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MITOCHONDRIAL DNA DAMAGE BY BLEOMYCIN

LORI O. LIM* and ALLEN H. NEIMS
Department of Pharmacology and Therapeutics, College of Medicine, University of Florida, Gainesville, FL 32610, U.S.A.

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Abstract—We have modified a specific and sensitive method of detecting different forms of mitochondrial DNA (mtDNA) and utilized it to study bleomycin (BLM)-induced mtDNA damage. Intact, nicked circular, and linear forms of mtDNA were separated by gel electrophoresis, detected by Southern blot hybridization, and characterized by size markers and alkali treatment. DNA from BLM-treated mitochondria from liver, lung, and L1210 tumors were all equally sensitive to damage by BLM. The extent of BLM-induced mtDNA damage was dependent on experimental conditions. *In vitro* incubation of isolated mitochondria with BLM showed that 100 μM BLM caused complete conversion of intact to nicked and linear forms. Scission of mtDNA was more extensive if mitochondria were lysed in the presence of BLM than after several washings to remove the drug from the incubation media. Isolated mtDNA was extremely sensitive to BLM such that 10 nM BLM caused loss of intact form. The extent of mtDNA damage by BLM was decreased by the addition of EDTA.

Bleomycin (BLM) is used in the treatment of certain types of squamous cell tumors and lymphomas [1]. However, its use is associated with serious pulmonary, skin, and bone marrow toxicity [2]. While it is well established that BLM causes nuclear DNA damage and cell death [3], there is little information on direct effects of BLM on mitochondria and mitochondrial DNA (mtDNA). After BLM treatment, mitochondrial damage, such as swelling [4], hypertrophy [5, 6], and decrease in number [7], has been observed. In 1976, Osieka *et al.* [8] reported that mouse fibroblasts treated with BLM for 24 hr exhibited mtDNA damage with conversion of closed circular to open circular forms.

Mitochondrial DNA is a closed circular double-stranded molecule consisting of 16 kilobase pairs, and it codes for various RNA species, cytochrome *b*, and subunits of cytochrome *c* oxidase, ATPase, and complex I of the respiratory chain [9, 10]. Published reports have indicated that the mtDNA may be an important target for several known mutagens and carcinogens [11–18]. Unlike nuclear DNA, mitochondrial DNA is not associated with histones, and a repair mechanism has not been demonstrated [19].

In this report, we present modifications of the method by Singh *et al.* [20] to determine mtDNA damage as well as studies of the effects of BLM on mtDNA under various experimental conditions. Our results show that BLM caused primarily single-stranded breaks of mtDNA. However, mtDNA in intact mitochondria was more resistant than isolated mtDNA to damage by BLM.

* Address all correspondence to: Lori O. Lim, Ph.D. Present address: Pesticide Research Laboratory, University of Florida, Gainesville, FL 32611.

† Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate; and BSA, bovine serum albumin.

METHODS

Materials. Unless specified, reagents were obtained from either the Sigma Chemical Co. (St. Louis, MO) or Bethesda Research Laboratories (Gaithersburg, MD).

Isolation of mitochondria. Liver and lung mitochondria were prepared from C57B/6J or DBA (7–10 weeks old) adult male mice obtained from Jackson Laboratories (Bar Harbor, ME). L1210 solid tumors were obtained by subcutaneously injecting murine leukemic L1210 cells (10^6 cells/0.1 ml) into the thigh of DBA mice and allowing the tumor to develop for 14 days. The tissues were homogenized with a Potter–Elvehjem homogenizer in a buffer containing 0.25 M sucrose, 3 mM Hepes†, and 1 mM EGTA, at pH 7.4. The homogenate was centrifuged at 900 *g* for 10 min. The mitochondrial fraction was prepared from the supernatant fraction by centrifugation at 9000 *g* for 10 min. The pellet was washed twice with the homogenization buffer. Protein was measured by the biuret method [21].

Mitochondrial respiration. Mitochondrial function was determined by respiration measured polarigraphically with an oxygen electrode connected to a YSI oxygen monitor (Yellow Springs, OH) [22]. Respiratory rates for state 4 (resting state), state 3 (ADP-stimulated) and dinitrophenol uncoupled state were determined at 30° in a reaction mixture (2 ml) containing 0.2 M sucrose, 20 mM KCl, 5 mM $MgCl_2$, 3 mM Hepes (pH 7.4), 5 mM potassium phosphate, 0.1 mM ADP, and 10 mM substrates (glutamate or succinate), in the presence or absence of 100 μM BLM. Bleomycin sulfate mixture (Sigma Chemical Co.) was dissolved in water. The extinction coefficient of bleomycin at 290 nm was $1.5 \times 10^4\ M^{-1}\ cm^{-1}$.

Incubation conditions for mitochondrial DNA studies. Isolated mitochondria (1 mg) were added to a reaction medium containing 250 mM sucrose, 20 mM KCl, 5 mM $MgCl_2$, 3 mM Hepes (pH 7.4), in

the presence or absence of BLM (0.1 μM to 100 μM), in a final volume of 250 μl, and incubated for 60 min at 37°. After incubation, mitochondria were washed once by centrifugation in a microcentrifuge at 12,000 *g* for 30 sec and resuspended in 250 μl of fresh reaction medium, unless otherwise specified. This washing procedure removed most of the free drug in that less than 0.25 μM (limit of detection by spectrophotometry) was found in the wash supernatant.

Isolated mtDNA (125 ng) was also incubated with BLM (0.01 to 10 μM) in the presence or absence of EDTA (1–3 mM with pH adjusted to 7.0) at 37°. After incubation, a dye solution containing 30% glycerol, 0.25% bromophenol blue, and 1 mM EDTA was added to the samples for electrophoresis.

Isolation of mitochondrial DNA. Mitochondria were solubilized by treatment with SDS (1%), 1 mM EDTA and 100 μg/mg proteinase K (Bethesda Research Laboratories) at 65° for 20 min. The mtDNA was extracted with 5X volume of phenol–chloroform–isoamyl alcohol (25:24:1) saturated with 0.1 M Tris and 0.2% β-mercaptoethanol, and then chloroform and isoamyl alcohol (24:1). The DNA was precipitated with ice-cold 95% ethanol, collected by centrifugation, and vacuum dried. The final DNA pellet was resuspended in 60 μl of 10 mM Tris (pH 7.4) and 1 mM EDTA buffer. MtDNA to be incubated directly with BLM was isolated by the procedure described above except that lysis was done in sodium dodecyl sulfate (SDS) without EDTA. and the final DNA pellet was resuspended in distilled water. The recovery of mtDNA by this procedure is greater than 80% based on 0.5 μg mtDNA per mg mitochondrial protein [23, 24] and comparison with a known amount of supercoiled pBR 322.

Detection of mitochondrial DNA. MtDNA (125 ng) was analyzed by electrophoresis in 0.7% agarose gels with 0.89 mM Tris–0.89 mM borate–10 mM EDTA buffer at 100 V for 3 hr. Following denaturation in 1.5 M NaCl and 0.5 M NaOH for 1 hr, and neutralization with 1 M Tris and 1.5 M NaCl for 1 hr, the gel was blotted onto a nitrocellulose filter for 16 hr [25]. After drying in an 80° oven for 2 hr, the blot was prehybridized for 6 hr at 68° in 30 ml of 6X SSC (3 M NaCl and 0.3 M sodium citrate), 0.6 ml of 50X Denhardt's solution (1% Ficoll 400, 1% polyvinylpyrrolidone, and 1% BSA), and 900 μg of heat-denaturated yeast tRNA. The blot was then hybridized for 16 hr at 68° in 15 ml of 6X SSC and 0.2 ml of 50X Denhardt's solution, 100 μg heat-denatured yeast tRNA, and 1 μg of heat-denatured biotinylated mtDNA probe. The mitochondrial DNA probe consisted of the entire mouse liver mtDNA genome with plasmid pSP64 as the vector cloned into *Escherichia coli* HB 101 (originally obtained from Dr. William Hauswirth, Department of Immunology and Medical Microbiology, University of Florida). For hybridization, the probe was nick-translated with dCTP, dGTP, dATP, and biotinylated dUTP (Bethesda Research Laboratories) in the presence of 200 pg DNase 1 and 2 units of *E. coli* DNA polymerase I at 16° for 90 min. After nick translation, the reaction was stopped by EDTA at a final concentration of 15 mM, and unincorporated nucleotides were removed by Sephadex G-50 spin column.

Mitochondrial DNA on the blot was detected by a colorimetric technique [26] adapted from Leary *et al.* [27]. This technique involved conjugating the hybridized biotinylated probe to streptavidin and then allowing the streptavidin to react with biotinylated alkaline phosphatase. Hybridized DNA was visualized as purple bands from the reduction of nitroblue tetrazolium and bromochloro-indolyl-phosphate to an insoluble precipitate. Limit of detection was 1 ng without correction for recovery and allowing only 1 hr for color development. For comparison, a duplicate blot with isolated mtDNA was hybridized to a ^{35}S-labeled dATP nick-translated probe and showed the identical banding pattern (data not shown).

Assignment of mtDNA forms (form I, closed circular; form II, nicked; and form III, linear) was based on a comparison of bands to size markers (pBR 322 supercoiled ladder 2–16 kb and lambda phage digested with Hind III linear fragments 0.5–23 kb, Bethesda Research Laboratories), alkali treatment (0.1 N NaOH for 5 min), heat treatment (90° for 5 min), and S1 nuclease (Bethesda Research Laboratories) digestion (1.2 units/125 ng mtDNA in a buffer containing 30 mM sodium acetate, 50 mM NaCl, and 1 mM $ZnSO_4$, pH 6, at 37° for 10 min).

Quantitation of hybridized DNA in terms of total mtDNA, represented as total area in arbitrary units as well as percent of total mtDNA in each form, was by scanning a negative film of the blot with a Zeineh Soft Laser Scanning Densitometer connected to an Apple Computer and Zeineh Videophoresis II program (BioMed Instruments, Inc., Fullerton, CA). Even though forms I and II were distinguished visually on the blot, they were not separable by densitometry and thus were quantified together. Form III DNA was from a single cut (16 kb) to the linear strand, and multiple hit fragments appeared as smears below form III.

RESULTS AND DISCUSSION

We have modified the method reported earlier from our laboratory [20] for the detection of damage to mitochondrial DNA. The procedures we present in this report offer several improvements: (1) the use of the entire mouse mtDNA genome as the probe, (2) the small amount of tissue required (as little as 0.25 mg of mitochondria may be used per assay), and (3) the use of non-radioactive biotinylated nucleotides for the nick translation of the probe.

By comparison with known size markers and various treatments of the mtDNA sample, the forms of mtDNA were easily distinguished. Double-stranded linear molecules (form III) were identified by comparing their location with those of lambda phage Hind III fragments 23 and 9 kb (Fig. 1a) and were confirmed by the appearance of a single band at the same location after restriction enzyme digestion with Bgl II which cleaves mtDNA at only one site (data not shown). With our electrophoresis conditions, form III migrated further than intact (form I) and nicked circles (form II). Form I was identified by matching its location with 16 kb supercoiled pBR 322. Both alkali and heat denaturation (Fig. 1b, lanes 2 and 3) converted nicked circles to single-stranded

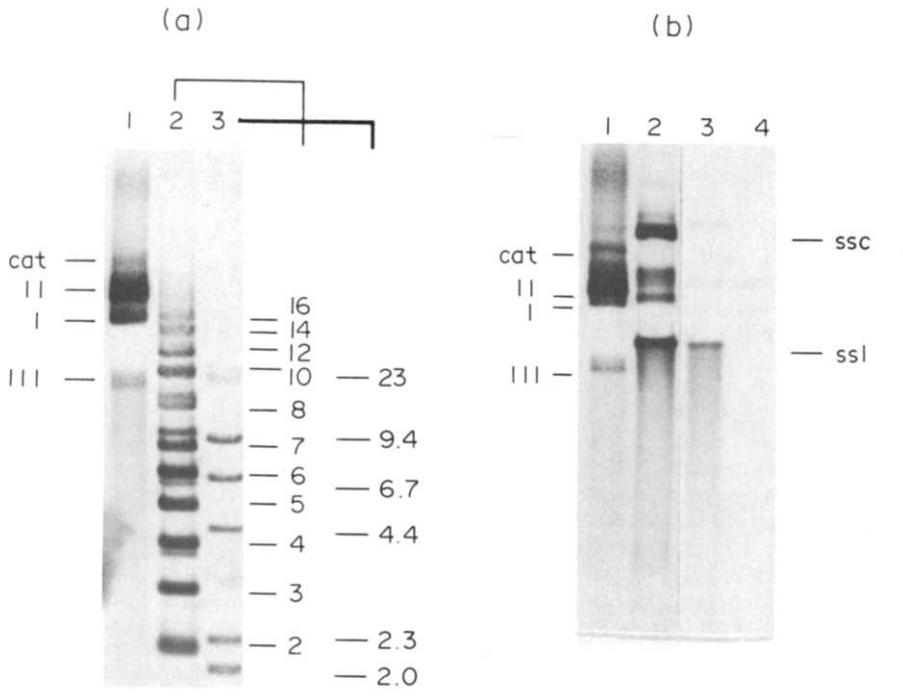

Fig. 1. Comparison of mitochondrial DNA with DNA markers and assignment of mitochondrial DNA forms. (a) Lane 1: isolated mouse (C57B/6J) liver mtDNA, Lane 2: a pBR 322 supercoiled ladder, and Lane 3: biotinylated lambda phage DNA digested with Hind III. (b) Isolated mouse liver mtDNA that was untreated (lane 1), alkali denatured (lane 2), heat denatured (lane 3), and heat denatured and then digested with S1 nuclease (lane 4). Forms of mtDNA are: I (intact circular), II (single nicked), III (double-stranded linear), ssc (single-stranded circular), ssl (single-stranded linear), and cat (catenates).

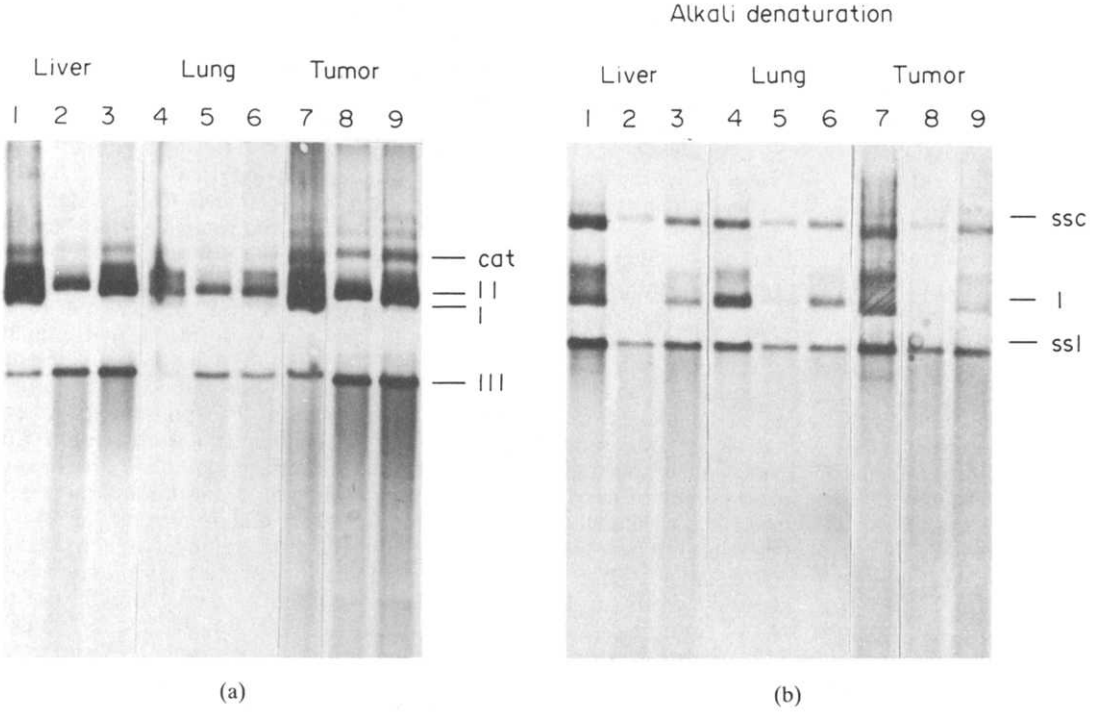

Fig. 2. Mitochondrial DNA from DBA mouse liver, lung, or tumor L1210 mitochondria treated with BLM. (a) Lanes 1, 4 and 7 are untreated controls. Lanes 2, 5 and 8 are BLM at 100 μM. Lanes 3, 6 and 9 are BLM at 10 μM. Mitochondria were isolated from the tissues as described in Methods. They were then incubated with BLM for 60 min at 37°. (b) MtDNA samples from (a) were alkali denatured before gel electrophoresis.

species with single-stranded circles migrating more slowly than single-stranded linears. The differentiation of single-stranded circle from linear was based on the analogy with the decreased mobility of the linear fragments from lambda phage-Hind III after alkali denaturation (data not shown). Further confirmation of the single-stranded forms involved S1 nuclease digestion of heat-denatured DNA, a procedure that resulted in the loss of the single-stranded forms (Fig. 1b, lane 4). The band which is labeled "cat" is tentatively identified as catenates, since these complex forms are known to migrate more slowly than forms I and II [28], although other forms such as oligomers may be present.

To begin to explore whether or not BLM may be directly toxic to mitochondria, we examined the effects of BLM on mitochondrial oxidative phosphorylation and mitochondrial DNA in different tissues, such as liver, and potential target tissues including lung and tumor. Respiration studies with isolated DBA mouse liver and tumour mitochondria and C57B/6J liver mitochondria treated with BLM up to 100 μM did not show any effect in the respiratory states (states 4, 3 and uncoupled state) using either glutamate or succinate as substrate (data not shown).

Mitochondrial DNA, on the other hand, was sensitive to BLM. With our procedures of isolation and detection of mtDNA, amounts of mtDNA recovered per mg mitochondrial protein from liver and tumor were similar, whereas that for lung was slightly less (Fig. 2). In all tissues, treatment of isolated mitochondria with BLM at 0.1 μM did not have an obvious effect, but strand breaks clearly demonstrated by loss of form I and increase in form III were apparent at higher concentrations. As concentration increased from 0.1 to 10 μM, there was a progressive increase of form III (Fig. 2a). And at 100 μM, complete conversion of mtDNA to nicked forms (form II) was detected (Fig. 2a, lanes 2, 5 and 8). Alkali denaturation confirmed the presence of primarily form II in these samples since no form I was observed (Fig. 2b, lanes 2, 5 and 8). Heat denaturation of BLM-treated DNA yielded the same banding pattern as alkali denaturation, indicating that BLM did not induce alkali labile sites (data not shown). Our results thus show that, with respect to strand scission, BLM caused primarily single-stranded scissions in mtDNA, although form III from direct double-stranded breaks may also be present. It should be noted that single-stranded scissions probably occurred at multiple sites on the double-stranded DNA molecule such that alkali denaturation resulted in a decrease in total mtDNA due to small fragments which are lost under the present electrophoresis conditions. Povirk *et al.* [29] showed that, while BLM causes mainly single-stranded damage in supercoiled Col E1 DNA, frequency of double-stranded breaks measured is higher than expected from single-stranded breaks in both strands at close proximity. Whether there is site specificity in the strand break, as observed in other forms of DNA, requires the isolation of form II band and analysis by restriction enzymes or site specific probes. We found neither tissue nor species difference in the sensitivity of mtDNA to BLM.

Mitochondrial DNA in the mitochondrion was more resistant to BLM damage than isolated mitochondrial DNA. With isolated mtDNA, 10 nM BLM caused complete conversion of mtDNA to form II (Fig. 3, lanes 2 and 4); 10^4 times more BLM (100 μM) was necessary to obtain the same extent of damage with intact mitochondria (Fig. 2, a and b, lane 2). Murray and Martin [30] reported a 30-fold increase in BLM (100 μM) required for the degradation of K562 human cell DNA *in situ* compared to that (3 μM) for isolated DNA *in vitro*. Our results also indicate that isolated mtDNA sensitivity to BLM is similar to that for other purified DNAs (for example, Refs. 31 and 32) under slightly different experimental conditions.

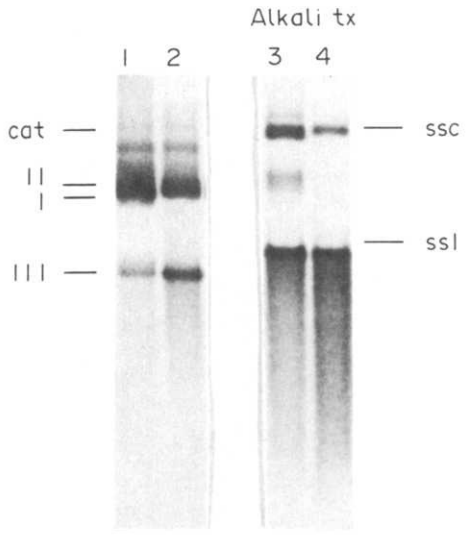

Fig. 3. Isolated mouse (C57B/6J) liver mtDNA treated with BLM and after alkali denaturation (lanes 3 and 4). Lanes 1 and 3 are untreated control, and lanes 2 and 4 are 10 nM BLM. Isolated mtDNA was incubated with BLM for 60 min at 37° and then loaded on agarose gel for electrophoresis.

Further studies showed that the resistance of mtDNA in intact mitochondria to BLM damage as measured by form III and fragments may be due to the possibility that BLM is not readily taken up into mitochondria. As shown in Fig. 4(a–c), untreated samples showed means of 20, 57, 9 and 13% in catenates, forms I and II, form III, and fragments respectively. The extent of damage to mitochondria by BLM depended on the presence of BLM in the media after incubation. The presence of BLM in solution after incubation (no wash) resulted in a greater degree of damage [complete loss of catenates, only 11% in forms I and II, and increases in both form III (19%) and fragments (62%)] than when unbound BLM was removed by repeated centrifugation (one to three washes) after incubation (Fig. 4, b and c). With one washing, 47% of total mtDNA was in form III and fragments. After the third washing, only 45% of total DNA was in form III (30%) and fragments (15%) (Fig. 4c) compared to 81% from no washing (Fig. 4a). Since there was no appreciable difference in the extent of damage after the first washing (47%) and the third washing (45%), the difference in percent as form III and fragments between the treated (45%) and control

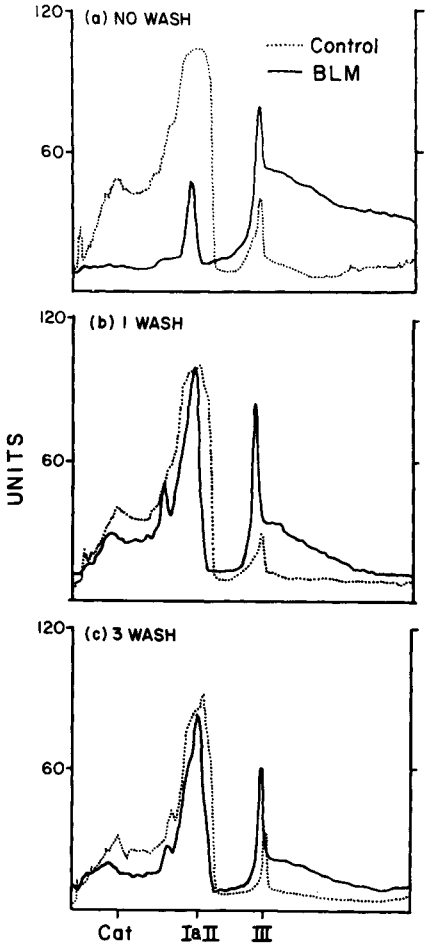

Fig. 4. Effects of washing of treated mouse (C57B/6J) liver mitochondria on the extent of mtDNA damage by BLM. Control and BLM (100 μM) treated mitochondria were either lyzed immediately (a, no wash) or washed by centrifugation and resuspension for one or three times before lysis and then processed for mtDNA isolation (b and c, respectively). The negative film of the blot was quantified by laser densitometry with the y-axis in arbitrary units. Total areas for control and BLM were 9184 and 8715, 7777 and 8320, and 5868 and 5848 for no wash, one wash, and three washes respectively.

(22%) then represented the damage that occurred during incubation. The extensive strand scission detected when BLM was not removed after incubation was then probably due to additional damage by BLM after lysis of the mitochondrial membrane. Thus, the most likely explanation for the decrease in sensitivity of mtDNA in mitochondria is that the mitochondrial membrane was a barrier to limit the uptake of BLM by the mitochondrion. Mitochondrial membrane as a barrier for chemicals has also been demonstrated for adriamycin where inhibition of mitochondrial respiration was greatly enhanced in mitochondria permeabilized with digitonin or osmotic shock [33]. We found that adriamycin under experimental conditions similar to those for bleomycin did not cause any strand scission in mtDNA after incubation with isolated liver, heart, or L1210 tumor mitochondria (data not shown).

In vitro studies were initiated to determine whether BLM-induced mtDNA damage involves metal ions as has been shown for DNA from other sources (for example, Refs. 34 and 35). Our results with isolated mtDNA showed that mtDNA damage by BLM was inhibited by EDTA. BLM (0.1 to 10 μM) caused total degradation and loss of mtDNA resulting in 100% in form III and fragments. While EDTA alone, up to 3 mM, had no effect on mtDNA (80% as forms I and II, and 3% as form III), addition of 1 mM EDTA prevented strand scission by 0.1 and 1 μM BLM. However, EDTA at 3 mM did not inhibit completely DNA damage by 10 μM BLM. Preliminary results with ferrous ammonium sulfate showed that BLM-induced mtDNA damage was enhanced (data not shown). These results are consistent with the hypothesis that BLM-induced DNA scission involves metal ions. The presence of EDTA probably chelates the iron required for the iron-catalyzed reactions in the formation of reactive oxygen intermediates. Further studies on the effects of iron, iron chelators, and oxygen radical scavengers are needed to clarify the mechanism of strand scission by BLM in mtDNA.

Our results showed that BLM at 10 μM caused damage to mtDNA from isolated mitochondria of both liver and known target tissues, lung and tumor. Even though this concentration is lower than the 140 μM required for mtDNA damage in mouse fibroblasts (L cells) exposed to BLM for 24 hr [8], it is much higher than the nanomolar concentration generally required for cytotoxicity. The lack of tissue specificity and resistance of mtDNA in intact mitochondria to BLM damage in the present study showed that BLM cytotoxicity is not due to mtDNA damage. Furthermore, there are a thousand or more copies of mtDNA in a cell and, at the present time, the extent of damage that is required for lethality of the exposed cells or its progeny is unknown.

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