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REDUCTION IN RABBIT SERUM AND PULMONARY ANGIOTENSIN CONVERTING ENZYME ACTIVITY AFTER SUBACUTE BLEOMYCIN TREATMENT

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Abstract—We studied morphological and biochemical alterations in the lungs of rabbits treated subcutaneously with bleomycin (5 mg/kg) three times weekly for 28 days. This treatment produced morphologically evident pulmonary damage characterized by the appearance of ciliated alveolar epithelial cells and endothelial blebs. No change in pulmonary collagen content was evident. Rabbits treated with bleomycin had significantly less pulmonary angiotensin converting enzyme activity than control animals, although no alterations in lung DNA, protein, glycosaminoglycans or hydroxyproline (an index of collagen) were observed. Serum angiotensin converting enzyme activity decreased with repeated bleomycin administration and was reduced by 42, 48 and 65 per cent from control animals 2, 3 and 4 weeks, respectively, after the initiation of drug treatment. These data provide biochemical evidence that subacute bleomycin administration damages the pulmonary endothelium in the absence of fibrosis and can reduce the serum activity of angiotensin converting enzyme in rabbits.

Bleomycin (BLM) is an effective antineoplastic agent, but its clinical utility is limited by its potentially lethal pulmonary toxicity [1]. This toxicity, which is specific to the air–blood barrier, initially appears as an inflammatory response that can ultimately develop into irreversible fibrosis. The pathogenesis of this drug-induced fibrosis is not clearly understood although the histological changes have been described in a large number of species, including man [1–5]. Examination of the biochemical effects of BLM in lungs may provide important information concerning the mechanism of BLM pulmonary toxicity.

Morphological studies [2, 6, 7] suggest that the earliest pulmonary cell type damaged by systemic BLM administration is the endothelium, although this has been disputed [8]. Thus, it is unclear if damage to the pulmonary endothelium after BLM treatment is an obligatory step in the sequence of events leading to lung fibrosis. Lung endothelium performs a variety of biochemical functions unrelated to respiration, such as hydrolysis of angiotensin I to angiotensin II by the peptidyl dipeptide hydrolase, angiotension converting enzyme (ACE; EC 3.4.15.1), and uptake and metabolism of biogenic amines [9, 10]. The lungs contain the highest specific activity of ACE in the body, excluding the epididymis and the testis [11], and it is likely that the pulmonary endothelium is the primary source of ACE activity in normal lungs [10]. The enzyme is associated with the luminal surface of the plasma membrane, and the endothelial cell plasma membrane has been hypothesized to be a possible source for serum ACE [12].

Very little is known about the pulmonary toxicity of BLM in rabbits even though they exhibit one of the most common acute toxic reactions associated with BLM administration in humans, namely pyrexia [13]. The non-respiratory functions of rabbit lung that are associated with the endothelium have been studied extensively both *in vivo* and *in vitro* and represent sensitive indices of pulmonary integrity (for review, see Ref. 9). Recent work in our laboratories [14] indicated that subacute administration of BLM to rabbits produced a marked reduction in the ability of the lungs to remove various biogenic amines. We have attempted to further characterize the biochemical changes that occur in rabbit lungs after systemic BLM administration and to assess whether the rabbit is a useful model for examining mechanisms responsible for bleomycin-induced pulmonary toxicity and for finding potential pharmacological means to mitigate the toxicity.

We therefore examined the effect of subacute BLM treatment on (a) rabbit pulmonary hydroxyproline (OH-Pro), glycosaminoglycans (GAG), DNA and protein content, and ACE activity to determine whether there was biochemical evidence of endothelial or parenchymal damage and (b) serum ACE activity to evaluate the potential usefulness of this enzyme as a biochemical marker of drug-induced lung injury.

MATERIALS AND METHODS

Animals and drugs. Male albino New Zealand rabbits, weighing between 2.0 and 3.6 kg, were provided with Purina rabbit chow and water *ad lib.* Clinical grade BLM (Blenoxane) (1.7 units/mg) was supplied by Dr. William T. Bradner of Bristol Lab-

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oratories (Syracuse, NY) and was dissolved in a phosphate-buffered 0.9% NaCl solution (8.0 g NaCl, 0.2 g KCl, 2.16 g $Na_2HPO_4 \cdot 7H_2O$, and 0.2 g KH_2PO_4 per liter H_2O, pH 7.4). Four animals served as untreated controls and two additional rabbits were injected with a solution of phosphate-buffered 0.9% NaCl three times weekly for 28 days; no difference in the pulmonary or serum values of interest in this study was observed between these two groups. Unless otherwise mentioned, the untreated rabbits served as control animals. Five additional rabbits received BLM, s.c., 5 mg/kg three times weekly for 28 days. This dose was chosen because, when calculated on the basis of surface area (83 mg/m^2), it is approximately equal to the dose (61 mg/m^2) that produces pulmonary fibrosis in mice after repeated BLM administration [2, 7, 15]. Two or three days after the last BLM injection each rabbit was anesthetized (urethane and allobarbital, 50 and 200 mg/kg, respectively), and the *in vivo* pulmonary clearance of various biogenic amines was studied [14]. After completion of the pulmonary clearance tests, the animal was killed with approximately 5 ml of air i.v. and the thoracic cavity was opened. Three pieces of lung (1 cm long × 0.3 cm thick) were removed from the subpleural region of the right lung for electron and light microscopy. Approximately 1–2 g of the lower right lung was homogenized and used for protein, DNA and ACE activity determinations. The upper portion of the right lung was utilized for the OH-Pro and GAG determinations. The dry/wet lung weights were obtained from the left lung after drying for 24 hr at 110°. Total pulmonary biochemical data were expressed based on the weight of both lungs.

Pulmonary DNA, protein and ACE activity. Both lungs from each rabbit were quickly removed, blotted, and weighed; approximately 1–2 g of the lower right lung was prepared as described previously for determining ACE activity [16]. Briefly, lung tissue was placed in an ice-cold glass tissue grinder containing 6 vol. of a solution of 500 mM K_2HPO_4, 300 mM NaCl, and 30 mM 1-*O*-*n*-octyl-β-D-glucopyranoside (pH 8.3). After homogenization the suspension was frozen immediately (−70°), thawed, and placed in a shaking water bath (4°) for 1 hr. During the shaking the sample was sonicated five times (10 sec each). The resulting preparation was centrifuged at 1000 *g* for 20 min (4°). The supernatant fraction was removed, and the pellet was resuspended in 500 mM K_2HPO_4 and 300 mM NaCl (pH 8.3) and sonicated twice (10 sec each). More than 80 per cent of both the total pulmonary protein, as measured by the method of Lowry *et al.* [17], and the total pulmonary DNA, as measured by the method of Burton [18], was found in the supernatant fraction. The activity of ACE was determined spectrophotometrically using hippuryl-L-histidyl-L-leucine as a substrate; more than 85 per cent of the total pulmonary ACE activity in control and treated rabbits was recovered in the supernatant fraction [15].

OH-Pro assay. Since pulmonary OH-Pro is derived almost exclusively from collagen [15], whole pulmonary collagen content was estimated by measuring OH-Pro content. A portion of the upper right lung (approximately 0.4 g) was removed, the weight was recorded, and then the sample was placed in a test tube that was sealed under vacuum (50 mTorr) with 2 ml of 6 M HCl–0.5% phenol (v/v) [18, 19]. The tube was heated for 20 hr at 110° and then evaporated to dryness on a Speed Vac concentrator (Savant Instruments, Inc., Hicksville, NY). The sample was resuspended in a 0.07 M sodium citrate buffer (pH 2.2) and filtered through a 0.45 μm Millipore filter (Millipore Co., Boston, MA). The OH-Pro concentration was determined by the spectrophotometric method of Woessner [19] and by amino acid analysis on a D500 Analyzer (Durrum Instrument Co., Sunnyvale, CA).

GAG assay. Pulmonary GAG were isolated by a modification of the method of Sobue *et al.* [20]. Approximately 2 g of the upper lobe of the right lung was defatted with 10 vol. of ice-cold acetone and then 5 vol. of ice-cold $CH_3OH:CHCl_3$ (1:3, v/v). The filtered, dried pulmonary tissue was resuspended in approximately 20 ml of 0.5 M Tris/HCl (pH 8.2) and incubated for 16 hr at 37° with 2.5 mg Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) and several drops of toluene. An additional 2.5 mg of Proteinase K was added to the flask and the suspension was incubated for an additional 8 hr. The resulting solution was adjusted to pH 12 with 6 M NaOH and, after incubation at 37° for 1 hr, was neutralized to pH 6–7 with 6 M HCl. To the digest we added 3 vol. of absolute ethanol containing 1.3% potassium acetate and allowed it to stand at −20° overnight. The solution was centrifuged at 6000 *g* for 20 min (4°) and the supernatant fraction, which contained no GAG, was discarded. The pellet was resuspended with approximately 20 ml of 6 mM Na_2SO_4 and the GAG content was assayed by a spectrophotometric technique that depends upon the formation of Alcian Blue–GAG complexes [21].

Serum ACE. Serum ACE activity was determined in venous blood from fourteen untreated rabbits. From the five rabbits receiving BLM (5 mg/kg) s.c. and the two rabbits receiving a phosphate-buffered 0.9% NaCl solution three times weekly, serum samples were obtained just prior to BLM or vehicle injections once or twice weekly. Thus, serum samples were withdrawn 2 or 3 days after the previous BLM treatment. The serum ACE activity of each sample was determined three or more times using a radiochemical assay based upon the formation of [glycine-1-^{14}C]hippuric acid from [glycine-1-^{14}C]hippuryl-L-histidyl-L-leucine [22] and did not vary more than 15 per cent. This technique has been shown to be more sensitive and less variable than spectrophotometric methods for determining serum ACE activity, since serum lipids and the extraction solvent do not interfere with the quantification of hippuric acid. The serum and pulmonary ACE activities of control and BLM-treated rabbits were also measured in the presence of a 0.2 μM concentration of the specific ACE inhibitor, 1-(D-3-mercapto-2-methyl-3-oxopropyl)-L-proline (SQ 14,225) [23] to ensure that other peptidase activites were not being assayed. The SQ 14,225 was supplied by Dr. Z. P. Horovitz of the Squibb Institute for Medical Research (Princeton, NJ).

Electron and light microscopy. Lung tissue from four control and four BLM-treated rabbits was prepared by the one-step fixation method of Simionescu and Simionescu [24]. Briefly, samples from the sub-

pleural region were removed, washed with cold 0.1 M cacodylate buffer (pH 7.4) and immersed in a 0.1 M cacodylate, 3 mM $CaCl_2$ solution (pH 7.4) containing 3 vol. of 5% paraformaldehyde and 3% gluteraldehyde, 2 vol. of 2% OsO_4 and 1 vol. of saturated, filtered lead citrate. The samples were fixed on ice for 60 min, stained with 1% tannic acid in a 0.1 M cacodylate solution (pH 6.8 to 7.0) for 20–30 min, rinsed with a 1% Na_2SO_4 and 0.1 M cacodylate solution, and then dehydrated with increasing concentrations of ethanol; the samples in 100% ethanol were washed twice for 20 min in propylene oxide, were allowed to stand overnight at room temperature in 50% propylene oxide:50% Epon (v/v), and were embedded in Epon for 24 hr at 60°. The embedded blocks were sectioned on a Bausch and Lomb automatic microtome equipped with a diamond knife. Silver sections were placed on Formvar and carbon-coated 100 mesh copper grids. The grids were then stained with lead citrate and examined using a Zeiss EM-9S-2 electron microscope. For light microscopic analysis, the embedded blocks were sectioned (1 μm), mounted on glass slides, and stained with toluidine blue. All chemicals were obtained from Ladd Research Industries, Inc. (Burlington, VT).

Statistical analyses. The significance of the differences between mean values was calculated with the aid of Student's *t*-test for unpaired variables; $P < 0.05$ was considered statistically significant.

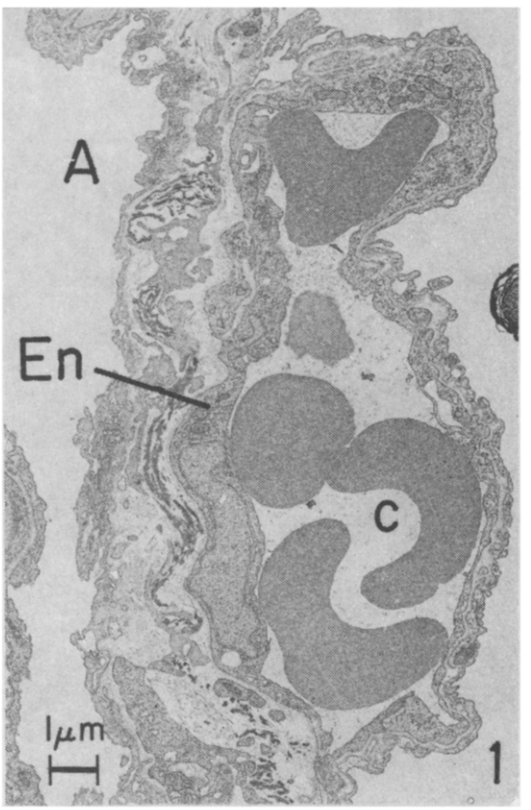

Fig. 1. Electron micrograph of the alveolar region of the lung from a control rabbit. Key: alveolus (A); capillary (C); and endothelial cell (En).

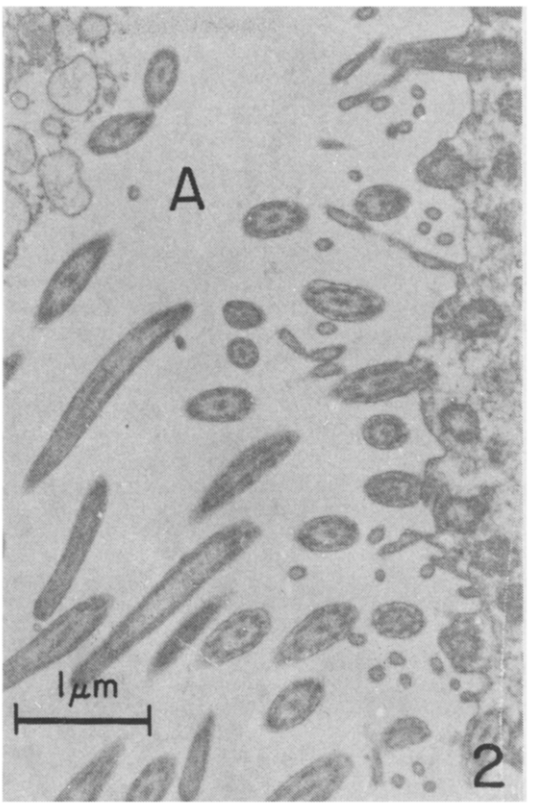

Fig. 2. Ciliated alveolar epithelial cell in the lung of a rabbit treated for 28 days three times weekly with 5 mg/kg of BLM. Alveolus (A).

RESULTS

Morphological damage. The most prominent morphological damage observed 30 days after repeated s.c. BLM injections was the appearance of ciliated alveolar epithelial cells (Figs. 1 and 2) similar to those reported to occur in the lungs of mice and humans exposed to BLM [1, 2, 6, 7]. An increase in the size and frequency of alveolar Type II epithelial cells was also noted along with extensive intraalveolar debris, the presence of alveolar macrophages, and alveolar Type I epithelial cell damage. The regions of damage frequently were focal, with the transition from normal-appearing lung tissue to damaged areas often being abrupt. In some sections, marked endothelial blebs were observed (Fig. 3), although endothelial detachment from the basement membrane was not seen in other areas examined. No evidence of an increase in collagen content was detected electron microscopically.

Pulmonary biochemistry. The mean initial body weight of the BLM-treated rabbits was not significantly different from that of the control group [2.51 ± 0.27 kg (S.E.) vs 3.14 ± 0.27 kg respectively]; after 4 weeks of treatment, however, the mean body weight of the BLM group was significantly less ($P < 0.01$) than that of the control group (1.92 ± 0.16 kg vs 3.14 ± 0.27 kg respectively). No significant differences in pulmonary wet weight and in total lung GAG, protein or DNA content were

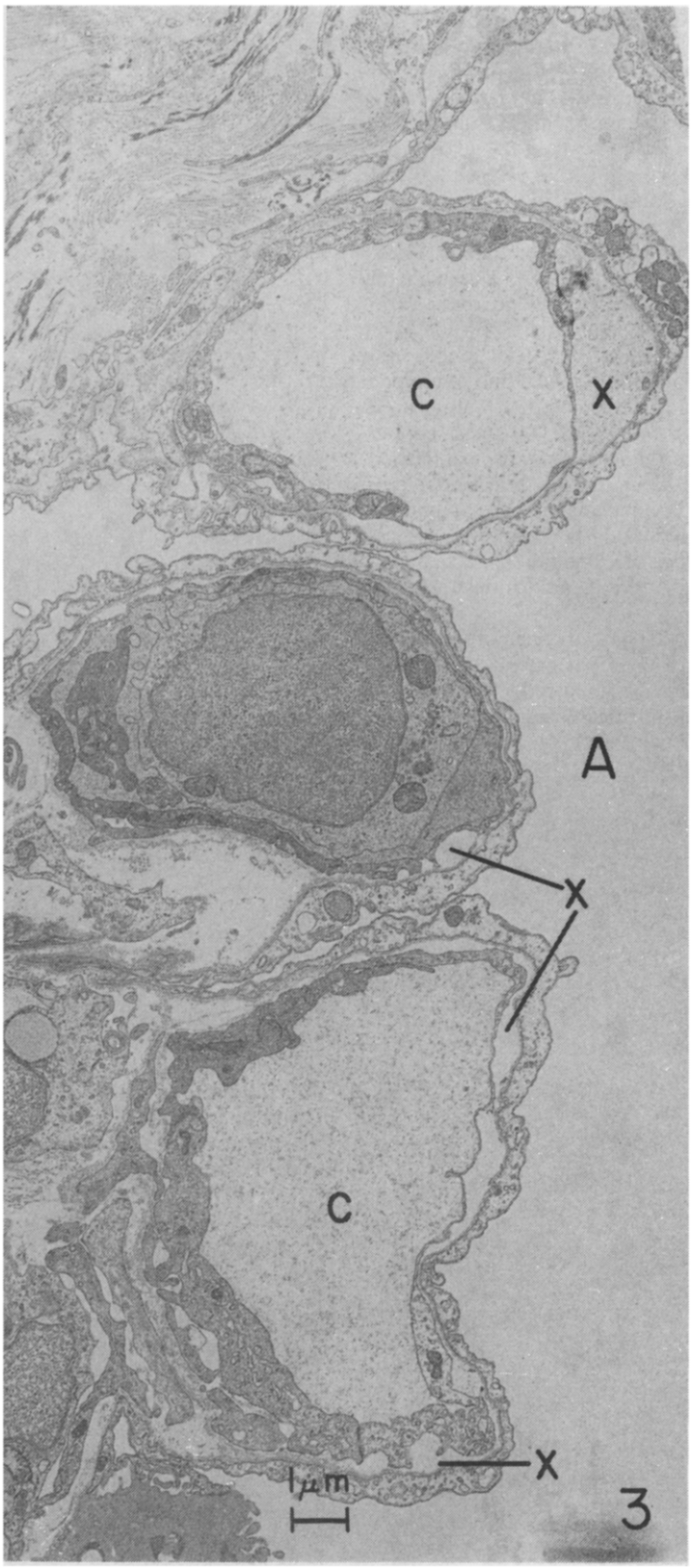

Fig. 3. Capillaries in the lungs of rabbits treated for 28 days three times weekly with 5 mg/kg of BLM. Several examples of endothelial detachment from the basement membrane or bleb formation (X) are seen. Key: capillary (C); and alveolus (A).

Table 1. Rabbit pulmonary biochemistry*

Indices	Control (N = 4)	Bleomycin (N = 5)
Wet weight of lungs (g)	9.64 ± 0.51	10.54 ± 0.65
Dry/wet weight	0.193 ± 0.010	0.170 ± 0.010
(Wet − dry)/dry weight	4.24 ± 0.29	4.96 ± 0.42
DNA (mg/lungs)	65.58 ± 7.04	59.74 ± 9.22
Protein (mg/lungs)	1058 ± 123	854 ± 89
OH-Pro (mg/lungs)	19.64 ± 2.49	19.64 ± 2.10
GAG (mg/lungs)	25.40 ± 6.69	21.17 ± 4.25†

* Rabbits were injected with BLM (5 mg/kg, s.c.) three times weekly for 28 days. The total pulmonary DNA, protein, OH-Pro and GAG were determined in both supernatant and 1000 *g* pellet fractions. Total pulmonary values were obtained by extrapolation and are based on total weight of both lungs. Values are means ± S.E.

† Lungs from three rabbits were measured.

Table 2. Pulmonary ACE activity in rabbits after BLM treatment*

Lung ACE	Control (N = 4)	Bleomycin (N = 5)
μmoles·min^{-1}·lungs^{-1}	34.73 ± 4.31	21.40 ± 1.74†
μmoles·min^{-1}·(g wet lungs)$^{-1}$	3.60 ± 0.40	2.10 ± 0.30†
μmoles·min^{-1}·(g dry lungs)$^{-1}$	18.77 ± 2.17	12.19 ± 1.18‡
nmoles·min^{-1}·(mg protein)$^{-1}$	33.58 ± 4.64	26.44 ± 4.10
nmoles·min^{-1}·(mg DNA)$^{-1}$	548.5 ± 89.4	393.2 ± 64.5

* Rabbits were injected s.c. with 5 mg/kg of BLM three times weekly for 28 days. Total ACE activity (μmoles of hippuric acid formed/min) from both the supernatant and pellet fractions was determined in triplicate from each animal. Statistical analyses were performed with the aid of Student's *t*-test. Values are means ± S.E.

† $P < 0.02$, compared to untreated controls.

‡ $P < 0.05$, compared to untreated controls.

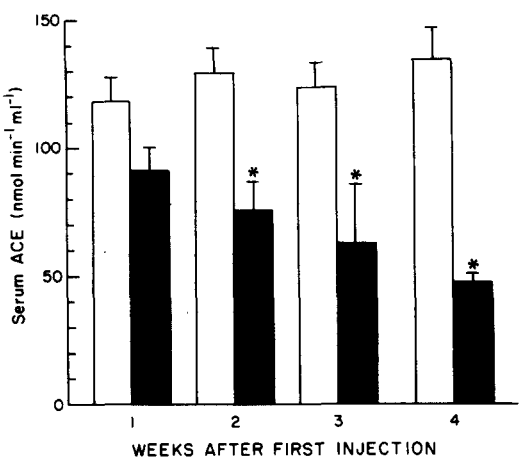

Fig. 4. Serum ACE activity of rabbits after treatment with BLM. Control rabbits (two) were injected with phosphate-buffered 0.9% NaCl solution and treated rabbits (five) with 5 mg/kg of BLM three times weekly. Serum samples were obtained once or twice each week from each animal and were assayed at least three times. The mean value of all animals is indicated. Control (open columns); BLM-treated (black columns). Bars; S.E. An asterisk (*) indicates $P < 0.05$, compared to control animal values.

detected (Table 1). There was no evidence of edema after BLM treatment since the dry/wet lung weights and the (wet − dry)/dry lung weights for both control and BLM-treated rabbits were not statistically different. No difference in the OH-Pro content in the lungs of rabbits compared to that in the lungs of control rabbits was observed spectrophotometrically (Table 1). When OH-Pro was measured by amino acid analysis, slightly lower amounts of OH-Pro were noted but again no difference in the pulmonary OH-Pro content in control and treated rabbits was detected [control 15.9 ± 3.78 (S.E.) mg/lungs; BLM-treated 14.06 ± 2.11 mg OH-Pro/lungs]. In contrast, lung ACE activity was reduced significantly after the treatment period (Table 2). We observed a decrease in total pulmonary ACE activity ranging from 35 to 40 per cent whether the data were normalized per lungs or per g wet weight or per g dry weight. Although the mean ACE activity per mg protein or per mg DNA was decreased in BLM-treated animals, it was not statistically different from the values obtained from control rabbits.

Serum ACE. The serum ACE activities were monitored throughout the treatment period in five BLM-treated rabbits. Two weeks after the initiation of drug administration, serum ACE activity was

Table 3. Inhibition of ACE by SQ 14,225*

	SQ 14,225	Serum ACE ($nmoles \cdot min^{-1} \cdot ml^{-1}$)	Lung ACE ($\mu moles \cdot min^{-1} \cdot lungs^{-1}$)
Control	−	162.7 ± 25.8 (3)	34.78 ± 4.45 (4)
	+	18.3 ± 1.6 (3)	9.10 ± 1.84 (4)
Bleomycin	−	47.2 ± 3.9 (3)	19.22 ± 0.94 (5)
	+	11.5 ± 1.6 (3)	6.84 ± 0.59 (5)

* Rabbits were treated s.c. with 5 mg/kg of BLM three times weekly for 28 days. Two or three days after the last BLM injection, the pulmonary and serum ACE activities were determined in the BLM-treated and untreated rabbits. The number of animals is indicated in parentheses. The concentration of SQ 14,225 [1-(D-3-mercapto-2-methyl-3-oxopropyl)-L-proline] was 0.2 μM. Values are means ± S.E.

reduced by 42 per cent from control values; further treatment resulted in a 48 and 65 per cent decrease in serum ACE activity, compared with control animals, 3 and 4 weeks, respectively, after the first BLM injection (Fig. 4). The serum ACE activity 4 weeks after drug treatment was 50 per cent lower than that seen in the same animals during the first week of BLM administration. The serum ACE activity of the vehicle-injected animals remained essentially constant throughout the treatment period and was not significantly different from that of fourteen untreated control rabbits [131.9 ± 32.9 (S.E.) $nmoles \cdot min^{-1} \cdot ml^{-1}$]. Although the serum protein concentration in BLM-treated rabbits was 25 per cent lower than that seen in the control animals (47.2 ± 3.65 mg/ml vs 63.7 ± 5.20 mg/ml respectively), the reduction was not statistically significant and the activity of serum ACE expressed per mg protein in BLM-treated rabbits was reduced by more than 60 per cent [2.42 ± 0.18 vs 0.94 ± 0.14 $nmoles \cdot min^{-1} \cdot (mg\ protein)^{-1}$; $P < 0.01$]. Thus, the reduction in serum ACE cannot be explained by a non-specific reduction in serum protein. Moreover, when sera from control and BLM-treated rabbits were mixed, the ACE activity assayed produced additive results. Similarly, extensive dialysis of sera from BLM-treated rabbits did not alter ACE activity. These data suggest that the reduced enzyme activity observed after BLM treatment was not due to the presence of a low molecular weight inhibitor of the enzyme.

To further characterize the reduction in ACE activity by BLM, we examined the effects of a specific ACE inhibitor, SQ 14,225 [23], on both serum and pulmonary ACE activities. As seen in Table 3, inclusion of 0.2 μM SQ 14,225 in the reaction mixture containing serum from control animals decreased serum ACE activity from 162.7 to 18.3 $nmoles \cdot min^{-1} \cdot ml^{-1}$. Similarly, serum ACE activity in BLM-treated rabbits was reduced from 47.2 to 11.5 $nmoles \cdot min^{-1} \cdot ml^{-1}$ by SQ 14,225. The pulmonary ACE activity in both control and BLM-treated rabbit lungs was reduced to less than 10 $\mu moles \cdot min^{-1} \cdot lungs^{-1}$ by 0.2 μM SQ 14,225.

DISCUSSION

An apparent increase in collagen is a prominent feature of the histopathology of pulmonary fibrosis, although in diffuse interstitial fibrosis a good correlation between concentration of lung collagen and lung histology or physiology is not always observed [5, 25]. The development of interstitial pulmonary fibrosis has been observed morphologically as the final toxic event in man and a variety of animals after subacute or chronic treatment with BLM [1–5]. By determining the OH-Pro content in the lungs of mice after repeated BLM administration, Sikic *et al.* [15] were able to quantify the pulmonary collagen content and, thus, the development of pulmonary fibrosis. McCullough *et al.* [5] found that OH-Pro content in the lungs of baboons treated chronically with BLM was elevated 6 months after the last drug injection. In the present study, subcutaneous administration of 5 mg/kg to rabbits three times weekly for 28 days did not result in an apparent increase in collagen, based both on electron microscopic observations and direct determination of OH-Pro content. The failure to see alterations in collagen content in rabbit lungs, however, does not imply that marked alterations in collagen synthesis or composition did not occur or that pulmonary fibrosis would not have developed at a later time. Other investigators [15, 25] have found that mice and rats require BLM administration for more than 4 weeks before significant increases in pulmonary OH-Pro content can be detected. Furthermore, Phan *et al.* [26] have reported that increases in the net rates of collagen synthesis occur several weeks prior to detectable elevation in pulmonary OH-Pro content in rats after intratracheal administration of BLM. Since fibrosis may involve destruction of Type I collagen with simultaneous deposition of Type III collagen [25], it is possible that alterations in the composition of pulmonary collagen may have been initiated at the time of our observations.

The interstitium of the pulmonary alveoli contains not only collagen but also a poorly defined amorphous ground substance. GAG comprise a group of macromolecules that are constituents of the pulmonary ground substance and that may play a fundamental role in the maintenance of lung structure, determination of lung mechanical properties, and interchange of nutrients and metabolites between the capillary and epithelial cells [27]. GAG are known to interact with collagen *in vitro* and may be important in collagen fibrillogenesis and insolubility [28]. Furthermore, BLM has been reported to increase pulmonary GAG content in the lungs of dogs at the time of fibrosis [4] and to stimulate the synthesis of GAG in cultured fibroblasts [29]. In the BLM-treated rabbits studied here, however, no increase in pulmonary GAG content was detected

28 days after BLM treatment. These data, in addition to the microscopic and OH-Pro results, suggest that significant fibrosis did not exist after 28 days of BLM treatment. In spite of the unaltered OH-Pro and GAG content, it is obvious from the electron micrographs (Figs. 2 and 3) that BLM administration to rabbits did result in pulmonary damage as manifested by the appearance of ciliated alveolar epithelial cells and by an increase in the number and size of Type II alveolar cells. These changes are similar to the prefibrotic morphological damage produced by BLM in other species [1–5]. It should be noted, however, that our electron microscopic studies were restricted to the subpleural region of the lungs and our biochemical determinations were done on only portions of the lungs; previous results [2, 8, 15, 25] have indicated that the initial pulmonary lesions associated with BLM administration in other species are focal and frequently subpleural. Thus, our evaluation of the morphological and biochemical damage may not represent the status of other regions in the lungs.

Although other investigators have reported similar epithelial morphological alterations in different species after BLM administration, some controversy exists over the action of BLM on pulmonary endothelium. Based on autoradiographic studies, BLM localizes in the pulmonary endothelium soon after injection [7]. Later, BLM-induced lesions in pulmonary arterial, capillary and venule endothelia, consisting of edema and bleb formation, can be observed electron microscopically [2]. Other investigators [8], studying mouse lungs at comparable times after BLM treatments, have not detected endothelial damage. It may be possible to determine the extent to which the pulmonary endothelium is damaged in some species by measuring the organ ACE activity. ACE is a glycoprotein associated with the luminal plasma membrane of lung capillary endothelial cells [10]. Reduction in pulmonary ACE has been reported after the acute systemic administration of some pulmonary toxins such as paraquat [30] and thiourea [31], but not after others, such as monocrotaline [32]. The variation in results with each agent may reflect (a) the individual sensitivity of the endothelial cells to the different toxins, (b) the ability of the endothelium to repair damage, or (c) the failure to measure total pulmonary ACE activity. In our study, by including 1-*O*-*n*-octyl-β-D-glucopyranoside, we have solubilized and measured the total ACE activity [15] and have demonstrated a 35 per cent decrease in pulmonary ACE activity after BLM treatment (Table 2).

In our studies, electron microscopic evidence of endothelial damage was observed in some sections (Fig. 3), but it was not a uniform finding. This may be due to the focal nature of lesions produced by BLM [2, 8, 15, 25]. The marked decrease in total lung ACE activity found after BLM treatment (Table 2) does, however, suggest the possibility of extensive endothelial damage. Although the pulmonary ACE activity was decreased when expressed per mg protein or per mg DNA, this difference was not statistically significant. This may be due to dynamic changes in the cell types residing in the lung after BLM treatment or to variability in protein or DNA determinations. Witschi [33] has suggested that the most satisfactory method to express the effects of pulmonary toxins on lung biochemistry is on a "per lung basis". Because the magnitude of the pulmonary ACE loss (13,350 nmoles · min^{-1} · $lungs^{-1}$) is so great, it is clear that the decrease cannot be explained completely either by an alteration in the pulmonary blood content or the change in blood ACE activity. Tom and Montgomery [25] have reported a decrease in rat pulmonary ACE after BLM administration and, in a preliminary report, Vats *et al.* [34] have observed a similar reduction in mouse lungs although Lazo [35] detected an increase in pulmonary and serum ACE activities in mice after subacute BLM administration. Newman *et al.* [36] have observed a decrease in lung ACE activity after a single intratrachel instillation of BLM in rats. Moreover, further *in vivo* studies with BLM-treated rabbits [14] indicated a marked reduction in the ability of the pulmonary endothelium to extract radiolabeled 5-hydroxytryptamine and norepinephrine, a proposed index of pulmonary endothelial integrity [37]. Thus, it appears that BLM can disrupt the biochemical functions of pulmonary endothelium prior to diffuse and widespread electron microscopic evidence of endothelial damage.

Although the origin of serum ACE is currently uncertain, it may originate by shedding from the vascular endothelium of the lung [12]. Hollinger *et al.* [31] found that, within 1 hr after treatment of rats with thiourea, a transient increase in serum ACE activity occurred concurrently with a decrease in lung ACE activity. Similar results have also been seen with paraquat [38]. Hollinger *et al.* [31, 38] have suggested that the elevation in serum ACE activity may represent the lost lung enzyme. In contrast, we observed a decrease in both pulmonary and serum ACE activities. In our study, however, the animals were treated for 28 days and we examined a subacute rather than an acute situation. Thus, the decrease in serum ACE seen in the BLM-treated rabbits may reflect the reduction in total pulmonary ACE. A recent study in mice [34] found that a decrease in serum and pulmonary ACE also occurs 9 weeks after BLM administration.

Clinically, the onset and the severity of BLM-mediated pulmonary damage are difficult to determine and are currently estimated by non-specific radiographic findings and pulmonary function tests which are often unreliable [39]. Monitoring serum ACE may be a useful tool for assessing the development of pulmonary toxicity to BLM: it is relatively easy to assay, requiring only small amounts (10 μl) of serum. Low serum ACE activity has also been observed in patients with pulmonary cancer and in a few individuals with non-Hodgkin lymphoma [40]. Grönhagen-Riska [40] has suggested that the low levels of serum ACE activity in patients with cancer may be due to the damage to the endothelium produced by chemotherapy or irradiation. Unfortunately, in all of the studies to date the type of antineoplastic agents, the irradiation schedule used, or the time after therapy when serum ACE levels were determined was not stated. Thus, it is possible that a number of antineoplastic agents, and irradiation therapy, may produce significant alterations in serum ACE activity when used clinically. Our results suggest that at least one agent, BLM, can lead to a marked reduction in serum and pulmonary ACE

activity in an animal model. Thus, serial monitoring of serum ACE activity in patients being treated with BLM may be useful in identifying impending pulmonary toxicity, perhaps at a time when the damage is reversible.

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REFERENCES

1. R. H. Blum, S. K. Carter and K. Agre, *Cancer, N.Y.* **31**, 903 (1973).
2. I. Y. R. Adamson and D. H. Bowden, *Am. J. Path.* **77**, 185 (1974).
3. C. W. M. Bedrossian, D. Greenberg, D. H. Yawn and R. M. O'Neal, *Archs Path. Lab. Med.* **101**, 248 (1977).
4. R. W. Fleischman, J. R. Baker, G. R. Thompson, U. H. Schaeppi, V. R. Iliveski, D. A. Cooney and R. D. Davis, *Thorax* **26**, 675 (1971).
5. B. McCullough, J. F. Collins, W. G. Johanson, Jr. and F. L. Grover, *J. clin. Invest.* **61**, 79 (1978).
6. Y. Aso, K. Yoneda and Y. Kikkawa, *Lab. Invest.* **35**, 568 (1976).
7. I. Y. R. Adamson and D. H. Bowden, *Am. J. Path.* **96**, 531 (1979).
8. A. W. Jones and N. L. Reeve, *J. Path.* **124**, 227 (1978).
9. C. N. Gillis, in *Vascular Neuroeffector Mechanism* (Eds. J. A. Bevan, T. Godfraind, R. A. Maxwell and P. M. Vahouette), p. 304. Raven Press, New York (1980).
10. J. W. Ryan and U. S. Ryan, *Fedn Proc.* **36**, 2683 (1977).
11. D. E. Cushman and H. S. Cheung, *Biochim. biophys. Acta* **250**, 261 (1971).
12. M. Das, J. L. Hartley and R. L. Soffer, *J. biol. Chem.* **252**, 1316 (1977).
13. C. A. Dinarello, S. B. Ward and S. M. Wolff, *Cancer Chemotherapy Rep.* (Part 1) **57**, 393 (1973).
14. J. D. Catravas, J. S. Lazo and C. N. Gillis, *J. Pharmac. exp. Ther.*, **217**, 524 (1981).
15. B. I. Sikic, D. M. Young, E. G. Mimnaugh and T. E. Gram, *Cancer Res.* **38**, 787 (1978).
16. J. S. Lazo and D. E. Quinn, *Analyt. Biochem.* **102**, 68 (1980).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
18. K. Burton, *Biochem. J.* **62**, 315 (1956).
19. J. F. Woessner, *Archs Biochem. Biophys.* **93**, 440 (1961).
20. M. Sobue, J. Takeuchi, K. Ito, K. Kimata and S. Suzuki, *J. biol. Chem.* **253**, 6190 (1978).
21. P. Whitman, *Biochem. J.* **131**, 343 (1973).
22. M. S. Rohrbach, *Analyt. Biochem.* **84**, 272 (1978).
23. M. A. Ondetti, B. Rubin and D. W. Cushman, *Science* **196**, 441 (1977).
24. N. Simionescu and M. Simionescu, *J. Cell Biol.* **70**, 608 (1976).
25. W. F. Tom and M. R. Montgomery, *Toxic. appl. Pharmac.* **53**, 64 (1980).
26. S. H. Phan, R. S. Thrall and P. A. Ward, *Am. Rev. resp. Dis.* **121**, 501 (1980).
27. A. L. Horwitz and R. G. Crystal, *J. clin. Invest.* **56**, 1312 (1975).
28. B. Obrink, *Eur. J. Biochem.* **34**, 129 (1973).
29. K. Otsuka, S. I. Murota and Y. Mori, *Biochim. biophys. Acta* **444**, 359 (1976).
30. R. A. Roth, K. B. Wallace, R. H. Alper and M. D. Baile, *Biochem. Pharmac.* **28**, 2349 (1979).
31. M. A. Hollinger, S. N. Giri, S. Patwell, J. E. Zuckerman, A. Gorin and G. Parsons, *Am. Rev. resp. Dis.* **121**, 373 (1980).
32. R. Huxtable, D. Ciaramitaro and D. Eisenstein, *Molec. Pharmac.* **14**, 1189 (1978).
33. H. Witschi, *Essays Toxic.* **6**, 125 (1975).
34. T. S. Vats, A. Molteni, L. Mattioli, D. Sobonya and R. Barth, *Fedn Proc.* **38**, 964 (1979).
35. J. S. Lazo, *Toxic. appl. Pharmac.*, **59**, 395 (1981).
36. R. A. Newman, P. J. Kimberly, J. A. Stewart and J. Kelley, *Cancer Res.* **40**, 3621 (1980).
37. J. D. Catravas and C. N. Gillis, *J. Pharmac. exp. Ther.* **213**, 120 (1980).
38. M. A. Hollinger, S. W. Patwell, J. E. Zuckerman, A. B. Gorin, G. Parsons and S. N. Giri, *Am. Rev. resp. Dis.* **121**, 795 (1980).
39. B. M. Lewis and R. Izbicki, *J. Am. med. Ass.* **243**, 347 (1980).
40. C. Grönhagen-Riska, *Scand. J. resp. Dis.* **60**, 83 (1979).