BIOLOGICAL PROPERTIES OF MACARBOMYCIN, AN ANTIBIOTIC CONTAINING PHOSPHORUS

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Macarbomycin is an antibiotic produced by *Streptomyces phaeochromogenes*, inhibitory mainly against Gram-positive bacteria including drug-resistant strains and with low toxicity. It is readily soluble in water and relatively stable over a wide pH range. Its antibacterial activity is markedly reduced by serum albumin. The antibiotic shows a moderate curative activity to staphylococcal infection in mice. When intravenously injected into mice, macarbomycin is excreted slowly. It is apparently not absorbed as such from the alimentary tract. By long-term oral administrations to baby and juvenile monkeys, macarbomycin promoted growth with no signs of toxicity.

As has already been reported by us[1], macarbomycin is an antibiotic containing phosphorus and belongs to the group containing moenomycin[2], prasinomycin[3] and diumycin[4]. Its acid hydrolysate contains D-glucosamine, D-glucose, UV-chromophore, three lipids and phosphorus. The antibiotic is clearly differentiated from moenomycin and prasinomycin by paper and thin-layer chromatography and by the fact that the acid hydrolysate of macarbomycin does not contain 6-deoxyglucosamine but it is closely related to diumycin. We tested macarbomycin for stability, inactivation by blood serum, distribution in organs after administration to mice, therapeutic effect *in vivo* and toxicity in mice. The biological characteristics of macarbomycin, moenomycin[5], prasinomycin[6] and diumycin[7] are similar. We also examined macarbomycin for growth-promoting effect in monkeys. This paper deals with the biological properties of macarbomycin *in vitro* and *in vivo*.

Materials and Methods

Mice: Male *ddY* mice weighing 18~20 g were used.

Enzymes: Alkaline phosphatase from calf intestinal mucosa, lipase, venom phosphodiesterase and bacterial amylase were purchased from Sigma Chemical Co., Nutritional Biochemicals Corp., Worthington Biochemical Corp. and Ueda Kagakukogyo Co., respectively. Protease from *Bacillus subtilis* var. *biotecus* was Nagarse manufactured by Nagase Sangyo Co. Phospholipase C was kindly supplied by Dr. OHSAKA of Department of Bacteriology II of our Institute.

Serum proteins: Human, bovine and horse serum albumins were purchased from Wako Pure Chemical Industries, Armour Pharmaceutical Co. and Nutritional Biochemicals Corp., respectively. Human γ-globulin and human fibrinogen were obtained from Green Cross Corp.

Macarbomycin: The potency of macarbomycin used was 4,250 units/mg, unless otherwise specified.

Test for stability of macarbomycin: Macarbomycin ammonium salt was dissolved in buffer solutions with pHs ranging from 2 to 10 at a concentration of 100 units/ml and after the pH of the solutions was checked and adjusted if needed, the solutions were heated at 60°C for 1 hour. The

antibiotic was also dissolved in 0.1, 0.2 and 0.5 N HCl or NaOH and heated in the same manner. After 1 hour heating, the solutions were immediately cooled and the pH adjusted to 7 to be submitted to potency assay. For stability test of dry powder of macarbomycin (5,000 units/mg), the antibiotic was heated for 3 hours at 60°, 80°, 90° and 100°C in an electric furnace.

Hydrolysis of macarbomycin by enzymes: The incubation mixture contained macarbomycin and the enzyme at 1,000 mcg/ml and 20 mcg/ml respectively in 0.05 M tris buffer of optimum pH for each enzyme. In the cases of alkaline phosphatase and phospholipase C, $MgCl_2$ was added at 0.005 M and in the case of amylase, $CaCl_2$ was added at 0.005 M. The activities of all the enzymes had been ascertained beforehand by hydrolysing suitable substrates at 2 mcg of enzyme/ml. The above mixtures were shake-incubated in a water bath at 37°C and after 1, 3, 6 and 20-hour incubation, aliquots of the mixtures were diluted 20 times with phosphate buffer (pH 7.0) to be assayed.

Assay method for potency: The disc-plate method or cylinder-plate method is used. Monolayer plates are used. Two-loopful growth of *Stapylococcus aureus* strain 193 cultured on nutrient agar for 18~24 hours is suspended in 10 ml of saline to give a homogeneous suspension and this is mixed with molten nutrient agar (pH 7.0) at a concentration of 1~2%. Ten ml of the mixture are immediately poured into a 9-cm Petri dish and spread to make a monolayer plate.

The potency of a standard batch of macarbomycin was designated as 1,000 units/mg, and 100 units/ml and 25 units/ml are adopted as the high and low concentrations of the standard solutions for the plate assay. The purest sample of macarbomycin at present has a potency of 5,000 units/mg. The diameter of the inhibition zone is influenced by the pH of the plate medium, that is, the lower the pH, the larger the diameter, but the diameter is hardly affected by the pH of the test solutions.

Quantitative assay of macarbomycin in organs: In a preliminary experiment, macarbomycin was found to be adsorbed in or bound to organs. When a mixture of 0.5 ml of macarbomycin solution at 200 units/ml and 0.5 g of organ homogenate, serum or urine of mice was shake-incubated at 37°C for 10 minutes, the potency of the macarbomycin in the mixture was reduced to around 10% except in the case of urine. If 5 ml of methanol was added to the mixture and shake-incubated at 37°C for 1 hour, 25~50% of the activity in the mixture was recovered. If 5 ml of methanol and 0.05 ml of 10% sodium citrate were added and shake-incubated, recovery was 70%.

On the basis of these results, the assay of macarbomycin in organs was carried out as follows: The organ homogenate was mixed with 10 volumes of methanol and 1/10 volume of 10% sodium citrate and after the mixture was shake-incubated at 37°C for 1 hour, the methanol extract was separated. After the methanol was evaporated out, the residue was submitted to the disc-plate assay. The quantity of macarbomycin in the test organ was calculated from the potency in the residue, its volume and the amount of the organ used.

Therapy against staphylococcal infection in mice: As the challenge organism, a virulent strain of *Staphylococcus aureus*, strain Smith was used. The procedure was the same as that described by YAMAZAKI[8].

Oral administration of macarbomycin in monkeys: Six laboratory-bred cynomolgus monkeys (*Macaca fascicularis*) were divided into 3 groups of 2 animals each. The first group was used to determine the effect of orally administered macarbomycin on body weight increase in a growth-retarded baby of 74 days of age. In this group, normal baby of about the same age was used as a control. The second group was used to test the growth-accelerating effect of the antibiotic in a nearly normally growing juvenile monkey aged 802 days. An 811-day-old monkey served as a control. In the third group, a normally growing infant monkey aged 157 days was used to test the growth-promoting effect of macarbomycin. A monkey of the same age was used as a control.

Twenty-five mg of macarbomycin was dissolved in 50 ml of distilled water to which was added 4 ml of honey. Two ml of the solution was orally administered to a test monkey by the use of a steel catheter of about 1 mm diameter each day before feeding, except that the second group was treated with 2 ml of a solution with a macarbomycin concentration seven times higher than the above described once a week during the last 4 months of the experimental period. In effect, the daily administered dose of macarbomycin is estimated at about 1 mg per animal. For the control

animals, 2 ml of a mixed solution of 50 ml of water and 4 ml of honey were given by the same method. The duration of experiment was 435, 445 and 278 days in groups 1, 2 and 3, respectively.

The monkeys were weighed once every week or every other week during the experimental period. Hematological and clinical biochemical determinations, including total leucocyte count (WBC), erythrocyte count (RBC), hematocrit value (Ht), total serum protein content (S-prot), serum glutamic oxalacetic transaminase activity (SGO-T), serum glutamic pyruvic transaminase activity (SGP-T) and blood urea nitrogen content (BUN), were conducted several times on arbitrary days of the experimental period.

In addition, blood, stool and urine were examined for the presence or absence of macarbomycin by the disc method once or twice through the experimental period. In group 1, the liver was biopsied to examine whether macarbomycin could be detected by the disc method 105 days after the onset of macarbomycin administration.

Results

Macarbomycin in aqueous solution is stable at pH 4~10 but unstable at a pH below 3 (Table 1). This is also true for macarbomycin in the cultured broth. The thermostability of dry powder of macarbomycin is shown in Table 2. The antibiotic is stable at 80°C and below. No decomposition occurs with enzymes such as alkaline phosphatase, phospholipase C, lipase, phosphodiesterase, protease and amylase.

Table 1. Stability of macarbomycin in aqueous solution

Activity retained		Activity retained	
pH 2	32.0%	0.1 N HCl	5.5%
pH 3	70.0	0.2 N HCl	2.9
pH 4	93.5	0.5 N HCl	0
pH 5	93.5	0.1 N NaOH	49.6
pH 6	100.0	0.2 N NaOH	16.8
pH 7	100.0	0.5 N NaOH	0
pH 8	100.0		
pH 9	96.0		
pH 10	94.0		

The concentration of macarbomycin: 100 units/ml. The solutions were heated at 60°C for 1 hour.

Table 2. Thermostability of macarbomycin in solid state

Temperature*	Activity retained
60°C	100%
80°C	100
90°C	100
100°C	7.55

* The samples of macarbomycin were heated for 3 hours in an electric furnace.

As described in the previous paper[1], macarbomycin inhibits Gram-positive bacteria, particularly *Staphylococcus aureus* including drug-resistant strains and some species of *Bacillus*, but it does not inhibit molds and yeasts even at 1,000 units/ml.

Inactivation of Macarbomycin by Blood Serum

The antibacterial activity of macarbomycin was markedly reduced by addition of blood serum or serum albumin. The antibacterial activity was examined against *Staphylococcus aureus* FDA 209P by bouillon dilution method in the presence and in the absence of blood serum or plasma proteins. As shown in Table 3, macarbomycin inhibits the growth of the organisms at 0.16 units/ml but in the presence of human or horse serum at 5, 10 and 20%, the activity was reduced to 1/62, 1/125 and 1/250, respectively. In pursuit of the activity-reducing factor, albumin, γ-globulin or fibrinogen was dissolved in saline at a concentration corresponding to that of each protein in plasma and the solution was used at 10% in place of the serum. The results are indicated in the same Table. γ-Globulin and fibrinogen had no influence but albumin evidently affected the activity of macarbomycin.

Distribution of Macarbomycin after a Single Administration to Mice

(1) Intraperitoneal administration

Macarbomycin was intraperitoneally injected into mice in a dose of 8,500 units/0.25 ml/mouse. At times ranging from 30 minutes to 24 hours following administration, 4 arbitrarily chosen mice were sacrificed to obtain urine, blood and organs. After being weighed, the organs were homogenized for the assay. The results are shown in Table 4. Macarbomycin, when intraperitoneally injected, was retained in the blood at high concentrations for a fairly long period of time and slowly excreted into the urine. The organs also showed long-lasting levels of the macarbomycin, particularly the liver and the lung maintained relatively high concentrations.

Table 3. Effect of serum and plasma proteins to activity* of macarbomycin

	Amount added (%)	MIC (units/ml)
Human serum	0	0.16
〃	5	10
〃	10	20
〃	20	40
Human serum albumin (3.4%)**	5	5
〃 〃	10	10
〃 〃	20	10
Horse serum albumin V(3.2%)**	10	5
Bovine serum 〃 (3.4%)**	10	10
Human γ-globulin (1.1%)**	10	0.08
Human fibrinogen (0.47%)**	10	0.16

* *S. aureus* FDA 209P (inoculation size 1 : 5,000)
** The concentration corresponds to that of each protein in plasma.

Table 4. Organ distribution of macarbomycin after injection in mice

	Macarbomycin content*							
	30 min.	1 hr.	2 hr.	3 hr.	5 hr.	7 hr.	9 hr.	24 hr.
Blood**	197.0	280.0	266.0	278.0	446.0	376.0	444.0	292.0
Urine**	5.6	27.2	60.0	82.0	84.2	153.0	135.1	32.8
Brain	1.7	0	3.7	8.2	2.9	5.6	6.5	3.3
Heart	1.8	5.2	9.6	11.5	7.1	8.6	6.8	9.1
Lung	12.2	11.5	16.2	28.6	30.0	27.7	24.4	16.9
Liver	10.3	12.8	14.6	32.3	28.8	49.2	29.3	66.0
Kidney	4.0	12.1	10.5	15.9	17.4	15.2	15.9	38.1
Spleen	2.9	6.2	4.7	4.6	10.8	10.4	12.7	8.2
Skeletal muscle***	0	10.7	11.1	18.2	18.7	17.6	22.3	15.3
Testis	0	4.8	5.3	7.5	6.1	9.0	8.2	17.8

Each mouse received 8,500 units of macarbomycin by the intraperitoneal route.
* The figures indicate the macarbomycin content (units) in the whole organ of one mouse, average of 4 mice.
** units/ml.
*** units/g.

(2) Oral administration

Macarbomycin was given orally to mice in a dose of 126,000 units/0.5 ml/mouse with the aid of a silver catheter. After 1, 3, 6, 9 and 24 hours, macarbomycin in urine, blood, brain, heart, lung, liver, kindney, spleen and skeletal muscle was assayed as described above. Four mice were used each time. No macarbomycin was detected in urine, blood or any of the organs tested.

Therapeutic Effect of Macarbomycin against Staphylococcal Infection

Mice intraperitoneally infected with a virulent strain of *Staphylococcus aureus* strain Smith were treated by a single administration of macarbomycin 2 hours after the infection. Intraperitoneal

Table 5. Body weight changes.

Group	Name of monkeys used	Age at the onset of the exp. (days)	Age at the end of the exp. (days)	Duration of the exp. (days)	Body weight at			From birth to the	
					Birth (g)	the onset of exp. (g)	the end of exp. (g)	Body wt. increase (g)	Body wt. increase (%)
				①	②	③	④	⑤	⑥
1 −E.	Y−44	74	509	435	230	350	1,560	120	52.2
1 −C.	Y−42	78	513		320	600	1,850	280	87.5
2 −E.	WY−2	802	1247	445	265	1,270	2,080	1,005	379.2
2 −C.	WY−1	811	1256		265	1,490	2,230	1,225	462.3
3 −E.	Y−56	157	435	278	360	810	1,600	450	125.0
3 −C.	Y−55	156	434		290	750	1,440	460	158.6

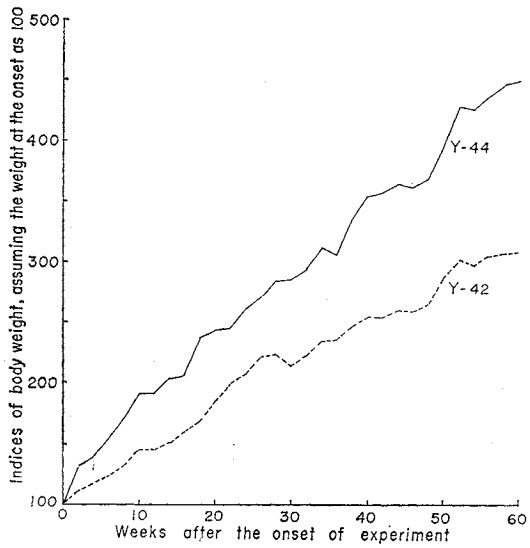
[Notes] ⑤=③−②, ⑥=⑤÷②×100, ⑦=⑤÷①
⑧=④−③, ⑨=⑧÷③×100, ⑩=⑧÷①
E: monkeys treated with macarbomycin
C: control monkeys

administration of 5,000 and 1,250 units/kg gave 100 and 50% protection, respectively. Subcutaneous injection of 50,000 and 25,000 units/kg gave 50% and 25% protection. Oral administration of 200,000 units/kg gave 25% protection. All the untreated control mice died 10~17 hours after challenge.

Toxicity of Macarbomycin

The LD_{50} of macarbomycin by intravenous injection was 750 mg/kg in mice. No delayed toxicity was observed. No toxic signs were noted after oral administration of 5,000 mg/kg to mice.

Fig. 1. Body weight curve of the monkeys of Group 1.



Growth-Promoting Effect of Macarbomycin by Oral Administration to Monkeys

(1) Body weight (Table 5)

Group 1: The macarbomycin-treated monkey (Y-44) was born having very small size and retarded markedly in his postnatal growth, compared with the control monkey (Y-42), that is, Y-44 gained only 1.62 g of body weight per day during the first 74 days after birth, while the daily body weight gain of Y-42 was 3.59 g for about the same period. After administration of macarbomycin, the growth rate of Y-44 was considerably accelerated as shown in Fig. 1. The rate of body weight increment during the experimental period was remarkably higher in Y-44 (345.7%) than in Y-42 (208.3%). However, the absolute weight

onset of exp.	During the exp. period		
Body wt. increase per day (g)	Body wt. increase (g)	Body wt. increase (%)	Body wt. increase per day (g)
⑦	⑧	⑨	⑩
1.62	1,210	345.7	2.78
3.59	1,250	208.3	2.87
1.25	810	63.8	1.82
1.51	740	49.7	1.66
2.87	790	97.5	2.84
2.95	690	92.0	2.48

increment was approximately equal in both animals.

Group 2: As shown in Table 5, macarbomycin-treated monkey, WY-2, was somewhat smaller than the control monkey, WY-1, at the onset of the experiment. WY-2 exceeded WY-1, however, in both the rate of body weight increment and the actual increment of weight throughout the experimental period, that is, the weight of WY-2 increased by 1.82 g per day and the body weight increase was 63.8 %, while WY-1 gained 1.66 g a day during the same period and his body weight increase was 49.7 %.

Group 3: Two monkeys of this group had grown very well for about 156 days after birth. The macarbomycin-treated monkey, Y-56, was a little heavier than the control, Y-55, at the onset

Table 6. Hematological and clinico-biochemical data

Group	Monkeys	No. of days after the onset of experiment	WBC ($\times 10^2$/mm^3)	RBC ($\times 10^4$/mm^3)	Ht (%)	S-prot. (g/dl)	SGO-T (K.U.)	SGP-T (K.U.)	BUN (mg/dl)
1	Y-44	100	145	681	46	7.4	N.D.	N.D.	N.D.
		148	150	602	45	7.8	N.D.	N.D.	N.D.
		233	156	530	41	6.2	45	23	15
		435	101	563	45	6.7	40	17	17
	Y-42	100	102	590	46	8.0	N.D.	N.D.	N.D.
		148	112	663	45	6.2	N.D.	N.D.	N.D.
		233	76	555	44	6.4	36	14	15
		435	106	591	46	7.4	45	16	5
2	WY-2	0	80	728	44	7.8	N.D.	N.D.	14
		78	103	568	41	7.0	42	14	20
		281	82	538	41	6.6	30	11	17
		419	103	627	43	6.4	30	15	15
	WY-1	0	169	636	45	8.0	N.D.	N.D.	13
		78	73	587	42	7.0	36	12	20
		281	120	560	44	7.0	36	12	15
		419	195	609	42	6.4	45	17	8
3	Y-56	0	114	495	43	6.4	38	12	10
		42	113	N.D.	41	6.0	42	7	15
		118	119	503	42	6.0	45	20	17
	Y-55	0	90	608	43	6.6	40	14	10
		42	176	N.D.	40	5.6	58	20	17
		118	118	549	43	6.6	46	13	16

of antibiotic administration. Both the body weight increase and the daily weight increment were larger in Y-56 than in Y-55 during tne experimental period of 278 days.

(2) Hematological and clinical biochemical results (Table 6)

Hematological and clinico-biochemical data are tabulated in Table 6. No pronounced difference was detected between the macarbomycin-treated monkeys and the controls.

(3) Detection of macarbomycin in blood, stool, urine and liver

No macarbomycin could be detected in blood and urine of the antibiotic-treated monkeys. However, stools of macarbomycin-administered monkeys sometimes contained the antibiotic.

No macarbomycin activity was demonstrated in the excised liver sample of Y-44 when examined 100 days after the onset of the antibiotic administration.

Discussion

Macarbomycin is phosphorus-containing antibiotic inhibiting mainly Gram-positive bacteria including drug-resistant strains. In the present study, we found that macarbomycin bound to serum albumin and to some substances, probably proteins, in the organs to give decreased activity. It is not thought, however, that the decrease is due to irreversible inactivation, because the activity is recovered, by extraction with methanol and sodium citrate. This binding can explain the long-lasting retention of macarbomycin in blood and organs and the retarded excretion.

The machanism of action of macabomycin was clarified by SUZUKI *et al.*[9] to be a specific inhibition of biosynthesis of the peptidoglycan in the bacterial cell wall. As can be expected from the mechanism, the toxicity of macarbomycin is low with an intravenous LD_{50} of 750 mg/kg in mice. Macarbomycin is not absorbed from the digestive canal and, accordingly, it demonstrates no toxicity when given orally even at 5,000 mg/kg. Moreover, macarbomycin shows an excellent growth-promoting effect on broilers and swine when orally administered with feed[1]. Thus, macarbomycin is considered to be useful as a feed aditive for domestic cattle rather than as a chemotherapeutic agent. The fact that *E. coli* strains carrying episomes are more sensitive to macarbomycin than the original bacteria having no episome[10] might make the application of macarbomycin as a feed additive useful for suppression of drug-resistant bacteria carrying R-factor.

In connexion with the use of the antibiotic as a feed additive, we examined macarbomycin for the growth-promoting effect on monkeys. Although the number of monkeys used in the present experiment is very small, the results obtained indicate that macarbomycin has a growth-promoting effect on both baby and juvenile cynomolgus monkeys. Furthermore, long-term oral administration of macarbomycin has not any detectable toxic effect on growing monkeys as shown by hematological and clinico-biochemical determinations.

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