[CANCER RESEARCH 35, 2015-2019, August 1975]

Binding of Bleomycin to DNA in Bleomycin-sensitive and -resistant Rat Ascites Hepatoma Cells[1]

Michiko Miyaki, Tetsuo Ono, Senji Hori, and Hamao Umezawa

Department of Biochemistry, Cancer Institute, Japanese Foundation for Cancer Research, Kami-Ikebukuro, Toshima-ku, 170 [M. M., T. O.] and Institute of Microbial Chemistry, Kami-Osaki, Shinagawa-ku, 141, [S. H., H. U.], Tokyo, Japan

SUMMARY

The ^{14}C activity of [^{14}C]bleomycin bound to DNA in bleomycin-sensitive rat ascites hepatoma cells (AH-66) was 8.7 times higher than in resistant cells (AH-66F) when the cells were incubated with [^{14}C]bleomycin. The difference in permeability to bleomycin was not significant; uptake of [^{14}C]bleomycin by the sensitive cells was only 1.2 times larger than that by the resistant cells, and the radioactivity incorporated into the nuclei of sensitive cells was only 1.3-fold greater. The bleomycin-inactivating enzyme level in the resistant cells was 3.5 times higher than in the sensitive cells, indicating that the antibiotic incorporated into the resistant cells was reduced in DNA-binding activity to a large extent. The level of protein-free thiol compound in the sensitive cells was 1.8-fold higher than in the resistant cells, suggesting a possible enhancement of bleomycin action by intracellular thiol compound as is found *in vitro*. These factors probably affect the DNA strand scission and the sensitivity of cells to this antibiotic.

Binding of [^{14}C]bleomycin to DNA *in vitro* was studied in the presence and the absence of dithiothreitol. A large portion of the radioactivity bound in the presence of dithiothreitol was unstable to acid, but the acid-resistant binding was also enhanced by this thiol compound.

INTRODUCTION

Bleomycin inhibits DNA synthesis (13) and causes disappearance of cells in mitosis (1, 6). It causes strand scission of DNA both *in vivo* and *in vitro* (3, 7-9, 12-15). This antibiotic is effective on squamous cell carcinoma (16) and, among rat ascites hepatomas, AH-66[2] is most sensitive (11). We reported that the extent of single-strand scission in DNA of the sensitive hepatoma (AH-66) was significantly larger than in the resistant hepatoma (AH-66F) after incubation of the cells with bleomycin (8). The difference in the number of strand breaks appeared to be responsible for the difference in sensitivity of the hepatoma cells to this antibiotic. Umezawa *et al.* (16) examined the distribution of [^{3}H]bleomycin in organs and concluded that the selective effect of bleomycin on squamous cell carcinoma was due to the high concentration of total bleomycin in the tumor and to the low activity of this tumor in inactivating bleomycin. In this study the incorporation of [^{14}C]bleomycin into nuclei and binding of the antibiotic to DNA were compared in AH-66 and AH-66F to investigate the reason for the difference in the number of strand breaks in these 2 hepatomas. The level of bleomycin-inactivating enzyme and the content of protein-free thiol were also measured.

MATERIALS AND METHODS

Incorporation of [^{14}C]Bleomycin into Subcellular Fractions. [^{14}C]Bleomycin (27 Ci/mg, M.W. 1400) was a gift from Nippon Kayaku Co., Tokyo, Japan. AH-66 or AH-66F cells were withdrawn from the abdominal cavity of a Donryu rat, suspended in Eagle's minimum essential medium (1.85×10^7 cells/ml), and incubated with [^{14}C]bleomycin, 100 to 150 μg/ml, at 37° for 60 min. The cells were washed 3 times with 0.85% NaCl solution, suspended in 5 mM calcium chloride, and disrupted in a Chaikoff homogenizer. To the homogenate was added 0.25 M sucrose, and the nuclei were sedimented by centrifugation at $2,000 \times g$ for 10 min, mitochondria were precipitated at $10,000 \times g$ for 10 min, and microsomes were pelleted at $40,000 \times g$ for 60 min. Subcellular fractions were dried and solubilized in Soluene (Packard Instrument Co., Downers Grove, Ill.) and the radioactivity was measured after addition of toluene scintillator.

Extraction and Purification of DNA from Hepatoma Cells. The nuclei (2×10^7/ml) were lysed in 1% sodium dodecyl sulfate: 10 mM EDTA (pH 8.0) at 20° for 30 min and the lysate was shaken twice with phenol saturated with 10 mM Tris-HCl:1 mM EDTA (pH 8.0). DNA was precipitated by addition of 2 volumes of ethanol and was treated with RNase A (Worthington Biochemical Corp., Freehold, N. J.), 50 μg/ml, and T_1 (Sankyo Co., Tokyo, Japan), 25 units/ml, at 37° for 60 min in 10 mM Tris-HCl:10 mM EDTA:150 mM NaCl (pH 7.5). The reaction mixture was shaken with phenol and DNA was precipitated 3 times by addition of the 0.54 volume of isopropyl alcohol. The solution of DNA in 10 mM Tris-HCl:10 mM EDTA (pH 7.5) was mixed with Cs_2SO_4 (3.9 g/6 ml) or CsCl (6 g/5 ml) and the mixture was centrifuged at 43,000 to 48,000 rpm for 40 to 44 hr at 20° in a Beckman 50 Ti rotor. Fractions were

[1] This work was supported in part by a research grant for cancer research from the Ministry of Education of Japan.

[2] The abbreviations used are: AH-66, a strain of rat ascites hepatoma carried in Donryu rats; AH-66F, a substrain of AH-66; TCA, trichloroacetic acid; DTT, dithiothreitol (threo-2,3-dihydroxy-1,4-dithiol butane).

Received February 3, 1975; accepted April 7, 1975.

collected from the bottom of the tube and aliquots were placed on Whatman No. 3MM paper discs that were subsequently washed with cold 5% TCA and ethanol, and the radioactivity was measured in toluene scintillator. In certain cases the discs were dried without washing. The amount of [^{14}C]bleomycin bound to DNA was determined by the ^{14}C radioactivity coincident with the peak of DNA (1 mg/ml = 20 A_{260}/ml) at a density of 1.44 in Cs_2SO_4 gradient.

Bleomycin-inactivating Activity in Hepatoma Cells. AH-66 or AH-66F cells (4.5×10^8) were suspended in 8 ml of 70 mM phosphate (pH 7.0) and disrupted sonically. The homogenate was centrifuged at 105,000 × *g* for 60 min and the supernatant was assayed for the inactivating enzyme. The reaction mixture (0.5 ml) containing 100 μg bleomycin B_2, 30 mM phosphate, 60 mM NaCl (pH 7.0), and 0.05 to 0.2 ml of the enzyme solution was incubated at 37° for 60 min and the residual bleomycin B_2 was determined by the method described (16) using *Bacillus subtilis* as an assay organism.

Thiol Content of Hepatoma Cells. The protein-free thiol content was measured by the method of Ellman (2). The cells (1 to 1.5×10^7) were sonically disrupted at 0° for 1 min in 1.5 ml of 5% sulfosalicylic acid and the homogenate was centrifuged for 5 min at 10,000 rpm. The supernatant (1 ml) was mixed with 4 ml reagent solution containing 0.5 mM 5,5'-dithio(2-nitrobenzoic acid), 500 mM phosphate, and 1 mM EDTA (pH 6.8), and thiol was determined from absorbance at 412 nm.

Binding of [^{14}C]Bleomycin to DNA *in Vitro*. Calf thymus DNA (1 mg/ml) was incubated with 15- or 30-μg/ml concentrations of [^{14}C]bleomycin in 10 mM Tris-HCl (pH 8.0) at 37° for 60 min in the presence or absence of 50 mM DTT. The reaction mixture was shaken with a mixture of 1% sodium dodecyl sulfate phenol, and DNA was pecipitated 3 times by ethanol and was centrifuged in Cs_2SO_4 or CsCl solution.

RESULTS

Incorporation of [^{14}C]Bleomycin into Subcellular Fractions of Hepatoma Cells. When bleomycin-sensitive AH-66 and bleomycin-resistant AH-66F cells were incubated with [^{14}C]bleomycin at 37° for 60 min, 0.1% of the total radioactivity in the medium was incorporated into the whole cells. The ratio of [^{14}C]bleomycin incorporated into AH-66 to AH-66F cells was 1.02:1.44. About 30 to 50% of the ^{14}C activity in the cells was incorporated into the nuclei, and 1 to 3% and 0.6% were distributed in the mitochondria and microsome fractions, respectively (Table 1). About 20% of the radioactivity incorporated into the nuclei was associated with the nuclear protein fraction when the cell lysate was treated with phenol. With these subcellular fractions no significant difference in the incorporation of ^{14}C activity was observed between AH-66 and AH-66F cells. However, the radioactivity bound to DNA in AH-66 cells differed from that in AH-66F cells as described below.

[^{14}C]Bleomycin Bound to DNA in Hepatoma Cells. The nuclear DNA was extracted with phenol after incubation of the cells (1.85×10^7 cells/ml) with [^{14}C]bleomycin (150 μg/ml) at 37° for 60 min, and it was purified by equilibrium centrifugation in Cs_2SO_4 solution. The peak of TCA-insoluble ^{14}C activity coincided with the peak of absorbance at 260 nm at density 1.44. About 0.7% of the [^{14}C]bleomycin incorporated into the cells was bound to DNA of AH-66 cells and 0.1% to DNA of AH-66F cells. The value of ^{14}C activity *versus* absorbance of DNA of AH-66 was significantly higher than that of AH-66F as shown in Chart 1, *a* and *b*. A peak of RNA was present at density 1.64 but the ^{14}C activity was very low even in the case of sensitive cells. The binding ratio was estimated at 1 mole bleomycin per 3.2×10^8 daltons DNA in the sensitive cells and less than 1 mole per 3.1×10^9 daltons DNA in the resistant cells. In the previous investigation (8) with alkaline sucrose gradient centrifugation, the size of bulk DNA in the sensitive cells was reduced to 160 S (5×10^8 daltons) and that of the resistant cells was reduced to 280 S (2×10^9) after the cells (2×10^6 cells/ml) were incubated for 30 min with bleomycin; 50 μg/ml. The number of scissions per strand of DNA was estimated at 8 for the sensitive cells, and 1 for resistant cells assuming that the size of single-stranded DNA was 4.4×10^9 daltons. Although the conditions for examining binding of bleomycin in these two experiments were somewhat different from the conditions for examining the size of

Table 1

Incorporation of [^{14}C]bleomycin into subcellular fractions of sensitive and resistant hepatomas

In Experiment 1, cells (1.87×10^7/ml) were incubated with 100 μg of [^{14}C]bleomycin per ml. In Experiment 2, cells (1.85×10^7/ml) were incubated with 150 μg of [^{14}C]bleomycin per ml.

Experiment	Subcellular fraction	^{14}C dpm/1 × 10^7 cells		Ratio (66:66F)
		AH-66	AH-66F	
1	Cell	2463 (100.0)	2423 (100.0)	1.02
	Nuclei	763 (30.9)	569 (23.5)	1.34
	Mitochondria	69 (2.8)	29 (1.2)	2.38
	Microsomes	15 (0.6)	15 (0.6)	1.00
2	Cell	3633 (100.0)	2524 (100.0)	1.44
	Nuclei	1501 (41.3)	1208 (47.8)	1.24
	Mitochondria	82 (2.2)	25 (1.0)	3.28
	DNA[a]	26 (0.7)	3 (0.1)	8.67

[a] dpm/100 μg of DNA, which was purified as described in "Materials and Methods" and banded in Cs_2SO_4 gradient.

broken DNA in the previous experiments, the size of DNA per [^{14}C]bleomycin molecule bound coincided to a reasonable degree with the size of the broken DNA (Table 2).

Level of Bleomycin-inactivating Enzyme in Hepatoma Cells. The 105,000 × *g* supernatant of homogenate of the hepatoma cells was assayed for the bleomycin-inactivating enzyme that releases 1 mole of ammonia from carboxyl amide of bleomycin B_2 or bleomycin A_2 and produces a compound with reduced activity for single-strand scission of DNA. The bleomycin-inactivating activity in the resistant cells was about 3.5 times greater than in the sensitive cells (Table 3). The results indicated that a large portion of the bleomycin that was incorporated into the resistant AH-66F cells would be inactivated before the molecules reach the DNA strands. The difference in inactivating ability would largely contribute to the difference in sensitivity of the 2 hepatomas to this antibiotic. Activity (0.47 unit) of the sensitive cells in inactivating bleomycin is lower than the lowest value from carcinomas sensitive to bleomycin reported previously (16), and the value of 1.46 in the resistant cells is close to the lowest activity of sarcoma resistant to bleomycin treatment.

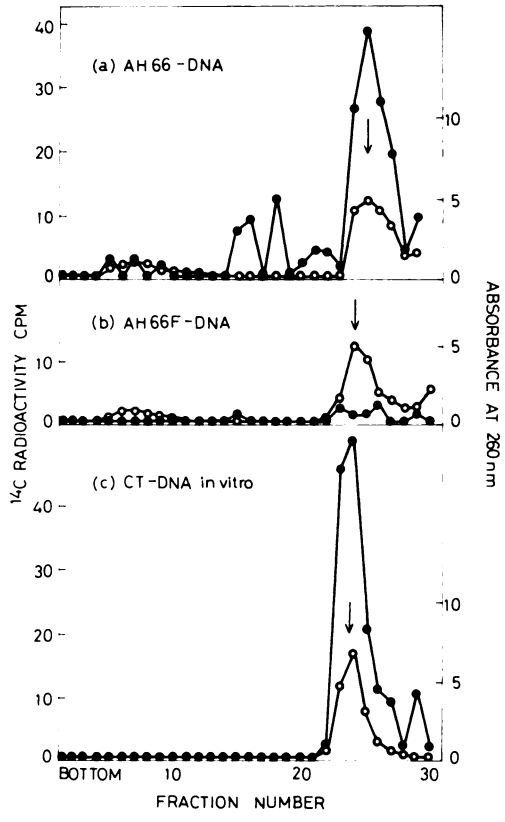

Chart 1. Buoyant density profile in Cs_2SO_4 gradient of DNA from the sensitive or resistant hepatoma exposed to [^{14}C]bleomycin. DNA was extracted from the cells, incubated with [^{14}C]bleomycin as described in "Materials and Methods," and centrifuged in Cs_2SO_4 gradient (*a* and *b*). Calf thymus DNA (*CT-DNA*) was incubated with [^{14}C]bleomycin in the absence of DTT and treated similarly to the DNA from hepatoma (*c*). *Arrow*, the position at a density of 1.44; O, absorbance at 260 nm when 0.1 ml of each fraction was diluted to 1 ml; ●, TCA-insoluble ^{14}C-activity in 0.1 ml of each fraction.

Table 2

Comparison of binding of [^{14}C]bleomycin to DNA and strand scission of DNA

	[^{14}C]Bleomycin bound to DNA[a]		
	^{14}C(dpm/100 μg DNA)	[^{14}C]Bleomycin (mole/dalton DNA)	Size of broken DNA[b] (daltons)
AH-66	25.6 ± 4.4[c]	$1/3.2 \times 10^8$	5×10^8
AH-66F	2.7 ± 1.3	$1/3.1 \times 10^9$	2×10^9

[a] Estimated from ^{14}C radioactivity coincident with the peak of DNA in Cs_2SO_4 equilibrium centrifugation.
[b] Estimated from the profile in alkaline sucrose gradient centrifugation analysis of cellular DNA (9).
[c] Mean ± S.E.

Table 3

Bleomycin-inactivating activity in the sensitive and resistant hepatoma

	Inactivated bleomycin formed (μg/mg protein/min)	Inactivated bleomycin formed (μg/1 × 10^7 cells/min)
AH-66	0.47[a]	2.86
AH-66F	1.46	10.10

[a] The cells were collected from the abdominal cavities of 7 to 8 rats and assayed for bleomycin-inactivating activity. The difference between duplicate experiments was within 3%.

Table 4

Content of nonprotein thiol in sensitive and resistant hepatomas

	Nonprotein thiol (nmoles/1 × 10^7 cells)	Protein (mg/1 × 10^7 cells)	DNA (μg/1 × 10^7 cells)
AH-66	302 ± 17[a]	5.7 ± 0.2	116 ± 12
AH-66F	172 ± 7	6.7 ± 0.2	122 ± 10

[a] Mean ± S.E.

Content of Thiol of Hepatoma Cells. DTT increased the binding of [^{14}C]bleomycin to DNA *in vitro* as indicated below, and thiol compounds have affected the decrease of melting temperature of DNA by bleomycin (10) and increased the number of strand scissions by this antibiotic *in vitro* (12, 14). The concentration of thiol in the nuclei seemed to affect the binding activity and strand scission in a similar manner. The content of protein-free thiol of the sensitive cells was 1.8-fold higher than that of the resistant cells (Table 4).

Binding of [^{14}C]Bleomycin to DNA *in Vitro*. [^{14}C]Bleomycin was incubated at 37° for 60 min with calf thymus DNA (1 mg/ml) in the presence or absence of 50 mM DTT. In the absence of the thiol, the activity bound to DNA after centrifugation in CsCl was about 0.05% of the [^{14}C]bleomycin in the medium. The binding ratio of [^{14}C]bleomycin to DNA was 1 mole per 1.1×10^8 daltons when DNA was incubated with the 15-μg/ml concentration of bleomycin, and it was 1 mole per 4.5×10^7 daltons with the 30-μg/ml concentration (Chart 2*b*). The radioactivity bound to DNA was stable to treatment with cold 5% TCA. It has been observed by Suzuki *et al.* (12) that

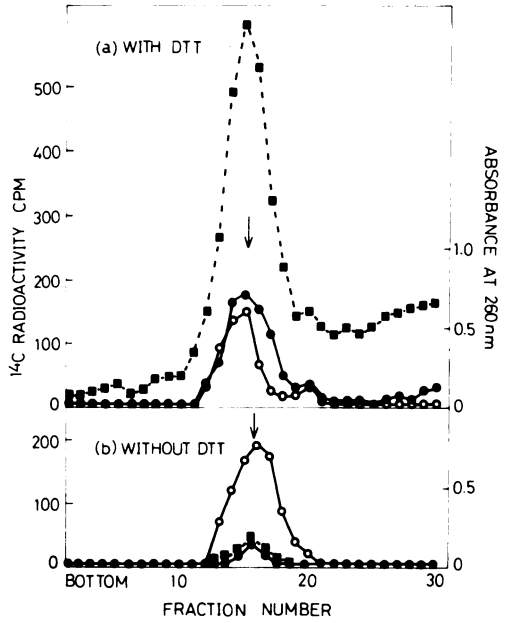

Chart 2. Buoyant density profile in CsCl gradient of calf thymus DNA incubated with [^{14}C]bleomycin. Calf thymus DNA was incubated with [^{14}C]bleomycin in the presence or absence of 50 mM DTT, under the conditions described in "Materials and Methods," and centrifuged in CsCl gradient. *Arrow*, the position at a density of 1.71; O, absorbance at 260 nm when 0.05 ml of each fraction was diluted to 1 ml; ■, total ^{14}C activity of 0.05 ml of each fraction; ●, TCA-insoluble ^{14}C activity of 0.05 ml of each fraction.

the amount of [^{3}H]bleomycin bound to DNA in the presence of 1 mM mercaptoethanol was 1.5 times higher than in the absence of mercaptoethanol after gel filtration through Sephadex. In this experiment addition of 50 mM DTT to the reaction mixture increased the binding to 20 times that which occurred without the thiol (Chart 2*a*). The radioactivity bound with DTT was so labile to acid that 70% of the activity was lost from the paper discs after washing with cold 5% TCA but the remaining radioactivity was still 6 times higher than that bound in the absence of DTT. The TCA-insoluble ^{14}C activity bound to DNA was further released by drastic treatment (Table 5). A large portion of the ^{14}C activity was lost by incubation of the complex at pH 4.0 or by heating at 100° in pH 7.5 buffer, while it was rather stable in alkaline solution and in high salt concentration such as in CsCl gradient. The residual activity after exhaustive treatment under the above conditions was 2 times higher than that of [^{14}C]bleomycin bound to DNA without DTT. The lability of [^{14}C]bleomycin bound to DNA with DTT is possibly related to the release of pyrimidine or purine base; Haidle *et al.* (4) have reported that the free bases were released from DNA by treatment of DNA with bleomycin in the presence of mercaptoethanol. The [^{14}C]activity bound in the absence of DTT was stable with the above treatment.

DISCUSSION

Bleomycin inhibits growth of ascites hepatoma AH-66 *in vivo* but not AH-66F (11). The number of single-strand scissions of DNA in AH-66 caused by bleomycin was about 8 times larger than in AH-66F after incubation of cells with this antibiotic (8). In this investigation a significant difference in the amount of [^{14}C]bleomycin bound to DNA was observed with the 2 hepatomas. The binding of bleomycin to DNA in AH-66 was 8.7 times larger than in AH-66F; the ratio of moles of bleomycin to DNA was 1:3.2 × 10^{8} daltons in AH-66 and 1:3.1 × 10^{9} in AH-66F. The similarity in binding and strand scission suggests that both phenomena occur at the same place. Despite the significant difference in binding and strand scissions, permeability of the sensitive cells to bleomycin was not so different from the resistant hepatoma. The bleomycin-inactivating activity in the sensitive cells was lower than the lowest activity of carcinomas that were sensitive to bleomycin, and the activity of AH-66F in inactivating bleomycin was close to the lowest activity of sarcomas resistant to bleomycin as reported previously (16). These results suggest that the sensitivity to bleomycin of the 2 hepatomas was dependent on the amount of the bleomycin-inactivating enzyme and thus on the amount of active bleomycin in the cells. Furthermore, the content of the thiol compound, which is kinown to enhance the strand scission of DNA by bleomycin *in vitro*, was 1.8-fold higher in the sensitive cells than in the resistant cells.

It is not clear whether the mechanism of binding or strand scission of DNA by bleomycin in the presence of thiol is similar to that in the absence of thiol. Even in the absence of thiol, bleomycin binds to DNA *in vitro* and causes strand scission, although the binding of [^{14}C]bleomycin to DNA is greatly enhanced by the addition of DTT. It has been suggested that thiol compounds react with the deoxyribosylpurine linkage in DNA giving a mercaptalated DNA the phosphodiester bond of which is easily hydrolyzed (5). In the presence of thiol compounds, 2 types of binding of bleomycin to DNA seem to occur. A large portion of [^{14}C]bleomycin bound to DNA in the presence of thiol compound was labile to acid. The other portion was stable

Table 5

Stability of binding of [^{14}C]*bleomycin to DNA in vitro*

Calf thymus DNA was incubated with [^{14}C]bleomycin *in vitro* and banded in CsCl gradient. The fraction of DNA was incubated under various conditions and the reaction mixture was placed on a filter paper disc that was then washed 3 times with cold TCA and ethanol.

Conditions	[^{14}C]Bleomycin (cpm/assay)	
	Bound to DNA with DTT	Bound to DNA without DTT
Total radioactivity	347 ± 27[a] (333)	33 (81)
TCA-insoluble activity		
control	104 ± 11 (100)	41 (100)
pH 4,[b] 37°, 30 min	45 ± 5 (43)	37 (90)
pH 7.5,[c] 37°, 30 min	100 ± 12 (96)	
pH 10,[d] 37°, 30 min	107 ± 15 (103)	
0.1 N NaOH	73 ± 8 (70)	36 (87)
pH 7.5, 100°, 5 min	39 ± 6 (37)	36 (87)
Dialysis	77 ± 11 (74)	36 (87)

[a] Mean ± S. E.
[b] Acetate buffer.
[c] Tris-HCl.
[d] 2-Amino-2-methylpropanol.

to acid and similar to that formed in the absence of thiol. ^{14}C activity bound in the presnce of thiol was 2 times higher than that bound in the absence of thiol even after treatment with acid, suggesting that acid-stable binding was also increased by thiol. When 1.9×10^7/ml cells were incubated with bleomycin (150 μg/ml) the concentration of [^{14}C]bleomycin in nuclei of the AH-66 cells was roughly estimated to be 0.4 μg/ml and that of DNA to be at 2 mg/ml. Then, binding *in vivo* seems to be more efficient than *in vitro* in the absence of thiol. This suggests a possible role of thiol compounds in cells. It is also likely that change in thiol content of nuclei during cell cycle may affect the sensitivity of cells to bleomycin.

ACKNOWLEDGMENTS

The authors are grateful to Dr. K. Horibata and Nippon Kayaku Co. for a generous gift of [^{14}C]bleomycin. We also thank T. Muto for technical assistance.

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