STUDIES ON THE MECHANISM OF ANTITUMOR EFFECT OF BLEOMYCIN ON SQUAMOUS CELL CARCINOMA

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Distribution of ^{3}H-bleomycin among organs of normal mice of various ages and old mice bearing skin carcinoma or sarcoma induced by 20-methylcholanthrene was studied and the ratios of the antibacterial activity to the radioactivity were examined. Higher concentration was shown in carcinoma than in sarcoma and in the former more than 50 % of ^{3}H-bleomycin remained in an active form exhibiting antibacterial activity. Slight or no activity was observed in sarcoma. The selective effect of bleomycin on mouse skin carcinoma induced by 20-methylcholanthrene was thus suggested to be due to the low ability of the tumor to inactivate bleomycin and probably also to the high uptake in this tumor. Differences were also observed among distributions of ^{3}H-bleomycin in lung and skin of mice of different ages. The higher concentration of its active form was shown in lung and skin of old mice at higher rate than in those of young ones.

An enzyme that releases one mole of ammonia from bleomycin and which inactivates bleomycin was extracted from tissues. The bleomycin inactivating activity of the enzyme protein was significantly lower in the protein extracted from squamous cell carcinoma of mice skin induced by 20-methylcholanthrene than in that extracted from sarcoma induced by the same chemical.

A group of new antitumor antibiotics produced by *Streptomyces verticillus* was discovered by UMEZAWA *et al.*[1] and named bleomycin. As reported by UMEZAWA *et al.*[2], bleomycins A_1, A_2, A_3, A_4, A_5, A_6, B_1, B_2, B_3, B_4 and B_5 were isolated. Thereafter, three more bleomycins (A_2'-a, A_2'-b and B_6) were found. These bleomycins resemble each other in physicochemical properties, and the chemistry of A_2 has been studied in the greatest ditail. As chemical information recently summarized by UMEZAWA[3], all bleomycins are unique glycopeptides consisting of 5 amino acids, an amine, L-gulose and 3-O-carbamoyl-D-mannose, and are different each other in the terminal amine moiety. Activities and toxicities of bleomycins were reported by ISHIZUKA *et al.*[4] A mixture of bleomycins containing bleomycin A_2 as the main component has been clinically studied in Japan by the Bleomycin Research Committee and the

therapeutic effect on squamous cell carcinoma, especially of the spino-cellular type, has been confirmed. Its effect on HODGKIN's disease and reliculosarcoma was also reported. The mode of action of bleomycin has been studied by SUZUKI *et al.*[4~6], KUNIMOTO *et al.*[7], NAGAI *et al.*[8~11], SHIRAKAWA *et al.*[12], and it has been confirmed that bleomycin reacts with DNA, resulting in strand scission, and that this action is promoted by a sulfhydryl compound or hydrogen peroxide. YAMAKI *et al.*[13] observed that action of the exo-type DNase is apparently stimulated by bleomycin, and MIYAKI *et al.*[14] observed inhibition of DNA ligase by a low concentration of bleomycin. DNA strand scission in intact cells has been confirmed by TERASIMA *et al*[15], and the cells in late G1 and early S phase are most sensitive to bleomycin[16]. Kinematographic study recently reported by MATSUDA *et al.*, Nipponkayaku Co., confirmed that HeLa cells in S phase are most sensitive to bleomycin. Therefore, it is likely that the primary action of bleomycin is its reaction with DNA causing strand scission and that this effect is enhanced by DNase and inhibition of DNA ligase. However, such information on the mode of action does not indicate the mechanism of the selective effect of bleomycin on squamous cell carcinoma.

In this paper, studies on the mechanism of the selective action on squamous cell carcinoma are reported.

Materials and Methods

^{3}H-Bleomycins: Bleomycin A_2 and bleomycin mixture which were prepared by Nipponkayaku Co. were tritiated and purified by CM-Sephadex chromatography as reported by UMEZAWA *et al*[2].) The ^{3}H-bleomycin mixture consisted of 48.5 % A_2, 27.0 % B_2, 11.8 % A_1, 3.4 % B_1, 3.9 % A_2' and 3.4 % B_4. Tritiated bleomycins decompose during storage and the decomposition can be shown by thin-layer chromatography using a solvent system of 10 % ammonium acetate-10 % ammonia-methanol (9 : 1 : 10). Therefore, the bleomycins were employed within two weeks after their purification.

Method of testing distribution of ^{3}H-bleomycin A_2 in organs of mice: A half ml of tritiated bleomycin in saline was subcutaneously injected, and 1 hour thereafter, the mice were killed by bleeding from the axilla vein. All organs were ground and extracted twice with 0.1 M phosphate buffer of pH 7.0 using the same weight as the organ. The radioactivity of each extract was determined by a Beckmann Liquid Scintillation System CPM-200 (LS-11). The antibacterial activity was determined by the cylinder plate method using *Bacillus subtilis* as the test organism and the corresponding bleomycin as the standard. The residues after the extraction of the ground organs were hydrolyzed in 1 N HCl in a sealed tube at 100°C for 18 hours and the radioactivity of the hydrolysate was determined. Radioactivities and antibaterial activities of feces, urine and serum were also examined.

Method of testing distribution of ^{3}H-bleomycin mixture in mice bearing carcinoma or sarcoma induced by 20-methylcholanthrene: As it was reported by ICHIKAWA *et al.*[17], a saturated solution of 20-methylcholanthrene in acetone which was kindly supplied by YOSHIDA and ODAJIMA, Sasaki Institute, Tokyo, was streaked twice a week for 10 weeks on the skin of 10-week-old mice. Twenty four weeks after the treatment (44 weeks after birth) a mouse bearing squamous cell carcinoma was used for the experiment. To another group of 10-week-old mice 0.1 ml of 20-methylcholanthrene suspension (10 μg/ml in 30 % Tween 80) was subcutaneously injected, and 17 weeks after the injection (27 weeks after birth) a mouse in which a skin sarcoma was induced was used for the experiment. The distribution of copper-containing ^{3}H-bleomycin mixture in organs and tumors of these mice was examined by the same method described above.

Inactivation of bleomycins A_2, A_5, B_2, B_4 by tissue homogenates: Liver, kidney, lung and skin of mice were homogenized with phosphate buffer (pH 6.8, 1/15 M) using 3 times the weight of each organ and centrifuged at 900 g. The supernatant was incubated for 3 minutes at 37°C and bleomycin was added at 1,000 μg/ml and further incubated at 37°C for 30 minutes. The residual bleomycin was determined by the cylinder-plate method described above.

Extraction of an enzyme to inactivate bleomycin: Liver (the total weight of liver was 23 g) from mice was homogenized with 70 ml of 1/15 M phosphate buffer, pH 6.8, and centrifuged at 900 g. The supernatant was centrifuged at 10,000 g. The supernatant (98 ml) was centrifuged at 105,000 g for 60 minutes. To the supernatant thus obtained, protamine (55.7 mg) was added and centrifuged at 10,000 g for 20 minutes. The enzyme in the supernatant was precipitated by 35~60 % saturation of ammonium sulfate and the precipitate was dissolved in 8.2 ml of phosphate buffer, 1/15 M, pH 7.2. It was then subjected to Sephadex chromatography (2.1×35.0 cm). The active fraction (10 ml) was treated by column chromatography using DEAE cellulose. The elution was carried out by raising the sodium chloride concentration in 5 mM phoshate buffer, pH 7.2, from 0 to 0.15 M. The total protein in the active fraction was determined by the FOLIN method.

Inactivation of bleomycins A_2, B_2, A_5 by the extracted enzyme: The enzyme prepared by Sephadex chromatography as described above was employed. The standard condition was as follows: 1.2 mg of the enzyme as protein was incubated with 100 μg/ml of bleomycin and after 60 minutes the residual bleomycin was determined by a paper disc method using *B. subtilis* as the assay organism.

Activity of protein of squamous cell carcinoma and sarcoma induced by 20-methylcholanthrene to inactivate bleomycin: Carcinoma and sarcoma were homogenized, and the enzyme solution was prepared by centrifugation at 10,000 g, 105,000 g, protamine treatment, and precipitation by 35~60 % saturation of ammonium sulfate as described above. The precipitate was suspended in 1/15 M phosphate buffer, pH 7.2, and its ability to inactivate bleomycin B_2 and the protein content were determined as described above. The enzyme activity required to inactivate 1 μg of bleomycin B_2 per minute was defined as one unit.

Results

Distribution of ^{3}H-Bleomycin A_2 among Organs of Mice

Using two mice each weighing 19 g, 2.0 mg of tritiated bleomycin A_2 (4.60×10^6 dpm in total) was subcutaneously injected and the syringe was then washed with water. Then, the residual radioactivity in the wash water was 0.93×10^6 dpm and therefore, the amount injected to two mice was 1.59 mg. The organs taken from two mice were combined, ground, extracted twice with 1/10 M phosphate buffer using the same weight as the organs and centrifuged at 3,000 r.p.m. for 20 minutes. The residue of each organ was hydrolyzed and radioactivity of the hydrolysate was determined. As shown in Table 1, totally 1.43 mg of the injected bleomycin was found in the extracts of organs, serum, urine and feces, and 0.16 mg was found in the hydrolysates of the residues. Thus, 100 % of the injected bleomycin as measured by radioactivity was recovered. From the results it can be seen that bleomycin in the hydrolysate is high in kidney, urinary bladder and skin. However, if we compare the amounts of bleomycin extracted with those remaining in the residues after the extraction, as shown in Table 2, there was no organ which contained particularly large amounts of

Table 1. Distribution of ^{3}H-bleomycin A_2* among phosphate buffer extracts of organs and excretes of two mice weighing 19 g

Organs *etc.*	By radioactivity			By antimicrobial activity		b/a
	Total in dpm ($\times 10^3$)	Total in μg of bleomycin (a)	μg/g	μg in total (b)	μg/g	
Liver	57.3	24.9	10.7	<0.50	<0.22	<0.020
Spleen	3.9	1.7	5.0	0.13	<0.38	0.076
Kidney	61.3	26.7	45.1	0.23	0.39	0.008
Testis	6.1	2.7	11.3	<0.08	<0.33	<0.030
Lung	7.8	3.4	9.7	0.64	1.82	0.188
Heart	3.4	1.5	8.8	<0.08	<0.47	<0.053
Brain	3.1	1.3	1.9	<0.09	<0.13	<0.069
Tongue	3.1	1.3	8.5	0.13	0.84	0.100
Urinary bladder	19.0	8.3	150.9	2.80	50.90	0.377
Eye	1.3	0.6	8.0	<0.05	<0.67	<0.083
Stomach	9.5	4.1	13.2	<0.08	<0.26	<0.020
Large intestine	39.9	17.3	12.7	<0.35	<0.26	<0.020
Small intestine	79.6	34.6	11.3	<0.74	<0.24	<0.021
Skelton	91.9	40.0	8.0	1.15	0.23	0.029
Head bone	12.4	5.0	6.0	<0.25	<0.28	<0.046
Muscle	119.5	52.0	7.2	<1.71	<0.24	<0.033
Peritoneum	69.6	30.3	26.8	<0.36	<0.32	<0.012
Diaphragm	2.8	1.2	9.8	<0.06	<0.49	<0.050
Skin	298.2	129.7	21.4	29.30	4.48	0.226
Omentum, fat and others	40.8	17.7	14.0	<0.35	<0.28	<0.020
Large intestine content	26.4	11.5	—	<1.57	—	<0.137
Small intestine content	70.0	30.4	—	<0.65	—	<0.021
Stomach content	53.3	23.2	—	<0.45	—	<0.019
Urine	2,240.5	974.1	—	1,064.00	—	1.092
Blood wash	2.4	1.0	—	0.14	—	0.140
Erythrocyte	7.4	3.2	—	1.17	—	0.366
Serum	24.7	10.7	—	9.60	—	0.897
Total	3,300	1,434.4				

* 2.60×10^6 dpm/mg ; 3.67×10^6 dpm was introduced subcutaneously into two mice ; The mice were killed 1 hour thereafter.

bleomycin in the residue after the extraction. The lowest amount was found in the eye. The amounts of bleomycin in urinary bladder and kidney vary in different experiments, due to contamination of urine containing bleomycin at high concentration. Therefore, the amounts of bleomycin shown in kidney and urinary bladder do not indicate the true values in the tissues of these organs. The results shown in Table 2 indicate that there is no organ which strongly binds bleomycin so that the bleomycin can not be extracted.

High concentrations of ^{3}H-bleomycin was measured by radioactivity in extracts of kindey, urinary bladder, skin and peritoneum. As already described, the values of bleomycin in kidney and urinary bladder do not indicate the true concentration in these tissues. The high concentrations in skin and peritoneum indicate the bleomycin is taken up by these tissues at higher concentrations than by the others. If the measurement of ^{3}H-bleomycin using antibaterial activity is designated as the active form, then as shown by the ratios of concentrations of the antibacterial activity to the radioactivity in Table 1, the active form is high in skin and lung. In the other

organs except tongue almost all ^{3}H-bleomycin as measured by the radioactivity had been converted to an inactive form.

Distribution of ^{3}H-Bleomycin Mixture in Mice Bearing Squamous Cell Carcinoma or Sarcoma Induced by 20-Methylcholanthrene

To a mouse weighing 32.0 g and bearing a squamous cell carcinoma 2.15 mg of ^{3}H-bleomycin mixture (17,492.25×10^3 dpm/mg) was subcutaneously injected using a microsyringe. One hour thereafter, the mouse was killed by bleeding from the axilla vein and each organ was weighed, ground and extracted twice with phosphate buffer of pH 7.0 using a weight equal to that of the organ. Radioactivities and antibacterial activities of the extracts were examined. Totally, 91.7 % of ^{3}H-bleomycin injected was recovered as radioactivity. The results are shown in Table 3. In this experiment, the local skin and the peritoneum of 1 cm in diameter around the area of injection were examined separately from other parts of these tissues. As shown in Table 3, high concentrations were shown in lung, skin and carcinoma, the figures being as 25.5 μg/g, 17.7 μg/g, and 15.9 μg/g respectively. As shown in Table 3, except for kidney and urinary bladder which is contaminated with urine, bleomycin which showed antibacterial activity was detected only in lung, skin and carcinoma. In serum, the concentration of bleomycin shown by antibacterial activity was higher than that shown by radioactivity measurements. Among bleomycins in the bleomycin mixture used, A_2 is the main component. Other bleomycins which have stronger antibacterial activity are thought to be differentially reduced in serum. In urine, the concentration of bleomycin shown by the radioactivity was slightly lower than that shown by the antibacterial activity.

Table 2. Amounts of ^{3}H-bleomycin A_2 in the residues* of ground organs of two mice after the phosphate buffer extraction

Organs	Total in dpm (×10^3)	Total in μg	μg/g	Amount in residue/ Total amount
Liver	25.8	11.2	4.8	0.31
Spleen	2.8	1.0	2.9	0.37
Kidney	13.8	6.0	10.1	0.18
Testis	0.9	0.4	1.7	0.13
Lung	2.7	1.2	3.4	0.26
Heart	0.4	0.2	1.2	0.12
Brain	1.8	0.8	1.2	0.39
Tongue	0.4	0.2	1.3	0.13
Urinary bladder	1.6	0.7	12.7	0.08
Eye	0.1	0.04	0.5	0.06
Stomach	1.7	0.7	2.3	0.15
Large intestine	8.6	3.7	2.7	0.18
Small intestine	14.4	6.3	2.1	0.15
Skelton	43.1	18.7	3.8	0.32
Head bone	6.7	2.9	3.2	0.35
Muscle	88.3	38.4	5.3	0.42
Peritoneum	18.8	8.2	7.2	0.21
Diaphragm	0.5	0.2	1.6	0.13
Skin	127.0	55.2	9.1	0.30
Omentum, fats and others	13.0	5.7	4.5	0.24
Total	371.9	161.74		

* The radioactivity of the hydrolysate of the residue was determined.

In another experiment, the identical quantity of the same ^{3}H-bleomycin mixture was subcutaneously injected into a mouse weighing 35.0 g and bearing a sarcoma induced by the subcutaneous injection of 20-methylcholanthrene. The radioactivities and antibacterial activities of extracts of organs and excretion were examined. Totally, 93.5 % of the injected radioactivity was recovered in the extracts. The result is shown in Table 4. Except for concentration in sarcoma, the results were substantially the same as that seen in the experiment with the mouse bearing squamous cell carcinoma. In the sarcoma, a much lower concentration (4.5 μg/g) than that in the skin (26.9 μg/g)

Table 3. Distribution of ^{3}H-bleomycin mixture* among organs and excretes of a mouse bearing carcinoma induced by 20-methylcholanthrene

Organs *etc.*	By radioactivity			By antimicrobial activity		b/a
	Total in dpm ($\times 10^3$)	Total in μg of bleomycin (a)	μg/g	μg in total (b)	μg/g	
Liver	142.3	17.5	10.2	<1.35	<0.79	<0.077
Kidney	271.2	33.4	66.1	11.55	22.87	0.346
Spleen	35.2	4.3	8.0	<0.62	<1.15	<0.144
Uterus	14.4	1.8	10.0	<0.16	<0.80	<0.089
Urinary bladder	27.7	3.4	117.2	1.30	44.82	0.382
Lung	41.5	5.1	25.5	1.55	7.75	0.304
Heart	9.7	1.2	8.6	<0.16	<1.15	<0.133
Eye	3.6	0.4	5.1	<0.17	<2.15	<0.425
Tongue	10.5	1.3	9.4	<0.19	<1.38	<0.146
Brain	16.6	2.0	4.7	<0.51	<1.20	<0.255
Diaphram	10.7	1.3	13.3	<0.27	<2.76	<0.208
Peritoneum	43.9	5.4	12.1	<0.49	<1.10	<0.091
Peritoneum around the injected place	209.6	25.8	73.9	4.65	13.90	0.180
Omentum, fat and others	86.3	10.6	10.8	<1.11	<1.13	<0.111
Stomach	17.4	2.1	6.2	<0.41	<1.20	<0.195
Small intestine	85.0	10.5	7.5	<1.46	<1.05	<0.139
Large intestine	51.2	6.3	6.4	<1.15	<1.16	<0.183
Skin	456.1	56.1	17.7	20.02	6.31	0.357
Skin around the injected place	771.4	95.0	135.7	56.25	80.36	0.592
Muscle	489.1	60.2	10.9	<7.61	<1.38	<0.126
Skelton	405.8	50.0	9.3	<7.49	<1.40	<0.150
Carcinoma	122.6	15.1	15.9	10.49	11.00	0.695
Gastric content	20.0	2.5	—	—	—	—
Small intestine content	166.9	20.5	—	—	—	—
Large intestine content	61.3	7.5	—	—	—	—
Serum	77.1	10.1	—	15.60	—	1.545
Erythrocyte	35.2	4.3	—	—	—	—
Urine	12,349.4	1,520.1	—	1,646.00	—	1.083
Total	16,031.7	1,973.8	91.7% recovery			

8.124×10^6 dpm ; 17,492.25×10^3 dpm (2.15 mg) was subcutaneously introduced into the mouse.

was observed and the active form of bleomycin was not detected in the sarcoma. Another minor difference from the previous experiment is that the concentrations shown by the antibacterial activity in serum and urine were slightly lower than those shown by the radioactivity.

The same experiments were repeated in the other two mice bearing carcinoma and the other two mice bearing sarcoma. The results of the distribution of ^{3}H-bleomycin in various organs were substantially same as those described in Tables 3 and 4. The distribution in carcinoma and sarcoma shown in Table 5 includes data from all the experiments. The total concentration shown by radioactivity was higher in the squamous cell carcinoma than in sarcoma. A more marked difference was found in the concentration of the active form which was significantly higher in the carcinoma than in the sarcoma.

Distribution of ^{3}H-Bleomycin Mixture in Mice of Different Ages

Distribution of ^{3}H-bleomycin mixture in mice weighing 19 g was reported in a previous paper by UMEZAWA *et al.*[18], and distribution of ^{3}H-bleomycin A_2 in mice

Table 4. Distribution of ^{3}H-bleomycin mixture* among organs and excretes of a mouse bearing sarcoma induced by 20-methylcholanthrene

Organs *etc.*	By radioactivity			By antimicrobial activity		b/a
	Total in dpm ($\times 10^3$)	Total in μg of bleomycin (a)	μg/g	μg in total (b)	μg/g	
Liver	162.8	20.0	9.1	<1.70	<0.77	<0.085
Kidney	124.2	15.3	29.1	10.00	19.05	0.654
Spleen	26.1	3.2	6.1	<0.56	<1.07	<0.175
Uterus	5.3	0.7	7.2	<0.18	<1.86	<0.257
Urinary bladder	23.3	2.9	161.1	0.98	54.34	0.338
Lung	52.9	6.5	26.5	3.61	14.70	0.555
Heart	11.1	1.4	7.7	<0.18	<0.99	<0.129
Eye	3.0	0.4	7.4	<0.19	<3.52	<0.475
Tongue	6.5	0.8	7.8	<0.22	<1.95	<0.275
Brain	19.6	2.4	5.8	<0.45	<1.09	<0.188
Diaphragm	10.0	1.2	12.9	<0.30	<3.23	<0.250
Peritoneum	54.3	6.7	13.4	<0.59	<1.18	<0.088
Peritoneum around the injected place	244.3	30.1	102.0	5.63	19.08	0.187
Omentum, fat and others	55.6	6.8	9.3	<0.11	<0.15	<0.016
Stomach	13.9	1.7	5.1	<0.35	<1.06	<0.206
Small intestine	59.2	7.3	8.1	<1.08	<1.20	<0.148
Large intestine	49.9	6.1	6.0	<1.10	<1.08	<0.180
Skin	659.3	81.2	26.9	20.06	6.65	0.247
Skin around the injected place	1,006.5	123.9	146.1	89.42	105.45	0.722
Muscle	449.9	55.4	12.3	<5.62	<1.25	<0.101
Skelton	348.9	42.9	8.2	<5.46	<1.05	<0.127
Sarcoma	124.9	15.4	4.5	<1.70	<0.50	<0.110
Gastric content	20.7	2.5	—	<1.35	—	<0.540
Small intestine content	135.0	16.6	—	<2.61	—	<0.157
Large intestine content	99.5	12.2	—	<2.03	—	<0.166
Serum	147.0	18.1	—	15.91	—	0.879
Erythrocyte	21.8	2.7	—	—	—	—
Urine	12,427.2	1,529.7	—	1,410.00	—	0.923
Total	16,362.7	2,014.1	93.5% recovery			

8.124$\times 10^6$ dpm ; 17,492.25$\times 10^3$ dpm (2.15 mg) was subcutaneously introduced into the mouse.

weighing 19 g was also described above. Comparing concentrations of active bleomycin in lungs of these mice with those of old mice bearing tumors, the former were found to be lower than the latter. Therefore, the distribution of ^{3}H-bleomycin mixture in mice of different ages was examined. Into each mouse of different ages, that is, 3 weeks after the birth, 5 weeks after the birth, and 28 weeks after the birth, 49.71~54.28 mg/kg of ^{3}H-bleomycin was subcutaneously

Table 5. Distribution of ^{3}H-bleomycin in squamous cell carcinoma and sarcoma induced by 20-methylcholanthrene at one hour after the subcutaneous injection

Exp.	Carcinoma		Sarcoma	
	Total BL μg/g	BL by the antibacterial activity, μg/g	Total BL μg/g	BL by the antibacterial activity
1	15.9	11.0	4.5	<0.5
2	22.7	17.2	13.7	1.9
3	17.9	13.7	14.4	2.8

To mice bearing carcinoma : 65.1 mg/kg, 64.6 mg/kg and 65.5 mg/kg were injected in Exp. 1, 2, 3.
To mice bearing sarcoma : 70.3 mg/kg, 70.6 mg/kg and 62.7 mg/kg were injected in Exp. 1, 2, 3.
Radioactivity of ^{3}H-bleomycin employed : 8.23$\times 10^6$ dpm/mg in Exp. 1, 8.23$\times 10^6$ dpm/mg in Exp. 2, 8.33$\times 10^6$ dpm/mg in Exp. 3.

Table 6. Distribution of ^{3}H-bleomycin mixture in liver, lung, skin, peritoneum of mice of various age

			Liver	Lung	Skin	Skin near the injected place	Peritoneum	Peritoneum near the injected place
Mouse, 3 weeks after the birth, weighing 10 g	μg/g by radioactivity	Exp. 1	10.1	10.8	10.6	88.2	22.5	46.4
		Exp. 2	12.5	8.2	14.6	90.9	23.6	49.0
	b/a	Exp. 1	<0.10	<0.28	<0.07	<0.20	<0.04	<0.03
		Exp. 2	<0.08	<0.21	<0.07	0.51	<0.03	<0.02
Mouse, 5 weeks after the birth, weighing 20 g	μg/g by radioactivity	Exp. 1	8.0	7.1	7.4	38.6	11.7	15.6
		Exp. 2	14.3	7.4	9.7	78.9	12.6	14.4
	b/a	Exp. 1	<0.11	0.29	0.11	0.44	<0.08	<0.05
		Exp. 2	<0.06	0.40	0.13	0.41	<0.07	<0.06
Mouse, 28 weeks after the birth, weighing 35 g	μg/g by radioactivity	Exp. 1	10.3	9.9	40.6	152.6	14.6	108.7
		Exp. 2	22.1	22.3	24.0	268.4	16.4	95.4
	b/a	Exp. 1	<0.10	0.64	0.46	0.76	0.22	0.25
		Exp. 2	<0.04	0.50	0.49	0.79	0.15	0.28

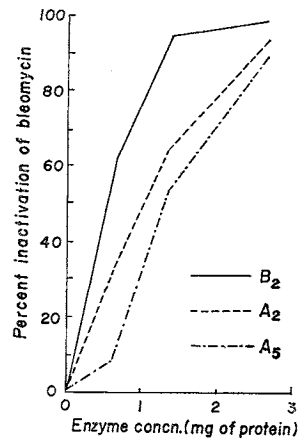
b is the concentration determined by the antimicrobial activity and a is that by the radioactivity and b/a indicates the ratio of the active bleomycin to the total bleomycin. In the Exp. 1, the specific activity of bleomycin was 6.70×10^{6} dpm/mg and the doses were as follows : 52.76 mg/kg for 3 weeks mouse, 49.71 mg/kg for 5 weeks mouse, 54.28 mg/kg for 28 weeks mouse. In Exp. 2, the specific activity of bleomycin was 6.56×10^{6} dpm/mg and doses were as follows : 52.77 mg/kg for 3 weeks, 5 weeks and 28 weeks mouse. It was subcutaneously injected and 1 hour thereafter, the concentrations of organs were determined by the radioactivity and the antibacterial activity.

Table 7. Inactivation of various bleomycins by homogenates of various organs of mice

Bleomycins	Remaining %				
	Lung	Skin	Liver	Spleen	Kidney
A_2	88.3	72.3	29.5	47.3	25.5
A_5	74.2	61.5	28.7	41.5	35.6
B_2	40.2	34.2	6.8	18.4	5.0
B_4	61.3	46.7	16.0	27.7	16.3

Fig. 1. Inactivation of bleomycins B_2, A_2 and A_5.

Reaction mixture : 100 μg/ml of bleomycin, 1/15 M phosphate buffer at pH 7.2 and mg of enzyme indicated. Incubation : at 37°C for 60 minutes.



injected and 1 hour thereafter, the distribution of ^{3}H-bleomycin in organs of the mice were determined as described above. No substantial differences were found in the distributions among organs of mice of different ages except in the case of lung, skin and peritoneum. The results obtained in liver, lung, skin and peritoneum are shown in Table 6. The organs of the youngest mice have the greatest ability to inactivate bleomycin. The ability to inactivate bleomycin was the weakest in the oldest mice of 28 weeks, and 50~64 % and 46~49 % of bleomycin remained in an active form in the lung and skin respectively. Moreover, a higher concentration was shown in the skin of the oldest mice than in that of younger ones, and in the peritoneum of the oldest mice 15~22 % of bleomycin was detected as the active form.

Extraction of A Bleomycin-Inactivating Enzyme and Isolation of Inactivated Bleomycin B_2

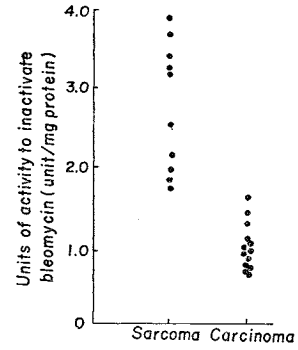
The percent of residual bleomycins after inactivation by tissue homogenates at

Table 8. Partial purification of the enzyme to inactivate bleomycin

	Total protein mg	Total[6] unit	The activity u/mg[7]	Yield %	Fold
Crude ext. 10,000 g[1]	1960	1984	1.01	100	1.0
105,000 g[2]	1393	1991	1.43	100	1.4
Protamine sulfate[3]	1400	1911	1.36	96.3	1.36
Ammonium sulfate 35~60 %	531	1715	3.2	86.4	3.2
Sephadex G-25[4]	314	850	2.7	42.9	2.7
DEAE cellulose[5]	70	810	11.6	40.8	11.5

1) 98 ml. 2) 81 ml. 3) Protamine (55.7 mg) was added and centrifuged at 10,000 g for 20 minutes. 4) Solvent: 1/15 M phosphate buffer at pH 7.2. 5) The elution was carried out by raising the concentration of sodium chloride in 5 mM phosphate buffer at pH 7.2 from 0 to 0.15 M. 6) One unit is the activity of inactivating 1 μg of bleomycin B_2 per min. 7) u/mg means u/mg as protein.

Fig. 2. Activity of the proteins obtained from squamous cell carcinoma and sarcoma induced by 20-methylcholanthrene in mice skin to inactivate bleomycin (one unit inactivates 1.0 μg of bleomycin B_2 in 1 minute).



37°C for 30 minutes are shown in Table 7. Among the bleomycins studied, namely A_2, A_5, B_2 and B_4, bleomycin B_2 is most rapidly inactivated. Among the homogenates of lung, skin, liver, spleen and kidney, the most rapid inactivation occurred in kidney and liver homogenates and the inactivation was the slowest in lung and skin homogenates.

The bleomycin-inactivating enzyme was extracted from homogenate of liver of mice by the procedure described in the previous section. The activity of the extract and the yield of the enzyme at each step of the extraction are shown in Table 8.

The optimum pH of enzyme was 7~7.5, and the optimum temperature was 37°C. The enzyme, purified by DEAE cellulose chromatography, was very unstable. Therefore, the enzyme obtained by Sephadex G-25 chromatography was employed for the experiments. The comparative inactivation of bleomycins A_2, A_5 and B_2 was studied and the results are shown in Fig. 1. Among these bleomycins, B_2 was most rapidly inactivated. After the inactivation, ammonia was determined using the CONWAY microdiffusion method followed by titration or by spectrometry after the ninhydrin reaction. One mole of ammonia was found to be released during the inactivation.

We attempted to isolate the inactivated bleomycin B_2 to find the chemical difference from bleomycin B_2. Two hundred mg of bleomycin B_2 was incubated with 40 ml of the enzyme solution (1,600 mg as protein) and 60 ml of 1/15 M phosphate buffer for one hour at 37°C. A hundred ml of methanol was added and the supernatant was concentrated to 20 ml. The inactivated and the residual bleomycins were adsorbed on IRC-50 resin in H^+ form and eluted with 0.05 N hydrochloric acid-acetone (1 : 1). After neutralization with IR-45 resin in OH^- form, the eluate was evaporated *in vacuo* to dryness. The powder was dissolved in 50 ml of distilled water and passed through a CM-Sephadex C-25 column (240 ml) and the inactivated bleomycin was eluted by raising the concentration of ammonium formate from 0.05 M to 1.0 M. The inactivated B_2 appeared in the fractions eluted at approximately 0.2 M. The eluate was treated by carbon chromatography using 0.02 M hydrochloric acid - n-propanol (1 : 1) to remove ammonium formate and the eluate was neutralized with IR-45 resin and evaporated to dryness,

yielding 132 mg of the inactivated bleomycin B_2.

A difference between the inactivated B_2 and bleomycin B_2 was found in the slightly lower basicity shown by high voltage electrophoresis. Hydrolysis in 6 N hydrochloric acid for 24 hours gave all the degradation products obtained from bleomycin B_2. As already described, during the enzymatic inactivation, one mole of ammonia was released. Therefore, the sugar moiety was tested by gas chromatography of the trimethylsilyl derivatives of the methanolysis products. The carbamoyl group was also found in the inactivated bleomycin B_2 These data suggest that ammonia would be released from the carboxyl amide, the presence of which was recently confirmed by TAKITA and MURAOKA in the Institute of Microbial Chemistry (unpublished data).

Specific Activity of Enzyme Proteins Extracted from Squamous Cell Carcinoma and Sarcoma Induced by 20-Methylcholanthrene

Carcinoma and sarcoma were induced by 20-mthylcholanthrene and half of each tumor was homogenized by the procedure described above. From the homogenate, the bleomycin-inactivating enzyme was extracted by centrifugation at 105,000 *g*, protamine treatment and precipitation with 35~60 % saturation of ammonium sulfate as described in the previous paragraph.

Preliminary experiments indicated that the bleomycin-inactivating enzyme existed in 105,000 *g* supernatant of homogenates of carcinoma and sarcoma and that the enzyme could be extracted by the same procedure as that used for extraction of the enzyme from liver. The yields of the extraction starting from homogenates of carcinoma and sarcoma were practically the same as that from liver homogenate.

The mean of the amount of the protein extracted from 13 carcinomas was 15.09 mg per g of carcinoma tissue, and the mean of the activity of carcinoma tissue calculated was 14.45 units per g of carcinoma tissue. One unit of the activity inactivates 1 μg of bleomycin B_2 per minute. Then mean of the amount of protein extracted from 10 sarcomas was 12.09 mg per g of sarcoma tissue, and the mean of the activity of sarcoma tissue calculated was 32.71 units per g of sarcoma tissue. Thus, the bleomycin-inactivating activity of sarcoma tissue was shown to be stronger than that of carcinoma. This was also shown by preliminary experiments testing bleomycin B_2-inactivating activity of 105,000 *g* supernatant of homogenates of 2 carcinomas and 2 sarcomas. The activity of the protein prepared from each of carcinomas and sarcomas is shown in Fig. 2. The other half of tumor was sent to the Department of Pathology, the First Tokyo National Hospital, for the histological diagnosis. As shown in Fig. 2, the activity of the protein obtained from 10 sarcomas was significantly higher than that of the 13 carcinomas examined. Only one exception which was not shown in the figure was found in one carcinoma which yielded a protein having an activity of 3.0 u/mg.

Discussion

As reported in previous papers[4-12], bleomycin causes strand scission of DNA *in vitro*, and the scission of DNA can also be shown *in vivo*[15]. At a low concentration bleomycin

inhibits DNA ligase. These effects are similar to the effects of radiation. This resemblance suggests that there may be human tumors which would be sensitive both to radiation and to bleomycin. However, the selective effect of bleomycin on squamous cell carcinoma was found to be due to another reason.

Before the clinical study of bleomycin was initiated, distribution of bleomycin among organs of mice was studied and a high concentration in lung was found. It was therefore thought that bleomycin might be effective against lung tumors. Later, the effect on penile cancer was observed by ICHIKAWA and a study of the distribution of bleomycin in mice showed high concentrations in the skin[19]. In these studies, concentrations of bleomycin were determined by the antibacterial activity.

ICHIKAWA and TAKEUCHI[17] have confirmed that bleomycin is effective against squamous cell carcinoma in mice skin induced by 20-methylcholanthrene, but not effective on sarcoma in mice skin induced by the same chemical. Now, as shown in Tables 3, 4, and 5, it was experimentally proved that the distribution of ^{3}H-bleomycin in the active form is significantly higher in the carcinoma than in the sarcoma. Moreover, the result suggests a higher distribution of total bleomycin in carcinoma than in sarcoma. Significantly higher concentration of active bleomycin in the carcinoma than in the sarcoma is thought to be due to the lower content of a bleomycin-inactivating enzyme in the former, because the activity of proteins extracted from the sarcoma was significantly higher than that extracted from carcinoma (Fig. 2). Thus, the results of the studies on distribution of radioactive bleomycin and on enzyme proteins inactivating bleomycin indicate that the selective effect of bleomycin on squamous cell carcinoma is dependent on the low activity of this tumor to inactivate bleomycin and probably also on the high distribution of total bleomycin in this tumor.

Study of distribution of bleomycin also gave useful information on toxicity of bleomycin. Among various organs, after the injection of ^{3}H-bleomycin, active bleomycin was higher in lung and skin than in other organs. Also, in *in vitro* experiments, inactivation of bleomycin was slowest in homogenates of lung and skin as compared with homogenates of liver, spleen and kidney. Moreover, the rate of inactivation was lower in old mice, 28 weeks after the birth, than in younger ones, 5 weeks or 3 weeks. These results suggest that toxicity from bleomycin would appear in skin and lung and more frequently in older patients. This is in accord with clinical observations.

The bleomycin-inactivating enzyme which was partially purified from mice liver was confirmed to release one mole of ammonia from carboxyl amide in bleomycin molecules. The induction of this enzyme by administration of bleomycin to mice could not be observed. The data indicate that this enzyme activity in lung and skin of young mice decreases with the age. Though it is not certain, this enzyme may have some physiological role.

Studies of mode of actions of antitumor compounds have indicated that antitumor compounds are generally very toxic to animal cells. Generally, they inhibit directly or indirectly nucleic acid or protein synthesis or interfere with the function of the cell membrane. Therefore, it is thought that such antitumor compounds can not be used for treatment of all kinds of human tumors. However, in the study of bleomycin, we found that even an antitumor compound with such characteristics had the possibility of effectiveness against particular types of human cancer. Cells in which an antitumor compound distributes at particularly high concentration, cells which have particularly high ability to activate an antitumor compound, cells which have particularly low ability to inactivate an antitumor compound, or cells in which an enzyme system relating to the action of the compound is particularly low or high, are selectively inhibited by such a compound, and a cancer composed of such cells having any of such abilities may be successfully treated by such an antitumor compound. Information of the distribution of antitumor compounds among organs and tissues of experimental animals, on inactivation and activation and on distribution in cells of certain type of experimental tumors can be extremely useful in predicting the type of human tumor which is likely to a response. Such a type of study on each

antitumor compound is thought to be one of ways to find a useful chemotherapeutic agents against human tumors.

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