MARIDOMYCIN, A NEW MACROLIDE ANTIBIOTIC

III. *IN VITRO* AND *IN VIVO* ANTIBACTERIAL ACTIVITY

MASAHIRO KONDO, TOKIKO OISHI, KIYOSHI ISHIFUJI
and KANJI TSUCHIYA

Biological Research Laboratories, Central Research Division,
Takeda Chemical Industries, Ltd., Osaka, Japan

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Maridomycin has been found to have a strong *in vitro* antibacterial activity against Gram-positive bacteria and some Gram-negative bacteria such as *Neisseria gonorrhoeae* and *Vibrio cholerae*. The antibiotic was more active at pH 9 than pH 6. The antibacterial activity was enhanced by decrease in bacterial inoculum size, but not influenced by the presence of horse serum. The *in vitro* bacterial resistance to maridomycin was enhanced stepwise by serial transfer, and cross resistance was observed between maridomycin and each of macrolide antibiotics tested. This antibiotic, however, was effective against clinically isolated macrolide-resistant group B and C staphylococci. Furthermore, maridomycin demonstrated bacteriostatic activity and its protein binding ratio examined by cellophane bag dialysis method was found to be low.

Maridomycin was as effective as leucomycin on Gram-positive bacterial infection in mice by subcutaneous, intraperitoneal or intravenous administration.

Maridomycin obtained from the culture filtrates of *Streptomyces hygroscopicus* No. B-5050 is a new macrolide antibiotic. The taxonomical studies of the producing organism,[1] isolation and physicochemical characteristics[2] and chemical structure[3,4] of the antibiotic have been reported previously.

The present paper deals with the *in vitro* properties of maridomycin, including antimicrobial spectrum, influence of medium pH, serum and inoculum size on activity, development of resistance, cross resistance, sensitivity distribution of staphylococci isolated from patients, bactericidal activity and the ratio of binding with serum protein. The therapeutic activity against experimental Gram-positive bacterial infection was also studied.

Materials and Methods

Antibiotics: Maridomycin was prepared by the method in the preceding papers,[1,2] and leucomycin (kitasamycin) was isolated from commercial preparations (Lot. LLA-810). Maridomycin and leucomycin were dissolved in methanol and then diluted with sterile distilled water for the *in vitro* studies. In the *in vivo* study, the antibiotics were suspended in 0.2 % carboxymethyl cellulose.

Determination of minimum inhibitory concentration: For the test of antibacterial activity of the antibiotic, stock cultures maintained on Trypticase soy agar (TSA) (BBL) or TSA supplemented with 10 % bovine blood (blood TSA) were used. Clinical isolates of staphylococci were kindly supplyed by Miss Y. SHIMIZU, Central Clinical Laboratory, Osaka University Hospital. The minimum inhibitory concentrations of the antibiotics were determined by the two-fold serial dilution technique using TSA or blood TSA as a test medium. One loopful of a suspension containing about 10^8 viable units per ml, cultivated for 18~24 hours on TSA or blood TSA, was streaked on each assay plate and the plates were incubated at 37°C for 18 hours. The minimum inhibitory concentration is determined as the lowest concentration at which the visible growth of the test organism is completely inhibited.

Development of resistance: *Staphylococcus aureus* FDA 209P was transferred successively every 48 hours into the next series of Trypticase soy broth (TSB) tubes containing the antibiotic. The organism was grown as same as the growth in control medium which contains no antibiotic.

Bactericidal activity: The viability of the microorganism in the presence of the drug was determined by the plate count technique. An 18-hour culture of *S. aureus* FDA 209P was suspended in TSB at the concentration of 1,000 times by TSB, and the antibiotic was added to give a concentration of 0.1, 1, 10 or 100 mcg/ml. An aliquot was withdrawn from each tube at 0, 2, 4, 6 and 8 hours after incubation at 37°C. Platings were made in duplicate at several dilutions to ensure reliable count. Colony counts were made after 48 hours of incubation.

Experimental infection in mice: Four-week old female CF_1/H mice weighing 18~22 g were infected intraperitoneally with 0.5 ml of a bacterial suspension.

S. aureus 308 A-1 was cultivated in the Brain Heart Infusion (BHI) broth overnight and diluted 10^{-1} with 5 % mucin. The challenge dose of each experiment was in the range of 17.8 to 31.6× LD_{50}.

Streptococcus pyogenes E-14 cultivated on blood TSA and the suspension of 2×10^{-3} mg/ml of the organism was diluted with nutrient broth. The bacterial suspension was further diluted 10^{-1} with 5 % mucin. The challenge dose of each experiment was in the range of 17.8 to 178×LD_{50}.

Diplococcus pneumoniae type I was cultivated on blood TSA overnight and the concentration of 2 mg/ml of bacterial suspension was diluted by 10^{-6} with nutrient broth. The challenge dose of each experiment was in the range of 31.6 to 316×LD_{50}.

Table 1. Antibacterial spectra of maridomycin and leucomycin

Organism		Medium	MIC in mcg/ml	
			Maridomycin	Leucomycin
Staphylococcus aureus	FDA 209 P	Trypticase soy agar	1.56	1.56
〃	308 A-1	〃	1.56	3.125
〃	1840	〃	3.125	1.56
Streptococcus pyogenes	E-14	Trypticase soy agar +10% bovine blood	0.39	0.78
〃	Dick	〃	0.39	0.78
〃	S-8	〃	0.2	0.78
〃	NY-5	〃	0.2	0.39
Streptococcus viridans		〃	0.39	0.39
Diplococcus pneumoniae	type I	〃	0.2	0.39
〃	type II	〃	0.1	0.39
〃	type III	〃	0.1	0.39
Corynebacterium diphtheriae		〃	0.1	0.39
Bacillus subtilis	PCI 219	Trypticase soy agar	0.78	1.56
Neisseria gonorrhoeae		Trypticase soy agar +10% bovine blood	3.125	0.78
Shigella flexneri	EW-10	Trypticase soy agar	>100	>100
Shigella sonnei	EW-33	〃	>100	>100
Salmonella typhosa	Boxhill-53	〃	>100	>100
Escherichia coli	NIHJ JC 1	〃	>100	>100
Vibrio cholerae	Inaba	〃	1.56	3.125
Klebsiella pneumoniae		〃	>100	>100
Proteus vulgaris		〃	>100	>100
Pseudomonas aeruginosa		〃	>100	>100
Candida albicans		〃	>100	>100

Inoculum size=One loopful of bacterial suspension (1 mg/ml)

Determination of therapeutic activity: The mice infected by the intraperitoneal route were given single subcutaneous, intraperitoneal, intravenous or oral administration of the antibiotic immediately after challenge. Numbers of dead animals by infection were recorded daily, and the 50 per cent effective dose (ED_{50} mg/kg) was determined 7 days after infection by the method of REED and MUENCH.[5]

Results

In Vitro Antibacterial Test

Antibacterial spectrum

The antibacterial spectrum and minimum inhibitory concentrations of maridomycin and leucomycin against certain Gram-positive and Gram-negative bacteria are summarized in Table 1.

Maridomycin and leucomycin exhibited similar spectra. Both antibiotics showed strong antibacterial activities against several strains of Gram-positive bacteria and *N. gonorrhoeae* and *V. cholerae* of Gram-negative bacteria. Other Gram-negative bacteria were insensitive.

Influence of Medium pH, Inoculum Size and Addition of Serum on the Activity

Table 2 shows the minimum inhibitory concentrations of maridomycin and leucomycin against *S. aureus* FDA 209P, 308 A-1 and 1840 inoculated on media ranging in pH from 6 to 9. The minimum inhibitory concentrations of maridomycin and leucomycin on pH 9 medium were about $^1/_8 \sim ^1/_{16}$ and $^1/_2 \sim ^1/_4$ of those on pH 6 medium, respectively. As shown in Table 3, the activities of the antibiotics were dependent on the inoculum size of the test organisms but relatively weak.

Presence of horse serum in the medium at 50 % did not affect the activities of the antibiotics (Table 4).

Table 2. Effect of medium pH on antibacterial activity of maridomycin and leucomycin

Organism	Medium pH	MIC in mcg/ml	
		Maridomycin	Leucomycin
S. aureus FDA 209P	6	3.125	3.125
	7	1.56	3.125
	8	0.78	1.56
	9	0.39	1.56
S. aureus 308 A-1	6	6.25	3.125
	7	3.125	3.125
	8	1.56	1.56
	9	0.39	0.78
S. aureus 1840	6	12.5	3.125
	7	3.125	1.56
	8	0.78	0.78
	9	0.78	0.78

Inoculum size=One loopful of bacterial suspension (10^8 v.u./ml)
Medium =Trypticase soy agar

Table 3. Effect of horse serum concentration in medium on antibacterial activity of maridomycin and leucomycin

Organism	Serum (%)	MIC in mcg/ml	
		Maridomycin	Leucomycin
S. aureus FDA 209P	0	1.56	0.78
	10	0.78	0.78
	20	0.78	1.56
	50	0.78	1.56
S. aureus 308 A-1	0	1.56	1.56
	10	1.56	1.56
	20	1.56	1.56
	50	1.56	1.56
S. aureus 1840	0	1.56	1.56
	10	1.56	1.56
	20	1.56	1.56
	50	0.78	1.56

Inoculum size=0.1 ml of bacterial suspension (10^7 v.u./ml)
Medium =Trypticase soy broth

Table 4. Effect of inoculum size on antibacterial activity of maridomycin and leucomycin (Trypticase soy agar)

Organism	Viable cell counts of inocula suspensions	MIC in mcg/ml	
		Maridomycin	Leucomycin
S. aureus FDA 209P	10^4/ml	0.39	0.39
	10^5	0.39	0.78
	10^6	0.39	0.78
	10^7	0.78	0.78
	10^8	1.56	0.78
S. aureus 308 A-1	10^4/ml	0.39	1.56
	10^5	0.78	1.56
	10^6	0.78	1.56
	10^7	0.78	1.56
	10^8	1.56	1.56
S. aureus 1840	10^4/ml	0.78	0.78
	10^5	0.78	1.56
	10^6	1.56	1.56
	10^7	1.56	1.56
	10^8	1.56	1.56

Inoculum size=One loopful of bacterial suspension

Table 5. Effect of inoculum size on antibacterial activity of maridomycin and leucomycin (Trypticase soy broth)

Organism	Viable cell counts of inocula suspensions	MIC in mcg/ml	
		Maridomycin	Leucomycin
S. aureus FDA 209P	10^4/ml	0.39	0.39
	10^5	0.78	0.78
	10^6	0.78	0.78
	10^7	0.78	0.78
	10^8	1.56	1.56
S. aureus 308 A-1	10^4/ml	0.78	1.56
	10^5	1.56	1.56
	10^6	1.56	1.56
	10^7	1.56	1.56
	10^8	3.125	1.56
S. aureus 1840	10^4/ml	0.78	1.56
	10^5	0.78	1.56
	10^6	1.56	1.56
	10^7	1.56	1.56
	10^8	3.125	1.56

Inoculum size=0.1 ml of bacterial suspension

Fig. 1. Patterns of development of resistance of *S. aureus* 209P to maridomycin and leucomycin

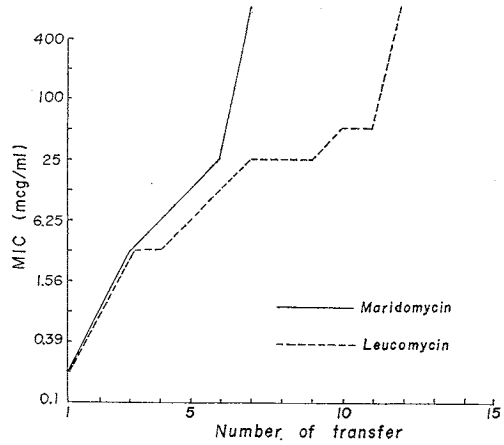
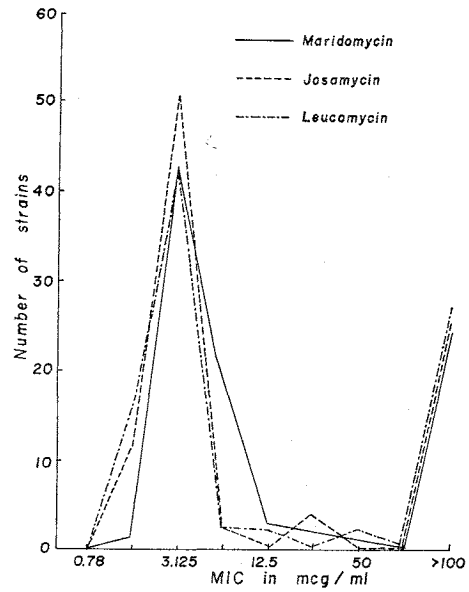


Fig. 2. Distribution of sensitivity of clinically isolated *S. aureus* against maridomycin, josamycin and leucomycin



Development of Resistance

The rate and degree of the resistance of staphylococci developed *in vitro* to maridomycin and leucomycin, as shown in Fig. 1, were nearly the same, and highly resistant strains were obtained after 6~11 transfers.

Cross Resistance

Cross resistance was studied with *S. aureus* FDA 209P resistant *in vitro* to maridomycin and several other macrolide antibiotics. The strains

Table 6. Cross resistant test among maridomycin and known macrolide antibiotics

Organism	MIC in mcg/ml					
	Marido-mycin	Josamycin	Leucomycin	Spiramycin	Triacetyl-oleando-mycin	Erythro-mycin
S. aureus FDA 209 P (parent)	1.56	3.125	1.56	3.125	3.125	0.78
R-Maridomycin	>400	>400	>400	>400	100	6.25
R-Josamycin	>400	>400	>400	>400	200	12.5
R-Leucomycin	>400	>400	>400	>400	200	25
R-Spiramycin	100	50	50	>400	100	12.5
R-Triacetyl-oleandomycin	100	50	50	100	>400	50
R-Erythromycin	50	200	50	>400	>400	>400

Table 7. Distribution of clinically isolated *S. aureus* against maridomycin and other macrolide antibiotics

MIC in mcg/ml	Distribution (number of strains)					
	Maridomycin	Josamycin	Leucomycin	Spiramycin	Triacetyl-oleandomycin	Erythromycin
>100	25	25	25	27	33	37
100	0	0	0	3	0	1
50	2	0	2	1	0	3
25	1	4	0	6	4	0
12.5	0	0	2	48	31	0
6.25	7	2	2	5	20	0
3.125	50	50	43	1	3	0
1.56	6	10	16	0	0	8
0.78	0	0	0	0	0	20
0.39	0	0	0	0	0	17
0.2	0	0	0	0	0	5

were made resistant by serial subcultures in TSB containing higher concentration of each antibiotics. The data presented in Table 6 indicated that mutual cross resistance present between maridomycin and each of several macrolide antibiotics.

Sensitivity of the Staphylococcus Strains Isolated Clinically

Maridomycin n concentrations of 1.56~6.25 mcg/ml was effective against 63 out of 91 clinically isolated staphylococcal strains. The growth in some of standard staphylococci was inhibited also in the concentration range from 1.56 to 3.125 mcg/ml. Three out of 91 strains were inhibited at concentrations of 25~50 mcg/ml of the antibiotic. The remaining 25 strains were not inhibited even at a concentration of 100 mcg/ml of maridomycin and other macrolide antibiotics tested (Table 7 and Fig. 2). As shown in Table 8, 2 strains (spiramycin-, triacetyloleandomycin- and erythromycin-resistant), 3 strains (triacetyloleandomycin- and erythromycin-resistant), 1 strain (spiramycin-resistant) and 8 strains (erythromycin-resistant) were found to be sensitive to maridomycin.

Bactericidal Activity

The bactericidal activities of maridomycin and leucomycin against *S. aureus* FDA 209P are shown in Figs. 3 and 4. The viability of the microorganisms cultivated in TSB containing various concentrations of the antibiotic was determined by plate count. The viable counts in logarithmic

Table 8. Effect of maridomycin and known macrolide antibiotics on clinically isolated macrolide antibiotics resistant *S. aureus*

Strain No.	Maridomycin	Josamycin	Leucomycin	Spiramycin	Triacetyl-oleandomycin	Erythromycin
5	>100	>100	>100	>100	>100	>100
8	>100	>100	>100	>100	>100	>100
9	>100	>100	>100	>100	>100	>100
11	>100	>100	>100	>100	>100	>100
16	>100	>100	>100	>100	>100	>100
18	>100	>100	>100	>100	>100	>100
20	>100	>100	>100	>100	>100	>100
22	>100	>100	>100	>100	>100	>100
26	>100	>100	>100	>100	>100	>100
35	>100	>100	>100	>100	>100	>100
45	>100	>100	>100	>100	>100	>100
48	>100	>100	>100	>100	>100	>100
50	>100	>100	>100	>100	>100	>100
52	>100	>100	>100	>100	>100	>100
54	>100	>100	>100	>100	>100	>100
60	>100	>100	>100	>100	>100	>100
61	>100	>100	>100	>100	>100	>100
64	>100	>100	>100	>100	>100	>100
67	>100	>100	>100	>100	>100	>100
70	>100	>100	>100	>100	>100	>100
71	>100	>100	>100	>100	>100	>100
83	>100	>100	>100	>100	>100	>100
88	>100	>100	>100	>100	>100	>100
91	>100	>100	>100	>100	>100	>100
93	>100	>100	>100	>100	>100	>100
63	50	25	50	>100	>100	50
76	50	25	50	>100	>100	>100
92	25	25	12.5	50	>100	50
30	6.25	25	12.5	100	>100	50
44	3.125	3.125	3.125	>100	>100	>100
25	6.25	3.125	3.125	12.5	>100	>100
75	6.25	3.125	3.125	12.5	>100	>100
68	3.125	1.56	3.125	12.5	>100	>100
56	3.125	3.125	3.125	100	12.5	0.78
1	6.25	3.125	3.125	12.5	25	>100
33	6.25	3.125	3.125	12.5	12.5	>100
4	3.125	3.125	3.125	12.5	25	>100
10	3.125	3.125	3.125	12.5	25	>100
28	3.125	1.56	3.125	12.5	12.5	>100
65	3.125	6.25	3.125	12.5	25	>100
89	3.125	3.125	1.56	12.5	12.5	>100
90	3.125	3.125	1.56	6.25	6.25	>100

Inoculum size = One loopful of bacterial suspension (10^8 v.u./ml)
Medium = Trypticase soy agar

Fig. 3. Bactericidal effect of maridomycin on *S. aureus* FDA 209P

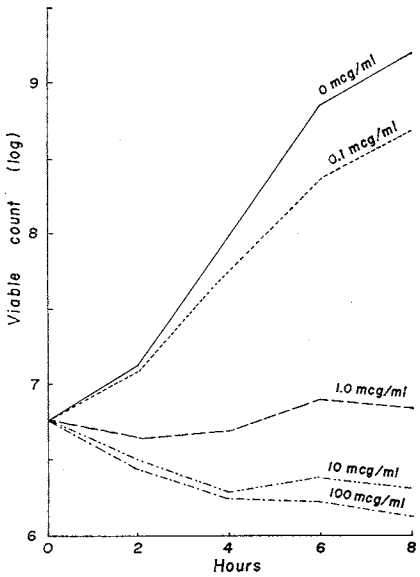


Fig. 4. Bactericidal effect of leucomycin on *S. aureus* FDA 209P

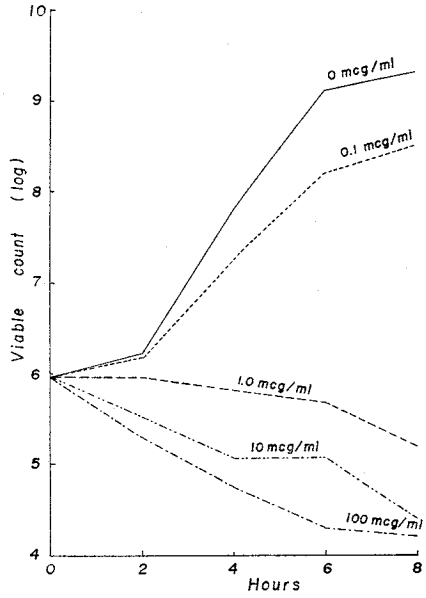


Table 9. Binding of maridomycin and leucomycin by horse serum

Antibiotic	Concentration before dialysis (mcg/ml)	Concentration (mcg/ml) after dialysis		Percent bound	
		Horse serum	Buffer	Experiment	Means
Maridomycin	10	7.0	7.9	11.4	12.22
		6.7	7.9	15.2	
		8.0	9.2	13.04	
		8.35	9.2	9.24	
Leucomycin	10	5.2	8.8	40.9	42.62
		5.7	8.8	35.2	
		4.5	9.0	50.0	
		5.0	9.0	44.4	

Visking cellophane bag, which contained 5 ml of horse serum or M/15 phosphate buffer (pH 8.0), was suspended in 10 ml of M/15 phosphate buffer (pH 8.0) containing 10 mcg/ml of maridomycin and leucomycin. Dialysis was conducted for 72 hours at 4°C. The percentage of binding of the maridomycin and leucomycin was calculated as follows:

$$\frac{(a-b)V}{aV} \times 100$$

V = Total volume
a = Concentration of antibiotic in dialysata after antibiotic solution dialysed against buffer
b = Concentration of antibiotic in dialysata after antibiotic solution dialysed against horse serum

scale are plotted against the time of exposure to the antibiotic.

When the antibiotics were added simultaneously, weak bactericidal activities were demonstrated at concentrations of 10 mcg/ml and 100 mcg/ml of maridomycin and leucomycin. At the concentration of 1 mcg/ml of maridomycin, the viable units did not vary for 8 hours and the same viable units as the control were still observed in the presence of both antibiotics at the concentration of 0.1 mcg/ml.

Binding with Horse Serum Protein

The results of the dialysis experiment are shown in Table 9. The binding ratio of maridomycin and leucomycin with horse serum protein slightly varied between individual experiments. At the concentration of 10 mcg/ml, the protein binding ratio of maridomycin was lower than that of leucomycin, *i.e.*, the protein binding ratio was calculated to be 12.22 % for maridomycin and 42.6 % for leucomycin, respectively.

In Vivo Antibacterial Test

The therapeutic activities of maridomycin and leucomycin on experimental infection in mice caused by *S. aureus* 308 A-1, *S. pyogenes* E-14 and *D. pneumoniae* type I are shown in Tables 10, 11 and 12. Against Gram-positive bacterial infections maridomycin showed the same therapeutic activity as leucomycin in subcutaneous, intraperitoneal and intravenous administration. In oral

Table 10. Therapeutic effect of maridomycin and leucomycin on *S. aureus* 308 A-1 infection in CF 1/H mice

Antibiotic		Maridomycin				Leucomycin			
In vitro sensitivity (mcg/ml)		1.56				3.125			
Administration route		SC	IP	IV	Oral	SC	IP	IV	Oral
ED_{50} in mg/kg	I	7.1	0.446	7.94	893	—	—	4.59	280
	II	6.45	—	11.2	649	—	0.25	12.4	325
	III	7.1	0.6	—	710	10.0	0.21	6.21	352
	IV	3.08	—	—	558	3.84	0.176	5.0	384
	V	—	0.66	10.0	—	7.04	0.181	6.21	—
	VI	3.85	0.125	8.93	649	5.96	0.096	6.21	281
	Average	5.52	0.458	9.52	691.8	6.71	0.183	6.77	324.4

Note: Five mice of each group were injected intraperitoneally with 0.5 % mucin which contains 1/10 volume of suspension of test organism.
Antibiotic was administered as a single dose immediately after challenge.

Table 11. Therapeutic effect of maridomycin and leucomycin on *S. pyogenes* E-14 infection in CF 1/H mice

Antibiotic		Maridomycin				Leucomycin			
In vitro sensitivity (mcg/ml)		0.39				0.78			
Administration route		SC	IP	IV	Oral	SC	IP	IV	Oral
ED_{50} in mg/kg	I	1.94	0.0725	2.1	171	2.79	0.0223	2.67	35.2
	II	—	0.0325	0.88	142	—	0.0562	3.1	—
	III	—	0.0447	—	141	—	0.0621	2.5	—
	IV	1.25	0.0176	1.0	119	1.44	0.0223	1.55	29.8
	V	1.49	0.0311	2.14	130	1.55	0.0352	2.18	62.1
	VI	0.96	0.071	1.25	100	2.23	0.0705	4.46	70.5
	Average	1.41	0.0449	1.47	133.8	2.00	0.0448	2.67	49.4

Table 12. Therapeutic effect of maridomycin and leucomycin on *D. pneumoniae* type I infection in CF 1/H mice

Antibiotic		Maridomycin				Leucomycin			
In vitro sensitivity (mcg/ml)		0.2				0.39			
Administration route		SC	IP	IV	Oral	SC	IP	IV	Oral
ED_{50} in mg/kg	I	80.64	11.20	140.8	446	55.8	25.00	100.0	400
	II	12.83	7.77	141.0	446	—	—	—	—
	III	28.10	8.13	43.5	352	20.0	6.25	50.0	446
	IV	—	—	—	—	22.3	11.70	35.2	446
	V	20.00	7.45	69.0	176	15.5	7.03	20.0	250
	VI	16.90	17.60	38.4	352	17.6	6.98	—	314
	Average	31.69	10.43	86.54	354.4	26.24	11.39	51.30	371.2

Note: Five mice of each group were injected intraperitoneally with 0.5 ml of bacterial suspension in TSB. Antibiotic was administered as a single dose immediately after challenge.

route, the therapeutic activity of maridomycin on staphylococcal or streptococcal infection was relatively low, compared with that of leucomycin. Against diplococcal infection, the therapeutic activities of both antibiotics were approximately equal in every route.

Discussion

Maridomycin at low concentrations was active against certain Gram-positive bacteria and its spectrum resembled to that of leucomycin. Furthermore, various *in vitro* antibacterial properties of maridomycin were similar to those of the macrolide antibiotics, *i.e.*, the antibacterial activity increased in high pH medium and slightly or not influenced by inoculum size and addition of serum. Maridomycin showed cross resistance to several macrolide antibiotics. However, the antibacterial activity of maridomycin was found against clinically isolated staphylococci including macrolide-resistant strains of group B and C classified by KONO *et al.*[6] This observation suggests that this antibiotic has no resistant-inducing activity. Furthermore, maridomycin has bacteriostatic activity rather than bactericidal activity.

Prominent therapeutic effect was observed against certain Gram-positive bacterial infection in mice. When maridomycin was given parenterally, the therapeutic dose is about the same as that of leucomycin. Studies in an attempt to increase therapeutic activity such as chemical modification of maridomycin are being carried out and the results will be present in the next paper.

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