BIOLOGICAL STUDIES ON INDIVIDUAL BLEOMYCINS

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Details of the effect on EHRLICH carcinoma of the individual bleomycins A_1, A_2, A_2', A_5, B_2, B_4 and B_6 and their distribution in organs of mice are reported. In this study, A_1, A_2, A_5 were found to have properties which might make them useful for treatment of tumors in skin of squamous cell carcinomas. The copper-free bleomycins had almost the same activities as the copper-containing forms against bacteria and EHRLICH carcinoma. Copper in the copper-containing bleomycins was shown to increase the toxicity and to increase the effect on vascular permeability by comparing copper-free and copper-containing A_2 and B_2. These observations suggest that copper-free bleomycins would be more useful for treatment of human tumors than copper-containing bleomycins.

Bleomycin, discovered by UMEZAWA *et al.*[1], is a mixture of peptide antibiotics separated into A group and B group by a Sephadex chromatography. They are separated into $A_1 \sim A_6$ and $B_1 \sim B_5$ by gradient chromatography on CM-Sephadex C-25[2]. Recently, other components, A_2' and B_6 were found. The chemistry of bleomycin suggests that they are unique peptides with new amino acids[3].

As reported by NAGAI *et al.*[4], SUZUKI *et al.*[5] and KUNIMOTO *et al.*[6], bleomycin A_2 lowers the melting temperature of DNA which has reacted with a sulfhydryl compound such as mercaptoethanol or glutathione, inhibits incorporation of ^{3}H-thymidine into DNA of intact cells of EHRLICH carcinoma, HeLa cells, *E. coli*, and A_2 or a bleomycin mixture, at a low concentration which shows slight inhibition on incorporation of ^{3}H-thymidine into DNA, inhibits cell division, causing enlargement of the carcinoma cells or elongation of the bacterial cells. As reported by ISHIZUKA *et al.*[7], a relatively high concentration of bleomycin is observed in the lung of mice, and twice-a-week subcutaneous injection in dogs causes hardening of palms and hair loss. As reported by ICHIKAWA *et al.*[8], a high concentration is observed in the skin of mice after injection. Recently, the therapeutic effect on squamous cell carcinomas of human, especially on the spinal cell type, was confirmed by the clinical studies of ICHIKAWA and the Clinical Committee on Bleomycin. The clinical studies not only confirmed the effect of bleomycin mixture but also found that a large dose caused hardening of skin and hair loss, and an extremely large dose caused pneumonia leading to fibrosis. Bleomycin has caused neither leukopenia nor an abnormal blood picture. As reported by ISHIZUKA *et al.*[7], bleomycin does not cause hemorrhage and ulcers in intestines and colon, the direct cause of death from mitomycin, actinomycin

and pluramycin. The therapeutic effect on squamous cell carcinoma of human and the side effects which appear in skin and lung are considered to be related to the high concentration in these tissues of bleomycin.

In this connection, the degree of inhibition of EHRLICH carcinoma, tissue distribution, and toxicity of each bleomycin must be determined to estimate the effect of each bleomycin on human squamous cell tumors and their side effects. The effects of bleomycin A_1, A_3, A_4, A_5, B_2 and B_4 on EHRLICH carcinoma were briefly reported by ISHIZUKA *et al.*[7] In the present paper, more details of the effect on EHRLICH carcinoma of A_1, A_2, A_2', B_2 and B_4 and their distribution in organs of mice are reported. As described by UMEZAWA *et al.*[1], bleomycins chelate copper, and are obtained as copper-containing forms when extracted from culture filtrates of *Streptomyces verticillus*. As reported by SUZUKI *et al.*[5] and NAGAI *et al.*[4], the effect of bleomycin A_2 lowering Tm of DNA is inhibited by Cu^{++}. The copper-free forms of the bleomycins have almost the same activities as the copper-containing forms in inhibition of bacteria and EHRLICH carcinoma. Therefore, in this paper the effect on EHRLICH carcinoma and the distribution in mouse organs are compared with copper-free and copper-containing antibiotics.

Materials and Methods

The copper-containing or copper-free bleomycins were prepared by Nihon Kayaku Co., using processes similar to those described by UMEZAWA *et al.*[1,2] Bleomycin A_1, A_2, A_2', A_5, B_2, B_4 and B_6 were obtained in sufficient amount for the present experiments. For comparison, a bleomycin mixture consisting of 55 % A_2, 5 % A_1, 5 % A_2 and 20 % B_2 which has been used for clinical study was also used. The effect on ascites type and a subcutaneous solid tumor of EHRLICH carcinoma was tested by a procedure similar to that of ISHIZUKA *et al.*[7] The distribution of bleomycin after the subcutaneous injection to mice was examined by the procedure described by ISHIZUKA *et al.*[7]

Results

Copper-Containing Bleomycins on Ascites Type of EHRLICH Carcinoma

After intraperitoneal inoculation of 2 million cells of EHRLICH ascites carcinoma, various doses of each bleomycin were injected for 10 days and the survival time of each mouse were observed until 50 days after the inoculation of the tumor cells.

The survival time in days of each mouse treated with various doses of copper-containing bleomycin A_1, A_2, A_2', B_1, B_2, B_4 and B_6 were shown in Tables 1 and 2.

Data on toxicity, and minimal effective doses of the various bleomycin are given in Table 3. These data suggest that among copper-containing bleomycins tested, A_2 and A_5 may be the most effective and least toxic. The activities against *Mycobacterium* 607 of each copper-containing bleomycin was tested by the cylinder plate method comparing with a sample of bleomycin mixture which had been arbitrarily designated as standard and the results were as follows: A_1 870 mcg/kg, A_2 569 mcg/mg, A_2' 1,092 mcg/mg, A_5 1,738 mcg/mg, B_1 440 mcg/mg, B_2 1,830 mcg/mg, B_4 5,898 mcg/mg, B_6 2,602 mcg/mg. The activities against *Mycobacterium* 607 are not parallel with the inhibition of the ascites form of EHRLICH carcinoma and the mouse toxicities.

Table 1. Effect of copper-containing bleomycins (A group) on ascites type of EHRLICH carcinoma in mice.

Dose mg/kg/day	Survival period in days*			
	Copper-containing A_1	Copper-containing A_2	Copper-containing A_2'	Copper-containing A_5
25.0	11 11 13 13 (12.0)	5 5 9 14 (8.2)	10 11 13 14 (12.0)	
12.5	>50>50>50>50 (>50.0)	11 11 11 22 (13.7)	13 17>50>50 (>32.5)	
10.0				16 43>50>50 (>39.7)
6.25	48>50>50>50 (>49.5)	14 32 39 44 (32.2)	21>50>50>50 (>42.7)	
5.0				>50>50>50>50 (>50.0)
3.12	48>50>50>50 (>49.5)	31>50>50>50 (>45.2)	21>50>50>50 (>42.7)	
2.5				35>50>50>50 (>46.2)
1.56	21 22>50>50 (>35.2)	29 33>50>50 (>40.2)	20 42>50>50 (>40.5)	
1.25				26>50>50>50 (>44.0)
0.78	15 18 25 38 (24.0)	22 36 40>50 (>37.0)	24 24>50>50 (>37.0)	
0.62				26 28>50>50 (>38.5)
0.39	23 45>50>50 (>42.0)	23 26 42>50 (>35.2)	17 24 30 43 (28.5)	
0.31				23 25 37>50 (>33.7)
0.19	12 14 14 28 (17.0)	19 24 32>50 (>31.2)		
0.15				24 48>50>50 (>43.0)
0.09	14 16 16 22 (17.0)	21 22 25 32 (25.0)		
0.08				19 21 21 21 (20.5)
0.04				17 17 17 22 (18.2)
Control	11 14 16 18 18 18 21 21 (17.1)	10 11 14 14 15 16 18 21 (14.8)	11 14 16 16 16 18 21 21 (16.6)	10 11 14 14 15 16 18 21 (14.8)

* Average survival days in parentheses.

Effect of Copper-Free A_2, B_2 and B_4 on the Ascites Form of EHRLICH Carcinoma

The bleomycin mixture which are used for clinical study is copper-free and consists of 50~65 % of A_2, 15~30 % of B_2 and 20~35 % of mixture of A_1 and A_2'. B_4 is more toxic than A_2 or B_2 and, therefore its content is controlled to be less than 3 % in the bleomycin mixture. These three copper-free bleomycins were assayed against the ascites form of EHRLICH carcinoma. The results are given in Table 4.

At the same time with the copper-containing forms were tested. The results are summarized in Table 5.

Table 2. Effect of copper-containing bleomycins (B group) on ascites type of EHRLICH carcinoma in mice.

Dose mg/kg/day	Survival period in days*			
	Copper-containing B_1	Copper-containing B_2	Copper-containing B_4	Copper-containing B_6
25.0	30 39>50>50 (>42.2)			
12.5	35 38>50>50 (>43.2)	12 14 14 42 (20.5)		11 12 12 12 (11.7)
6.25	17 31>50>50 (>37.0)	7 25 35 36 (25.7)	15 17 24 24 (20.0)	18>50>50>50 (>42.0)
3.12	14 19 22 24 (19.7)	12 25>50>50 (34.2)	25 31 39 44 (34.7)	11>50>50>50 (>40.2)
1.56	18 21>50>50 (>34.7)	12 29>50>50 (35.2)	29>50>50>50 (>44.7)	39 39>50>50 (>44.5)
0.78	16 17 18 23 (18.5)	19>50>50>50 (>42.2)	29 32>50>50 (>40.2)	43>50>50>50 (>48.2)
0.39	17 17 21 22 (19.2)	18 21 21 23 (20.7)	26>50>50>50 (>44.0)	18 18 24>50 (>27.5)
0.19			25 25 31>50 (>32.7)	13 13 16 18 (15.0)
0.09			21 23 23 32 (24.7)	
Control	10 14 15 15 16 16 19 20 (15.6)	10 14 15 15 16 16 19 20 (15.6)	11 14 14 18 19 19 19 21 (17.2)	11 14 16 16 16 18 21 21 (16.6)

* Average survival days in parentheses.

Table 3. Effect of copper-containing bleomycins on ascites from of EHRLICH carcinoma and their lethal or toxic doses to mice.

Bleomycins	Minimal effective dose* mg/kg/day for 10 days		Maximal dose showing no toxicity mg/kg/day	Lethal dose for 10 days
	++	+		
A_1	0.39	0.39	12.5	25.0
A_2	0.19	0.09	3.12	12.5
A_2'	0.78	0.39	6.25	25.0
A_5	0.15	0.15	5.0	>10.0
B_1	1.56	1.56	12.5 or 25.0	>25.0
B_2	0.78	0.78	3.12	>12.5
B_4	0.39	0.39	1.56	≧ 6.25
B_6	0.78	0.39	6.25	12.5

* ++ means the ratio of the mean survival period of mice treated to that without treatment is higher than 2; + means, this ratio is higher than 1.5.

As shown by the experiment described above, copper-free A_2 and B_2 have lower toxicity than the copper-containing forms. The same result was observed in a bleomycin mixture in which A_2 was the main component (50~65 %) and B_2 was the second component (15~30 %). The results of testing the survival periods of mice treated with copper-free and copper-containing bleomycin mixture were shown in Table 6.

The bleomycin mixture in the copper-free form has more than 4 times less toxicity than that in the copper-containing form. The activity against the ascites form of EHRLICH carcinoma was not different.

Table 4. Effect of copper-free A_2, B_2 and B_4 on the ascites form of EHRLICH carcinoma in mice.

Dose (mg/kg/day)	Survival period (days)		
	Copper-free A_2	Copper-free B_2	Copper-free B_4
25.0	21 23 48>50 (>35.5)	22 37>50>50 (>39.7)	
12.5	21 42 46>50 (>39.7)	23 40 47>50 (>40.0)	
6.25	45 46>50>50 (>47.7)	40>50>50>50 (>47.5)	11 18 25 30 (21.0)
3.12	35>50>50>50 (>46.2)	36>50>50>50 (>46.5)	31 42 42>50 (>41.2)
1.56	40 43>50>50 (>45.7)	23>24>50>50 (>36.7)	37>50>50>50 (>46.7)
0.78	21 37>50>50 (>39.5)	28>50>50>50 (>44.5)	29>50>50>50 (>44.7)
0.39	33 37 44>50 (>41.0)	19 23 33>50 (>31.2)	25 26 36>50 (>34.2)
0.19	21 26>50>50 (>36.7)		18 23 28 37 (26.5)
Control	10 11 14 14	10 14 15 15	11 14 17 18
	15 16 18 21 (14.8)	16 16 19 20 (15.6)	19 19 19 21 (17.2)

* Average survival days in parentheses.

Effect of Copper-Free and Copper-Containing A_2 on the Subcutaneous Solid Form of EHRLICH Carcinoma

Table 5. Effect of copper-free and copper-containing A_2, B_2 and B_4 on ascites type of EHRLICH carcinoma.

Bleomycins	Minimal effective dose mg/kg/day		Dose causing no toxicity	Lethal dose
	++	+		
Cu-A_2	0.19	0.09	3.12	>12.5
A_2	0.19	<0.19	12.5	>25.0
Cu-B_2	0.78	0.78	3.12	>12.5
B_2	0.78	0.39	12.5	>25.0
Cu-B_4	0.39	0.39	1.56	≧ 6.25
B_4	0.39	0.39	3.12	≧ 6.25

As described by ISHIZUKA *et al.*[7], 2 million cells of EHRLICH carcinoma taken from the ascites form were subcutaneously inoculated and daily intraperitoneal injection of bleomycin was started 24 hours after the inoculation and continued for 10 days. Fifteen days after the inoculation each tumor was cut out and weighed. The results are shown in Tables 7 and 8. Bleomycin A_2 showed similar effect with the copper-containing and copper-free forms.

Effect of Copper-free and Copper-containing Bleomycin A_2 on Ascites Form of the YOSHIDA Rat Sarcoma

Rats (Donryu, 80~100 g) were intraperitoneally inoculated with 1×10^5 cells of

Table 6. Effect of copper-free and copper-containing bleomycin mixture on ascites form of EHRLICH carcinoma in mice.

Dose (mg/kg/day)	Survival period (days)*	
	Copper-free bleomycin mixture	Copper-containing bleomycin mixture
25.0	22 28 >50 >50 (>37.5)	5 5 7 7 (6)
12.5	11 45 >50 >50 (>39.0)	11 12 14 14 (12.7)
6.25	42 >50 >50 >50 (>48.0)	14 28 29 31 (25.5)
3.13	>50 >50 >50 >50 (>50.0)	37 46 >50 >50 (>45.7)
1.56	33 37 41 >50 (>40.2)	23 33 >50 >50 (>39.0)
0.78	22 24 26 >50 (>30.5)	22 30 >50 >50 (>38.0)
0.39	17 25 35 >50 (>31.7)	23 25 26 47 (30.2)
Control	14 15 16 17	14 15 16 17
	18 19 20 21 (17.5)	18 19 20 21 (17.5)

* Average survival days in parentheses.

Table 7. Effect of copper-free bleomycin A_2 on the subcutaneous solid form of EHRLICH carcinoma.

No.	Daily dose in mg/kg and weight of each tumor (mg)							
	12.5	6.25	3.12	1.56	0.78	0.39	0.19	0
1	25	80	155	170	250	490	900	1,000
2	30	105	165	200	280	550	1,000	1,100
3	33	121	185	213	290	600	1,150	1.200
4	42	133	200	215	300	700	1,190	1,430
5	73	143	201	240	300	750	1,230	1,680
6	76	147	215	255	305	790	1,250	1,730
7	115	152	218	265	395	800	1,370	2,800
8	120	167	228	305	530	800	1,510	2,820
9	—	—	—	—	—	—	—	3,100
10	—	—	—	—	—	—	—	4,000
Mean	64.2	131.0	195.8	232.8	331.2	685.0	1,200.0	2,080.0
Inhibition %	97.0	93.8	90.6	88.9	84.1	67.1	42.2	0

Table 8. Effect of copper-containing bleomycin A_2 on the subcutaneous solid form of EHRLICH carcinoma.

No.	Daily dose in mg/kg and weight of each tumor (mg)						
	6.25	3.12	1.56	0.78	0.39	0.19	0
1	tox.	90	180	280	400	700	1,000
2	tox.	110	200	300	500	800	1,100
3	15	125	215	320	500	800	1,200
4	25	156	225	355	480	880	1,430
5	30	190	228	410	600	1,200	1,680
6	50	210	232	480	700	1,500	1,730
7	68	228	290	500	850	1,500	2,800
8	106	230	362	520	905	1,700	2,820
9	—	—	—	—	—	—	3,100
10	—	—	—	—	—	—	4,000
Mean	49.0	167.3	241.5	395.6	494.2	1,141.2	2,080.0
Inhibition %	97.7	91.6	88.4	81.0	76.3	45.2	0

YOSHIDA rat sarcoma cell and 24 hours thereafter the intraperitoneal injection of bleomycin was started daily for 7 days and the survival period of rats was observed. Three rats were used for each dose. The results are given in Table 9.

Bleomycin A_2 in copper-free and copper-containing forms showed only a slight effect on YOSHIDA rat sarcoma.

Absorption and Excretion of Copper-free Bleomycin A_2 in Rabbits after the Subcutaneous Injection

Copper-free bleomycin A_2 was subcutaneously injected in a rabbit (2.5 kg) at a dose of 50 mg/kg (125 mg/rabbit). The urine was taken continuously from the bladder by a canula and 2 ml of blood was taken at various times after the injection from the vein. The results are shown in Table 10. A high blood level of 60 mcg/ml was observed at 30 minutes after the injection and decreased gradually thereafter. Concentrations of 1,800~4,400 mcg/ml were observed in urine for 6 hours after the injection. In urine taken for 8 hours after the injection 69.0 % of the injected bleomycin A_2 was excreted. The concentration in urine seemed to decrease

rapidly after 6 hours. The cxretion of the copper-containing forms was reported by ISHIZUKA *et al.*[7]. Copper-free A_2 seems to be more completely excreted in 8 hours than the copper-containing form. The urine taken after the injection of the copper-free bleomycin was not bluish colored suggesting that the copper-free bleomycin was excreted in the copper-free form.

In order to determine whether bleomycin A_2 in the urine did not contain cupric ion, the urine was evaporated *in vacuo* and bleomycin in the urine was extracted with methanol. The extract was identified as bleomycin A_2 by thin layer chromatography using Silica gel G. (solvent system: 10% ammonium acetate - 10% ammonia : methanol; 9 : 1 : 10). Cupric ion was not detected in the excreted bleomycin A_2 with 0.5% rubeanic acid. Therefore, injected bleomycin A_2 was excreted without convertion to the copper-containing form.

Table 9. Effect of copper-free and copper-containing A_2 on ascites form of the YOSHIDA rat sarcoma.

Dose mg/kg/day	Survival period (days)*							
	Copper-free				Copper-containing			
10.0	13	13	13	(13.0)	14	14	15	(14.3)
2.5	12	12	13	(12.3)	9	10	11	(10.0)
0.62	10	11	12	(11.0)	10	10	11	(10.3)
0	9	9	9		9	9	9	
	10	11	11	(9.8)	10	11	11	(9.8)

* Average survival days in parentheses.

Table 10. Absorption and excretion of copper-free bleomycin A_2 in rabbit.

(Rabbit 2.5 kg, Bleomycin A_2: 50 mg/kg (125 mg/body))

Time	Urine			Blood mcg/ml
	Vol. (ml)	mcg/ml	mg in total	
30 min.	1.3	12.5	0.02	60.0
1 hr.	3.7	1,800	6.66	44.0
2	4.1	2,500	10.25	44.0
washed	12.4	510	6.32	
3	5.6	4,400	24.64	36.0
washed	11.3	300	3.39	
4	17.5	440	7.70	36.0
6	10.0	2,100	21.00	18.0
8	15.0	420	6.30	12.0
Total			86.26 Recovery: 69.0%	

Distribution of Copper-containing Bleomycin A_1, A_2, A_2', A_5, A_6, B_1, B_2, B_4, B_6 and Copper-free A_2 and B_2 among Organs of Mice

Bleomycins have been shown to be effective on squamous cell carcinoma of human and the toxicity appeared in the skin (hardening and hair loss) and the lung (pneumonia leading to fibrosis). The effect on human squamous cell carcinoma and the side effects which were observed in cases treated with high doses are considered to be related to the tissue or organ distribution of bleomycins. Therefore, the distribution of each bleomycin on various tissues of mice was examined to keep in choosing the best bleomycin for treatment of squamous cell carcinoma of humans. For this purpose, copper-containing bleomycin A_1, A_2, A_5, A_6, B_1, B_2, B_4, B_6 were injected at the dose of 50 mg/kg to mice. One and 3 hours thereafter, four mice were sacrified in each case and skin, peritoneum, liver, spleen, kidney, lung and uterus were taken and ground with glass homogenizer in 1/10 M phosphate buffer of pH 6.8. The bleomycins in the extract were determined by the paper disc plate method using *B. subtilis* as the test organism and the corresponding bleomycin as the standard. The results are shown in Tables 11 and 12.

The highest skin concentration was observed with A_2. The concentration in

Table 11. Distribution of copper-containing bleomycin A_1, A_2, A_2', A_5, A_6 after the subcutaneous injection of 50 mg/kg to mice.

	Organs	A_1		A_2		A_2'		A_5		A_6	
		1 hr	3 hrs	1 hr	3 hrs	1 hr	3 hrs	1 hr	3 hrs	1 hr	3 hrs
mcg/g	Skin	5.2	<5.2	25.0	0.6	<2.1	<1.5	6.5	<0.1	<0.2	<0.4
	Peritoneum	8.5	<4.0	5.5	<1.7	<1.4	<1.5	4.8	<0.1	<0.2	<0.2
	Liver	<3.6	<3.5	<1.7	<1.7	<3.0	<1.5	<0.1	<0.1	<0.1	<0.1
	Spleen	<3.3	<4.2	<1.7	<1.9	<1.4	<1.5	<0.2	<0.2	<0.1	<0.3
	Kidney	<3.3	<4.4	0.9	<1.7	<1.3	<1.3	5.8	<0.1	8.2	2.5
	Lung	<3.0	<4.4	0.7	<1.8	2.0	<1.5	0.6	<0.1	<0.1	<0.1
	Uterus	<2.7	<6.0	2.6	<1.8	1.7	<1.4	1.8	<0.1	1.4	<0.2
mcg/ml	Serum	52.0	15.5	40.0	0.8	36.0	<0.1	80.0	<0.1	400.0	3.2
	Urine	2,500	1,600	1,600	1,000	3,600	200	15,000	4,500	1,250	1,000

The assay method used was sensitive to the following concentrations: A_1 2.0 mcg/ml, A_2 1 mcg/ml, A_2' 0.8 mcg/ml, A_5 0.1 mcg/ml, A_6 0.1 mcg/ml.

Table 12. Distribution of copper-containing bleomycin B_1, B_2, B_4, B_6 after the subcutaneous injection of 50 mg/kg to mice.

	Organs	B_1			B_2		B_4		B_6	
		30 min	1 hr	3 hrs	1 hr	3 hrs	1 hr	3 hrs	1 hr	3 hrs
mcg/g	Skin	<7.1	<4.9	<2.5	<1.4	<1.6	9.1	<0.2	0.5	<0.3
	Peritoneum	<4.1	<4.0	<6.6	5.6	<1.2	4.3	<0.3	<0.3	<0.4
	Liver	<5.4	<5.2	<5.7	<1.3	<1.4	<0.1	<0.2	<0.3	<0.4
	Spleen	<3.5	<4.0	<6.0	<0.9	<1.4	0.3	<0.2	<0.4	<0.3
	Kidney	9.5	<5.4	<3.4	<1.3	<1.4	10.1	2.5	2.0	3.1
	Lung	10.9	<4.1	<4.2	1.0	<1.0	9.0	1.1	0.6	0.5
	Uterus	<4.0	<2.3	<3.4	1.2	<1.5	9.1	0.3	0.5	<0.3
mcg/ml	Serum	70.0	14.5	0.0	46.0	1.25	75.0	8.0	86.0	7.6
	Urine	3,400	5,500	10.0	4,600	1,500	3,500	1,600	1,900	1,150

The assay method used was sensitive to the following concentrations: B_1 3.0 mcg/ml, B_2 0.78 mcg/ml, B_4 0.1 mcg/ml, B_6 0.2 mcg/ml.

Table 13. Distribution of copper-free bleomycin A_2 and B_2 among organs of mice after the subcutaneous injection of 50 mg/kg.

	Organs	A_2			B_2		
		30 min	1 hr	3 hrs	30 min	1 hr	3 hrs
mcg/g	Skin	19.7	5.3	<1.6	1.2	<1.1	<1.2
	Peritoneum	1.1	1.2	<1.0	<1.6	<1.2	<1.2
	Liver	<1.2	<1.8	<1.4	<1.4	<1.5	<1.5
	Spleen	<1.5	<1.4	<1.7	<1.5	<1.5	<1.5
	Kidney	2.3	1.7	<1.4	3.2	0.8	<1.4
	Lung	2.3	0.8	<1.4	1.6	1.0	<1.6
mcg/ml	Serum	25.0	42.0	<0.8	43.0	43.0	0.7
	Urine	1,680	7,200	800	5,000	5,000	3,500

The assay method used was sensitive to the following concentrations: A_2 0.78 mcg/ml, B_2 0.78 mcg/ml.

peritoneum was high with A_1, A_2, A_5, B_2 and B_4. In liver and spleen all bleomycins showed only very low concentrations. The concentration in kidney was high with A_5, A_6, B_1, B_4 and B_6 and highest with B_4. A high dose (20 mg/kg) of bleomycin mixture containing B_4 as the main component injected into dogs resulted in kidney damage. With A_2, reversible liver function reduction was observed in the dogs. The concentration in lung was relatively high with A_2', B_1 and B_4. It was especially high with B_1 and B_4. The concentration in uterus was high with A_2, A_5, A_6, B_2 and B_4 and especially so with B_4.

The ratio of the concentration in skin to that in lung is as follows: A_1 trace/5.2; A_2 0.9/25.0; A_5 0.6/6.5; A_6 tr/tr; B_1 10.0/0; B_2 1.0/tr; B_4 9.0/9.1; B_6 0.6/0.5. If the lung toxicity of bleomycin is due to its high concentration in lung, then the bleomycin having a lower ratio would be useful for treatment of squamous cell carcinoma of human. From this view point, A_1, A_2 and A_5 are favorable and A_6, B_1, B_2, B_4, especially B_1, are unfavorable.

The concentration in urine is the highest in the case of A_5. Bleomycin A_5 is thus considered to be most rapidly excreted in urine. Among the copper-containing bleomycins the minimal effective dose on ascites type of EHRLICH carcinoma was smallest for A_2 and A_5. If the toxicity of copper-containing A_5 and A_2 to mice are compared, A_5 has the lower toxicity. Bleomycin A_2 or a bleomycin mixture containing A_2 as the main component has been studied clinically, but A_5 is considered to be another bleomycin worthy of clinical study.

The distribution of copper-free bleomycin A_2 and B_2 after the subcutaneous injection to mice was examined by a similar procedure and the results are shown in Table 13. If the data in Table 13 are compared with the data in Table 10 or 12 of copper-containing forms, then no substantial differences are seen between copper-free and copper-containing forms, except the higher concentration of copper-free forms in urine. With both A_2 and B_2, and copper-free forms are more rapidly excreted in urine. As already described, copper-free A_2 has lower toxicity than copper-containing A_2. The lower toxicity of copper-free A_2 may be due to the rapid excretion into urine. However, there is no difference in the antitumor effects of copper-free and copper-containing A_2 on the subcutaneous solid form and ascites form of EHRLICH carcinoma. Moreover, a slightly stronger effect was observed in copper-free A_2 against the ascites form of EHRLICH carcinoma. In this connection, it is interesting that the reaction of bleomycin A_2 with DNA in the presence of a mercaptoethanol was found by NAGAI *et al.* to be inhibited by cupric ion.

Effect of Copper-containing Bleomycin A_1, A_2, A_5, B_1, B_2, B_4, B_6 and Copper-free A_2, B_2, B_4 on the Vascular Permeability in Rabbit Skin

One hundred mcg of bleomycin in 0.1 ml of saline was intracutaneously injected to the rabbit skin and immediately, Evans blue (10 ml of 1 % solution) was injected intravenously into a rabbit. Thirty minutes thereafter the diameters of the blue zone of the skin was measured. As a control, 0.005 mcg of bradykinin was injected. The results are shown in Table 14. Copper-containing bleomycin A_2, A_5, B_4, B_6 and

bleomycin mixture containing A_2 as the main component and B_2 as the second component raised the vascular permeability, but copper-containing A_1, B_1, B_2 did not. Copper-containing A_5 is a little weaker than copper-containing A_2. The order of the effect in the copper-containing forms was $B_4>B_6>A_2>A_5>A_1=B_1=B_2$. The order of the effect on ascites type of EHRLICH carcinoma shown in Table 3 is $A_2\fallingdotseq A_5>A_1=B_4=A_2'=B_6=B_2>B_1$. The order of the effect on the vascular permeability is not parallel with the effect on the ascites form of EHRLICH carcinoma. The increase in vascular permeability may be related to the venous damage at the site of injection of bleomycin B_4 and B_6, with weak antitumor activity, low skin concentration, and high concentration in kidney are considered to be unfavorable for treatment of squamous cell carcinoma.

Table 14. The effect of copper-containing and copper-free bleomycins on the vascular permeability in rabbit skin.

	Bleomycins	Dose (mcg)	Zone (mm)
Copper-containing	A_1	100	0
	A_2	//	12.0
	A_5	//	8.0
	B_1	//	0
	B_2	//	0
	B_4	//	17.0
	B_6	//	14.0
	mixture	//	9.0
Copper-free	A_2	//	0
	B_2	//	0
	B_4	//	17.0
	mixture	//	0
Bradykinin		0.005	16.0

The increase in vascular permeability is not observed with copper-free A_2 and copper-free B_2. This observation also suggests that copper-free bleomycins are preferable for treatment of human cancer. However, it is interesting that copper in B_4 shows no influence on the vascular permeability. As already described, the copper-free A_2 and B_2 are less toxic to mice than the copper-containing forms but copper in B_4 dose not influence its toxicity.

Discussion

The order of the effect of copper-containing bleomycins on ascites type of EHRLICH carcinoma is, as shown in Table 3, $A_2\fallingdotseq A_5>A_1$, $B_4>A_2'$, $B_6>B_2$, B_1 and the toxicity to mice is in the order of A_2, B_2, $B_4>A_2'$, A_5, $B_6>A_1$, B_1. In this respect A_2 or A_5 is considered to be best for treatment of tumors. The concentration in lung is high in cases of B_4 and B_6 and the concentration in skin is high with A_1, A_2, A_5 and B_4. Thus, A_1, A_2, A_5 are expected to be useful for treatment of tumors in skin or of squamous cell carcinoma. A_2, A_5, B_4 and B_6 increased the vascular permeability of rabbit skin. From the data above described B_4 and B_6 are considered to be undesirable for treatment of human tumors.

The copper in the copper-containing bleomycins was shown to be not necessary for antitumor activity by testing copper-free and copper-containing bleomycin A_2 and B_2. The copper-containing forms, moreover, have increased toxicity and increased vascular permeability in the case of bleomycin A_2 and B_2. However, copper in bleomycin B_4 has no effect on the toxicity to mice, on the vascular permeability and on the activity against EHRLICH carcinoma. These observations suggest that copper-free bleomycins are more useful for treatment of human tumors than copper-containing forms. It is also interesting that copper-free forms are more rapidly excreted than the copper-containing forms.

At present, copper-free bleomycin A_2 or bleomycin mixture containing A_2 as the main component are studied clinically, but A_5 is considered to be worthy of clinical study. Especially it is interesting to study of copper-free A_5.

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