ON THE MECHANISM OF ACTION OF BLEOMYCIN: SCISSION OF DNA STRANDS *IN VITRO* AND *IN VIVO*

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The bleomycins, water-soluble basic glycopeptide antibiotics produced by *Streptomyces verticillus*[1,2], exhibit antitumor and antibacterial activity[3], and therapeutic effects on human squamous cell carcinoma have been reported[4]. They inhibit DNA synthesis in *E. coli*, EHRLICH carcinoma, and HeLa cells[5]. *In vitro*, bleomycin A_2 or the other bleomycins react with DNA which has previously been treated with a sulfhydryl compound, and cause a decrease in its melting temperature (Tm)[6]. In the reactions *in vitro* and *in vivo*, strand scission in DNA has been confirmed by sucrose density gradient centrifugation analysis, and the results are presented in this communication.

Bleomycin A_2, copper-free, was supplied by Dr. TAKITA, Institute of Microbial Chemistry. DNA from HeLa cells which has been labelled with ^{32}P was prepared by the sodium dodecylsulfate(SDS)-phenol method and purified by methylated bovine serum albumin column chromatography. DNA of *E. coli* was prepared according to the method of MARMUR[7]. Changes in molecular sizes of DNA treated with bleomycin A_2 *in vitro*, before or after dialysis, were analyzed by sucrose density gradient centrifugation.

In the *in vivo* experiments, *E. coli* 15 T$^-$ cells labelled with ^{14}C-thymine were incubated with bleomycin A_2 for 30 minutes at 37°C. The cells were suspended in a solution containing SDS (1 %) and EDTA (0.05 M), which was shaken with an equal volume of 90 % phenol for 10 minutes in the cold. EDTA, which prevents the effect of bleomycin on DNA in the presence of a sulfhydryl compound[6], was added to avoid DNA degradation by the antibiotic during extraction. The aqueous layer was subjected to sedimentation analysis. HeLa cells cultured in EAGLE MEM medium[8] supplemented with 10 % calf serum were labelled with ^3H-thymidine for 20 hours and incubated with bleomycin A_2 for 6 hours at 37°C. EDTA (0.05 M) was then added, and the DNA was extracted and analyzed according to the method of TERASIMA and TSUBOI[9].

As is shown in Fig. 1, incubating of ^{32}P-labelled DNA from HeLa cells with bleomycin A_2 at 37°C in Tris buffer (pH 7.6)

Fig. 1. Effect of bleomycin A_2 on sedimentation patterns of DNA of HeLa cells *in vitro*.

^{32}P-labelled DNA from HeLa cells (about 40 μg/ml) was incubated for 2 hours at 37°C with bleomycin A_2 as indicated in 50 mM Tris (pH 7.6) buffer supplemented with 1 mM 2-mercaptoethanol. The incubation mixture (0.2 ml), before or after dialysis against the same buffer for several hours at 4°C, was layered on 4.6 ml of alkaline (pH 12.5) or neutral (pH 7.5) sucrose density gradient solution (5~20 %) and sedimented at 50,000 rev./min. for 2 hours at 20°C in a SW 50 L rotor of a model L2-65B BECKMAN ultracentrifuge. The fractions were collected from the bottom of the tubes, and DNA was precipitated in each fraction with 5 % trichloroacetic acid using bovine serum albumin as carrier. The precipitate was suspended in 2 N NH_4OH and the radioactivity was measured in a GM counter.

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

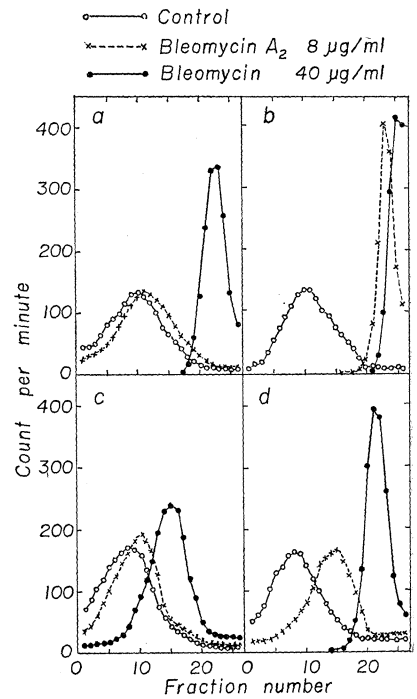
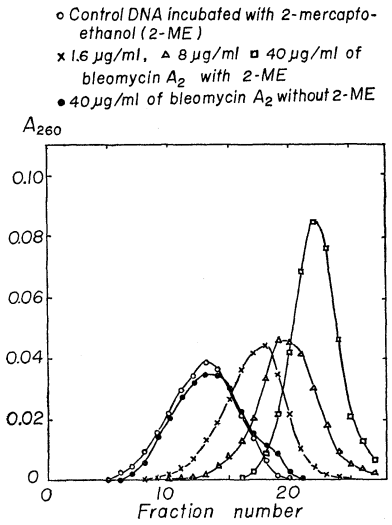


Fig. 2. Influence of 2-mercaptoethanol on the bleomycin activity *in vitro*.

E. coli B DNA (250 μg/ml) was incubated with bleomycin A_2 in 50 mM Tris buffer (pH 7.6) in the absence or presence of 1 mM 2-mercaptoethanol for 2 hours at 37℃, and dialyzed against the same buffer for 16 hours in the cold. The DNA samples (0.2 ml) were centrifuged in alkaline sucrose density gradient solution (5~20%) at 50,000 rev./min. for 120 min. at 20℃. Absorbance at 260 mμ was measured after addition of 0.8 ml of water to each fraction. In the absence of bleomycin A_2, the identical sedimentation pattern of DNA was obtained with or without 2-mercaptoethanol.



with 1 mM 2-mercaptoethanol decreased its sedimentation rate, but no radioactivity was found in the cold TCA-soluble fraction in any samples. The amounts of slow sedimenting DNA increased when the reaction mixture was dialyzed against the incubation medium for several hours at 4°C. The effect was more marked in alkaline than neutral sucrose solutions. These results suggest that single strand scission occurred in DNA treated with the antibiotic. Similar results were obtained with DNA of *E. coli*. As illustrated in Fig. 2, the strand breaks were demonstrated in DNA of *E. coli* treated with bleomycin A_2 even at the concentration of 1.6 μg/ml in Tris buffer supplemented with 1 mM 2-mercaptoethanol, but no significant change was observed in DNA treated with 40 μg/ml of the antibiotic in the absence of 2-mercaptoethanol. The result showed that the sulfhydryl compound was necessary for bleomycin A_2 to cause scission in DNA strand as in the case of decreasing Tm of DNA[6]. The grade of the strand scission was parallel to the concentration of bleomycin A_2 employed.

Fig. 3. Effect of bleomycin A_2 on DNA of growing *E. coli* 15T⁻ cells.

E. coli 15T⁻ cells, labelled with ^{14}C-thymine (0.05 μc/ml, 24 mc/mM) for 120 min., were incubated in the growth medium containing bleomycin A_2 for 30 min. at 37℃. The cells were collected by contrifugation and DNA was extracted by sodium dodecylsulfate-phenol method. After centrifugation at 36,000 rev./min. for 2 hours at 4℃ in alkaline sucrose density gradient solution in SW 50 L rotor fractions were collected and the DNA was precipitated as described in Fig. 1. The radioactivity was measured in a low background windowless gas flow counter.

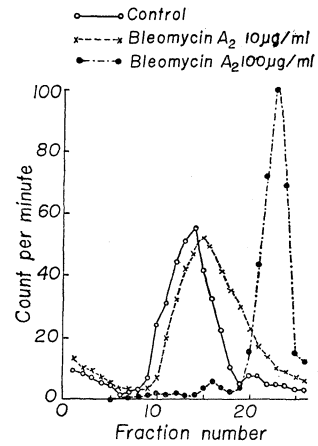
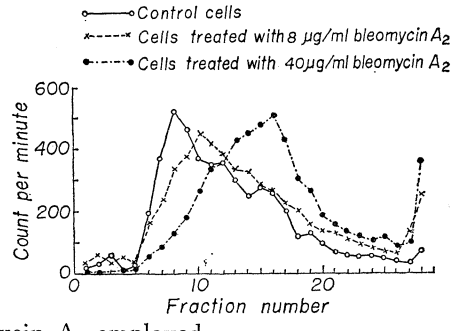


Fig. 4. Effect of bleomycin A_2 on DNA of growing HeLa cells.

HeLa cells, labelled with ^{3}H-thymidine (0.05 μc/ml, 1,850 mc/mM) for 20 hours, were incubated with bleomycin A_2 for 6 hours at 37℃. The cells were collected by centrifugation and suspended in cold PBS containing 0.05 M EDTA. The cell suspension, 0.1 ml (1×10^4 cells) was layered on 4.6 ml of alkaline sucrose density gradient solution (pH 12.5, 5~20%) overlaid with 0.2 ml of 2% SDS solution and centrifuged at 36,000 rev./min. for 90 min. at 20℃.



Single-strand scission of DNA was also observed in growing *E. coli* 15T⁻ cells, incubated with bleomycin A_2 at 10 or 100 μg/ml for 30 minutes (Fig. 3). A similar breakage occurred in HeLa cells incubated with the antibiotic at 8 or 40 μg/ml for 6 hours (Fig. 4). Unless EDTA was added to

the cell suspension, more marked scission of DNA was demonstrated. The enhancement of DNA degradation seemed to occur during the extraction procedure.

The results presented in this paper indicate that in the presence of a sulfhydryl compound *in vitro*, bleomycin binds to DNA, and causes single-strand scission. A similar reaction is induced by bleomycin in growing bacterial and mammalian cells. The scission of DNA may be the cause of the inhibition of thymidine incorporation into DNA of growing cells and the inhibition of cell division which have been reported in a previous paper[5].

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