Inhibition of Collagen Accumulation by Glucocorticoids in Rat Lung after Intratracheal Bleomycin Instillation

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ABSTRACT
Male Fischer 344 rats were given a single lung instillation of bleomycin sulfate (0.6 units/100 g). Some animals were treated 24 hr after bleomycin administration with triamcinolone diacetate. Steroid treatment was continued on alternate days for 4 weeks. At the end of 4 weeks, the lungs of rats receiving bleomycin alone had two-fold increases of both prolyl hydroxylation activity and proteinaceous hydroxyproline as compared to control values. The lungs of bleomycin-treated rats which received 4 mg of triamcinolone diacetate per kg on alternate days had a 33% increase of prolyl hydroxylation activity and a 37% increase of proteinaceous hydroxyproline content as compared to control values. Lung prolyl hydroxylation activity and proteinaceous hydroxyproline content of bleomycin-treated animals receiving glucocorticoid (8 mg/kg) on alternate days were the same as control values. The results indicate that alternate day administration of the synthetic glucocorticoid triamcinolone diacetate blocks lung collagen accumulation following a single intratracheal dose of bleomycin to rats.

INTRODUCTION
The bleomycins are a family of glycopeptides isolated from Streptomyces verticillus and used as antineoplastic agents (12, 13, 37). Bleomycin is an effective therapeutic agent for Hodgkin's lymphoma (5), testicular carcinoma (29, 30), and squamous cell carcinomas (14).

The use of bleomycin in cancer therapy is hampered by toxic effects, with the major adverse effect being interstitial pneumonitis followed by fibrosis, which occurs both in humans and animals (1-4, 7, 31, 38). The lung pathology observed histologically in humans is similar to that in animals and does not differ significantly from interstitial pneumonitis and fibrosis induced by other lung toxins (7). Fibrotic lungs can be induced in rats by a single i.t. injection of bleomycin (22, 34-36) and does not require repeated treatments. This model has the advantage of requiring a single dose of bleomycin to obtain the same histological changes observed by repeated systemic administration. The major biochemical changes of collagen metabolism in lungs of bleomycin-treated animals are increased prolyl hydroxylation activity (15), collagen synthesis (6, 22), and accumulation of proteinaceous hydroxyproline (10, 15, 33, 35, 36).

Glucocorticoids have been recently shown both in vivo and in cell culture to selectively decrease collagen polypeptide synthesis (9, 17, 20, 21, 23, 24, 26, 27). This may account for their beneficial therapeutic effect in chronic inflammatory diseases. These steroids have been used to ameliorate the symptoms of pulmonary fibrosis and other lung diseases in humans (32). The results reported herein demonstrate that administration of the synthetic fluorinated glucocorticoid triamcinolone diacetate prevents the accumulation of collagen in lungs following a single intratracheal administration of bleomycin to rats.

MATERIALS AND METHODS
Male Fischer 344 rats (120 g) were purchased from Charles River Breeding Laboratories (Portage, Mich.). Protease-free bacterial collagenase was obtained from Advanced Biofactures (Lynbrook, N. Y.). The collagenase used in all experiments was protease free and did not digest tryptophan-labeled Escherichia coli protein. Bleomycin sulfate was a gift from Bristol Laboratories (Syracuse, N. Y.). Triamcinolone diacetate was obtained from Lederle Laboratories (Pearl River, N. Y.). Sodium methohexital was purchased from Eli Lilly Co. (Indianapolis, Ind.). [5-3H]Proline (25 Ci/mmol) was purchased from Amerahs/ Searle Corp. (Arlington Heights, Ill.).

Rats were kept in cages covered with filter tops to minimize risk from airborne pathogens and were provided chow and water ad libitum. All animals were maintained at least 1 week prior to bleomycin instillation at which time they weighed between 180 and 190 g.

Animals were anesthetized by an i.p. injection of sodium methohexital (4.0 mg/100 g). Bleomycin sulfate (0.6 unit/100 g) was solubilized in sterile 0.9% NaCl solution, and 0.25 ml/rat was instilled i.t. by a syringe fitted with a modified feeding tube. Control animals were given 0.25 ml of sterile 0.9% NaCl solution. Starting 24 hr after bleomycin instillation, animals receiving triamcinolone diacetate were given i.p. injections every other day.

Rats were killed by decapitation and bled. The lungs were removed and dissected free of central bronchi and vessels, rinsed in cold 0.9% NaCl solution, blotted, weighed, and minced. Mincing of the lung tissue served a 2-fold purpose: to remove blood from the tissue and to provide a more homogeneous sample for prolyl hydroxylation activity and proteinaceous hydroxyproline determination. There were no differences of prolyl hydroxylation activity, soluble protein, or proteinaceous hydroxyproline between perfused lung and those not perfused but minced and washed in 0.9% NaCl solution.

The 20,000 × g supernatant of a homogenate of minced lung tissue was prepared for determination of prolyl hydroxylation activity by the tritium release assay (11) as described previously (20). The protein of the 20,000 × g supernatant fraction was assayed by the method of Lowry et al. (16). Another portion of minced lung tissue was used to determine total lung tissue proteinaceous hydroxyproline as described previously (9) by the method of Prokop and Udenfriend (25). Collagen and noncollagen protein syntheses were determined using lung minces (0.3 g) incubated for 2 hr with 100 μCi of radioactive proline by the collagenase digestion assay as described previously (20), a modification of the method of Miller and Udenfriend (19).

RESULTS
Rats treated with glucocorticoid (4 mg/kg) alone gained weight at a decreased rate as compared to control rats (Chart
1. The 8-mg/kg triamcinolone group did not gain or lose weight. Decreased weight gain and weight loss at higher doses are major toxic effects of glucocorticoid treatment. Rats treated with bleomycin alone or plus glucocorticoid lost weight within the first 2 weeks and then gained weight thereafter.

Total lung prolyl hydroxylase activity, proteinaceous hydroxyproline, and supernatant protein were determined 1, 2, and 4 weeks after bleomycin instillation (Charts 2 to 4). Enzyme activity peaked at 1 week and decreased by 4 weeks to approximately 120% above control after bleomycin treatment. Total lung proteinaceous hydroxyproline of bleomycin-treated rats increased to approximately 125% above control during the 4-week period. At the end of 4 weeks, the rat lungs receiving bleomycin alone had a 55% increase of collagen synthesis and a 58% increase in noncollagen protein synthesis (Chart 5). Similar increases of lung collagen synthesis following bleomycin administration have been reported (6, 22). Although triamcinolone given daily has been shown to selectively decrease collagen synthesis in skin, calvaria, and lung of neonates given steroid alone (9, 18, 20, 26), this is not the case when the steroid is given on alternate days at a lower dose level to much older rats. However, triamcinolone treatment did block the elevated rate of lung collagen synthesis induced by bleomycin administration. The amount of proteinaceous hydroxyproline is directly related to collagen content since less than 5% of this amino acid represents elastin, the only other major hydroxyproline-containing protein in lung (8, 10).

The elevations of both prolyl hydroxylase (Chart 2) and total proteinaceous hydroxyproline content (Chart 3) are greater than the elevation of soluble protein (Chart 4) at all time points, indicating a specific elevation of collagen biosynthetic parameters.
A group of rats receiving a single intratracheal instillation of bleomycin was injected i.p. with 4 mg of triamcinolone per kg every other day for 4 weeks. After 4 weeks, the total lung prolyl hydroxylase activity of rats receiving steroid was 33% increased above control value (Chart 2). Also at 4 weeks, proteinaceous hydroxyproline was increased by 37% in the lungs of rats receiving bleomycin and the glucocorticoid (Chart 3). Complete inhibition of bleomycin-induced prolyl hydroxylase activity and hydroxyproline accumulation was observed in rats receiving 8 mg of triamcinolone per kg for 4 weeks (Charts 2 and 3). In all rats receiving bleomycin plus either dose of glucocorticoid, supernatant protein was decreased as compared to rats treated with bleomycin alone (Chart 4). Rats instilled with 0.9% NaCl solution and treated at either dose of triamcinolone diacetate every other day for 4 weeks had less lung prolyl hydroxylase activity and proteinaceous hydroxyproline than did control rats.

Time course data are also expressed per mg protein (Tables 1 and 2). Prolyl hydroxylase activity was significantly elevated at all time points in bleomycin-treated rats (Table 1). Triamcinolone given at 4 mg/kg blocked bleomycin-induced prolyl hydroxylase activity after 4 weeks. When rats were treated at 8 mg/kg, the prolyl hydroxylase elevation was completely blocked after 2 and 4 weeks treatment. Hydroxyproline content of lung per mg protein was significantly elevated at 2 and 4 weeks after bleomycin treatment. Bleomycin-treated rats given 8 mg/kg on alternate days had lung hydroxyproline content equivalent to control values at 2 and 4 weeks after bleomycin. Triamcinolone given alone at 4 mg/kg significantly decreased hydroxyproline per mg protein at 1 and 2 weeks while having no effect at 4 weeks. This latter effect may be a function of decreased lung protein content. Similar decreases in hydroxyproline content were also observed after treating bleomycin rats with steroid at 4 mg/kg at 1 and 2 weeks, although at 4 weeks hydroxyproline content was increased about control value. The first 2 changes may also be due to significant increases in total lung protein content. Since the amount of total lung protein is significantly different from control value in many groups (Chart 4), normalization of data per whole lung is better than per mg protein.

**DISCUSSION**

Pulmonary fibrosis is a toxic effect of bleomycin treatment. The molecular mechanism(s) involved is not known. However, the histological progression of fibrosis is similar in humans and animals (3, 4, 7). The histological changes which occur in the lung with bleomycin-induced fibrosis are the same whether the drug is administered systemically or i.t. (1, 33, 34). Systemic administration requires larger and repeated doses of bleomycin to induce the fibrotic lung effect, whereas i.t. injection does not (1, 15, 33, 34). Furthermore, the amount of bleomycin and the

**Table 1**

<table>
<thead>
<tr>
<th>Week</th>
<th>Control</th>
<th>Bleomycin (4 mg/kg)</th>
<th>Triamcinolone (4 mg/kg)</th>
<th>Triamcinolone + Bleomycin (4 mg/kg)</th>
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<td></td>
<td>1.66 ± 0.10b</td>
<td>3.27 ± 0.20b</td>
<td>1.94 ± 0.38</td>
<td>3.67 ± 0.25b</td>
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<td>1.33 ± 0.06</td>
<td>2.26 ± 0.11b</td>
<td>1.28 ± 0.33</td>
<td>3.60 ± 0.07b</td>
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<tr>
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<td>1.96 ± 0.10</td>
<td>2.91 ± 0.21b</td>
<td>1.75 ± 0.09</td>
<td>2.26 ± 0.13</td>
</tr>
</tbody>
</table>

- Mean ± S.E.
- Statistically significant at p < 0.05.

**Table 2**

<table>
<thead>
<tr>
<th>Week</th>
<th>Control</th>
<th>Bleomycin (8 mg/kg)</th>
<th>Triamcinolone (8 mg/kg)</th>
<th>Triamcinolone + Bleomycin (8 mg/kg)</th>
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</thead>
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<td></td>
<td>0.06 ± 0.01b</td>
<td>0.07 ± 0.002</td>
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<tr>
<td></td>
<td>0.07 ± 0.003</td>
<td>0.16 ± 0.01b</td>
<td>0.08 ± 0.004</td>
<td>0.09 ± 0.005b</td>
</tr>
</tbody>
</table>

- Mean ± S.E.
- Statistically different at p < 0.05.
number of systemic administrations needed to obtain the fibrotic effect in the lung induces toxicity in other tissues (33). Administration i.t. confines the toxicity to the lung (15). Both prolyl hydroxylase activity and hydroxyproline content of lung are elevated after a single i.t. injection of bleomycin (10, 15, 33, 36). Lung explants of bleomycin-treated rats incorporated more radioactive proline into collagen as compared to control values (6, 22). Thus, increased collagen synthesis may account for at least part of the accumulation of collagen in bleomycin-instilled lung.

The selective inhibitory effect of glucocorticoids on collagen synthesis in connective tissues and in isolated fibroblasts is well documented. Steroid treatment results in a greater inhibition of collagen synthesis than total noncollagen protein synthesis (20). The glucocorticoid inhibitory effect of collagen synthesis is not due to a drug-induced alteration of precursor pool specific activity. Polysomes isolated from the dermis of steroid-treated animals synthesize collagen in the wheat germ lysate system at a reduced rate (18). Furthermore, poly(A) mRNA isolated from these polysomes synthesized less collagen as compared to poly(A) mRNA isolated from control polysomes (26). The ability of triamcinolone diacetate to inhibit lung collagen accumulation following a single i.t. dose of bleomycin may be due to the ability of this glucocorticoid to selectively decrease collagen polypeptide synthesis. This selective inhibitory effect of glucocorticoids on collagen synthesis is further supported by the fact that glucocorticoids do not decrease polysomal poly(A) mRNA for prolyl hydroxylase in skin or in lung.

It would be premature and potentially harmful for clinical oncologists to conclude from this paper that patients receiving bleomycin should also be treated with large doses of glucocorticoids.

ACKNOWLEDGMENTS

We thank Dr. Jason Kelley for teaching K. M. Sterling, Jr., the bleomycin instillation technique.

REFERENCES

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