A NOVEL METHOD FOR EVALUATING THE OUTER MEMBRANE PERMEABILITY TO β-LACTAMASE-STABLE β-LACTAM ANTIBIOTICS

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A novel method is described which allows estimation of the outer membrane permeability to β-lactamase-stable β-lactams by determining antibiotic concentrations in the periplasm. The method is based on measurement of the inhibiting activity of β-lactamase-stable β-lactams on the hydrolysis of a substrate by periplasmic β-lactamase. Application of the method to carbenicillin revealed that the high level of resistance to carbenicillin of an *Escherichia coli* strain acquiring the plasmid encoding ampicillin resistance resulted from the poor ability of carbenicillin to penetrate the outer membrane of *E. coli*.

In Gram-negative bacteria the outermost layer of the envelope, the outer membrane, acts as a barrier that β-lactam antibiotics encounter on their way to their target enzymes localized on the cytoplasmic membrane. Accordingly, the ability of a β-lactam to penetrate the outer membrane is considered to be one of the major factors determining its antibacterial activity against Gram-negative bacteria. Among the several methods devised for assessing the outer membrane permeability to β-lactam antibiotics, the most reliable were found to be those that utilize the susceptibility of β-lactams to β-lactamase which is localized in the space between the outer and cytoplasmic membranes termed the periplasm[1~4]. In early reports, membrane permeability was expressed as crypticity, that is the fold increase in hydrolytic activity of β-lactamase toward an antibiotic subsequent to disruption of an intact bacterial suspension. Recently, ZIMMERMANN and ROSSELET refined this type of evaluation by showing that the estimation of diffusion rate of β-lactam across the outer membrane was possible on the assumption that the diffusion of an antibiotic is balanced by its hydrolysis by β-lactamase in the periplasm[4]. However, this method possesses a serious disadvantage in that it is not applicable to β-lactamase-stable β-lactams, especially since most of the newly developed β-lactam antibiotics are classified in this group. This report describes a novel method applicable to the β-lactamase-stable β-lactams, which was derived from a modification of the procedure devised by ZIMMERMANN and ROSSELET.

Materials and Methods

1. Bacterial strains

E. coli CSH2 (F^-, *nal, metB*) and its transconjugant possessing the TEM-type β-lactamase-encoding plasmid[5], *E. coli* CSH2/RKl, were generously provided by Dr. T. YOKOTA of Juntendo University.

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2. Antibiotics

The antibiotics used were carbenicillin, ampicillin (Beecham Pharmaceuticals, Betchworth, Surrey, U.K.) and cephaloridine (Glaxo Research Ltd., Greenford, Middlesex, U.K.).

3. Antibiotic susceptibility testing

The minimum inhibitory concentrations (MIC) of the test antibiotics were determined by the agar dilution method with the use of a multipoint inoculator[6]. The inocula used were one hundred-fold dilutions of overnight cultures in trypticase soy broth (BBL, Cockeysville, U.S.A.). After incubation at 37°C for 20 hours, the lowest concentration that inhibited macroscopic colonial growth was regarded as the MIC.

4. Preparation of intact and sonicated cell suspensions

The test strain was grown at 37°C for 20 hours in heart infusion broth (Difco, Detroit, U.S.A.), harvested by centrifugation for 10 minutes at $5,000 \times g$, washed once with 0.067 M potassium phosphate buffer (pH 7.0) supplemented with 0.01 M $MgCl_2$ and resuspended in the same ice-cold buffer to give an optical density to its thirty-fold dilutions at 320 nm of 0.3. Part of this suspension was sonicated in an ice-water bath for 5 minutes at maximum power with an ultra-sonic disruptor (Tomy Seiko Co., Ltd.). The supernatant of the intact cell suspension was prepared by centrifugation for 10 minutes at $9,000 \times g$ followed by filtration through a Millipore filter (HA, 0.45 μm).

5. Preparation of β-lactamase

The test strain was grown at 37°C for 20 hours in heart infusion broth, harvested by centrifugation, washed once and resuspended in 0.067 M potassium phosphate buffer (pH 7.0). The cell suspension was sonicated for 5 minutes at maximum power and then centrifuged for 1 hour at $10,000 \times g$. The resultant supernatant was applied to a Sephadex G-100 column and eluted with 0.067 M potassium phosphate buffer. The enzyme fractions were pooled and stored at -20°C. This partially purified enzyme preparation was used to determine the kinetic parameters for the test β-lactams.

6. Assay of β-lactamase activity

β-Lactamase activity was determined by direct spectrophotometric assay or microiodometric assay according to SAWAI *et al.*[3] Spectrophotometric assay was performed with a Hitachi 200-20 spectrophotometer equipped with a thermostatted cell holder. The enzyme solution (0.1 ml: intact cells, sonicate and supernatant of cell suspension or purified preparation) was mixed in a 1-cm quartz cuvette with the substrate and/or inhibitor and 200 μmoles of phosphate buffer (pH 7.0) to make a final volume of 3.0 ml and incubated at 37°C. Each component of the reaction mixture was preincubated in a water bath at 37°C. The hydrolysis rate of the β-lactam ring was followed by a change in ultraviolet absorption at 240 nm for penicillins and at 320 nm for cephaloridine. Microiodometric assay was performed when the hydrolysis rate of carbenicillin was determined in the intact cell suspension. The substrate dissolved in 0.95 ml of 0.067 M phosphate buffer (pH 7.0) containing 0.01 M $MgCl_2$ was preincubated for 5 minutes at 37°C. Carbenicillin was then hydrolyzed by adding 0.05 ml of intact cells suspended in the phosphate buffer containing $MgCl_2$. The reaction mixtures were incubated at 37°C with vigorous shaking. After incubation for 30 minutes, the reaction was stopped by adding 0.5 ml of 0.15 M sodium tungstate dissolved in 2 M acetate buffer (pH 4.0). The mixtures were diluted with the same tungstate solution to reduce the carbenicillin concentration to 66.7 μM, and a 1.5 ml portion was added to 1.5 ml of starch-iodine reagent[3]. After standing for exactly 20 minutes at 25°C, the absorbance of the reaction mixtures was measured at 595 nm with a Hitachi 200-20 spectrophotometer. For the control, sodium tungstate was added to the carbenicillin solution before adding the intact cells to make mixtures having the same composition as those of the reaction mixtures. The absorbance of the control mixtures was measured. The amount of hydrolyzed substrate in the reaction mixtures was calculated from the iodine consumption which was determined experimentally. Errors resulting from the enzymes which leaked out from the intact cells were corrected using β-lactamase activity of the supernatants. In all assays of β-lactamase activity, the substrate consumed during the enzyme reaction was less than 20% of the added substrate.

7. Enzyme kinetic parameters

MICHAELIS constant (K_m), maximum rate of substrate hydrolysis (V_{max}) and inhibitor constant (K_i) were calculated from a plot of 1/v versus 1/s (LINEWEAVER-BURK plot).

8. Procedure for calculation

The concentration of a β-lactamase stable β-lactam in the periplasm of β-lactamase synthesizing cells may be determined according to the following procedure. It depends on inhibition of the periplasmic β-lactamase activity by a test β-lactam. The procedure consists of two steps for determining each of the following parameters: (1) permeability coefficient of a substrate, (2) concentration of a test β-lactam.

(1) Determination of substrate permeability coefficient:

This is determined according to the procedure of ZIMMERMANN and ROSSELET[4]. The substrate concentration in the periplasm (S_p) is obtained by application of the MICHAELIS-MENTEN equation.

$$S_p = \frac{v_{intact} \cdot K_m}{V_{max} - v_{intact}} \quad (A)$$

where v_{intact}, K_m and V_{max} are the velocity of β-lactam hydrolysis by intact cells, the MICHAELIS constant and the maximum velocity, respectively. Then, the permeability coefficient (C) can be derived by the following equation on the assumption that the rate of antibiotic diffusion across the outer membrane equals the rate of β-lactam hydrolysis.

$$C = \frac{v_{intact}}{S_0 - S_p} \quad (B)$$

where S_0 is a given substrate concentration outside the cells.

(2) Determination of β-lactam concentration in the periplasm:

When cells are incubated with both substrate and test β-lactam, the concentration of a substrate in the periplasm (S_p') is calculable by the following equation using the value of C determined as described above. This equation is based on the same assumption as equation (B).

$$S_p' = S_0 - \frac{v_{intact}'}{C} \quad (C)$$

where v_{intact}' is the rate of hydrolysis of a substrate by intact cells in the presence of a test β-lactam. v_{intact}' can be determined experimentally. Assuming that a test β-lactam competitively inhibits the hydrolysis of a substrate by periplasmic β-lactamase, the following relationships can be obtained from the MICHAELIS-MENTEN equation used for competitive inhibition:

$$v_{intact}' = \frac{S_p' \cdot V_{max}}{S_p' + K_{m\,app}} \quad (D')$$

and

$$K_{m\,app} = K_m\left(1 + \frac{I_p}{K_i}\right) \quad (E')$$

These equations can be rearranged as follows:

$$K_{m\,app} = \frac{S_p'(V_{max} - v_{intact}')}{v_{intact}'} \quad (D)$$

and

$$I_p = \frac{K_i(K_{m\,app} - K_m)}{K_m} \quad (E)$$

where $K_{m\,app}$, K_m, V_{max} and K_i are the apparent MICHAELIS constant, MICHAELIS constant and maximum velocity of a substrate and inhibitor constant of a test β-lactam, respectively. The values of K_m, K_i and V_{max} are determined experimentally using the sonicates as enzyme preparation. Accordingly, the concentration of a test β-lactam in the periplasm (I_p) is determined by inserting the value of $K_{m\,app}$ into the equation (E), while $K_{m\,app}$ is given by equation (D).

Results

Determination of the Concentration of Carbenicillin in the Periplasm of an *E. coli* Strain Possessing Plasmid Encoding Ampicillin Resistance

The method described above was used to estimate the ability of carbenicillin to penetrate the outer membrane of *Escherichia coli* and compared with the method of ZIMMERMANN and ROSSELET. *E. coli* CSH2 strain possessing TEM-type β-lactamase-encoding plasmid was incubated with various concentrations of carbenicillin together with 1 mM cephaloridine as a substrate. Concentrations of carbenicillin in the periplasm were determined according to the procedure described in Materials and Methods and compared with those of carbenicillin, ampicillin and cephaloridine which were calculated according to the method of ZIMMERMANN and ROSSELET (Table 1). The concentrations of carbenicillin in the periplasm corresponding to the lower concentrations outside the cells could not be estimated because of its rather weak inhibiting activity or its low ability to penetrate the outer membrane.

In comparison of our novel method with that of ZIMMERMANN and ROSSELET, periplasmic concentrations of carbenicillin in both methods were in good agreement when the concentration in the medium was 1,000 or 5,000 μM. When the concentration in the medium was 25,000 μM, however there was a difference between the values determined by two methods. Judging from the inhibitor constant of carbenicillin to the β-lactamase (12 μM), the inhibition of hydrolysis of cephaloridine by carbenicillin seemed to be too strong and the change of hydrolysis rate of cephaloridine seemed to be too small against a wide change of periplasmic concentration of carbenicillin when the concentration of carbenicillin in the medium was more than 25,000 μM. Similar findings were obtained by the method of ZIMMERMANN and ROSSELET: it was difficult to determine the periplasmic concentration higher than the MICHAELIS constant because the change of hydrolysis rate of carbenicillin was small against a wide change of periplasmic concentration of carbenicillin.

The concentrations of ampicillin and cephaloridine in the periplasm corresponding to the higher concentrations outside the cells could not be determined since the absorptions of these antibiotics were beyond the limit of measurement of the spectrophotometer. However, it was possible to compare the concentrations of the three β-lactams in the periplasm at an outside concentration of 1 mM. The ratios of the concentration of carbenicillin in the periplasm (which was measured by our novel method) to that of ampicillin and cephaloridine were 0.52 and 0.004 respectively. A β-lactam concentration

Table 1. Concentrations of the β-lactams in periplasm of *E. coli* CSH2/RK1.

Concentration in medium (μM)	Concentration in periplasm (μM)			
	Novel method	Method of ZIMMERMANN and ROSSELET		
	CBPC[a]	CBPC[b]	ABPC[c]	CER[c]
25,000	22	66	ND[d]	ND
5,000	6.0	5.1	ND	ND
1,000	1.4	1.56	2.7	330
200	ND	ND	1.8	55

Abbreviations: CBPC, carbenicillin; ABPC, ampicillin; CER, cephaloridine.
a: Details are described in Materials and Methods.
b: Determined by microiodometry.
c: Determined by direct spectrophotometry.
d: Not determined.

Table 2. Comparison of MICs and kinetic constants of the β-lactams.

Antibiotic	MIC (μg/ml)		Kinetic constants			
	R+ [a]	R− [b]	V_{max} [c]	K_m (μM)	K_i (μM)	K_m/V_{max} [d]
Carbenicillin	51,200	6.25	100	31	12	100
Ampicillin	12,800	6.25	870	65	—	24
Cephaloridine	100	3.13	1410	970	—	222

a: *E. coli* CSH2/RK1.
b: *E. coli* CSH2.
c, d: Expressed as percentage of the value for carbenicillin.

in the periplasm of β-lactamase synthesizing cells is approximately proportional to its concentration outside the cell provided that the antibiotic concentration in the periplasm is below the MICHAELIS constant. The relationship can be derived from the equations (A) and (B) assuming $V_{max} \gg v_{intact}$ and $S_0 \gg S_p$, and expressed as $S_p = (K_m/V_{max}) \cdot C \cdot S_0$. Accordingly, the ratio of the concentration in the periplasm corresponding to the fixed outside concentration between β-lactams is inversely proportional to the ratio of the outside concentration giving the definite concentration in the periplasm. Provided that the susceptibilities of the target enzymes to the test β-lactams are the same, the ratio between their minimum inhibitory concentration (MIC) against a test strain is conceived to be inversely proportional to the ratio between their concentrations in the periplasm corresponding to the fixed outside concentration. Table 2 shows the MICs of the test β-lactams against the test strains of *E. coli* and the kinetic constants for the test β-lactams of the β-lactamase from the test strain. Since the MICs of carbenicillin, ampicillin and cephaloridine against the parent strain free of the R-plasmid are quite similar, any contribution of activities of the β-lactams against the target enzymes to the difference in their MICs against the plasmid possessing strain can be disregarded. The ratios of MICs of carbenicillin against the plasmid harboring strain to those of ampicillin and cephaloridine were 0.25 and 0.002. These ratios were not explained solely by the stability of the antibiotics to the plasmid-mediated β-lactamase. Stability of a β-lactam to β-lactamase in the range of low antibiotic concentrations effective against target enzymes can be evaluated by K_m/V_{max}. The ratio of this index of carbenicillin to those of ampicillin and cephaloridine were 0.24 and 2.2, respectively. In contrast, the ratio of MICs coincided quite well with that of the experimentally determined antibiotic concentrations in the periplasm, as was expected. This close correlation also provides evidence of the validity of the novel method described in this report. Furthermore, the concentration of carbenicillin in the periplasm of the test strain was low although the hydrolysis rate was smaller than that of ampicillin by plasmid-mediated β-lactamase. This indicates that carbenicillin had poor ability to penetrate the outer membrane. The high level of resistance to carbenicillin conferred by the plasmid encoding TEM-type β-lactamase is explained by the synergism between the poor outer membrane permeability to carbenicillin and the plasmid-mediated β-lactamase.

Discussion

Recent studies on the outer membrane of Gram-negative bacteria have shown that hydrophilic solutes penetrate the outer membrane through water-filled pores constituted by proteins, named porins, which restrict entry by both size and hydrophobicity of the solutes[7,8,9]. β-Lactam antibiotics are also considered to penetrate the outer membrane in this way since NIKAIDO *et al.* demonstrated the

porin-dependent penetration of cephaloridine by using porin-deficient mutants[10]. In several methods for assessing the outer membrane permeability to β-lactams, mutation altering in outer membrane permeability or EDTA treatment was employed to disrupt the permeability barrier[11,12]. However, cells obtained as described above are not considered to be completely free from the barrier because increased outer membrane permeability was reported to result from the reorganization of the membrane with production of exposed phospholipid bilayer regions which permit the penetration of hydrophobic molecules[13]. Since a concentration causing 50% growth inhibition (IC_{50}) against strains treated as above would depend on the hydrophobicity of a test antibiotic, the ratio of IC_{50} of the intact cells to that of the treated cells does not seem to be an exact index of the ability of an antibiotic to penetrate the outer membrane. Another method utilizing the incorporation of radiolabeled β-lactams is unsatisfactory because of the high level of nonspecific adsorption. The method devised by ZIMMERMANN and ROSSELET surpasses the others in that it allows quantitative evaluation of the permeability coefficients of β-lactams which is constant and is independent of other parameters of β-lactams such as concentration, stability to β-lactamase or affinity of target enzymes[4]. Nevertheless, this method possesses a disadvantage in that it is not applicable to β-lactams stable to β-lactamases. This report describes a modification of the method which allows its application to β-lactamase-stable β-lactams. The validity of this novel method was examined in comparison with the method of ZIMMERMANN and ROSSELET using the moderate stability of carbenicillin to TEM-type β-lactamase. This novel method was reliable provided that the periplasmic concentration of a tested β-lactam was not higher than the inhibitor constant. This method can determine the concentration of a β-lactamase-stable β-lactam in the periplasm but not its permeability coefficient. However, rough values of the coefficient can be estimated by determining the stability of a β-lactam to the β-lactamase of the test strain (K_m/V_{max}) since the concentration of a β-lactam in the periplasm approximates to $(K_m/V_{max})\cdot C\cdot S_0$ on the condition that it is below the MICHAELIS constant for the β-lactam. This method was used to estimate the ability of carbenicillin to penetrate the outer membrane of a strain of *E. coll* harboring a plasmid encoding TEM-type β-lactamase. The discrepancy between the moderate stability of carbenicillin to TEM-type β-lactamase and the high level of resistance of plasmid-harboring strains to the antibiotic was previously discussed by YAMAMOTO and YOKOTA[13]. Our results indicate that the high level of carbenicillin-resistance of the strain acquiring the plasmid results from the poor ability of carbenicillin to penetrate the outer membrane of *E. coli*.

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