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Murine strain differences in pulmonary bleomycin metabolism

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Bleomycin (BLM*) is an important antitumor agent that also produces scleroderma-like skin changes and potentially fatal pulmonary fibrosis [1]. The mechanism(s) of these side-effects has not been clearly established, but may be caused by BLM-mediated radical abstraction of DNA or membranes sites. Protection from toxicity may be accorded by intracellular metabolism of BLM to the less active deamidoBLM (dBLM) form by the enzyme BLM hydrolase [2–4]. The metabolite produced by BLM hydrolase is approximately 1% as efficient as the parent compound in producing oxygen radicals, DNA strand scissions, tumor cytotoxicity, or pulmonary fibrosis in animal models [4].

* Abbreviations: BLM, bleomycin; BLM A_2, bleomycin A_2; dBLM, deamidobleomycin; dBLM A_2, deamidobleomycin A_2; PBS, phosphate-buffered saline; and i.t., intratracheal.

The relevance of this enzymatic inactivation for *in vivo* protection from BLM-induced lung fibrosis or for tumor resistance has not been firmly established. Umezawa *et al.* [5] has shown in mice that *in vitro* enzyme activity, as measured indirectly by bioassay, is high in such tissues as liver, kidney, and bone marrow. These organs are known to be resistant to the toxic effect of BLM in animals and humans [1, 3, 5]. Conversely, BLM hydrolase activity appears to be low in lung and skin homogenates. Bioassays of BLM, however, are relatively insensitive and are affected by endogenous and exogenous inhibitory or stimulatory factors [3, 6]. Thus, the levels of BLM hydrolase in organs should be examined with methods that allow direct measurement of metabolite formation.

Some inbred strains of mice exhibit natural resistance to BLM-induced pulmonary fibrosis [7, 8]. BALB/c mice express only low levels of pulmonary collagen synthesis and content following the same intratracheal (i.t.) dose of BLM that produces extensive lung collagen synthesis and content in C57BL/6 mice [7]. A similar murine strain difference in pulmonary sensitivity has been seen with BALB/c and C57BL/6 mice when BLM is administered by continuous subcutaneous infusion [8] or by i.v. or i.p. injections [9, 10]. Lung collagen is slightly lower in DBA/2 mice than C57BL/6 mice after i.t. BLM injection [7] but equal to that of C57BL/6 mice after multiple i.v. injections [9]. Although it has been proposed that the strain variation is at least partly immunologically mediated [9, 11, 12], non-immunological factors may also regulate pulmonary sensitivity to BLM [9, 12]. Because our previous results suggested that tissue BLM hydrolase may play a role in protecting organs from BLM [3], we examined *in vitro* pulmonary BLM hydrolase activity in these inbred murine strains to test the hypothesis that the differential pulmonary response in mice to BLM is determined by the level of this inactivating enzyme. In addition, *in vitro* BLM hydrolase activity was assayed in other organs reported to be sensitive (skin) or resistant (liver, kidney) to the toxic effects of BLM.

Materials and Methods

Animals. Female C57BL/6N, DBA/2N, and BALB/cN mice were obtained from the NIH animal care facility (Frederick, MD). All animals were maintained in the Yale University School of Medicine animal care facility and fed *ad lib.* Animals were 8–10 weeks of age and weighed 20–23 g at the time of experiments.

Drugs. Blenoxane was supplied by the Bristol Myers Co. (Wallingford, CT). BLM A_2 constitutes 65–70% of the clinical Blenoxane mixture [4] and was used in the copper-free form in all experiments as a substrate for BLM hydrolase. BLM A_2 and dBLM A_2 were also used as standards for HPLC analysis. BLM A_2 and dBLM A_2 were prepared as previously described [3]. BLM A_2 and dBLM A_2 standards and prepared organ samples were analyzed by HPLC as previously described [10] using a 650-10S flow-fluorescence spectrophotometer (Perkin–Elmer, Norwalk, CT) and recording with a Shimadzu (Kyoto, Japan) C-RIA integrator.

Bleomycin hydrolase preparation. Mice were killed by an i.p. injection of 100 mg/kg of pentobarbital sodium, the aorta was cut, and lungs were perfused with 3 mL of ice-cold phosphate-buffered saline (PBS) (pH 7.2) injected via the right ventricle. Lungs, liver, and kidneys were removed and rinsed with ice-cold PBS to remove blood. An approximate 2 × 3 cm area of ventral skin was removed from the shaved abdominal wall of the animals. Then, 2–4 mL of a 0.1 M sodium phosphate-buffered solution (pH 7.2) was added to the organs, and tissues were homogenized with an Ultra-Turrax Tissumizer (Tekmar, Cincinnati, OH). Organ samples were then processed for BLM hydrolase isolation by collecting the purified post-microsomal supernatant fraction [13]. Protein content was determined [14] and the sample was then stored at −70° until used.

Bleomycin hydrolase assay. BLM hydrolase activity in organ preparations was determined as previously described [13] by measuring the rate of formation of the dBLM A_2 metabolite from the parent BLM A_2 compound. Analysis of dBLM A_2 formation was performed by HPLC as noted above. Peak identification and standard curves for BLM A_2 and dBLM A_2 were generated by the injection of purified BLM A_2 and dBLM A_2 standards either alone or with the sample reaction mixture. 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.

Data analysis. Analysis of organ BLM hydrolase activity was done using a one-way analysis of variance (ANOVA). Significant differences between groups were assessed by Tukey's test for multiple comparison [15].

Results

The activity of BLM hydrolase *in vitro* was assayed in lung, liver, kidney, and skin samples from C57BL/6, DBA/2, and BALB/c mice (Fig. 1). We found a significant difference in lung BLM hydrolase activity among the inbred murine strains. BALB/c mice had the highest levels of pulmonary BLM hydrolase activity, 2.6-fold ($P < 0.001$) greater than C57BL/6 mice, and 1.6-fold more than DBA/2 ($P < 0.05$). The mean pulmonary BLM hydrolase activity in DBA/2 mice was 1.6-fold greater than that in C57BL/6 mice ($P = 0.07$). This pattern in lung BLM hydrolase activity correlates inversely with pulmonary sensitivity to i.v., i.t. and subcutaneous BLM [8–10, 12].

Among the other organs sampled, the rate of dBLM A_2 formation was greatest in liver samples from all three murine strains, with BLM hydrolase specific activities elevated 2- to 5-fold over those from corresponding lung homogenates. There was no significant strain variation in liver BLM hydrolase activity among the murine strains.

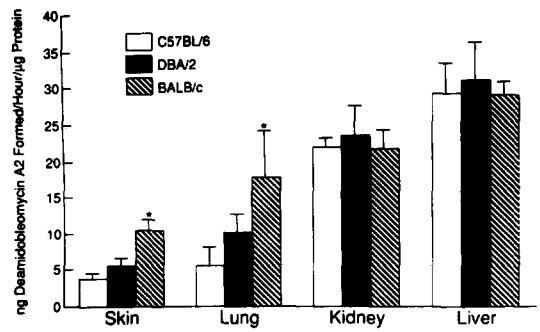

Fig. 1. Bleomycin hydrolase activity *in vitro* in skin, lung, kidney, and liver homogenates from murine strains. Organ post-microsomal fractions were incubated with bleomycin A_2 for 60–120 min and then assayed by HPLC for deamidobleomycin A_2 metabolite formation. Pulmonary values are means ± SEM from ten C57BL/6 mice, eight DBA/2 mice, and fourteen BALB/c mice; the mean values for other organs were obtained from five animals in each group. An asterisk (*) indicates significant differences of BALB/c mice versus either DBA/2 mice ($P < 0.05$) or C57BL/6 mice ($P < 0.001$). There were no significant differences among murine strains for kidney or liver BLM hydrolase activity.

Kidney samples displayed up to 3-fold greater BLM hydrolase activity than lung specimens but, as with liver, no murine strain variation was demonstrated. Skin samples exhibited the lowest amount of BLM hydrolase activity among organs studied. A murine strain variation in BLM hydrolase activity similar to that found in lung samples was also noted. BALB/c mice had the greatest amount of skin BLM hydrolase activity, over 2.5-fold of that in C57BL/6 mice ($P < 0.001$), and more than 1.5-fold of that found in DBA/2 mice ($P < 0.05$).

Discussion

In this study we have examined whether naturally occurring resistance to BLM-induced pulmonary fibrosis in inbred murine strains correlates with a high level of tissue BLM hydrolase activity. Murine strain variation in sensitivity to BLM-induced toxicity has been described with the administration of BLM by the intratracheal route [7] as well as following systemic administration [8–10, 12]. In particular, we have noted that BALB/c mice are more resistant to BLM-induced pulmonary fibrosis than C57BL/6 mice [8, 10].

Our results comparing the *in vitro* activity of BLM hydrolase in lung post-microsomal fractions from the murine strains showed a significantly elevated (greater than 2.5-fold) level of BLM hydrolase activity in the resistant BALB/c strain versus the sensitive C57BL/6 mice. BALB/c also had significantly greater activity than DBA/2, a strain noted to have a slightly attenuated fibrotic pulmonary response to intratracheal BLM compared to C57BL/6 [8]. DBA/2 mice had a mean pulmonary BLM hydrolase activity less than that of BALB/c mice but greater than C57BL/6 mice. Thus, an inverse correlation exists between *in vitro* lung BLM hydrolase activity and sensitivity to BLM-induced lung fibrosis.

Overall, tissue levels of BLM hydrolase were highest in liver and kidney, and lowest in lung and skin. This pattern of organ enzyme activity varies inversely with known sensitivities of these organs to BLM [1–3], and confirms previous findings of BLM hydrolase activity by radioimmunoassay [5].

Of interest was the finding in our model of an overall lower level of skin BLM hydrolase activity, as well as a murine strain variation in skin BLM hydrolase activity similar to that of lung activity. Clinically apparent skin sensitivity to BLM has been reported in humans with manifestations ranging from alopecia to skin thickening and Raynaud's phenomenon [16, 17]. It is probable that the etiology for skin sensitivity to BLM may also, as in lungs, be related to a lack of tissue inactivating enzyme activity. While the cutaneous response to BLM in animal models has not been reported, it may be possible to address this issue using measures of collagen gene or protein expression in skin specimens [18].

In summary, we have demonstrated that levels of pulmonary BLM hydrolase activity differ and correlate inversely with murine sensitivity to BLM-induced pulmonary fibrosis. In addition, the distribution of tissue BLM hydrolase levels correlates inversely with the spectrum of BLM organ toxicity. Efforts aimed at increasing local lung BLM hydrolase activity may also be of value in limiting BLM lung toxicity.

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ANDREW E. FILDERMAN*†
JOHN S. LAZO‡

* *Pulmonary and Critical Care Section*
New England Deaconess Hospital
Boston, MA 02215; and
‡ *Department of Pharmacology*
University of Pittsburgh
School of Medicine
Pittsburgh
PA 15261, U.S.A.

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† Reprint requests should be addressed to: Andrew E. Filderman, M.D., Pulmonary and Critical Care Section, Palmer 117, New England Deaconess Hospital, 185 Pilgrim Road, Boston, MA 02215.

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