ON THE MECHANISM OF ACTION OF BLEOMYCIN

STRAND SCISSION OF DNA CAUSED BY BLEOMYCIN AND ITS BINDING TO DNA *IN VITRO*

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Strand scission of DNA caused by bleomycin A_2 (BLM-A_2) and binding of the antibiotic to DNA was studied *in vitro*, examining the results of sucrose density gradient centrifugation and Sephadex G100 column chromatography. BLM-A_2 caused strand scission of both single and double stranded DNA of *E. coli* or HeLa cells at the concentration of 1.6 μg/ml, but not that of ribosomal and transfer RNAs, in the presence of 1 mM 2-mercaptoethanol. When 2-mercaptoethanol was omitted, no strand scission was observed. The action of the antibiotic was inhibited by addition of 0.1 mM Cu^{++}, Co^{++}, Zn^{++}, and 1 mM ETDA. By dialysis of the reaction mixture, after the incubation, double stranded DNA treated with BLM-A_2 was partly converted to single strand and strand scission was more markedly demonstrated. Binding of ^{3}H-BLM-A_2 to DNA was observed regardless of the presence or absence of 2-mercaptoethanol and amounts of bound BLM-A_2 to single stranded DNA was about twice higher than to the double stranded. Addition of Cu^{++}, Zn^{++}, EDTA, or phleomycin inhibited binding of BLM-A_2 to DNA but that of actinomycin, mitomycin or pluramycin did not show any effect.

The bleomycins, water-soluble basic glycopeptide antibiotics, produced by *Streptomyces verticillus*, exhibit antitumor and antibacterial activity[1], and have been employed for treatment of human squamous cell carcinoma. There are more than 13 bleomycins which have novel structures chelating with copper. The chemical structures of constituents of bleomycin A_2 (BLM-A_2) have been reported by TAKITA *et al.*[2] and its molecular weight is about 1,400.

As reported in a previous paper[3], BLM-A_2 inhibits DNA synthesis in growing cells of *E. coli*, EHRLICH carcinoma and HeLa cells. *In vitro*, bleomycins react with DNA in the presence of a sulfhydryl compound, and show the effect of decreasing the melting temperature (Tm) of DNA[4]. Furthermore, as the results of the reaction *in vitro* and *in vivo*, strand scission in DNA occurs[5,6,7]. In this paper, more detailed study is reported on strand scission of DNA which occurs *in vitro* by treatment of BLM-A_2 and on binding of the antibiotic to DNA.

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Materials and Methods

Materials

BLM-A_2 (copper-free, lot F_4) and universally labeled ^{3}H-BLM-A_2 (copper-free, 1.2×10^4 dpm/μg) were supplied by Drs. T. TAKEUCHI and T. TAKITA, Institute of Microbial Chemistry, Tokyo. Pluramycin was supplied by Dr. K. MAEDA, National Institute of Health, Tokyo. Phleomycin (lot a-9331-648) was obtained from Bristol Laboratories, Syracuse, N.Y., and actinomycin D was from Merck and Co., Rahway, N.J. Mitomycin C is a product of Kyowa Hakko Kogyo, Co., Tokyo. ^{14}C-Thymine (24 mc/mM) and ^{32}Pi were purchased from Daiichi Pure Chemicals Co., Tokyo.

DNA of *Escherichia coli* B was prepared by the method of MARMUR[8]. Preparation of ^{14}C-thymine-labeled DNA from *E. coli* 15T$^-$ cells was made according to the method previously described[6] (specific activity 560 cpm/μg). ^{32}P-Labeled DNA was prepared by the sodium dodecylsulfate-phenol method from HeLa cells which were cultured in EAGLE-MEM medium[9] supplemented with 10 % calf serum. ^{32}Pi (0.7 μc/ml in the medium) was incorporated for 24 hours and DNA was purified by methylated bovine serum albumin column chromatography.

Treatment of DNA with the Antibiotics

Unless otherwise stated, 250 μg/ml of DNA was incubated with the antibiotics at the various concentrations for 2 hours at 37°C in 50 mM Tris (pH 7.6) supplemented with 1 mM 2-mercaptoethanol (Tris-ME buffer), and in some cases it was dialyzed against the same buffer for 16 hours at 4°C.

Sucrose Density Gradient Centrifugation Analysis

The DNA samples (0.2 ml) were layered on the top of 4.6 ml of an alkaline (0.3 M NaOH, 0.7 M NaCl, and 2 mM EDTA, pH over 12.5) or neutral (0.02 M potassium phosphate buffer, and 0.1 M NaCl, pH 7.5) sucrose density gradient solution (5~20 %). Centrifugation was carried out in an SW 50 L rotor of a Beckman L2-65B centrifuge at 50,000 rev./min. for 120 minutes at 20°C. The fractions (0.2 ml) were collected from the bottom of the tubes, and the absorbance at 260 mμ was measured after addition of 0.8 ml of water to each fraction. The radioactivity of ^{32}P-DNA or ^{14}C-DNA in each fraction was determined with Aloka GM counter or windowless gas flow counter, respectively, after being precipitated with cold 5 % TCA using bovine serum albumin as carrier (200 μg/tube), dissolved in 2 N NH_4OH and dried on a planchet.

Hydroxylapatite Column Chromatography Analysis

BLM-A_2-treated ^{14}C-DNA (20 μg), before or after dialysis, was mixed with unlabeled native and heat-denatured *E. coli* B DNA (400 μg each) as internal references and chromatographed on 0.9×4.0 cm column of hydroxylapatite according to the method of BERNARDI[10]. Elution was made with a linear gradient of potassium phosphate (pH 6.8) from 0.01 to 1.0 M at 4°C and 2 ml fractions were collected. Optical density in each fraction was measured at 260 mμ and the radioactivity was determined as mentioned above.

Binding of ^{3}H-BLM-A_2 to DNA

The mixture of *E. coli* B DNA (250 μg/ml) and ^{3}H-BLM-A_2 (100 μg/ml) was incubated in several conditions at 37°C for 2 hours (total 0.5 ml) and, before or after dialysis, applied to Sephadex G100 column (1.2×18 cm) previously equilibrated with the incubation buffer. The column was eluted with the same buffer. One ml of each fraction was collected and read at 260 mμ to measure the DNA peak. The radioactivity of ^{3}H-BLM-A_2 was determined in Packard scintillation counter (dioxan-PPO-POPOP system). The amounts of ^{3}H-BLM-A_2 bound to DNA was calculated from the radioactivity at the peak of DNA on chromatogram.

Results

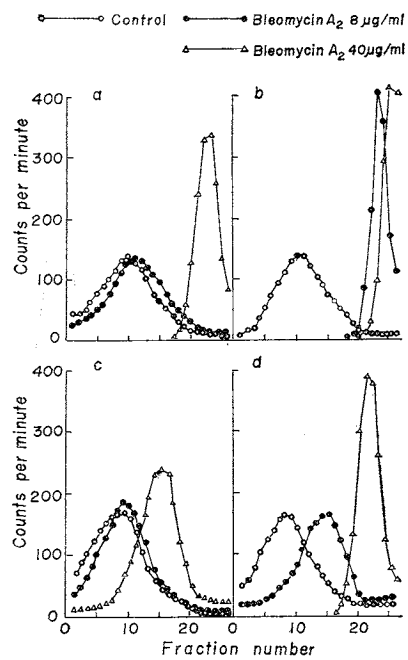
Effect of BLM-A_2 on Sedimentation Patterns of DNA

^{32}P-Labeled DNA from HeLa cells incubated with BLM-A_2 in Tris-ME buffer at 37°C for 2 hours was subjected to alkaline or neutral sucrose density gradient centrifugation analysis (Fig. 1a and c). The DNA treated with 40 μg/ml of the antibiotic sedimented more slowly than the control DNA and this effect was more markedly shown in alkaline than neutral sucrose solution, though little change was observed at 8 μg/ml of BLM-A_2 in both alkaline and neutral solutions. The change in sedimentation pattern of DNA treated with the antibiotic was observed more markedly at 8 and 40 μg/ml when the reaction mixture was dialyzed against the incubation medium (Fig. 1b and d). No radioactivity was found in the cold TCA-soluble fraction in all samples. These results suggest that single strand scission occurred in DNA strand incubated with the BLM-A_2 and the scission in DNA was promoted by dialysis. Similar results were also obtained with double stranded and heat-denatured DNA of *E. coli* B, but not with ribosomal RNA or transfer RNA. It indicated that the strand scission caused with BLM-A_2 is highly specific to DNA.

Fig. 1. Effect of BLM-A_2 on sedimentation patterns of DNA

^{32}P-labeled DNA from HeLa cells (about 40 μg/ml) was incubated for 2 hours at 37°C with BLM-A_2 in Tris-ME buffer. The incubation mixture, before or after dialysis, was layered on alkaline or neutral sucrose gradient solution and sedimented at 50,000 rpm for 2 hours at 20°C.

a and b: alkaline sucrose centrifugation (a before, b after dialysis)
c and d: neutral sucrose centrifugation (c before, d after dialysis)



Influence of 2-Mercaptoethanol on Bleomycin Activity

As already described, a sulfhydryl compound or H_2O_2 was necessary for bleomycin to decrease the Tm of DNA and cause single strand scission of DNA[4,5,6,7]. As shown in Fig. 2, the strand breaks were observed in DNA of *E. coli* B treated with BLM-A_2 even at the concentration of 1.6 μg/ml in Tris-ME buffer and this effect was parallel to the concentration of the antibiotic employed. But little change was observed in DNA treated with 40 μg/ml of the antibiotic in the absence of 2-mercaptoethanol. Sedimentation pattern of the control DNA incubated in the absence of 2-mercaptoethanol was the same as that of control DNA incubated with 2-mercaptoethanol.

Hydroxylapatite Column Chromatography Analysis of BLM-A_2-treated DNA

Heat-denatured and native DNA as internal references appeared at 0.14 M and at 0.24 M phosphate concentrations, respectively, with the elution of linear gradient of Pi concentration from the hydroxylapatite column. And ^{14}C-labeled DNA incubated with BLM-A_2 (40 μg/ml) for 2 hours at 37°C appeard in the same position as native DNA

Fig. 2. Influence of 2-mercaptoethanol on BLM-A_2 activity

E. coli B DNA (250 μg/ml) was incubated with BLM-A_2 in Tris or Tris-ME buffer for 2 hours at 37℃, and dialyzed against the same buffer for 16 hours in the cold. Alkaline sucrose density gradient centrifugation was carried out at 50,000 rpm for 120 minutes.

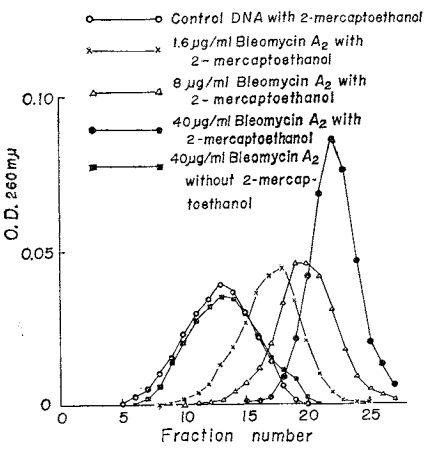


Fig. 3. Hydroxyapatite column chromatography analysis of BLM-treated DNA

A procedure is mentioned in the text.

o——o Left: ^{14}C-DNA of *E. coli* 15 T⁻ incubated with BLM-A_2 (40 μg/ml)
Right: ^{14}C-DNA of *E. coli* 15 T⁻ incubated with BLM-A_2 (40 μg/ml) and dialyzed

•·····•: *E. coli* B DNA (a mixture of native and heat-denatured) as internal reference

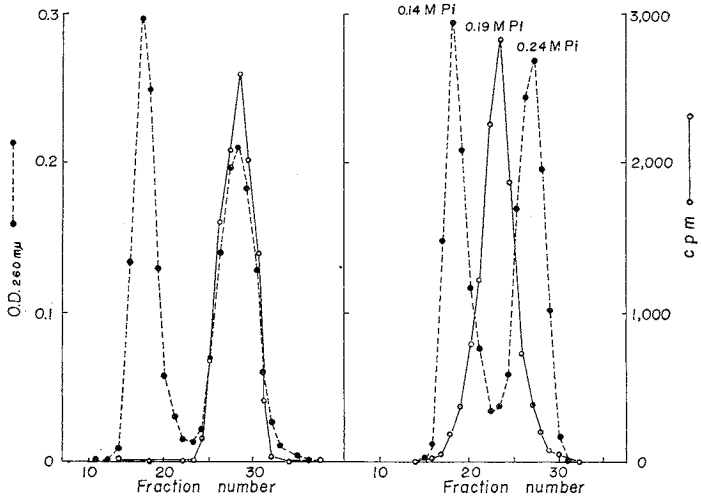


Fig. 4. Influence of divalent cations and EDTA on the DNA-breaking activity of BLM

E. cloi B DNA (250 μg/ml) was incubated with BLM-A_2 (40 μg/ml) in Tris-ME buffer in the presence of metal ions or EDTA and dialyzed against the same buffer.

A pattern with Co (0.1 mM) is similar to that with Zn.

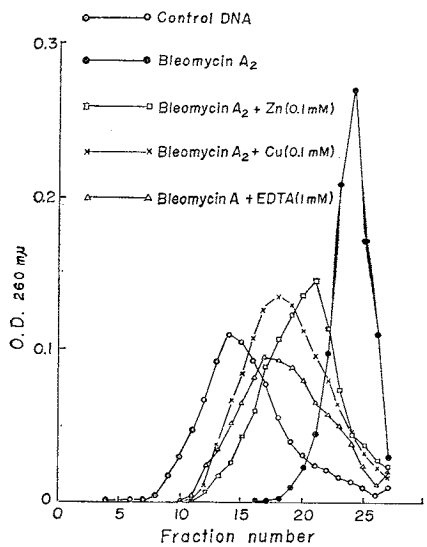


Table 1. Bind of ^{3}H-labeled BLM-A_2 to DNA

DNA	Incubation medium		Amount of ^{3}H-BLM-A_2 bound to DNA: cpm / 550 μg DNA	Molar ratio BLM-A_2: Nucleotide
Native	Tris-ME	inc.	1,106	1 : 350
		dial.	1,668	1 : 230
Heat-denatured	Tris-ME	inc.	2,357	1 : 165
		dial.	2,476	1 : 155
Native	Tris-H_2O_2	inc.	1,721	1 : 225
Native	Tris	inc.	958	1 : 405
		dial.	1,097	1 : 355
r-RNA	Tris-ME	inc.	45	1 : 8,800

inc. : The reaction mixture was incubated at 37℃ for 2 hours.
dial. : The reaction mixture was dialyzed against 300 volumes of incubation medium after incubation.
The data were obtained from Sephadex G 100 column chromatography.

(0.24 M Pi conc.), but after the reaction mixture was subjected to dialysis DNA peak appeared in the earlier fraction than native DNA and in the later fraction than heat-denatured DNA (Fig. 3). These results suggest that double stranded DNA incubated with BLM-A_2 in these conditions may still retain double strandness at neutral pH, though single strand breaks occur in the molecules. And after dialysis, double strandness may be destroyed, considerable part of the molecules being converted to the single

Fig. 5. Isolation of the BLM-DNA complex by gel filtration of Sephadex G100 column chromatography

A procedure is mentioned in the text.

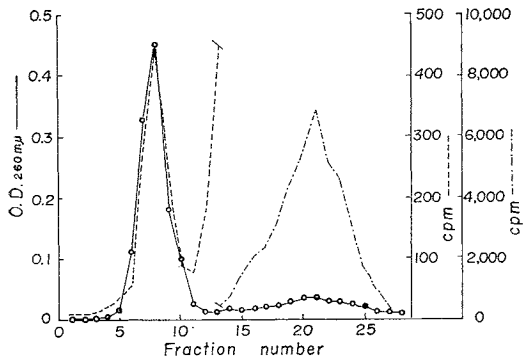


Table 2. Influence of divalent cations, EDTA, and antibiotics on binding of BLM-A_2 to DNA

Treatment	Amount of ^{3}H-BLM-A_2 bound to DNA	
	cpm / 50 μg DNA	Percent of control
Control	1,106	100 %
+ $CuCl_2$ 0.1 mM	636	58
+ $ZnSO_4$ 0.1 mM	626	57
+ EDTA 1 mM	289	26
+ Phleomycin	682	62
+ Pluramycin	1,218	111
+ Mitomycin C	1,077	92
+ Actinomycin D	1,204	108

Control: Native DNA of *E. coli* B and ^{3}H-BLM-A_2 was incubated in Tris-ME buffer.
Antibiotics were used at the concentration of 100 μg/ml each.

stranded and increasing the strand scission.

Influences of Divalent Cations and EDTA on the DNA-breaking Activity of BLM-A_2

As reported previously[4], divalent cations such as Cu^{++}, Zn^{++} and Co^{++} at the concentration of 0.1 mM and EDTA at 1 mM completely reversed the Tm-decreasing activity of BLM-A_2. As seen in Fig. 4, the addition of $CuCl_2$, $ZnSO_4$, $CoCl_2$ (0.1 mM each) or EDTA (1 mM) to the incubation mixture inhibited the activity of BLM-A_2 to cause strand scission in DNA molecule. But 0.1 mM of EDTA or $MgCl_2$ did not show such effect (not indicaded here).

Binding of ^{3}H-BLM-A_2 to DNA

In order to demonstrate the interaction of BLM-A_2 with DNA, the mixture of ^{3}H-BLM-A_2 and *E. coli* B DNA treated under several conditions was subjected to Sephadex G100 column chromatography. Typical chromatogram was shown in Fig. 5; Native DNA of *E. coli* (250 μg/ml) and ^{3}H-BLM-A_2 (100 μg/ml) were incubated in Tris-ME buffer at 37°C for 2 hours and applied to the column. DNA and free BLM were separated by gel filtration with the appearance of DNA-BLM complex. From the radioactivity seen in DNA peak, amount of BLM bound to DNA was calculated (1 μg of ^{3}H-BLM-A_2 was 1,811 cpm in scintillation counter). In this case, 1,106 cpm of ^{3}H-BLM-A_2 was bound to 50 μg of native DNA and the binding ratio was one molecule of BLM per 350 DNA nucleotides.

In Tables 1 and 2, the results are summarized. As seen in Table 1, the binding ratio in Tris-ME buffer was increased about 50 % when the incubation mixture was dialyzed. About twice amount of BLM was bound to heat-denatured DNA than to native DNA, though the binding ratio was not increased by dialysis. Without 2-mercaptoethanol, about 90 % of BLM-A_2 compared with the case with 2-mercaptoethanol was bound to DNA and slightly increased by dialysis. When 1 mM of hydrogen peroxide was added to the incubation mixture in place of 2-mercaptoethanol, bound BLM-A_2 was increased about 50 %. BLM-A_2 could hardly bind to ribosomal RNA.

As shown in Table 2, addition of Cu^{++}, Zn^{++}, or EDTA to Tris-ME buffer at the concentration at which the strand scission in DNA caused by BLM-A_2 was inhibited (Fig. 4), also inhibited the binding of BLM-A_2 to DNA. Moreover, addition of phleomycin (100 μg/ml), which is chemically related to bleomycin, lowered the amount of BLM-A_2 bound to DNA, but the other antibiotics, pluramycin, mitomycin C, or actinomycin D, which are all known to bind to DNA, did not affect binding of BLM to DNA.

These facts obtained by gel filtration analysis indicated that (1) BLM-A_2 binds to both single and double stranded DNA, though the amount of binding to the former is about twice than to the latter. (2) Binding of BLM-A_2 is highly specific to DNA. (3) Without 2-mercaptoethanol or hydrogen peroxide, BLM-A_2 can bind to DNA. (4) Divalent cations such as Cu^{++}, Zn^{++}, or EDTA prevent the binding of BLM-A_2 to DNA. (5) Mode of binding of BLM-A_2 to DNA is the same or similar to that of phleomycin but different from pluramycin, mitomycin C, or actinomycin D.

Discussion

As reported in previous papers,[4,5,6,7] interaction of bleomycin and DNA was demonstrated by thermal denaturation and strand scission of DNA by sedimentation in the presence of a sulfhydryl compound or hydrogen peroxide. In this paper, correlation of strand scission caused by BLM-A_2 and binding of BLM-A_2 to DNA was studied.

DNA strand scission was observed by sucrose density gradient centrifugation analysis and binding of BLM-A_2 to DNA was analyzed by Sephadex G100 column chromatography. Both reactions are highly specific to DNA, occur in both single and double stranded DNA, and are inhibited by Zn^{++}, Co^{++}, Cu^{++} and EDTA.

BLM-A_2 did not cause single strand scission in DNA without 2-mercaptoethanol but in the same conditions binding of BLM-A_2 to DNA was observed with about 90 % of binding efficiency of that with 2-mercaptoethanol. From analysis of Tm-decreasing activity[4], the sequence of the reaction was shown that the reaction may occur first between DNA and sulfhydryl compound and then BLM-A_2. These results suggest that there are two types of binding of BLM-A_2 to DNA: one is binding to DNA modified with sulfhydryl group or hydrogen peroxide, leading to strand scission or decrease of Tm, and the other is binding to DNA presumably not modified with no sequential reaction.

About twice as much BLM-A_2 binds to single stranded DNA as to native DNA. This finding is in accordance with the result that after dialysis native DNA treated with BLM-A_2 binds about 50 % more than before dialysis, because dialyzed DNA was partly changed to the single stranded as shown in Fig. 3.

Mode of inhibitory effects of metal ions such as Zn^{++}, Co^{++}, and Cu^{++} on bleomycin activity remains to be determined. One possibility is that they might react with sulfhydryl group. But the fact that they inhibit binding of BLM-A_2 to DNA while BLM-A_2 binds to DNA without sulfhydryl compounds suggests that these metal ions may act, in addition, directly on the BLM-A_2 molecule. Bleomycin is a chelating agent and may react with these metals. Addition of EDTA to the mixture of BLM-A_2 and DNA diminished BLM activity. In another experiment, BLM-A_2 did not exhibit its activity even in the presence of 2-mercaptoethanol when citrate buffer was used instead of Tris buffer. These facts suggest that the chelating activity of BLM-A_2 competes with that of other chelating agents and they weaken the biological activity. In other words, the chelating activity of BLM is necessary for its activity.

The reason of increase in strand scission by dialysis also remains to be determined. However, it may partly reflect the fact that single stranded part in BLM-A_2-treated

DNA molecules is increased by dialysis and that more BLM-A_2 bind to single strand DNA than to double strand.

Molar ratio of DNA nucleotide to BLM-A_2 in binding was 350 : 1 when 250 μg/ml of native DNA and 100 μg/ml BLM-A_2 were used and binding was observed by Sephadex column chromatography. However, the real number of BLM-A_2 molecules bound to DNA may be much more than that, because the release of bound BLM-A_2 from DNA may occur during running of column chromatography. In preliminary experiment by equilibrium dialysis, a 15 : 1 molar ratio was observed in the same conditions.

Many antibiotics have been known to bind to DNA *in vitro* and *in vivo* resulting in inhibition of nucleic acid synthesis or in degradation of DNA. Actinomycin D[11], mitomycin C[12], pluramycin[13], chromomycin A_3[14], daunomycin[15], nogalamycin[16], and anthramycin[17] are in this group. Compared with these drugs, the interaction of BLM-A_2 and DNA is unique in three points: (1) They increase Tm of DNA but BLM-A_2 decreases it; (2) BLM is the only one to cause single strand scission of DNA; (3) The affinity of BLM to single strand DNA is stronger than to double stranded DNA, while for other antibiotics double strandness or helical secondary structure is necessary for the binding to DNA. The fact that binding of BLM to DNA is not affected by addition of actinomycin D, mitomycin C or pluramycin reflects the difference mentioned above, although mechanism of binding of BLM-A_2 to DNA is not clear yet. Phleomycin, a compound related to BLM, inhibited the binding of BLM-A_2 to DNA. It means that its binding site on DNA is similar to that of BLM-A_2. However it should be noted that phleomycin increases Tm of poly dAT[18], though BLM-A_2 does not significantly affect it[4].

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