SYNERGISTIC EFFECTS OF A MACROLIDE AND A CELL WALL-AFFECTING ANTIBIOTIC ON *PSEUDOMONAS AERUGINOSA IN VITRO* AND *IN VIVO*

1. COMBINED EFFECTS OF A MACROLIDE AND A PEPTIDE ANTIBIOTIC

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Synergistic effects of peptide and macrolide antibiotics against *Pseudomonas aeruginosa* were investigated *in vitro* and *in vivo*. Synergistic effects were evaluated by estimating the number of viable bacteria at varying intervals in the logarithmic growth phase. These bacteria were treated concurrently with polymyxin B (PL) at the final concentration of 1.56 U/ml and with 9,3''-di-*O*-acetylmidecamycin (MOM) at varying concentrations. Synergistic effect was observed when PL was used with MOM at 3.13 and 12.5 μg/ml respectively. When MOM at 50 μg/ml was used with PL, the viable bacterial count was reduced to below 1/300 of the control to which PL alone had been added. Thus, the synergistic effect was remarkable. Similar results were obtained when colistin methanesulfonate (CL) was used instead of PL.

Subsequently, attempts were made to determine if this action could also be found in *in vivo* experiments using mice. PL or CL was injected intramuscularly and midecamycin (MDM) or MOM was administered once or repeatedly by the oral route. Simultaneously, *Pseudomonas aeruginosa* strain IFO 3455 was inoculated intraperitoneally to mice. In the case of treatment once or repeatedly using both PL and MDM or MOM, the survival rate of infected mice increased significantly compared to single treatment by PL alone. Thus, the synergistic effects were demonstrated in four experiments. (The significance levels for the experiments were $P=0.070$, 0.015, 0.042 and 0.024).

Similar results were obtained when strain No. 5 was used to infect mice ($P=0.0096$, 0.0027).

When CL and MOM were given to mice once prior to infection with strain No. 5, synergistic effects were obtained as well ($P=0.010$, 0.034).

YAMAMOTO and HOMMA[1,2] isolated L-forms from *Pseudomonas aeruginosa* strains and demonstrated a remarkable difference in the susceptibility to antibiotics between L-forms and their parent strains. In particular, the L-forms show remarkable susceptibility to macrolide antibiotics such as midecamycin (MDM) and its derivative, 9,3''-di-*O*-acetylmidecamycin (MOM). YAMAMOTO and HOMMA[3] reported isolating unstable L-forms from patients with chronic respiratory tract infections, urinary tract infections and mastitis due to *P. aeruginosa*, who were undergoing treatment with β-lactam antibiotics.

The factors which convert *P. aeruginosa* strains into L-forms *in vivo* include antibiotics, enzymes such as lysozyme, complement and antibody. KAWAHARAJO, KASAI and HOMMA[4] conducted *in vivo* and *in vitro* experiments to investigate the synergistic effects between MDM or MOM and carbenicillin (CBPC) against *P. aeruginosa* and showed that MDM or MOM acts effectively on spheroplasts induced *in vitro* by CBPC. This indicates that there is synergistic action between the two kinds of antibiotics.

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Similarly, the survival rate was significantly higher in mice treated with CBPC and MDM or MOM than in those treated with CBPC alone. Thus synergistic action between CBPC and MDM or MOM was observed.

In the present experiment, we investigated *in vivo* and *in vitro* synergistic effects between MDM or MOM and PL,[5] an antibiotic which causes a fissure in the surface layer of *P. aeruginosa* caused by binding of lipopolysaccharides (LPS) to the lipid A site.

Materials and Methods

Strains

P. aeruginosa IFO 3455 and strain No. 5 were provided by Dr. KAZUYUKI MORIHARA (Shionogi Research Laboratories, Osaka) and Dr. YASUKIYO NAKASE (The Kitasato Institute).

Antibiotics

Midecamycin (MDM) and its derivative, 9,3″-di-*O*-acetylmidecamycin (MOM) were used as the macrolide antibiotics (Meiji Seika Kaisha, Ltd., Tokyo). For the *in vitro* experiment, MDM and MOM were dissolved in 1/20 volume of ethanol and dimethylsulfoxide (DMSO), respectively. For the *in vivo* experiment, MDM and MOM were suspended in 0.5% hydroxypropylmethyl cellulose or 0.5% gum arabic solution. Polymyxin B sulfate (PL, Taito Pfizer Co., Tokyo) and colistin methanesulfonate (CL, Kaken Chemicals Co., Tokyo) were dissolved in distilled water or physiological saline solution.

Culture Media

Tryptic soy broth (TSB, Difco), Tryptic soy agar (TSA, Difco), Brain heart infusion broth (BHIB, Difco) and Brain heart infusion agar (BHIA, Difco) were used.

Mice

Four-week-old *dd*Y (Shizuoka Agricultural Cooperative Association for Laboratory Animals) and ICR (Japan Charles River Inc.) male mice (SPF) were used.

Test for Antibiotic Susceptibility

The minimal inhibitory concentration (MIC) was estimated by the tube dilution method. One tenth ml of a 1,000-fold dilution (about 10^5 cells/ml) of bacteria cultured overnight in TSB was inoculated to 2 ml of serial two-fold dilutions of each antibiotic prepared with BHIB. MIC was expressed by the lowest concentration at which no turbidity occurred after 18 hours incubation at 37°C.

In Vitro Synergistic Effects

The number of viable cells were estimated by plate count of ten-fold serial dilution on BHIA plates. To the medium containing about 10^5 bacterial cells per ml in logarithmic phase, PL and MOM were added. The mixture was incubated by shaking at 37°C. One, 3 or 6 hours after addition of antibiotics, samples were removed from each tube and diluted with BHIB. Such diluted bacterial solution of a fixed volume was spread over the surface of BHIA plates. The number of colony forming units (CFU) was calculated after incubation at 37°C overnight as the viable bacteria. When CL was used, the procedure was the same as in the case of PL. The controls were cultured in the same manner with no addition of antibiotic or the addition of PL, CL or MOM. Synergistic effects were evaluated by comparing the CFU of the bacterial cultures with the two kinds of antibiotics and the controls.

In Vivo Synergistic Effects

The bacterial strain to be used for injection was cultivated overnight at 37°C on TSA slants and suspended in TSB. Mice were injected intraperitoneally with 0.5 ml of the bacterial suspensions. For the single administration, 0.5 ml of a macrolide (MDM or MOM) was given orally once 1 hour prior to administration of the bacterial inoculum after which 0.2 ml of a peptide antibiotic (PL or CL) was injected intramuscularly. For the repeated administration, 0.2 ml of a macrolide was administered orally 2 hours before administration of the bacterial inoculum and the peptide antibiotic was injected in-

tramuscularly immediately after administration of the inoculum. The injection was repeated every 2 hours for 24 hours.

The survival rate of the animals was observed 7 days after injection. The significant difference between the groups administered only one antibiotic and the groups administered both kinds of antibiotics was determined based on the number of animals surviving on the 7th day, using Fisher's exact method.

Results

The potential synergistic effect was examined *in vitro*. To the IFO 3455 strain at 4.6×10^5 CFU/ml, PL at a final concentration of 1.56 U/ml ($\frac{1}{4}$MIC) and MOM at the final concentration of 3.13, 12.5 or 50 μg/ml (below the MIC) were added simultaneously, and their synergistic effect was examined based on the time course changes of CFU (Fig. 1). Decrease in CFU depended on the concentration of MOM in the cultures to which PL and MOM had been added. CFU was 2.0×10 CFU/ml in the culture to which PL and 50 μg/ml MOM had been added, as compared to 5.5×10^3 CFU/ml in the culture 1 hour after the addition of PL alone. Thus, CFU in the culture to which both PL and 50 μg/ml MOM had been added was about 1/300 that of the culture to which only PL had been added, demonstrating a remarkable synergistic effect. CFU following the single addition of 50 μg/ml MOM alone was 1.1×10^6/ml and that without any antibiotic was 1.2×10^6/ml. CFU tended to increase under these conditions.

Fig. 1. Synergistic effect of polymyxin B and 9,3''-di-*O*-acetylmidecamycin against growth of *P. aeruginosa* IFO 3455.

PL was added to the bacterial solution (4.6×10^5) to give a final concentration of 1.56 U/ml ($\frac{1}{4}$ MIC). MOM was then added to give the concentrations shown below. Samples were taken 1, 3 and 6 hours after addition of the antibiotic, and the number of viable bacteria estimated by CFU. The ordinate shows the number of viable bacteria and the abscissa the incubation time.

a: No antibiotic (control). b: MOM at 50 μg/ml (control). c: PL at 1.56 U/ml (control). d: PL at 1.56 U/ml plus MOM at 3.13 μg/ml. e: PL at 1.56 U/ml plus MOM at 12.5 μg/ml. f: PL at 1.56 U/ml plus MOM at 50 μg/ml.

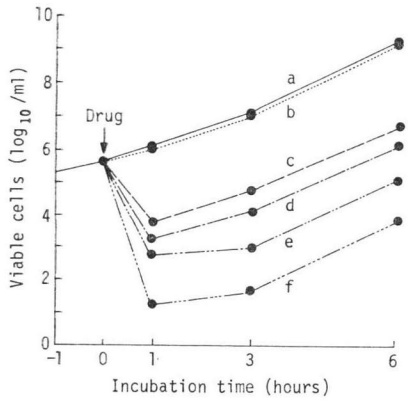


Fig. 2. Synergistic effect of colistin and 9,3''-di-*O*-acetylmidecamycin against growth of *P. aeruginosa* IFO 3455.

CL was added to the bacterial solution (3.5×10^5 CFU/ml) to give a final concentration of 0.78 μg/ml ($\frac{1}{4}$ MIC) and MOM was added as indicated below. For other conditions, see note to Fig. 1.

a: No antibiotic (control). b: MOM at 50 μg/ml (control). c: CL at 0.78 μg/ml (control). d: CL at 0.78 μg/ml plus MOM at 3.13 μg/ml. e: CL at 0.78 μg/ml plus MOM at 12.5 μg/ml. f: CL at 0.78 μg/ml plus MOM at 50 μg/ml.

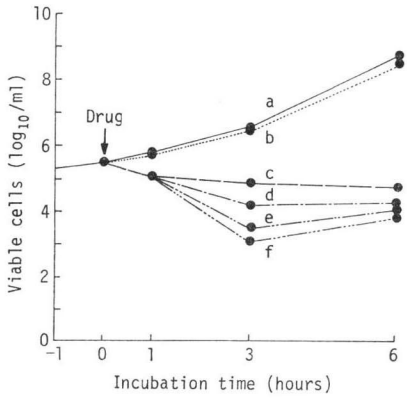


Table 1. Synergistic effects of polymyxin B and midecamycin or 9,3''-di-*O*-acetylmidecamycin against mice infected with *Pseudomonas aeruginosa* IFO 3455.

Experiment No.	Mice	Inoculated amount (Viable bacteria per mouse)	Number of times antibiotics administered	Group	Dose (Once per mouse)	Protection rate (%) (Surviving mice/total mice)	Probability value (P)
1	*dd*Y	3.6×10^7 (1 MLD*)	1 time	A	PL 2000 U plus MOM 10 mg	9/10(90)	A: B P=0.070
				B	PL 2000 U	5/10(50)	
				C	MOM 10 mg	0/10(0)	
				D	No antibiotic	0/10(0)	
2	ICR	3.3×10^7 (2 MLD)	5 times	A	PL 500 U plus MDM 2 mg	13/15(86.7)	A: C P=0.015
				B	PL 500 U plus MOM 2 mg	12/15(80)	
				C	PL 500 U	7/16(43.8)	B: C P=0.042
				D	MDM 2 mg	0/15(0)	
				E	MOM 2 mg	0/15(0)	
				F	No antibiotic	0/12(0)	
3	*dd*Y	5.5×10^7 (2 MLD)	7 times	A	PL 500 U plus MOM 2 mg	11/20(55)	A: B P=0.024
				B	PL 500 U	4/20(20)	
				C	MOM 2 mg	0/20(0)	
				D	No antibiotics	0/20(0)	

* MLD: Minimum lethal dose.

Note to Table 1: Viable bacteria were inoculated intraperitoneally to mice. A macrolide antibiotic was given orally at the indicated amount one hour before the inoculation of viable bacteria in the case of single administration of the macrolide and peptide and 2 hours before bacterial inoculation in the case of repeated administrations. Immediately after the bacterial inoculation, a peptide antibiotic was injected intramuscularly. The control received only a macrolide or a peptide antibiotic or received a solution containing no antibiotics. The animals were observed for 7 days and the protective rate was calculated based on the rate of surviving mice to total number used in the experiment. The probability was also calculated for comparison.

Both 0.78 μg/ml ($\frac{1}{4}$ MIC) of CL and various concentration of MOM (below the MIC) were added to the bacterial culture at 3.5×10^5 CFU/ml and the potential synergistic effect was examined based on the CFU the same as in the case of PL (Fig. 2). The CFU observed 3 hours after the addition of CL was 8.4×10^4 CFU/ml. However, it was 1.1×10^3 CFU/ml following the addition of both CL and MOM at 50 μg/ml. When both were added, the CFU decreased to about 1/80 of that after addition of only CL or MOM. Thus, synergistic effect was demonstrated. CFU tended to be high in the culture to which 50 μg/ml MOM only was added and in the culture to which no antibiotic was added.

Subsequently, the *in vivo* synergistic effect was studied in mice infected with *P. aeruginosa*. The effect of the antibiotics on the survival rate was examined. Table 1 shows the survival rate and probability (P) in experiments using varying amounts of inoculum and antibiotics and different mouse strains.

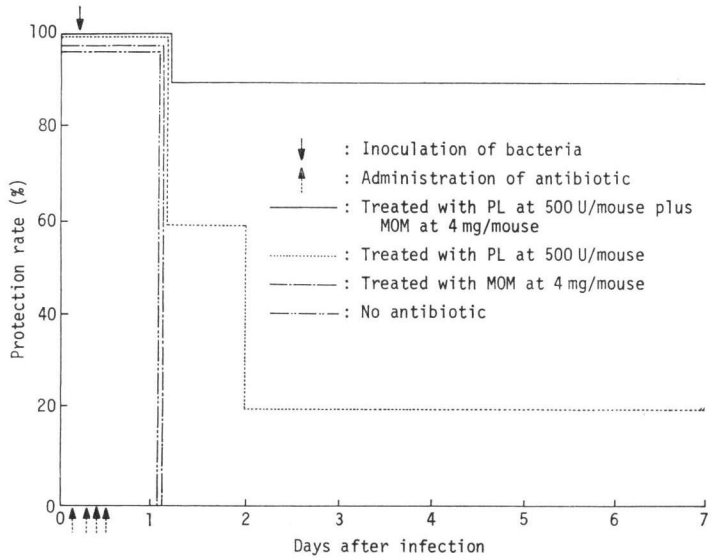
In experiments using the strain IFO 3455, the potential synergistic effect between PL and MDM or MOM was examined. In the first experiment, a single dose of MOM at 10 mg/mouse and of PL at 2000 U/mouse was administered. In the second experiment, MDM or MOM at 2 mg/mouse and PL at 500 U/mouse were injected five times, and in the third experiment, MOM at 2 mg/mouse and PL at 500 U/mouse were injected seven times. In all three groups, the number of surviving animals was significantly higher compared to the group treated with PL alone. Thus, the synergistic effect between PL and MOM was clearly observed (P=0.070, 0.042, 0.024 in the first, second and third experiments, respectively). When MDM instead of MOM was administered concurrently with PL 5 times, the number of surviving animals was also significantly higher than in the group administered only a single antibiotic.

Table 2. Synergistic effects of polymyxin B or colistin and 9,3''-di-*O*-acetylmidecamycin against mice infected with *Pseudomonas aeruginosa* No. 5.

Experiment No.	Mice	Inoculated amount (Viable bacteria per mouse)	Number of times antibiotics administered	Group	Dose (Once per mouse)	Protection rate (%) (Surviving mice/total mice)	Probability value (P)
4	ICR	4.8×10^{6} (1 MLD*)	1 time	A	CL 4 mg plus MOM 10 mg	7/10 (70)	A: B P=0.010
				B	CL 4 mg	1/10 (10)	
				C	MOM 10 mg	0/10 (0)	
				D	No antibiotic	0/10 (0)	
5	*dd*Y	2.0×10^{5} (5 MLD)	1 time	A	CL 4 mg plus MOM 10 mg	8/10 (80)	A: B P=0.034
				B	CL 4 mg	3/10 (30)	
				C	MOM 10 mg	0/10 (0)	
				D	No antibiotic	0/10 (0)	
6	ICR	6.1×10^{6} (2 MLD)	4 times	A	PL 500 U plus MOM 4 mg	11/20 (55)	A: B P=0.0096
				B	PL 500 U	3/20 (15)	
				C	MOM 4 mg	0/20 (0)	
				D	No antibiotic	0/20 (0)	
7	*dd*Y	4.1×10^{6} (100 MLD)	4 times	A	PL 500 U plus MOM 4 mg	9/10 (90)	A: B P=0.0027
				B	PL 500 U	2/10 (10)	
				C	MOM 4 mg	0/10 (0)	
				D	No antibiotic	0/10 (0)	

* MLD: Minimum lethal dose.
Remark: See note to Table 1.

Fig. 3. Protection rate in mice infected with *P. aeruginosa* No. 5 following 4 administrations of polymyxin B and 9,3''-di-*O*-acetylmidecamycin.
See Experiment 7 of Table 2 for experimental conditions.



Thus, the synergistic effect was also observed for MDM and PL (P=0.015).

Table 2 shows the synergistic effect in mice infected with strain No. 5 under varying conditions of inoculum, frequency of dosing and animal strain. In the fourth and fifth experiments, MOM at 10 mg/

mouse and CL at 4 mg/mouse were administered once, and in the sixth and seventh experiments, MOM at 4 mg/mouse and PL at 500 U/mouse were administered four times. In the group treated once with MOM and CL, the number of surviving animals was significantly higher than the group treated with CL alone, indicating a synergistic effect (P=0.010, 0.034). In the group treated with MOM and PL four times, a remarkable synergistic effect was also observed (P=0.0096, 0.0027). The control mice in all experiments treated with either no antibiotic or a macrolide antibiotic alone died on the first day of experiment.

One example of protection rate after challenge is shown in Fig. 3. All 7 experiments showed similar trends in protection rate.

Discussion

It is known that PL, a peptide antibiotic, binds specifically with lipid A of LPS.[5] KOIKE, IIDA and MATSUO[6] observed by electron microscopy *P. aeruginosa* treated with PL, which binds specifically with LPS, and CL at optimal concentrations and noted the formation of blebs on the cellular surface and cracks in some parts of the cell wall. Furthermore, cracks were also observed in the cell membrane as incubation with the antibiotic was prolonged.

In our experiments, remarkable synergistic effects were observed both *in vitro* and *in vivo* between PL or CL and MDM or MOM. These synergistic effects seem to be partly due to the fact that PL and CL impair the cell wall, allowing macrolides to permeate the cytoplasm. YAMAMOTO and HOMMA[2] and KAWAHARAJO and coworkers[4] reported that L-forms and spheroplasts are susceptible to macrolide antibiotics. When the cell wall is impaired by a peptide antibiotic (PL or CL), a macrolide can permeate the cytoplasm and exert its action. In this regard, the mechanism of the action of MOM is presently under investigation using ^{14}C-labelled MOM.

NISHINO and NAKAZAWA[7] reported the formation of cracks in the cellular outer membrane of *P. aeruginosa*. We performed experiments assuming that macrolide antibiotics can pass through the damaged cell wall to the cytoplasm and exert their action. The validity of this assumption was tested in the present study and the results described herein.

The finding that peptide antibiotics such as PL and CL as well as β-lactam antibiotics[4] act synergistically with macrolides is significant, not only from the standpoint of basic studies but also in the selection of antibiotics for therapy in the future.

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