RELEVANCE OF *IN VITRO* ANTIBACTERIAL ACTIVITIES AND PHARMACOKINETIC PROPERTIES OF ANTIPSEUDOMONAL β-LACTAM ANTIBIOTICS TO THEIR THERAPEUTIC EFFECTS ON URINARY TRACT INFECTION CAUSED BY *PSEUDOMONAS AERUGINOSA* P 9 IN MICE

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The therapeutic effects of seven antipseudomonal β-lactam antibiotics on experimental urinary tract infection caused by *Pseudomonas aeruginosa* P 9 in mice were compared, and the results were analyzed in relation to their *in vitro* antibacterial activities and pharmacokinetic properties.

The CD_{50} values were (mg/kg): cefsulodin, 6.19; cefoperazone, 162; sulbenicillin, 167; ticarcillin, 184; azlocillin, 121; mezlocillin, 390; and piperacillin, 227. Cefsulodin was more active than the other antibiotics not only in therapeutic effects but also in *in vitro* antibacterial effects evaluated according to growth inhibitory, bactericidal, and bacteriolytic activities. It also penetrated and persisted well in the kidney of mice. The therapeutic effects of cefoperazone, azlocillin, and piperacillin were much less than expected from their *in vitro* antibacterial activities; the CD_{50} values were more than 18-fold as large as that of cefsulodin, whereas the differences of their MIC values were less than four-fold.

In the past several decades, epidemics of severe life-threatening infectious diseases of bacterial origin have decreased in frequency concomitant with the conspicuous progress in chemotherapy. However, infections caused by bacteria previously regarded as avirulent have become frequent, and these organisms have received increasing attention because of their low susceptibility to various chemotherapeutic agents. *Pseudomonas aeruginosa* is one of these bacteria.

Penicillins such as carbenicillin and sulbenicillin or aminoglycosides, represented by gentamicin have been used for the treatment of infections caused by *P. aeruginosa*, but these drugs may have deficits with respect to potency or side effects[1,2]. Based on the need for more potent and safer antipseudomonal agents, several antipseudomonal β-lactam antibiotics have been developed[3~7].

In this study, the therapeutic effects of seven currently available antipseudomonal β-lactam antibiotics on experimental urinary tract infection caused by *P. aeruginosa* P 9 in mice were compared, and the results were analyzed in relation to their *in vitro* antibacterial activities and pharmacokinetics in mice.

Materials and Methods

Antibiotics

Cefsulodin was prepared at Takeda Chemical Industries, Ltd., Osaka, Japan. Cefoperazone (Pfeizer GmbH, Karlsruhe, Germany), sulbenicillin (Takeda Chemical Industries, Ltd., Osaka, Japan), ticarcillin (Beecham Pharmaceuticals, Worthing, England), azlocillin, mezlocillin (Bayer, Leverkusen, Germany), and piperacillin (Toyama Chemical Co., Tokyo, Japan) were obtained from commercial sources.

Bacteria

P. aeruginosa P 9 with type G O-antigen was supplied by HOMMA[5]. Forty eight strains of *P. aeruginosa* were chosen at random from our collection of clinical isolates. The organisms were grown in King A broth[9] at 37°C for 16 hours with shaking, and used for the inoculum.

Testing of Antibiotic Susceptibility

The minimal inhibitory concentrations (MICs) of the agents against *P. aeruginosa* P 9 were determined by means of the standard two-fold serial dilution method using agar or broth medium. In the agar dilution method, one loopful (2 mm in diameter) of a bacterial suspension, diluted to 10^5 or 10^8 colony-forming units (cfu)/ml with Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville Md.), was streaked for a length about 2 cm on Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md.). In the broth dilution method, 0.5 ml of an antibiotic solution was inoculated with 4.5 ml of a 10^5 cfu/ml bacterial suspension in TSB. The microtitration system was used to estimate the susceptibilities of clinical isolates. A 0.1 ml volume of antibiotic solution in TSB was inoculated with 1.5 μl of a bacterial suspension to yield an inoculum of 10^5 cfu/ml. The MIC was defined as the lowest concentration of antibiotic that showed no visible growth of the organisms after overnight incubation at 37°C. After the MIC was determined by the broth dilution method, one loopful of the culture in each tube was inoculated on TSA plates containing no antibiotic. The lowest concentration of antibiotic associated with no growth after overnight incubation of the plate was defined as the minimal bactericidal concentration (MBC).

Viable Counts and Turbidimetric Studies

Ten-milliliter volumes of broth containing appropriate concentrations of antibiotic were inoculated with bacteria from overnight cultures to yield an initial concentration of 10^7 cfu/ml. The cultures were incubated with shaking at 37°C, and at various intervals a portion of them was removed. Cfu was estimated by the plate count method, and opacity was measured with a Coleman Junior II spectrophotometer.

Microscopy: Antibiotic-induced morphological changes in bacteria were examined by light microscopy using stained specimen after four hours of exposure in the turbidimetric system.

Therapeutic Test

The urinary tract infection was produced as previously described[5]. CF#1/K (Kyoto Herbal Garden, Takeda Chemical Industries, Ltd.) female mice weighing 20 to 22 g were used. *P. aeruginosa* P 9 cultivated overnight with King A broth was diluted to 1/6 with the same medium, and 0.05 ml of the bacterial suspension which contained 10^7 cfu was inoculated transurethrally into the bladder. On day three after the inoculation, mice with a bacteriuria of more than 10^5 cfu/ml were selected and subjected to chemotherapy. Antibiotics of serial two-fold dilution were administered subcutaneously to groups of mice twice a day (9 am and 5 pm) for ten days. Each group was consisted of more than ten animals. Infected but untreated mice served as controls. The mice were killed on the day following completion of medication and were examined for gross renal pathology and for bacterial recovery from the kidney. Both kidneys were removed aseptically and homogenized in four ml of sterile distilled water. Serial ten-fold dilutions of the homogenates were plated on NAC agar (Eiken Chemical Co., Ltd., Tokyo, Japan).

The dose of antibiotic required to eradicate bacteria from the kidneys of 50% of the mice (CD_{50}) was calculated by the probit method[10].

Antibiotic Assay

The antibiotics were administered subcutaneously to CF#1/K female mice weighing 20 to 22 g at a dose of 100 mg/kg. Groups of four animals were killed for antibiotic assay at various times up to eight hours after the administration. Urine was obtained by compression of the bladder through the external abdominal wall, and was diluted 20 times with a 0.1 M phosphate buffer solution (PBS, pH 7.0). Blood was collected from the axillary artery and vein with a small volume of heparin, and plasma was separated by centrifugation. The mice were killed by bleeding, and the kidneys from each mouse were removed and homogenized with four volumes of PBS. The homogenates were centrifuged and the

supernatant was collected. The concentration of antibiotic in each specimen was assayed by the agar well method with *P. aeruginosa* NCTC 10490 for cefsulodin, with *Bacillus subtilis* ATCC 6633 for sulbenicillin, ticarcillin, azlocillin, and mezlocillin, and with *Micrococcus luteus* ATCC 9341 for cefoperazone and piperacillin.

Results

MICs and MBCs

Cefsulodin showed the most potent antibacterial activities against *P. aeruginosa* P 9 (Table 1). The activities of cefoperazone, azlocillin, and piperacillin were similar, and their MICs and MBCs were two to four times as large as those of cefsulodin. Sulbenicillin, ticarcillin, and mezlocillin were less active than the others. The MBCs of all the agents were identical to or twice their MICs, and the activity of an individual antibiotic was influenced slightly by inoculum size. The proportion of clinical isolates inhibited at 3.13 μg/ml or lower were (%): cefsulodin, 75; cefoperazone, 42; sulbenicillin, 4; ticarcillin, 2; azlocillin, 29; mezlocillin, 2; and piperacillin, 63. With every agent tested, except ticarcillin, the concentration that inhibited the growth of most clinical isolates of *P. aeruginosa* was identical to the MIC against strain P 9 (Fig. 1).

Bactericidal Activity

All the agents tested showed a distinct bactericidal activity at concentrations corresponding to the MIC (determined by broth dilution method) or higher, and the number of viable cells began to decrease after two hours of incubation (Fig. 2). Cefsulodin showed a similar bactericidal effect over a wide concentration range, and the reduction rate of the viable counts during the six hours of incubation period was greater than that obtained with the other antibiotics. The bactericidal rates of cefsulodin, sulbenicillin, ticarcillin, and azlocillin for the first three hours of incubation were relatively higher than those of cefoperazone, mezlocillin, and piperacillin. With 1,600 μg/ml of sulbenicillin and 800 μg/ml of ticarcil-

Fig. 1. Susceptibility of 48 clinical isolates of *P. aeruginosa* to antipseudomonal β-lactam antibiotics. Arrow indicates the MIC of each antibiotic against strain P 9.
CFS, cefsulodin; CPZ, cefoperazone; SBPC, sulbenicillin; TIPC, ticarcillin; AZPC, azlocillin; MZPC, mezlocillin; PIPC, piperacillin.

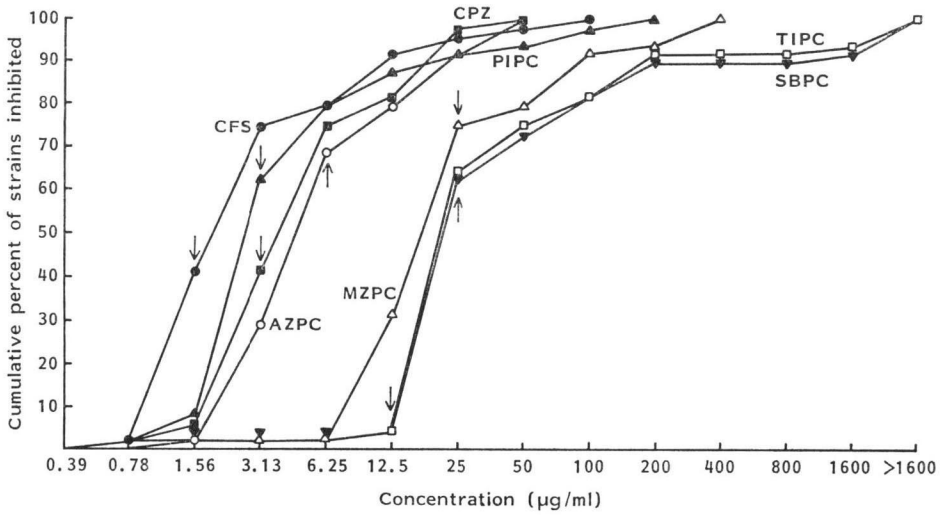
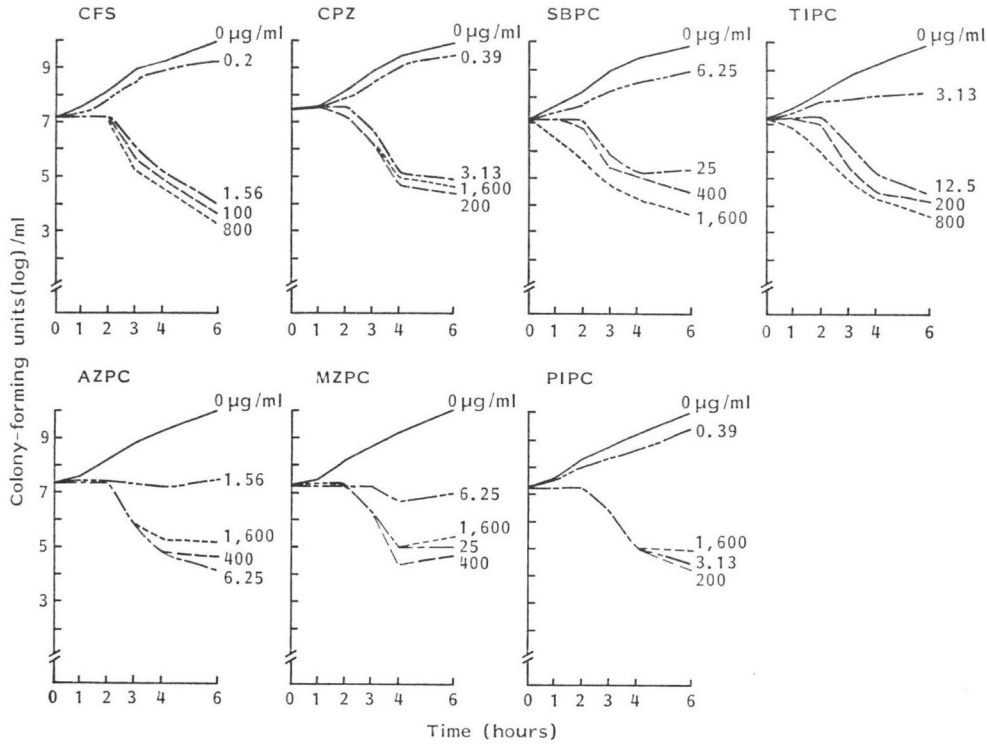


Fig. 2. Effects of antipseudomonal β-lactam antibiotics on viability of *P. aeruginosa* P 9.
See Fig. 1 for abbreviations.



lin, the decrease of viable cells occurred within one hour of treatment. Contrariwise, the bactericidal activities of cefoperazone, azlocillin, and piperacillin were reduced at higher concentrations.

Turbidimetric Study

The opacity of the *P. aeruginosa* P 9 culture continued to increase after the addition of the antibiotics (Fig. 3). After three to four hours of incubation, growth ceased, and then opacity decreased slightly at concentrations corresponding to the MIC or higher. Except for sulbenicillin and ticarcillin, growth inhibitory and bacteriolytic effects were similar over a wide range of the concentration. The opacity did not increase with 1,600 μg/ml of sulbenicillin and 800 μg/ml of ticarcillin.

Table 1. *In vitro* antibacterial activities of antipseudomonal β-lactam antibiotics against *P. aeruginosa* P 9.

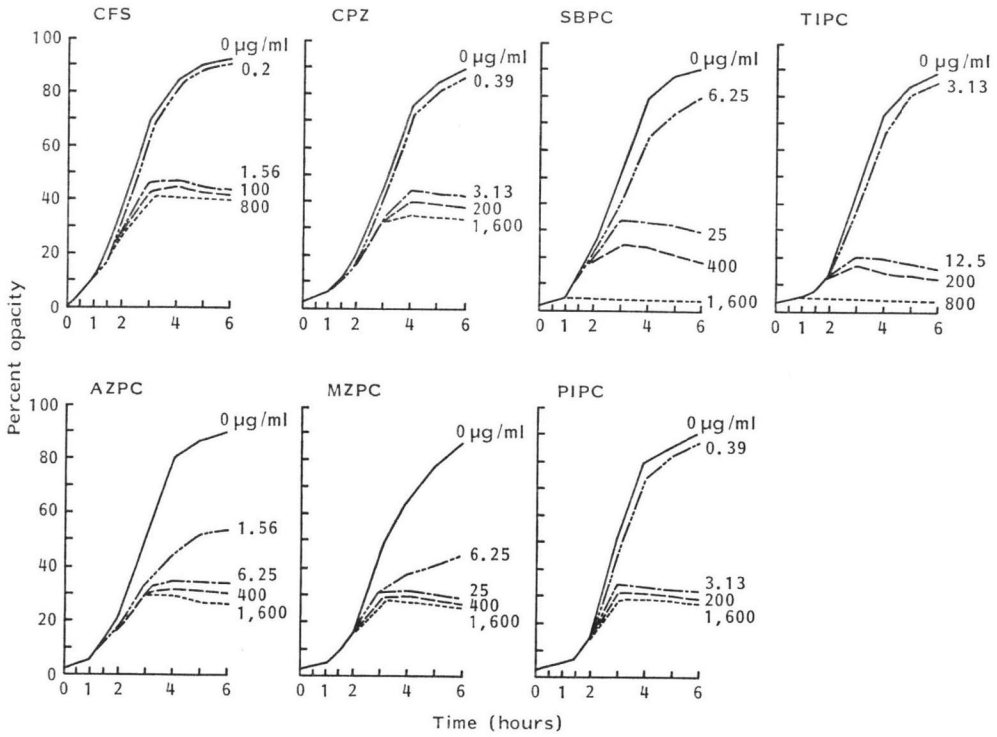
Antibiotic	Agar MIC (μg/ml)		Broth	
	10^8	10^5	MIC (μg/ml)	MBC (μg/ml)
Cefsulodin	3.13	0.78	1.56	1.56
Cefoperazone	6.25	3.13	3.13	6.25
Sulbenicillin	25	12.5	25	50
Ticarcillin	25	6.25	12.5	25
Azlocillin	6.25	1.56	6.25	6.25
Mezlocillin	12.5	6.25	25	25
Piperacillin	3.13	1.56	3.13	3.13

Table 2. Therapeutic effects of antipseudomonal β-lactam antibiotics on urinary tract infection caused by *P. aeruginosa* P 9 in mice.

Antibiotic	CD_{50}* (mg/kg)
Cefsulodin	6.19 (3.57~10.5)
Cefoperazone	162 (48.8~3210)
Sulbenicillin	167 (89.7~ 340)
Ticarcillin	184 (108 ~ 489)
Azlocillin	121 (47.6~ 461)
Mezlocillin	390 (236 ~ 961)
Piperacillin	227 (136 ~ 745)

* CD_{50}, 50% clearance dose, administered subcutaneously twice a day for 10 days starting on day 3 after infection. Figures in parentheses indicate 95% confidence limit.

Fig. 3. Effects of antipseudomonal β-lactam antibiotics on growth curve of *P. aeruginosa* P 9.
See Fig. 1 for abbreviations.



Morphological Response

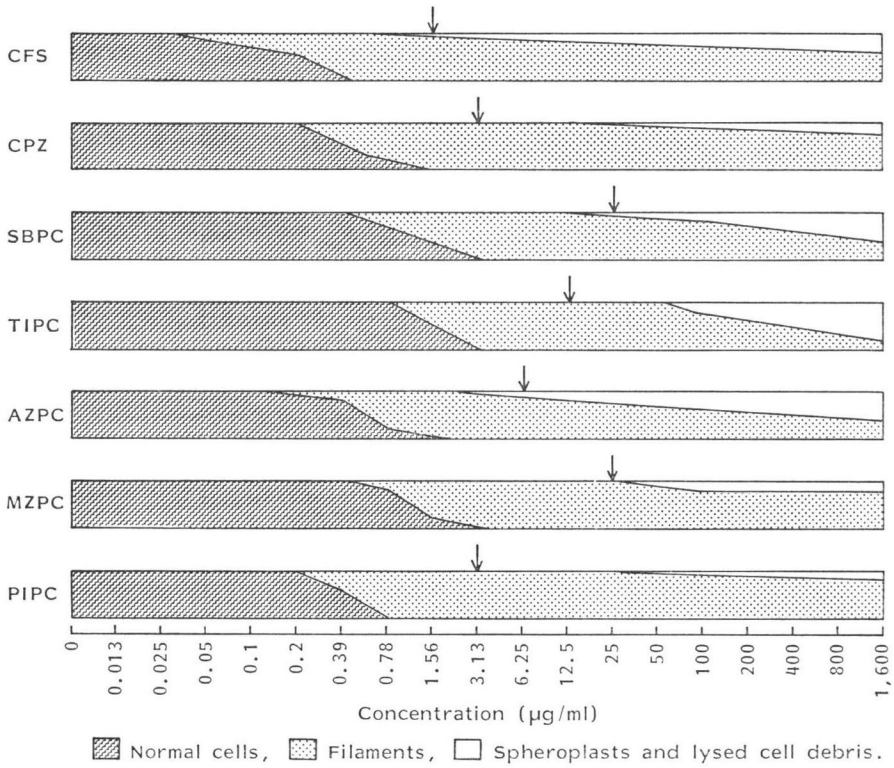
All the antibiotics induced elongation of the cells at concentrations below the MICs; this effect was strongest with cefsulodin (Fig. 4). The degree of elongation was remarkable at concentrations around the MIC, and filaments as long as 60 to 80 μm were formed. Cefsulodin and azlocillin evoked cell lysis even at concentrations below the MICs. At higher concentrations of sulbenicillin and ticarcillin, spheroplast-like structure and lysed cells were frequently observed. Only a small proportion of the cells lysed after exposure to cefoperazone, mezlocillin, and piperacillin even at concentrations much higher than their respective MICs.

Therapeutic Effects

The therapeutic effects of cefsulodin, based on the clearance rate of the infecting organisms from the kidney, was superior to those of the other antibiotics (Table 2). Treatment with 6.25 mg/kg of cefsulodin resulted in eradication of the infecting organisms in the kidneys of more than a half the mice; the eradication rate increased with higher dose levels. In all treatment there were mice which had gross renal lesions, but from which no bacteria were recovered. No correlation was seen between dosage and the mean renal bacterial counts calculated for the positive cases. The therapeutic effects of cefoperazone, azlocillin, and piperacillin were much lower than expected from their *in vitro* activities; their CD_{50} values were more than 18-fold as large as that of cefsulodin, whereas the differences of their MIC values were less than four-fold.

Fig. 4. Morphological response profiles of *P. aeruginosa* P 9 exposed to antipseudomonal β-lactam antibiotics for four hours.

Arrow indicates the MIC of each antibiotic. See Fig. 1. for abbreviations.



Antibiotic Levels in Plasma, Kidney and Urine

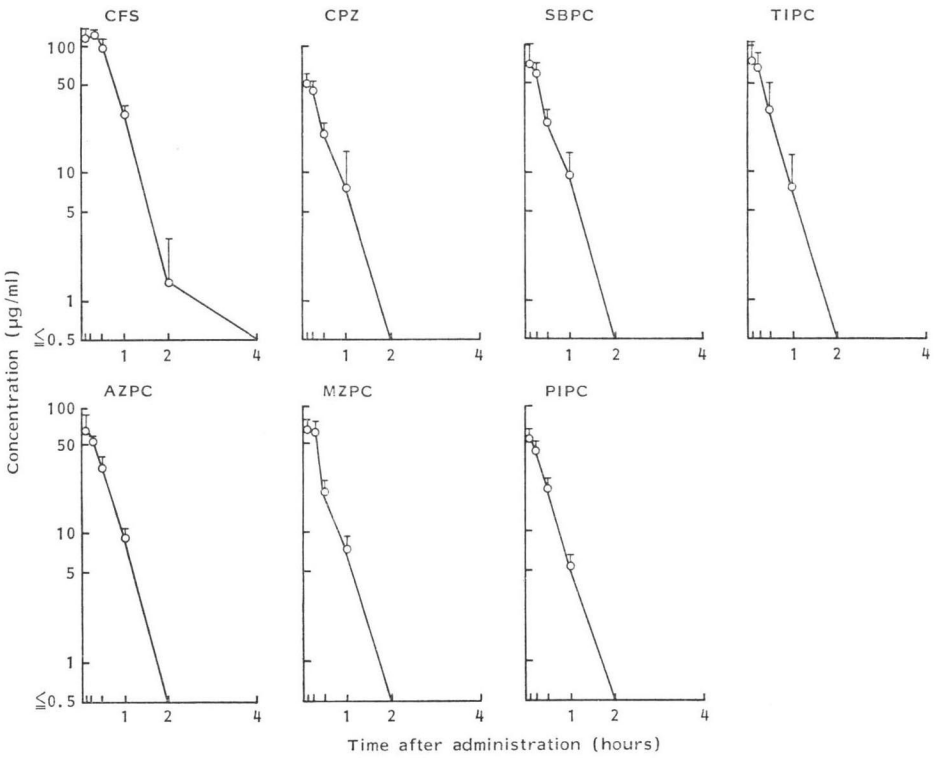
After a single subcutaneous administration of 100 mg/kg, the peak plasma level was attained at 15 minutes for cefsulodin and at six minutes for the rest (Fig. 5). Cefsulodin showed the highest peak level (124 μg/ml), followed by ticarcillin, sulbenicillin, mezlocillin, azlocillin, piperacillin, and cefoperazone in descending order. Except for cefsulodin, plasma levels declined rapidly and became undetectable at two hours after administration.

The peak renal levels were attained at six minutes for cefoperazone and ticarcillin, and at 15 minutes for the other antibiotics (Fig. 6). Piperacillin showed the highest peak level (177 μg/g), followed by cefsulodin, ticarcillin, sulbenicillin, azlocillin, cefoperazone, and mezlocillin in descending order. The duration of the renal concentration differed considerably among the antibiotics tested: sulbenicillin, ticarcillin, and azlocillin were excreted rapidly, and were undetectable at two hours; cefoperazone, mezlocillin, and piperacillin were detected at two hours, and disappeared by four hours; cefsulodin remained the longest, and a concentration of 4.3 μg/g, which exceeds the MIC of this antibiotic against *P. aeruginosa* P 9, was detected at four hours after administration.

The peak urinary levels were attained at 30 minutes for all the antibiotics (Fig. 7). Cefsulodin showed the highest peak level (14,500 μg/ml), followed by ticarcillin, sulbenicillin, piperacillin, azlocillin, cefoperazone, and mezlocillin in descending order. The levels in the urine were 40 to 500 times as high as those in the kidney, and cefsulodin, which had the longest duration, was detected at a concentration of

Fig. 5. Plasma levels of antipseudomonal β-lactam antibiotics after a single subcutaneous administration of 100 mg/kg in mice.

Each point represents the mean (±SD) of results from four animals. See Fig. 1 for abbreviations.



155 μg/ml at six hours after administration.

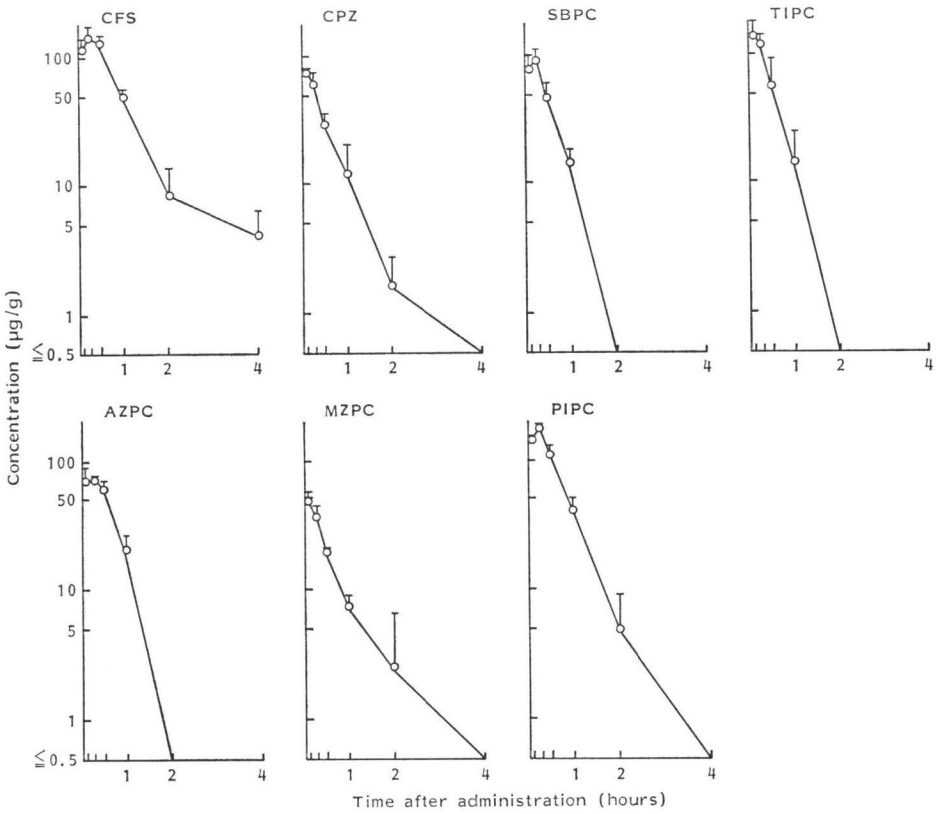
Discussion

Among the seven antipseudomonal β-lactam antibiotics tested, cefsulodin showed the most potent antibacterial activities against *P. aeruginosa* P 9 *in vitro*. The activities of cefoperazone, azlocillin, and piperacillin were intermediate, and those of sulbenicillin, ticarcillin, and mezlocillin were the least potent. The relative antibacterial activities of the seven agents obtained against strain P 9 were also noted with the clinical isolates, and these results agree with those previously reported[11,12]. All the agents tested demonstrated a distinct bactericidal activity at concentrations corresponding to the MIC or higher. Viable cell counts began to decrease prominently after two hours of incubation, whereas the opacity of the culture kept increasing. Therefore, lysis of the cells may not necessarily be required for the loss of their viability. Contrariwise, the bactericidal activities of cefoperazone, azlocillin, mezlocillin, and piperacillin were reduced at concentrations exceeding the MIC; the degree of reduction and the concentration at which it occurred differed from one agent to another. The paradoxical reduction of the bactericidal activity of penicillin against *Staphylococcus* or *Streptococcus* at higher concentrations is known as "Eagle effect"[13]. Recently, this effect was also seen with penicillin against some strains of Gram-negative rods such as *Proteus, Haemophilus*, and *Escherichia coli*[14]. Although the precise mechanism of the "Eagle effect" is unknown, it has been speculated that, in *S. aureus*, a high concentration of penicillin probably inhibits autolytic activities of the cells resulting in their inability to lyse[15,16].

Microscopy revealed that the increase in opacity of the culture during which viable cell counts decreased progressively resulted from an extreme elongation of individual cells. This elongation occurred

Fig. 6. Renal levels of antipseudomonal β-lactam antibiotics after a single subcutaneous administration of 100 mg/kg in mice.

Each point represents the mean (±SD) of results from four animals. See Fig. 1 for abbreviations.



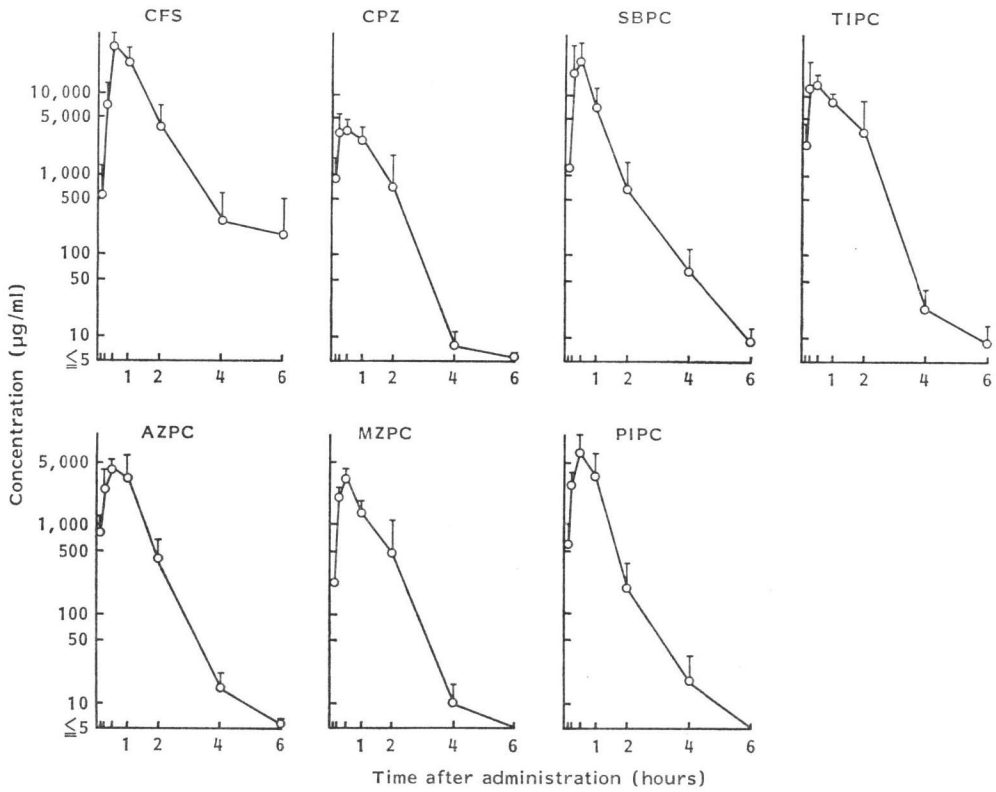
over a wide range of concentration, and filaments up to 80 μm appeared. A close correlation between morphological responses of bacterial cells exposed to β-lactam antibiotics and the affinity profiles of β-lactam antibiotics for penicillin-binding proteins (PBPs) was proposed[17], and the functions of PBP-1, -2, and -3 as the lethal targets in *E. coli* have been elucidated[15~20]. The elongation of the cells results from the inhibition of PBP-3. NOGUCHI *et al.* examined the affinity profiles of various β-lactam antibiotics for the PBPs of *P. aeruginosa* NCTC 10490, and found that antipseudomonal agents such as cefsulodin, sulbenicillin, ticarcillin, piperacillin, and apalcillin had the highest affinity for PBP-3 which, like PBP-3 of *E. coli*, functions in septation[21]. CURTIS *et al.* also reported that the α-sulfocephalosporins, including cefsulodin, showed the highest affinity for the PBP-3 of *P. aeruginosa* 18s SAI^{-}[22]. Therefore, it is conceivable that the antipseudomonal β-lactam antibiotics used in this study have a high affinity for the PBP-3 of *P. aeruginosa* P 9.

In the experimental urinary tract infection study, the therapeutic effects of cefoperazone, azlocillin, and piperacillin were lower than expected from their *in vitro* antibacterial activities; their CD_{50} values were more than 18-fold as large as that of cefsulodin, whereas the differences of their MIC values were less than four-fold. The bactericidal activities of cefoperazone, mezlocillin, and piperacillin for the first three hours of incubation were relatively lower than those of cefsulodin, sulbenicillin, ticarcillin, and azlocillin. The bacterial killing for a short period seems to be an important factor for therapeutic efficacy, because renal levels of antibiotics persist only for a limited period.

After a single subcutaneous administration of 100 mg/kg to mice, renal levels of sulbenicillin, ticarcillin, and azlocillin decreased rapidly, and became undetectable two hours later. Cefoperazone, mezlocillin, and piperacillin were detected at two hours, and disappeared by four hours. Cefsulodin attained

Fig. 7. Urinary levels of antipseudomonal β-lactam antibiotics after a single subcutaneous administration of 100 mg/kg in mice.

Each point represents the mean (±SD) of results from four animals. See Fig. 1 for abbreviations.



a high renal level, and a concentration of 4.3 μg/g, which exceeds the MIC of this agent against *P. aeruginosa* P 9, was detected four hours later.

Cefsulodin showed an excellent therapeutic effect on urinary tract infection caused by *P. aeruginosa* P 9 in mice, and its superiority was also noted in antibacterial activity *in vitro* and in pharmacokinetic property in mice. These results suggest that cefsulodin is a promising agent in the treatment of urinary tract infections caused by *P. aeruginosa*.

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