Bifunctional Intercalation of Antitumor Antibiotics BBM-928A and Echinomycin with Deoxyribonucleic Acid. Effects of Intercalation on Deoxyribonucleic Acid Degradative Activity of Bleomycin and Phleomycin†

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ABSTRACT: The binding of peptide antitumor antibiotics, BBM-928A and echinomycin, to superhelical PM2 DNA and the effects of the resulting conformational changes of DNA on the DNA-degradative activity of two related antitumor antibiotics, bleomycin A_2 and phleomycin D_1, have been studied. The bifunctional intercalative mode of the DNA binding of BBM-928A concluded previously from viscometric and fluorometric studies has been confirmed by gel electrophoretic analysis. Under the employed electrophoretic conditions, DNA-bound BBM-928A showed little dissociation whereas echinomycin and ethidium bromide showed partial and nearly complete dissociation, respectively. BBM-928A induced neither single-strand nor double-strand breaks in DNA. Competitive binding studies by fluorescence changes suggested that binding sites on DNA molecules for BBM-928A (or echinomycin) may differ from those for ethidium bromide, since binding to DNA by the two drugs was not competitive even at saturating concentratons. The lack of such a competition between the two drugs is not consistent with the neighbor-exclusion principle. The DNA-degradative activity of both bleomycin A_2 and phleomycin D_1 increased with the removal of the negative superhelicity of DNA by the BBM-928A intercalation and decreased with the formation of positive superhelical turns induced by high concentrations of BBM-928A. Thus the degradative activity of both bleomycin A_2 and phleomycin D_1 is sensitive in a similar manner to the degree of superhelicity rather than the double helicity of DNA, although there are differences between these two drugs in interaction with DNA.

BBM-928 (Okhuma et al., 1980; Tomita et al., 1980; Huang et al., 1980) is a new family of actinoleukin-like antibiotics containing two substituted quinoline chromophores which are linked by a cyclic decadepsipeptide. BBM-928A, one of the BBM-928 family, shows potent antitumor activities against P388 and L1210 leukemia, B16 melanoma, Lewis carcinoma, and sarcoma 180 cells, with a potency approximately 3-fold greater than that of the structurally related antibiotic echinomycin and 100–300-fold that of mitomycin (Okhuma et al., 1980).

Using viscometric and fluorometric measurements, we have recently demonstrated (Huang et al., 1980) that BBM-928A intercalated bifunctionally with DNA, involving both quinoline chromophores. The apparent DNA binding affinity constant of BBM-928A was 1.39×10^7 M^{-1} and approximately one BBM-928A molecule was bound per five to six DNA base pairs at saturation. An unwinding angle of 43° was observed for the intercalation of one BBM-928A molecule with DNA.

In the present study, we have confirmed the DNA-intercalative mode of BBM-928A by the use of agarose gel electrophoresis to assay the effects of BBM-928A on the covalently closed supercoiled DNA of PM2 phage. For comparison, the effects of echinomycin on PM2 DNA have also been studied. Echinomycin has been shown to intercalate bifunctionally with DNA at low ionic strength (Wakelin & Waring, 1976).

Although BBM-928A, echinomycin, and ethidium bromide (EB)[1] are all DNA intercalators, only BBM-928A and echinomycin demonstrated antitumor activities. The cyclic structure which possesses a 2-fold rotational symmetry (Keller-Schierlein et al., 1959; Sobell et al., 1971) and the bifunctionality of the DNA intercalation may provide BBM-928A or echionomycin with unique sites or modes of DNA binding which are different from those of EB, which shows little base/sequence preference in binding to DNA. The preliminary evidence presented in this study suggests that BBM-928A and echinomycin may bind to DNA at sites differing from those of EB.

We have also studied the effects of helical and superhelical changes of DNA induced by intercalation of BBM-928A on the DNA-degradative activities of two glycopeptidic antitumor antibiotics, bleomycin (BLM) A_2 and phleomycin (PLM) D_1, for the reasons that (1) superstructures of DNA may be related to gene activities and it is of interest to study the combined effects of these two different types of antitumor agents on DNA and (2) BBM-928A may serve as a structural probe to examine the suggested differences between BLM A_2 and PLM D_1 in interaction with DNA (Huang et al., 1981).

Materials and Methods

Materials. BBM-928A, echinomycin, bleomycin A_2, and phleomycin D_1 were obtained from the Bristol Laboratories, Syracuse, NY. Covalently closed, circular, supercoiled form I DNA of PM2 bacteriophage was isolated as previously described (Strong & Hewitt, 1975). Only DNA preparations with more than 85% of DNA in form I were used. The relaxed form I^0 PM2 DNA was prepared by treatment of form I DNA with the calf thymus topoisomerase (Mong et al., 1980). Ethidium bromide, Tris, borate, Na_2EDTA, β-mercaptoethanol, and calf thymus DNA were obtained from Sigma

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[1] Abbreviations: EB, ethidium bromide; BLM A_2, bleomycin A_2; PLM D_1, phleomycin D_1; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate.

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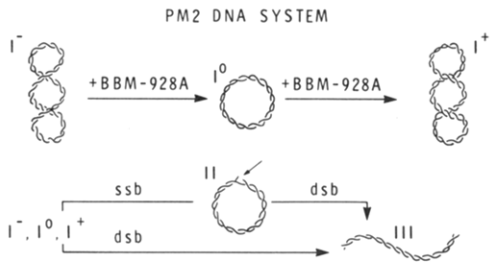

FIGURE 1: PM2 DNA system used for studies of the BBM-928A-induced superhelical changes of PM2 DNA and of the DNA-degradative activity of bleomycin A_2 and phleomycin D_1. I^- (or I), native, negatively supercoiled PM2 DNA; I^0, relaxed DNA with virtually no supercoils; II, nicked, relaxed DNA; III, linear DNA; ssb, single-strand break; dsb, double-strand breaks. See text for detailed explanation.

Chemical Co., St. Louis, MO. Agarose-ME was purchased from Miles Laboratories, Elkhart, IN.

Treatment of PM2 DNA with BBM-928A or Echinomycin. Form I or form I^0 PM2 DNA was incubated with varying amounts of BBM-928A or echinomycin in a buffer solution (final volume 0.15–0.20 mL) containing 50 mM borate (pH 9.5) and 66 mM NaCl for 30 min at room temperature. The BBM-928A- or echinomycin-treated PM2 DNA was then analyzed by gel electrophoresis with or without the treatment of BLM A_2 or PLM D_1.

DNA-Breakage Activity of BLM A_2 and PLM D_1. To the untreated or BBM-928A-treated form I or form I^0 DNA in 50 mM borate buffer (pH 9.5) containing 66 mM NaCl were added β-mercaptoethanol (25 mM) and a constant amount (17–30 nM) of BLM A_2 or PLM D_1 to start the DNA-degradative activity. After 30 min at room temperature, the reaction was terminated with the addition of Na_2EDTA (25 mM) and analyzed by gel electrophoresis.

Agarose Gel Electrophoresis. Aliquots of untreated or intercalation treated PM2 DNA (0.8–1.5 μg), with or without degradation by BLM A_2 or PLM D_1, were electrophoresed in a 0.9% slab gel for 6–10 h at room temperature with a 40 mM Tris-HCl buffer containing 5 mM sodium acetate and 1 mM EDTA, pH 7.8. The electrophoresed gels were stained with 0.5 μg/mL EB and photographed with a Polaroid CU-5 Land camera equipped with a no. 8 Kodak Wratten gelatin filter (Eastman Kodak Co., Rochester, NY) and type 665 Land films while excited with a transilluminator (Ultraviolet Products, Inc.). For gels electrophoresed in the presence of saturating amounts of EB, both the gels and the electrophoretic buffer contained 0.5 μg/mL EB.

Quantitation of Single-Strand and Double-Strand DNA Breaks by Densitometric Scannings of Negative Films of Gels. EB-stained gel patterns of the drug-treated PM2 DNA were scanned with a recording Transidyne General densitometer equipped with an automatic computing integrator. As illustrated in Figure 1, the production of the form II and form III DNA from the form I (or I^-) or form I^0 DNA was used to calculate the extent of single-strand and the double-strand breaks, respectively, according to the procedures described previously (Lloyd et al., 1978a,b; Huang et al., 1981; Mirabelli et al., 1980). In this procedure the difference in the intensity of the maximal EB staining between form I and form II DNA has been properly corrected. For minimization of the effects of the variation in the intensity of the EB staining of DNA with different superhelicities (Morgan et al., 1979) on the calculation of the DNA breakage activity of BLM A_2 or PLM D_1, the DNA preparations treated with increasing concentrations of BBM-928A, but in the absence of BLM A_2 or PLM D_1, were used as the undegraded DNA controls.

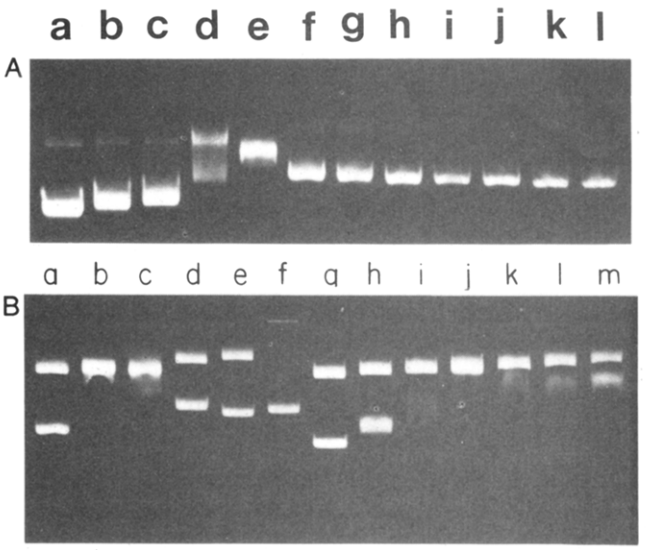

FIGURE 2: (A) Agarose gel electrophoretic pattern of the DNA products after treatments of PM2 form I DNA preparation with increasing concentrations of BBM-928A. The [BBM-928A] to [DNA] ratios were 0 (a), 0.025 (b), 0.0375 (c), 0.050 (d), 0.0625 (e), 0.075 (f), 0.0875 (g), 0.100 (h), 0.125 (i), 0.150 (j), 0.175 (k), and 0.200 (l). DNA migrated from top to bottom. DNA was stained with ethidium bromide. (B) The gel electrophoretic pattern of DNA after treatment of a mixture (lane g) of form I (60%) and form II (40%) DNA with increasing concentrations of BBM-928A (lanes a–f) or echinomycin (lanes h–m). BBM-928A to DNA concentration ratios were 0.022 (a), 0.044 (b), 0.055 (c), 0.077 (d), 0.110 (e), and 0.220 (f). Echinomycin to DNA concentration ratios were 0.022 (h), 0.044 (i), 0.055 (j), 0.077 (k), 0.110 (l), and 0.220 (m).

Binding Studies by Fluorescence Changes. The enhancement of the EB fluorescence at 600 nm by the untreated or the BBM-928A-treated calf thymus DNA (33.9 μM, as nucleotide-P residues) was measured in a 10 mM Tris-HCl buffer (pH 8.4) containing 100 mM NaCl, with an excitation wavelength of 510 nm (both slits; 4 nm) in an Amico SPF-500 ratio spectrofluorometer equipped with an X–Y recorder. The treatment of DNA by BBM-928A or echinomycin was performed for 6 min at room temperature. The determination of the concentrations of EB, BBM-928A, and echinomycin has been described (Huang et al., 1980).

The quenching effects of the EB-treated calf thymus DNA on the fluorescence of BBM-928A at 500 nm were measured with an excitation wavelength at 400 nm. The proper selection of these two wavelength pairs eliminated any mutual fluorescence interference between EB and BBM-928A. Uncorrected fluorescence was reported in the present study.

Under the conditions employed, experiments with the DNA-induced EB fluorescence enhancement at a constant [EB] to [DNA] ratio of 0.25 indicated that up to an EB concentration of 50 μM (or 200 μM DNA), the fluorescence enhancement is linearly increased with the increase in the EB concentration. Thus, the current study employing low EB concentrations (0–20.3 μM) would not be subject to concentration-dependent complications, such as the "inner filter effect".

Results

Gel Electrophoretic Analysis of the Intercalation of BBM-928A with DNA. Figure 2A shows the gel electrophoretic patterns of the covalently closed, supercoiled form I PM2 DNA treated with increasing amounts of BBM-928A. The untreated DNA preparation (lane a) contains 87% form I DNA and 13% nicked, relaxed, circular form II DNA, as measured by densitometric scans. Treatment with increasing

concentrations of BBM-928A (lanes b–l), at [BBM-928A] to [DNA] ratios of 0.025–0.200, caused a decrease followed by a restoration in the electrophoretic mobility after reaching a minimum at a BBM-928A to DNA concentration ratio range of 0.050–0.063 (lane d or lane e). This concentration ratio for minimal electrophoretic mobility approximates the ratio of 0.054 we have reported previously (Huang et al., 1980) to induce the maximal viscosity of the BBM-928A-treated DNA. The decrease in the electrophoretic mobility of DNA, like the increase in the viscosity, resulted from the removal of the negatively supercoiled turns of form I DNA upon the intercalation of BBM-928A. The completely relaxed form I^0 DNA displayed the least mobility, and addition of more BBM-928A supercoiled the relaxed DNA into positively supercoiled forms, resulting in an increased electrophoretic mobility. When assayed with a gel electrophoretic system containing saturating concentration of EB, all DNA products which had been treated with increasing concentrations of BBM-928A were converted to positively supercoiled forms, with the DNA treated with high concentrations of BBM-928A moving slightly ahead of the untreated DNA (data not shown).

It is also clear from Figure 2A that little increase in the amount of form II DNA or the production of the linear form III DNA was observed as a result of the treatment of DNA with BBM-928A throughout the concentration range used. Thus under these conditions BBM-928A caused no detectable single-strand or double-strand DNA breaks, which would produce forms II and III DNA, respectively.

Figure 2B shows the effect of the treatment with increaseing concentrations of echinomycin on the electrophoretic mobility of PM2 DNA when compared to DNA treated with BBM-928A. In this experiment, the untreated DNA preparation (lane g) contained both form I (60%) and form II (40%) DNA, and the relative gel mobility of these two DNA forms could be easily observed. With a [BBM-928A] to [DNA] ratio of 0.055, form I DNA was almost completely relaxed, and higher ratios (lanes d–f) caused formation of positive superhelical turns in form I DNA (cf. Figure 2A). At a very high ratio (0.22) (lane f), the mobility of form II DNA increased significantly, probably as a result of denaturation. Low concentrations of echinomycin reduced the mobility of the treated form I DNA, but even very high concentrations of echinomycin (ratio of 0.11 or 0.22) failed to induce a complete relaxation or the formation of positive supercoils in form I DNA.

Fluorescence Studies of the DNA Binding for BBM-928A, Echinomycin, and Ethidium Bromide. The DNA bands shown in Figure 2 were stained with EB which is a typical monofunctional intercalator. Clearly the intensity of the EB staining of DNA was only slightly reduced even when the DNA was treated with high concentrations of BBM-928A. This implies that pretreatment of DNA with BBM-928A, which intercalates with DNA with high avidity, only slightly affected the subsequent intercalation of EB. The slight reduction in EB fluorescence intensity in DNA treated with high BBM-928A concentrations could be simply the result of a partial absorption, especially in the UV region, by BBM-928A of the excitation light from a transilluminator which was used for photography of the gels. To confirm this, we selected appropriate fluorescence excitation–emission wavelength pairs for either EB or BBM-928A to observe the effects of pretreatment of DNA with one drug on the subsequent binding of the other drug. The observation that upon binding to DNA, the fluorescence of EB was enhanced but that the BBM-928A was quenched (Huang et al., 1980) was employed to allow such a study. Figure 3A shows effects of BBM-928A treatment

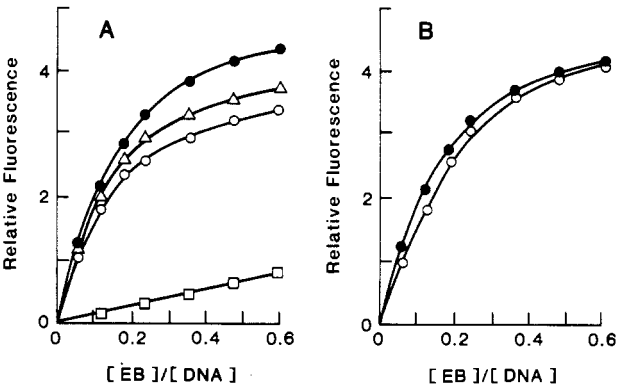

FIGURE 3: Effect of the pretreatment of calf thymus DNA with BBM-928A (A) or echinomycin (B) on the DNA-induced enhancement of ethidium bromide fluorescence. After increasing amounts of ethidium bromide were added to a fixed amount of untreated or drug-treated DNA (33.9 μM), the fluorescence intensity at 600 nm was measured with excitation at 510 nm. (A) (□) Ethidium bromide in the absence of DNA; (●) ethidium bromide in the presence of DNA; (△) DNA pretreated with BBM-928A at [BBM-928A]/[DNA] = 0.125; (○) DNA pretreated with BBM-928A at [BBM-928A]/[DNA] = 0.307. (B) (●) DNA; (○) DNA pretreated with echinomycin at [echinomycin]/[DNA] = 0.153.

of DNA on the binding of EB to DNA studied by using the fluorescence enhancement at 600 nm with an excitation at 510 nm. At this wavelength pair BBM-928A has neither excitation nor absorption activity. The results show that the EB fluorescence enhancement induced by BBM-928A-treated DNA (at a BBM-928A to DNA concentration ratio of 0.13 or 0.30) was only slightly lower (16% or 27%) than that induced by the untreated DNA.

Fluorescence of BBM-928A was quenched upon binding to DNA (Huang et al., 1980). Since EB had no excitation activity at 400 nm, the fluorescence of BBM-928A at 500 nm was observed with an excitation at 400 nm. Addition of DNA at [BBM-928A] to [DNA] ratios of 0.051 and 0.022 reduced the fluorescence intensity to 55% and 28%, respectively, and further addition of EB at an [EB] to [DNA] ratio of 0.028 or 0.056 had little effect on the DNA-quenched fluorescence (data not shown). These observations suggest that EB did not replace the BBM-928A already bound to DNA and are thus consistent with the high DNA-binding affinity (1.90×10^7 M^{-1}) of BBM-928A (Huang et al., 1980).

Figure 3B shows that the pretreatment of DNA with echinomycin, at a drug to DNA ratio of 0.20, did not significantly affect the ability of DNA to induce fluorescence of EB. Thus, the bifunctional intercalator echinomycin, like BBM-928A, did not compete with EB for DNA-binding sites.

Effects of DNA Conformational Changes Induced by BBM-928A on DNA-Degradation Activity of Bleomycin A_2. Figure 4A shows the gel electrophoretic assays, and Figure 4B shows the quantitative analyses of the responses of the DNA-breakage activity of bleomycin A_2 to the BBM-928A-induced conformational changes, in the superhelix as well as the double helix, of form I PM2 DNA. Of the untreated DNA preparation (lane a in Figure 4A), 85% was in form I DNA (major band) and 15% in form II (minor band). When DNA (114 μM) was treated with 17.2 nM BLM A_2 in the absence of BBM-928A treatment (lane b), 56% of the DNA was converted to form II due to single-strand breaks and 11% to form III DNA due to double-strand breaks. Only about 33% of the DNA remained as form I (Figure 4B). Pretreatment of DNA with BBM-928A at concentrations increasing to a drug to DNA ratio of 0.05–0.06 (lanes c to e or f) increased the production of both form II (to 75–80%) and form III DNA

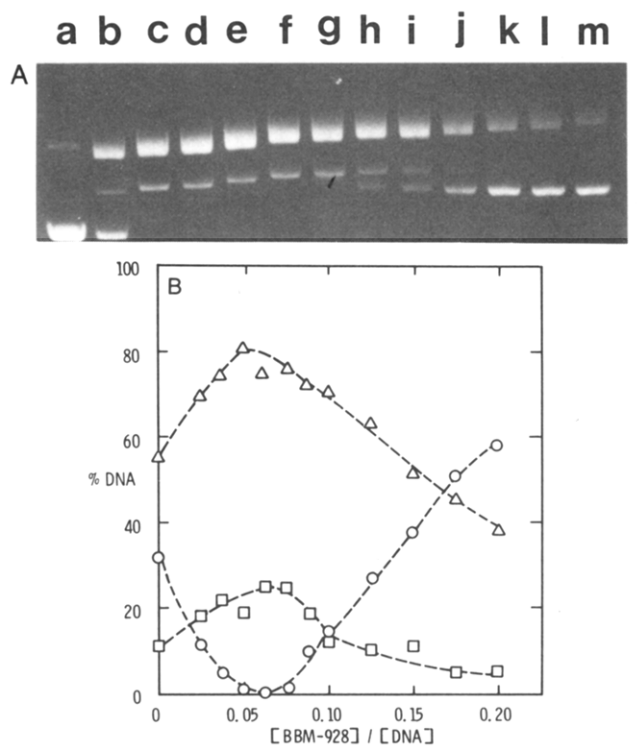

FIGURE 4: (A) Gel electrophoretic pattern of the bleomycin A_2 digested DNA products. Prior to bleomycin A_2 digestion, PM2 form I DNA was pretreated with increasing concentrations of BBM-928A at [BBM-928A] to [DNA] ratios of 0 (b), 0.025 (c), 0.0375 (d), 0.050 (e), 0.0625 (f), 0.075 (g), 0.0875 (h), 0.10 (i), 0.125 (j), 0.150 (k), 0.175 (l), and 0.20 (m). Lane a shows the PM2 form I DNA preparation without treatment of either BBM-928A or bleomycin A_2. DNA migrated from top to bottom of the gel pattern. Digestion of DNA (113.6 μM) with bleomycin A_2 (17.2 nM) was performed at room temperature for 30 min. (B) Quantitative analysis of bleomycin A_2 induced production of form II DNA (single-strand break) and of form III DNA (double-strand break) from form I DNA. Data were taken from the results shown in Figure 4A. The untreated form I DNA (lane a in Figure 4A) contains 85% DNA in form I and 15% in form II. (○) Remained form I DNA after bleomycin A_2 digestion; (△) form II DNA; (□) form III DNA.

(to 20–25%) at the expense of form I DNA (reduced to 0–2%), although the BLM A_2 concentration was maintained at a constant (17.2 nM) level. This ratio range corresponds to the ratio range 0.050–0.063 (Figure 2A) required for BBM-928A to completely remove the negatively supercoiled turns of the form I PM2 DNA, producing the relaxed form I^0 DNA. At higher concentrations, BBM-928A reduced the production of both the form II and form III DNA (lanes g–m), resulting in a high retention of the undegraded form I DNA. These changes corresponded to the formation of positively supercoiled turns of DNA induced by higher concentrations of BBM-928A. Similar types of response were observed when 10.2 nM BLM A_2 was used to degrade DNA (data not shown).

Electrophoretic mobility of the undegraded, residual form I DNA was reduced by treatment with BBM-928A (cf. Figure 2A). Such a reduction might sometimes result in comigration of the residual form I DNA with either form II or form III DNA in gels and thus might affect the accuracy of quantitation of each DNA species. To solve this problem, a sufficiently high concentration of EB (0.5 μg/mL) was added to both the gel and the electrophoresis buffer. This converted all form I DNA molecules to positive superhelical form which migrated with a mobility similar to that of the native, negatively superhelical form I DNA and thus was separated from form II or form III DNA. Results of such assays (not shown) were similar to those shown in Figure 4B.

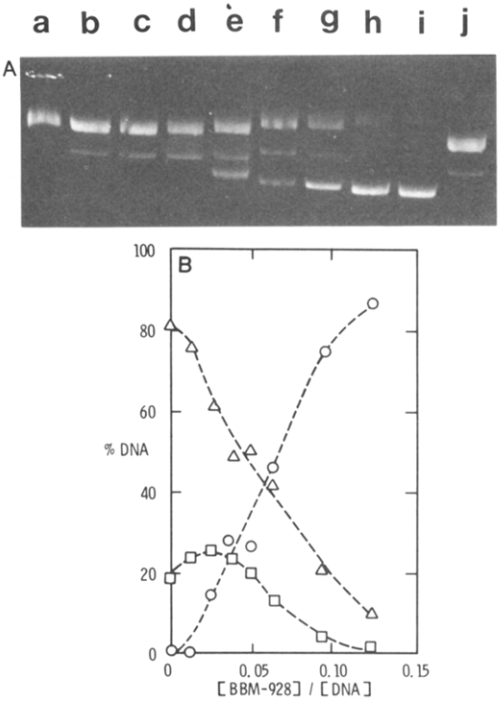

FIGURE 5: (A) Digestion by bleomycin A_2 (25.7 nM) of the isolated PM2 form I^0 DNA (113.6 μM, lane a) pretreated with increasing concentrations of BBM-928A. Ratios of [BBM-928A] to [DNA] were 0 (b and j), 0.0125 (c), 0.025 (d), 0.0375 (e), 0.050 (f), 0.070 (g), 0.090 (h), and 0.125 (i). DNA migrated from top to bottom. The fast-moving DNA band is the residual form I^0 DNA which became positively supercoiled upon intercalation of BBM-928A. (B) Quantitative analysis of the conversion of form I^0 to form II and form III DNA shown in Figure 5A. Analysis with EB-containing gels (not shown) indicated that the form I^0 DNA preparation contained 95% DNA in form I^0 and 5% DNA in form II. (○) Residual form I^0 DNA; (△) form II DNA; (□) form III DNA.

That the effects observed are due to conformational variations in the DNA was confirmed in an experiment (not shown) in which form I, form I^0, and an equimolar mixture of both were treated with BLM A_2 (11.5 nM) under equivalent conditions. In this experiment, 33% of the form I, 50% of the form I^0, and 37% of the form I + form I^0 of the mixture were degraded. These results provide additional confirmation that relaxed form I^0 DNA is more sensitive than form I DNA to degradation by BLM A_2, irrespective of whether it is induced by chemical or enzymatic means.

Effects of the BBM-928A Induced Conformational Changes of Form I^0 DNA on DNA-Degradative Activity of Bleomycin A_2. The decrease in the BLM A_2 induced production of the form II and form III DNA when form I DNA was treated with higher concentrations of BBM-928A (Figure 4) may be the consequence of (1) an inhibitory effect of high concentrations of BBM-928A on the DNA-degradative activity of BLM A_2 or (2) a response to the formation of positive supercoils. To differentiate these two possiblities, we have employed purified form I^0 PM2 DNA which was prepared by treatment of form I DNA with isolated calf thymus topoisomerase. The addition of any amount of BBM-928A would supercoil the form I^0 DNA into positive superhelical form. Figure 5A shows the gel pattern and Figure 5B shows the quantitative analysis of the effects of the treatment of form I^0 DNA with low concentrations of BBM-928A on the BLM A_2 activity. These results indicated that low concentrations of BBM-928A caused an immediate decrease in the production of form II DNA and a decrease, after a brief increase, of the production of form III DNA, with an increasing retention of the undegraded form I^0 DNA which was obviously in positive

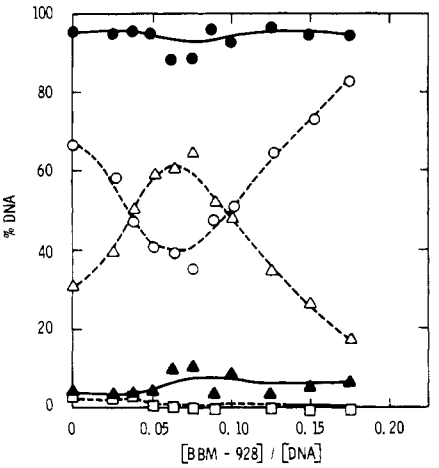

FIGURE 6: Quantitative analysis of the conversion of BBM-928A-pretreated form I DNA (114.5 μM) to form II and form III DNA by phleomycin D_1 (18.5 nM) digestion. The pretreatment of DNA with BBM-928A (concentrations as shown) and the subsequent digestion by phleomycin D_1 were performed at room temperature for 30 min. (○) Residual form I DNA after phleomycin D_1 digestion; (△) form II DNA after phleomycin D_1 digestion; (□) form III DNA after phleomycin D_1 digestion; (●) form I DNA before phleomycin D_1 digestion; (▲) form II before phleomycin D_1 digestion.

superhelical form because of BBM-928A intercalation. These results are very similar to those observed for BLM A_2 activity (Figure 4B) when form I DNA was pretreated with BBM-928A at [BBM-928A] to [DNA] ratios greater than 0.05. These results thus suggest that the decrease in BLM A_2 activity is due to the formation of positive superhelical turns in DNA.

Effects of BBM-928A-Induced DNA Conformational Changes on DNA-Degradative Activity of Phleomycin D_1. Experiments similar to those for BLM A_2 described in parts A and B of Figure 4 were performed with phleomycin D_1, and the results are shown in Figure 6. These results show that the DNA-degradative activity of PLM D_1, which produced mainly single-strand breaks (Huang et al., 1981), responded to the BBM-928A-induced DNA conformational changes in a manner similar to that of BLM A_2, which produced both single-strand and double-strand breaks. This was further confirmed with the use of form I^0 DNA (data not shown), the degradation of which by PLM D_1 declined immediately upon addition of low concentrations of BBM-928A, in a manner similar to that shown in Figure 5.

Discussion

Previous studies by viscometry and fluorometry (Huang et al., 1980) suggested a bifunctional intercalation of BBM-928A with DNA involving both quinoline chromophores. Present results of the gel electrophoretic analysis of the BBM-928A-treated superhelical PM2 DNA confirm this mode of binding since BBM-928A induced a decrease, followed by a restoration, in the gel electroporetic mobility of the superhelical DNA (Figure 2A). The pattern of the mobility changes of DNA induced by BBM-928A was a mirror image of that of the viscosity changes (Huang et al., 1980). These changes are typically induced by increasing concentrations of DNA intercalators and are the results of the removal of the negative superhelical turns of the native form I DNA followed by the acquisition of the positive superhelical turns. The gel electrophoretic studies show that the critical ratio of [BBM-928A]/[DNA] required to completely relax the form I DNA into form I^0 DNA was 0.05–0.06, which was comparable to the critical ratio of 0.050–0.063 obtained from viscometric studies and thus confirms the unwinding angle of 43° previously reported for BBM-928A (Huang et al., 1980).

The observation of similar critical concentration ratios obtained from viscometric and electrophoretic assays further suggests that during the process of electrophoresis, little dissociation of bound BBM-928A from DNA occurred. This probably is the first report of such a case for a DNA-intercalative agent, probably as a result of a very high affinity constant (1.93×10^7 M^{-1}) for the DNA binding of BBM-928A (Huang et al., 1980). The absence of the dissociation of DNA-bound BBM-928A under electrophoretic conditions should allow us to use gel electrophoresis techniques for further studies of the nature of interactions between DNA and BBM-928A. Under the same electrophoretic conditions, most of the bound EB molecules are dissociated from DNA, unless EB is present in both the gel and the buffer (Espejo & Lebowitz, 1976).

Echinomycin binds to DNA bifunctionally at low ionic strengths (Wakelin & Waring, 1976). During electrophoresis, partial and marked dissociation of DNA-bound echinomycin molecules might have occurred at low and high drug concentrations, respectively, so that high echinomycin concentrations failed to restore the gel mobility of the treated DNA (Figure 2B). This is consistent with a relatively moderate DNA-binding affinity of $(1–6) \times 10^6$ M^{-1} for echinomycin (Wakelin & Waring, 1976).

The results of the gel electrophoretic analysis of the BBM-928A-treated DNA further suggest that under our conditions, BBM-928A, even at relatively high concentrations, showed little DNA-degradation activity since no production of either form II or form III DNA was observed (Figures 2A and 6).

The present study shows that the DNA-degradative activity of both BLM A_2 and PLM D_1 responded in a similar manner to the BBM-928A-induced conformational changes in superhelicity but not in the double helicity of the circular DNA. The activity was maximal when superhelical DNA was completely relaxed and declined with the formation of either negative or positive superhelical turns. This is particularly interesting in view of the fact that the interaction of PLM D_1 with DNA differs from that of BLM A_2 in several regards and that PLM D_1 induces essentially no double-strand breakage of DNA (Huang et al., 1981).

In many cells, DNA exists in superhelical forms in higher ordered structures such as nucleosomes and their polynucleosomal arrays (Olins & Olins, 1974; Hewish & Burgoyne, 1973; Oudet et al., 1975; Germond et al., 1975; Finch & Klug, 1976; Worcel, 1978) or nucleoids (Cook & Brazell, 1976; Luchnik & Glasser, 1980). Nucleoids contain superhelical DNA constrained in a manner similar to circular DNA. Changes in these superstructures of DNA have been suggested to be related to certain gene activities.

For example, superstructural changes may be related to transcriptional activities (Weintraub & Groudine, 1976; Piper et al., 1976; Garel & Axel, 1977), replication (Mattern & Painter, 1979; Mattern & Scudiero, 1981), and differentiation (Luchnik & Glasser, 1980). With the remarkable response of the DNA-degradative activity of BLM A_2 or BLM D_1 to the relaxing effect of BBM-928A on DNA superstructures, the treatments of cells with these two types of antitumor drugs as in combination chemotherapy would no doubt produce significant biological effects, which remain to be carefully studied.

The unique, cyclic, 2-fold symmetric structure, the strong bifunctional intercalation, and the high antitumor activity of BBM-928A made it interesting to compare the mode of its DNA intercalation with that of EB, a monofunctional inter-

calator which is virtually inactive against tumors. Although the activity of BLM A_2 and PLM D_1 responded to the DNA superhelical changes induced by BBM-928A in a similar fashion, we have previously observed (C.-H. Huang et al., unpublished results) that only the activity of BLM A_2 responded to superhelical changes induced by EB. These observations thus suggested that there may be at least some subtle differences, which could be probed and detected by the use of PLM D_1, in the superhelical changes induced by the two intercalators. These differences may possibly be related to the unique structure, the bifunctional intercalation, or the different binding sites (discussed below) of BBM-928A when compared to EB.

A possible correlation of the antitumor activity of BBM-928A with its intercalation with DNA remains to be established. Such a correlation has been suggested for echinomycin (Katagiri et al., 1975). If such a correlation is valid for BBM-928A, it is possible that the antitumor activity may be related to certain specific types of intercalation by BBM-928A at specific sites on DNA molecules differing from those of EB. Indeed, our present data (Figure 3) suggest that the binding of EB and BBM-928A (or echinomycin) to DNA was not competitive even at saturating concentration. A mode of intercalation differing from that of EB has been suggested for a number of other DNA intercalators with a medium or low unwinding angle as compared to that of EB (Sobell et al., 1977; Miller & Pycior, 1979).

The lack of competition in DNA binding between EB and BBM-928A or echinomycin at nearly saturating concentrations seems to violate the neighbor-exclusion principle (Crothers, 1974), which sets an upper limit for the binding of a monofunctional intercalator such as EB at a drug to DNA concentration ratio of 0.25 and a bifunctional intercalator such as BBM-928A or echinomycin at a ratio of 0.125. At these ratios, there will be two DNA base pairs between two adjacent intercalating sites and two base pairs are needed for every intercalating site. The limit for EB has been consistent with experimental results (Olins, 1969; Angerer & Moudrianakis, 1972; Huang & Baserga, 1976; also see Figure 3A,B). Binding studies showed that 11 nucleotides were required for the binding of one BBM-928A molecule (Huang et al., 1980). This means that for the intercalation of each quinoline chromophore, two to three base pairs are needed. The lack of competition in DNA binding between EB and BBM-928A at high concentrations is puzzling. A large contribution of nonintercalative bindings such as ionic bindings is not expected since the fluorescence studies of the competitive binding were performed in a relatively high ionic strength (100 mM NaCl), which would reduce the ionic interaction considerably. Furthermore, the results in Figure 2A indicate that the dissociation of BBM-928A molecules bound to DNA was insignificant during the gel electrophoresis which would break up most ionic interactions.

The question whether the intercalation of a bifunctional intercalator obeys the neighbor-exclusion principle is still open. Violation of this principle by echinomycin (Wakelin & Waring, 1976) and other bifunctional intercalators such as certain diacridine derivatives (Le Pecq et al., 1975) has been reported. By calculations of the binding curves of echinomycin using a statistical model based on the assumption that echinomycin at high concentrations produced mainly monofunctional intercalation, Shafer (1980) was able to show no violation of the neighbor-exclusion prinicple by echinomycin. While a similar type of assumption can be applied to the intercalation of BBM-928A alone, it may still be difficult to explain the lack of competitive binding between BBM-928A (or echinomycin) and EB at saturating concentrations.

Our observation that the BBM-928A relaxed PM2 DNA was more sensitive to the BLM A_2 activty differs from those reported by Lloyd et al. (1978b) and Povirk et al. (1979). Lloyd et al. (1978b) found that form I PM2 DNA was slightly more sensitive than isolated I^0 DNA to the clinical mixtures of bleomycins, and Povirk et al. (1979) reported the same preference to argue in favor of the presence of the intercalation of the bithiazole moiety with DNA. Currently, we have no clear explanation for this discrepancy. However, we want to emphasize that our conclusions were derived from studies of the effects on the BLM A^2 activity with systematic changes of superhelicity of the same DNA preparation. In our hands, we observed some variations in the DNA-degradative activity of BLM A_2, depending upon the amount of form II and form III DNA in the form I and form I^0 DNA preparations. We have observed that the concentration of BLM A_2 to cause breakage of 50% of DNA molecules could vary from 15 to 32 nM in different form I DNA preparations (Huang et al., 1981). This type of variation may complicate any direct comparison between form I and form I^0 DNA preparations. Nevertheless, our experiment showed that form I^0 DNA is more sensitive than form I when treated with BLM A_2 under the same conditions. This is consistent with our conclusion based on the studies of the BBM-928A-relaxed PM2 DNA.

Furthermore, the present system using BBM-928A to induce superhelical changes encompasses the changes in negative as well as positive superhelicities of DNA. Intercalator-free circular DNA preparations with different degrees of negative superhelicity can be prepared by the treatment of topoisomerase in a manner similar to that for our form I^0 DNA, but in the presence of varying amounts of EB, which was subsequently removed. However, such a procedure cannot produce positive superhelical DNA forms. One of our purposes of the present study is to examine the effects of BBM-928A-induced DNA conformational changes on the activity of BLM. The presence of BBM-928A during the digestion of DNA by BLM may raise the possibility that BBM-928A at low concentrations may directly stimulate and, at high concentrations, inhibit the degradative activity of BLM (Figure 4), regardless of the superhelical state of DNA. However, the results shown in Figure 5 indicated that whether BBM-928A stimulated or inhibited the BLM A_2 activity depended more on the decrease or increase in superhelicity of DNA rather than on the changes of the BBM-928A concentration. The handness of superhelicity seemed to be irrelevant.

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