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

2. COMBINED EFFECTS OF A MACROLIDE WITH A FOSFOMYCIN AND AN AMINOGLYCOSIDE ANTIBIOTIC

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Synergistic effects of the cell wall-affecting antibiotics, dibekacin (DKB) and fosfomycin (FOM) and a macrolide antibiotic, midecamycin (MDM) or its derivative 9,3″-di-*O*-acetylmidecamycin (MOM) against *Pseudomonas aeruginosa* were investigated *in vitro* and *in vivo*. Synergistic effects were evaluated by estimating the number of viable bacteria at varying intervals after the two kinds of antibiotics were added to the logarithmic phase of the bacterial solution. Six hours after addition of antibiotic, the viable bacterial count of the culture treated with FOM and MOM underwent 2 log reduction compared to that which treated with FOM alone. Thus synergistic effect was significant. The number of viable bacteria treated with DKB and MDM showed slight reduction at 3 hours after addition of the two antibiotics and a marked reduction was noted after 20 hours compared with the control.

Synergistic action was also demonstrated in *in vivo* experiments using mice. Three experimental mouse infection models, intraperitoneal infection, subcutaneous infection with carrageenan solution and burn infection were used. FOM was administered subcutaneously. DKB was administered intramuscularly. MDM or MOM was administered by the oral route. In all three experiments the survival rate of infected mice treated with FOM and MOM increased significantly compared to control mice. Similar synergistic effect was also obtained with DKB and MDM.

In previous papers, the authors reported that compared to treatment with a single antibiotic, combined treatment of a macrolide with cell wall-affecting antibiotics such as a peptide antibiotic (polymyxin B and colistin methansulfonate) or a β-lactam antibiotic (carbenicillin) against *P. aeruginosa* decreased the *in vitro* viable bacterial count and increased significantly the survival rate of mice infected with viable bacteria.[1,2]

NISHINO and NAKAZAWA[3] observed in electronmicroscopic studies "cracks" which had formed in the cell wall outer membrane of *P. aeruginosa* which had been suspended in a culture medium containing a small quantity of dibekacin (DKB). KAHAN *et al.*[5] demonstrated that fosfomycin (FOM) inhibits the early stage of cell wall synthesis of bacteria resulting in bactericidal action.

Thus in this paper, synergistic effects of FOM and MOM (9,3″-di-*O*-acetylmidecamycin)*** or DKB and MDM (midecamycin) against *P. aeruginosa* were investigated first in *in vitro* experiments and then in *in vivo* experiments.

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*** 9,3″-Di-*O*-acetylmidecamycin: New name, midecamycin acetate is proposed for INN.

Materials and Methods

Bacterial Strains

P. aeruginosa strain IFO 3455 was provided by Dr. KAZUYUKI MORIHARA, Shionogi Research Laboratories, Osaka. *P. aeruginosa* strain No. 5 was kindly supplied by Dr. YASUKIYO NAKASE, The Kitasato Institute, Tokyo. *P. aeruginosa* strain MSK-079 was obtained from the collection of this laboratory.

Animals

Four (16~18 g) or six-week-old (23~25 g) female *dd*Y mice, SPF, (Shizuoka Agricultural Co-operative Association for Laboratory Animals) were used.

Culture Media

Tryptic soy broth (TSB), Tryptic soy agar (TSA), Brain heart infusion broth (BHIB), Brain heart infusion agar (BHIA) and Nutrient broth (NB) purchased from Difco Laboratories Inc. were used.

Antibiotics

Dibekacin sulfate (DKB) and fosfomycin disodium (FOM) were kindly provided by Meiji Seika Kaisha, Ltd., Tokyo. They were used as the cell wall-affecting antibiotics. Midecamycin (MDM) and its derivative, 9,3''-di-*O*-acetylmidecamycin (MOM), also provided by Meiji Seika Kaisha, Ltd., were used as the macrolide antibiotics. For the *in vitro* experiment, MDM was dissolved in 0.5% ethyl alcohol and MOM in dimethylsulfoxide (DMSO), both at a final concentration of 1,000 μg/ml. For the *in vivo* experiment, MDM and MOM were suspended in 0.5% gum arabic and hydroxylpropylmethyl cellulose solution, respectively. FOM and DKB were dissolved in distilled water or physiological saline for both *in vitro* and *in vivo* experiments.

Antibacterial Susceptibility Testing

MIC (minimum inhibitory concentration) was estimated by the broth dilution methods as described previously.[2] Antibacterial activity of FOM was determined using NB medium. For the test with DKB and the macrolide antibiotic, BHIB medium was used.

Measurement of Viable Cells

A cell wall-affecting and a macrolide antibiotic were added immediately to the logarithmic phase of the bacterial culture. As controls, a cell wall-affecting antibiotic alone, a macrolide antibiotic alone or no antibiotic was added in the same manner. NB medium was used for the investigation of synergy against FOM and MOM. BHIB medium was used for the investigation of synergy against DKB and MDM. After addition of antibiotics, the mixture was incubated at 37°C with shaking. At the times indicated in the text, samples were taken and 10 fold-serial dilution performed with NB or BHIB medium. The viable cells were estimated by inoculating the culture on BHIA plates. The number of colony forming units (CFU) was calculated as the viable bacteria. Synergistic effect was evaluated by comparing CFUs of the test bacterial cultures treated with the two kinds of antibiotics and those of the controls.

Infection and Treatment of Antibiotics

Bacteria to be used for injection were cultivated overnight at 37°C on TSA slant medium and suspended in TSB medium. For the intraperitoneal infection, mice were injected with 0.5 ml of bacterial suspension containing more than MLD (minimum lethal dose) live bacteria. In the subcutaneous infection, mice were injected in the back with 2 ml of 0.5% carrageenan solution containing viable bacteria. The MLD of strains IFO 3455 and No. 5 in the intraperitoneal infection experiments were 3.7×10^7 CFU/mouse and 3.8×10^5 CFU/mouse, respectively. The MLD of strain MSK-079 in the subcutaneous infection experiment was 2.6×10^8 CFU/mouse. For the burn infection, a burned mouse model was used according to STIERITZ and HOLDER.[4] Mice were anesthetized by intraperitoneal injection of pentobarbital sodium. One ml of ethyl alcohol was infused within a range of 1.0×1.5 cm to each mouse, the back skin of which had been depilated and burned by ignition. This was repeated twice. The burned back was inoculated subcutaneously with 0.2 ml of bacterial suspension. In this burn infection experiment, MLD of strain IFO 3455 was 1.0×10^1 CFU/mouse.

Following infection, 0.1 ml of the cell wall-affecting antibiotic, FOM or DKB solution, was given subcutaneously or intramuscularly in the femoral region, and 0.2 ml of the macrolide antibiotic, MDM or MOM suspension, was given orally. In the controls, a cell wall-affecting antibiotic alone, a macrolide antibiotic alone or a soultion containing no antibiotic was administered. The antibiotics were administered repeatedly at intervals of 2 hours from the day of bacterial inoculation to the second day after bacterial inoculation. In the intraperitoneal and burn infections, survival rates of the animals were observed 7 days after infection. In the subcutaneous infection, survival rates were observed 10 days after infection. 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 or 10th day, using FISHER's exact method.

Results

In Vitro Synergistic Effects

MICs of FOM against strains No. 5, MSK-079 and IFO 3455 were 12.5 μg/ml, and that of DKB against strain IFO 3455 was 3.13 μg/ml. MOM and MDM did not show antibacterial activity against strains No. 5, MSK-079 and IFO 3455 because the MICs were over 100 μg/ml.

The potential synergistic effect was examined *in vitro*. Fig. 1 shows the synergistic effect of FOM and MOM against strain No. 5. FOM and MOM at final concentrations of 6.25 μg/ml (1/2 MIC) and 50 μg/ml were added simultaneously to the bacterial solution at 2.1×10^5 CFU/ml. The viable bacterial number was examined 1, 3 and 6 hours after addition of antibiotics. The number of viable bacteria was 1.6×10^3 CFU/ml in the culture to which both FOM and MOM had been added, as compared to 4.7×10^4 CFU/ml in the culture 6 hours after the addition of FOM alone. Thus the number of viable bacteria in the culture to which both FOM and MOM had been added underwent 1 log reduction compared to the culture to which FOM alone had been added, demonstrating a significant synergistic effect. The viable bacterial counts of the cultures to which a single dose of 50 μg/ml MOM alone was added and to which no antibiotic was added were both 1.5×10^9 CFU/ml. The growth of the culture containing MOM tended to increase under these conditions.

Fig. 1. Synergistic effect of fosfomycin and 9,3''-di-*O*-acetylmidecamycin against growth of *P. aeruginosa* No. 5.

FOM was added to the bacterial solution (2.1×10^5 CFU/ml) to give a final concentration 6.25 μg/ml (1/2 MIC) and MOM was then added to give a final concentration of 50 μg/ml. Samples were taken 1, 3 and 6 hours after addition of the antibiotics. The number of colony forming units (CFU) was calculated as the viable bacteria. The ordinate shows the number of viable bacteria and the abscissa the incubation time.

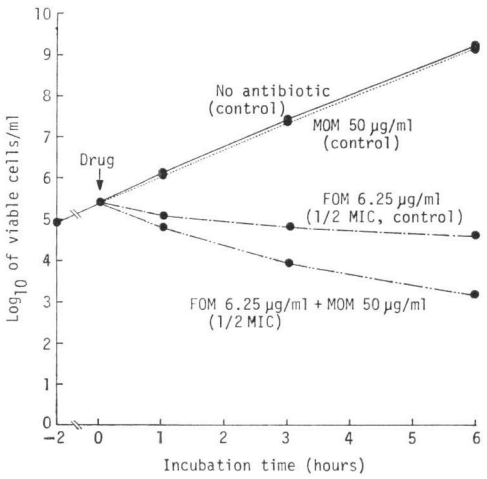


Fig. 2 shows the synergistic effect of FOM and MOM against strain MSK-079. Both FOM and MOM were added to the bacterial culture at 7.8×10^4 CFU/ml to the same final concentrations used in the experiment with strain No. 5. The synergistic effect was examined based on the number of viable bacteria at the same time after addition of antibiotics as in the case with strain No. 5. The number of viable bacteria observed 6 hours after the addition of FOM

Fig. 2. Synergistic effect of fosfomycin and 9,3″-di-*O*-acetylmidecamycin against growth of *P. aeruginosa* MSK-079.

FOM was added to the bacterial solution (7.8×10^4 CFU/ml) to give a final concentration of 6.25 (1/2 MIC) and MOM was added to give a final concentration of 50 μg/ml. For other conditions, see note to Fig. 1.

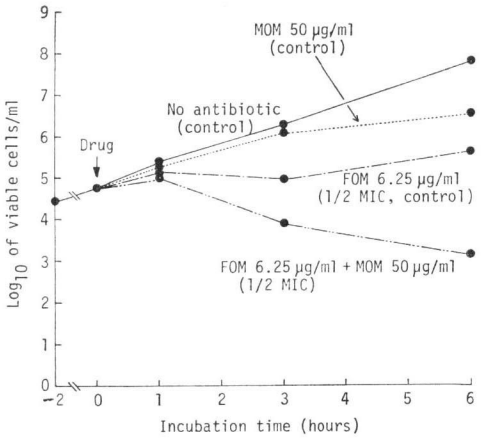
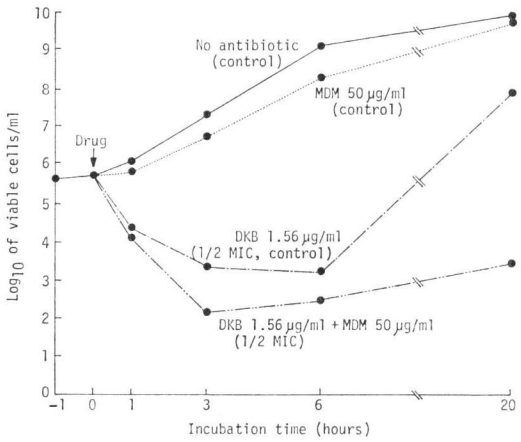


Fig. 3. Synergistic effect of dibekacin and midecamycin against growth of *P. aeruginosa* IFO 3455.

DKB was added to the bacterial solution (6.6×10^5 CFU/ml) to give a final concentration of 1.56 μg/ml (1/2 MIC). MDM was then added to give a final concentration of 50 μg/ml. Samples were taken 1, 3, 6 and 20 hours after addition of the antibiotics. For other conditions, see note to Fig. 1.



only was 5.4×10^5 CFU/ml. However, it was 2.2×10^3 CFU/ml following the addition of both FOM and MOM. Thus the number of viable bacteria in the culture containing both FOM and MOM underwent 2 log reduction compared to that which contained FOM only, and significant synergistic effect was demonstrated. The number of viable bacteria following the single addition of MOM alone was 2.9×10^6 CFU/ml. The number of viable bacteria in the culture with no antibiotic was 7.0×10^7 CFU/ml. Thus the number of viable bacteria tended to decrease in the culture to which MOM alone had been added.

Fig. 3 shows the synergistic effect of DKB and MDM against strain IFO 3455. DKB at a final concentration of 1.56 μg/ml (1/2 MIC) and MDM at a final concentration of 50 μg/ml were added simultaneously to the bacterial culture at 6.6×10^5 CFU/ml. One, 3, 6 and 20 hours after addition of antibiotics, their synergistic effect was evaluated by estimating the number of viable bacteria. The number of viable cells was 1.5×10^2 CFU/ml in the culture to which DKB and MDM had been added, as compared to 2.3×10^8 CFU/ml in the culture 3 hours after the addition of DKB alone. Moreover, the viable cells in the culture with both DKB and MDM was observed to decrease as compared with the culture with only DKB. At 20 hours after addition of antibiotics, the number of viable cells in the culture to which both DKB and MDM had been added was 3.5×10^3 CFU/ml as compared to 9.1×10^7 CFU/ml in the culture containing DKB alone. Thus a marked synergistic effect was demonstrated. The growth of the culture containing DKB alone showed a tendency of inhibition until 6 hours, but 20 hours after the addition of the antibiotic, bacteria grew again at the same rate as noted in the culture without antibiotic.

In Vivo Synergistic Effects

In vivo synergistic effect was studied in mice infected with *P. aeruginosa*. Antibiotics were admini-

Table 1. Synergistic effect of fosfomycin and 9,3''-di-*O*-acetylmidecamycin against *P. aeruginosa* strain No. 5 or MSK-079 infection in mice.

Experiment No.	Infective strain	Inoculated amount (viable bacteria per mouse)	Infection model*2	Number of times antibiotics administered	Group	Dose (once per mouse)	Protection rate (%)*3	Probability value
1	No. 5	1.6×10^8 (40 MLD*1)	i.p.	6 times on day of infection	A	FOM 0.2 mg+ MOM 0.0125 mg	80(8/10)	A : B p=0.08
					B	FOM 0.2 mg	40(4/10)	
					C	MOM 0.0125 mg	0(0/10)	
					D	No antibiotic	0(0/10)	
2	No. 5	1.3×10^8 (30 MLD)	i.p.	7 times on day of infection	A	FOM 0.2 mg+ MOM 0.025 mg	100(20/20)	A : B p=0.02
					B	FOM 0.2 mg	75(15/20)	
					C	MOM 0.025 mg	0(0/10)	
					D	No antibiotic	0(0/10)	
3	MSK-079	2.9×10^8 (1 MLD)	s.c. with 0.5% carrageenan	9 times over three days of infection	A	FOM 0.1 mg+ MOM 0.4 mg	93.3(14/15)	A : B p=0.02
					B	FOM 0.1 mg	53.3 (8/15)	
					C	MOM 0.4 mg	0(0/10)	
					D	No antibiotic	0(0/10)	

*1 MLD: Minimum lethal dose.

*2 i.p.: Intraperitoneal infection. s.c.: Subcutaneous infection.

*3 Parentheses indicate surviving mice per total mice.

Viable bacterial solution was inoculated to mice. MOM in the amounts indicated was administered orally followed by FOM administered subcutaneously in the amounts indicated. FOM and MOM were administered repeatedly before and after the inoculation of viable bacteria. The controls received only MOM or FOM or a solution containing no antibiotic. The animals were observed for 7 or 10 days and the protection rate was calculated based on the rate of surviving mice to total number used in experiment. The probability (p) was also calculated for comparison.

stered to mice infected with bacteria and their synergistic effect was examined by observation of the number of surviving mice.

Table 1 shows *in vivo* synergistic effects of FOM and MOM against strain No. 5 or MSK-079 infection in mice. In the first experiment, 40 MLD of bacterial suspension was injected to mice intraperitoneally. FOM at 0.2 mg/mouse and MOM at 0.0125 mg/mouse were administered a total of 6 times on the day of infection with strain No. 5, *i.e.*, once before infection, once immediately after bacterial inoculation and four times after that. In the second experiment, mice were injected with 30 MLD bacterial suspension FOM at 0.2 mg/mouse and MOM at 0.025 mg/mouse were administered a total of 7 times in a manner similar to that of experiment 1. In the third experiment, mice were injected with 0.5% carrageenan solution containing MLD bacterial suspension of strain MSK-079. FOM at 0.1 mg/mouse and MOM at 0.4 mg/mouse were administered a total of nine times over three days of subcutaneous infection, *i.e.*, once before injection of bacteria, once immediately after infection, 3 times on the day of bacterial inoculation, twice on the second day of infection, and twice on the third day. In the intraperitoneal infection, the number of surviving animals in the group treated with FOM and MOM was significantly higher than in the group treated with FOM alone. Thus synergistic effect between FOM and MOM was clearly observed ($p=0.08$, 0.02). Similar synergistic effect of FOM and MOM was observed in the subcutaneous infection ($p=0.02$).

Fig. 4. Survival rates of mice infected with *P. aeruginosa* MSK-079 following 9 administrations of fosfomycin and 9,3″-di-*O*-acetylmidecamycin.
See Experiment 3 of Table 1 for experimental conditions.

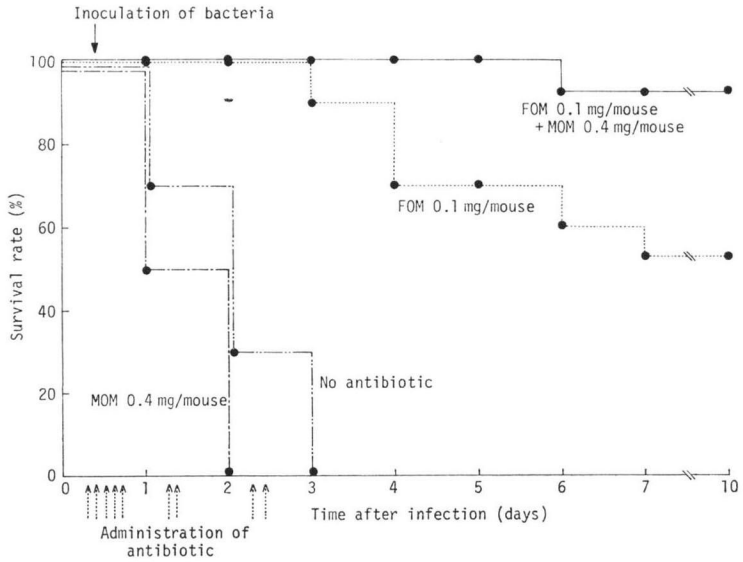
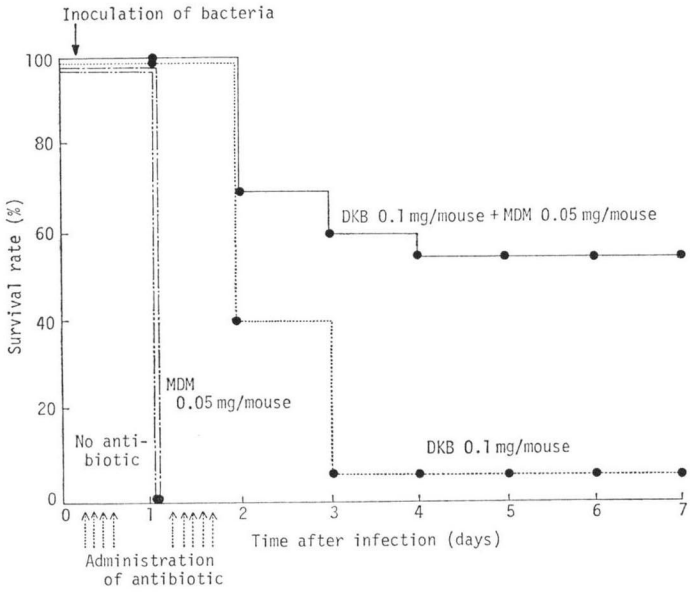


Fig. 5. Survival rate in mice infected with *P. aeruginosa* IFO 3455 following 9 administration of DKB and MDM. See Experiment 7 of Table 2 for experimental conditions.



In experiment 1, the control mice treated with only the macrolide antibiotic died as did the control mice treated with no antibiotic on the first day of bacterial inoculation. Experiment 2 showed similar survival rates. In experiment 3, the control mice treated with a macrolide or no antibiotic died on the third day after bacterial infection. Fig. 4 shows the change of survival rates of mice infected with strain MSK-079 following 9 administrations of FOM and MOM.

Table 2. Synergistic effect of dibekacin and midecamycin against mice infected with *P. aeruginosa* IFO 3455.

Experiment No.	Inoculated amount (viable bacteria per mouse)	Infection model	Number of times antibiotics administered	Group	Dose (once per mouse)	Protection ratio (%)*2	Probability value
4	7.5×10^7 (2 MLD*1)	i.p.	5 times on the 1st day of infection	A	DKB 0.1 mg+ MDM 2 mg	40(4/10)	A: B p=0.04
				B	DKB 0.1 mg	0(0/10)	
				C	MDM 2 mg	0(0/5)	
				D	No antibiotic	0(0/5)	
5	9.8×10^4 (9,800 MLD)	Burn infection	3 times on the 1st day and 5 times on the 2nd day of infection	A	DKB 0.1 mg+ MDM 0.2 mg	60(6/10)	A: B p=0.08
				B	DKB 0.1 mg	20(2/10)	
				C	MDM 0.2 mg	0(0/5)	
				D	No antibiotic	0(0/5)	
6	1.1×10^5 (11,000 MLD)	Burn infection	3 times on the 1st day and 5 times on the 2nd day of infection	A	DKB 0.1 mg+ MDM 0.2 mg	53.3(16/30)	A: B p=0.03
				B	DKB 0.1 mg	26.7(8/30)	
				C	MDM 0.2 mg	0(0/10)	
				D	No antibiotic	0(0/10)	
7	1.2×10^5 (12,000 MLD)	Burn infection	4 times on the 1st day and 5 times on the 2nd day of infection	A	DKB 0.1 mg+ MDM 0.05 mg	55(11/20)	A: B p=0.0006
				B	DKB 0.1 mg	5(1/20)	
				C	MDM 0.05 mg	0(0/20)	
				D	No antibiotic	0(0/20)	

*1 MLD: Minimum lethal dose.

*2 Parentheses indicate surviving mice per total mice.

Viable bacterial solution was inoculated to mice. MDM in the amounts indicated was administered orally followed by DKB administered intramuscularly in the amounts indicated. DKB and MDM were administered repeatedly before and after the inoculation of viable bacteria. For other conditions, see note to Table 1.

Table 2 shows *in vivo* synergistic effects of DKB and MDM against mice infected with strain IFO 3455. In the fourth experiment, bacterial suspension containing 2 MLD live cells were injected to mice intraperitoneally. Antibiotic treatment with 0.1 mg/mouse DKB and 2 mg/mouse MDM was performed 5 times on the day of infection; once prior to the bacterial inoculation and 4 times at intervals of 2 hours after. In the fifth experiment, MDM at 0.2 mg/mouse was administered 1 hour before the 9,800 MLD bacterial inoculation at the burn site and DKB at 0.1 mg/mouse was injected immediately after the bacterial inoculation. The same amount of DKB and MDM was administered 2 more times at intervals of 2 hours on the first day after bacterial inoculation. The sixth experiment was done using the same procedure as the fifth experiment but using a larger number of mice and 11,000 MLD bacterial inoculum. In the seventh experiment, DKB at 0.1 mg/mouse and MDM at 0.05 mg/mouse were administered a total of 8 times; once prior to the 12,000 MLD bacterial inoculation at the burn site and 3 times after the inoculation at 2-hour intervals on the day and 5 times on the first day after bacterial inoculation. In the intraperitoneal infection, the number of surviving animals of the group administered DKB and MDM repeatedly was significantly higher than the group treated with DKB alone, indicating a synergistic effect ($p=0.04$). Synergistic effect between DKB and MDM was also observed in the burn infection model ($p=0.08$, 0.03, 0.0006).

The control mice in all experiments died on the first day of the experiment. One example of survival rate after challenge is shown in Fig. 5. All 4 experiments showed similar trends.

Discussion

The authors previously reported that synergistic effects between a cell wall-affecting antibiotic, such as polymyxin B and colistin methansulfonate, and a macrolide antibiotic against *P. aeruginosa* were observed *in vitro* and *in vivo*.[2] However, the use of peptide antibiotics has been limited in clinical medicine because of its side effects. Because DKB, an aminoglycoside antibiotic, is more commonly used clinically and affects the cellular surface of bacteria, the synergistic effect of DKB and the macrolide antibiotic MDM was investigated in the present experiments.

As for FOM, KAHAN *et al.*[5] confirmed that it inhibits the early stage of cell wall synthesis of bacteria and demonstrates bactericidal action. This indicates that FOM produces spheroplasts as does the penicillin group of antibiotics. KAWAHARAJO *et al.*[1] reported that a macrolide antibiotic showed antibacterial activity against spheroplasts of *P. aeruginosa* induced by carbenicillin. Thus it seems that the synergistic action of FOM and MOM is probably similar to that of carbenicillin and a macrolide antibiotic.

In the case of chronic respiratory tract infection due to *P. aeruginosa,* the usual administration of antibiotics to patients over a long period of time does not result in a complete cure. In this study, it was confirmed that the combined use of a very small amount of MOM or MDM and cell wall-affecting antibitoic, FOM or DKB, was effective against experimental mouse infection due to *P. aeruginosa.* MASHIMO *et al.*[6] reported that the combined use of β-lactam antibiotics and MOM against chronic infections due to *P. aeruginosa* is an effective method of chemotherapy. In some cases *P. aeruginosa* were eradicated and in others clinical symptoms improved.

The results of this investigation indicate that the combined use of cell wall-affecting antibiotic, FOM or DKB, and a macrolide antibiotic, MOM or MDM, may be useful for treating chronic respiratory tract infection due to *P. aeruginosa*.

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