A SIMPLE METHOD FOR TESTING THE EFFICACY OF A β -LACTAMASE INHIBITOR AGAINST β -LACTAMASE-PRODUCING GRAM-NEGATIVE BACTERIA

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The synergistic effect of a β -lactam antibiotic in combination with a β -lactamase inhibitor (cloxacillin) on Gram-negative bacteria producing various types of β -lactamases was studied with respect to the properties of the enzymes by using a set of bacterial strains we had developed previously for evaluation of β -lactamase stability of β -lactam antibiotics¹⁾. Cloxacillin showed strong synergy in combination with ampicillin or cephalosporins against typical cephalosporinase-producing bacteria, but not against penicillinase-producing bacteria. Analysis of the results indicated that the efficacy of a competitive inhibitor such as cloxacillin is greatly influenced by the following factors: the affinity of the inhibitor for the enzyme, the difference between the affinity of the inhibitor for the enzyme and that of the combined antibiotic for the enzyme, and the ability of the inhibitor to penetrate the bacterial outer membrane. It was also demonstrated that this information concerning the efficacy of an inhibitor could easily be obtained by using the set of bacterial strains and the methods described.

Many Gram-negative bacteria produce β -lactamases, and the enzyme production is well known to be closely associated with resistance to β -lactam antibiotics. This fact has created interest in inhibitors of the enzyme as antibiotic-protecting agents.

In a previous paper¹⁾, we described a set of Gram-negative bacterial strains which produce nine kinds of β -lactamases whose enzymological and physiological properties were examined in detail. These strains afford a simple method for the evaluation of β -lactamase stability of β -lactams. In the present paper, we describe the use of these bacterial strains as a simple tool for measuring the efficacy of an inhibitor in protecting the antibiotics from various kinds of β -lactamases. It is known that oxacillin, cloxacillin, and dicloxacillin inhibit β -lactamases of Gram-negative bacteria²⁾, and these semisynthetic penicillins are also stable to hydrolytic activity of various types of β -lactamases except oxacillin-hydrolyzing penicillinases^{3,4)}. We used cloxacillin as the inhibitor, and we evaluated the synergistic effect of a combination of the inhibitor with ampicillin, cephalothin, or cephaloridine on β -lactamase-producing bacteria.

Materials and Methods

Bacterial Strains

The bacterial strains used in this study were selected from the set of bacterial strains reported previously¹⁾. *Escherichia coli* ML1410 RGN14 and *E. coli* ML1410 RGN823 harbor R plasmids, RGN14 and RGN823, respectively^{5,6)}. These plasmids mediate the constitutive synthesis of type Ia and type Ib penicillinases^{7,9)}, respectively (corresponding to TEM-1 and TEM-2 penicillinases of MATTHEW's classification⁸⁾). *Klebsiella pneumoniae* strains GN69 and GN118 produce a species-specific penicillinase whose enzymological properties are similar to those of the type I penicillinases¹⁰⁾. *Proteus mirabilis* strains are producers of so-called carbenicillin-hydrolyzing penicillinase mediated by R plasmids¹⁾. The ten other bacterial strains are constitutive producers of species-specific cephalosporinases. The

enzyme of *P. vulgaris* is a unique cephalosporinase with relatively broad substrate specificity, active even on penicillins, and differing from typical cephalosporinases of other bacteria¹¹⁾. All the β -lactamases mentioned above are produced constitutively and located in the periplasmic space. Substrate profiles of these β -lactamases were reported in a previous paper¹⁾, and more details on their properties will be published subsequently.

β -Lactam Antibiotics

β -Lactam antibiotics employed in this study were kindly provided by the following pharmaceutical companies: ampicillin, cephalothin and cloxacillin, Meiji Seika Kaisha Ltd., Tokyo, Japan; cephaloridine, Torii Pharmaceutical Co., Tokyo, Japan.

Preparation of β -Lactamases

Type Ib penicillinase was purified by a procedure reported previously⁸⁾; cephalosporinase of *P. morgani* 1510 was obtained according to the procedure of FUJII-KURIYAMA *et al.*¹²⁾ Cephalosporinase of *P. vulgaris* GN 76/C-1 was purified by adsorption and elution on CM-Sephadex column and subsequent gel filtration on a Sephadex G-75 column. *P. vulgaris* GN76/C-1 is the parental strain of GN76/C-1/1 and GN76/C-1/3, and is a higher producer of the enzyme (2.4 units/mg dry weight bacteria).

Measurement of β -Lactamase Activity

The kinetic measurement of the β -lactamase reaction was performed by the microiodometric assay method of NOVICK¹³⁾ with slight modifications. The inhibitory effect of cloxacillin on the enzyme activity was measured under two different conditions. In one case, soluble β -lactamase in 50 mM phosphate buffer (pH 7.0) was the enzyme preparation, and in the other case, intact cells suspended in 13 mM phosphate buffer (pH 7.0) containing 0.68% NaCl and 1 mM MgSO₄ were used as the enzyme preparation.

Synergy Assay

The test for synergy was carried out by the checkerboard method on agar, and the synergistic effect was expressed as the fractional inhibitory concentration (FIC) index described by DOUGHERTY *et al.*¹⁴⁾. The FIC index for the combination of drugs A and B is calculated as follows.

$$\text{FIC} = \frac{\text{MIC of A in combination}}{\text{MIC of A alone}} + \frac{\text{MIC of B in combination}}{\text{MIC of B alone}}$$

Overnight cultures of the bacterial strains grown in nutrient broth at 37°C were diluted 1/100 with fresh broth; 5 μ l of the bacterial suspension (about 3×10^8 cells per ml) was spotted on heart infusion agar containing one drug or a combination of two drugs using an inoculum replicating device (Microplanter, Sakuma Factory, Tokyo, Japan). Minimum inhibitory concentration (MIC) was measured after incubation at 37°C for 18 hours. All the media were products of Eiken Chemical Co., Tokyo, Japan.

Results

Synergistic Effect on β -Lactamase-producing Bacteria

The values of the FIC index measured by the combination of cloxacillin and one of the β -lactams (ampicillin, cephalothin, or cephaloridine) against 15 strains are shown in Table 1 together with the β -lactamase activity of the test strains and the MICs of the drugs alone.

The results show high FIC values for the penicillinase-producing strains in all combinations tested, suggesting little synergistic effect on these bacteria. In contrast, the inhibitor exhibited a powerful synergistic effect on all the cephalosporinase-producing bacteria except the *P. vulgaris* strains. It was an interesting fact that the inhibitor exhibited a weak effect on the *P. vulgaris* strains; it is known that these strains produce a cephalosporinase which has some penicillinase activity^{1, 11)}. These observations elucidate a close relationship between the synergistic effect of an inhibitor on the β -lactamase-producing bacteria and the nature of the enzyme.

Table 1. Synergistic activities of cloxacillin in combination with three β -lactam antibiotics.

Strains	β -Lactamase activity (units)*		MIC (μ g/ml)				FIC index**		
	Penicillinase	Cephalosporinase	APC	CER	CET	CLX	(APC: CLX)	(CER: CLX)	(CET: CLX)
<i>E. coli</i> ML1410 RGN823	16.7		> 3200	25	25	1600	≤ 1.0 (3200: 800)	1.0 (12.5: 800)	0.75 (12.5: 400)
<i>E. coli</i> ML1410 RGN14	0.6		800	6.3	12.5	800	0.75 (200: 400)	1.0 (3.1: 400)	0.38 (3.1: 100)
<i>K. pneumoniae</i> GN69	1.11		800	12.5	6.3	400	0.75 (200: 200)	0.56 (6.3: 25)	0.63 (3.1: 50)
<i>K. pneumoniae</i> GN118	0.05		50	3.1	3.1	400	0.75 (12.5: 200)	0.75 (1.6: 100)	0.63 (1.6: 50)
<i>P. mirabilis</i> N-29	2.0		800	25	6.3	400	0.50 (200: 100)	0.63 (12.5: 50)	1.0 (3.1: 200)
<i>P. mirabilis</i> N-29/2	0.28		12.5	6.3	6.3	200	0.50 (3.1: 50)	1.0 (3.1: 100)	0.75 (1.6: 100)
<i>E. coli</i> 255		0.72	800	50	800	400	0.14 (12.5: 50)	0.19 (6.3: 25)	0.16 (25: 50)
<i>C. freundii</i> GN346		24.2	800	200	800	400	0.16 (25: 50)	0.13 (12.5: 25)	0.16 (25: 50)
<i>C. freundii</i> GN346/16-10		4.0	200	100	800	200	0.19 (12.5: 25)	0.19 (12.5: 12.5)	0.27 (50: 50)
<i>E. cloacae</i> 363		24.9	800	800	3200	400	0.16 (25: 50)	0.16 (25: 25)	0.27 (50: 100)
<i>E. cloacae</i> 363/1-3		10.6	400	400	3200	200	0.28 (12.5: 50)	0.19 (25: 25)	0.27 (50: 50)
<i>P.morganii</i> 1510		2.68	200	400	1600	200	0.09 (6.3: 12.5)	0.09 (12.5: 12.5)	0.08 (25: 12.5)
<i>P.morganii</i> 1510/3		0.07	50	50	200	50	0.16 (6.3: 1.6)	0.38 (6.3: 12.5)	0.09 (6.3: 3.1)
<i>P. vulgaris</i> GN76/C-1/1		1.8	800	1600	800	400	0.75 (200: 200)	0.38 (400: 50)	0.56 (200: 25)
<i>P. vulgaris</i> GN76/C-1/3		0.03	50	200	100	200	0.75 (25: 50)	0.5 (50: 50)	0.31 (6.3: 50)

* Units/mg dry weight of bacteria, one unit of penicillinase or cephalosporinase activity was defined as that amount of enzyme which hydrolyzed one μ mole of benzylpenicillin (penicillinase) or cephaloridine (cephalosporinase) in 1 minute at pH 7.0 and 30°C.

** Numerical values in parenthesis are the concentrations (μ g/ml) of β -lactam and cloxacillin resulting in the most effective synergy.
Abbreviations: APC; ampicillin, CER, cephaloridine, CET; cephalothin, CLX; cloxacillin.

Inhibitory Activity of Cloxacillin against the β -Lactamases

In order to better understand the relationship between the synergistic effect of cloxacillin on the β -lactamase-producing bacteria and the sensitivity of the enzyme to the inhibitor, the inhibitory activity of cloxacillin against three typical β -lactamases, *i.e.*, type Ib penicillinase, typical cephalosporinase of *P. morganii*, and the *P. vulgaris* cephalosporinase with broad substrate specificity, was measured. The assay was performed using both the soluble extracellular enzymes and the intact cells which carry the β -lactamase in the periplasmic space. Cephaloridine, which is a common and favorable substrate of the three β -lactamases^{8,11,12} and known to have high ability to pass through the outer membrane of Gram-negative bacteria¹³, was employed as substrate.

Fig. 1 (A) shows that cloxacillin is a powerful inhibitor of the typical cephalosporinase, but a poor inhibitor of penicillinase. Inhibition of *P. vulgaris* cephalosporinase activity by cloxacillin was also demonstrated although it was significantly weaker compared with the inhibition of typical cephalosporinase. Similar patterns were observed when the intact cells were used (Fig.

1B), though the inhibitor concentration required for marked inhibition was about 10^4 times higher than in the case of the soluble enzyme. This fact may be attributed to the poor ability of cloxacillin to pass through the outer membrane of Gram-negative bacteria.

Affinity of β -Lactamases for the β -Lactams

The enzyme constants for the interaction of three kinds of β -lactamases with cloxacillin, ampicillin, and cephaloridine are shown in Table 2. These values were calculated from Lineweaver-Burk plots (data not shown), and expressed as the K_m value or the K_i value. A high affinity between the typical cephalosporinase and cloxacillin was observed. In contrast, cloxacillin exhibited remarkable low affinity for the penicillinase. The affinity of the *P. vulgaris* cephalosporinase for the inhibitor was intermediate between the typical cephalosporinase and penicillinase.

Cloxacillin acted as a competitive inhibitor of the enzyme reaction catalyzed by the three kinds of β -lactamases studied. The difference in affinity for the enzyme between the inhibitor and the substrate was well expressed by the ratio of the K_i value of the inhibitor to the K_m (or K_i) value of the drug. These ratios strongly suggested a close relationship between the synergistic effect of the combined agents on the β -lactamase-producing bacteria and the affinity of the agents for the enzyme: the lower the K_i /

Fig. 1. Inhibitory effect of cloxacillin on the activity of the extracellular soluble β -lactamases and those in the periplasmic space of intact cells.

One ml of the reaction mixture containing 50 μ M cephaloridine, concentration of cloxacillin as indicated and the enzyme preparation was incubated for 15 minutes at 30°C, and then the amount of the hydrolyzed substrate was measured by the microiodometric method. In the case of the soluble enzyme the reaction medium was 50 mM phosphate buffer (pH 7.0), and in the case of intact cells, the medium was 13 mM phosphate buffer (pH 7.0) containing 0.68% NaCl and 1 mM MgSO₄.

(A) Effect on the soluble enzymes, type Ib penicillinase from *E. coli* ML 1410 RGN 823 (○), typical penicillinase from *P. morganii* 1510 (●), cephalosporinase from *P. vulgaris* GN 76/C-1 (△).

(B) Effect on the enzymes in intact cells, *E. coli* ML1410 RGN823 (○), *P. morganii* 1510 (●), *P. vulgaris* GN76/C-1 (△).

Table 2. K_m and K_i values for β -lactamase - β -lactam interaction.

β -Lactams	β -Lactamases							
	<i>E. coli</i> ML1410 RGN823			<i>P. vulgaris</i> GN76/C-1			<i>P. morganii</i> 1510	
	K_m (μM)	K_i^* (μM)	K_i/K_m^{**}	K_m (μM)	K_i (μM)	K_i/K_m	K_m (μM)	K_i/K_m (K_i/K_i)
Cephaloridine	500		6.0×10^{-2}	60		1.4×10^{-3}	214	2.0×10^{-6}
Ampicillin	32		0.94	4		2.2×10^{-2}	8	(5.3×10^{-5})
Cloxacillin		30			8.6×10^{-2}			4.2×10^{-4}

* K_i values were determined by using cephaloridine as substrate.

** K_i/K_m is the ratio of K_i of cloxacillin to K_m of cephaloridine or ampicillin. In the case of ampicillin hydrolysis by the *P. morganii* cephalosporinase, the ratio K_i/K_i was calculated because ampicillin is hardly hydrolyzed by the cephalosporinase.

K_m (or K_i/K_i) ratio the greater the synergistic effect.

Discussion

By using a well-known β -lactamase inhibitor, cloxacillin, we have demonstrated a practical application of the set of Gram-negative bacteria to the inhibitor evaluation. This study has shown that antibacterial synergy of the combination of cloxacillin and usual β -lactams varies with the type of β -lactamase produced. This may also be the case for other β -lactamase inhibitors which act as competitive inhibitors. The set of bacterial strains prepared by us comprises strains producing nine kinds of β -lactamases, representing the principal ones of Gram-negative bacteria¹⁾. These strains and the methods described in this paper offer useful information for the development of new inhibitors.

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