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Quantitative Measurement of Single- and Double-Strand Breakage of DNA in *Escherichia coli* by the Antitumor Antibiotics Bleomycin and Talisomycin

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We developed an assay in which single-strand breakage (ssb) and double-strand breakage (dsb) of intracellular DNA by chemical agents can be accurately quantitated and differentiated. *Escherichia coli* cells containing plasmid pBR322 DNA were incubated with the antitumor antibiotics bleomycin A_2 (BLM A_2) or talisomycin A (TLM A). The plasmid DNA was isolated and then analyzed by electrophoresis on 1% agarose gels to separate the following conformational forms of plasmid DNA: (i) native, covalently closed, super helical, form I; (ii) nicked, relaxed circular, form II; and (iii) double-strand broken, linear, form III. Quantitation by densitometric analysis of the gels showed that BLM A_2 and TLM A were equally active in terms of the concentrations of drug necessary to reduce equivalent amounts of form I DNA in the cells, whereas in vitro (using isolated pBR322 DNA as a drug substrate) twofold more TLM A than BLM A_2 was required to produce an equivalent amount of reduction in form I DNA. TLM A produced more intracellular dsb than did BLM A_2. The intracellular dsb activities (dsb/ssb ratio) measured from BLM A_2 and TLM A were equivalent to those measured for the respective agents when isolated pBR322 DNA was used as the substrate. In *E. coli* both ssb and dsb were repaired, but TLM A damage was repaired more slowly and to a lesser extent, which may reflect the relative frequency of dsb.

The bleomycins (BLM) are a group of glycopeptide antibiotics which were first isolated by Umezawa et al. (36) as copper complexes from culture media of *Streptomyces verticillus*. BLM was found to be a potent antibiotic against a variety of microorganisms (10) and active against several neoplasms in humans, both as a single agent and in combination chemotherapy (7). The primary mechanism of action of BLM cytotoxicity appears to be interaction with and subsequent breakage of cellular DNA (3, 9; for reviews see references 35 and 39).

Talisomycin (TLM) is an antitumor antibiotic related structurally to BLM, which contains two new amino acids and a unique sugar, 4-amino-4,6-dideoxy-L-talose that have not been found previously in the BLM complex (24). A TLM congener, TLM S_{10} is the first bleomycinic acid-substituted BLM analog to enter clinical trials (R. Comis, R. Gaver, S. Ginsberg, B. Poiesz, A. Louie, S. D. DiFino, A. Scalzo, Abstr. Proc. Am. Assoc. Cancer Res. **25**:116). Although the mechanism by which TLM produces DNA breaks appears to be similar to BLM, i.e., complex formation with Fe(II) and oxygen and subsequent free radical formation (2, 15, 33), a number of differences between these two groups of antitumor antibiotics have been reported, including differences in their respective antibacterial and antifungal activities (11), in vivo animal antitumor activities (1, 24, 29), pharmacokinetics (37), and cell-cycle phase specificities (18).

Studies from our laboratory and others have demonstrated that the structural alterations near the bithiazole in TLM, which distinguish it structurally from BLM, result in a reduction of the total DNA breakage activity when using isolated PM2 DNA as a substrate (19, 33), enhanced production of double-strand breaks (dsb) relative to single-strand breaks (ssb) (19), as well as a change in the site and sequence specificities of DNA breakage (13, 17, 19, 20–23, 34). These observed differences may have important implications as to their respective cytotoxic and antitumor activities. Therefore, it is important to determine whether these observations, made using isolated DNA, accurately reflect the interactions of these drugs with intracellular DNA.

In this paper, we describe a method whereby the interactions of TLM and BLM with a specific DNA substrate were compared by using both isolated and intracellular DNA. The utility of this method for study of the cellular repair of drug-induced DNA damage was also investigated and the results obtained are discussed with respect to cytotoxic properties of these two structural groups of antibiotics.

MATERIALS AND METHODS

pBR322 DNA isolation. Native plasma pBR322 DNA was amplified in and isolated from *Escherichia coli* JA221 (*hsdM*$^+$ *hsdR lacY leuB6 trpE5 recA1*) by the procedures described by Clewell and Helinski (6). The mutation at the *recA* locus in JA221 was verified by the enhanced sensitivity of this strain to UV and X-irradiation (4) relative to RR1, which is a *recA*$^+$ *E. coli* strain.

Analysis of the degradation of isolated pBR322 DNA. BLM A_2 and TLM A (Bristol Laboratories, Syracuse, N.Y.) were incubated with 2 μg of pBR322 DNA in a buffer containing 10 mM Tris-hydrochloride (pH 7.5), 10 mM NaCl, and 40 mM dithiothreitol in a final volume of 50 μl. This solution was incubated for 30 min at 37°C. The reaction was termi-

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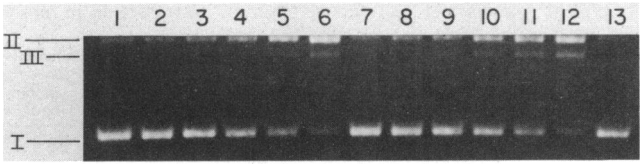

FIG. 1. Agarose gel electrophoresis of pBR322 DNA after incubation with TLM A and BLM A_2 (as described in the text). Lanes: 1, 7, and 13, pBR322 incubated in the absence of drug; 2 through 6, treated with BLM A_2 at of 5, 10, 25, 50 and 100 nM, respectively; 8 through 12, treated with TLM A at of 10, 20, 50, 100, and 200 nM, respectively. I, II, and III (on left), positions of respective forms of pBR322 in the gel.

nated by the addition of 50 μl of solution containing 30 mM Na_2EDTA, 70% glycerol, and 0.05% (wt/vol) bromphenol blue. Samples (50 μl) of the pBR322 DNA-drug reaction mixtures were electrophoretically separated on 1% agarose gels. Electrophoresis was performed in 0.04 M Tris-hydrochloride–0.005 M sodium acetate–1 mM EDTA (pH 7.8) and 5 V/cm for 10 h at room temperature. After electrophoresis, the gels were stained in the dark in electrophoresis buffer containing 0.5 μg of ethidium bromide per ml for a minimum of 2 h. The order of migration of DNA (fastest to slowest) under these conditions was covalently closed, circular DNA (form I); double-strand broken, linear DNA (form III); and single-strand broken (relaxed), circular DNA (form II).

Quantitative measurements of the amount of DNA that had migrated into the gel were based on the fluorescence enhancement after ethidium bromide intercalation. After electrophoresis and ethidium bromide incubation, as described above, the slab gels were photographed on a UV plate. The negative films of the gels were scanned with a RFT model 2955 scanning densitometer (Transidyne General Corp., Ann Arbor, Mich.), and the relative positions and amounts of each of the DNA forms were recorded. Quantitation of the three forms of DNA produced after drug treatment was obtained by measuring the area under the recorded peaks corresponding to the position on the agarose gel for forms I, II, and III pBR322 DNA as determined by control samples (no drug). A correction was made for the decreased fluorescence intensity of form I due to the topological restriction in ethidium bromide binding relative to forms II and III DNA (19).

Analysis of degradation of intracellular pBR322 DNA. *E. coli* containing plasmid pBR322 was grown by the procedure described by Clewell and Helinski (6). After amplification of the plasmid in the presence of chloramphenicol (200 μ/ml), the cells were centrifuged at 3,000 × *g* for 10 min. The cells were then suspended in L broth media (1:20 ml, original culture) and incubated in the presence of increasing concentrations of drug for 30 min at 37°C, after which time deferoxamine mesylate was added to a concentration of 10 mM to inactivate the extracellular TLM or BLM analogs (32) and was present at 10 mM concentration throughout the DNA isolation. The plasmid DNA was isolated by the method of Ratzkin and Carbon (28). The samples were maintained at 0°C during the plasma isolation. The isolated DNA was then separated by agarose gel electrophoresis, and the topological forms of pBR322 DNA were quantitated as described above.

Analysis of repair of drug-induced DNA damage. Cells were incubated with the drugs as described above; however, after a 30-min incubations, the cells were immediately centrifuged at 0°C, and the cell pellet was suspended in L broth media and incubated at 37°C with shaking. At time intervals up to 30 min, samples were removed from the cell suspension, KCN was added to 20 mM to prevent further strand-rejoining repair (26), and the cell mixture was quick-frozen in an ethanol-dry ice bath. When all samples were collected, the cell mixtures were thawed quickly at 60°C, and the plasmid DNA was isolated and analyzed as described above.

RESULTS

Breakage of isolated pBR322 DNA. Agarose gel electrophoresis of pBR322 DNA which was first treated with increasing concentrations of TLM A and BLM A_2 demonstrated the progressive conversion of form I DNA to form II and form III DNA induced by these agents (Fig. 1). Quantitative analysis by densitometry of the ssb and dsb production by TLM A and BLM A_2 treatment shown in Fig. 1 was performed, and the results are shown in Fig. 2. The results demonstrate that at concentrations of drug required to produce equivalent reductions in form I DNA, TLM A consistently produced approximately twice as many dsb than did BLM A_2 (Table 1). This twofold increase in the double-strand breakage activity of TLM relative to BLM was consistently reproduced with different batches of pBR322 DNA and in multiple experiments. Moreover, in contrast to the 12-fold greater sensitivity of PM2 DNA to breakage by BLM A_2 as compared with that by TLM A (19), pBR322 DNA is only 2-fold more sensitive to BLM A_2 than it is to TLM A. TLM $S_{10}b$ (an analog of TLM A in which the carboxyl terminal amine is replaced with putrescine) demonstrated equivalent total pBR322 DNA breakage and also had a twofold increase in dsb activity, relative to BLM A_2.

Breakage of intracellular pBR322 DNA. *E. coli* containing pBR322 plasmids was incubated in the presence of BLM A_2 and TLM A, after which plasmid DNA was isolated and separated on agarose gels as described above. Incubation of the cells with increasing concentrations of TLM A (Fig. 3A, lanes 2 through 6) produced a decrease in form I DNA and an increase in forms II and III compared with cells incubated

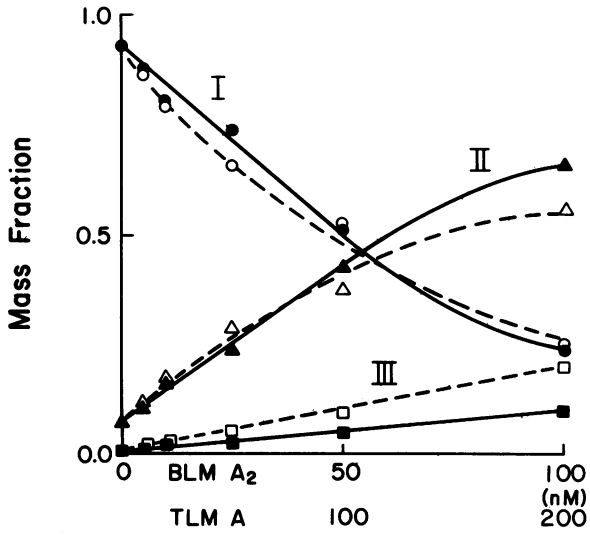

FIG. 2. Mass fractions of pBR322 DNA conformational isomers after treatment with TLM A (open symbols, broken lines) or BLM A_2 (closed symbols, solid lines). Data were obtained from gel pattern shown in Fig. 1. Symbols: ○, form I DNA; △, form II DNA; and □, form II DNA.

TABLE 1. Increases in forms II and III pBR322 DNA after treatment of TLM A and BLM A_2 to cause 50 and 75% reduction in mass fraction of form I DNA

Plasmid DNA type	Drug	Increase in mass fraction[a]:							
		At 50% reduction of form I				At 75% reduction of form I			
		Concn	II	III	III/II	Concn	II	III	III/II
In *E. coli*	BLM A_2	12 μM	0.42	0.08	0.19	24 μM	0.63	0.12	0.19
	TLM A	13 μM	0.35	0.15	0.43	27 μM	0.51	0.24	0.47
Isolated	BLM A_2	55 nM	0.40	0.06	0.15	100 nM	0.58	0.10	0.17
	TLM A	117 nM	0.33	0.13	0.39	194 nM	0.47	0.20	0.43

[a] Increases are calculated by subtracting the mass fractions of form II and III DNA in controls (no drug treatment) from the mass fractions observed at indicated drug concentrations and are expressed in arbitrary units resulting from densitometer scans. Area was quantitated automatically by the densitometer for peaks corresponding to forms of DNA (see text).

in the absence of drug (lane 1). Similar results were obtained with BLM A_2 (Fig. 3B).

Quantitative analysis of the data shown in Fig. 3A and B was performed, and the results are displayed in Fig. 4. The untreated *E. coli* cells contained approximately 99% form I pBR322 DNA and 1% form II. Form III DNA was not detectable. The results demonstrate that, at equivalent concentrations, the extent of reduction of form I DNA by the two drugs was similar. However, TLM A consistently produced more form III DNA than did BLM A_2. In *E. coli* a 50% reduction of form I DNA occurred at 12 μM BLM A_2 and at 13 μM TLM A (Table 1). At both 50 and 75% reductions of form I DNA, the ratio of form III to form II DNA produced was approximately 2 to 2.5 times greater for TLM A than it was for BLM A_2. It is also seen in Table 1 that this 2- to 2.5-fold increase in the ratio of form III to form II DNA produced by TLM A relative to BLM A_2 observed when *E. coli* carrying the pBR322 plasmid was treated is consistent with the results obtained with isolated pBR322 DNA. Although TLM A and BLM A_2 demonstrated equivalent activities in terms of the degradation of form I pBR322 DNA within cells, BLM A_2 was approximately twofold more active than TLM A in degrading isolated form I DNA (Table 1). The total plasmid DNA recovered in each of the preparations (controls and drug treated) was equivalent (±5%), as determined by densitometric scanning and summation of the three conformational forms of plasmid in each of the lanes.

With increasing concentrations of either drug, concomitant increase in what appears to be *E. coli* chromosomal DNA (labeled C) contaminating the plasmid DNA preparations was observed (Fig. 3A and B). Chromosomal DNA does not appear in the preparations from untreated cells because it is attached to the cell membrane (6), and the procedure used to prepare the plasmid removes those complexes from the samples by centrifugation. The smearing pattern, which increased in intensity and length with increasing concentrations of drug, originated from a position in the agarose gel (labeled C in Fig. 3A and B) which was equivalent to the migration distance of *E. coli* DNA isolated by the procedure of Marmur (16) (Fig. 5). Therefore, the smear produced at high concentrations of either drug indicates fragmentation of the *E. coli* chromosomal DNA by the drugs at two or more locations between chromosomal DNA attachment sites on the cellular membrane.

To verify that the DNA breakage observed in these in vivo experiments was the result of lesions occurring intracellularly and not due to DNA breakage by the drugs during the DNA isolation procedure, the following experiments were performed. Either BLM A_2 or TLM A (100 μM) was added immediately before cell lysis to samples which had been previously incubated in the absence of drug (controls). Under these conditions no degradation of plasmid or chromosomal DNA was observed, i.e., the amounts and relative proportions of the forms of DNA were equivalent to that

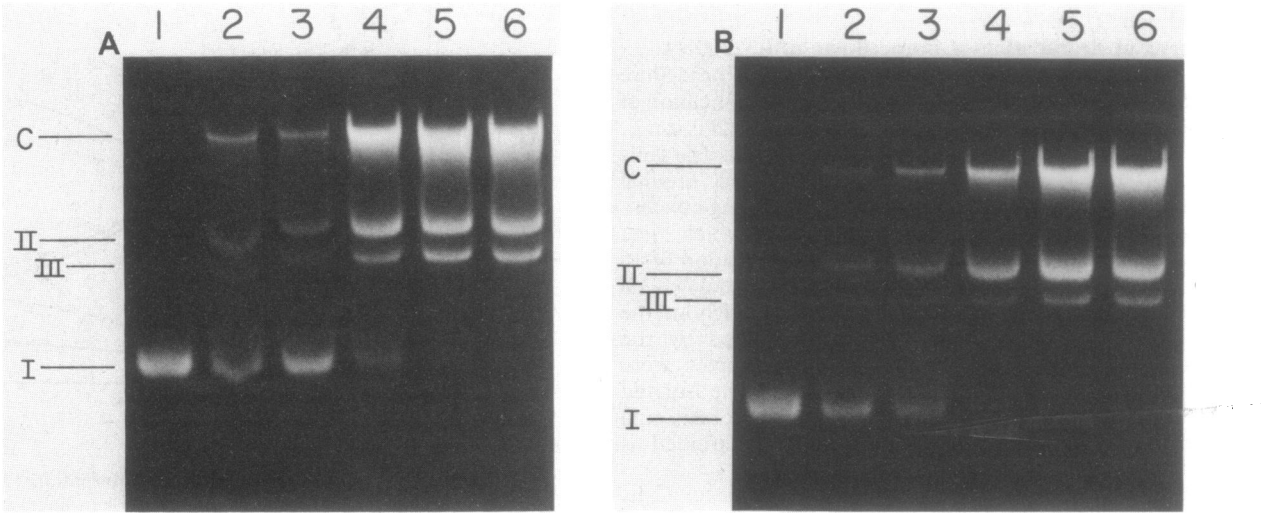

FIG. 3. Degradation of pBR322 DNA within *E. coli* incubated in the presence of TLM A (A) or BLM A_2 (B). *E. coli* was incubated in the presence of various concentrations of drug for 30 min, and the plasmid DNA was then isolated and analyzed by agarose gel electrophoresis (see text). Lanes: 1, control (no drug); 2, 5 μM of drug; 3, 10 μM; 4, 25 μM; 5, 50 μM; 6, 100 μM.

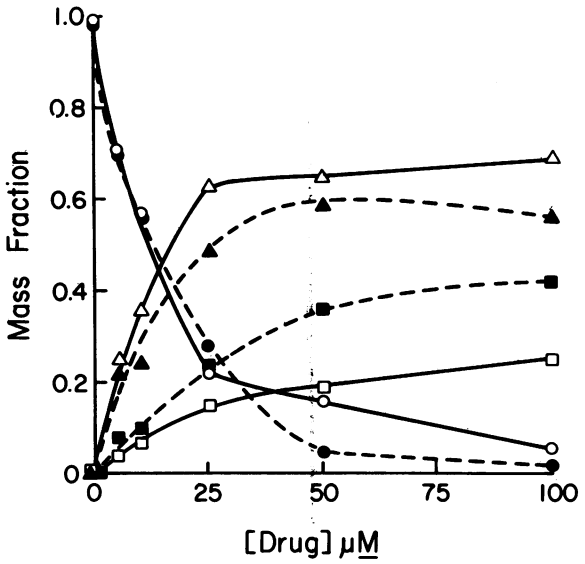

FIG. 4. Effect of increasing concentrations of TLM or BLM on plasmid pBR322 in vivo. Data shown in Fig. 3 were analyzed to determine the mass fraction of DNA in each form (○ and ●, form I; △ and ▲, form II; □ and ■, form III) as a function of increasing concentrations of TLM A (closed symbols) and BLM A_2 (open symbols).

observed for samples in which no drug had been added (data not shown).

Cellular repair of drug-induced DNA damage. To investigate the influence of DNA repair mechanisms on the results obtained in the TLM- and BLM-intracellular DNA breakage assays, the following experiments were performed. Cells were treated with various concentrations of drug, followed by posttreatment incubation at 37°C and subsequent isolation of plasmid DNA. The results of an experiment in which cells were pretreated with 35 and 50 μM of TLM A and BLM A_2, respectively, are shown in Fig. 6A and B. With increasing time of post-TLM A incubation, an increasing amount of form I DNA and corresponding decrease in the amounts of forms II and III DNA were observed (Fig. 6A, lanes 2 through 8). Figure 6B shows similar evidence of increasing amounts of form I DNA during postincubation in drug-free medium after BLM A_2 treatment. The amounts of plasmid DNA in each of the lanes in Fig. 6A and B were quantitated by densitometric scanning. When equivalent amounts of initial reduction of form I DNA (85% reduction) were produced by either drug, the amount of form I DNA which is present with increasing time of postincubation in drug-free medium is significantly greater in the BLM-pretreated *E. coli* than in the TLM-treated *E. coli* (Fig. 7A). Essentially, all of the BLM-induced plasmid DNA damage was repaired after 30 min, as evidenced by a return to control levels of forms I, II, and III DNA (Table 2), whereas in the TLM A-treated cells the amount of form I DNA returned to 57% of the control level within 30 min. Figure 7B (and Table 2) shows the results of experiments in which the initial level of BLM A_2- and TLM A-induced breakage of plasmid DNA was reduced relative to that shown in Fig. 6A and B. At this level of DNA breakage (60% reduction in form I DNA) the amount of form I DNA, which appeared after increasing the time of incubation in drug-free medium, was only slightly (but consistently) greater in the BLM A_2-treated *E. coli*.

These experiments, designed to investigate the repair of drug-induced DNA damage, were performed under conditions in which no significant de novo plasmid DNA synthesis

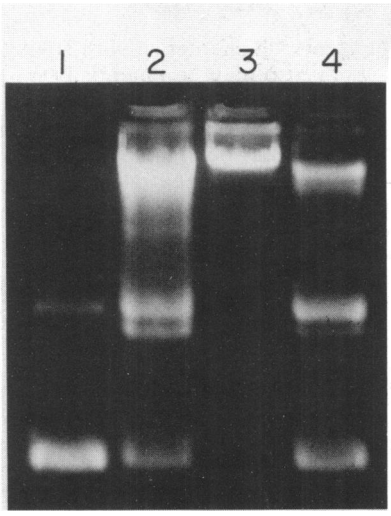

FIG. 5. Agarose gel electrophoresis of DNA isolated from *E. coli* containing plasmid (incubated in the presence of drug) and isolated *E. coli* chromosomal DNA. pBR322 DNA isolated from *E. coli* which was incubated in the presence of (lanes): 1, no drug; 2, TLM A; 3, *E. coli* chromosomal DNA; 4, BLM A_2. See text for details.

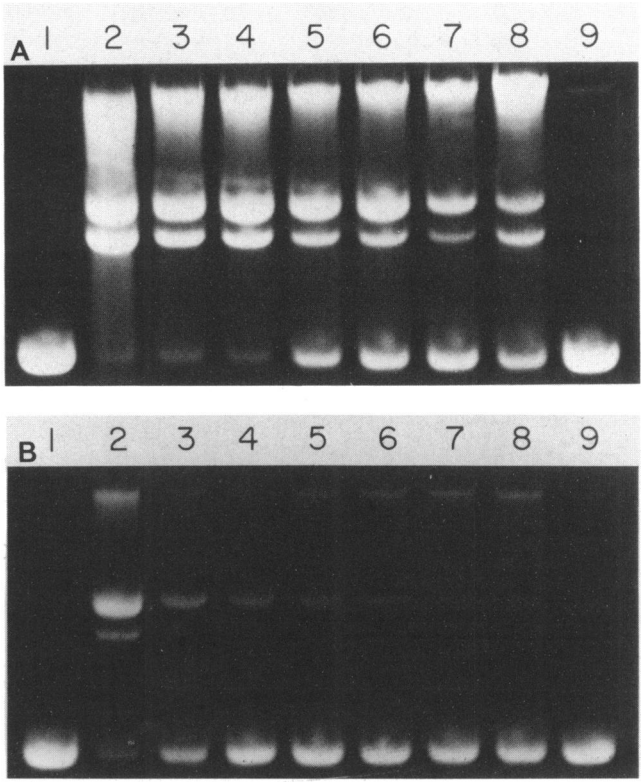

FIG. 6. Repair of plasmid DNA after incubation of *E. coli* with 35 μM TLM A (A) or 50 μM BLM A_2 (B) for 30 min. Cells were then collected, suspended in fresh medium, and incubated at 37°C for various periods of time. After the post-drug incubation periods, the plasmid DNA was isolated and analyzed on agarose gels (as described in the text). Lanes: 1, control (after 30-min drug incubation period); 9, control (after 30-min post-drug incubation); (2 through 8 correspond to DNA from cells treated with drug) 2, immediately after 30-min drug incubation; 3, at 0 min; 4, 2 min; 5, 5 min; 6, 10 min; 7, 20 min; 8, 30 min of post-drug incubation.

occurred during the 30 min of post-drug incubation. This was evidenced by measurement of DNA concentrations in the final plasmid preparation in control samples by absorbancy at 260 nm, uptake of [^{3}H]thymidine in drug-treated (and control) samples of plasmid isolate, and as measured by densitometric scanning of agarose gels to assess the recovery of plasmid in each experimental preparation. By these parameters, no increase in DNA concentrations (±5%) was

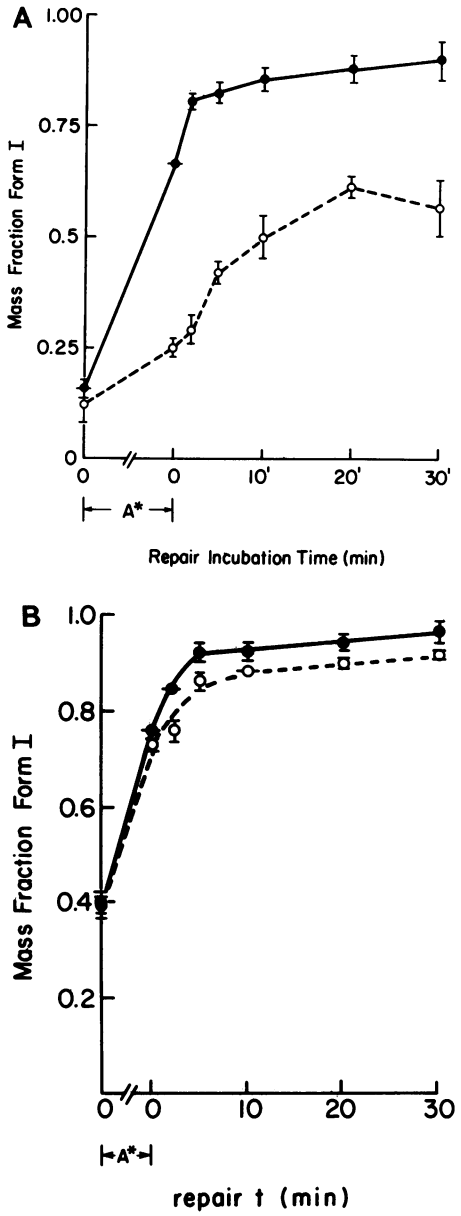

FIG. 7. Mass fraction of form I DNA with increasing post-drug incubation period. (A) Data was obtained from densitometric scans of gels shown in Fig. 6A and B (and two additional experiments) in which the initial reduction in form I DNA produced by BLM A_2 and TLM A was 85% (see text for details). (B) Data was obtained from densitometric scans of gels in which the initial reduction in form I DNA produced by BLM A_2 and TLM A was 60% (see text for details). Symbols: ●, BLM A_2, ○, TLM A. Standard deviations are indicated by bars. A*, time at which cells were removed from medium containing drug and suspended in fresh medium (15 min at 0°C).

TABLE 2. Mass fraction of forms I, II, and III pBR322 DNA measured at 0 and 30 min of post-drug incubation

Drug	Concn (μM)[a]	Time (min)[b]	Mass fraction[c]		
			I	II	III
None		0	0.97 ± 0.01	0.03 ± 0.01	0
None		30	0.98 ± 0.01	0.02 ± 0.02	0
BLM A_2	20	0	0.40 ± 0.02	0.49 ± 0.06	0.11 ± 0.03
BLM A_2	20	30	0.97 ± 0.02	0.02 ± 0.01	0.01 ± 0.00
BLM A_2	50	0	0.15 ± 0.03	0.65 ± 0.04	0.19 ± 0.02
BLM A_2	50	30	0.91 ± 0.04	0.05 ± 0.01	0.04 ± 0.01
TLM A	20	0	0.39 ± 0.04	0.43 ± 0.03	0.18 ± 0.04
TLM A	20	30	0.92 ± 0.01	0.05 ± 0.01	0.03 ± 0.02
TLM A	35	0	0.12 ± 0.04	0.55 ± 0.04	0.33 ± 0.03
TLM A	35	30	0.56 ± 0.01	0.25 ± 0.02	0.23 ± 0.04

[a] Drug concentrations indicate micromolar amount of drug incubated with cells before posttreatment incubations.

[b] t = 0 data is that measured immediately after addition of deferoxamine mesylate; t = 30 data is that measured after 30-min post-drug incubation.

[c] Form I, II, and III mass fractions were calculated from quantitative analysis of gels such as shown in Fig. 6. Experiments were run in triplicate, and the standard deviations are indicated (±). See Table 1, footnote *a*, for explanation of units.

observed. Monitoring of cell replication by absorbancy at 600 nm indicated no significant increase in cell number during the 30-min period. Furthermore, it has previously been reported that BLM causes an inhibition in replicative-type DNA synthesis both in vivo and in vitro (14, 25, 39). Therefore, the increase in the mass fraction of form I DNA observed during the post-drug incubation period does not appear to be due to de novo synthesis of plasmid DNA.

The repair of the damage produced in the DNA by both BLM A_2 and TLM was not restricted to the plasmid but also occurred in the chromosomal DNA. With increasing time of postincubation, the smearing pattern corresponding to the fragmented chromosomal DNA becomes less intense and corresponds to increasingly higher-molecular-weight forms of DNA (Fig. 6A and B). This pattern would appear to reflect a repair of fragmented chromosomal DNA induced by both drugs.

DISCUSSION

A variety of agents which interact with DNA can produce ssb or dsb or both. dsb may be potentially more cytotoxic than ssb. Therefore, as BLM-induced direct dsb in vitro (19, 27) the examination of intracellular dsb is an important problem, but one that has been difficult to approach experimentally. Earlier studies with neutral sucrose sedimentation provide some evidence for induction of dsb of bleomycin and for their subsequent repair (31). However, because of the supralethal bleomycin doses used and the lack of quantitative data on the number of breaks produced, few conclusions can be drawn regarding the relationships between dsb, repair of the damaged DNA, and cytotoxicity. Strong and Crooke (33) reported that treatment of Novikoff hepatoma ascites cells in vitro with TLM or BLM resulted in breakage of intracellular DNA and that TLM was less potent than BLM. However, the methodology used in these experiments (alkaline sucrose density gradient centrifugation) did not allow for the distinction between alkaline labile breaks, ssb, and dsb. Our earlier reported observation that the TLM possessed a greater degree of dsb activity than BLM (as assessed by using plasmid DNA as the substrate) (19) has led us to explore systems which would allow for the accurate measurement and comparison of ssb and dsb produced

intracellularly by these agents. The methodologies presented in this paper allow for an accurate quantitation of the intracellular single- and double-strand breakage potential of these agents as well as a means of accessing the ability of a cell to repair the damaged DNA. The choice of substrate also provided for a method whereby results of intracellular DNA breakage can be directly compared with those observed on isolated DNA (Table 1).

Previous studies have indicated that most double-strand breaks in preparations of isolated DNA produced by BLM do not result from accumulation of random ssb (27). We have previously reported that the interaction of TLM with isolated PM2 DNA results in more dsb per molecule of form I DNA degraded than that resulting from BLM (19). The present study demonstrates that the increased double-strand breakage activity of TLM observed with isolated pBR322 also occurred when cells containing the plasmid are treated with these drugs.

There was a significant difference in the potency of both compounds with respect to total plasmid breakage activity as measured in vitro (isolated pBR322) and in vivo (in *E. coli*) (Table 1). For example, as determined by the concentration of drug required to reduce the amount of form I DNA by 50%, BLM A_2 was approximately 200-fold more potent in vitro than in vivo. Although TLM A also demonstrated greater DNA breakage activity in vitro than in vivo, the 100-fold difference was less than that measured for BLM. This difference in relative in vitro to in vivo DNA breakage activities for the two drugs may reflect differences in their cellular permeabilities, cellular metabolism (i.e., activation and inactivation), or access to breakage-sensitive sites on DNA.

The results of experiments designed to investigate the repair of drug-induced damage to DNA indicate that the breakage produced by both TLM A and BLM A_2 is repaired in *E. coli*. As observed by the decrease in form II and form III DNA and concomitant increase in form I DNA during the post-drug incubation period, it appears that both ssb and dsb are repaired, followed by super helical twisting of the DNA by DNA gyrase (for review see reference 8) (Fig. 6). An alternative explanation is that the reduction in forms II and III DNA represents nuclease digestion of these particular forms of DNA, and the increase in form I DNA represents de novo synthesis of plasmid DNA. However, this explanation is unlikely as no detectable plasmid synthesis occurred during the 30-min postincubation period in cells which were initially incubated in the absence (Fig. 6, lanes 1 and 9) or presence of drug.

These studies indicate that the rate of repair of DNA damage differs after incubation with either TLM A or BLM A_2. The damaged DNA in cells treated with BLM A_2 was repaired more rapidly than that treated with TLM A (Fig. 6 and 7) even though the initial amount of DNA breakage produced by each drug was equivalent. This more rapid rate of repair of DNA breakage in BLM A_2-treated cells relative to TLM A-treated cells might be explained by differences in the double-strand breakage activities of these two drugs. Both ssb and dsb were repaired to some degree within 30 min of recovery incubation (Table 2). However, there appears to be an inverse relationship between the amount of dsb produced initially by the drug and the capacity of the cells to repair the damaged DNA to its native covalently closed form within 30 min. At the concentrations of drug which produced approximately an 85% decrease in form I DNA (50 μM BLM and 35 μM TLM), BLM produced more form II and less form III than did TLM. After 30 min of post-drug incubation, the mass fraction of form I DNA returned to 0.91 or 0.56 in the cells treated initially with BLM or TLM, respectively. When cells were treated at concentrations of drug which produced equivalent levels of form III DNA (50 μM BLM and 20 μM TLM), again the initial amount of form II DNA was greater in the BLM-treated cells, but within 30 min of post-drug incubation, the mass fraction of form I DNA had returned to approximately equivalent levels in both the TLM- and BLM-treated cells.

Although these results do not provide an absolute explanation for the respective rates of repair of TLM- and BLM-induced DNA damage, it does appear that the ability of cells to repair the plasmid DNA to its initial covalently closed, super helical form may be related to the extent of double-strand breakage produced by either drug. However, the different site and sequence specificities of breakage of DNA evidenced for BLM A_2 and TLM A may also be involved in an explanation of the different intracellular rates of repair of their respectively produced DNA damage. We have recently observed that the site and sequence specificities of breakage of BLM A_2 and TLM A are equivalent at drug concentrations which produced low levels of breakage of covalently closed super helical (form I) pBR322 DNA (i.e., approximately one break per plasmid molecule) (21). However, as the number of breaks per DNA molecule increased, the site and sequence specificities of breakage of BLM A_2 were different from those of TLM A. Regardless of the actual mechanistic explanation, the decreased rate of repair of the DNA damage produced by TLM A may, in part, explain the more potent antitumor activity of TLM A relative to BLM A_2 (1, 24). Thus, one might speculate that further modification of TLM (i.e., modification of the L-talose sugar moiety) may yield new agents with increased double-strand breaking activity or different site and sequence specificities or both, resulting in a comparatively enhanced antitumor activity.

The DNA repair experiments presented in this paper were done with *E. coli* JA221, which contains several mutations (see above), one of which is in the *recA* gene. The *recA* protein is the product of a gene that was discovered by Clark and Margulies on the basis of its indispensability for *E. coli* recombination (5). Mutations in the *recA* gene cause a reduction in genetic recombination by as much as 10^6, high sensitivity to UV and X-irradiation, loss of UV mutability, and extensive "reckless" degradation of DNA after UV or X-irradiation (4). In *E. coli* cells, repair of DNA dsb produced by gamma irradiation, requires an active *recA* gene and the presence of another DNA duplex that has the same base sequence as the broken double helix (12).

The role of the *recA* gene in the repair of DNA damage has led numerous investigators to study its possible function in the repair of BLM-induced DNA damage. Yamagami et al. (38) reported that *recA*$^-$, *recB*$^-$, and *recC*$^-$ mutants were fourfold more susceptible than wild types to BLM as determined in a colony-forming assay. Ross and Moses (30) also reported that the *recA* gene product is required for normal survival after BLM exposure, but that the *recB* and *recC* gene products are not. However, a number of reports have concluded that *recA*$^-$ strains are as susceptible as wild types to BLM. Yamamoto and Hutchinson (40) have reported evidence indicating that the role of the *recA* gene in the repair of BLM A_2-induced DNA damage may be a function of the metabolic state of the cell. The results of the experiments reported in this paper indicate that both ssb and dsb produced by BLM A_2 or TLM A are repaired in *E. coli* cells that are *recA*$^-$. These results, as well as those of others

discussed above, indicate that the response of *recA*-deficient mutants of *E. coli* to BLM and TLM may differ from the response to X-irradiation. If the effects of these drugs and ionizing radiations are ascribed to DNA strand breaks and to bases released from the DNA, significant differences between the drugs and X-irradiations must nevertheless exist. A possible explanation is that the drugs must pass through the cell wall to interact with DNA. However, a more direct explanation may be that the cellular repair processes respond to the type of DNA damage produced by these drugs at specific sites (or confined to relatively small areas) of the genome differently than to damage from X-rays randomly distributed through the DNA.

Further studies with various repair-deficient mutants as well as BLM- and TLM-resistant and -susceptible strains of *E. coli* in this assay system may aid in understanding the relationship between the cytotoxic effects of these drugs and the DNA lesions they produce.

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