REACTION OF BLEOMYCIN WITH DNA
STRAND SCISSION OF DNA IN THE ABSENCE OF SULFHYDRYL OR PEROXIDE COMPOUNDS

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The effect of bleomycin on the infectivity and the molecular integrity of bacteriophage $\phi X$174 DNA was examined by means of the spheroplast assay and sucrose density gradient centrifugation.

Bleomycins caused degradation of the DNA, both in the single and double stranded forms even in the absence of 2-mercaptoethanol or hydrogen peroxide, although the reaction was accelerated by the addition of either of the compounds. All the bleomycin compounds examined, $A_2$, $A_5$ and $B_2$, were effective for the reaction but copper-chelated $A_2$ was not. The reaction was inhibited by the addition of EDTA*.

Bleomycins are antibiotics which are produced by *Streptomyces verticillus* and have antitumour and antibacterial activities1,2,3. It has been reported that bleomycins inhibit DNA synthesis rather than protein or RNA synthesis in *Escherichia coli*, *Ehrlich* carcinoma and HeLa cells4. They cause strand scissions in DNA of growing *E. coli*, and HeLa cells4. They bind to DNA and decrease the melting temperature ($T_m$) and the molecular size in the presence of sulfhydryl compounds or hydrogen peroxide, *in vitro*5,7,8. It has been suggested that sulfhydryl compounds or hydrogen peroxide may take part in the *in vivo* action of bleomycin5,8.

In the study of effects of bleomycin on the growth of bacteriophage $\phi X$174, which has a single-stranded circular DNA (phage DNA) and also produces double-stranded circular DNA called replicative form (RF)** in the host cell during the development, the degradation of the single-stranded phage DNA and the double-stranded RF DNA was observed *in vitro* in the absence of sulfhydryl compounds or hydrogen peroxide. The results are presented in this paper.

Materials and Methods

The following bleomycins were used: mixture (lot. F-7, $A_2$; 69.0%, $B_2$; 28.8%, $A_4$; 1.5%, $B_1$; 1.5%, $A_5$; 0.9%, $A_1$; 0.5%). $A_2$ (lot. F1-8), $A_5$ (lot. 3, spd), $A_2$(Cu) (lot. F1-8, copper-chelated), $B_2$ (lot. 32). All bleomycins were kindly offered by Nippon Kayaku

* EDTA : ethylenediaminetetraacetic acid.
** RF : RF-I (both strands are closed) and RF-II (one or both strands are nicked).
Co., Tokyo. (Methyl-\( ^3 \text{H} \)) thymidine (specific activity 15.5 c/m mole) was purchased from Schwarz Bioresearch, Inc. Bacterial and phage strains were given to us by Dr. R. L. Sinsheimer. Zone Sedimentation and Radioactivity Measurement

DNA or its reaction mixture (in 0.2~0.6 ml) was layered on a 5~20 % sucrose density gradient in 0.05 M tris~0.5 M sodium chloride~1 mM EDTA (pH 7.6, Tris~saline~EDTA buffer) and centrifuged at 4°C in an SW 27 rotor of a Beckman L2-65B centrifuge at 25,000 r.p.m. for 16 hours. Fractions were collected from the bottom of tubes. The DNA in each fraction was precipitated with cold 5 % (final concentration) trichloroacetic acid (TCA) after adding 100 \( \mu \)g of herring sperm DNA as a carrier. Precipitates containing \( ^3 \text{P} \) or \( ^3 \text{H} \)-DNA were collected on glass fibre paper (Whatman GF/C), dried and counted in an Aloka scintillation counter (toluene base scintillator).

Bioassay

The biological activity of the phage DNA was measured by the spheroplast assay as described by Guthrie et al.\(^{10}\)

Preparation of Phage DNA

\( ^3 \text{P} \)-Labeled \( \phi X174 \ am3 \) (lysis defective mutant) was prepared essentially according to the procedure of Sinsheimer et al.\(^{11}\) \( ^3 \text{P} \)-Labeled \( \phi X174 \ am3 \) DNA was prepared by the method of Guthrie et al.\(^{10}\). The phage DNA was purified by sucrose density gradient centrifugation, if necessary. Fractions containing \( ^3 \text{P} \)-phage DNA were pooled and the DNA was precipitated with 2.5 volumes of ethanol after the addition of \( \text{tRNA} \) (20 \( \mu \)g/ml) or the cold phage DNA (80 \( \mu \)g/ml) as a carrier. Precipitates were dissolved in 0.05 M tris buffer (pH 7.6 or 7.4).

Preparation of \( ^3 \text{H} \)-Labeled RF DNA

\( E. \ coli \ HF4704\) (her\(^{-}\), thymine requiring) was grown in TPG-2A medium\(^{11}\) containing 5 \( \mu \)g/ml of thymine to about 1\( \times \)10\(^8\) cells/ml at 37°C with aeration. The cells were incubated after the addition of 30 \( \mu \)g/ml of mitomycin C for 10 minutes without aeration, collected by centrifugation and resuspended in the fresh medium containing 2 \( \mu \)g/ml of thymine. The cells were infected with \( \phi X174 \ am3 \) and at the same time 0.02 \( \mu \)c/ml of \( ^3 \text{H} \)-thymidine and 30 \( \mu \)g/ml of chloramphenicol were added. The infected cells were incubated further for 3 hours under vigorous aeration, then collected, washed and resuspended in 0.05 M tris buffer (pH 8.1). The cells were lysed by lysozyme~EDTA treatment\(^{10}\), followed by freezing and thawing. The lysate was shaken with an equal volume of phenol saturated with 0.05 M tris buffer (pH 8.1) and the aqueous phase was recovered after separation by centrifugation. Remaining phenol in the aqueous phase was removed by the extraction with ether. After adding 0.1 volume of 3 M sodium acetate (pH 5.5), 2.5 volumes of isopropanol and 10 \( \mu \)g/ml of yeast \( \text{tRNA} \), the solution was allowed to stand in a freezer overnight. The precipitate was collected by centrifugation.

Results

Effects of Bleomycin A\(_2\) on Infectivity of Single-stranded Phage DNA to Spheroplast

If \( \phi X174 \) phage DNA, single-stranded and closed, is opened by one strand scission or suffers chemical modification in some base residues by the action of hydroxylamine or other reagents, its infectivity to \( E. \ coli \) spheroplast is known to be lost. Therefore this spheroplast assay system gives a very sensitive detection method for DNA alteration. The effect of bleomycin A\(_2\) on the infectivity of the single-stranded phage DNA to the spheroplast is shown in Fig. 1. The inactivation of phage DNA by 1 \( \mu \)g/ml of bleomycin A\(_2\) was observed in the presence of neither 2-mercaptopoethanol nor hydrogen peroxide.
Fig. 1. Inactivation of phage DNA by bleomycin A₂ as revealed by spheroplast assay.

The phage DNA (about 1×10⁻³ µg/ml) was incubated with bleomycin A₂ in 0.05 M tris buffer (pH 7.6) at 37°C. A portion of reaction mixture at the time indicated was transferred and diluted into ice-cold 0.05 M tris buffer (pH 8.1) containing yeast tRNA (2 µg/ml). Dilution was performed until the bleomycin present caused no effect on the spheroplast with respect to the infectivity of the phage DNA. The biological activity of the treated phage DNA was measured in the spheroplast assay as described by Guthrie et al. ⁷)

![Graph](image)

Degradation of Single-stranded or Double-stranded DNA by Bleomycin in the Absence of 2-Mercaptoethanol or Hydrogen Peroxide

Effects of three different types of bleomycin, A₂, A₅ and B₂, on the phage DNA were examined by means of sucrose density gradient centrifugation. As shown in

Fig. 2. Effects of bleomycin A₂ and A₂(Cu) and the inhibition of the bleomycin activity by EDTA.

The phage DNA (1×10⁻³ µg/ml) was incubated with bleomycin in 0.05 M tris buffer (pH 7.6) at 37°C for 90 minutes. The reaction mixture was layered on a 5~20% sucrose density gradient in Tris-saline-EDTA buffer and centrifuged as described in materials and methods. Each fraction was collected in a planchet, dried and counted in an Aloka GM counter.

A. a. untreated  b. bleomycin A₂(Cu) (5 µg/ml)  c. bleomycin A₂ (5 µg/ml) + EDTA (2.2 mM)

![Graph](image)
Fig. 3. Effects of various bleomycins on the sedimentation behaviour of the phage DNA.

The phage DNA (about 0.7 μg/ml) was incubated with bleomycin in 0.05 M tris buffer (pH 7.4) at 37°C for 120 minutes. The reaction mixture was centrifuged and counted as described in the legend of Fig. 2.

a. untreated  
b. bleomycin B₂ (50 μg/ml)  
c. bleomycin A₆ (50 μg/ml)

Fig. 4. Effect of bleomycin on double-stranded RF DNA.

The RF DNA (about 3 x 10⁻² μg/ml) was incubated with bleomycin in 0.05 M tris buffer (pH 7.6) at 37°C for 90 minutes. The DNA in the reaction mixture was precipitated in the cold with the addition of 0.1 volume of tRNA (1 mg/ml), 0.1 volume of 3M sodium acetate (pH 5.5) and 2 volumes of ethanol and then redissolved in 0.05 M tris buffer (pH 7.6). The dissolved DNA was centrifuged and counted as described in materials and methods.

a. untreated  
b. bleomycin mixture (50 μg/ml)

c. RF-I  
d. RF-II

Fig. 5. Effect of 2-mercaptoethanol on bleomycin activity.

The phage DNA (about 1 x 10⁻² μg/ml) was incubated with bleomycin mixture in 0.05 M tris buffer (pH 7.6) at 37°C. A portion of the sample at the time indicated was added with carrier DNA to make a final concentration of 100 μg/ml and with cold TCA at a final concentration of 5%. The precipitates were collected on glass fibre paper, dried and counted in a scintillation counter.

a. untreated  
b. bleomycin mixture (3.4 mg/ml)  
c. bleomycin mixture (3.4 mg/ml) +2-mercaptoethanol (1 mM)

Action of Bleomycin in Cooperation with 2-Mercaptoethanol or Hydrogen Peroxide

The amount of TCA-insoluble portion from the DNA treated with bleomycin decreased more rapidly in the presence of 2-mercaptoethanol than in the absence of that (Fig. 5). The effect of hydrogen peroxide on the DNA degradation by bleomycin is illustrated in Fig. 6. The sedimentation pattern of phage DNA treated with 3.3 μg/ml of bleomycin A₂, at which concentration only a little change was caused in the absence of hydrogen peroxide, showed a remarkable degradation upon the further addition of hydrogen peroxide.

Fig. 6. Effect of hydrogen peroxide on double-stranded RF DNA.

The RF DNA (about 3 x 10⁻² μg/ml) was incubated with bleomycin in 0.05 M tris buffer (pH 7.6) at 37°C for 90 minutes. The DNA in the reaction mixture was precipitated in the cold with the addition of 0.1 volume of tRNA (1 mg/ml), 0.1 volume of 3M sodium acetate (pH 5.5) and 2 volumes of ethanol and then redissolved in 0.05 M tris buffer (pH 7.6). The dissolved DNA was centrifuged and counted as described in materials and methods.

a. untreated  
b. bleomycin mixture (50 μg/ml)  
c. bleomycin mixture (50 μg/ml) +hydrogen peroxide (1 mM)
0.03 mM of hydrogen peroxide. The pattern of DNA treated with hydrogen peroxide only was the same as that of untreated DNA.

Ineffectiveness of Copper-chelated Bleomycin and Inhibition by EDTA of the Bleomycin Action

The copper-chelated bleomycin has been reported not to bring about the observable strand scission of DNA even in the presence of 2-mercaptoethanol. It is also known that the strand scission of DNA with bleomycin in the presence of 2-mercaptoethanol is inhibited by an addition of EDTA. We examined whether these effects held also for the bleomycin action on the phage DNA without 2-mercaptoethanol. As shown in Fig. 2A, copper-chelated bleomycin A2 did not cause the observable degradation of DNA in the absence of 2-mercaptoethanol, while bleomycin A2 freed from copper by the dithizon treatment recovered the activity (not indicated here). The activity of bleomycin A2 was inhibited rapidly by the addition of EDTA in the absence of 2-mercaptoethanol as shown in the sedimentation (Fig. 2B) or in the spheroplast assay experiment (Fig. 1). These results are consistent with those previously reported.

Discussion

The biological activity of bleomycins has been discussed in connection with DNA degradation. 3H-Labeled bleomycin has been reported to bind to DNA and to cause strand scission and degradation of DNA in the presence of 2-mercaptoethanol or hydrogen peroxide but not in their absence in vitro even though it binds to DNA. These conclusions were deduced from the observed change in melting temperature or sedimentation patterns. In our studies it was found that, even in the absence of 2-mercaptoethanol or hydrogen peroxide, bleomycin(s) inactivated the biological activity of phage DNA and moreover degraded both the phage DNA and the double-stranded RF DNA. 2-Mercaptoethanol or hydrogen peroxide only enhanced the degradation of DNA by bleomycin. Consistent with the results of previous authors, the chelation of copper to bleomycin or the addition of EDTA to the reaction mixture inhibited the in vitro activity of bleomycin in the present system.

φX174 Phage DNA and RF DNA used in our experiments possess several advantages for the detection of alteration in their molecular integrity. For example, the formation of only a single nick in the DNA strand may be easily revealed, because the circular closed native strand and the resulted linear open strand can be separated from each other by zone sedimentation. Moreover the spheroplast assay can afford a very sensitive detection method for DNA alteration as mentioned above. Presumably, the sensitivity of our
system detects strand scissions not detected previously.

Although the biological activity of bleomycin in vivo might be considered in connection with sulfhydryl or peroxide compounds, their stimulation of DNA degradation by bleomycin seems not to be indispensable, at least in vitro.

References