REDUCED ACTIVITY OF DNA POLYMERASE PREPARED FROM BLEOMYCIN-TREATED CANCER CELLS

TOYOTA KOBAYASHI

Department of Oral Surgery and Biochemistry,
Chiba University School of Medicine,
Inohanamachi, Chiba City, Chiba, Japan

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Rat-ascites-hepatoma AH 130 cells were incubated at 37°C for 1 hour with bleomycin at the concentration of 1~30 μg/ml. Then, DNA polymerase was prepared from the cells. The enzymatic activity was about half of the control value. Thymidine kinase similarly prepared from bleomycin-treated cells, did not show such a marked inhibition in their activities. *In vitro* addition of bleomycin to the DNA polymerase assay system did not produce any appreciable inhibition. Moreover, the antibiotic stimulated the enzymatic activity when native DNA was used as template.

An antitumor antibiotic bleomycin was isolated from *Streptomyces verticullus* by UMEZAWA *et al.* in 1966[1]. Bleomycin was observed to exhibit a significant anticancer activity, especially on squamous cell carcinoma[2]. In the field of oral surgery, the antibiotic is very effective for the treatment of tongue cancer or soft tissue cancer[3].

The mode of action of bleomycin has been extensively studied by UMEZAWA and his colleagues[4,5,6]. DNA synthesis was most markedly inhibited by bleomycin. However, RNA synthesis was not significantly affected. The antibiotic also interferred with protein synthesis.

Recently, KANAMOTO[7] in our department, studied the *in vitro* effect of bleomycin on DNA polymerase. The antibiotic did not show any appreciable effect on the enzymatic activity when heat-denatured DNA was used as template. Moreover, bleomycin stimulated the enzymatic activity as high as twice the control when native calf thymus DNA was used as template. These results suggested to us that the mode of action of bleomycin *in vivo* might be different from that *in vitro*.

The present paper deals with the comparative study of the *in vivo* and *in vitro* effects of bleomycin on the enzymatic activities of DNA polymerase, thymidine kinase and DNA-dependent RNA polymerase.

Materials and Methods

Chemicals:

(1) Non-radioactive nucleoside triphosphates, adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP), and uridine triphosphate (UTP) were obtained from Boehringer and Soehne, Mannheim, Germany. Deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), and calf thymus DNA were purchased from the Sigma Chemical & Co., St. Louis, Mo., U. S. A.

Bleomycin complex and bleomycin A_2 were the generous donation of Nihon Kayaku & Co., Tokyo, Japan, to Prof. Y. MIURA.

(2) Radioactive compounds: ^{3}H-thymidine triphosphate (^{3}H-TTP, specific activity, 12 c/m mole) and ^{14}C-cytidine triphosphate (^{14}C-CTP, specific activity, 161 mc/m mole) were purchased from Schwarz Bioresearch Inc., Orangeburg, N. Y., U. S. A.

^{14}C-thymidine (^{14}C-Tdr), having a specific activity of 50 mc/m mole, was obtained from Daiichi Kagaku, Tokyo, Japan.

Incubation of cancer cells: The AH 130 strain of rat-ascites-hepatoma was inoculated into peritoneal cavity of female Donryu-strain rats weighing about 150 g. The hepatoma cells were collected by low-speed centrifugation from ascitic fluid 5~7 days after the inoculation. The harvested cells were washed once with saline and incubated under oxygen for 60 minutes at 37°C in plastic vessels containing 5 volumes of EAGLE's minimum essential medium[8], supplemented with 10 % calf serum and 10 % 0.2 M Tris-HCl buffer (pH 8.0). Bleomycin was added as a solution in the Tris-buffer. When the incubation was over, the cells were thoroughly washed several times with saline in order to remove bleomycin.

Preparation of crude enzyme solution:

(1) DNA polymerase (deoxyribonucleic acid nucleotidyl-transferase, EC 2. 7. 7. 7.): This enzyme was extracted according to the method originally described by MUKUNDAN *et al.*[9] Hepatoma cells were homogenized with Teflon-pestle homogenizer in 4 volumes of a solution of 0.35 M sucrose containing 0.004 M $MgCl_2$ and 0.035 M $KHCO_3$. Then, the homogenate was centrifuged at 105,000 × *g* for 2 hours. The supernatant fraction was used as the crude enzyme preparation. The assay of enzymatic activity was performed by measuring the incorporation of ^{3}H-TTP into an acid-insoluble product. The reaction mixture contained the following components, as described by BOLLUM[10]: 10 μmoles of phosphate buffer (pH 7.3), 2 μmoles of $MgCl_2$, 0.25 μmole of β-mercaptoethanol; 3.75 mμ moles each of dATP, dCTP and dGTP, 0.04 mμ mole of ^{3}H-TTP (0.5 μc), 120 μg of calf thymus DNA and 0.05 ml of the enzyme preparation. Incubation of the above-mentioned mixtures in a total volume of 0.25 ml was performed for 30 minutes at 37°C and an aliquot of 0.05 ml was placed on a Whatman No. 3 MM filter disc which was washed with 0.1 M $Na_4P_2O_7$ before use[11]. The disc was treated by the procedure of BOLLUM[10]. The radioactivity on the disc was measured by a scintillation spectrometer. All assays were performed in duplicate. Heat-denatured DNA was prepared by keeping a solution of DNA at 100°C for 10 minutes, then cooling immediately in an ice bath. The hyperchromic shift by this procedure was approximately 20 %. Protein content of the enzyme preparation was determined by biuret reaction[12].

(2) Thymidine kinase (ATP: thymidine 5'-phosphotransferase, EC 2. 7. 1. 21): The method of preparation and assay of this enzyme was originally described by EKER[13] and modified by FUJII *et al.*[14] Cancer cells were homogenized with 0.2 M Tris-HCl buffer (pH 8.0) and a 2 % homogenate was centrifuged at 8,000 × *g* for 30 minutes. The supernatant solution was used as crude enzyme solution. The assay system consisted of 200 μl of 0.2 M Tris-HCl buffer (pH 8.0), 25 μl of 100 mM $MgCl_2$, 25 μl of 100 mM ATP, 25 μl of 1 mM ^{14}C-Tdr (0.5 μc), and 200 μl of crude enzyme solution. The incubation was carried out at 37°C for 30 minutes. After heating at 100°C for 5 minutes, 0.1 ml of the supernatant of the assay system was placed on a disc of DEAE cellulose filter. After successive washing with 0.001 M ammonium formate, water and 95 % ethyl alcohol, the radioactivity of phosphorylated thymidine remaining on the disc was measured by a scintillation spectrometer. All assays were performed in triplicate. Protein content of the enzyme solution was determined by LOWRY's method[15].

(3) RNA polymerase (nucleosidetriposphate: RNA nucleotidyltransferase, EC 2. 7. 7. 6.)

1) Aggregate enzyme: The aggregate enzyme was prepared from the nuclear fraction of hepatoma cells according to the method originally described by WEISS[16]. Hepatoma

cells were homogenized with 4 volumes of 0.25 M sucrose containing 3.3 mM $CaCl_2$. The isolation of nuclear fraction was carried out by the slightly modified method of MURAMATSU[17]. The isolated nuclear fraction was suspended in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol, 0.5 % sodium deoxycholate and 35 % glycerol. The activity of RNA polymerase was expressed as $\mu\mu$mole of ^{14}C-CTP incorporated into RNA per mg of enzyme protein. The assay system consisted of 20 μmoles of Tris-HCl buffer (pH 8.0), 0.1 μmole each of ATP, GTP and UTP, 2 mμ moles of ^{14}C-CTP (25 μc/μmole), 0.2 μmole of dithiothreitol, 3 μmoles of $MgCl_2$ and 0.05 ml of the enzyme preparation in total 0.20 ml. The incubation was carried out at 37°C for 10 minutes.

2) Soluble enzyme: The soluble RNA polymerase was prepared from the nuclear fraction of hepatoma cells according to the method described by ROEDER and RUTTER[18]. The nuclear suspension was adjusted to 0.01 M Tris-HCl (pH 7.9), 1.0 M sucrose, 0.005 M $MgCl_2$ and 0.005 M dithiothreitol. Ammonium sulfate (4 M, adjusted to pH 7.9 with ammonia) was added to bring the concentration to 0.3 M. The viscous solution was sonicated in 20 ml aliquots for 60 second periods with a sonicator (Umeda Sonore 150) until the viscosity decreased to a point at which the solution readily formed drops at the tip of a pipet. The suspension was then rapidly mixed with 2 volumes of 0.05 M Tris-HCl (pH 7.9), 25 % (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM ethylenediamine tetraacetate and 0.5 mM dithiothreitol. The precipitate was collected by centrifugation for 60 minutes at 105,000 × g and resuspended in dithiothreitol. After dialysis for 3 hours against dithiothreitol containing 0.05 M ammonium sulfate, the dialysate was centrifuged at 105,000 × g for 60 minutes. The supernatant was immediately subjected to assay for RNA polymerase activity.

Results

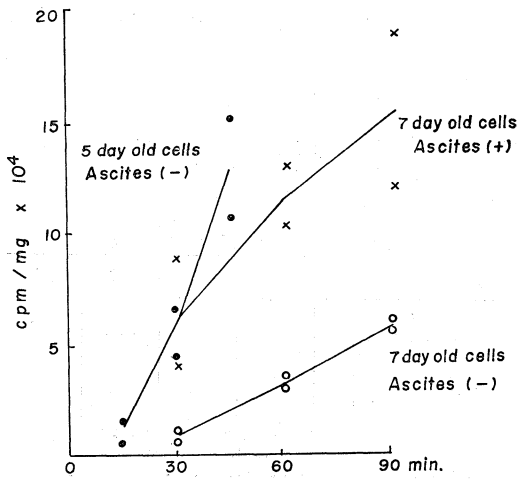
Preliminary Experiments

(1) Incorporation of ^{3}H-Tdr into DNA during *in vitro* incubations of hepatoma cells.

Fig. 1 shows the time course of the incorporation of ^{3}H-Tdr into DNA by hepatoma cells. When ascitic fluid was added to the incubation medium instead of calf serum, a higher rate of the incorporation was observed. A similar higher incorporation was also noticed when 5 day-old cells were incubated instead of 7 day-old cells. However, larger differences were found between the duplicate samples when the rate of incorporation was high. Thus, the 60-minute incubation of 7 day-old cells without addition of ascitic fluid was used as the standard condition. Using this standard condition, when 2 μg/ml of bleomycin complex was added to the incubation medium, the incorporation of ^{3}H-Tdr into DNA was 53 % of the control. (Mean of the duplicate experiments).

Fig. 1

The incubation of cancer cells was carried out in the medium described in the text. Fifty μc of ^{3}H-Tdr was added in each vessel. DNA was extracted by SCHMIDT-THANNHAUSER's method and the amount of DNA was determined by diphenylamine method.



(2) Time course of the enzymatic reaction of polymerases.

Preliminary time course experiments

revealed that DNA polymerase activity maintained a constant rate during the first 30 minutes, whereas RNA polymerase activity was constant only over the first 10-minute period. Therefore, the incubation was limited to 30 minutes for DNA polymerase and to 10 minutes for RNA polymerase.

DNA Polymerase

(1) Direct action of bleomycin on DNA polymerase.

Table 1 shows the *in vitro* action of bleomycin on DNA polymerase system. When native DNA was used as template, the activity of DNA polymerase was remarkably stimulated, whereas, when heat-denatured DNA was used as template, practically no appreciable change was observed in the enzymatic activity.

(2) Indirect action of bleomycin on DNA polymerase.

Fig. 2 demonstrates the activity of DNA polymerase prepared from the hepatoma cells incubated with bleomycin complex or bleomycin A_2. In all cases, considerably reduced activities of DNA polymerase were observed. At the concentration of 10 μg/ml of bleomycin, the activity was about 84 % of the control when heat-denatured DNA was used as the template, and 65 % of the control when native DNA was used as the template.

In order to know if similar reduced activities of other enzymes concerning the synthesis of nucleic acids would be observed, thymidine kinase and DNA-dependent RNA polymerase were prepared from the hepatoma treated with bleomycin and their activities were compared with the controls.

Fig. 2

The incubation of hepatoma cells was carried out as described in the text. The enzyme preparations used in the experiment with bleomycin complex and that used in the experiment with bleomycin A_2 are extracted from the different pools of hepatoma cells. Thus, it is unable to compare directly the absolute values on the ordinate (the values obtained without bleomycin-pretreatment).

Activity of DNA polymerase was expressed as $\mu\mu$ moles of ^{3}H-TTP incorporated into DNA during 30 minutes incubation per mg of enzyme protein.

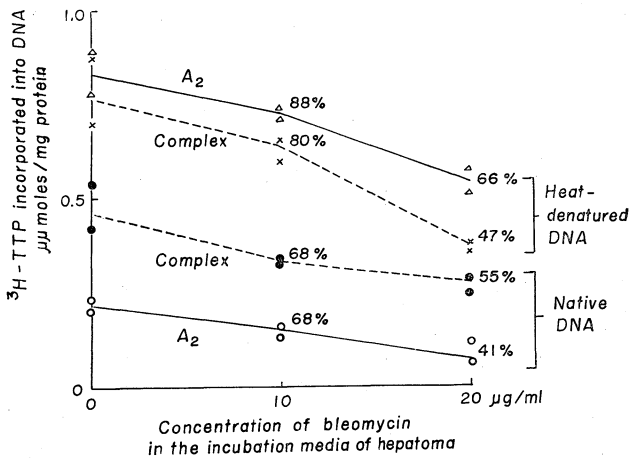


Table 1. *In vitro* effect of bleomycin complex on DNA polymerase prepared from hepatoma

Template	Concentration of bleomycin (μg/ml) and DNA polymerase activity (% of control)			
	0	8	12	20
120 μg of native DNA	100	210	224	230
120 μg of heat-denatured DNA	100	94	90	86

DNA polymerase activity was measured by the method described in the text. Bleomycin was added just before the addition of ^{3}H-TTP to the incubation medium.

Table 2. Thymidine kinase activity prepared from bleomycin-treated hepatoma

Concentration of bleomycin A_2	Thymidine kinase activity mμ moles/mg protein/30 min.
0 μg/ml	0.900 ± 0.053
1	0.923 ± 0.041
5	0.966 ± 0.048
10	0.850 ± 0.057

The hepatoma cells were 7 days-old. Thymidine kinase activity was expressed as mμ moles of ^{14}C-Tdr phosphorylated during 30 minutes by 1 mg of enzyme protein.

Thymidine Kinase

As shown in Table 2, almost no change in the extractable thymidine kinase activity was observed when the hepatoma was incubated with 1~10 μg/ml of bleomycin.

The direct addition of 2~20 μg/ml of bleomycin to the assay system of thymidine kinase from a 26-hour regenerating liver, resulted in an activity about 92 % of that in the control.

These data indicate that the activity of thymidine kinase prepared from bleomycin-treated cells seems almost unaffected by the preincubation with bleomycin.

RNA Polymerase

The activity of aggregated RNA polymerase prepared from the nuclei of bleomycin-treated cells was compared with the control. As shown in Table 3, the activity was impaired to some extent: in one experiment, 69 % of the control; in another, 84 % of the control. In these cases, however, the enzyme system used was so-called "aggregate enzyme" which contains bleomycin-treated DNA.

To avoid the effect of the pretreatment of bleomycin on DNA, RNA polymerase without DNA (soluble enzyme) should then be used with calf thymus DNA as the template. As shown in Table 4, the enzyme prepared from the hepatoma cells incubated in medium containing 20 μg/ml of bleomycin A_2, showed a distinct decrease in the activity to about 50 percent of the control.

Table 3. The activity of RNA polymerase (aggregate enzyme) prepared from bleomycin-treated hepatoma

Concentration of bleomycin complex	RNA polymerase activity μμ moles of ^{14}C-CTP incorporated into RNA per mg of protein during 10 minutes	
	Experiment I	Experiment II
0 μg/ml	33.25 (100 %)	36.63 (100 %)
2	27.82 (84 %)	25.31 (69 %)

RNA polymerase (aggregate enzyme) was prepared and assayed according to the methods described in the text. All assays were carried out in duplicate.

Table 4. The activity of soluble RNA polymerase prepared form bleomycin-treated hepatoma

Concentration of bleomycin complex	RNA polymerase activity μμ moles of ^{14}C-CTP incorporated into RNA per mg of protein during 10 minutes
0 μg/ml	19.91 (100 %)
20	9.01 (47 %)

Soluble RNA polymerase was prepared from 7 day-old hepatoma as described in the text. When the enzyme was assayed, 120 μg of calf thymus DNA was added as the template.

Discussion

Concerning the method of the present experiment, the author should like to emphasize the following points:

(1) In a series of preliminary experiments, the author found that the activities of DNA polymerase prepared from the different pools of hepatoma cells varied so widely that the standard deviation exceeded over 5 % level. However, when the activities of DNA polymerase prepared from the same pooled cells, were simultaneously determined, the standard deviation did not exceed over 5 % level. Thus, it should be noticed that the comparison of DNA polymerase activities were made with the aliquots prepared from the same pooled cells.

(2) One might consider that the washing of hepatoma cells would not be sufficient to eliminate bleomycin from the hepatoma cells. However, if 20 μg/ml of bleomycin would still remain in the washed cells, the preparation of DNA polymerase should contain 4 μg/ml of bleomycin, because the 20 % homogenate was prepared with sucrose solution. Based on the data shown in Table 1, an *in vitro* addition of 4 μg/ml of bleomycin would

not change in the DNA polymerase activity with heat-denatured DNA as template or even it would stimulate DNA polymerase when native DNA was used as template. However, as a matter of fact, the DNA polymerase activity prepared from bleomycin-treated hepatoma actually showed a marked decrease as shown in Fig. 2; thus, one might assume that, after the washing procedure, there was practically no remaining bleomycin in the DNA polymerase prepared from bleomycin-treated hepatoma.

The current theory on the mode of action of bleomycin is nick formation in DNA strands: Bleomycin combines with DNA, then makes nicks. There are several papers of evidence for this line. For example, SUZUKI *et al.*[19] reported specific scission of DNA strands by bleomycin. They found small fragments of DNA after bleomycin treatment. In addition, the melting temperature of bleomycin-treated DNA was remarkably decreased in the presence of sulfhydryl compounds[20,21] or hydrogen peroxide[22]. The apparent stimulation of DNA polymerase by *in vitro* addition of bleomycin[7] may be interpreted as active repair of nicks which occurred in DNA strands.

UMEZAWA *et al.*[4,5,6] have already reported that bleomycin caused a marked inhibition of the incorporation of ^{14}C-leucine into protein.

Furthermore, TERASHIMA[23] noticed that the most bleomycin-sensitive period during the life cycle of the synchronized culture of HeLa cells was the late G_1 and the very beginning of S periods. These periods are coincident with the time of the intracellular biosynthesis of DNA polymerase. The intracellular synthesis of thymidine kinase preceeds, usually by several hours, the synthesis of DNA polymerase. Concerning RNA polymerase, it is rather hard to determine the period of biosynthesis; however, the activity of RNA polymerase is usually high during the G_1 period.

The fact that DNA polymerase and RNA polymerase prepared from bleomycin-treated hepatoma decreased in their activity, may be interpreted as an inhibition of enzyme protein synthesis if many of the hepatoma cells were in the late G_1 and the early S period. However, this was not the case because the hepatoma used in this experiment consisted of randomly growing cells. Another possible reason why DNA polymerase and DNA-dependent RNA polymerase prepared from bleomycin-treated hepatoma showed reduced activities, may be a stimulation of DNA-decomposing enzymes by bleomycin-treatment. However, deoxyribonucleases exist mostly in the particulate fraction of the cells. As the DNA polymerase used in this experiment was prepared from the supernatant fraction of the cells, it might not be contaminated by deoxyribonuclease.

Thus, at the present stage, it is hard to give a reasonable interpretation of the selective reduction of the activities of DNA and RNA polymerase prepared from bleomycin-treated hepatoma. Further experiments are required.

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