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Cobalt Chelate of Bleomycin. II. Binding to Deoxyribonucleic Acid of Ehrlich Solid Tumor in Mice[1]

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Cobalt chelate of bleomycin (Co-BLM) bound deoxyribonucleic acid (DNA) was purified from Ehrlich solid tumor of mice administered with Co-BLM by the extraction from the purified nuclei. In both ^{57}Co-BLM and Co-BLM (^{14}C), the DNA was taken in 99% purity, and the decrease in the specific radioactivity of the Co-BLM=DNA complex was minimized in the course of the purification procedure.

The complex was stable in the Sephadex G-100, hydroxyapatite columns and neutral sucrose gradient solution. Moreover, this was stable in the electrophoretic conditions. However, Co-BLM dissociated from DNA by acid, alkaline or heat treatment.

Keywords——bleomycin; Co-bleomycin; cobalt chelate; bleomycin-metal complex; purification of Co-bleomycin bound DNA; characterization of Co-bleomycin bound DNA; DNA-antibiotics complex; radiopharmaceuticals; tumor scanning agents

In 1972, we proposed cobalt chelate of bleomycin (Co-BLM) as a promising tumor specific scanning agent.[3a,b] In the tumor tissues, Co-BLM was concentrated in the cell nuclei, whereas BLM, other metal chelates of BLM and unchelated Co (II) were present in the subcellular fractions,[4] and the distribution of Co-BLM in the tumor tissue was in accord with deoxyribonucleic acid (DNA) contents of each fraction. BLM is known to bind to DNA.[5a,b] These suggest that Co-BLM binds to DNA in tumor cell nuclei.

This paper describes the purification and characterization of Co-BLM-A_2 bund DNA from Ehrlich solid tumor nuclei of mice administered with Co-BLM.

Experimental

Purification of Nuclei——Co-BLM-A_2 (labeled with ^{57}Co or ^{14}C) was prepared in the manner as described previously.[4] One hour after *i.v.* injection of Co-BLM-A_2 to mice bearing Ehrlich solid tumor, the tumor tissue was excised and homogenized in 0.25 M sucrose solution containing 3.3×10^{-3} M $CaCl_2$ (hereafter designated as A sol.). This 20% preparation was centrifuged at $700 \times g$ for 10 min in a refrigerated centrifuge (Hitachi 18-PR 3). The precipitate (crude nuclear fraction) was suspended in A sol. and centrifuged. The pellet was suspended to 3—5% concentration (v/v) in 2.1 M sucrose solution and centrifuged in a refrigerated ultracentrifuge (Beckman L-2, T-30 roter) at 5×10^4 rpm for 60 min. The supernatant solution was decanted. The clear jelly-like pellet at the bottom of the tube was suspended in A sol. and centrifuged at $700 \times g$ for 10 min.

Extraction of Nucleoprotein——Approximately 20 volumes of 0.14 M NaCl solution was added to the nuclear pellet and allowed to stand for at least 12 hr at 0°. After centrifugation the pellet was suspended in about 10 volumes of 1 M NaCl solution. After being kept at 0° for about 24 hr, the suspension was centrifuged at $15000 \times g$ for 15 min. The supernatant was the nucleoprotein fraction.

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Deproteinization of the Purified Nucleoprotein Fraction——On the nucleoprotein saline solution, approximately 2 volumes of cold ethanol was layered gently. The nucleoprotein spooled on a glass rod with a gentle stirring as a thread-like precipitate. After washing with excess ethanol, the precipitate was dissolved in 1 M NaCl solution. The solution was shaken with an equal volume of $CHCl_3$–isoamyl alcohol (24:1) for 15 min and centrifuged. Deproteinization was repeated until very little protein was seen. The supernatant was mixed with ethanol. The DNA thus obtained was dissolved in 0.15 M NaCl solution containing 0.015 M sodium citrate (hereafter denoted as SSC solution of DNA).

Chromatography on Sephadex G-100——The DNA solution (863 μg/ml) was placed on a Sephadex G-100 column (1.7×37.5 cm) and was eluted at a rate of 6 ml/hr with distilled water. Each 2 ml fraction was collected. After counting of radioactivity, 0.2 ml of each fraction was submitted to DNA analysis.

DNA, Ribonucleic Acid (RNA) and Protein Determination——Diphenylamine method[6] was adopted for DNA determination with deoxyribose as a standard. RNA was determined with Webb's method.[7] Lowry's method was employed for protein determination.

Radioactivity——Radioactivity was measured with a welltype scintillation counter (Ten EA 14 Kobekogyo Co. LTD) for ^{57}Co and with a liquid scintillation counter (Aloka LS-502, Aloka Co. LTD) for ^{14}C.

Sucrose Density Gradient Centrifugation Analysis——After gel filtration, the DNA fraction was concentrated and layered on the top of 4.6 ml of an alkaline (0.3 M NaOH, 0.7 M NaCl an 2×10^{-3} M ethylenediamine tetraacetic acid (EDTA), pH over 12.5) or neutral (0.02 M potassium phosphate buffer containing 0.1 M NaCl, pH 7.5) sucrose density gradient solution (5—20%). Centrifugation was carried out in a SW 50.1 roter of Beckman L-2 65B centrifuge at 45000 rpm for 120 min at 20°. The fractions (7 drops) were collected from the bottom of the tubes and the absorbance at 260 nm was measured after addition of 3 ml of distilled water to each fraction.

Dialysis of the Purified DNA——After gel filtration, 1 ml of Co-BLM-A_2 bound DNA solution (DNA; 200 μg/ml) was dialyzed 48 hr at 0° against one of the following solutions; 1×10^{-2} M EDTA, 2 M NaCl, $HgCl_2$ or $AgNO_3$ (400 μg/ml) solution and distilled water.

Chromatographic Analysis on Hydroxyapatite Column[9]——One ml of the purified DNA solution (1500 μg/ml) was placed on hydroxyapatite column (1.7×20 cm) equilibrated with 1×10^{-3} M phosphate buffer and eluted with a linear gradient of potassium phosphate (pH 6.8) from 1×10^{-3} M to 5×10^{-1} M at room temperature at a rate of 3 ml/hr. One ml of the DNA solution (1500 μg/ml) was heated at 95° for 10 min and was chromatographed on hydroxyapatite column in the conditions mentioned above. Two ml of distilled water was added to each fraction and the absorbance of the solution at 260 nm was recorded.

Treatment of the Purified DNA with Perchloric Acid——To 0.5 ml of the purified DNA (100 μg/ml), 0.5 ml of 2 N perchloric acid was added under ice-cooling. The precipitate was filtrated on a millipore filter (pore size; 0.22 μm).

The Binding of Co-BLM to the Heat Denatured DNA——The DNA purified from Ehrlich ascites tumor cells according to Colter, *et al.*[10] was dissolved in SSC solution (50 μg/ml). To 1 ml of the solution, 1 ml of 0.2 M phosphate buffer containing 2% formaldehyde was added and the mixture was heated at 95° for 10 min. After cooling at 0°, 1 ml of ^{57}CO-BLM-A_2 aqueous solution (50 μg/ml) was added to the heat denatured DNA solution. The solution was incubated at 37° for 30 min. This mixture was dialyzed against distilled water at 4° for 24 hr.

Polyacrylamide Gel Diskelectrophoresis of the Purified DNA[11a,b]——On 4% polyacrylamide gel (1×12 cm), 2.5% gel (1×1 cm) was layered. 50 μl of 2.1 M sucrose solution of the DNA purified from Ehrlich tumor tissue was layered on the gel. The current was adjusted to 2 mA/tube in 1×10^{-2} M Tris-glycine buffer (pH 6.8). Bromphenol blue (BPB) was used as a tracking dye. Gel was stained with 0.01% of toluidine blue or was cut into 0.5 cm pieces for the measurement of radioactivity.

Results

Purification of Co-BLM-A_2 Bound DNA

Approximately 64% of the radioactivity (^{57}Co) was lost in the process of nucleoprotein extraction. The loss of the DNA was about 46%. Radioactivity-DNA ratios (R) in the purification procedure are listed in Table I. The ratio of (R) of the nucleoprotein fraction

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to that of the crude nuclear fraction was 0.66 (Table I). When Co-BLM-A_2 (^{14}C) was used, it was 0.80 (Table II). After deproteinization, ^{57}Co-BLM-A_2 and Co-BLM-A_2 (^{14}C) bound DNA preparations were of approximately 99% purity. These were RNA free and protein content was about 1%.

Table I. Radioactivity-DNA Ratios (R) in the Purification Procedures of the DNA form Ehrlich Solid Tumor of ^{57}Co-BLM Injected Mouse

	Radioactivity (R.A) ($\times 10^4$ cpm)	DNA (mg)	$(R)=\frac{\text{R.A}}{\text{DNA}}$ ($\times 10^4$)	$\frac{(R)\text{ of 2 or 3}}{(R)\text{ of 1}}$
1. crude nuclear fraction	78.7	27.3	2.89	—
2. nucleoprotein fraction	28.4	14.9	1.90	0.66
3. purified DNA	20.6	9.9	2.08	0.71

Table II. Radioactivity-DNA Ratio (R) in the Purification Procedures of the DNA from Ehrlich Solid Tumor of Co-BLM(^{14}C) Injected Mouse

	Radioactivity (R.A) ($\times 10^{-2}$ μCi)	DNA (mg)	$(R)=\frac{\text{R.A}}{\text{DNA}}$ ($\times 10^{-3}$)	$\frac{(R)\text{ of 2 or 3}}{(R)\text{ of 1}}$
1. crude nuclear fraction	7.3	10.1	7.2	—
2. nucleoprotein fraction	2.9	5.0	5.8	0.80
3. purified DNA	2.1	4.8	4.4	0.61

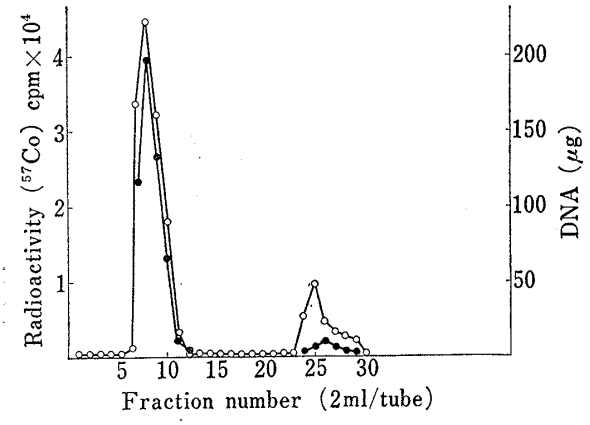

Fig. 1. Chromatogram of ^{57}Co-BLM-A_2 Bound DNA Purified from Ehrlich Solid Tumor on Sephadex G–100

Charged sample: DNA; 863 μg radioactivity; 1.63×10^5 cpm.
Conditions: described in the experimental.
○——○: radioactivity.
●——●: DNA.

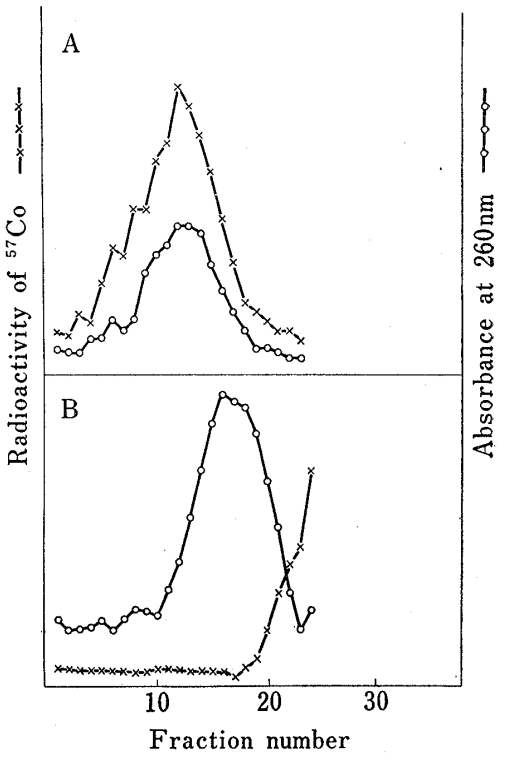

Fig. 2. Sucrose Density Gradient Centrifugation Analysis of ^{57}Co-BLM-A_2 Bound DNA Purified from Ehrlich Solid Tumor

A: neutral, B: alkaline,
conditions: described in the experimental.

Gel Chromatography of the Purified DNA

Figure 1 illustrates that ^{57}Co and DNA were present in the same fraction and indicates ^{57}Co-BLM binds to the DNA *in vivo*.

Binding Ratio of Co-BLM-A_2 to Nucleotides of the DNA

The base composition of Ehrlich ascites tumor cell DNA is 0.49 in purine and 0.51 in pyrimidine.[10] The purified DNA bound with Co-BLM-A_2 (^{14}C) was 4.8 mg and deoxyribose content was 0.90 mg. The total nucleotides were approximately 8.3×10^{18}. From these data, one molecule of Co-BLM-A_2 bound per approximately 5400 nucleotides in the DNA *in vivo*.

Sucrose Density Gradient Centrifugation Analysis

Sucrose density gradient centrifugation analysis patterns of Co-BLM-A_2 bound DNA purified from Ehrlich solid tumor are shown in Fig. 2. In alkaline sucrose solution, the radioactivity of ^{57}Co-BLM-A_2 dissociated from the DNA, while in neutral solution, the radioactivity remained bound to the DNA.

Hydroxyapatite Column Chromatography Analysis

In the chromatograms of Co-BLM-A_2 bound DNA purified from Ehrlich solid tumor on hydroxyapatite column, the radioactivity of ^{57}Co-BLM-A_2 eluted together with the DNA. On the other hand, in heat denatured DNA, the radioactivity was found in different fraction from DNA (Fig. 3).

Effect of Perchloric Acid on the Complex

After the treatment of Co-BLM-A_2 bound DNA with perchloric acid, the radioactivity was no longer detectable in the precipitate of the DNA. Most of the radioactivity was in the filtrate and it was dialyzed out into distilled water.

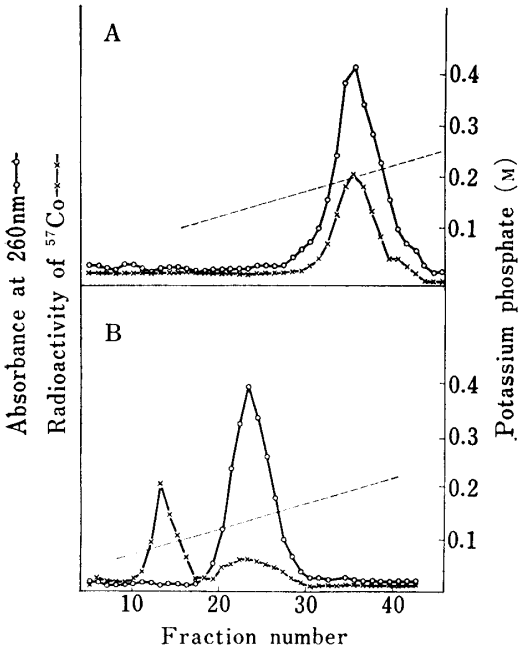

Fig. 3. Chromatograms of ^{57}Co-BLM-A_2 Bound DNA Purified from Ehrlich Solid Tumor on Hydroxyapatite

A: native, B: heat denatured,
conditions: described in the experimental.

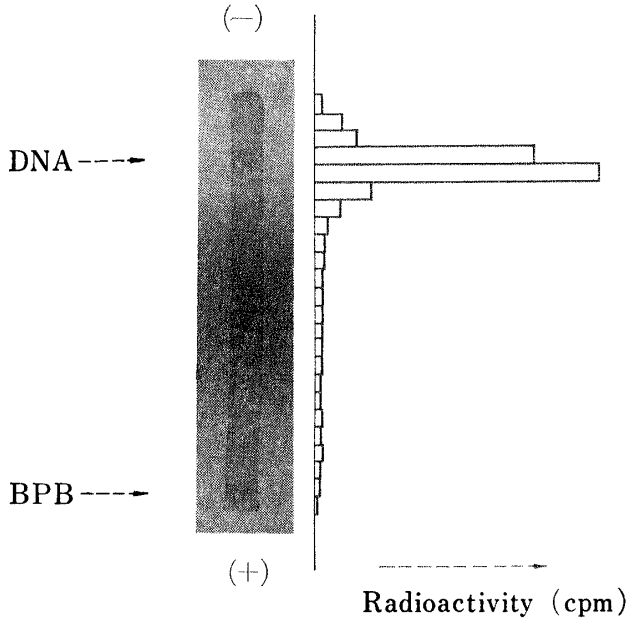

Fig. 4. Disk Electrophoretic Pattern of ^{57}Co-BLM-A_2 Bound DNA Purified from Ehrlich Solid Tumor on Acrylamide Gel

The concentration of gel; 4% in Tris-HCl buffer, pH 8.6 Gel was stained with toluidine blue.
0.5 pieces of gel was submitted for the measurement of radioactivity before staining.

The Binding of Co-BLM-A_2 to the Heat Denatured DNA *in Vitro*

About 90% of radioactivity remained in a Visking tube after dialysis, which indicated that ^{57}Co-BLM-A_2 bound to the heat denatured DNA.

Polyacrylamide Gel Diskelectrophoresis of the Purified DNA

In the electrophoresis of the purified DNA, there was a single broad toluidine blue stained band and the radioactivity of ^{57}Co was on the DNA band. No radioactivity was detected outside the band (Fig. 4).

Discussion

It has been well recognized that BLM binds to DNA and caused single strand breaks.[5a,b] Its affinity to DNA decreases in the presence of divalent cations, such as copper (II) and zinc (II). Copper (II) inhibited the single strand break activity of BLM.[12] The effects of Co (II) on the BLM action have not been well elucidated so far.

The radioactivity-DNA ratio (*R*) in the process of DNA purification (Table I) is a measure of affinity of Co-BLM-A_2 to DNA. There was no marked decrease in (*R*) This shows no considerable release of the radioactivity from DNA during purification procedure. Almost the same (*R*) were obtained in ^{57}Co-BLM-A_2 and Co-BLM-A_2 (^{14}C). It is concluded that most of Co-BLM-A_2 bind firmly to DNA, but not to nuclear protein and other components in tumor tissue, and inorganic cobalt is not liberated. A number of antibiotics have been shown to bind to DNA in nuclei and the biological implications of the phenomena have been discussed.[13] Actionmycin and Daunomycin formed complexes with DNA and the complexes dissociated in the presence of Hg (II) or Ag (I) ions.[14] The Co-BLM-A_2=DNA complex obtained in the present investigation was stable in the presence of Hg (II) or Ag (I) ions. This suggests the mode of binding to DNA may be somewhat different in Co-BLM-A_2 from other antibiotics. Though the Co-BLM-A_2=DNA complex was fairly stable in 2 N NaCl solution, it is dissociated by alkaline, acid or heat treatment. It is possible that the unwinding contributes to the dissociation of Co-BLM-A_2 from the complex. Co-BLM-A_2 did bind to the single strand DNA as described above. The fact suggests the structures of higher order of DNA have nothing to do with the binding of Co-BLM, though the complex formed *in vivo* and *in vitro* may not be exactly the same.

The bond formed between Co-BLM and DNA in tumor cell nuclei may be non-covalent one. It is likely to assume a ternary complex in which cobalt coordinates to both BLM and to DNA, probably to its bases. Comparison of chamical properties between DNA complexes with Co-BLM and BLM and the implications to their biological activities are the subjects of further studies.

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