ENTEROBACTER CLOACAE OUTER MEMBRANE PERMEABILITY TO CEFTIZOXIME (FK 749) AND FIVE OTHER NEW CEPHALOSPORIN DERIVATIVES

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(Received for publication January 9, 1980)

The ability of ceftizoxime to penetrate the outer membrane was compared with those of five other new cephalosporins: cefotiam, cefuroxime, cefotaxime, cefmetazole and cefoxitin, using a clinical isolate of *Enterobacter cloacae* as a test strain. Estimation of permeability was performed by a method utilizing the inhibitory activities of the cephalosporins against β-lactamase located in the periplasm. Of the cephalosporins tested, both ceftizoxime and cefmetazole gave remarkably high concentrations in the periplasm, several times higher than those of cefotaxime and cefoxitin and ten or more times higher than those of cefuroxime and cefotiam. The approximate permeability coefficient of ceftizoxime was also several times higher than those of cefotiam and cefmetazole and over ten times higher than those of cefoxitin, cefuroxime and cefotaxime.

The incidence of infections caused by cephalosporin-resistant Gram-negative bacilli, whose resistance to cephalosporins is due to their β-lactamase production, has increased. Intensive effort in current cephalosporin research is being directed towards finding new cephalosporin derivatives which are highly stable to β-lactamases. A series of extremely β-lactamase-stable cephalosporin derivatives has been developed. However, on investigating the actions of these cephalosporin derivatives, there is difficulty in assessing their abilities to penetrate the outer membrane of Gram-negative bacteria. This is because the most reliable assays of outer membrane permeability utilize the hydrolyzing activity of periplasmic β-lactamase[1~4] and consequently is not applicable to β-lactamase-stable derivatives. We have recently developed a novel method for evaluating the outer membrane permeability to β-lactamase-stable β-lactam antibiotics by utilizing their inhibitory activity against β-lactamase[5]. The purpose of this study was to compare the permeability of *Enterobacter cloacae* to ceftizoxime, a new cephalosporin developed in our research laboratories with those of other recently developed β-lactamase-stable cephalosporins using our recently devised method.

Materials and Methods

Bacterial strains

The test strain used in the experiments on outer membrane permeability was *Enterobacter cloacae* No. 91 which was isolated in this laboratory from a clinical specimen. The 68 strains of *E. cloacae* used for antibiotic susceptibility testing were also clinical isolates.

Antibiotics

The antibiotics used were ceftizoxime (FK 749, Fujisawa Pharm. Co., Ltd., Osaka, Japan), cefo-

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tiam (SCE 963, Takeda Chem. Ind., Osaka, Japan), cefuroxime (Glaxo Res., Ltd., Greenford, U.K.), cefotaxime (HR 756, Hoechst-Roussel, West Germany), cefmetazole (CS 1170, Sankyo Co., Ltd., Tokyo, Japan), cefoxitin (Merck Institute, Rahway, U.S.A.) and cephaloridine (Eli Lilly Co., Indianapolis, U.S.A.)

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) containing 0.01 M $MgCl_2$ and resuspended in the same ice-cold buffer to give an optical density of 9.0 at 320 nm. This preparation was used as the intact cell suspension. A 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., Tokyo, Japan) and used as the sonicated cell suspension. 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).

Preparation of β-lactamase

The test strain was grown at 37°C for 20 hours in heart infusion broth (Difco), 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 centrifuged for 1 hour at $10,000 \times g$. The resulting supernatant was applied to a Sephadex G 100 column and eluted with 0.067 M potassium phosphate buffer (pH 7.0). The enzyme fractions were pooled and stored at -20°C. This partially purified enzyme preparation was used to determine the kinetic parameters of the test cephalosporins.

Assay of β-lactamase activity

β-Lactamase activity was determined by direct spectrophotometric assay using a Hitachi 200-20 spectrophotometer equipped with a thermostatted cell holder. The enzyme solution (0.1 ml; intact cells, sonicate, supernatant of cell suspension or purified preparation) was mixed in a 1-cm quartz cuvette with substrate and/or inhibitor and 200 μmoles of phosphate buffer (pH 7.0) to 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 velocity of hydrolysis of the β-lactam ring was followed by the change in absorption of cephalosporins at 260 or 320 nm.

Measurement of outer membrane permeability to cephalosporins

The concentration of cefotiam in the periplasm was determined according to the method of ZIMMERMANN and ROSSELET[4] since this cephalosporin was fairly susceptible to the β-lactamase of the test strain. In this method the β-lactam concentration in the periplasm (S_p) is calculated using the MICHAELIS-MENTEN equation rearranged as shown below.

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

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. The method also enables the permeability coefficient of a β-lactam (C) to be estimated using the following equation.

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

where S_0 is a given substrate concentration outside the cells. The other cephalosporin concentrations in the periplasm were determined according to the method described in detail previously[5]. It depends on inhibition of the periplasmic β-lactamase activity for a substrate (cephaloridine was used in this study) by the test β-lactam. An outline of the procedure is described below. First, the permeability coefficient of a substrate (C_{sub}) was calculated as described above. Next, the concentration of a substrate in the periplasm (S_p') when cells were incubated with both substrate and test cephalosporin was calculated using the following equation.

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

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

$$v_{\text{intact}}' = \frac{S_p' \cdot V_{\max}}{S_p' + K_{m\,app}} \quad \text{and} \quad K_{m\,app} = K_m\left(1 + \frac{I_p}{K_i}\right)$$

where $K_{m\,app}$, K_m, $V_{\max}$ and K_i are the apparent MICHAELIS constant, MICHAELIS constant, maximum velocity of a substrate, and inhibitor constant of a test cephalosporin respectively. These equations can be rearranged as follows;

$$I_p = \frac{K_i(K_{m\,app} - K_m)}{K_m} \quad \text{and} \quad K_{m\,app} = \frac{S_p'(V_{\max} - v_{\text{intact}}')}{v_{\text{intact}}'} \qquad \text{(D)}$$

The values of K_m, K_i and $V_{\max}$ were determined experimentally using the sonicates as enzyme preparation while S_p' was obtained as described above. Accordingly, the concentration of a test cephalosporin in the periplasm (I_p) was calculated using equation D.

Antibiotic susceptibility testing

The minimal inhibitory concentrations (MIC) of test antibiotics were determined by the agar dilution method using Heart Infusion Agar (Difco)[6]. Undiluted or one hundred-fold dilutions of overnight culture in Trypticase Soy Broth (BBL, Cockeysville, U.S.A.) were inoculated with the use of the multipoint inoculator. After incubation at 37°C for 20 hours, the lowest concentration that inhibited macroscopic colonial growth was regarded as the MIC.

Results and Discussion

The antibacterial activity of cephalosporin against Gram-negative bacteria is mainly determined by its stability to β-lactamase, its ability to penetrate outer membrane and its activity against target enzymes. The ability of ceftizoxime to penetrate the bacterial outer membrane was compared with those of the other newly developed cephalosporins, using a clinical isolate of *E. cloacae* as a test strain. *E. cloacae* was selected for a test strain because this species is a typical non-cephalosporin susceptible Gram-negative organism at which recent developments of new cephalosporins have been aimed. Table 1 shows the concentrations of the cephalosporins in the periplasm of the test strain when cells were exposed to 10 and 100 μM of each cephalosporin. Since cefotaxime was very intensely active against the β-lactamase of the test strain, the rate of hydrolysis of a substrate by intact cells was much decreased so that one could not determine exactly when cells were exposed to 100 μM of the antibiotic, nevertheless the hydrolysis substrate, namely cephaloridine, was increased more than ten-fold in its MICHAELIS constant. Accordingly, the concentration of cefotaxime in the periplasm corresponding to the outside concentration of 100 μM was not shown in the table. Among the cephalosporins tested, ceftizoxime and cefmetazole gave remarkably high concentrations in the periplasm.

Table 1. Concentrations of the cephalosporins in the periplasm of *Enterobacter cloacae*.

Antibiotic	Concentration in medium (μM)	Concentration in periplasm (μM)[a]
Ceftizoxime	10	7.4
	100	62
Cefotiam	10	0.07
	100	1.0
Cefuroxime	10	0.9
	100	1.4
Cefotaxime	10	4.8
	100	–[b]
Cefmetazole	10	8.0
	100	61
Cefoxitin	10	6.1
	100	16

a) Test strain was *Enterobacter cloacae* No. 91. Values are averages of duplicate measurements.
b) Not determined due to the high inhibiting activity of cefotaxime against the β-lactamase.

The periplasmic concentrations of ceftizoxime were 7.4 and 62 μM when cells were exposed to 10 and 100 μM of the antibiotic, respectively. These values were several times higher than those of cefotaxime and cefoxitin and ten or more times higher than those of cefuroxime and cefotiam.

The permeability coefficient (C) of a cephalosporin is expressed by the following equation.

$$C = \frac{S_p}{S_0 - S_p}\left(\frac{V_{max}}{K_m + S_p}\right) \quad \text{(E)}$$

where S_0, S_p, K_m and V_{max} are the concentration outside the cells, the concentration in the periplasm, the MICHAELIS constant and the maximum velocity of a test cephalosporin, respectively. This relationship can be derived from the equations (A) and (B) by canceling v_{intact}. Considering that the rates of hydrolysis of the test cephalosporins were extremely slow, together with the fact that the K_i values for the antibiotics were markedly low, the K_m values for the test cephalosporins approximate to their K_i values. Meanwhile, the values of V_{max} of the test cephalosporins approximated to the rates of hydrolysis at a substrate concentration of 100 μM since the K_m values for the test cephalosporins appeared to be much below that concentration. Accordingly, the approximate values of the relative permeability coefficients can be obtained by inserting the approximate value of K_m and V_{max} into equation (E). As shown in Table 2, the approximate permeability coefficient of ceftizoxime was significantly superior to the permeability coefficients of the other cephalosporins. The permeability coefficient of ceftizoxime was several fold superior to those of cefotiam and cefmetazole, and tens or more fold superior to those of cefoxitin, cefuroxime and cefotaxime. The antibacterial activity of ceftizoxime against *E. cloacae* was the most potent of the cephalosporins tested as shown in Table 3. The results of this study indicate that the superior ability of ceftizoxime to penetrate the outer membrane partly contributes to its high antibacterial activity.

Table 2. Approximate permeability coefficients of the cephalosporins.

Antibiotic	V_{max}[a]	K_i (μM)	Approximate permeability coefficient[c]
Ceftizoxime	0.81	0.54	1.00
Cefotiam	56	54[b]	0.40
Cefuroxime	0.10	0.017	0.04
Cefotaxime	0.10	0.024	0.06
Cefmetazole	0.23	0.15	0.40
Cefoxitin	0.10	0.17	0.09

a) Approximate values estimated by the velocity of hydrolysis at a substrate concentration of 100 μM. Values expressed in percentage of the velocity of hydrolysis of cephaloridine.
b) This value represents K_m as an exception.
c) Relative values expressed in ratio to that of ceftizoxime.

Table 3. Mean MICs of the cephalosporins against 68 strains of *E. cloacae*.

Antibiotic	Mean MIC (μg/ml)	
	10^0*	10^{-2}
Ceftizoxime	43	1.04
Cefotiam	>400	28.5
Cefuroxime	>400	100
Cefotaxime	53	1.7
Cefmetazole	>400	300
Cefoxitin	>400	340

* Inoculum size: Inocula used were undiluted overnight culture (10^0) or its centesimal dilution (10^{-2}).

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