Bleomycin Hydrolase Activity and Cytotoxicity in Human Tumors

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ABSTRACT

A sensitive new method to assay bleomycin (BLM) metabolism was developed using an ion-paired, reverse-phase high-pressure liquid chromatography technique in conjunction with fluorescence detection that allowed levels of BLM less than 20 ng/ml to be detected. The metabolism of bleomycin B₂ in homogenates from benign and malignant human tumors was studied, and all 14 tumors were capable of metabolizing bleomycin to desamidobleomycin B₂. Metabolites other than desamidobleomycin B₂ were not detected. BLM hydrolase activities in individual tumors varied more than 7-fold. The importance of BLM hydrolase in limiting the therapeutic effectiveness of BLM was examined by measuring BLM hydrolase activity and the response of human tumors to BLM in culture. Response to BLM in culture was measured by dissociating human tumors to form single-cell suspensions and exposing the cells to 0.05 or 1 μg/ml of BLM for 1 hr. Colony formation after BLM treatment was determined in soft agar and, when compared to that of untreated cells, varied by more than 100-fold. No correlation was observed, however, between BLM hydrolase activity and response to BLM in soft agar. Thus, human tumors can metabolize BLM, and while BLM hydrolase activity may be important in tumor resistance to the drug, these data suggest that either (a) the enzyme activity in the tumor homogenate does not reflect that in the clonogenic cells or (b) other mechanisms of resistance may be operative.

INTRODUCTION

BLM³ can be metabolized in murine tissues by an aminopeptidase B-like enzyme activity, called BLM hydrolase, that hydrolyses the carboxamide group of the ε-aminoalanine moiety in BLM converting it to the corresponding desamidobleomycin (22). Umezawa et al. (20–22) and others (1, 2, 12) have suggested that BLM hydrolase activity may be a major determinant in tumor responsiveness and drug toxicity. In human tumors, however, BLM hydrolase activities have not been characterized, in part, because of the lack of rapid and sensitive methods. Previously, most investigators relied upon bioassays to measure the inactivation of BLM (13, 22). We now describe a sensitive, ion-paired, reverse-phase HPLC technique that allowed us to assay the BLM hydrolase activity in a variety of human tumors. To evaluate the importance of this enzyme activity in limiting the therapeutic effectiveness of BLM, we compared the BLM hydrolase activity in human tumors to their responsiveness to BLM in culture.

MATERIALS AND METHODS

Drugs and Materials. BLM (Blenoxane) and copper-free BLM B₂ (Lot 15681-4-04-A) were obtained as gifts from Dr. William T. Bradner (Bristol Laboratories, Syracuse, N.Y.). The copper-free metabolite of BLM B₂, BLM dB₂ (Lot 600028), was kindly supplied by Dr. A. Fujii (Nippon Kayaku Co. Ltd., Tokyo, Japan). For BLM hydrolase assays, BLM B₂ was isolated from Blenoxane using the methods of Fujii et al. (9) and Roy et al. (17). Blenoxane (50 to 100 mg) was dissolved in glass-distilled H₂O, applied to a CM-Sephadex C-25 column (1.5 × 30 cm), and eluted at 4°C with a linear gradient of 50 to 750 mM NH₄OOCO (pH 6.5) at 30 ml/hr. Absorbance in each fraction was measured at 254 nm, and the fractions containing BLM B₂ were pooled, lyophilized, desalted on a Sephadex G-10 column (0.8 × 80 cm), and eluted at 4°C with H₂O. The amount of BLM B₂ was calculated on the basis of an extinction coefficient at 254 nm of 1.3 × 10⁴ M⁻¹ cm⁻¹ that we obtained for Blenoxane in a 0.1 M sodium phosphate-buffered solution (pH 7.2). The purity of the BLM B₂ was evaluated with our HPLC method and greater than 95% comigrated with authentic BLM B₂, CM-Sephadex C-25 (particle size, 40 to 125 μm) and Sephadex G-10 (particle size, 40 to 125 μm) were purchased from Pharmacia Fine Chemicals, Inc. (Piscataway, N.J.).

Spectrometric Measurements and HPLC System. Uncorrected fluorescence spectra of copper-complexed BLM B₂ and BLM B₂ were recorded at 25°C with a Perkin-Elmer 650-10S fluorospectrophotometer. A Beckman Model 332 HPLC system was equipped with a Perkin-Elmer 650-10S flow fluorospectrophotometer and a Shimadzu CR-1A integrator. Fluorescence was monitored with an excitation of 287 nm (10-nm bandpass) and an emission of 355 nm (10-nm bandpass). In some experiments, a Uvicord S detector (254 nm) was used. BLM B₂ and BLM dB₂ standards (10 to 125 μg/ml) for HPLC were resuspended in 0.1 M phosphate buffer (pH 7.2) to which 1 mM CuSO₄ was added to improve the HPLC peak resolution (14) and stored at −20°C. Samples were eluted from a Whatman Partisil PXS 10/25 C₅-column (4.6-mm inside diameter × 250 mm) with a mobile phase of CH₃OH:CH₃CN:H₂O:CH₃COOH (225:75:692:8) containing 2 mM heptane sulfonic acid and 25 mM triethylamine (pH 5.5). All HPLC solvents were obtained from Burdick and Jackson Laboratories (Muskegon, Mich.). Triethylamine was purchased from J. T. Baker Chemical Co. (Phillipsburg, N.J.) and heptane sulfonic acid from Eastman Kodak Co. (Rochester, N.Y.). The capacity factor, k', was calculated for both BLM B₂ and BLM dB₂ as the ratio of the adjusted retention time of the individual component to the retention time of the unretained component.

Tumor Samples. Tumor samples were obtained from women undergoing routine clinical treatment (Table 1). All patients except Patients H and I had received no prior therapy. Patient H was treated previously with pelvic irradiation and Patient I received melphalan initially, then doxorubicin-HCl and cis-diaminedichloroplatinum, and finally 5-fluorouracil and hexamethylmelamine. The sterile surgical samples were placed on ice in the operating room and transported directly to the laboratory where they were trimmed free of normal and necrotic elements and either processed immediately for BLM hydrolase activity and soft-agar growth (Patients A, B, and M) or refrigerated overnight, according to the method of Pavelic et al. (18), in sterile...
BLM Metabolism and Cytotoxicity

Table 1: Patient information and histological diagnosis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Primary site</th>
<th>Stage</th>
<th>Histological appearance</th>
<th>Specimen site</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>48</td>
<td>Vagina</td>
<td>III</td>
<td>Squamous cell (moderately differentiated)</td>
<td>Vagina</td>
</tr>
<tr>
<td>B</td>
<td>58</td>
<td>Ovary</td>
<td>IAa</td>
<td>Clear cell</td>
<td>Ovary</td>
</tr>
<tr>
<td>C</td>
<td>71</td>
<td>Ovary</td>
<td>III</td>
<td>Undifferentiated</td>
<td>Ovary</td>
</tr>
<tr>
<td>D</td>
<td>70</td>
<td>Colon</td>
<td>Bb</td>
<td>Adenocarcinoma</td>
<td>Colon</td>
</tr>
<tr>
<td>E</td>
<td>63</td>
<td>Ovary</td>
<td>III</td>
<td>Serous</td>
<td>Omentum</td>
</tr>
<tr>
<td>F</td>
<td>68</td>
<td>Ovary</td>
<td>III</td>
<td>Mixed mesodermal</td>
<td>Ovary</td>
</tr>
<tr>
<td>G</td>
<td>63</td>
<td>Stomach</td>
<td>IVc</td>
<td>Adenocarcinoma (moderately differentiated) to well differentiated</td>
<td>Omentum</td>
</tr>
<tr>
<td>H</td>
<td>58</td>
<td>Cervix</td>
<td>Recurrent</td>
<td>Squamous cell (poorly differentiated)</td>
<td>Omentum</td>
</tr>
<tr>
<td>I</td>
<td>59</td>
<td>Ovary</td>
<td>Recurrent</td>
<td>Adenocarcinoma</td>
<td>Peritoneal implant</td>
</tr>
<tr>
<td>J</td>
<td>50</td>
<td>Ovary</td>
<td>Recurrent</td>
<td>Serous</td>
<td>Colon</td>
</tr>
<tr>
<td>K</td>
<td>42</td>
<td>Cervix</td>
<td>IV</td>
<td>Adenocarcinoma (moderately differentiated)</td>
<td>Cervix</td>
</tr>
<tr>
<td>L</td>
<td>43</td>
<td>Paracolpos</td>
<td></td>
<td>Schwannoma (benign)</td>
<td>Paracolpos</td>
</tr>
<tr>
<td>M</td>
<td>70</td>
<td>Vagina</td>
<td>II</td>
<td>Squamous cell (moderately differentiated)</td>
<td>Vagina</td>
</tr>
<tr>
<td>N</td>
<td>68</td>
<td>Ovary</td>
<td>IIC</td>
<td>Endometrioid adenosquamous cell</td>
<td>Ovary</td>
</tr>
</tbody>
</table>

* International Federation of Gynecologists and Obstetricians staging system.
* Modified Dukes classification.
* Modified from TNM system (American Joint Committee for Cancer Staging and End Results Reporting).

BLM Hydrolyase Assay. Human tumor tissue was homogenized for BLM hydrolyase activity using the procedures of Yoshio et al. (24) for rat tissue. Rat liver was used as a reference tissue in our study and was obtained from 250- to 280-g male Sprague-Dawley rats. Rat liver and human tumor tissue were rinsed several times with an ice-cold 0.1 M sodium phosphate buffer solution (pH 7.2) to remove any blood or excess fluid, blotted dry with gauze, and weighed on an ice-cold Petri dish. The tissue was minced with scalpels, transferred to a Teflon-glass homogenizer, diluted with 2 volumes of 0.1 M sodium phosphate-buffered solution (4°), and homogenized with an Ultra-Turrax Tissue-mixer (Tekmar Co., Cincinnati, Ohio). The sample was homogenized further with 5 to 10 strokes of a Teflon pestle, cooled on ice, and homogenized again with 5 to 10 strokes. The sample was centrifuged at 105,000 x g for 60 min at 4°. Low-molecular-weight BLM hydrolase was assayed in a final volume of 500 μl by incubating various amounts of homogenate protein (0.1 to 0.8 mg; 100 μl) in 0.1 M sodium phosphate buffer solution (pH 7.2) for various times (0.5 to 8 hr) with 50 μg of copper-free BLM B2 per ml (400 μl in 0.1 M sodium phosphate buffer solution). The reaction mixture was incubated in a shaking water bath at 37° and stopped by placing it on ice and adding 500 μl ice-cold methanol. The sample was centrifuged at 15,600 x g for 3 min, and 5 μl of 200 mM CuSO4 were added to the resulting supernatant fraction (final concentration, 1 mM) prior to injection into the HPLC column. Recovery for BLM B2 was between 85 and 120%. All samples were incubated for 2 or more times and at 2 or more protein concentrations. Reaction conditions were constructed so that in almost all reactions less than 20% of the substrate, BLM B2, was metabolized. All samples were assayed within 45 days of homogenization. In 4 human tumor samples examined, no change in BLM hydrolyase activity was found after 15 days of freezing (−70°) and a 0 to 20% decrease in activity was noted after 70 days at −70°. No BLM hydrolyase activity was detected in the low-molecular-weight material that passed through the Centriflo cones, and pellets from human tumor homogenates contained less than 1% of the total BLM hydrolyase activity observed in the high-molecular-weight material of the 105,000 x g supernatant fraction. Protein in the 105,000 x g supernatant fractions was measured using the method of Bradford (4) and DNA by the method of Burton (7).

Soft-Agar Assay. At the time tumor samples were prepared for BLM hydrolyase assays, a portion of the tumor was minced into small pieces (1 to 2 cu mm) with sterile scalpels and was added to an enzyme cocktail containing RPMI 1640, 0.7% collagenase (CLS II, 138 units/mg; Millipore Corp., Freehold, N.J.), 0.02% deoxyribonuclease (1200 units/ml; Worthington Biochemical Corp., Freehold, N.J.), and 0.005% Pronase (56.3 proteinase units kaken/mg, B grade; Calbiochem-Behring Corp., La Jolla, Calif.). The tissue was incubated at 37° with slow agitation (60 to 70 rpm) for 1 to 2 hr until greater than 50% single cells were observed microscopically. The cell suspension was then passed sequentially through a 200-mesh metal screen and surgical gauze and examined for single cells. After all cell aggregates had been removed, the cell suspension was centrifuged at 150 x g for 3 to 4 min. The supernatant fraction was discarded, and the cell pellet was resuspended in RPMI 1640. The cell number and cell viability, on the basis of exclusion of 0.5% trypan blue, was determined. The viability ranged from 54 to 96%. Cells were exposed at 37° for 1 hr to 0.05 or 1 μg of BLM per ml or to equivalent amounts of the vehicle, a 0.1 M sodium phosphate buffer solution (pH 7.2). This concentration of BLM was approximately 1 and 20%, respectively, of the maximum plasma concentration x time exposure achievable in patients (3). After drug exposure, the cells were centrifuged at 150 x g for 3 to 4 min, the supernatant fraction was removed, and the cell pellet was washed with a solution of Connaught Memorial Research Laboratories Medium 1066 containing 15% heat-inactivated (56° for 45 min) fetal bovine serum and penicillin (100 units/ml) and streptomycin (100 μg/ml). After centrifugation at 150 x g for 3 to 4 min, the cell pellet was resuspended in the Connaught Memorial Research Laboratories Medium 1066 solution with 0.3% agar and was cultured in triplicate in 35-mm Petri dishes using a modification of the 2-layer soft-agar conditions of Hamburger et al. (10). The cells were placed (1 × 103 to 5 × 106 viable cells/dish) upon an underlayer consisting of 1 ml of McCoy's medium with 10% heat-inactivated fetal bovine serum, 5% horse serum, 1 mg sodium pyruvate, 2 mM glutamine, and 0.5% agar. Cultured dishes were examined immediately after plating for cell aggregates.

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The cells were incubated at 37° in 5% CO₂ in a humidified atmosphere for 3 weeks and examined for colonies using an inverted or dissecting microscope at ×40 or ×20, respectively. Aggregates containing more than 30 cells were considered to be colonies, and growth was defined as the presence of 15 or more colonies in dishes with untreated cells. The number of colonies in untreated cultures in these experiments ranged from 17 to 704.

RESULTS

Fluorescence Detection and HPLC Separation. The fluorescence excitation maximum for copper-complexed BLM B₂ (25 µg/ml) in the HPLC mobile phase was 297 nm and the emission maximum was 353 nm (spectra not shown). BLM dB₂ had a similar excitation maximum, but the emission maximum was shifted slightly to 355 nm. With an excitation wavelength of 297 nm and an emission wavelength of 355 nm, we observed with the HPLC system that BLM B₂ was separated readily from BLM dB₂ with the K' of the parent compound and its metabolite being 5.2 and 4.0, respectively (Chart 1). The separation was very dependent upon the organic:aqueous ratio in the mobile phase with small changes in the ratio resulting in major differences in the K'. The fluorescence intensities for both BLM B₂ and BLM dB₂ were similar, and both compounds displayed linear integrated peak areas over at least a 10-fold concentration range (Chart 2). Less than 2 ng of both BLM B₂ and BLM dB₂ could be detected easily with flow fluorescence. When compared to UV detection, flow fluorescence detection was at least 10-fold more sensitive than UV absorption at 254 nm. Since Yoshioka et al. (24) had previously described the enzyme activity in rat liver, this tissue was examined first. When 105,000 x g supernatant fractions from homogenates of rat liver (200 µg of protein) were incubated at 37° for 30 min with 50 µg of BLM B₂ per ml, a peak appeared with the same retention time as BLM dB₂ and with a concentration, as determined by the integrated area, corresponding to that lost in the BLM B₂ peak (Chart 3, A and B). Furthermore, the peak was not present in the reaction mixture containing BLM B₂, which was not incubated (Chart 3B), nor was it seen in the homogenates incubated for 30 min without BLM B₂ (Chart 3C). The peaks, which appeared prior to 5 min, were contributed by homogenate components but were well separated from the BLM peaks. Thus, rat liver homogenates could metabolize BLM B₂, and the formation of BLM dB₂ could be assayed easily with this HPLC technique.

Human ovarian tumor (Patient B) was homogenized and prepared as described previously for rat liver, and the sample was incubated for 2 to 8 hr with 50 µg of copper-free BLM B₂ per ml (A and D), by not incubating but including BLM B₂ (B and E), or by incubating at 37° without substrate (C and F). The activity was heat sensitive, being lost completely in boiled samples. The BLM dB₂ peak was not observed in the reaction mixture that was incubated without BLM B₂, but a peak at 4 min did appear after incubation, which was derived from a component in the homogenate (Chart 3F). Thus, human tumors did have the capacity to metabolize BLM. No metabolism or loss of BLM dB₂ was observed when BLM dB₂ was incubated at 37° for 18 hr with the human tumor homogenate. Since high molar ratios of DNA to BLM might quench BLM fluorescence (8), we examined whether there was sufficient
DNA in the homogenate to alter BLM metabolism or fluorescence intensity. The average DNA content in the 105,000 × g supernatant fraction of homogenates was 51.8 μg/ml, and preincubation of the reaction mixture for 30 min at 25° with 20 μg/ml deoxyribonuclease I (2100 units/mg; Worthington Biochemical Corp.) did not increase either the recovery of or the metabolism of BLM B2 (data not shown). Thus, it appears unlikely that endogeneous DNA interfered markedly with the reaction or the assay.

**BLM Hydrolase and Soft-Agar Assays.** BLM hydrolase activity was measured in homogenates of 14 human tumors to determine the range of activities in different patients. With all tumor homogenates, 2 or more incubation times were examined to ensure that the reaction was linear with time. Chart 4 illustrates the activities found in 2 representative tumors compared to that seen with rat liver. Once the reaction was found to be linear with time, the activity in each tumor was assayed at 2 or more protein concentrations. Product formation depended upon protein concentration for all tumors assayed, and the results from 3 representative tumor homogenates are compared to rat liver homogenates in Chart 5. BLM hydrolase activity from a variety of patients who differ in age and tumor type was compared to the response to BLM, as measured in soft agar (Table 2). BLM hydrolase activity when normalized to protein varied more than 7-fold in tumor samples from different patients and was found in both benign and malignant tumor tissue. In all human samples examined, the BLM hydrolase activity was less than that seen with rat liver (109 ng/min/mg protein) using the same substrate concentration. When normalized to tumor homogenate DNA, BLM hydrolase activity varied more than 100-fold, and 3 samples had more activity than that seen in rat liver (50.8 ng/min/μg DNA). The large differences in protein:DNA ratios in individual tumors presumably reflect the varied sources, tumor types, cellularities, growth rates, and viabilities. Regardless of how the enzyme activity was normalized, BLM hydrolase activity did not correlate with viability, as measured by trypan blue, or the ability of tumors to grow in soft agar. The response to BLM was deter-

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**Chart 4.** Time dependency for the formation of BLM dB2 by homogenates of rat liver and human tumors. Rat liver and human tumors were homogenized and centrifuged at 105,000 × g for 1 hr. Low-molecular-weight material was removed from the resulting supernatant fraction and incubated with 50 μg of BLM B2 per ml for various periods of time. ●, rat liver (200 μg protein); ○, Patient B (400 μg protein); □, Patient H (800 μg protein).

**Chart 5.** Product formation and protein amount. The 105,000 × g supernatant fraction of rat liver and human tumor homogenates were incubated for 1 to 2 hr and for 2 to 4 hr, respectively. ●, rat liver; △, Patient B; □, Patient H; ○, Patient I.

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**Table 2**

<table>
<thead>
<tr>
<th>Patient</th>
<th>BLM hydrolase</th>
<th>Colons</th>
<th>Colony formation (% of untreated control) in the presence of BLM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/min/mg protein</td>
<td>ng/min/μg DNA</td>
<td>% viability</td>
</tr>
<tr>
<td>A</td>
<td>62.9 ± 1.3 b</td>
<td>75.4 ± 1.6 b</td>
<td>48</td>
</tr>
<tr>
<td>B</td>
<td>50.3 ± 6.3</td>
<td>114.3 ± 14.3</td>
<td>54</td>
</tr>
<tr>
<td>C</td>
<td>47.2 ± 5.0</td>
<td>20.3 ± 2.1</td>
<td>55</td>
</tr>
<tr>
<td>D</td>
<td>38.1 ± 9.8</td>
<td>2.4 ± 0.6</td>
<td>72</td>
</tr>
<tr>
<td>E</td>
<td>37.9 ± 3.4</td>
<td>76.8 ± 6.9</td>
<td>93</td>
</tr>
<tr>
<td>F</td>
<td>36.3 ± 1.4</td>
<td>5.8 ± 0.2</td>
<td>83</td>
</tr>
<tr>
<td>G</td>
<td>35.3 ± 2.4</td>
<td>14.1 ± 0.8</td>
<td>96</td>
</tr>
<tr>
<td>H</td>
<td>25.9 ± 3.6</td>
<td>19.9 ± 2.9</td>
<td>92</td>
</tr>
<tr>
<td>I</td>
<td>24.3 ± 1.7</td>
<td>4.6 ± 0.3</td>
<td>74</td>
</tr>
<tr>
<td>J</td>
<td>22.9 ± 2.1</td>
<td>36.3 ± 3.3</td>
<td>85</td>
</tr>
<tr>
<td>K</td>
<td>19.7 ± 3.5</td>
<td>25.1 ± 4.5</td>
<td>94</td>
</tr>
<tr>
<td>L</td>
<td>15.4 ± 2.9</td>
<td>0.9 ± 0.2</td>
<td>96</td>
</tr>
<tr>
<td>M</td>
<td>9.0 ± 0.0</td>
<td>3.5 ± 0.0</td>
<td>92</td>
</tr>
<tr>
<td>N</td>
<td>8.6 ± 0.8</td>
<td>4.1 ± 0.4</td>
<td>93</td>
</tr>
</tbody>
</table>

Viability based upon trypan blue exclusion of dissociated cells prior to drug treatment and growth in soft agar.

Mean ± S.D. (N = 3 to 8).

NG, no growth.

ND, not determined.
mined by comparing colony formation in soft agar after a 1-hr exposure to 2 different concentrations of BLM. In the 7 ovarian tumor samples examined, 4 formed colonies in culture with 2 (Patients I and J) exhibiting more than a 50% inhibition in colony formation after BLM treatment in culture. Seven samples were obtained from the area of the squamous epithelium of the lower female pelvic region: 2 vaginal and 2 cervical squamous cell carcinomas; a metastatic gastric and a metastatic colonic adenocarcinoma; and a benign schwannoma. Five of the 7 samples formed colonies, including a benign schwannoma, and one sample (Patient H) displayed a 98% decrease in clonal formation after exposure to the drug. Although benign tumors are not generally believed to grow well in soft agar, previous work by Bradley et al. (5) demonstrated that some nonmalignant tissues could form colonies in soft agar, and our work confirmed their findings. Although BLM response among the tumors varied more than 100-fold, no correlation between BLM hydrolase activity and response to BLM in soft agar was observed.

**DISCUSSION**

The cytotoxic mechanism of action of BLM has not been completely established, but it is believed to be related to the action of the drug on the functional and physical integrity of DNA (11, 19). One major determinant of DNA damage should be the length of time DNA is exposed to BLM. This exposure time may depend upon the cellular uptake and the rate of BLM inactivation. Work by Miyaki et al. (12) and Akiyama et al. (1, 2) indicated that inactivation was the important factor limiting the antitumor activity of BLM. Umezawa et al. (22) suggested that inactivation of BLM was mediated by an aminopeptidase B-like activity located in the 105,000 x g supernatant fraction of organ homogenates, which was called BLM hydrolase. Although the enzyme activity cleaved the N-terminal region of that inactivation of BLM was mediated by an aminopeptidase B-like activity located in the 105,000 x g supernatant fraction of organ homogenates, which was called BLM hydrolase. Al though the enzyme activity cleaved the N-terminal region of BLM, differences in the C-terminal region apparently were important since BLM B2 appeared to be a better substrate than either bleomycin A2 or A5 (22, 23). Purification of the murine liver enzyme to homogeneity was not successful because the activity was unstable, and the possibility of more than one enzyme activity has not been excluded.

HPLC methods to separate BLM from the deaminated metabolite have been described by Yoshioka et al. (24) and Muraoka (14), which utilize UV absorption detection (254 nm). However, because a large number of cellular components absorb at 254 nm, care must be taken to ensure that the sample is free of contaminating and comigrating substances. Chien et al. (8) reported previously that the bithiazole ring of BLM imparts it with fluorescence properties, and in our HPLC mobile phase, both BLM B2 and BLM dB2 displayed excitation maxima of 297 nm and emission maxima of 353 and 355 nm, respectively. The technique described here combines the selectivity and sensitivity of fluorescence spectroscopy with the speed of HPLC. This assay, which can be conducted with less than 100 mg of tissue, is at least 10 times more sensitive than previously described techniques and allowed us to establish that a variety of different human tumors do have the capacity to metabolize BLM. Furthermore, as with rat liver homogenates, the BLM hydrolase activity appears to reside almost exclusively in the high-molecular-weight (greater than 25,000) material of the 105,000 x g supernatant fraction of human tumor samples. Human tumor homogenates also do not seem capable of metabolizing BLM dB2.

BLM inactivation may not always reflect BLM hydrolase activity. The 2 principle inactivation assays, antibacterial activity (22) and inhibition of DNA polymerase I (13), presumably require DNA strand breakage, which is influenced by a number of molecular species including divalent metal ions (17, 21) and which can be repaired by exonuclease III and, perhaps, other enzymes (15). Yoshioka et al. (24) reported that in rat heptoma cells BLM could be inactivated by a substance other than BLM hydrolase, although work by others (1) has not confirmed its presence in Chinese hamster ovary cells. Muller et al. (13), using an in vitro DNA polymerase assay, found that human carcinomas from the head and neck region could inactivate BLM. Our results indicate that a variety of human tumors can metabolize BLM and all seem to have activities/mg protein that are less than that seen in normal rat liver. In contrast to the results of Muller et al. (13) who found that the level of BLM inactivation varied according to the stage of tumor differentiation, we observed in our study of tumors from the female pelvic region no obvious relationship between differentiation and BLM hydrolase activity. Patients A and M, for example, both had moderately differentiated squamous-cell carcinomas and had widely different enzyme activities. Furthermore, both benign and poorly differentiated tumors were found with BLM hydrolase activities intermediate in value to the activities observed in moderately differentiated tumors. Because the histological appearance of a cancer may vary depending upon the site of tumor biopsy, more extensive studies should be undertaken to determine the relationship between BLM hydrolase activity and BLM inactivation in human tumors and their relationship to degree of differentiation.

Since the metabolism of BLM may be an important determinant limiting the antitumor activity of BLM, we examined a limited number of human tumors for their responsiveness to BLM in culture. Clonal assays have been extremely useful in evaluating both murine and human tumor responsiveness to the cytotoxic actions of anticancer agents (6, 10, 16, 18). Although all the human tumors were capable of metabolizing BLM, no correlation was observed between BLM hydrolase activity and sensitivity of human tumors to BLM, as measured by colony formation in soft agar (Table 2). Of the 9 tumors that formed colonies, 4 (Patients H, I, J, and K) showed more than a 50% decrease in the number of colonies after BLM treatment, and all of these samples had intermediate BLM hydrolase levels. It seems unlikely that this lack of correlation was related to the use of clinical-grade BLM (Blenoxane) in the soft-agar assay, since the other major components of the clinical mixture also appear to be metabolized by this enzyme activity, although perhaps at a somewhat slower rate (22, 23). It is difficult to relate these data to clinical responsiveness, however, since inhibition of colony formation in soft agar may not always be tantamount to a clinical response and since none of our patients were treated with BLM. Further work with patients that are to be treated with BLM should be conducted to determine the usefulness of this BLM hydrolase assay for predicting response in vivo.

Work with Chinese hamster ovary cells (1) suggested that BLM hydrolase may be responsible for resistance to BLM since resistant cells had elevated levels of BLM hydrolase. In our human tumor samples, the range in both BLM hydrolase activ-
It must be noted, however, that the Chinese hamster ovary cells used in the previous studies (1) were continuously cultured cell lines and had a much higher cloning efficiency than our human tumor cells. Since human tumors contain clonogenic and nonclonogenic tumor cells, in addition to normal cells, it is possible that the BLM hydrolase activity of the clonogenic cells was markedly different from the nonclonogenic cells. Furthermore, the enzyme activity may be located partially in the extracellular material that was digested during the single-cell preparations. Alternatively, other mechanisms of resistance may be operative. Yoshioka et al. (24) reported that AH-66 rat hepatoma cells contained a low-molecular-weight substance that inactivated BLM but which was not BLM hydrolase. Akiyama et al. (1), however, were unable to confirm the presence of this substance in Chinese hamster ovary cells. Although no BLM hydrolase activity was detected in the low-molecular-weight material of the 105,000 × g supernatant fraction of human tumor samples, the presence of other inactivating mechanisms in human tumor tissue cannot be excluded. It is also possible that human tumors have a variety of mechanisms which alter the toxicity of BLM, such as endogenous inhibitors of the enzyme activity, increased DNA repair, poor BLM uptake, or varying intracellular concentrations of sulfhydryl compounds or Fe(II) (14). The sensitive HPLC technique described here, which requires relatively small amounts of tissue, should be useful in further evaluating the importance of BLM hydrolase in modulating the toxicity of BLM to both malignant and normal host tissues.

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