

Lack of metabolism as the biochemical basis of bleomycin-induced pulmonary toxicity

(bleomycin hydrolase/high-pressure liquid chromatography/lung fibrosis/fluorescence/aminopeptidase)

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ABSTRACT The biochemical basis for bleomycin-induced pulmonary toxicity was studied *in vitro* and *in vivo* with an improved HPLC system. The *in vitro* metabolism of bleomycin A₂ to desamido-bleomycin A₂ was measured in tissue homogenates from a species sensitive (mice) and relatively resistant (rabbits) to the pulmonary fibrogenic properties of bleomycin. Lung tissue from mice lacked detectable bleomycin hydrolase activity, whereas rabbit lung tissue homogenates had high levels of the enzyme activity, equaling that seen in rabbit kidneys and spleen. Injection of radiolabeled bleomycin A₂ into mice demonstrated that only a small percentage of the total dose was taken up by any organ and that extensive metabolism of this drug occurred within 1 hr in liver, kidneys, and spleen but not in lungs *in vivo*. In addition, metabolites other than desamido-bleomycin A₂ were prominent, and their relative amounts increased with time. Mice injected subcutaneously with bleomycin A₂ developed pulmonary fibrosis, while animals treated with equivalent doses of desamido-bleomycin A₂ did not, indicating that this metabolite is not as toxic to the lungs as is the parent compound. These results provide direct evidence that metabolism plays a major role in determining the toxic potential of bleomycin to the lungs.

Bleomycin (BLM) is a glycoprotein that is extensively used in combination with other anticancer agents because of its relative lack of hematological and gastrointestinal toxicity. However, pulmonary toxicity is common with BLM and limits its therapeutic utility (1). Life-threatening, irreversible pulmonary fibrosis is associated with high cumulative doses of BLM; thus, it is important to understand the biochemical basis of its lung toxicity. Furthermore, BLM may be a useful prototype for studying the general mechanism by which other substances, including anticancer drugs, cause pulmonary toxicity.

Cellular toxicity from BLM exposure is believed to result from interactions between the drug and DNA (2). Although considerable attention has been directed toward understanding these interactions, comparatively little is known about the biochemical mechanism responsible for tumor sensitivity and host toxicity to this drug. BLM can be metabolized by an aminopeptidase B-like enzyme activity, BLM hydrolase, that acts on the carboxamide group of the β -aminoalanine moiety in BLM, converting it to the corresponding desamido-BLM (3). Umezawa *et al.* (3) have postulated that desamido-BLM is less toxic than the parent compound and that BLM hydrolase activity may be a major determinant of host organ toxicity because bioassay results indicate that lungs of mice are less capable of inactivating BLM than are other organs.

Previous experiments (4-7) indicated that repeated injections of mice with 5-10 mg of BLM per kg of body weight for 4 weeks or more produced pulmonary fibrosis as detected by

an increase in lung hydroxyproline content and by histological analyses, whereas a similar injection protocol failed to yield fibrosis in rabbits (8). The experiments reported here examined the relative BLM hydrolase activities in various organs of these two species to evaluate the hypothesis of Umezawa *et al.* (3). A modification of our recently described sensitive HPLC system for detection of BLM B₂ and desamido-BLM B₂ (9) was used. In addition, the metabolism of radiolabeled BLM A₂ in organs of mice *in vivo* and the pulmonary toxicity of the only previously described metabolite of BLM A₂, desamido-BLM A₂ (BLM dA₂), were determined.

MATERIALS AND METHODS

Drugs. BLM (Blenoxane) and Cu-free BLM A₂ that were used as HPLC standards were obtained from W. T. Bradner (Bristol Laboratories, Syracuse, NY). The Cu-free metabolite of BLM A₂, BLM dA₂ (lot TN-A-029), that was used as a standard was supplied by A. Fujii (Nippon Kayaku, Tokyo, Japan). The BLM used in the enzyme assays and *in vivo* studies was obtained from Blenoxane by the procedures of Roy *et al.* (10). The BLM dA₂ injected into mice was synthesized by incubating 15 mg of Cu-free BLM A₂ for 24 hr at 37°C with 5 ml (125 mg of protein) of the rabbit liver homogenate used for BLM hydrolase activity assays. After 24 hr, the reaction mixture was centrifuged through a CF-25 Centriflo cone (Amicon) at 1,000 × g for 30 min at 4°C to remove any unreacted BLM A₂ and the product, BLM dA₂. The material remaining in the cone was resuspended twice with 6 ml of glass-distilled water and centrifuged. The filtrates were pooled and purified on a CM-Sephadex column by the method of Roy *et al.* (10). Based upon HPLC and TLC analyses, the reaction yielded BLM dA₂ that was >93% pure. [³H]BLM A₂ (specific activity, 32 mCi/mmol; 1 Ci = 3.7 × 10¹⁰ Bq) was synthesized by the method of Roy *et al.* (10); the radiolabeled material was identical to the unlabeled BLM A₂ on CM-Sephadex chromatography, HPLC, and TLC and was metabolized to BLM dA₂ with rabbit liver homogenates.

Spectroscopic Measurements and the HPLC System. Uncorrected fluorescence spectra of Cu-complexed BLM A₂ and BLM dA₂ were recorded at 25°C with a Perkin-Elmer 650-10S fluorescence spectrophotometer. BLM A₂ and BLM dA₂ were separated by using a Whatman Partisil PXS 10/25 C₈ column (4.6 mm × 250 mm) with a mobile phase and flow rate described in the legend to Fig. 1. The capacity factor, K', was calculated for both BLM A₂ and BLM dA₂ as the ratio of the adjusted retention time of the individual component to the retention time of the unretained components.

In Vitro Studies. Organs were removed from male albino New Zealand rabbits weighing between 2.8 and 3.0 kg or from

female C57Bl/6N mice (Charles River Kingston, Stone Ridge, New York) weighing between 20 and 24 gm and were rinsed with an ice-cold 0.1 M sodium phosphate-buffered solution (pH 7.2) to remove blood. After the organs were weighed, 2 vol of a 0.1 M phosphate-buffered solution were added, and the tissue was homogenized for 10 sec with an Ultra-Turrax Tissumizer (Tekmar, Cincinnati, OH). The sample was homogenized further with 5–10 strokes of a Teflon pestle, cooled on ice, and homogenized again with 5–10 strokes. The sample was centrifuged at $20,800 \times g$ for 45 min at 4°C . Low molecular weight material was removed from the resulting supernatant fraction with a CF-25 Centriflo cone (Amicon) by centrifugation at $1,000 \times g$ for 30 min at 4°C . The concentrated solution was resuspended to its original volume with ice-cold 0.1 M sodium phosphate solution and concentrated a second and third time with the Centriflo cone. The resulting solution was removed from the cone, and the cone was washed twice with the 0.1 M phosphate-buffered solution. The concentrated solution and the washes were combined and stored at -70°C until use. The metabolism of BLM A_2 *in vitro* was assayed as described for BLM B_2 (9) except that $120 \mu\text{g}$ of Cu-free BLM A_2 per ml was used. More than 90% of the total organ BLM hydrolase activity was located in the high molecular weight material of the $105,000 \times g$ supernatant fraction in all organs examined. Protein in the homogenates was determined by the method of Bradford (11).

In Vivo Studies. [^3H]BLM A_2 ($4.5 \mu\text{Ci}$) in 0.25 ml of phosphate-buffered saline (NaCl/KCl/ P_i ; 137 mM NaCl/2.7 mM KCl/8 mM Na_2HPO_4 /1.5 mM KH_2PO_4 , pH 7.4) was injected subcutaneously into female C57Bl/6N mice weighing between 20 and 24 gm. At various times urine was collected and mice were killed by cervical dislocation. Serum was obtained from blood taken by cardiac puncture. After the descending aorta was cut, the lungs were perfused with NaCl/KCl/ P_i until free of blood and then removed. The liver, spleen, and kidneys also were removed. All organs were washed with ice-cold NaCl/KCl/ P_i to remove blood, weighed, and homogenized in 1 or 2 ml of ice-cold 0.1 M phosphate-buffered solution. To the resulting homogenate, 0.1 vol of 6 M trichloroacetic acid was added, and the sample was shaken and centrifuged at $15,600 \times g$ for 1 min. The supernatant fraction was neutralized with an equal volume of 0.5 M tri-*n*-octylamine in 1,1,2-trichlorotrifluoroethane (12), and the aqueous layer was injected on the HPLC with 2 mM CuSO_4 , which was included to improve peak resolution (9).

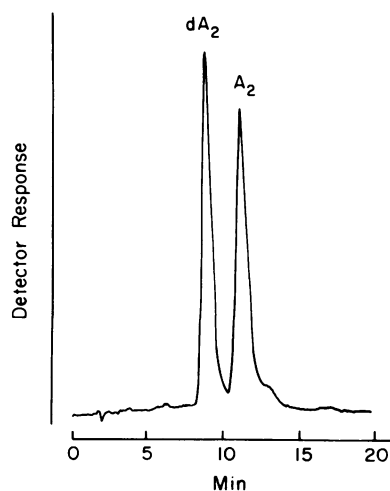


FIG. 1. HPLC separation. Samples ($800 \mu\text{g}$) of BLM A_2 and dA_2 standards were applied and eluted with $\text{CH}_3\text{OH}/\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{CH}_3\text{COOH}$, 2,175:725:7,020:80 (vol/vol), containing 2 mM heptanesulfonic acid and 25 mM triethylamine (pH 5.5). Flow rate was 1.4 ml/min.

Fractions (0.3 min) were collected and counted by liquid scintillation techniques and corrected for counting efficiency by using external standard techniques. An injection volume of $500 \mu\text{l}$ was used for all samples and between 1,000 and 50,000 dpm were applied onto the HPLC column. HPLC fractions obtained from samples with low amounts of radioactivity were counted four or five times to ensure accurate determinations. Some samples (namely, serum, urine, and kidneys) that had a high radioactive content were diluted to $500 \mu\text{l}$ with 0.1 M phosphate-buffered solution to maintain a constant injection volume. Addition of [^3H]BLM A_2 to organs from untreated mice indicated a recovery of [^3H]BLM A_2 of $>98\%$.

Pulmonary fibrosis was evaluated histologically and by measuring lung hydroxyproline content (5). Mice were injected subcutaneously twice weekly with NaCl/KCl/ P_i or 10 mg of either BLM A_2 or dA_2 per kg. After 6 wk, the mice were killed and the lungs were removed and weighed. The post caval lobe was washed with NaCl/KCl/ P_i and placed in ice-cold 0.1 M cacodylate-buffered solution containing 2.5% glutaraldehyde. Lung tissue was examined for collagen content both light-microscopically after staining with Masson's trichrome and biochemically with methods previously described (4, 8).

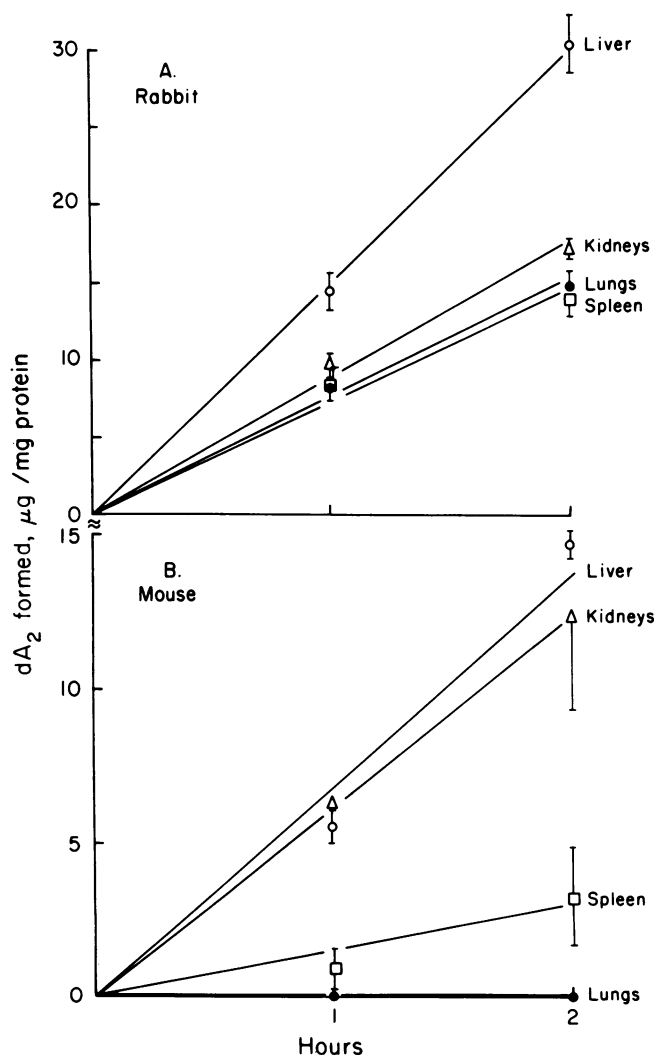


FIG. 2. BLM hydrolase activity *in vitro*. The BLM hydrolase activity in the high molecular weight material of the $105,000 \times g$ supernatant fraction from rabbit (A) and mouse (B) organs was measured. BLM A_2 ($60 \mu\text{g}$) was added to $600 \mu\text{g}$ of protein and incubated for 1 or 2 hr. BLM dA_2 was separated from BLM A_2 by HPLC, and the amount was determined by spectrofluorescence. Each value is the mean \pm 1 SEM of 3–5 determinations.

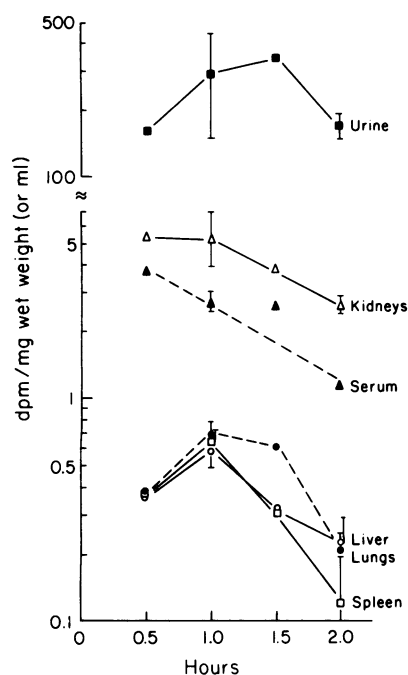


FIG. 3. Radioactivity in organs of mice after subcutaneous injection of $[^3\text{H}]\text{BLM A}_2$. Values at 1 and 2 hr were obtained from 3 or 4 mice, and other values were determined from a single mouse. (Bars = 1 SEM.)

RESULTS

Fluorescence Detection and the HPLC System. The fluorescence excitation maximum for Cu-complexed BLM A_2 (25 $\mu\text{g}/\text{ml}$) in the HPLC mobile phase was 297 nm, and the emission maximum was 353 nm. BLM dA_2 had a similar excitation maximum, but the emission maximum was shifted slightly to 355 nm. With an excitation wavelength of 297 nm (10-nm bandpass) and an emission wavelength of 355 nm (10-nm bandpass), we observed with the HPLC system that BLM A_2 was separated readily from BLM dA_2 , with the K' of the parent com-

pound and its metabolite being 4.0 and 3.3, respectively (Fig. 1). The fluorescence intensities for both BLM A_2 and dA_2 were similar, and both compounds displayed linear integrated peak areas over at least a 10- to 15-fold concentration range.

In Vitro Assays. The formation of BLM dA_2 from BLM A_2 was determined in the high molecular weight material from organs at 1 and 2 hr (600 μg of protein per reaction; 0.5 ml). No metabolite other than BLM dA_2 was detected. In other experiments, various concentrations of protein were used to ensure that enzyme was limiting in the 2-hr incubation. With rabbit tissues, liver homogenates contained the highest amount of enzyme activity, whereas spleen, kidneys, and lungs had lower but similar BLM hydrolase activity (Fig. 2A). In contrast, the BLM hydrolase activity in mouse lung homogenates was below the detection limits of our assay (Fig. 2B). Mouse liver, spleen, and kidney homogenates all had detectable levels of BLM hydrolase activity.

In Vivo Results. The total radioactivity in selected organs, serum, and urine of mice at various times after injection of $[^3\text{H}]\text{BLM A}_2$ was measured. Urine contained at least 10-fold more radioactivity than serum at all times examined (Fig. 3). Consistent with previously reported values for the serum half-life of BLM A_2 (13), a serum half-life of ≈ 50 min was observed for $[^3\text{H}]\text{BLM A}_2$. The radioactivity in kidneys greatly exceeded that seen in liver, spleen, and lungs at all time points examined, and at 1 hr there was 8-fold more radioactive material in the kidneys than in any of these other organs. In liver, lungs, and spleen, peak levels of radioactivity appeared at ≈ 1 hr, and each of these organs had similar dpm per mg of wet weight.

To evaluate the nature of the radioactivity in each organ of mice, we next used our HPLC system. One hour after subcutaneous treatment with $[^3\text{H}]\text{BLM A}_2$, $>95\%$ of the total radioactivity in serum and urine comigrated with authentic $[^3\text{H}]\text{BLM A}_2$ (fractions 34–36; Fig. 4 A and B). In kidney samples (Fig. 4C), prominent peaks appeared at fractions 34–36 (BLM A_2) and at fractions 28–29 (BLM dA_2). Additional peaks also were seen in the void volume (fractions 12–14) and at fractions 25–26. In liver tissue (Fig. 4D), both $[^3\text{H}]\text{BLM A}_2$ and dA_2 were

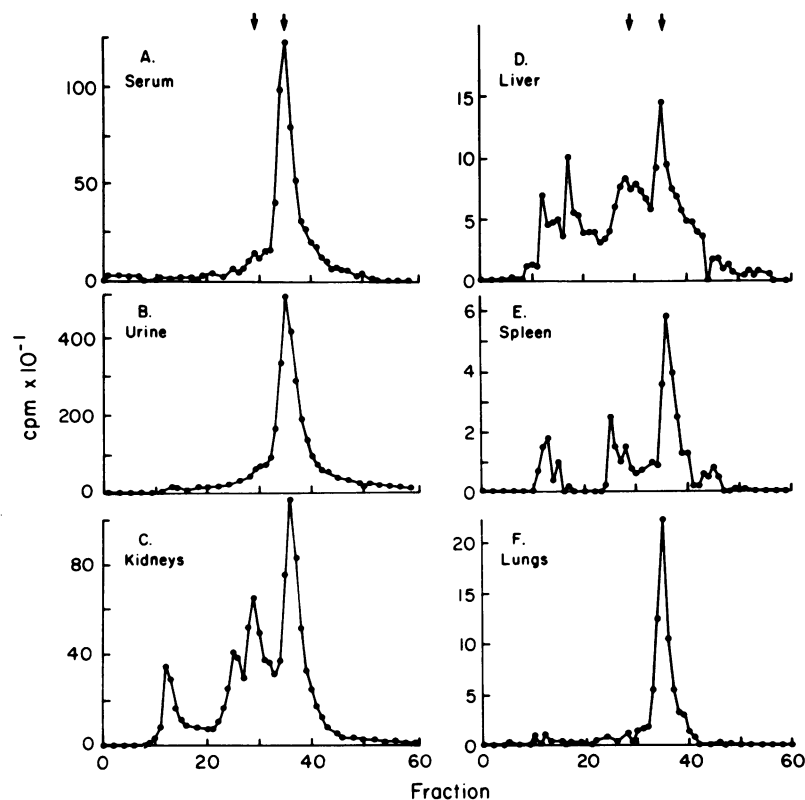


FIG. 4. Representative HPLC radioactivity profiles from organ homogenates of a mouse treated with $[^3\text{H}]\text{BLM A}_2$. One hour after a subcutaneous injection of 4.5 μCi (1×10^7 dpm) of $[^3\text{H}]\text{BLM A}_2$, urine was collected and the mouse was killed. After serum was obtained, organs were removed and homogenized in a 0.1 M phosphate-buffered solution, and the radioactivity was extracted. Samples (500 μl) were injected onto the HPLC column and eluted at 1.4 ml/min. Fractions (0.3 min) were collected and counted by liquid scintillation techniques. Similar results were obtained from three other mice. Arrows indicate the location of BLM dA_2 and A_2 .

detected as were radioactive peaks between these known peaks, in the void volume, and between the void volume and the [^3H]-BLM dA₂ peak. Spleen homogenates from mice also had multiple peaks, with one occurring after the parent compound (fractions 43–47; Fig. 4E). In lung samples, however, the only prominent peak was [^3H]BLM A₂, indicating no major metabolism in that organ at 1 hr (Fig. 4F). When organ homogenates from untreated mice were mixed with [^3H]BLM A₂ and injected onto the HPLC system, the only peak observed was one that comigrated with [^3H]BLM A₂.

The HPLC profile of the metabolites in organs changed with time after injection. For example, in kidney samples a decrease in BLM A₂ relative to the other metabolites was seen over the 2-hr period (Fig. 5 A–D). In particular, peaks at fractions 12–14, 25–26, and 27–29 increased greatly in size. No major metabolite peaks were seen in urine or serum samples at any of these time points, whereas in lung tissue very small peaks characteristic of BLM dA₂ and at fractions 12–14 were noted at 2 hr. To demonstrate that the unknown peaks were indeed true

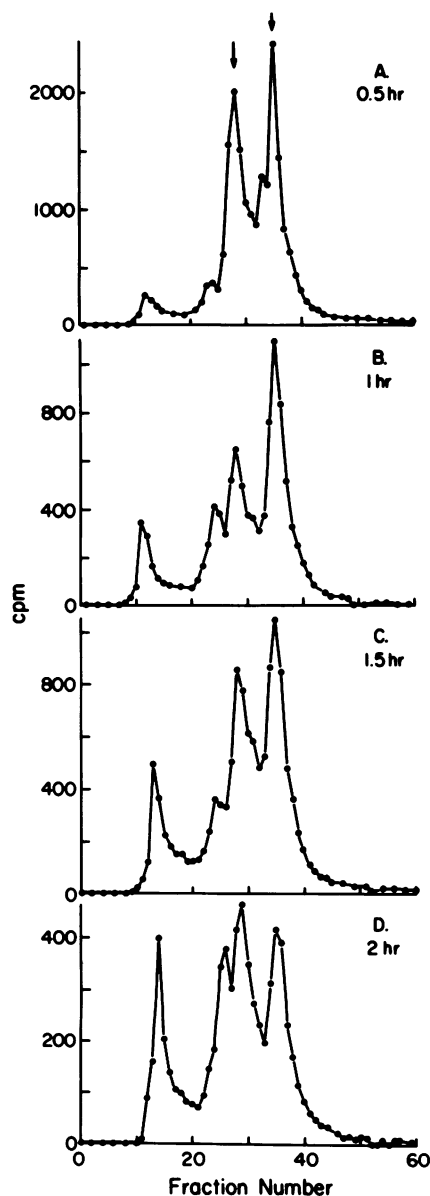


FIG. 5. HPLC radioactivity profiles from mouse kidneys at various times. Kidney homogenates from mice treated 0.5 hr (A), 1 hr (B), 1.5 hr (C), or 2 hr (D) were extracted, injected onto HPLC, and eluted at 1.4 ml/min. Fractions (0.3 min) were collected and counted. Arrows indicate the location of BLM dA₂ and A₂ standards.

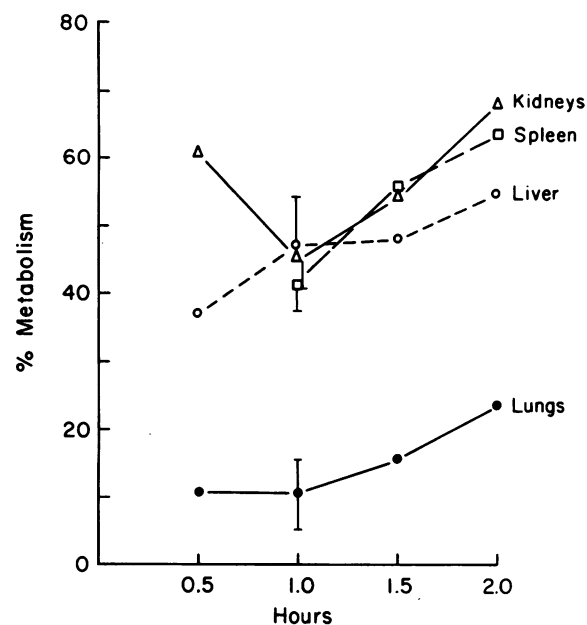


FIG. 6. Metabolism of [^3H]BLM A₂ *in vivo*. The percentage of the total radioactivity that was not [^3H]BLM A₂ (namely, fractions 32–41) was calculated in organs at various times by HPLC. One-hour values are the mean results from four mice. All other time-point values were obtained from a single mouse. (Bar = 1 SEM.)

metabolites of [^3H]BLM A₂, we pooled the peaks located in the void volume (fractions 12–16) and applied them to a CM-Sephadex column (9). The radioactivity was eluted as a single peak with 50 mM NH₄COOH (pH 6.5), whereas both BLM A₂ and dA₂ required higher concentrations of NH₄COOH to be eluted. Thus, this peak appeared to be a new metabolite(s) of BLM A₂.

The percentage of [^3H]BLM A₂ metabolism in organs was studied at various times after drug injection by determining the percentage of radiolabel found outside of the [^3H]BLM A₂ peak on the HPLC profiles (Fig. 6). Liver, spleen, and kidneys showed extensive metabolism at 1 hr, with >45% of the drug being metabolized. In contrast, only 10% of the drug in lungs appeared as metabolites. The metabolite formation increased in all organs over time, with liver, spleen, and kidneys having 55% or more of the radiolabel as metabolites at 2 hr. In contrast, the maximum metabolism seen in mouse lungs *in vivo* was only 23%.

To evaluate the ability of the only previously known metabolite of BLM A₂, BLM dA₂, to cause pulmonary fibrosis, we injected mice for 6 weeks twice weekly with NaCl/KCl/P_i or 10 mg of BLM A₂ or dA₂ per kg. Previous studies (5, 6) indicated that this dose schedule of BLM produced increases in lung hydroxyproline, a marker of collagen content, and histological evidence of increased collagen. In the lungs of mice treated with 10 mg of BLM A₂ per kg, there was clear evidence

Table 1. Pulmonary hydroxyproline content after repeated treatment with BLM A₂ or dA₂

	Hydroxyproline, μg per lung	Body weight, g
Control	165.1 ± 9.1	19.8 ± 0.8
BLM A ₂	215.7 ± 21.0*	14.7 ± 0.3*
BLM dA ₂	176.7 ± 5.5	19.2 ± 0.4

Mice were treated subcutaneously twice a week for 6 weeks with NaCl/KCl/P_i or 10 mg of BLM A₂ or dA₂ per kg of body weight. Each hydroxyproline value is the mean ± 1 SEM obtained from the right and left lungs of five mice minus the post caval lobe. The post caval lobe weight was the same percentage of the total weight of the lungs (10%) in all groups. Statistical comparisons between groups were performed with the Wilcoxon rank sum test. *, $P < 0.05$.

of fibrosis demonstrated by a 31% increase in the pulmonary hydroxyproline content (Table 1) and by an increased presence of collagen observable after staining with Masson's trichrome (data not shown). In contrast, a similar treatment with BLM dA₂ did not cause fibrosis as defined by our biochemical and morphological methods, although some lung consolidation and hypercellularity was seen in the lungs of mice treated with BLM dA₂. BLM A₂ was quite toxic to mice as seen by the significant decrease in total body weight; this was not observed in mice treated with BLM dA₂.

DISCUSSION

Umezawa *et al.* (3, 14) hypothesized that pulmonary toxicity occurred in lungs rather than other organs because the lungs were deficient in their ability to inactivate BLM. This conclusion was based upon observations with a bioassay that measured antimicrobial activity. Other investigators (13) indicated, however, that microbiological bioassays for BLM content are not only laborious but also insensitive. More important, in a bioassay some organs that do not display drug-induced toxicity appeared unable to inactivate BLM *in vitro*. Consequently, the relationship between antimicrobial activity and host organ toxicity is not clear. Umezawa *et al.* (3, 14) also suggested that inactivation was due to the formation of a deaminated compound by an aminopeptidase B-like enzyme activity, BLM hydrolase. The deaminated form of BLM is the only reported metabolite and, although it is thought to be less potent in causing DNA strand breaks (15), its toxicity, particularly to lung tissue, has not been studied previously.

Recently, HPLC systems have been used to study BLM metabolism, but these have relied upon UV absorption, which lacked the sensitivity to measure nanogram quantities of drug. The HPLC system described here for BLM A₂ is at least 10-fold more sensitive than those not using spectrofluorescence (9) and permits measurement of enzyme activity with less tissue or substrate. Using this HPLC system, we have observed that in organ homogenates from a species that has been shown to be sensitive to BLM-induced pulmonary fibrosis (4-7), the lung BLM hydrolase activity is at least 5- to 15-fold less than that seen in other organs that do not demonstrate frank evidence of toxicity. In addition, the BLM hydrolase activity in the lung homogenates of a resistant species, rabbits, was similar to several other organs in that animal and significantly greater than that seen in mouse lung homogenates. Using much higher substrate concentrations of BLM A₂, Yoshioka *et al.* (16) have reported that, in rat lung homogenates, the BLM hydrolase activity was lower than that seen in kidneys and liver but not in spleen or intestinal tissue. Unfortunately, the relative sensitivity of rat to BLM-induced pulmonary fibrosis compared to other species has not been determined. Based upon our results, it seems likely that interspecies differences occur in sensitivity to drug-induced pulmonary toxicity and pulmonary BLM hydrolase content.

To evaluate directly the mechanism responsible for BLM-induced pulmonary fibrosis *in vivo*, we utilized the recently described method for the synthesis of intrinsically radiolabeled BLM A₂ (9). It is apparent that BLM is only poorly taken up by tissues because, in all organs examined, <1% of the total drug injected was found in any organ. Because spleen and liver tissue contained almost identical amounts of radioactivity as in lungs and because kidneys had 8-fold more radioactivity, it seems unlikely that preferential uptake of BLM can explain organ toxicity.

When a dose of BLM A₂ sufficient to produce pulmonary fibrosis after repeated administration was injected as a single

dose, extensive metabolism was noted within 0.5 hr after drug injection. Lungs from mice consistently demonstrated a significantly lower amount of drug metabolism than liver, spleen, or kidneys at all time points examined. It appears that a number of new metabolites of BLM A₂ can be formed in large quantities in liver, spleen, and kidneys, but the chemical nature of these metabolites is not known nor is the relationship between the formation of BLM dA₂ and these other metabolites clear. However, deamidation of BLM-like compounds is known to reduce by 100-fold their ability to cause single-strand DNA breaks *in vitro* (15). In addition, our results indicate that BLM dA₂ is much less potent in producing pulmonary fibrosis than is the parent compound. Thus, high levels of BLM hydrolase activity in lung tissue would protect the lungs from the toxic effects of BLM. This appears to occur in rabbit lungs. Therefore, the results of this study indicate (i) that BLM A₂ is extensively metabolized in most tissues examined, (ii) that metabolites other than BLM dA₂ are formed *in vivo*, and (iii) that, based upon *in vitro* and *in vivo* data, the sensitivity of the lungs to BLM-induced toxicity can best be explained by the lack of BLM hydrolase activity.

One of the major problems with the clinical use of BLM is identification of patients that may be susceptible to drug-induced fibrosis. No reliable methods to identify patients at risk are available. Once pulmonary fibrosis occurs, it is difficult to control and may be irreversible and life-threatening (1). The HPLC technique we have described for measuring BLM hydrolase activity *in vitro* is both sensitive and rapid; <100 mg of tissue is required. Therefore, it may be possible in the future to biopsy human lungs and assay BLM hydrolase activity prior to therapy to determine if the enzyme activity is related to the sensitivity of each patient to BLM-induced pulmonary fibrosis.

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