EFFECTS OF BLEOMYCIN A_2 ON DEOXYRIBONUCLEASE, DNA POLYMERASE AND LIGASE REACTIONS

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Bleomycin A_2 caused degradation of DNA in the intact cells of *E. coli* $15T^-$ and stimulated breakdown of DNA by DNase in the crude extract of *E. coli* B. On the other hand, the incorporation of dGMP into acid-insoluble fraction by partially purified *E. coli* B DNA polymerase was stimulated by bleomycin A_2 for a short period after the addition of the antibiotic, and thereafter degradation of the product was observed. Digestion of salmon sperm DNA by pancreatic DNase was also stimulated by the antibiotic. An inhibitory effect of bleomycin A_2 on polynucleotide ligase prepared from T_4 phage-infected *E. coli* B was observed. The above *in vivo* and *in vitro* effects of bleomycin A_2 can be explained if it causes strand scission of DNA. The stimulation of DNase reaction may also be related to the DNA strand scission by bleomycin.

As reported in previous papers, bleomycins inhibit DNA synthesis in growing cells of *E. coli*, EHRLICH carcinoma and HeLa[1], cause scission of DNA strand *in vivo* and *in vitro*[2,3], and decrease the melting temperature of DNA in the presence of sulfhydryl compounds[4,5] or hydrogen peroxide[6].

The results concerning the effect of bleomycin A_2 on DNA polymerase reaction, the stimulation of degradation of DNA by nuclease, and the inhibition of polynucleotide ligase are presented in this paper.

Materials and Methods

Materials: Copper-free (lot F_4) and copper-saturated (Cu 5.1 %) bleomycin A_2 were supplied by Dr. T. TAKITA, Institute of Microbial Chemistry, Tokyo. Phleomycin (lot a-9331-648) was prepared by Bristol Laboratories, Syracuse, N. Y. Pluramycin was supplied by Dr. K. MAEDA, National Institute of Health, Tokyo. DNA of *E. coli* B and *E. coli* $15T^-$ were prepared according to the method of MARMUR[7]. ^{14}C-Thymine-labeled DNA of *E. coli* $15T^-$ was prepared as described previously[4]. Salmon sperm DNA and pancreatic deoxyribonuclease (E. C. 3, 1, 4, 5) were obtained from Calbiochem, Los Angeles, and deoxyribonucleotide triphosphates were from Sigma Chemical Co., St. Louis. dGTP-8-^{14}C (12 mC/mM) and thymine-2-^{14}C (24 mC/mM) are commercial products of Schwartz Bioresearch Inc., Orangeburg, N. Y. and Daiichi Pure Chemical Co., Tokyo, respectively.

Degradation of DNA in intact cells of *E. coli* $15T^-$: After incubation with ^{14}C-thymine for 2 hours, *E. coli* $15T^-$ cells were washed twice with a large volume of Tris-

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minimum medium[8] and suspended in a growth medium containing unlabeled thymine (10 μg/ml). The cell suspension (0.1 ml, about 1×10^7 cells, 400 cpm in cold TCA-insoluble fraction) was incubated with various concentrations of bleomycin, phleomycin or pluramycin at 37°C for 5, 15, 30 and 60 minutes. The incubation was stopped by addition of 2 ml of cold 5 % TCA. The precipitate was washed twice with cold 5 % TCA. The supernatant after TCA precipitation and the washing were combined, and TCA was removed by extraction with ether. The radioactivity in the cold TCA-soluble fraction was measured by a windowless gas-flow counter.

Assay of DNA polymerase and DNase reaction: *E. coli* B was harvested at the logarithmic phase of growth in nutrient broth, sonicated at 10 KHz for 10 minutes, and centrifuged at $105,000\times g$ for 120 minutes. The supernatant fraction was used as the crude enzyme source. DNA-primed DNA polymerase from *E. coli* B was partially purified by the method of RICHARDSON *et al.*[9], including streptomycin precipitation, autolysis, $(NH_4)_2SO_4$ fractionation, acid precipitation, ethanol precipitation and chromatography on DEAE cellulose. The incubation mixture of DNA polymerase reaction contained 12.5 mμmoles of $MgCl_2$, 0.25 mμmole of 2-mercaptoethanol, 5 mμmoles of dATP, dCTP, TTP and ^{14}C-dGTP, 10 μg of *E. coli* B DNA and 0.1 unit of the partially purified or crude enzyme (40 μg as protein) in a total volume of 0.25 ml. To test DNase activity, cold dGTP and ^{14}C-thymine labeled *E. coli* 15T$^-$ DNA were employed in place of ^{14}C-dGTP and *E. coli* B DNA. After incubation at 37°C, the reaction mixture was chilled in ice, 0.5 mg of bovine serum albumin was added, and the precipitate formed by the addition of 0.25 ml of 10 % PCA was collected. The precipitate was washed twice with 2 ml of 5 % PCA. The radioactivity of the residue was counted by a windowless gas-flow counter.

Chase experiment in EHRLICH carcinoma cell lysate: The reaction mixture containing $105,000\times g$ supernatant of EHRLICH cell lysate as the crude DNA polymerase solution (100 μg as protein), 20 μg of salmon sperm DNA, 6 mμmoles of dATP, dCTP and TTP, 2 mμc (160 $\mu\mu$moles) of ^{14}C-dGTP, and 2 μmoles of ATP in a total volume of 0.2 ml was incubated at 37°C for 5 minutes. Immediately after the incubation 16 mμmoles of ^{12}C-dGTP and bleomycin A_2 were added to the reaction mixture and the incubation was continued for 35 minutes at 37°C. The reaction was stopped by the addition of 0.2 ml cold 10 % TCA. The precipitate was washed with 5 % TCA and the radioactivity of TCA-insoluble fraction was measured by a windowless gas-flow counter.

Pancreatic DNase reaction: The digestion was performed as described in the legend of Fig. 3.

Assay of polynucleotide ligase reaction: Polynucleotide ligase was prepared from T_4 phage-infected *E. coli* B according to the method of WEISS and RICHARDSON[10] and the reaction was carried out by the method of ANDO *et al.*[11] The product of the enzymic reaction was examined by alkaline sucrose density gradient centrifugation analysis.

Results

Degradation of DNA in Intact Cells of *E. coli* 15T$^-$

As presented in Table 1, in the intact cells of *E. coli* 15T$^-$ DNA degradation into the acid-soluble fraction was negligible in the control group during the incubation, but the addition of bleomycin A_2 (copper-free or -saturated) or phleomycin caused marked degradation of DNA and the amounts of degradation products formed were proportional to the concentration of the antibiotics and the incubation time. About 10 % of total DNA was degraded by bleomycin A_2 (copper-free) at a concentration of 100 μg/ml in 15 minutes and 28 % in 60 minutes. Copper-free and copper-saturated bleomycin A_2 showed almost the same effect on DNA degradation at 100 μg/ml but at

10 μg/ml the former exhibited a stronger effect. The most rapid and strongest degradation was observed with phleomycin; at 100 μg/ml about 10% of DNA was degraded into the acid-soluble fraction in 5 minutes and 54% in 60 minutes and even at 10 μg/ml about 40% of DNA was degraded into the acid-soluble in 60 minutes. Pluramycin has been reported to inhibit DNA and RNA polymerase reactions by binding with DNA[12,13,14], however at 10 μg/ml it did not show an effect on DNA degradation in 60 minutes.

Table 1. DNA degradation by antibiotics in intact cells of *E. coli* 15T⁻

Treatment		Radioactivity in cold TCA-soluble fraction (cpm per tube)			
		5 min.	15 min.	30 min.	60 min.
Control		0	1	1	4
BLM A_2(-Cu)	100 μg/ml	14	41	80	114
	10 μg/ml	10	17	46	85
	1 μg/ml	4	10	13	13
BLM A_2 Cu-saturated	100 μg/ml	7	29	71	124
	10 μg/ml	1	11	10	33
	1 μg/ml	4	9	2	13
Phleomycin	100 μg/ml	35	119	199	216
	10 μg/ml	7	51	92	158
	1 μg/ml	4	6	11	14
Pluramycin	10 μg/ml				3

The procedure is described in the text. Total radioactivity is 400 cpm per tube at zero time.

DNA Degradation by the Crude Extract of *E. coli* B

When the crude extract of *E. coli* B was used as the enzyme source, 89% inhibition of DNA polymerase reaction by 40 μg/ml of bleomycin A_2 was observed in 30 minutes at 37°C. The inhibitory effect of bleomycin A_2 was completely reversed by the addition of 10^{-4} M of Cu^{+2} or Zn^{+2} or 10^{-3} M of EDTA. The results coincide with the effect of the antibiotic on melting temperature of DNA reported in a previous paper[4]. In the same reaction medium the fate of the DNA added as the primer was investigated. As shown in Fig. 1, primer DNA became acid-soluble more rapidly in the presence of bleomycin A_2. It suggests that the antibiotic stimulates the

Fig. 1. DNA degradation by the crude extract of *E. coli* B in the presence and absence of bleomycin A_2.

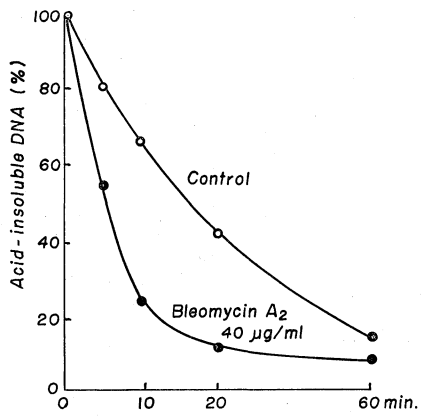
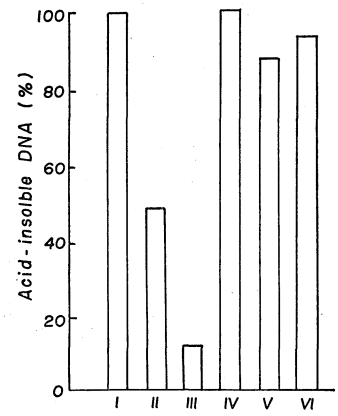


Fig. 2. DNA degradation in a medium with or without the crude extract of *E. coli* B and the effects of bleomycin A_2 and pluramycin.

I Zero time
II Control (20 minutes incubation at 37℃)
III plus BLM A_2 40 μg/ml
IV plus PLM 10 μg/ml
V minus cell extract
VI minus cell extract, plus BLM A_2 40 μg/ml



degradation of DNA by DNase in the crude extract of *E. coli* B. Fig. 2 shows the effects of the antibiotics on DNA breakdown in the same medium with or without the cell extract. Marked degradation of DNA was observed in the presence of both the cell extract and bleomycin A_2. Pluramycin, which is known to bind with DNA, protected DNA from degradation by the cell extract.

Effect of Bleomycin A_2 on Pancreatic DNase Action

Salmon sperm DNA was incubated with pancreatic DNase in the presence or absence of bleomycin A_2. DNA breakdown was measured by the increase of absorbancy at 260 mμ. Two μg/ml of bleomycin A_2 significantly stimulated the degradation of DNA by the DNase and the stimulatory effect of the antibiotic was remarkable at the concentration of 20 μg/ml. Without the enzyme bleomycin A_2 showed a slight increase of absorbancy in 120 minutes. The results are presented in Fig. 3.

In Vitro DNA Synthesis in the Presence of Bleomycin A_2

In the reaction of the partially purified DNA polymerase prepared from *E. coli* B an interesting result was obtained. Bleomycin A_2 significantly stimulated the incorporation of ^{14}C-dGMP into acid-insoluble fraction in a short period and thereafter showed a marked degradation of the product. During the period of marked stimulation by bleomycin A_2 the incorporation of dGMP increased linearly and the slope of the increase rate was much steeper than in the absence of the antibiotic (Fig. 4 a). Breakdown of the primer DNA was studied simultaneously in the same reaction mixture without nucleotides. As illustrated in Fig. 4 b, during the first 10 minutes the DNA remained acid-insoluble at the same level with or without the antibiotic and thereafter degradation occurred more rapidly in the presence of bleomycin A_2.

Similar results were obtained regarding the fate of the reaction product of DNA polymerase in the extract of EHRLICH carcinoma cells. Incorporation of

Fig. 3. Effect of bleomycin A_2 on pancreatic DNase action.

A : Salmon sperm (native) DNA 25 μg/ml alone in the medium containing Tris-HCl (pH 7.5) 5 mM, $MgCl_2$ 5 mM, KCl 50 mM and 2-mercaptoethanol 3 mM
B : A plus BLM A_2 2 μg/ml
C : A plus BLM A_2 20 μg/ml
D : A plus DNase I 10^{-2} μg/ml
E : A plus DNase I 10^{-2} μg/ml and BLM A_2 2 μg/ml
F : A plus DNase I 10^{-2} μg/ml and BLM A_2 20 μg/ml

The increase of optical density at 260 mμ during the incubation was plotted.

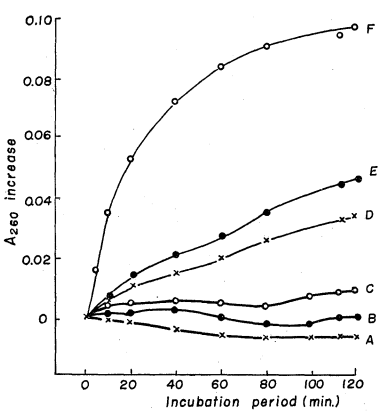


Fig. 4. Effect of bleomycin A_2 on DNA polymerase reaction (a) and breakdown of primer DNA (b).

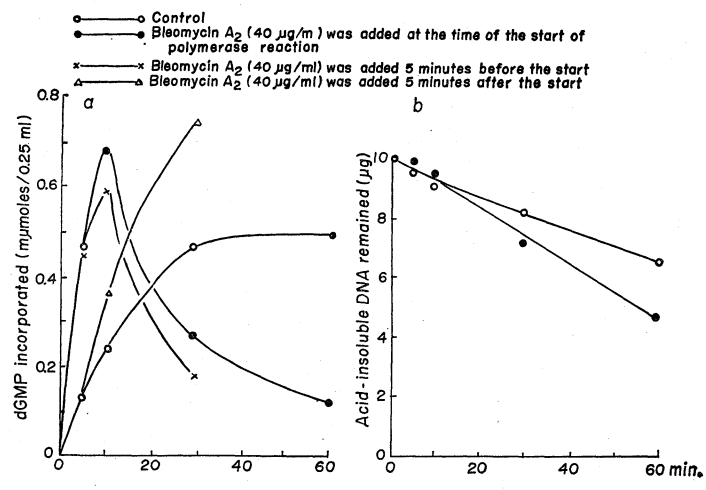


Fig. 5. Chase experiment testing the effect of bleomycin A_2 on DNA polymerase reaction of EHRLICH carcinoma cell lysate.

A : minus BLM A_2 (control)
B : plus BLM A_2 1.6 μg/ml
C : plus BLM A_2 8 μg/ml
D : plus BLM A_2 40 μg/ml
The details are described in the text.

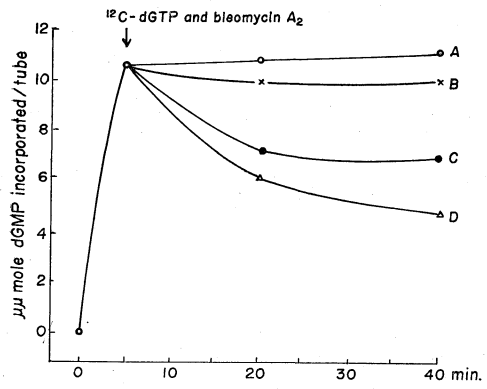
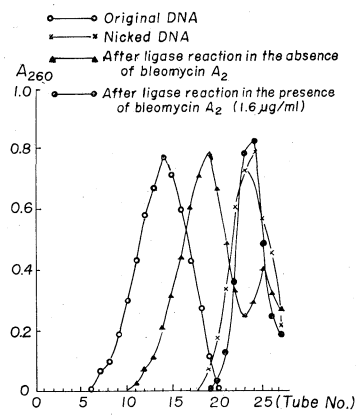


Fig. 6. Effect of bleomycin A_2 on polynucleotide ligase reaction.

Nicked DNA (pancreatic DNase treated) of *E. coli* B was incubated with polynucleotide ligase (DEAE cellulose fraction) at 37℃ for 30 minutes. Alkaline sucrose density gradient centrifugation (5~20 %, 0.3 M NaOH, 0.7 M NaCl, and 2 mM EDTA, pH over 12.5) was carried out at 50,000 r.p.m. for 120 minutes at 20℃ in a SW 50L rotor of Beckman L2 65B ultracentrifuge.



the radioactive precursor into acid-insoluble product in the DNA polymerase reaction was reduced by the addition of a large amount of cold precursor. Bleomycin A_2 was introduced simultaneously to the reaction mixture. As shown in Fig. 5, the radioactivity of the acid-insoluble fraction was reduced by the addition of the antibiotic and this decrease was more marked at higher concentrations of the antibiotic. It indicates that the antibiotic stimulates the degradation of the reaction products.

Effect on Polynucleotide Ligase Reaction

Single strand breaks were introduced into DNA molecules by pancreatic DNase. As shown in Fig. 6, nicked DNA (substrate of polynucleotide ligase) sedimented more slowly during the alkaline sucrose density gradient centrifugation analysis than original DNA. After polynucleotide ligase reaction it sedimented more rapidly than before the reaction and sedimentation pattern became close to the original ones, indicating that the strand scission was repaired by the enzyme. But in the presence of bleomycin A_2 at a concentration of 1.6 μg/ml the reaction was completely inhibited. However, it must be noted that 2-mercaptoethanol was contained in the reaction mentioned above at a concentration of 6.6 mM and under these conditions strand scission would be caused in substrate DNA by bleomycin A_2. Therefore there remains a possibility that the polynucleotide ligase reaction *per se* is not inhibited by the antibiotic.

Discussion

Bleomycin A_2 has been reported to bind with DNA[3] resulting in strand scission[2,3,5,6] and decrease of the melting temperature[4,5,6] in the presence of sulfhydryl compounds or hydrogen peroxide. The former effect is an endonucleolytic activity of the antibiotic. This may increase the priming activity of DNA for DNA polymerase as in the case of pancreatic DNase[15]. On the other hand the strand scission caused by bleomycin A_2 is thought to make the DNA molecule more sentisive to the action of exo-type DNases.

Partially purified DNA polymerase of *E. coli* B has been known to be accompanied by some exo-type DNase. This significant stimulation of DNA polymerase reaction at the early stage and the inhibition at the late period can be well explained by degradation of the primer and product DNA by bleomycin A_2 (Figs. 4 and 5). In the experiment using crude extract as an enzyme source, bleomycin A_2 showed strong inhibition of DNA polymerase reaction after 30 minutes of incubation. However, the time course of synthesis and degradation of DNA revealed that the inhibition might be caused by stimulated breakdown of the product. As the cell extract contains several kinds of exo- and endo-type DNases, primer DNA and product DNA seem to be affected by bleomycin A_2 and easily broken to the acid-soluble form by the enzymes. Stimulation of DNA breakdown by pancreatic DNase shown in the presence of the antibiotic supports this consideration. On the contrary DNA degradation by cell extract was counteracted by pluramycin which is known to stabilize DNA structure[12].

In the intact cells of *E. coli* $15T^-$ bleomycin A_2 and phleomycin caused marked degradation of DNA into acid-soluble fragments. This indicates that the same effect of bleomycin A_2 as shown *in vitro* may also occur *in vivo*. However, phleomycin inhibits the DNA polymerase reaction and increases melting temperature of DNA[16,17]. It will be interesting to study in more detail the differences in the mode of action of both antibiotics *in vivo* and *in vitro*.

From the above it is concluded that the inhibition of DNA synthesis by bleomycin A_2 *in vivo* and *in vitro* is due to strand scission and stimulation of the DNase reaction. Bleomycin A_2 inhibits the polynucleotide ligase reaction. This inhibition can also be explained by the effect of the antibiotic causing strand scission of DNA, though more details remain to be studied.

Recently, LUCIA and CAIRNS[18] raised a question concerning the significance of DNA polymerase in DNA biosynthesis and the apparatus operating *in vivo* for DNA synthesis is not yet completely established. Therefore, the direct effect of bleomycin A_2 on DNA synthesis *in vivo* remains to be determined, and bleomycin A_2 may be a useful tool to elucidate the mechanism of *in vivo* DNA synthesis.

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