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Toxicological Approaches to Streptothricin Antibiotics. I. Implications of Delayed Toxicity in Mice[1]

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In order to investigate the mode of the delayed toxicity of streptothricin antibiotics, racemomycin-A and -D were administered intravenously to mice in a surviving dose. Antimicrobial activity levels in serum declined very rapidly. Localized distribution of antibiotics was conspicuous in the kidneys, and binding rate of the antibiotics with serum proteins *in vitro* was related to β-lysine units in the molecule. Mice administered with antibiotics showed gradual decrease in their body weight, and they further suffered prominent damages in their liver, spleen, and kidneys. These lesions seemed to kill the mice by the delayed toxicity. The mice that died by the lethal doses of the antibiotics were considered to be due to the respiratory paralysis.

Keywords——Streptothricin antibiotics; delayed toxicity; acute toxicity; distribution of the antibiotics; nephrotoxicity; electrostatic affinity of the antibiotics; retention of the antibiotics

Streptothricin antibiotics, produced by several *Streptomyces* species,[3] show a strong growth inhibition against gram-positive and gram-negative bacteria, mycobacteria, fungi, and virus.[4,5] However, these antibiotics cannot be used clinically because of their typical delayed toxicity,[4,6] that is, toxicity occurred afterward without accumulation, in mice when administered by a single injection. Therefore, the mode of the delayed toxicity should be elucidated.

Streptothricin antibiotics are differentiated from each other by the number of β-lysine units (Fig. 1), and the number of β-lysines in their molecule is related directly to their acute toxicity.[4] The delayed toxicity in mice was demon-

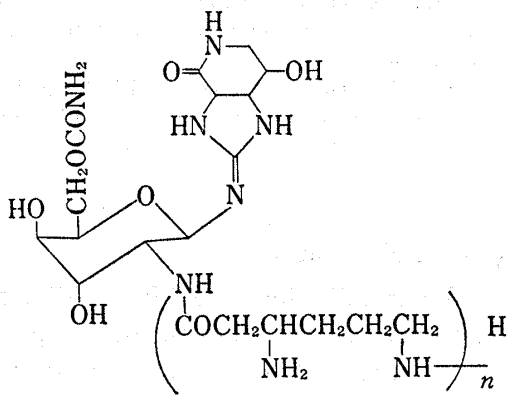

Fig. 1. Structure of Racemomycin

Racemomycin-A: $n=1$
Racemomycin-C: $n=2$
Racemomycin-B: $n=3$
Racemomycin-D: $n=4$

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strated in all the antibiotics of this class. In order to clarify the mode of delayed toxicity, two antibiotics, racemomycin-A and -D were used in the present work.

Materials and Methods

Animal——Male ddY mice weighing 24—25 g were used for the experiments, except for the experiment (26—27 g) described in Table II.

Antibiotics——Racemomycin-A hydrochloride[4] was isolated according to the method previously reported[7] and racemomycin-D hydrochloride[8] was obtained from the culture broth of *Streptomyces lavendulae* OP-2.[9]

Administration and Dose——The antibiotic was dissolved in distilled H_2O and administered intravenously from the tail vein of a mouse with a single dose of various amounts of antibiotics (dose of racemomycin-A were 200 mg/kg for distribution of various organs, 100 mg/kg for blood level and urinary excretion. That of racemomycin-D were 40 mg/kg for distribution of various organs, 20 mg/kg for blood level and urinary excretion).

Antimicrobial Test——Antimicrobial activity was determined by the standard disk assay method employing a plate (Nutrient agar, Eiken Chemical Co., Tokyo) seeded with *Bacillus subtilis* PCI-219 (10^6 spores/ml). The plate was stood for 2 hr at 5° and incubated at 37° for 18 hr. In the determination of blood levels of antimicrobial activity, a diffusion time of 5 hr was adopted. Calibration curves were obtained for racemomycin-A at concentrations of 5 to 500 μg/ml and for racemomycin-D 1 to 500 μg/ml.

Blood Levels of the Antibiotic——At the prescribed time after administration, mice were anesthetized with ether, and blood samples were drawn from the heart.

Serum Preparation——Serum sample was obtained from blood drawn from vena cave.

Binding Rate of the Antibiotic to Serum Protein——The equilibrium dialysis method of Kaneyo, *et al.*[10] was employed. The cellulose tube (Arthur H. Thomas Co. USA) containing the antibiotic aq. solution (250 μg/ml, 12 ml) and mouse serum (3 ml) was dialyzed against M/15 sodium phosphate buffer (pH 7.4, 40 ml) at 37° for 4 hr. Antimicrobial activity of the outer solution was determined with a reference to the control experiment.

Urinary Excretion of the Antibiotic——At 24 hr after administration of the antibiotic, the total volume of urine from mice which were housed in restraint cages was measured and the antimicrobial activity of urine was determined.

Organ Sample——Organs (Table I and II) washed with saline and weighed on a microbalance from each 8 mice were excised after they were killed by bleeding from the carotid artery.

Results

I General Toxicity

General Findings and LD_{50} of Mice after Intravenous Administration of Racemomycins——When racemomycins at the surviving doses depicted in Fig. 2 and Table II were each administered intravenously into mice from the tail vein, restlessness, labored respiration, and projection of eyeballs were observed as signs of acute poisoning. The signs disappeared about 1 min after administration. Thereafter mice showed decrease of autonomic movements, crouching with closed eyes.

LD_{50} value of racemomycin-A was 300 mg/kg,[11] that of racemomycin-D was 9.44 mg/kg.[8]

Changes in Body and Organ Weight——First, the body weight of mice administered each antibiotic by a single injection was traced during 48 hr. As shown in Table I, decrease in body weight was clearly observed. Several organs were excised. Weight of the liver and spleen decreased undoubtedly with time.

In addition, some manifestation of symptoms was observed at 48 hr after administration. The kidney turned white in both experiments. The lung and adrenal gland showed congestion, and the intestine was bleeding.

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TABLE I. Change in Body Weight of Mice after Dosing with Racemomycins (RM) and Variation of Weight of Several Organs

Body and organ	Experiment	Weight: Time after administration (hr) 1	3	5	24	48
Whole body (g)	Control	27.35±1.85	27.88±1.53	27.56±2.01	28.75±1.79	30.05±1.36[a]
	RM-A	26.95±2.06	26.96±1.70	29.71±0.99[b]	25.55±1.62[c]	24.25±1.44
	RM-D	27.20±1.63	27.06±0.55	28.39±1.64	25.13±2.17	24.88±1.25
Brain	Control	1.56±0.10	1.63±0.15	1.51±0.06	1.60±0.17	1.52±0.07
	RM-A	1.59±0.25	1.64±0.12	1.52±0.05	1.54±0.10	1.48±0.06
	RM-D	1.55±0.11	1.62±0.13[d]	1.54±0.13	1.57±0.18	1.50±0.17
Lung	Control	0.85±0.10	0.87±0.11	0.75±0.10	0.84±0.06	0.79±0.07
	RM-A	0.83±0.04	0.79±0.04	0.81±0.05	0.80±0.05	0.86±0.07
	RM-D	0.78±0.11	0.84±0.08	0.83±0.09	0.78±0.08[b]	0.82±0.07
Heart	Control	0.49±0.06	0.47±0.03	0.52±0.08	0.48±0.06	0.47±0.03
	RM-A	0.50±0.05	0.48±0.08	0.50±0.03	0.52±0.04	0.46±0.02
	RM-D	0.47±0.07	0.50±0.08	0.49±0.04	0.53±0.05	0.54±0.07[b]
Liver	Control	5.20±0.40	5.17±0.38	5.29±0.40	5.50±0.32	5.51±0.28
	RM-A	5.23±0.58	4.91±0.43	5.19±0.14	5.09±0.18[c]	4.62±0.32[c,d]
	RM-D	5.38±0.64	5.00±0.35	5.10±0.18	4.93±0.37[c]	4.83±0.20[c,d]
Adrenal gland (mg)	Control	24.39±4.06	24.21±4.29	25.62±4.31	23.77±6.21	23.45±5.46
	RM-A	24.92±1.25	24.25±1.95	23.38±1.25	22.35±2.54	24.74±3.66
	RM-D	24.10±1.97	22.74±1.45	22.94±1.34	21.91±2.86	23.76±4.71
Spleen	Control	0.43±0.04	0.41±0.05	0.42±0.04	0.43±0.04	0.40±0.04
	RM-A	0.47±0.08	0.45±0.04	0.43±0.02	0.38±0.03[c,d]	0.29±0.05[a,c]
	RM-D	0.43±0.06	0.45±0.11	0.48±0.05	0.42±0.05	0.31±0.01[a,c]
Kidneys	Control	1.55±0.11	1.58±0.10	1.56±0.14	1.54±0.16	1.47±0.08
	RM-A	1.49±0.12	1.41±0.19[b]	1.52±0.11	1.56±0.07	1.50±0.10
	RM-D	1.46±0.13	1.52±0.10	1.53±0.11	1.48±0.10	1.67±0.10[a,c]
Testicle	Control	0.84±0.07	0.85±0.05	0.79±0.04	0.85±0.07	0.80±0.05
	RM-A	0.81±0.07	0.79±0.07	0.85±0.08	0.86±0.08	0.78±0.05
	RM-D	0.81±0.13	0.82±0.06	0.75±0.09	0.82±0.06	0.83±0.09

Each value represents the mean ± SE of 6 mice.
Dose: Racemomycin-A 200 mg/kg, Racemomycin-D, 40 mg/kg.
Statistical significance: *a*) $p<0.01$ (value of 1 hr), *b*) $p<0.05$ (control), *c*) $p<0.011$ (control), *d*) $p<0.05$ (value of 1 hr).

II Blood Levels, Distribution in Several Organs and Urinary Excretion of Antibiotics

Blood Levels of Antibiotics——Fig. 2 illustrates the blood antimicrobial activity after intravenous administration of racemomycin-A and -D in mice. The blood concentration values declined very rapidly as the time passed, and the antimicrobial activity in both experiments was very low at 1 hr, and racemomycin-A was not detectable at 2 hr.

Distribution of the Antibiotic in Several Organs——Distribution of antimicrobial activity after intravenous administration of the antibiotics was examined and result is shown in Table II. Antimicrobial activity from racemomycin-A was detectable in only the kidney and that from racemomycin-D was mainly in the kidney and in the liver, spleen to some extent. 48 hr after the administration,

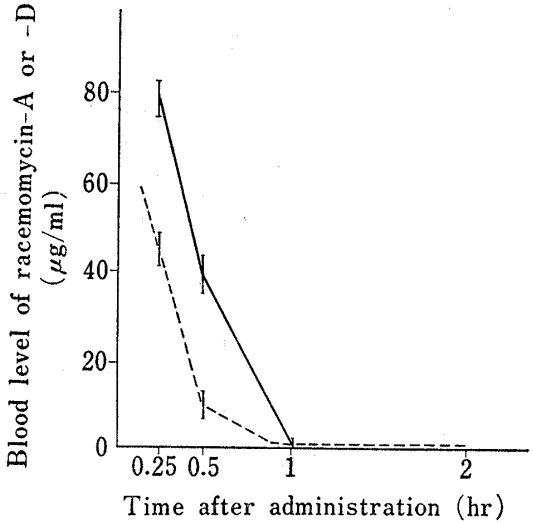

Fig. 2. Apparent Blood Level as Antimicrobial Activity of Racemomycin-A (——) and -D (----) in Mice after Intravenous Administration (Racemomycin-A 200 mg/kg, Racemomycin-D 40 mg/kg)
Plotting: mean value ± SE (n=4).

antimicrobial activity of racemomycin-A was not detectable in the kidney, while that of racemomycin-D was found in the kidney. Antimicrobial activity from racemomycin-D was detectable to a little extent in the lung and adrenal gland at later periods, in spite of dosing a lower dose than that of racemomycin-D. These distribution profiles of the antibiotics were reproducible.

Table II. Distribution of Antimicrobial Activity in Organs of Mice after Intravenous Administration of the Antibiotics

Organ	Residual antimicrobial activity (μg/g)									
	Racemomycin A					Racemomycin-D				
	Time after administration (hr)									
	1	3	5	24	48	1	3	5	24	48
Brain	—	—	—	—	—	—	—	—	—	—
Lung	—	—	—	—	—	—	—	—	—	+
Heart	—	—	—	—	—	—	—	—	—	—
Liver	—	—	—	—	—	2	2	2	2	2
Adrenal gland	—	—	—	—	—	—	—	—	+	+
Spleen	—	—	—	—	—	2	2	2	2	3
Kidney	200	69	100	29	—	59	58	105	12	3
Bladder	—	—	—	—	—	—	—	—	—	—
Testicle	—	—	—	—	—	—	—	—	—	—
Stomach	—	—	—	—	—	—	—	—	—	—
Small intestine	—	—	—	—	—	—	—	—	—	—
Large intestine	—	—	—	—	—	—	—	—	—	—

Dose: Racemomycin-A, 200 mg/kg, Racemomycin-D, 40 mg/kg.
Values are mean of 8 mice.
— not detected, + detected.

Table III. Urinary Recovery of Antimicrobial Activity after Intravenous Administration of the Antibiotics in Mice

Antibiotic	Concentration in urine (μg/ml)	Recovery (%)
Racemomycin-A	205	8
Racemomycin-D	181	36

Mice were dosed intravenously with 100 mg/kg of racemomycin-A or 20 mg/kg of racemomycin-D in water. Recovery in from excretion during 24 hr after administration.

Table IV. *In Vitro* Binding Ratio of the Antibiotics with Serum Protein of Mice

Antibiotic	Residual antimicrobial activity (μg/ml)		Binding rate[c] (%)
	Experiment[a]	control[b]	
Racemomycin-A	88	100	12
Racemomycin-D	34	54	37

a) Sample+Serum protein of mice
b) Sample+H_2O
c) Binding rate (%) $(b-a/b)\times 100=(\%)$

Urinary Excretion of the Antimicrobial Activity——Table III shows the result of urinary excretion of antimicrobial activity from racemomycin-A or -D within 24 hr. The recovery of antimicrobial activity from racemomycin-A was much lower than that of racemomycin-D.

III Binding Rate of the Antibiotic to Serum Protein

Binding rate of the antibiotic to serum protein was obtained from the *in vitro* experiment. As shown in Table IV, the binding rate of racemomycin-A was 12%, while that of racemomycin-D was 37%.

Discussion

It is common knowlegde that antibiotics of the streptomycin–neomycin group produce the diminished renal function, nephrotoxicity.[12] Racemomycins also seemed to have the same mode, that is, a high antimicrobial activity from racemomycin-A and -D was observed, especially in the kidneys (Table II). Active principle in serum, some organs, and urine seemed to be corresponding intact antibiotic, from bioautogram against *B. subtilis* PCI-219. Although data are not shown, binding of these antibiotics with the homogenized tissue of the kidney was almost negligible. Therefore, retention of the antibiotic in the kidneys after administration may be the result of electrostatic affinity, not by a covalent bond.[13] Racemomycin-D was retained more than racemomycin-A in some organs, in spite of the low dose of the former used. In addition, the binding of racemomycin-D with serum protein *in vitro* was three times higher than that of racemomycin-A (Table IV). These results clearly indicate that number of β-lysine unit in the molecule is related to these retention and binding profiles. Thus these profiles *in vivo* may also reflect as acute toxicity of the antibiotics. The higher concentration of the antibiotic in the kidneys of mice reached 5 hr after administration in both experiments. These concentration levels were reproduced. From these data, we can presume that antibiotics administered are retained in some organs, especially in the kidney, in mice and this leads to their diminished function for a relative short period. Histopathological investigation for the effect of racemomycins will be made later.

Antimicrobial activity in the serum and organs disappeared practically 48 hr after administration of racemomycin-A. Recovery of antimicrobial activity in the urine after treatment with racemomycin-A was low (Table III). This fact suggests that main part of the antibiotic seemed to be excreted into urine in an inactive form. On the other hand, racemomycin-D in a lower dose than racemomycin-A was excreted into urine in a high level, despite higher retention of the former in the organs. This fact suggests that racemomycin-D is more stable than racemomycin-A through the metabolic pathways. One of the chemically inactive structures of racemomycin-A is its open lactam form[14] and it displayed stronger acute toxicity in mice than the original antibiotic. The other structure is the N-acetyl derivative in the molecule.[15] The structural features of the inactive substances as metabolites in urine or distributed in the whole body should be studied by means of the tracer technique.

The common partial structures of streptothricins are streptolidine, D-gulosamine, β-lysine, and carbamoyl groups. The carbamoyl group is also involved in the molecules of polyoxines, bleomycin, mitomycins, and geldanamycin, but these antibiotics showed no delayed toxicity to test animals. Recently, streptothricin-like antibiotics involving glycine,[16] N-formimino-

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glycine,[17] or sarcosine[18] instead of β-lysine were isolated. These antibiotics also revealed delayed toxicity. From these respects, carbamoyl and β-lysine moieties seemed to be unrelated to this toxicity. Detailed experiments on various antibiotics[19] with low delayed toxicity belonging to this class may lead to further elucidation of the mechanism of the toxicity.

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