Inhibition of Bleomycin-Induced Pulmonary Fibrosis by Cobra Venom Factor

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A single dose of cobra venom factor, a known, potent depletor of serum complement, has recently been reported to be highly effective in inhibiting lung collagen deposition at 7 days after bleomycin treatment. The present study shows that this is associated with suppression of the bleomycin-induced increase in collagen synthesis, down to virtually normal levels. There is also a concomitant reduction in tissue free proline pool size. No significant effects are noted in noncollagenous protein synthetic rates. This inhibitory effect is not seen at 30 days after bleomycin and is correlated with a return to normal levels of serum complement hemolytic activity. These data suggest an important role for complement activity in the lung's fibrotic response to bleomycin. (Am J Pathol 1982, 107:025-028)

BLEOMYCIN is an antitumor antibiotic that is highly effective against squamous cell carcinomas, but has a major drawback in causing pulmonary toxicity at high cumulative doses. This toxicity is manifested as an interstitial pneumonitis developing ultimately into pulmonary fibrosis.[1-3] In the rat, a single intratracheal instillation causes increased lung collagen synthesis and deposition.[4,5] These changes are accompanied by an interstitial infiltrate composed of neutrophils, lymphocytes, plasma cells, and macrophages, which became sparse at 1 week, except for the mononuclear infiltrate.[4] Lung lavage fluid also contains increased numbers of neutrophils and lymphocytes.[6]

The sequence of events suggests an initial cell recruitment phase whose significance in the overall pathogenetic mechanism of pulmonary fibrosis still remains to be evaluated. Leukocyte chemotaxis is known to be mediated by complement-derived chemotactic factor(s). Complement is also important for other immunologic responses, such as the Arthus reaction,[7] allergic phenomena,[7] and delayed hypersensitivity.[8] Since various manifestations of immune responsiveness are involved in the pulmonary fibrotic response, in this study we have abrogated the complement system by intravenous administration of cobra venom factor (CoF) and analyzed its impact on increased lung collagen synthesis as a result of bleomycin instillation.

Materials and Methods

Animals

Experiments were all performed with male Fisher 344 inbred strain of rats (175-200 g), with no morphologic evidence of pulmonary disease as assessed by the sacrificing of a few animals and examination of their lungs by light microscopy. Intratracheal administration of bleomycin (1.5 units/animals) was as described previously.[4] Control animals underwent sham tracheostomy. Animals were divided into four groups: A) control, untreated animals, B) animals given CoF alone, C) animals that received bleomycin only, and D) animals treated with CoF and bleomycin. Fifty units of CoF was given intravenously via the tail vein 2 hours before bleomycin instillation. No repeat dose was attempted to avoid the complications of antibody production to CoF and long-term or chronic absence of complement. Each group consisted of 6 animals.

Supported by Veterans Administration Research Funds and NIH Grant HL-23192. Sem H. Phan is a Research Associate of the Veterans Administration.

Accepted for publication November 9, 1981.

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0002-9440/82/0408-0025$00.70

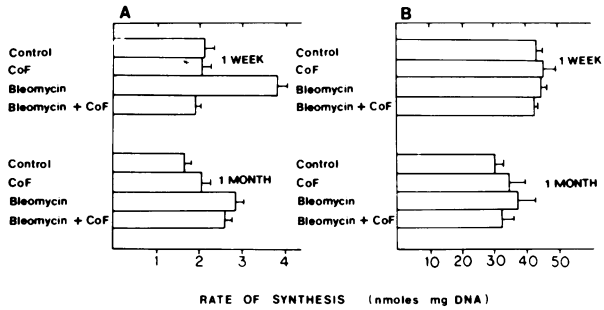

Figure 1 – Effect of CoF on lung protein synthesis. Collagenous (**A**) and noncollagenous (**B**) protein syntheses were expressed as nanomoles of proline incorporated by short-term lung explant cultures per milligram total DNA, in 6 hours of pulsing with ^{3}H-proline. Data points are means ± 1 standard deviation; $n = 6$. Only the group treated with bleomycin alone is significantly different ($P < 0.05$) in rate of lung collagen synthesis from the control group at 1 week. At 1 month, the bleomycin and the bleomycin + CoF groups are both significantly ($P < 0.05$) elevated over the control group in collagen synthesis, and the bleomycin + CoF group over the CoF group.

Reagents

Bleomycin (Blenoxane®) was a generous gift of Bristol Laboratories (Syracuse, NY). CoF, free of phospholipase A_2, was purchased from Cordis Laboratories (Miami, Fla). Bacterial collagenase (CLSPA grade) was obtained from Worthington (Freehold, NJ). L-2,3-[^{3}H]-proline (20–40 Ci/mmol) was from New England Nuclear (Boston, Mass). All other reagents were of analytic reagent grade.

Collagen Synthesis Assay

Lung collagen synthesis was assayed *in vitro* by incubation of minced rat lung in media containing radioactive proline and measurement of the rate of incorporation into collagenase-sensitive trichloroacetic-acid(TCA)-precipitable material. Minced lung was prepared as described previously.[5] Following homogenization with a Polytron (Brinkmann Instruments, Westbury, NY), the samples were centrifuged, and the pellet was washed twice with 10% TCA.[1] The supernatants were all pooled and stored for total free proline assay and free proline radioactivity determination as previously described.[5] The final pellet was then resuspended in 0.1 N NaOH and digested with purified bacterial collagenase as described by Peterkovsky and Diegelmann.[9] Collagenase-sensitive counts represent collagenous protein synthesis, while collagenase-insensitive counts represent non-collagenous protein synthesis. Rates of synthesis were expressed as nanomoles proline incorporated/mg DNA in 6 hours of incubation. We derived these by dividing the incorporated radioactivity by the specific radioactivity of the tissue free proline pools.

Analytic Determinations

Total lung DNA content was determined as described previously.[5] Free proline in the pooled supernatants was determined according to Troll and Lindsey[10] as previously modified.[5] Radioactivity was determined with Beckman MP scintillation cocktail (Beckman Instruments, Palo Alto, Calif) with a [^{3}H] counting efficiency of 35%.

Assay for Serum Complement

This was performed using a standard protocol (hemolytic unit of complement, CH_{50}) according to Kabat and Mayer.[11] Animals were bled from the inferior vena cava under ketamine anesthesia. Serum samples were obtained after clotting at 4 C. CH_{50}'s dropped to less than 5% of normal levels approximately 2 hours after injection of CoF and persisted at these low levels for 4 days before gradually returning to normal levels by 7 days.

Statistical Analysis

Data obtained from the various animal groups were compared for statistically significant differences between groups by using Student's *t*-test.

Results

Effects on Lung Collagen and Protein Synthesis

Having determined the efficacy of the CoF in depleting serum complement, the effect on bleomycin-induced increases in lung collagen synthesis was then examined. As previously observed,[5] bleomycin caused marked increases in the rate of lung collagen synthesis at both 7 and 30 days after treatment (Figure 1A), while noncollagenous protein synthesis was relatively unaffected (Figure 1B). When CoF was given just prior to bleomycin treatment, the bleomycin-induced increase in collagen synthesis was not seen, indicating total suppression by treatment with CoF at 7 days after bleomycin instillation. At 30 days after bleomycin instillation, however, no significant suppression by CoF was noted, and was not entirely unexpected, since the effect on CH_{50}'s of the CoF lasted for only 7 days. CoF alone had no significant effects on collagenous or noncollagenous synthesis. These data suggest that our previous observation of the effectiveness of CoF in inhibiting the bleomycin-induced increased lung collagen content at Day 7, but not at Day 30 after bleomycin instillation,[12] was due to this effect on collagen synthesis.

Selectivity of Effects on Lung Protein Synthesis

Normal lung mince usually uses approximately 1% of its protein synthetic machinery for collagen synthesis. This parameter, referred to as the percentage of collagenous protein synthesis, was obtained with the following formula:

$$\% = \frac{C}{C + 5.04\,(NC)} \times 100\%,$$

where C = collagenous counts per minute (cpm) (sensitive to collagenase), NC = noncollagenous cpm (resistant to collagenase), and 5.04 represents a correction factor for the average lower content of proline in noncollagenous proteins. Bleomycin treatment caused this parameter to go up from 0.87 in normal controls to 1.50, a 72% increase, at Day 7. This persisted at Day 30 (Figure 2), but to a lesser extent. CoF treatment again prevented the bleomycin-induced increase at Day 7, but not at Day 30. This effect, together with the data in Figure 1, indicate that the bleomycin-induced increase in the percentage of collagenase protein synthesis was due to increased collagen synthesis, while noncollagenous synthesis was unaffected; and furthermore, CoF specifically prevented this increase, without affecting noncollagenous synthesis.

Effects on Tissue-Free Proline Pools

Bleomycin has been reported to cause marked increases in the size of the tissue-free proline pools.[13] In this study, the elevation was by 93% at 1 week and by 232% at 1 month above control levels (Figure 3).

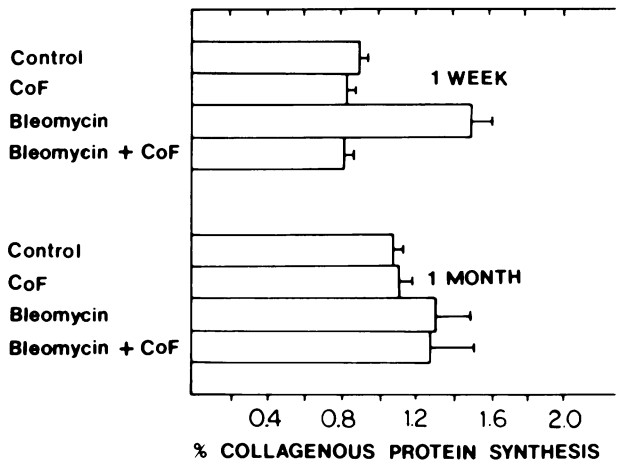

Figure 2—Selectivity of CoF effect on lung protein synthesis. The percentage of collagen synthesis was calculated as described in the text. Each data point represents the mean ± 1 standard deviation; $n = 6$. Only the bleomycin group is significantly different ($P < 0.005$) from the control group at 1 week. No differences were noted at 1 month.

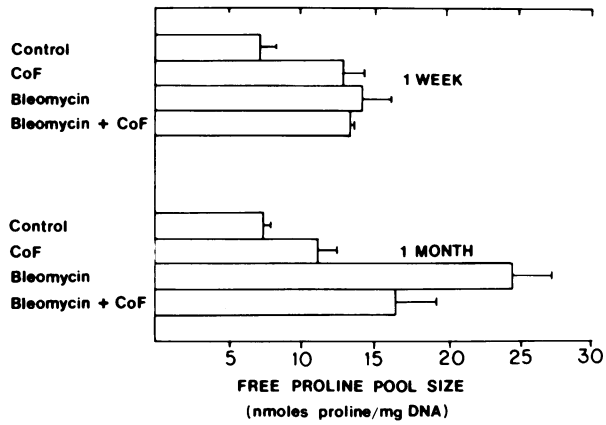

Figure 3—Effect of CoF on tissue-free proline pools. Proline pools were expressed as nanomoles free proline per mg total DNA in the lung mince sample. Each data point represents the mean ± 1 standard deviation, $n = 6$. All groups were significantly different ($P < 0.05$) from the control group at 1 week and 1 month. At 1 month, however, the effect of bleomycin + CoF was not significantly different from CoF.

CoF treatment alone curiously caused a 60–70% elevation over control levels. At 7 days after bleomycin, no difference was apparent between the groups receiving CoF alone and CoF plus bleomycin. Thus, despite the fact that no significant difference was noted between groups receiving bleomycin alone and CoF plus bleomycin, CoF was effective in preventing the *bleomycin*-induced increase in proline pool size. This effect was more apparent at 1 month. While bleomycin alone caused a 232% elevation above the values in untreated control animals, animals treated with bleomycin plus CoF had values only 49% above those of animals receiving CoF alone. Furthermore, the latter increase was not statistically significant. Thus, CoF was highly effective in preventing the marked bleomycin-induced increase in tissue-free proline pool size, although CoF itself caused a 60–70% increase.

Discussion

CoF is a very effective decomplementing agent.[14,15] It is an anionic glycoprotein with a molecular weight of 144,000.[16] A single intravenous dose causes depletion of serum complement lasting up to 4–5 days as determined by C3 and CH_{50} levels.[14] Serum complement components and their derivatives are important components of the inflammatory and immune mediator systems.[7,8,17,18] Because of their important role in these events (some of which are present in the response to bleomycin), examining the effect on the bleomycin-induced fibrogenic response of abrogating the complement system would provide some insight into the relationship or association between inflam-

mation (pneumonitis), the immune system, and pulmonary fibrosis.

The data derived from this study showed that CoF was highly effective in preventing bleomycin-induced increases in lung collagen synthesis but failed to do so when its effect on CH_{50} levels waned. This effect was specific for collagen synthesis with no effect on noncollagen synthesis. Thus, the preventive effect was directly dependent on abolition of serum complement activity. On the other hand, the effect on tissue proline pool size was longer lasting and not as dependent on persistent reduction in CH_{50} levels. This is an interesting observation, since free proline pool size is usually directly correlated with the intensity of the fibrogenic response as assayed by total tissue collagen content.[19,20] In our case, bleomycin-induced increases in lung collagen synthesis and deposition[12] were not significantly affected by CoF at 1 month, despite the persistent suppression of bleomycin-induced proline pool size expansion, thus suggesting that increased collagen synthesis is not directly dependent on increased proline pool size.

Due to the multiplicity of functions that have been attributed to serum complement components and their derivatives, it would be impossible from our data to decipher whether one or all of these functions are necessary for full expression of the fibrotic response to bleomycin. Depletion of neutrophils with anti-neutrophil serum was ineffective in ameliorating or preventing fibrosis.[12] This would seem to indicate that CoF was effective in preventing the fibrotic response by abrogating a complement-dependent inflammatory and/or immune function(s) other than those involved in recruiting neutrophils (ie, chemotaxis) and enhancing neutrophil function (ie, phagocytosis, lysosomal degranulation, etc.).

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Acknowledgment

We would like to acknowledge the excellent technical assistance of Darlene D'Amato and the secretarial assistance of Jeny Breakstone.