SYNERGISTIC EFFECT OF CEPHALEXIN WITH MECILLINAM

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In vitro and *in vivo* synergistic effects of cephalexin and mecillinam against *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* sp., *Serratia marcescens* and *Proteus* sp. were demonstrated and their action mechanism were also discussed.

The growth curve after the exposure of cephalexin and mecillinam at the concentrations at which these antibiotics had no effects when given alone showed a decrease of the turbidity and the presence of a bactericidal effect. In experimental infection in mice, the combination of both drugs showed a synergistic effect and excellent therapeutic effect. The blood concentration ratio of cephalexin to mecillinam was coincident with the concentration ratio of these antibiotics at which the synergistic effect was observed *in vitro*. Phase-contrast and scanning electron micrographs of bacterial cells exposed to the combination of cephalexin and mecillinam showed somewhat elongated bacteria and formation of spindle cells with swelling in the central part. A leakage of the cellular contents from part of the swelled cell wall was observed by transmission electron microscope. Cephalexin showed an affinity for penicillin binding proteins (PBPs)-1a and 3 in *Escherichia coli* and mecillinam showed an affinity for PBP-2. When these antibiotics were used concurrently, they exerted an additive effect to increase the affinity for PBPs. The lytic activity was increased much more after the combination of two antibiotics than after a single exposure.

Recently, the relationship between penicillin binding proteins (PBPs) and morphological changes of the bacterial cells has called much attention to the mechanism of penicillin action. In 1975, SPRATT[1] reported six proteins localized on the cytoplasmic membrane of *Escherichia coli*, which have affinity for β-lactam antibiotics and suggested that PBP-1, PBP-2 and PBP-3 were essential for the elongation of the cell, determination of morphology and cell division, respectively. This led to the demonstration of the correlation between PBPs and morphological changes of the cell. PBP-1 was further separated into components of PBP-1a and PBP-1bs. If PBP-1, PBP-2 and PBP-3 are inhibited by drugs, every inhibitory action causes lysis of bacteria, formation of ovoid cells and elongation of the cell, respectively. Cephalexin has a strong affinity for PBP-3 of *E. coli*, and the cells exposed to cephalexin formed filamentous cells[2]. Mecillinam has a unique affinity for PBP-2, and the cells exposed to mecillinam formed ovoid cells[3,4].

Accordingly, we were interested in the difference of morphological changes of *E. coli* exposed to cephalexin and mecillinam and studied the morphological changes of the cells and the changes of antibacterial activities after the exposure of the combination of these two antibiotics. We also discuss the mechanism of this combination action.

Materials and Methods

Bacterial Strains

One strain of *Staphylococcus aureus*, 19 strains of *E. coli*, 13 strains of *Klebsiella pneumoniae*, 5 strains of *Enterobacter aerogenes*, 6 strains of *Enterobacter cloacae*, 11 strains of *Serratia marcescens*, 11 strains of *Proteus vulgaris*, 12 strains of *Proteus mirabilis*, 11 strains of *Proteus morganii* and 1 strain of

Proteus rettgeri were used for the experiment.

Antibiotics

Cephalexin (CEX) was supplied by Shionogi Pharmaceuticals Co., Ltd., mecillinam (MPC) and pivmecillinam (PMPC) were from Takeda Pharmaceuticals Co., Ltd., and benzylpenicillin (PCG) was from Meiji Seika, Ltd. ^{14}C-Benzylpenicillin (^{14}C-PCG) was provided by the Radiochemical Center (Amersham, England).

Synergistic Effect

The synergistic effect between CEX and MPC was first examined by the checkerboard titration method using heart infusion agar (Nissui). For the bactericidal effect of the combination of CEX with MPC, the bacteria were cultured by Biophotometer BIO-LOG II (JASCO) using heart infusion broth (Nissui), and the drug was added to the log-phase culture. Time-course changes of the turbidity and viable counts were determined.

Therapeutic Effect on Experimental Infection

E. coli ST-0198 or *K. pneumoniae* KC-1 which were mixed with the same amount of 6% gastric mucin (Orthana-Kemisk-Fabrik-A/S) and inoculated intraperitoneally into 10 *dd*Y male mice in each group. CEX, PMPC or both were administered orally 2 hours after the bacterial inoculation and the survival rate was determined 7 days later. The ED_{50} was calculated according to the LICHFIELD-WILCOXON method[5].

Blood Concentration

CEX or PMPC was administered orally to *dd*Y male mice. Blood was collected from four mice per group at different time intervals, and the serum was separated. For the measurement of CEX, *Micrococcus luteus* ATCC 9341 was used as a test organism and antibiotic medium 8 (Difco) was used[6]. For the determination of MPC, *E. coli* NIH J was used as the test strain[7].

Observation by Phase-contrast Microscopy

The film agar containing the drug was prepared on a slide glass, covered with a cover glass on which the bacterial solution was spread and then embedded with paraffin. This preparation was subjected to observation by phase-contrast microscope (Nikon, Japan).

Observation by Electron Microscopy

The drug was added to a culture of log-phase growth and the bacteria were collected at different time intervals. They were fixed by KELLENBERGER's method[8] and dehydrated in a graded series of ethanol. For scanning electron microscopy, the bacteria were dried using the critical point drying method[9] and evaporated with carbon and gold. The surface structure of the bacteria was observed with a JSM-35 (JEOL, Ltd., Japan). For transmission electron microscopy, the bacteria were dehydrated, embedded with epoxy resin according to LUFT's method[10] and sectioned with ultramicrotome 4801 A (LKB, Sweden). The preparation was stained with uranyl acetate and lead citrate[11] and subjected to observation by Akashi S-500 (Japan).

Formation of Spheroplasts

The formation of spheroplasts was observed in the bacteria exposed to the drug for 4 hours in heart infusion broth (stabilized) containing 20% sucrose and 0.2% $MgCl_2$ or in the same medium containing no sucrose and $MgCl_2$ (shocked).

Affinity for Penicillin Binding Proteins

The affinity of the antibiotics for penicillin binding proteins in *E. coli* K-12 was observed by the competition method with ^{14}C-PCG according to SPRATT's method[1].

Lytic Activity in the Buffer

The decrease of the turbidity was measured at the wave length of 550 nm after the bacterial solution was suspended in the 50 mM phosphate buffer (pH 6.0, 7.0 and 8.0), which had been exposed to the drug for 25 minutes at the log-phase growth.

Results

Examination of Synergistic Effect by the Checkerboard Titration Method

Table 1 shows the synergistic effect between CEX and MPC against various bacteria. A bacterial suspension of 10^6 cells/ml was inoculated using a platinum loop. The arrow in the table shows the combination ratio of CEX to MPC at which the synergistic effect occurs and represents the ratio of CEX to MPC showing the minimum fractional inhibitory concentration index (min. FIC index)[12]. The minimum FIC index was less than 0.5 in 16 out of 21 strains and in the range of 0.531 to 0.75 in the remaining 5 strains. Synergistic effects between CEX and MPC were observed against a total of 21 strains. The combination ratio of CEX to MPC showing synergistic effects varied depending on the strain and the combination ratio varied greatly even with the same strain. The synergistic effect was frequently observed in the combination ratio of CEX to MPC of 8: 1 and 64: 1.

Susceptibility of Various Bacteria to the Combination of CEX and MPC at 8: 1 and 32: 1

As a synergistic effect between CEX and MPC was observed by the checkerboard titration method, the presence of synergistic effects against various bacteria was determined at a constant combination ratio of 8: 1 or 32: 1. The results are shown in Tables 2 and 3 and Fig. 1 (inoculum size; 10^8 cells/ml). Tables 2 and 3 show the susceptibility distribution against clinical isolates when the combination ratio was 8: 1 and 32: 1, respectively. Fig. 1 shows the cumulative susceptibility distribution against 89 strains. The

Table 1. Combination effect of CEX and MPC on various organisms.

Organisms \ Ratio CEX / MPC	≥2048 1	1024 1	512 1	256 1	128 1	64 1	32 1	16 1	8 1	4 1	2 1	1 1	1 2	1 4	1 8	1 16	1 32	1 64
1. *S. aureus* 209-P JC													←*→					
2. *E. coli* NIH JC-2							←*→											
3. *E. coli* NIH	←*→																	
4. *E. coli* K-12				←*→														
5. *E. coli* No.29				←*→														
6. *E. coli* ST-0198						←*→												
7. *E. coli* KC-14					←*→													
8. *E. coli* 167				←*→														
9. *K. pneumoniae* KC-1						←*→												
10. *K. pneumoniae* NCTC 9632								←*→										
11. *K. pneumoniae* 178	←*→																	
12. *S. marcescens* IFO 3736					←*→													
13. *S. marcescens* T-55	←*→																	
14. *E. aerogenes* KC-1							←*→											
15. *E. cloacae* NCTC 9394							←*→											
16. *P. vulgaris* OX-19					←*→													
17. *P. vulgaris* 101				←*→														
18. *P. mirabilis* 1287					←*→													
19. *P. mirabilis* 181						←*→												
20. *P. morganii* Kono	←*→																	
21. *P. rettgeri* NIH 96					←*→													
No. of minimum FIC index			1	2	1	2	4	2	4	1	1		1	2				
No. of addition and synergism	3	7	7	11	9	14	16	16	12	11	11	10	9	9	7	5	4	3

Medium: heart infusion agar.

* Represents the ratio of minimum FIC index of CEX to that of MPC.
Horizontal arrows indicate the range of the synergistic effect.

Table 2. Susceptibility of several bacterial species to the combination of CEX and MPC.

Organisms	Drug	MIC (μg/ml) 0.19	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100	200	400	800	>800	Total strains
	CEX	0.025	0.05	0.1	0.19	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100	200	400	800	>800	
E. coli	CEX						2	15	2										
	MPC			2	2	6		1				2	1			2		3	19
	CEX + MPC			6	6	1	3	3											
K. pneumoniae	CEX						10	1	2										
	MPC					1											2	10	13
	CEX + MPC			1	9	1	1		1										
Enterobacter sp.	CEX								1						10				
	MPC				1		1									2	2	5	11
	CEX + MPC				1	3		2	1				1	1	2				
S. marcescens	CEX													1	10				
	MPC																	11	11
	CEX + MPC											1	1	8	1				
P. vulgaris	CEX									2		5	2		2				
	MPC														1		4	6	11
	CEX + MPC					1	1	3	1	2	1				2				
P. mirabilis	CEX							5	5	2									
	MPC														2	2	8		12
	CEX + MPC						2	10											
P. morganii	CEX									1			2	7	2				
	MPC																2	10	12
	CEX + MPC							2	1	1	1	2	2	2	1				

CEX:MPC = 8:1
Inoculum size: 10^8 cells/ml

Table 3. Susceptibility of several bacterial species to the combination of CEX and MPC.

Organisms	Drug	MIC (μg/ml) 0.78	1.56	3.13	6.25	12.5	25	50	100	200	400	800	>800	Total strains
	CEX	0.025	0.05	0.1	0.19	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100	200	400	800	>800	
E. coli	CEX				2	15	2												
	MPC			2	2	6		1				2	1			2		3	19
	CEX + MPC		4	5	5	5													
K. pneumoniae	CEX				10	1	2												
	MPC					1											2	10	13
	CEX + MPC			10	2	1													
Enterobacter sp.	CEX						1						10						
	MPC				1		1									2	2	5	11
	CEX + MPC				1	2		2	2		1	1	2						
S. marcescens	CEX											1	10						
	MPC									2								11	11
	CEX + MPC											5	4						
P. vulgaris	CEX							2		5	2		2						
	MPC														1		4	6	11
	CEX + MPC				2	1	2	1	3				2						
P. mirabilis	CEX					5	5	2											
	MPC														2	2	8		12
	CEX + MPC			2	6	4													
P. morganii	CEX							1			2	7	2						
	MPC																2	10	12
	CEX + MPC						4		1	1	3	2	1						

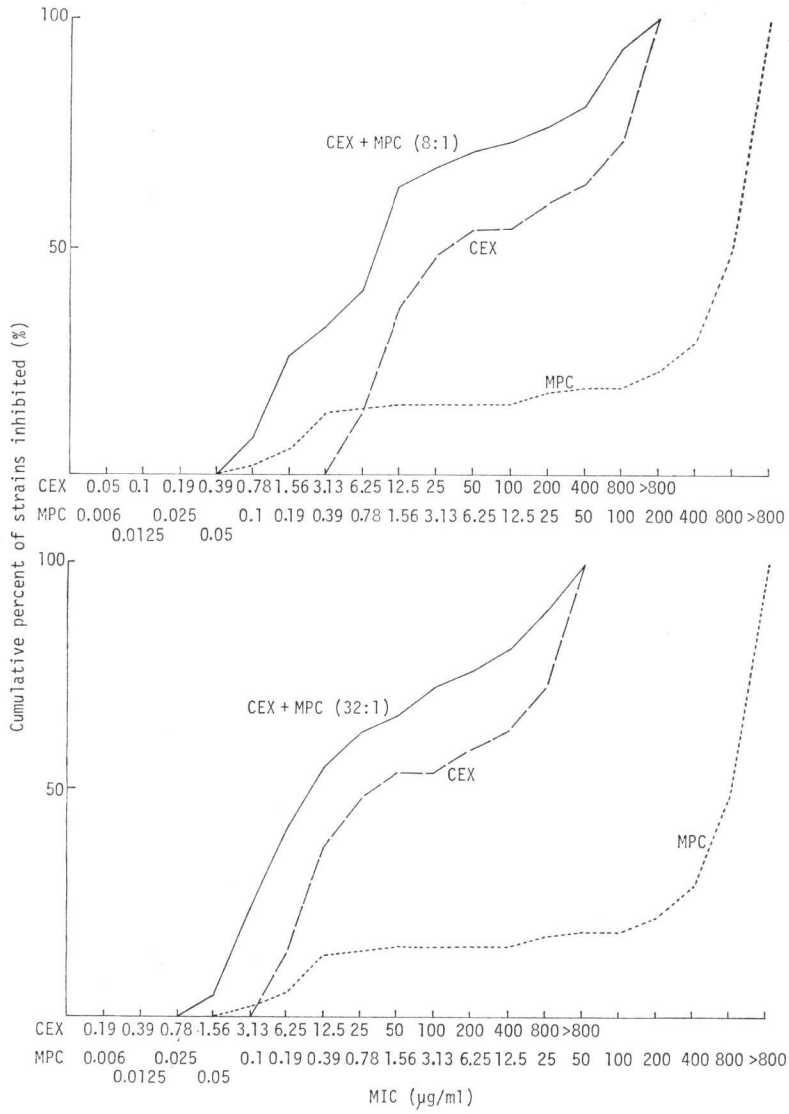
CEX:MPC = 32:1
Inoculum size: 10^8 cells/ml

addition of MPC increased the antibacterial activity of CEX by 2 to 32-fold and 2 to 16-fold at the combination ratio of 8: 1 and 32:1, respectively. Similar synergistic effects were observed when the inoculum size was 10^6 cells/ml. Based on the FIC index, potentiation was observed; about 50% at the combination ratios of 8: 1 and 32: 1 (inoculum size; 10^8 cells/ml) and about 60% and 45% at the combination ratio of 8: 1 and 32: 1, respectively (inoculum size; 10^6 cells/ml). When additive effects were included, a total of 80% showed combination effects.

Lysis and Bactericidal Effect under the Combination of CEX and MPC

Figs. 2 and 3 show the lytic activity and bactericidal effects of the combination of the drugs against *E. coli* ST-0198 at 2.5×10^6 and 1.6×10^7 cells/ml. When the cells were exposed to the combination of

Fig. 1. Cumulative percentage of 89 strains susceptible to the combination of CEX and MPC. The inoculum size was 10^8 cells/ml.



CEX and MPC, the turbidity and viable count decreased significantly at either inoculum size, which indicates that the synergistic effect occurs between CEX and MPC.

Therapeutic Effects on Experimental Infection in Mice

Table 4 shows ED_{50} of the drug against experimental infections in mice. For *E. coli* ST-0198 infection, two drugs were given at the combination ratio of 8: 1 (CEX: MPC) and for *K. pneumoniae* KC-1 infection, they were given at the combination ratio of 4: 1. This combination ratio was determined based on the ED_{50} of each antibiotic given alone. The ED_{50} of the combination of antibiotics was less than that of either antibiotic. The FED (fractional effective dose) index was 0.55 for *E. coli* ST-0198 and 0.33 for *K. pneumoniae* KC-1.

Fig. 2. Combination effect of CEX and MPC on the growth curve of *E. coli* ST-0198.
The drug was added at the time shown by the arrow.
1, Control. 2, MPC 0.1 μg/ml. 3, CEX 6.25 μg/ml. 4, MPC 0.05 μg/ml. 5, CEX 3.13 μg/ml. 6, CEX 6.25 μg/ml+MPC 0.1 μg/ml. 7, CEX 3.13 μg/ml+MPC 0.05 μg/ml.

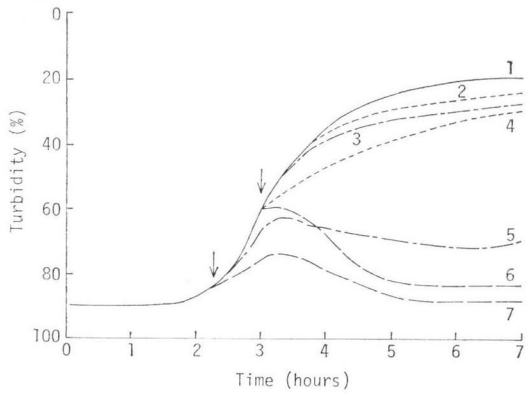
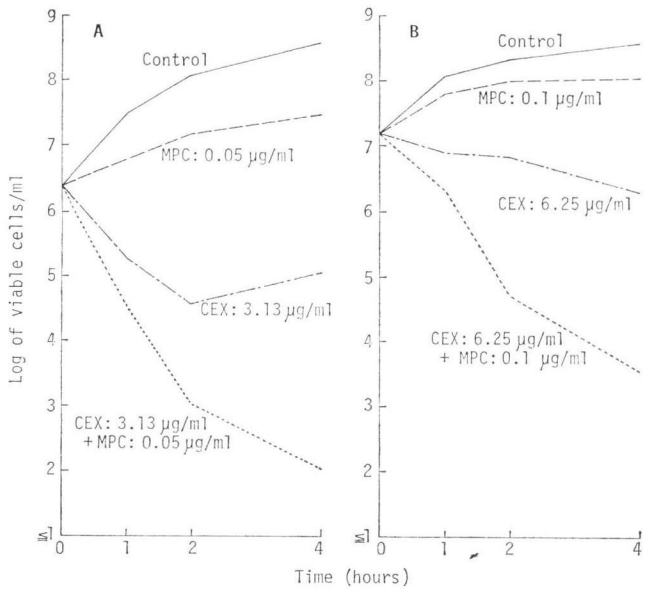


Fig. 3. Combination effect of CEX and MPC on the viability of *E. coli* ST-0198.
(A) shows the changes of viable cells when the drug was added to the culture of 2.5×10^6 cells/ml.
(B) shows the changes of viable cells when the drug was added to the culture of 1.6×10^7 cells/ml.



Blood Concentration

Table 5 shows the blood concentration ratio of CEX to MPC at 15, 30, 60, 90, 120 and 180 minutes in mice. CEX was given orally at doses of 2, 0.5 and 0.125 mg/mouse and PMPC was given orally at doses of 2, 0.125 and 0.0156 mg/mouse. Either drug reached the peak level 15 minutes later. When the drug was given at the minimum effective doses required for 100% survival (0.125 mg/mouse of CEX and 0.0156 mg/mouse of PMPC for *E. coli* ST-0198 infection, and 0.5 mg/mouse of CEX and 0.125 mg/mouse of PMPC for *K. pneumoniae* KC-1 infection), the blood concentration ratio of CEX to MPC was

Table 4. Combination effect of CEX and MPC on experimental infections with *E. coli* ST-0198 and *K. pneumoniae* KC-1 in mice.

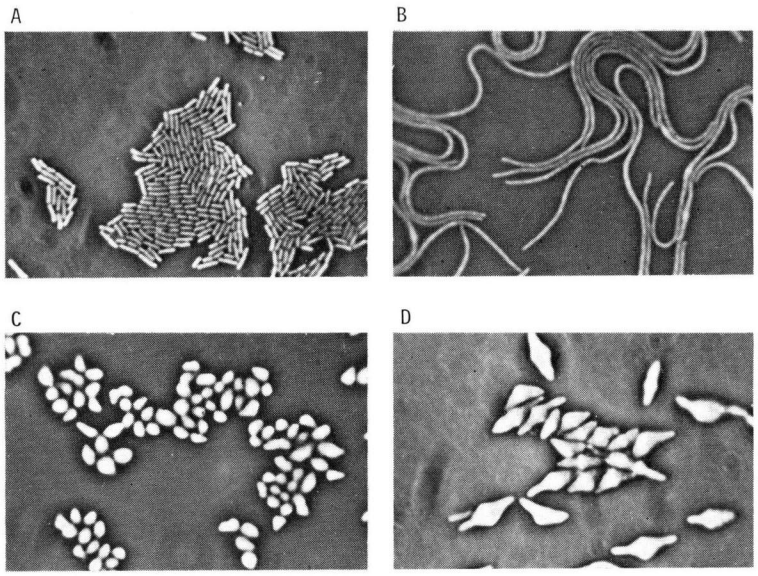
Organisms	ED_{50} (mg/mouse)			Ratio[a]
	CEX	MPC	CEX+MPC	
E. coli ST-0198	0.15	0.017	0.039+0.0049	(8 : 1)
K. pneumoniae KC-1	0.90	0.27	0.16 +0.040	(4 : 1)

[a] The combination ratio of CEX to MPC.

Table 5. The ratio of serum levels of CEX and MPC after single oral dose to mice.

Dose (mg/mouse)			The ratio of serum levels of CEX to MPC					
CEX : PMPC	CEX	PMPC	15 min.	30 min.	60 min.	90 min.	120 min.	180 min.
1 : 1	2.0	2.0	0.84	3.78	4.11	1.94	1.77	3.33
	0.125	0.125	1.31	2.21	2.29	2.50	—	—
4 : 1	0.5	0.125	5.12	7.93	10.0	6.67	3.81	10.0
8 : 1	0.125	0.0156	6.73	15.24	27.5	20.0	—	—

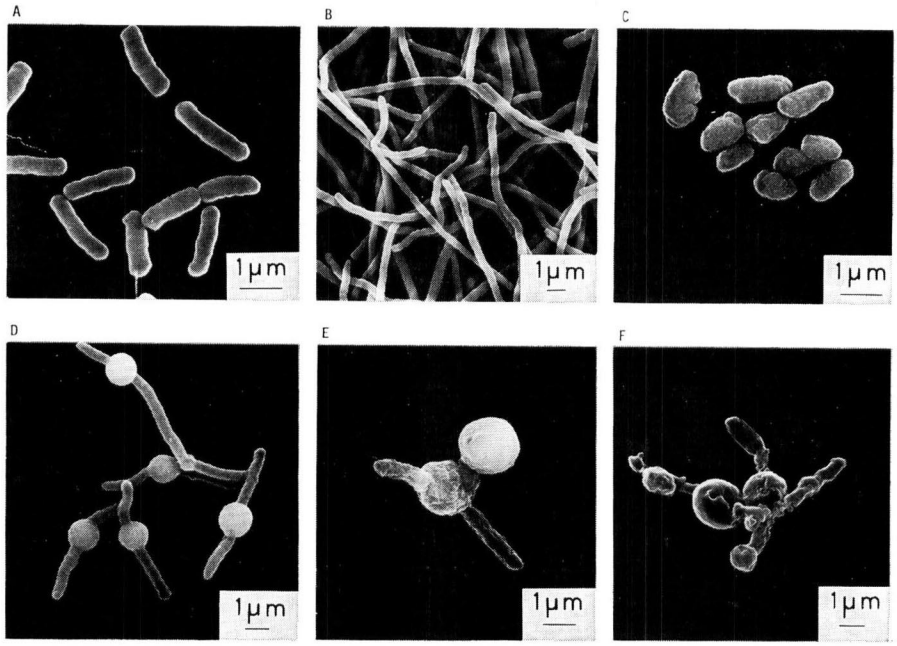
Fig. 4. Phase-contrast micrographs of *E. coli* ST-0198 exposed to CEX, MPC and the combination for 2 hours.
(A) Normal cells.
(B) Filamentous cells were observed following the exposure to CEX at 3.13 μg/ml.
(C) The formation of ovoid cells was observed after the addition of MPC at 0.05 μg/ml.
(D) The formation of spindle cells was observed in the presence of CEX (3.13 μg/ml) and MPC (0.05 μg/ml).



between 6.73: 1 and 27.5: 1 (average, 17.4: 1) for the former case and between 3.81: 1 and 10: 1 (average, 7.3: 1) for the latter case, respectively. These ratios are in the range at which the *in vitro* synergistic effect was observed.

Fig. 5. Scanning electron micrographs of *E. coli* ST-0198 exposed to CEX, MPC and the combination.

(A) Normal cells.
(B) *E. coli* cells exposed to CEX at 3.13 μg/ml for 2 hours.
(C) *E. coli* cells exposed to MPC at 0.05 μg/ml for 2 hours.
(D) *E. coli* cells exposed to the combination of CEX (3.13 μg/ml) and MPC (0.05 μg/ml) for 1 hour.
(E) *E. coli* cells exposed to the combination of CEX (3.13 μg/ml) and MPC (0.05 μg/ml) for 2 hours.
(F) *E. coli* cells exposed to the combination of CEX (3.13 μg/ml) and MPC (0.05 μg/ml) for 4 hours.



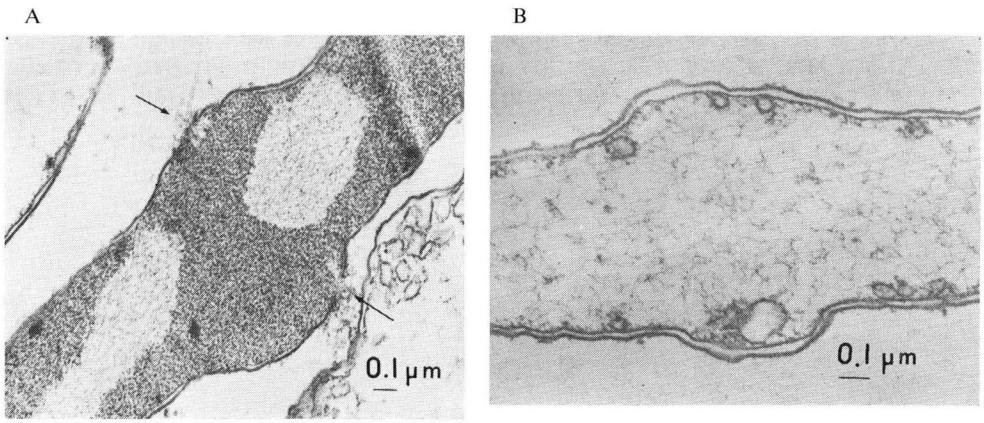
Observation of Morphological Changes by Phase-contrast Microscopy

Fig. 4 shows the morphological changes of *E. coli* ST-0198 exposed to the drug for 2 hours by phase-contrast microscopy. CEX at 3.13 μg/ml caused formation of filamentous cells and MPC at 0.05 μg/ml caused formation of ovoid cells. The combination of both drugs induced swelling of the central part of the bacterial cells and formation of spindle cells. Similar changes were seen both in *E. coli* K-12 and *K. pneumoniae* KC-1.

Observation of Morphological Changes by Electron Microscopy

Fig. 5 shows the observation by the scanning electron microscope. Fig. 5-A shows the normal *E. coli* ST-0198 cells observed with the scanning electron microscope. Figs. 5-B and 5-C indicate the morphological changes of the cells exposed to the combination of CEX at 3.13 μg/ml and MPC at 0.05 μg/ml for 2 hours. Figs. 5-D, 5-E and 5-F show the elongation and swelling of the central part of the bacteria which were exposed to the combination of the two drugs. Formation of a spheroplast-like structure was observed after exposure to both drugs for 2 hours and lyzed cells were observed after the exposure for 4 hours. Figs. 6-A and 6-B show transmission electron micrographs of the changes of the internal structure of the bacteria. In Fig. 6-A, the overlap of the bulge part with the cytoplasmic mem-

Fig. 6. Ultrathin sections of *E. coli* ST-0198 exposed to the combination of CEX (3.13 μg/ml) and MPC (0.05 μg/ml) for 1 hour.



brane is not seen, and the break of the cell wall and the leakage of cellular contents are seen (arrow). No special changes were seen in the cytoplasm. As shown in Fig. 6-B, membrane-like structures of lyzed bacteria were encountered frequently.

Formation of Spheroplasts

No significant difference was found between the osmotic shocked culture and stabilized culture of the control or bacteria exposed to CEX or MPC alone. However, when the bacteria were exposed to the combination of CEX and MPC, a significant difference was seen between the two cultures, and spheroplast formation was increased (Fig. 7). These results are consistent with the observation of morphological changes.

Fig. 7. Comparison of the viable counts of stabilized and osmotically shocked cultures.
The left side of this figure shows the viable bacterial counts of *E. coli* ST-0198 exposed to the drug for 4 hours at the initial bacterial count of 10^7 cells/ml and the right shows the viable count at the initial count of 10^6 cells/ml. A: stabilized culture, B: osmotically shocked culture.

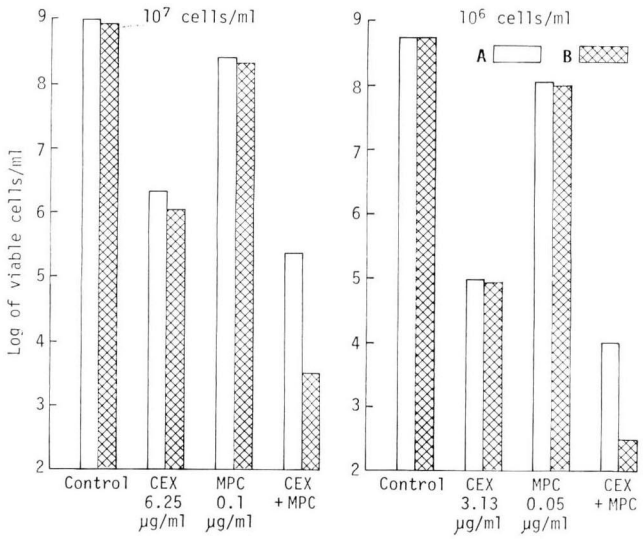


Fig. 8. Gel electrophoretic patterns of ^{14}C-benzylpenicillin binding proteins in *E. coli* K-12.

The membrane was treated with various β-lactam antibiotics at 30°C for 10 minutes and then treated with ^{14}C-PCG at 30°C for 10 minutes.

(A) CEX at 12.5 μg/ml, (B) CEX at 3.13 μg/ml, (C) CEX at 0.78 μg/ml, (D) control, (E) CEX at 0.78 μg/ml and MPC at 0.025 μg/ml, (F) CEX at 3.13 μg/ml and MPC at 0.1 μg/ml, (G) CEX at 12.5 μg/ml and MPC at 0.4 μg/ml, (H) MPC at 0.025 μg/ml, (I) MPC at 0.1 μg/ml, (J) MPC at 0.4 μg/ml and (K) MPC at 1.0 μg/ml.

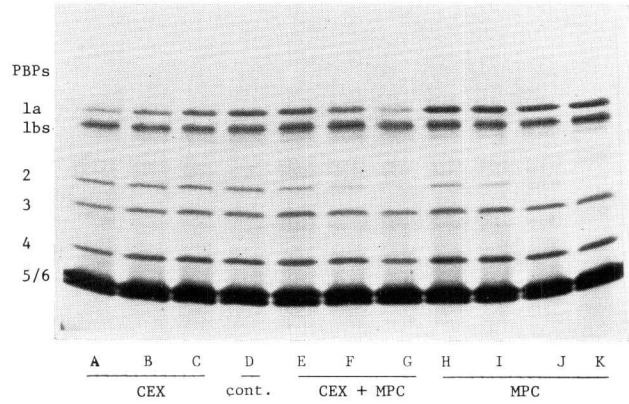
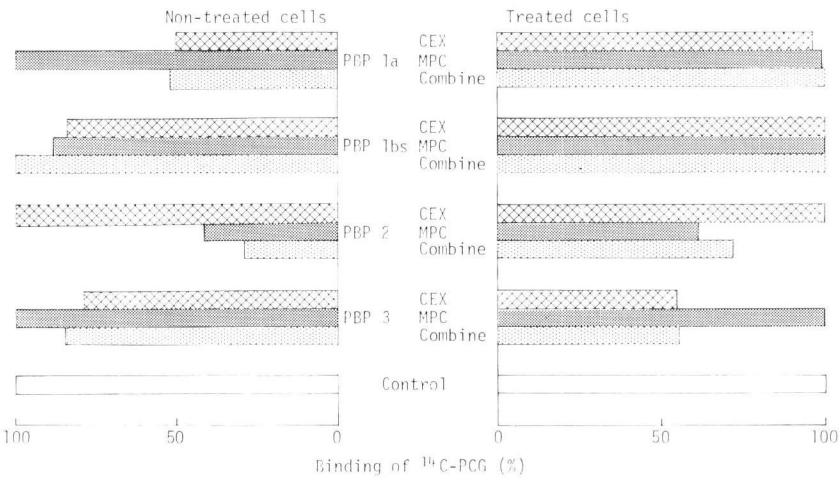


Fig. 9. Affinity of antibiotics for penicillin binding proteins from *E. coli* K-12.

For the non-treated cells, affinity was studied using the competition method with ^{14}C-PCG in the *E. coli* K-12 membrane. For the treated cells, the bacterial cells were exposed to CEX at 3.13 μg/ml, MPC at 0.1 μg/ml or both for 1 hour, and the affinity was measured by the competition method with ^{14}C-PCG in the membranes of the cells with morphological changes.

The control was defined to be 100%.



Affinity for Penicillin Binding Proteins

Fig. 8 shows the affinity for PBPs based on the competition with ^{14}C-PCG. When the bacteria were exposed to CEX, affinity for PBP-1a, 3, 1bs and 4 was observed, while exposure to MPC showed affinity for PBP-2. The affinity was measured using Dual-wavelength TLC scanners-90 (Shimadzu, Japan) and expressed as a percentage of the control, as shown in the left half of Fig. 9. The right half

Fig. 10. Effect of CEX, MPC and the combination on lysis of *E. coli* ST-0198 in phosphate buffer.

The lysis rate in 50 mM phosphate buffer was determined in *E. coli* ST-0198 exposed to the drug for 25 minutes and the lysis activity is expressed as the percent of the control at 0 minute (control, 100%).

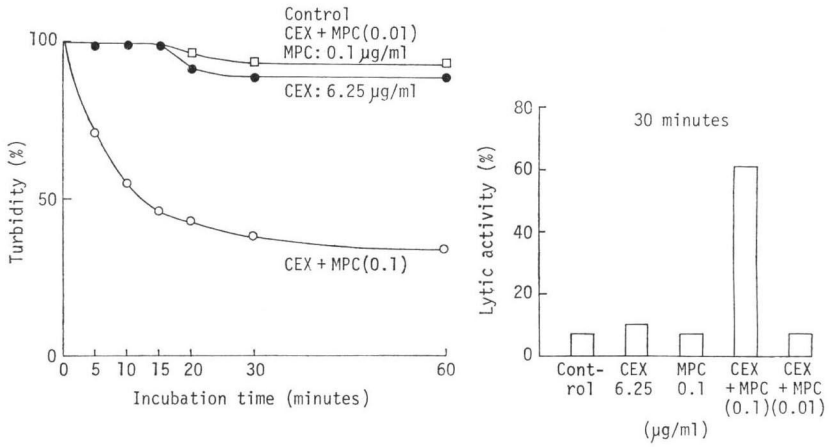
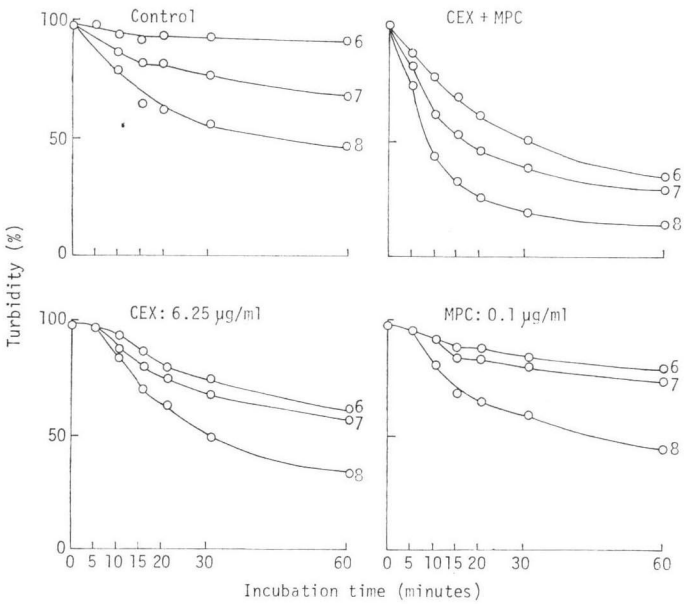


Fig. 11. Effect of CEX, MPC and the combination on cell lysis of *E. coli* ST-0198 in various pH of phosphate buffer.

The cell lysis of the bacteria exposed to the drug in 50 mM phosphate buffer at pH 6, 7 and 8 is expressed as the relative percentage of the control value at 0 minute (control, 100%).

The values 6, 7 and 8 in the figure represent the pH values.



of Fig. 9 shows the affinity of ^{14}C-PCG using the cytoplasmic membrane of *E. coli* K-12 cells with morphological changes after the exposure to each drug for one hour. When two drugs were used concomitantly, an affinity similar to that in the presence of single antibiotic was demonstrated.

Lysis in Phosphate Buffer

The lysis of *E. coli* ST-0198 was determined after the incubation in the drug-containing medium for

25 minutes at 37°C. The cells underwent the lysis rapidly 5 minutes after the exposure to both drugs at concentrations where each drug did not show lytic activity by itself, and about 60% of lytic activity was observed 30 minutes later (Fig. 10). Fig. 11 shows the lysis rate of the bacteria in 50 mM phosphate buffer at different pH values. In alkaline pH, the lytic activity was increased. Normal bacteria did not undergo lysis in the acid pH. When both drugs were combined, the lysis was accelerated even in the acid pH as compared with a single exposure.

Discussion

Various combinations of antibiotics and chemotherapeutic agent are now used in order to obtain an extended antibacterial spectrum, to increase antibacterial activity, to prevent the appearance of resistant bacteria, to decrease adverse reactions and to potentiate their therapeutic effects. There have been many reports on the synergistic action between mecillinam and penicillin or cephalosporin[13~16]. However little information is available about the mechanism of the synergistic action.

We studied the synergistic action between cephalexin and mecillinam which have mutually different actions on the morphology of *E. coli*. First, we determined whether there is synergistic action against various bacteria using the checkerboard titration method. The results showed that the combination of the drugs exerted synergistic actions against all strains. However, the ratio of CEX to MPC at which synergistic action occurred varied greatly depending on the strains. To confirm further the synergistic action between both drugs, antibacterial action was determined in the presence of both drugs. Bactericidal activities against various bacteria were significantly increased when both drugs were used concurrently at concentrations at which either drug showed no bactericidal action by itself. When the intervals of addition of two drugs were changed, a synergistic bactericidal effect appeared at every interval. We also studied *in vivo* synergistic action between two drugs against experimental infection in mice. FED indices against *E. coli* and *K. pneumoniae* were 0.55 and 0.33, respectively. The *in vivo* concentration ratio of CEX to MPC was 17.4: 1 or 7.3: 1; which was in the range of concentration at which *in vitro* synergistic action was observed. These results support a view that the synergistic effect of both drugs in fact occurs *in vivo*. The mechanism of synergistic effect of these two drugs was studied using *E. coli*.

At present, various factors such as the permeability to the outer membrane, stability to β-lactamase, affinity of antibiotics for the penicillin binding proteins (PBPs), and induction of a bacteria autolytic enzyme have been suggested for the determinants of antibacterial activity of β-lactam antibiotics. Especially, affinity for PBPs and induction of autolysin have received extensive attention. In the present study, we studied morphological changes of the bacteria exposed to the combination of cephalexin and mecillinam. The observations of morphological changes revealed filamentous cells caused by cephalexin and ovoid cells caused by mecillinam. The combination of both drugs induced formation of spindle cells. When the bacteria were exposed to the combination of both drugs, a bulge appeared at the central part of the elongated cells, and extrusion of a spheroplast-like structure from the bulge and lysis was observed. GREENWOOD[17] demonstrated by scanning electron microscopy that there were two action sites for penicillin in the *E. coli* and a bulge was formed into the filamentous cells. He thought that either a filamentous cell or ovoid cell was formed since cephalexin and mecillinam had only one action site. SPRATT[1] reported that the effects of β-lactam antibiotics on the cell division, cell elongation and cell shape of *E. coli* were attributable to the penicillin binding proteins-1, 2 and 3. He confirmed a close correlation between penicillin binding proteins and morphology of the bacteria, and studied the function of penicillin binding proteins extensively. MATSUHASHI *et al.*[18,19] suggest a correlation between penicillin binding proteins and cell division. As the combination of cephalexin with mecillinam caused an increase of bactericidal effect and lytic activity, there is a possibility that the combination of both drugs increases the affinity for other types of penicillin binding proteins which are not identical with those for which each antibiotic has affinity. However, it is demonstrated that two antibiotics have an additive affinity for penicillin binding proteins 2 and 3. The combination of mecillinam and cephalexin stimulates the formation of spheroplasts. The bacteria seem to easily undergo osmotic shock lysis when exposed to the combination of these drugs, and it is suggested that spindle cells may be involved in the lysis

of the bacteria. In the experiment on cell lysis in a phosphate buffer, the lytic activity was higher when exposed to the combination than when exposed to either antibiotic alone. In normal cells, the lysis occurred in the alkaline pH, while the lysis occurred in the acid pH when the bacteria were exposed to cephalexin or mecillinam. When the bacteria were exposed to the combination of both drugs, the lysis occurred in either pH, indicating that the combination of both drugs induces activation of one or more enzymes involved in the lysis. Based on these results, it is concluded that cephalexin and mecillinam show a synergistic effect against *E. coli* through inhibiting penicillin binding proteins 3 and 2 and cause the formation of spindle cells. As the morphology of spindle cells is hardly maintained and the cell wall of the bulge part is easily broken, the spheroplast is formed in the bulge and osmotic shock occurs. When the bacteria were exposed to the drug for short time (the time during which no morphological change was induced), the lysis of the cell was strongly induced. This may contribute to the synergistic effect of both drugs.

Recently, TOMASZ *et al.*[20~22] studied the relationship between penicillin binding proteins and lysis or cell death in *E. coli* and suggested three hypotheses; in their hypotheses, penicillin binding protein-1 was involved in cell death but the correlation between penicillin binding proteins 2 or 3 and cell death was not clarified.

The function of various penicillin binding proteins and the mechanism of bacterial lysis are now studied extensively using β-lactam tolerant mutants and PBP-deficient strains of *E. coli*[23,24]. It would be interesting to study how the inhibition of penicillin binding proteins 2 and 3 is involved in bacteriolysis.

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