SYNERGISTIC ACTIVITY OF AMPICILLIN AND CLOXACILLIN

PROTECTIVE EFFECT OF CLOXACILLIN ON ENZYMATIC DEGRADATION OF AMPICILLIN BY PENICILLINASE, AND THERAPEUTIC ACTIVITY OF MIXTURES OF AMPICILLIN AND CLOXACILLIN

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A mixture of ampicillin (aminobenzyl penicillin, AB-PC) and cloxacillin (methylchlorophenylisoxazolyl penicillin, MCI-PC) showed an excellent bactericidal activity even at low concentrations in which either of the penicillins alone did not have any activity against a clinically isolated strain of *Escherichia coli*, resistant to both of these penicillins. In this case, the microbial degradation of AB-PC, caused by penicillinase (PC-ase), was strongly inhibited in the presence of MCI-PC. The enzymatic hydrolysis of AB-PC by extracellular and cell-bound PC-ase, obtained from an AB-PC resistant strain of *Staphylococcus aureus*, was also inhibited in the presence of MCI-PC. Against that, under comparable conditions, penicillin G (PC-G) was almost completely destroyed by cell-bound PC-ase from this organism. An *in vivo* synergism was observed when mixtures of AB-PC and MCI-PC were applied for the treatment of mice challenged with a clinically isolated strain of *E. coli* resistant to both of these penicillins. Similar results were obtained in the treatment of mice experimentally infected with a strain of *Staphylococcus aureus*, which is highly resistant to AB-PC, but sensitive to MCI-PC.

A decrease in the rate of hydrolysis of methicillin (dimethoxyphenyl penicillin, DMP-PC) by staphylococcal penicillinase (PC-ase) was first noted by ROLINSON and his co-workers[1]. At the same time, GOUREVITCH *et al.*[2] demonstrated that the reaction of staphylococcal PC-ase with PC-ase resistant penicillins, such as DMP-PC and oxacillin (methylphenylisoxazolyl penicillin), led to an inactivation of the enzyme, and that there was a direct relationship between the amount of DMP-PC decomposed and the degree of inactivation of the enzyme.

The present investigation was undertaken to study the protective effect of cloxacillin (methylchlorophenylisoxazolyl penicillin, MCI-PC), a PC-ase resistant penicillin, on the enzymatic hydrolysis of ampicillin (aminobenzyl penicillin, AB-PC) by PC-ase obtained from some clinically isolated bacteria. Furthermore, the effect

of MCI-PC *in vitro* was confirmed by the fact that the therapeutic activity in mice was enhanced by the combination of both penicillins.

Materials and Methods

1. Substances used: AB-PC (Beecham), MCI-PC (Beecham) and potassium penicillin G (PC-G) were used in these experiments. The commercial PC-ase preparation (Tokyo Kenbikyoin) had an activity of 400,000 u/vial.

2. Organisms used and preparation of extracellular and cell-bound PC-ase: *Staphylococcus aureus* No. 39 was isolated from a patient at the Institute for Medical Science, and *Staph. aureus* T-5 and *Escherichia coli* T-20 were supplied by Toho University. *E. coli* No. 11 was isolated from a patient at Kyoto Municipal Hospital.

The extracellular and cell-bound PC-ases were prepared as follows: Brain-heart infusion broth (80 ml) was inoculated each with one loopful of AB-PC resistant strains of *Staph. aureus* or *E. coli* isolated from patients, then incubated with shaking at 37°C for 4 hours. Each culture was centrifuged (13,000×*G*, 20 min.) and the supernatant was used as the extracellular PC-ase. The sedimented cells were washed three times with 0.1 M phosphate buffer (pH 7.0) and resuspended in this buffer (80 ml). The cell suspensions were sonicated for 30 minutes (*E. coli*) or 90 minutes (*Staph. aureus*). The supernatant from the centrifugation (1,500×*G*, 15 min.) was used as the cell-bound PC-ase.

3. Incubation of penicillins with enzymes: Sufficient AB-PC solution (10,000 mcg/ml) and/or MCI-PC solution (10,000 mcg/ml) was added to tubes containing 10 ml solution of the commercial PC-ase (10,000 u/ml) in 0.1 M phosphate buffer (pH 7.0), to give the desired final concentration of penicillin(s) after adjustment with buffer to a total volume of 20 ml. The mixtures were allowed to react with shaking for 1 or 3 hours at 37°C. After termination of the reaction by boiling in a water bath for 8 minutes, an aliquot of the mixture was used for the determination of the rate of penicillins inactivation.

In experiments with PC-ase prepared from staphylococci or *E. coli*, 1 ml of AB-PC solution (10,000 mcg/ml) and/or 1 ml of MCI-PC solution (5,000 mcg/ml) was mixed with 18 ml of the extracellular or the cell-bound PC-ase solutions. Eventually the resulting mixtures were adjusted with buffer to a total volume of 20 ml, and were incubated for different periods of time at 37°C.

4. Microbioassay: The rates of hydrolysis, in the mixture of both penicillins, were determined by a disc method using *E. coli* NIHJ for AB-PC and *Staph. aureus* No. 20 for MCI-PC. *Staph. aureus* No. 20 is resistant to AB-PC but sensitive to MCI-PC, so MCI-PC in the mixture can be assayed without being disturbed by AB-PC.

5. Determination of viable cell count: Nutrient broth containing AB-PC with or without MCI-PC at the concentrations mentioned below was inoculated with an overnight culture of *E. coli* No. 11 to give a viable cell count of 10^7 cells/ml, and was incubated with shaking in water-bath for 24 hours at 37°C. Small samples were withdrawn at intervals, and tenfold serial dilutions of the samples were mixed with nutrient agar in petri dishes. Colonies of viable cells were counted after the incubation (24 hours, 37°C).

(1) AB-PC 40 mcg/ml (2) MCI-PC 40 mcg/ml
(3) AB-PC (20 mcg/ml)+MCI-PC (20 mcg/ml)

6. Preincubation of PC-ase with MCI-PC: The cell-bound PC-ases obtained from *Staph. aureus* No. 39 and *E. coli* T-20, were incubated with MCI-PC for different time intervals preceding the experiment. AB-PC (1 mg/ml) or PC-G (1 mg/ml) as substrates were added to the mixture which was incubated for 1 hour at 37°C. The rates of hydrolysis of these substrates were determined by the iodometric method.

7. Experimental treatment of mice infected with some clinically isolated strains of *E. coli* and *Staph. aureus*: The combined activity of AB-PC and MCI-PC *in vivo* was studied by using mice infected with the clinically isolated strains of *E. coli* and *Staph. aureus*. Seven mice (ICR-strain, 21~23 g) of each group were challenged intravenously

(*Staph. aureus*) or intraperitoneally (*E. coli*) with 0.5 ml of the bacterial suspensions (viable cell count; *E. coli* 5×10^8, *Staph.* 5×10^6). The penicillins were subcutaneously administered 1 and 4 hours after the infection. The mice treated were observed for 14 days, and the therapeutic effect of the penicillins was expressed as CD_{50}.

Results

1. Bactericidal Activity of Both Penicillins in Combination against a Clinically Isolated Strain of *E. coli* and Microbial Degradation of AB-PC

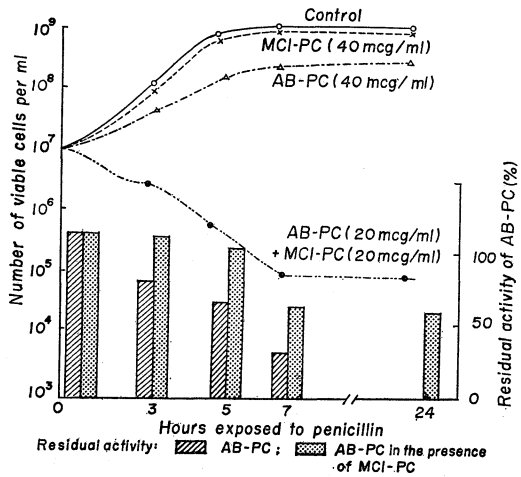
The bactericidal activities of AB-PC, MCI-PC and the 1 : 1 combination of these penicillins against a highly resistant strain of *E. coli* No. 11 (minimum inhibitory concentration: AB-PC, MCI-PC 400 mcg/ml) were compared. The results are shown in Fig. 1.

When AB-PC or MCI-PC respectively was added to the media at a concentration of 40 mcg/ml, the bacterial growth was not inhibited since the viable cell counts increased similar to that of the control. On the other hand, in the presence of AB-PC (20 mcg/ml) and MCI-PC (20 mcg/ml), each concentration corresponding to one-twentieth of the MIC, the viable cell count in the medium decreased to 10^5 cells/ml from 10^7 cells/ml after incubation. These results indicate that the bactericidal activity is synergistically enhanced by the combination of AB-PC and MCI-PC.

The rate of microbial degradation of AB-PC was simultaneously determined. As shown in Fig. 1, AB-PC was not substantially degraded by *E. coli* No. 11 in the presence of MCI-PC during 3- or 5- hours of incubation, but AB-PC was markedly inactivated in the absence of MCI-PC.

It is suggested from these results that the enhanced bactericidal activity of the combination of AB-PC and MCI-PC is due to inactivation of PC-ase by MCI-PC.

Fig. 1. Bactericidal activity of AB-PC and MCI-PC against a clinically isolated strain of *E. coli* (No. 11) and microbial degradation of AB-PC.
MIC: AB-PC 400 mcg/ml; MCI-PC >400 mcg/ml



2. Protective Effect of MCI-PC on Enzymatic Degradation of AB-PC by PC-ase

(1) Commercial penicillinase

The protective activity of MCI-PC on the degradation of AB-PC was studied with a commercial PC-ase. The results given in Table 1 demonstrate that AB-PC alone was almost completely destroyed after 3 hours of incubation, while MCI-PC alone at concentrations of 250 or 500 mcg/ml, was scarcely affected under the same condition. On the other hand, the degradation of AB-PC in the presence of MCI-PC at different combination (1 : 1, 1 : 2, 2 : 1) was considerably inhibited in comparison with that of

AB-PC alone. However, the relationship between the extent of degradation of AB-PC and the optimal proportion of both penicillins mixed could not be established under this condition.

(2) Staphylococcal penicillinase

(a) Extracellular penicillinase: According to the procedure described above, the protective effect of MCI-PC on the enzymatic degradation of AB-PC by the extracellular PC-ase, was studied (Table 2).

AB-PC (500 mcg/ml), added to the supernatant of centrifuged culture medium, was rapidly degraded by extracellular PC-ase to an extent of 68 %, 93 % and 99 % after 0.5, 1 and 2 hours of incubation. However, MCI-PC (250 mcg/ml) did not suffer any decrease in activity under these conditions.

Table 1. Protective effect of MCI-PC on enzymatic degradation of AB-PC by commercial penicillinase

	Penicillins	Substrates	Rates of degradation**	
			1 hr.	3 hrs.
PC-ase* 5,000 u/ml	AB-PC 500 mcg/ml + MCI-PC 500 mcg/ml	AB-PC	25 %	55 %
		MCI-PC	0	7
	AB-PC 500 mcg/ml + MCI-PC 1,000 mcg/ml	AB-PC	39	48
		MCI-PC	0	0
	AB-PC 500 mcg/ml + MCI-PC 250 mcg/ml	AB-PC	45	54
		MCI-PC	0	8
	AB-PC 500 mcg/ml alone	AB-PC	58	83
	MCI-PC 500 mcg/ml alone	MCI-PC	0	0
	MCI-PC 250 mcg/ml alone	MCI-PC	0	9

* Tokyo Kenbikyoin 400,000 u/vial
** Disc method

Table 2. Protective effect of MCI-PC on enzymatic degradation of AB-PC by staphylococcal penicillinase (No. 39)

Enzyme	Penicillins	Rates of degradation*		
		0.5 hr.	1 hr.	2 hrs.
Extracellular	AB-PC 500 mcg/ml	68 %	93 %	99 %
	MCI-PC 250 mcg/ml	0	0	0
	AB-PC 500 mcg/ml + (MCI-PC 250 mcg/ml)	0	0	52
Cell-bound	AB-PC 500 mcg/ml	30	96	99
	MCI-PC 250 mcg/ml	0	0	8
	AB-PC 500 mcg/ml + (MCI-PC 250 mcg/ml)	22	32	46

* Disc method

While AB-PC (500 mcg/ml), incubated with extracellular PC-ase in the presence of MCI-PC (250 mcg/ml), was not affected within 1 hour of incubation, 52 % of the initial amount was degraded after 2 hours incubation. These results indicate that MCI-PC prevents the enzymatic degradation of AB-PC by extracellular PC-ase, which was released from a PC-resistant strain of *Staph. aureus* (No. 39).

(b) Cell-bound penicillinase: The incubation of AB-PC (500 mcg/ml) with cell-bound PC-ase of *Staph. aureus* No. 39 resulted in an extensive inactivation of this penicillin after 1~2 hours of incubation, whereas MCI-PC was largely unaffected under the same condition. When AB-PC (500 mcg/ml) was incubated with the enzyme in the presence of MCI-PC (250 mcg/ml), the degradation of AB-PC was markedly inhibited in comparison with its destruction in the absence of MCI-PC. Thus, the antibiotic activity of AB-PC was diminished by 32 % in 1 hour or 46 % in 2 hours.

3. Protective effect of MCI-PC from Inactivation of AB-PC and PC-G by PC-ase

In order to determine whether the protective effect of MCI-PC from enzymatic inactivation of AB-PC also applies to PC-G, the rates of the enzymatic degradation of AB-PC and PC-G were compared in the presence of MCI-PC (Table 3). When the inhibitor (MCI-PC, 4 mg/ml) was added at zero time to the incubation mixture of the substrate (AB-PC, 1 mg/ml) with PC-ase (0.02 mg/ml), a marked difference was found between the rates of hydrolysis of AB-PC and PC-G by the cell-bound PC-ase from *Staph. aureus* No. 39; while PC-G was almost completely hydrolysed (residual activity, 3 %), 53 % of the original activity of AB-PC was left intact under this condition. When PC-ase was preincubated with MCI-PC for 5~20 minutes before addition of the substrates, the difference of protection from inactivation between the two penicillins largely disappeared.

Table 3. Inactivation of staphylococcal β-lactamase (No. 39) by contact with MCI-PC prior to addition of AB-PC or PC-G

Preincubation (minutes)	Substrates and residual activity*	
	AB-PC	PC-G
60	92 %	92 %
40	82	84
20	75	63
10	75	61
5	65	58
0	53	3

* Iodometric assay

Table 4. Inactivation of *Escherichia coli* (T-20) β-lactamase by contact with MCI-PC prior to addition of AB-PC or PC-G

Preincubation (minutes)	Substrates and residual activity*	
	AB-PC	PC-G
60	79 %	57 %
40	76	57
20	74	55
10	69	55
5	66	55
0	64	55

* Iodometric assay

Table 5. Therapeutic activity of AB-PC and MCI-PC in combination on experimentally infected mice

Treatment*	CD_{50} dose of total penicillins (mg/kg)	
	E. coli No. 11**	*Staph. aureus* T-5**
AB-PC alone	>1,740	>600
MCI-PC alone	> 870	350~469
AB-PC+MCI-PC (2 : 1)	330	347
AB-PC+MCI-PC (1 : 1)	507	424

* Penicillins were injected subcutaneously 1 and 4 hours after challenge.
** MIC: *Staph. aureus* T-5 (AB-PC, 50 mcg/ml; MCI-PC, 0.5 mcg/ml)
E. coli No. 11 (AB-PC, 400 mcg/ml; MCI-PC, >400 mcg/ml)

Preincubation of cell-bound PC-ase from *E. coli* T-20 with MCI-PC (Table 4), did not result in much more protection of AB-PC, compared with the corresponding experiments with PC-ase from *Staph. aureus* No. 39. Again, however, AB-PC was protected more effectively than PC-G.

4. Therapeutic Activity of a Combination of AB-PC with MCI-PC against Experimental Infection in Mice

Mice were challenged with *E. coli* No. 11 which was highly resistant to both penicillins (MIC: AB-PC 400 mcg/ml, MCI-PC>400 mcg/ml). As shown in Table 5, when AB-PC alone, subcutaneously administered twice after infection, resulted in a

CD_{50} value of >1,740 mg/kg, indicating that AB-PC is ineffective in the treatment of the mice infected with this strain. The administration of MCI-PC alone was also not effective (CD_{50}, >870 mg/kg) under the same condition. However, the CD_{50} value resulting from the combination of AB-PC with MCI-PC (2 : 1) was 330 mg/kg and 507 mg/kg respectively in the case of 1 : 1 combination of these penicillins. Thus, the therapeutic effect of both penicillins in combination was superior to that obtained with AB-PC or MCI-PC alone. This indicates an *in vivo* synergism between AB-PC and MCI-PC against a PC-resistant strain of *E. coli*.

In the treatment of mice infected with *Staph. aureus* T-5, which is highly resistant to AB-PC but sensitive to MCI-PC, the administration of MCI-PC alone showed a considerable effect (CD_{50}, 350~469 mg/kg), but a treatment with AB-PC alone had no effect (CD_{50}, >600 mg/kg). On the other hand, when AB-PC and MCI-PC were given in combination (2 : 1), the therapeutic effect (CD_{50}, 347 mg/kg) was comparable to that of MCI-PC alone, and a similar effect was obtained with the 1 : 1 combination of both penicillins.

In view of the fact that the MCI-PC portion of the AB-PC/MCI-PC mixtures, administered to the animals, represented only about a half or one third of the total dose of penicillin, it seems to be justified to explain the therapeutic effects, obtained with the mice infected with *Staph. aureus* T-5, by a true synergism between the two penicillins.

Discussion

It is known that some penicillins of the isoxazolyl group prevent the enzymatic degradation of some PC-ase sensitive penicillins by inactivating PC-ase.[1,2,3,4] In the present study, it was found that the enzymatic degradation of AB-PC *in vitro* was also prevented by MCI-PC.

A trial to clarify the enhancement of the therapeutic activity *in vivo* by a combination of an isoxazolyl penicillin and AB-PC was already carried out by BACH *et al.*[5], who noted a synergism between ampicillin and dicloxacillin in the treatment of mice infected with *Shigella flexneri* under a limited condition, such as the repeated administration of the penicillins (4 times) at an early stage of infection. In the experimental infection with *Proteus morganii* NCTC-235, able to hydrolyze cephaloridine, O'CALLAGHAM[6] found an *in vivo* synergism of cephaloridine and some cephalosporin derivatives, resistant to β-lactamase, by the repeated administration of both cephalosporins (5 times) after infection.

In the present study, AB-PC and MCI-PC administered in combination showed an enhanced therapeutic activity in mice infected with some AB-PC resistant strains of *Staph. aureus* and *E. coli*. In our experiments, the synergistic activity of AB-PC and MCI-PC against *E. coli* was more pronounced than that reported previously for this organism by the other researchers. In this regard, however, the difference in sensitivity of the bacteria used, properties of PC-ase produced, and other factors should be taken into account. Furthermore, if the synergistic activity of AB-PC and MCI-PC in the infected mice is based on the inhibiting effect of MCI-PC against PC-ase from the bacteria, further studies remain to be done with respect to a correlation between the distribution of PC-ase in the infected animal and the degradation of AB-PC *in vivo* by the distributed enzyme.

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