum obtained from bleomycin-treated animals (7 days prior to sacrifice) on lung collagen synthesis *in vitro*. Addition of either serum up to 20% (v/v) had no effect on collagen synthesis (Figure 7), indicating that the inhibitory activity was not derived from serum present in the extracts.

Table 1—Effect of Fractionated Lung Extracts on Lung Collagen Synthesis*

	Protein (mg/ml)	Rates of protein synthesis†	
		Collagenous	Noncollagenous
No addition		405 ± 41	7.20 ± 0.93
Control			
V_1	0.05	301 ± 52	6.21 ± 0.20
V_2	1.72	177 ± 19	4.58 ± 0.16
V_3	1.70	393 ± 21	10.01 ± 0.31
V_4	0.30	411 ± 17	9.42 ± 0.22
Bleomycin			
V_1	0.15	203 ± 39	4.59 ± 0.32
V_2	2.10	291 ± 31	7.71 ± 0.79
V_3	1.60	414 ± 19	9.31 ± 0.99
V_4	0.25	425 ± 20	8.46 ± 1.01

* Lung extracts (from control and bleomycin-treated animals) were fractionated by gel filtration. Fractions were collected and pooled into four major fractions labeled V_{1-4} (see Figure 6). Each was then tested for effects on lung collagen synthesis by direct addition into the collagen synthesis assay mixture at the doses indicated in milligrams per milliliter.

† Expressed as picomoles proline incorporated per milligrams DNA in three hours of incubation. Data expressed as the mean ± 1 SD, with $N = 3$.

Effect of Bleomycin

To rule out the possibility that the inhibitory effects were due to residual bleomycin in the bleomycin-treated lung extracts, addition of this drug to the *in vitro* assay system was tested for inhibitory effects. The results shown in Figure 8 are consistent with the known stimulatory effects of bleomycin added *in vitro* to fibroblasts in culture.[19,20] There was slight stimulation at doses up to 0.25 U/ml, followed by inhibition at higher doses—presumably due to direct toxicity effects. The stimulation was partially due to decreased DNA content upon bleomycin addition *in vitro* and when thus corrected was not statistically significant.

Effect of PGE_1 Analog

Since PGE_1 and other agents that stimulate intracellular cAMP production have been reported to inhibit collagen production by fibroblasts in culture,[17]

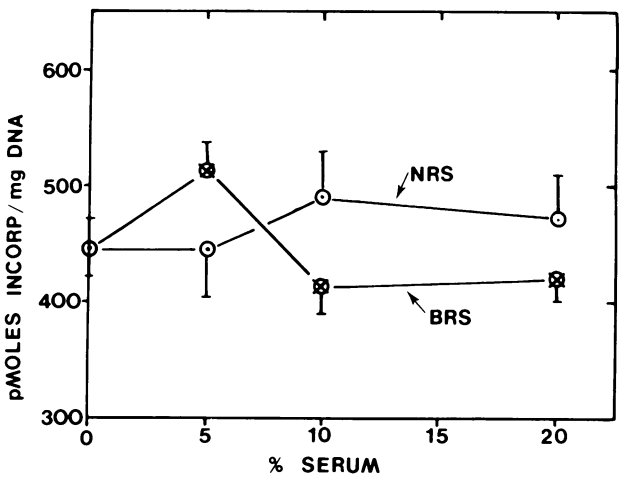

Figure 7—Effect of serum. Blood as obtained from bleomycin-treated and untreated control animals via the inferior vena cava while under ketamine anesthesia. Serum samples were obtained after clotting overnight at 4 C. "BRS" refers to serum from bleomycin-treated (7 days prior to sacrifice) rats and "NRS" from control untreated rats. Dose of serum is expressed as volume %. Each data point represents the mean ± 1 SD, with $N = 5$. All data points were not statistically different (t test) from the data point at 0% dose.

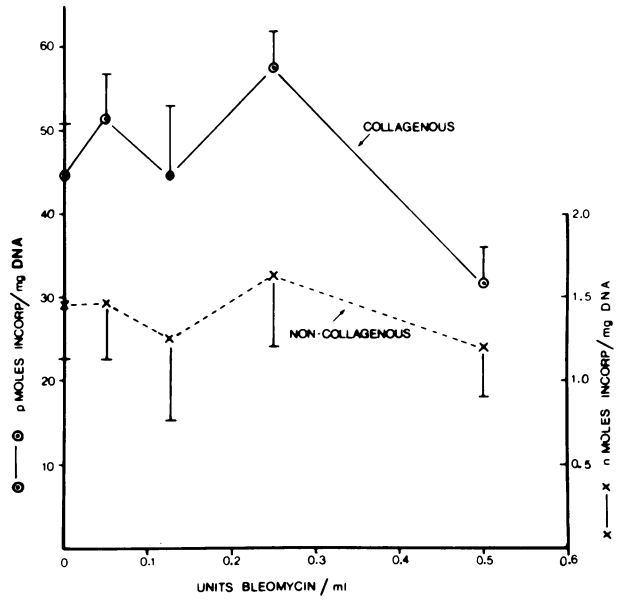

Figure 8—Effect of bleomycin *in vitro*. Bleomycin was added directly in the collagen synthesis assay mixture as described in Materials and Methods. Noncollagenous protein represented the counts incorporated into collagenase-resistant proteins. Each data point represents the mean ± 1 SD, with $N = 4$. No statistically (t test) significant differences were noted between the data points and their respective 0% dose values.

we tested the effect of 15-M-PGE_1 to see whether it could mimic or resemble the dose-response curve generated by the lung extracts. Figure 9 shows a similar inhibitory dose-response curve for collagen synthesis by the 15-M-PGE_1. Noncollagenous protein synthesis was essentially unaffected by this treatment, suggesting that a PGE_1 type of mediator may be responsible for the inhibitory activity in normal lung extracts. The ineffectiveness of dialysis in removing such activity and association of activity with molecules with apparent molecular weight > 150,000 may be due to tight binding to some carrier protein(s). However, the loss of activity upon trypsinization of the lung extracts is more difficult to explain.

Effect of Spleen Extracts

Since there is the suggestion that mononuclear cells regulate fibroblast proliferation and metabolism, spleen extracts were examined for inhibitory activity of lung collagen synthesis. Table 2 shows that spleen extracts from both normal and bleomycin-treated animals are inhibitory to lung collagen synthesis by approximately 25% at a 5% (v/v) dose. There was no statistically significant difference between normal and bleomycin-treated spleen extracts, in contrast to the

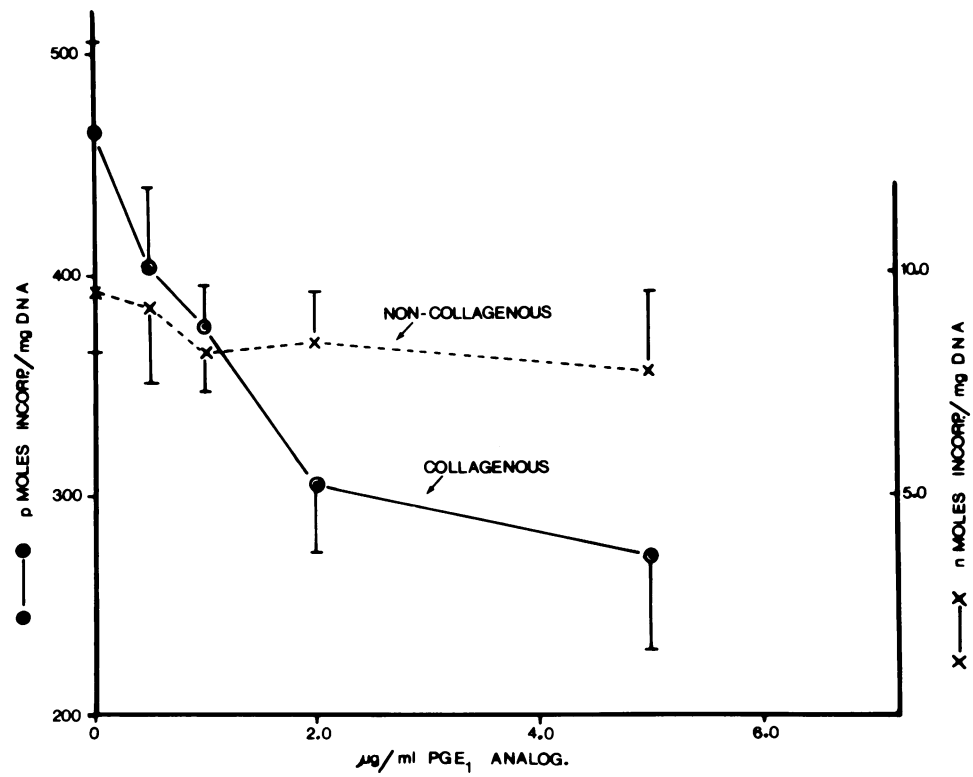

Figure 9—Effect of 15-M-PGE_1. 15-M-PGE_1 was added directly into the incubation medium just prior to pulsing with radioactive proline. Each data point represents the mean ± 1 SD, with $N = 4$. Collagenous synthetic rates were significantly lower (t test) than in its absence in the presence of 15-M-PGE_1 at doses >1.0 µg/ml ($P < 0.05$ and $P < 0.01$ at 2 and 5 µg/ml). Noncollagenous rates were not significantly (t test) affected by 15-M-PGE_1.

Table 2—Effect of Soluble Extracts From Spleen on Lung Protein Synthesis*

Extracts added	Concentrations (%)†	Rates of synthesis†	
		Collagenous	Noncollagenous
None	—	501 ± 30	8.0 ± 1.81
Control spleen	5	385 ± 53	6.4 ± 1.60
Bleomycin-treated spleen	5	310 ± 91	6.4 ± 1.20
Control lung	5	355 ± 45	5.5 ± 0.80
Bleomycin-treated lung	5	502 ± 31	9.4 ± 1.2

* Soluble spleen and lung extracts were prepared from control and bleomycin-treated animals and tested for effects on lung protein synthesis. Extracts were added directly into the assay mixture in the doses indicated.

† Data expressed in picomoles proline incorporated per milligrams DNA in 3 hours of incubation. Data points represent the mean ± 1 SD, with $N = 4$.

lung extracts. The loss of inhibitory regulatory factors appeared to be localized in the lung in bleomycin-induced lung injury.

Discussion

Tissue injury resulting in a fibrotic response usually is preceded by an inflammatory response, although an inflammatory response does not necessarily result in excessive connective tissue deposition (eg, bronchial pneumonia with complete resolution). In view of this, it is crucial that we have an understanding of how initiation or triggering of the fibrotic response takes place before we can begin to unravel the problem of why a fibrotic response, with occasional fatal consequences, develops following some inflammatory events, but not in others, although the intensity of inflammation may appear to be worse. In an attempt to pursue this goal, we have used a model of interstitial pneumonitis with consequent pulmonary fibrosis, to investigate the mechanism that activates the fibrotic response.

Since endotracheal bleomycin-induced pulmonary fibrosis is associated with increased collagen synthesis, beginning at 4 days after instillation and lasting for 4–6 weeks,[9] we chose to start the investigation by examining lung mince extracts from 7-day bleomycin-treated as well as those of normal control animals for any effects on lung collagen synthesis in a lung explant culture system that we have used previously.[9] In view of reports in the literature indicating that lymphocytes and macrophages, when appropriately stimulated, would release substance(s) into their medium that would stimulate cultured fibroblasts to proliferate and synthesize more collagen,[12,14] the initial expectation was to see stimulatory activity in the bleomycin lung extracts. It was thus somewhat surprising to see inhibitory activity, especially at the higher doses of bleomycin-treated lung extract. The statistically insignificant stimulation at the lower doses was not highly reproducible; furthermore, we were not able to detect any stimulatory activity in any of the fractions obtained from the Sephacryl S-200 column, suggesting that a lack of inhibitory factors, rather than the masking of a stimulatory factor by other inhibitory factors, is the best explanation for the noted increase in lung collagen synthesis when treated with bleomycin. The inhibitory activity, present in larger amounts in normal lung extracts, was heterogeneous, as shown in Figure 6 and Table 1. It was composed of at least two different entities with molecular weight > 150,000 and with differing specificities with respect to inhibition of collagenous versus noncollagenous protein synthesis. Normal and bleomycin-treated rat serums, as well as bleomycin added *in vitro*, had no similar detectable activity. *In vitro* addition of bleomycin to cultured fibroblasts had an opposite effect by stimulating collagen synthesis.[19,20] An analog of PGE_1, on the other hand, mimics the dose-response curve seen with normal lung extracts. However, the high molecular weight and proteinaceous nature of the lung extract activity would seem to exclude PGE_1 as the mediator responsible for the inhibitory activity seen in normal lung extracts.

The data presented here suggest that in endotracheal bleomycin injury, a significant decrease in inhibitory regulatory activity, normally present in normal untreated lungs, is partially responsible for the noted increase in collagen synthesis. The normal inhibitory activity may be similar to that produced by cultured mononuclear cells and directed at isolated cultured fibroblasts.[10,11] The known direct effects of bleomycin on fibroblast proliferation and collagen synthesis[19,20] would further contribute to the observed increase in lung collagen synthesis. The response in the lung with respect to loss of inhibitory regulatory activity appears to be localized to the site of the injury and is not seen in the spleen.

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