Biochemical Pharmacology, Vol. 37, No. 6, pp. 1111–1116, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
Pergamon Press plc

ALTERATIONS IN PULMONARY PROTECTIVE ENZYMES FOLLOWING SYSTEMIC BLEOMYCIN TREATMENT IN MICE*

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(*Received* 19 *June* 1987; *accepted* 11 *August* 1987)

Abstract—Repeated bleomycin administration in animals and humans produces significant lung fibrosis. The pathogenesis of this toxicity may be multifactorial, but it appears to be initiated through the production of radical oxygen species by an activated bleomycin–iron–oxygen ternary complex. Protection of lung tissue from bleomycin-induced toxicity may occur through both specific metabolic inactivation of bleomycin by the enzyme bleomycin hydrolase, as well as by such non-specific antioxidants as catalase and the glutathione system. The effect of chronic, systemic administration of bleomycin on the activities and levels of these enzymes and proteins in pulmonary tissue is unknown. C57BL/6 mice were injected subcutaneously with saline, non-fibrogenic (2 mg/kg) and fibrogenic (10 mg/kg) doses of bleomycin twice-weekly for 6 weeks. Animals were killed at 0, 1.5, 3, and 6 weeks after initiation of bleomycin treatment. Catalase activity was increased more than 50% at 3 weeks in the low-dose animals, and was decreased over 40% at 6 weeks in the high-dose animals. Total lung glutathione levels were unaffected in both groups, although glutathione reductase activity was increased significantly (over 2-fold) at 1.5 and 3 weeks in the high-dose animals. At 6 weeks glutathione reductase was increased 7- and 12-fold in low and high-dose animals respectively. Glutathione peroxidase activity also was elevated more than 2-fold above control values at 6 weeks in both sets of animals. There was no evidence of induction of bleomycin hydrolase activity at any time point. Rather, bleomycin hydrolase activity was decreased significantly to 30 and 40% of control values at 3 and 6 weeks, respectively, in mice receiving the fibrogenic doses of bleomycin. These results demonstrate that chronic, systemic administration of non-fibrogenic and fibrogenic doses of bleomycin produces changes in activity of lung antioxidant defense mechanisms. The early loss of lung bleomycin hydrolase activity may contribute to the pathogenesis of bleomycin-induced pulmonary toxicity following repeated drug exposure.

Bleomycin (BLM) interacts with oxygen and Fe(II) to form an "activated" ternary BLM complex, which is thought to produce both tumor destruction and lung fibrosis [1]. Oxygen species with radical characteristics are generated that cause both DNA strand scission and lipid peroxidation [2]. Although unresolved questions exist concerning the chemical nature and diffusability of the radical species generated by BLM, antioxidants such as catalase (CAT), superoxide dismutase (SOD), and those generated by the glutathione (GSH) system may afford at least partial protection from BLM-induced toxicity. Thus, these substances may detoxify several of the putative radicals produced by BLM in the lungs. Additionally, specific protection from BLM toxicity may also occur through the action of BLM hydrolase (BH), which inactivates BLM [3]. The enzyme product, deamido BLM (dBLM), is approximately 1% as efficient as the parent BLM compound in producing oxygen radicals, DNA strand scission, lung fibrosis, or weight loss in an animal model [4].

Exposure of animals to conditions that generate extensive oxygen radicals (e.g. 85 to 100% O_2) induce antioxidant substances [5]. The effects of BLM on these pulmonary defense systems are less clear. Previous studies have suggested that BLM can modify catalase and glutathione shuttle enzyme activity when injected directly into the lungs via the trachea [6]. This route of administration, however, bypasses the potentially important pulmonary endothelial protective barrier, causes significant peribronchial damage and inflammation, and may produce a pathologic lesion that is histologically distinguishable from that seen following systemic BLM therapy [7]. Furthermore, a number of antineoplastic agents that do not cause pulmonary fibrosis when given systemically produce pulmonary fibrosis with intratracheal administration [8].

Therefore, we examined whether repeated, systemic administration of BLM alters the activities and amounts of pulmonary antioxidant defense systems at varying intervals during low- and high-dose BLM administration. The higher 10 mg/kg BLM dose results in a gradual increase in lung collagen, as measured by hydroxyproline, with significant

* Supported by National Institutes of Health Grants (CA-01012; CA-25883), American Cancer Society Grant CH-316, and a grant from the Parker B. Francis Foundation.

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accumulations seen by week 6 of BLM administration [9]. BLM doses approximating the lower 2 mg/kg dose have not been found to result in significant lung fibrosis at similar time points [9, 10]. It is known that BLM may directly induce such enzymes as $(Na^+–K^+)$ATPase [11] and prolyl hydroxylase [12], or directly inhibit other enzymes including DNA polymerase [13], tyrosine hydroxylase [14], and dopamine β-hydroxylase [15]. Since the effect of chronic BLM administration on the protective enzyme BH is unknown, we also evaluated the effect of repeated systemic BLM treatment on pulmonary BH activity. Both the low and high doses of BLM were studied for potential induction or inhibition of BH activity.

METHODS

Animals. Female C57BL/6 mice were obtained from the NIH animal facility (Frederick, MD). Animals were maintained in the specific pathogen-free animal care facility at the Yale University School of Medicine and fed *ad lib*. At the start of experiments, mice were 8–10 weeks of age and weighed between 20 and 23 g.

Treatment protocol. Animals were injected with either saline (100 μl), low-dose BLM (2 mg/kg), or high-dose BLM (10 mg/kg) subcutaneously twice-weekly for 6 weeks. Clinical-grade Blenoxane (Bristol Myers Co., Syracuse, NY) dissolved in normal saline was used for injections. A total volume of 100 μl was injected subcutaneously in the abdominal region of each animal. Five animals from each group were killed at 0, 1.5, 3, and 6 weeks. Lung samples were placed in 1.0 ml of 0.1 M sodium phosphate buffer (pH 7.2) and homogenized (Ultra-Turrax Tissumizer, Tekmar, Cincinnati, OH) prior to enzyme assays. Half of the lung homogenate was utilized for BH measurement, and the remaining half was used for CAT, total GSH, glutathione peroxidase (GPO), and glutathione reductase (GR) assays.

Bleomycin hydrolase. Lung homogenates were centrifuged at 20,800 *g* for 45 min at 4°. The resulting supernatant fraction was centrifuged at 105,000 *g* for 60 min at 4°. The post-microsomal supernatant fraction, containing greater than 90% of the BH activity [4], was then further purified using CF-25 Centriflo cones (Amicon, Danvers, MA) centrifuged at 1000 *g* for 30 min at 4° to remove low molecular weight material. This procedure was repeated a second time using 0.1 M phosphate-buffered saline as a rinsing solution. Protein concentration in the resulting retentate was determined by the method of Bradford [16], and the enzyme sample was stored at −70° until further use.

BH activity was determined by measuring the rate of formation of the dBLM A_2 metabolite from the parent BLM A_2 compound when reacted with aliquots of lung preparations. BLM A_2, which constitutes 65–70% of the clinical Blenoxane mixture [17], was purified from Blenoxane as previously described [4] and used in the copper-free form in all experiments as a substrate for BH. BLM A_2 and dBLM A_2 were also used as standards for high-pressure liquid chromatography (HPLC) analysis. The dBLM A_2 standard was prepared as described by Lazo and Humphreys [4]. The prepared lung homogenates (0.8 to 1.6 mg protein) were reacted in a final volume of 500 μl with 50 μg of BLM A_2 for 120–360 min at 37°. The reaction was stopped by the addition of 0.5 ml of ice-cold methanol, and the sample was centrifuged at 15,600 *g* for 10 min. Aliquots (0.1 to 0.25 ml) of the sample were then analyzed by reverse-phase HPLC.

BLM A_2 and dBLM A_2 standards (50 μg/ml) for HPLC analysis were suspended in double-distilled water and stored at −20°. Standards and organ samples were converted to the copper form by adding 1/3 to 1/5 volume of 7.5 mM $CuSO_4$ prior to injection on HPLC. Samples were eluted from a Rainin (Woburn, MA) Microsorb C8 column (4.6 mm inside diameter × 250 mm) using a Beckman (Fullerton, CA) model 332 HPLC system with a mobile phase of $CH_3OH:CH_3CN:H_2O:CH_3COOH$ (160:72:760:8) containing 2 mM heptane sulfonic acid and 25 mM triethylamine. The pH of the mobile phase was adjusted to 5.5. Flow rate was 1.0 ml/min. Uncorrected fluorescence spectra of copper-complexed BLM A_2 and dBLM A_2 were recorded at 25° with a Perkin–Elmer (Norwalk, CT) 650-10S flow-fluorescence spectrophotometer and a Shimazdu (Kyoto, Japan) C-RIA integrator. Fluorescence was monitored with an excitation wavelength of 297 nm (10 nm band pass) and an emission wavelength of 355 nm (10 nm band pass).

BH activity in lung samples was determined by measuring the rate of formation of the dBLM A_2 metabolite from the parent BLM A_2 compound. Areas under peaks were calculated with the Shimadzu C-RIA integrator. Standard curves for BLM A_2 and dBLM A_2 were generated daily. Peak identification was confirmed by coinjection of BLM A_2 and dBLM A_2 standards with the reaction mixtures. For all calculations of the rate of dBLM A_2 formation, incubation times were used that yielded linear product formation with time and caused less than 10% substrate metabolism.

Catalase activity. CAT activity was measured by the method of Feinstein [18], using 1.5% sodium perborate as substrate for CAT. Eight milliliters of the 0.1 M $NaBO_3 \cdot 4H_2O$ solution (pH 6.8) and 1.5 ml of 67 mM phosphate buffer (pH 6.8) were added to a series of 125-ml Erlenmeyer flasks. The flasks were allowed to equilibrate at 37° for 15 min. After equilibration, 0.5 ml of distilled water was added to the first flask, and 100 μl of the 105,000 *g* supernatant fraction from the lung homogenate was added to the other flasks. The reaction was stopped after exactly 5 min by the addition of 10 ml of 2 M H_2SO_4. Each flask was then tritrated at room temperature with 50 mM $KMnO_4$. The volume required to tritrate each sample was subtracted from the H_2O blank. The amount of catalase in each sample was determined by comparison with a standard curve generated with a solution of pure catalase (2500 units/mg solid, 1 unit will decompose 1.0 μmol of H_2O_2 per min at pH 7.0 at 25°; Sigma, St. Louis, MO).

Total glutathione. Total lung GSH content was measured by the method of Griffith [19]. All solutions contained 125 mM phosphate buffer and 6.3 mM EDTA, and were adjusted to pH 7.5. The

following solutions were added to a cuvette: 700 μl of 0.3 mM NADPH and 100 μl of 6 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB). The 105,000 *g* supernatant fraction was diluted 1:10 with 0.1 M phosphate buffer (pH 7.2). This diluted supernatant (100 μl) was then added to a cuvette, and the reaction was initiated by addition of 10 μl glutathione reductase (50 units/ml, 1 unit will reduce 1.0 μmol of oxidized glutathione per min at pH 7.6 at 25°; Sigma). The linear increase in absorbance at 412 nm was monitored continuously over 10 min, and total GSH was measured by comparison to a standard curve generated by known amounts of GSH (0.6 to 2.6 μg; Sigma). GSH concentration was determined by measurement of the slope of the increase in absorbance.

Glutathione peroxidase. GPO activity was measured by the method of Paglia and Valentine [20]. The following solutions were added to a cuvette: 2.58 ml of 50 mM phosphate buffer (pH 7), 100 μl of 8.4 mM NADPH, 10 μl of glutathione reductase (100 units/ml), 10 μl of 1.125 M NaN_3, and 100 μl of 0.15 M reduced GSH. To each cuvette, 100 μl of the 105,000 *g* supernatant fraction was then added. The reaction was initiated by addition of 100 μl of 2.2 mM H_2O_2. The conversion of NADPH to $NADP^+$ was followed by continuous monitoring of the linear decrease in absorbance at 340 nm. GPO activity was calculated by comparison of slopes to a standard curve generated with a solution of pure GPO (100 units/mg, 1 unit will catalyze the oxidation by H_2O_2 of 1.0 μmol of reduced glutathione to oxidized glutathione per min at pH 7.0 at 25°; Sigma).

Glutathione reductase. GR was measured by the method of Goldberg and Spooner [21]. The following solutions were added to a cuvette in order: 2.60 ml of 0.12 M phosphate buffer (pH 7.2), 100 μl of 15 mM EDTA, 100 μl of 65.3 mM oxidized glutathione (GSSG), 100 μl of 105,000 *g* supernatant. The reaction was initiated by the addition of 50 μl of 9.6 mM NADPH in 1% $NaHCO_3$. The decrease in NADPH was monitored by the linear decrease in absorbance at 340 nm. GR was calculated by comparison to a standard curve generated by known amounts of GR (100 units/mg; Sigma).

Statistics. To facilitate comparisons among different biochemical measurements, data were depicted as a percent of control in all figures. At each time point, statistical analysis of the low-dose treatment group versus control and of the high-dose group versus control was done using Student's *t*-test. A $P < 0.05$ was considered to represent a significant difference between groups.

RESULTS

C57BL/6 mice, an inbred murine strain sensitive to BLM-induced pulmonary fibrosis [22], received saline, low-dose, or high-dose BLM subcutaneously twice-weekly over a 6-week period. Lung tissue was then assayed for CAT, GSH, GR, GPO and BH at 0, 1.5, 3, and 6 weeks. Five animals were analyzed per each treatment group at each time point. No deaths occurred, but there was a 12% decrease in body weight in high-dose mice at 6 weeks (data not shown). In addition, there was a 16% increase in lung weight at the 6-week time point in high-dose animals. There were no significant changes in body weight or lung weight in saline or low-dose mice at any of the time points tested.

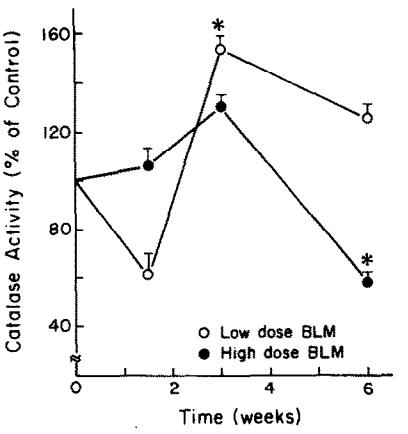

Fig. 1. Pulmonary catalase activity in C57BL/6 mice receiving low-dose (2 mg/kg) or high-dose (10 mg/kg) bleomycin subcutaneously twice-weekly for 6 weeks. Lungs were removed at various times after initiation of bleomycin treatment and were homogenized; the catalase activity was measured in the post-microsomal supernatant fraction. Catalase activity is expressed as a percentage ± SEM of the pulmonary activity found in saline-treated control animals. The pooled average catalase activity for all control mice was 492 units/lungs. A total of five animals per treatment was examined in each group at each time point. Key: (*) $P < 0.05$ vs control.

No significant changes in CAT activity were seen at 1.5 weeks in either the low-dose or high-dose mice (Fig. 1). CAT activity, however, was increased over

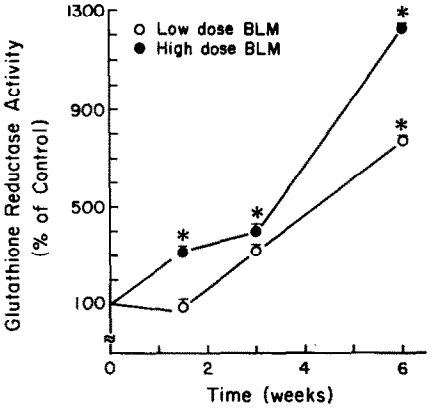

Fig. 2. Pulmonary glutathione reductase activity in C57BL/6 mice receiving low-dose (2 mg/kg) or high-dose (10 mg/kg) bleomycin subcutaneously twice-weekly for 6 weeks. Lungs were removed at various times after initiation of bleomycin treatment and were homogenized; the glutathione reductase activity was measured in the post-microsomal supernatant fraction. Glutathione reductase activity is expressed as a percentage ± SEM of the pulmonary activity found in saline-treated control animals. The pooled average glutathione reductase activity for all control mice was 0.1 unit/lungs. A total of five animals was examined in each group at each time point. Key: (*) $P < 0.05$ vs control.

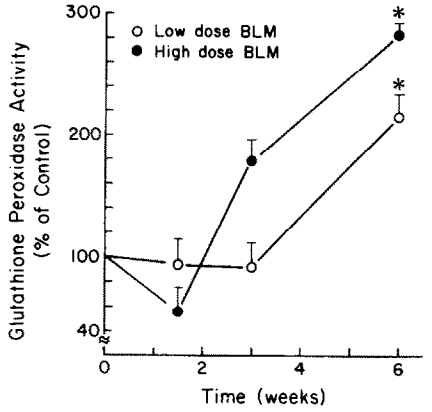

Fig. 3. Pulmonary glutathione peroxidase activity in C57BL/6 mice receiving low-dose (2 mg/kg) or high-dose (10 mg/kg) bleomycin subcutaneously twice-weekly for 6 weeks. Lungs were removed at various times after initiation of bleomycin treatment and were homogenized; the glutathione peroxidase activity was measured in the post-microsomal supernatant fraction. Glutathione peroxidase activity is expressed as a percentage ± SEM of the pulmonary activity found in saline-treated control animals. The pooled average glutathione peroxidase activity for all control mice was 0.6 unit/lungs. A total of five animals was examined in each group at each time point. Key: (*) $P < 0.05$ vs control.

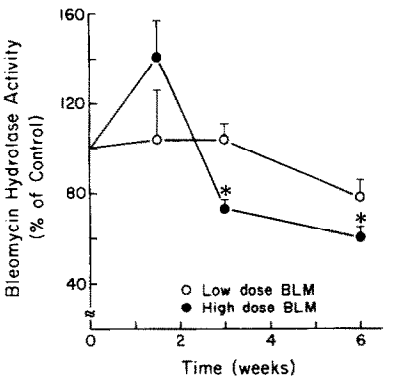

Fig. 5. Pulmonary bleomycin hydrolase activity in C57BL/6 mice receiving low-dose (2 mg/kg) or high-dose (10 mg/kg) bleomycin subcutaneously twice-weekly for 6 weeks. Lungs were removed at various times after initiation of bleomycin treatment and were homogenized; the bleomycin hydrolase activity was measured in the post-microsomal supernatant fraction. Bleomycin hydrolase activity is expressed as a percentage ± SEM of the pulmonary activity found in saline-treated control animals. The pooled average bleomycin hydrolase activity for all control mice was 11.9×10^{-4} μg deamido bleomycin A_2 formed/hr/mg protein. A total of five animals was examined in each group at each time point. Key: (*)$P < 0.05$ vs control.

1.5-fold above saline-treated control in the low-dose animals at 3 weeks and decreased over 1.5-fold from saline-treated control at 6 weeks in high-dose mice.

Marked changes in both GR and GPO were seen in each BLM treatment group. A significant elevation in pulmonary GR activity occurred as early as 1.5 weeks after the initial treatment with the high-dose BLM (Fig. 2), and greater increases were noted at the later time points. GR activity of the high-dose animals compared to controls was 3-fold greater at 1.5 weeks, over 3-fold greater at 3 weeks, and almost 13-fold greater at 6 weeks. GPO activity (Fig. 3) was not changed significantly in either BLM treatment group until the 6-week time point when activity was increased significantly in both low-dose and high-dose groups. Low-dose animals displayed over 2-fold greater activity at this time point, while high-dose mice showed 2.8-fold greater activity. Despite these significant changes in GSH-shuttle enzyme activity, there were no significant changes in total GSH levels in either treatment group at any time point (Fig. 4).

There were no significant changes in pulmonary BH levels in mice receiving non-fibrogenic doses of BLM at any of the time points (Fig. 5). Lung BH activity, however, was decreased 30% at 3 weeks in the high-dose group, and 40% at 6 weeks after initiation of drug treatment.

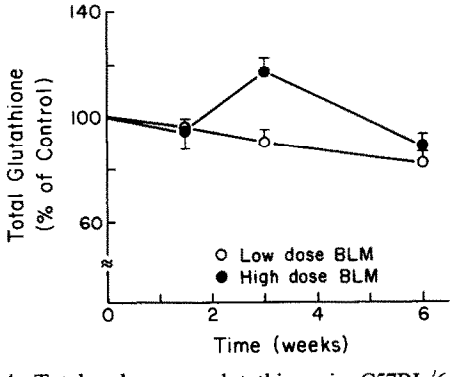

Fig. 4. Total pulmonary glutathione in C57BL/6 mice receiving low-dose (2 mg/kg) or high-dose (10 mg/kg) bleomycin subcutaneously twice-weekly for 6 weeks. Lungs were removed at various times after initiation of bleomycin treatment and were homogenized; total glutathione was measured in the post-microsomal supernatant fraction. Glutathione is expressed as a percentage ± SEM of the pulmonary levels found in saline-treated control animals. The pooled average total glutathione for all control mice was 0.5 μmol/lungs. A total of five animals was examined in each group at each time point.

DISCUSSION

BLM therapy can result in serious pulmonary toxicity in almost half of the patients receiving the drug [2]. The mechanism of this adverse effect is most likely related to the production of activated oxygen species by BLM with resulting DNA strand scission, lipid peroxidation or other cellular injury [2]. Protection from BLM pulmonary toxicity could occur through the action of such non-specific antioxidants as CAT, the GSH system, SOD, or through specific metabolic inactivation of BLM by the enzyme BH [23]. We have established previously that pulmonary BH activity correlates inversely with sensitivity of rabbits or the inbred BALB/c murine strain to BLM-induced lung fibrosis [4, 24].

Our data indicate that systemic BLM administration resulted in perturbations in several pulmonary antioxidant defense mechanisms. The timing

of these changes, although later than that seen with the i.t. route [6], occurred prior to the development of pulmonary fibrosis. Changes in antioxidant defenses were seen as early as 1.5 weeks into high-dose BLM treatment; this is well before the expected onset at 6 weeks of significant lung fibrosis [9]. Of interest is the demonstration of changes in lung antioxidants in animals given the lower non-fibrogenic dose of BLM. These changes included an increase in CAT activity at 3 weeks and a decrease at 6 weeks. GPO and GR activities were increased significantly at 6 weeks, without any changes in overall lung GSH levels. While we did not measure the oxidized GSSG form of GSH, or mixed disulfides, GSSG usually accounts for only a small fraction of GSH present in cells [24]. The action of BLM on GSH and the GSH system appears unique among the anticancer agents that produce lung injury. BCNU has been found to inhibit lung GR and not to alter total lung GSH [25], whereas cyclophosphamide increases total lung GSH levels [26]. The increase in GSH-shuttle enzyme activity after BLM implies that this potential antioxidant system is activated by both chronic low-dose and high-dose BLM administration with a resulting increase in the capacity of detoxification by this pathway. Whether the GSH system acts solely as a protective factor from BLM-induced toxicity is unclear. The GSH system may play a role in detoxifying reactive peroxide radicals to their corresponding alcohol or by maintaining adequate reduced GSH levels in tissue for detoxification [27]. It has been argued by others, however, that GSH may promote toxicity by providing reducing equivalents to the oxidized Fe(III)–BLM molecule [28], allowing for cyclic oxidation reduction of the Fe(III)–BLM molecule and catalytic production of activated oxygen species by Fe(II)–BLM [29].

Of significance were results indicating that BLM diminished activity of the pulmonary protective enzyme bleomycin hydrolase in mice receiving the fibrogenic dosage of drug. No significant changes in lung BH activity were seen with the low-dose mice. While the decrease in activity in the high-dose group was found at a point when fibrosis is expected to occur, significant inhibition of BH activity was also demonstrated at the 3-week time point prior to the onset of significant lung fibrosis. The mechanism of this suppression in lung BH activity is unknown. It is unlikely to be due simply to a relative increase in the amount of acellular proteinaceous material (e.g. collagen) since increases in other enzymes were found at both 3 and 6 weeks. It is possible that a change in the cell populations of the lung could affect BH content. While we cannot completely exclude this explanation, it should not be related solely to changes in alveolar or interstitial cell populations, as both interstitial fibroblasts and alveolar macrophage-cell types which accumulate during BLM-induced lung injury have been shown to have significant amounts of BH activity [30]. BLM has been shown to inhibit directly other enzyme activity including DNA polymerase [13], tyrosine hydroxylase [14] and dopamine β-hydroxylase [15]. While we have not found *in vitro* inhibition of bleomycin hydrolase by bleomycin in previous studies, it is possible that the *in vivo* production of oxygen radicals, either by BLM or a secondary accumulation of inflammatory cells, has a deleterious effect upon this enzyme activity.

In conclusion, we have shown that chronic systemic non-fibrogenic and fibrogenic BLM treatment in C57BL/6 mice resulted in changes in antioxidant defense mechanisms. An increase in activity of these antioxidants as a response to the presence of oxygen radical species may be important in protection of the lung from BLM toxicity. However, the finding of a significant decrease in lung BH activity both prior to and during the expected onset of pulmonary fibrosis may contribute to enhanced pulmonary damage with repeated doses of BLM, and thus may represent an important pathogenetic mechanism in BLM-induced pulmonary fibrosis.

Acknowledgements—We wish to thank Betty Brown for her excellent editorial assistance, and Drs. Dale Hoyt, John Schisselbauer, and Jim Harrison for review of the manuscript.

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