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Paradoxical Antibacterial Activities of β-Lactams against *Proteus vulgaris*: Mechanism of the Paradoxical Effect

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Fifteen β-lactam antibiotics were divided into four classes based on their antibacterial actions and β-lactamase-inducing activities in *Proteus vulgaris*. One of these groups, which included cefmenoxime, ceftriaxone, cefuzonam, and cefotaxime, showed a clear paradoxical antibacterial activity against *P. vulgaris*. This group showed growth-inhibitory activity at relatively low concentrations, up to certain limits. These cephalosporins have, as a common moiety, an aminothiazolyl-oxyimino group in the 7-acyl side chain and have high β-lactamase-inducing activities and low stabilities against the β-lactamase. In a mutant strain incapable of inducing β-lactamase, however, the paradoxical antibacterial activity was not observed. These findings suggest that β-lactamase plays an essential role in the paradoxical antibacterial effect in *P. vulgaris*. We conclude that the induction of a large amount of β-lactamase and the low stability against β-lactamase may account for the paradoxical antibacterial activity in *P. vulgaris*.

It is generally assumed that antibacterial activity is proportional to the drug concentration. However, several exceptional observations have been reported (1, 2, 7). Eagle and Musselman (2) have observed that the activity of penicillin G against gram-positive cocci falls off as its concentration is increased. Similar paradoxical effects of antibiotics have been reported (1, 7). In these reports, the mechanisms of the paradoxical phenomena have not been explained.

In our previous paper (4), we reported that cefmenoxime showed paradoxical antibacterial activity against several strains of *Proteus vulgaris* and that this paradoxical effect was dependent on the β-lactamase inductivity and the stability against the β-lactamase that was induced.

Cefmenoxime is one of the new type of cephalosporins and is modified in its 7-acyl side chain with an aminothiazolyl group (10). The cephalosporins with aminothiazolyl groups are not as stable against the β-lactamase specific to *P. vulgaris* (13). Thus, if the paradoxical effect is actually dependent only on the enzymatic characteristics (of the β-lactam antibiotic), the paradoxical theory should be applicable to other aminothiazolyl cephalosporins.

In this study, we explored this paradoxical mechanism in *P. vulgaris* by using 15 β-lactam antibiotics and confirmed a causal relationship between the paradoxical antibacterial effect and the enzymatic characteristics of the β-lactam antibiotic.

MATERIALS AND METHODS

Bacterial strains. *P. vulgaris* 11, a strain that has been described previously (4), and *P. vulgaris* 11-S, a β-lactamase-noninducing mutant of *P. vulgaris* 11, were used in this study. *P. vulgaris* 11-S was derived by mutagenesis with selection, based on its susceptibility to cephaloridine and ampicillin.

A logarithmic-phase culture of *P. vulgaris* 11 was treated with 25 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml for 20 min at 37°C. The washed and treated cells were inoculated in sensitivity broth (Eiken Chemical, Tokyo, Japan) and incubated for 3 h at 37°C. The washed cells were plated onto sensitivity disk agar (Eiken Chemical). After overnight growth of the culture, colonies were replicated onto sensitivity disk agar supplemented with cephaloridine, ampicillin, or rifampin at 10 or 100 μg/ml. Cephaloridine- and ampicillin-susceptible and rifampin-resistant colonies were picked and purified by restreaking them onto the same selection plates. Among these colonies, the lowest β-lactamase-inducing mutant was selected and used as *P. vulgaris* 11-S (Table 1).

The electrofocusing patterns of penicillin-binding proteins and outer membrane preparations were compared with those from the parent strain by the methods of Spratt and Pardee (14) and Tokunaga et al. (15). No alterations in electrophoretic patterns were observed between these two strains (data not shown). The mutation frequency was about 10^{-8}.

Antibacterial agents. The antibacterial agents used in this study were cephaloridine, cephalothin, and moxalactam (Shionogi, Osaka, Japan); cefotiam and cefmenoxime (Takeda Chemical Industries, Osaka, Japan); cefuroxime and ceftazidime (Nippon Glaxo, Tokyo, Japan); ceftriaxone (Nippon Roche, Tokyo, Japan); cefotaxime (Hoechst Japan, Tokyo, Japan); cefuzonam (Lederle Japan, Tokyo, Japan); cefoperazone, piperacillin, and cefbuperazone (Toyama Chemical, Tokyo, Japan); ceftizoxime (Fujisawa Pharmaceutical, Osaka, Japan); and cefmetazole (Sankyo, Tokyo, Japan).

Susceptibility testing. The broth dilution method was used for susceptibility testing (4). An overnight culture of *P. vulgaris* in sensitivity broth (Eiken Chemical) was inoculated into the same medium containing doubling dilutions of β-lactam antibiotics to give an inoculum size of 10^6 cells per ml. The test tubes were incubated at 37°C for 18 h. The MICs were determined as the lowest concentrations at which visible growth was first inhibited.

Effect of clavulanic acid on the paradoxical effect. The broth dilution method mentioned above was used to study the effect of clavulanic acid on the paradoxical effect. An overnight culture of *P. vulgaris* 11 in sensitivity broth was diluted to 10^6 cells per ml with the same broth containing serial doubling dilutions of each of the cephalosporin antibi-

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TABLE 1. Susceptibility and β-lactamase inducibility of *P. vulgaris* 11 and *P. vulgaris* 11-S

Bacterial strain	MIC (μg/ml) [a]				β-Lactamase induction (U/mg of protein) at the following inducer concn (μg/ml)[b]:						
	CMX	ABPC	CER	Rif	0	0.10	0.39	1.56	6.25	25	100
P. vulgaris 11	0.20	>200	>200	>200	0.005	0.020	0.11	0.35	1.68	1.86	2.43
P. vulgaris 11-S	0.006	1.56	1.56	>200	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.006

[a] Abbreviations: CMX, cefmenoxime; ABPC, ampicillin; CER, cephaloridine; Rif, rifampin.
[b] Enzymatic activity was determined in units per milligram of protein by using cephaloridine as the substrate. Cefmenoxime was used as the β-lactamase inducer.

otics and clavulanic acid. After 18 h of incubation at 37°C, visible growth was observed.

Preparation of β-lactamase. β-Lactamase was prepared by a previously described method (4). A final concentration at 10 μg of cefmetazole per ml was added into the logarithmic-phase culture of *P. vulgaris* as a β-lactamase inducer. After 2 h of incubation, the cells were harvested and washed twice. The bacterial cells were disrupted and centrifuged at 100,000 × *g* for 60 min at 4°C. β-Lactamase was purified on CM-Sephadex C-50 (3).

Assay of β-lactamase. The β-lactamase activity was determined by the modified microiodometric method of Novick (8) for piperacillin or the spectrophotometric method (12) for cephalosporin antibiotics. The amount of enzyme that hydrolyzed 1 μmol of the substrate in 1 min at 30°C in 0.05 M buffer solution was defined as 1 U of enzyme activity. The maximum rate of hydrolysis (relative V_{max}) and the affinity for the β-lactams (K_m or K_i) were determined by Lineweaver-Burk plotting (5). The relative V_{max} values are expressed in percent, with the V_{max} of cephaloridine being taken as 100. For the determination of K_i, cephalothin was used as the substrate.

Induction activity for the β-lactamase. The β-lactamase-inducing activity was determined by a previously described method (4). An overnight culture of *P. vulgaris* in sensitivity broth was diluted 20-fold with the same medium and incubated with shaking at 37°C for 2 h. Then, various concentrations of antibiotics were added as the β-lactamase inducers. Incubation was continued for 1 h. The cells were harvested and washed twice. The cells were disrupted and centrifuged at 15,000 × *g*. The supernatant was used as the crude β-lactamase preparation, and β-lactamase activity was determined with cephaloridine as the substrate. The concentration of protein was determined by the method of Lowry et al. (6).

RESULTS

Antibacterial activity and β-lactamase inducer activity. The 15 β-lactams used in this study were divided into four classes based on growth-inhibitory activity (Table 2) and β-lactamase-inducing activity (Table 3).

Group A included the cephalosporins cephaloridine, cephalothin, cefotiam, and cefuroxime, which did not inhibit the growth of *P. vulgaris* 11 at any concentration up to 100 μg/ml.

Group B included cefmenoxime, ceftriaxone, cefuzonam, and cefotaxime. These are well-known as broad-spectrum cephalosporins. In the parent strain, these agents showed the paradoxical antibacterial activity and did not inhibit bacterial growth at higher concentrations but inhibited growth at lower concentrations. However, these showed dose-dependent and very strong antibacterial activities against *P. vulgaris* 11-S.

Piperacillin and cefoperazone were classified into group C, and these had proportional antibacterial activities against both strains of *P. vulgaris* 11 and 11-S.

Group D cephems also had dose-dependent and significantly strong antibacterial activities against both *P. vulgaris*

TABLE 2. Growth-inhibitory effects of β-lactam antibiotics on *P. vulgaris*

Group	β-Lactam	*P. vulgaris* 11		*P. vulgaris* 11-S	
		Paradoxical effect[a]	MIC (μg/ml)[b]	Paradoxical effect	MIC (μg/ml)
A	Cephaloridine	−	>100	−	6.25
	Cephalothin	−	>100	−	3.13
	Cefotiam	−	>100	−	0.78
	Cefuroxime	−	>100	−	1.56
B	Cefmenoxime	+	0.39	−	0.025
	Ceftriaxone	+	6.25	−	0.013
	Cefuzonam	+	0.10	−	0.013
	Cefotaxime	+	0.20	−	0.025
C	Cefoperazone	−	0.78	−	0.025
	Piperacillin	−	0.78	−	0.05
D	Ceftizoxime	−	0.10	−	0.025
	Ceftazidime	−	0.10	−	0.05
	Cefbuperazone	−	0.20	−	0.20
	Cefmetazole	−	6.25	−	3.13
	Moxalactam	−	0.20	−	0.20

[a] +, Paradoxical antibacterial activity; −, proportional antibacterial activity.
[b] The MIC was determined as the lowest concentration at which visible growth was first inhibited.

TABLE 3. Inducer activity of β-lactam antibiotics for formation in *P. vulgaris* 11

Group	Antibiotics	β-Lactamase activity (U/mg of protein) at the following inducer concn (μg/ml)[a]:						
		0	0.10	0.39	1.56	6.25	25	100
A	Cephaloridine	<0.01	0.09	0.17	0.33	0.58	1.20	1.52[b]
	Cephalothin		0.11	0.09	0.31	0.49	0.83[b]	0.64
	Cefotiam		0.07	0.10	0.30	0.49	0.95	1.43[b]
	Cefuroxime		0.10	0.25	0.53	0.65	0.82	1.18[b]
B	Cefmenoxime		0.02	0.11	0.35	1.68	1.86	2.43[b]
	Ceftriaxone		<0.01	0.48	0.90	1.23	2.21[b]	1.12
	Cefuzonam		0.01	0.06	0.32	0.63	0.91[b]	0.69
	Cefotaxime		0.09	0.69	1.83	1.82	2.83[b]	2.00
C	Cefoperazone		<0.01	<0.01	<0.01	0.02	0.11	0.14[b]
	Piperacillin		<0.01	<0.01	0.03	0.15	0.27	0.39[b]
D	Ceftizoxime		<0.11	0.37	1.13[b]	1.00	0.98	0.93
	Ceftazidime		0.07	0.08	0.08	0.26	1.94[b]	1.20
	Cefbuperazone		0.01	0.36	0.80[b]	0.63	0.35	0.44
	Cefmetazole		0.04	0.29	0.52	0.54[b]	0.45	0.20
	Moxalactam		0.05	1.48	1.57[b]	0.93	0.15	0.08

[a] Enzymatic activity was determined in units per milligram of protein by using cephaloridine as the substrate.
[b] The peak β-lactamase activity.

11 and *P. vulgaris* 11-S. This group was composed of some 7-aminothiazolyl-oxyimino cephalosporins such as ceftizoxime and ceftazidime and some 7-methoxy cephalosporins or oxacephems, such as cefmetazole, cefbuperazone, and moxalactam.

The β-lactamase-inducing activities of group A, B, C, and D β-lactams in the culture broth of *P. vulgaris* are shown in Table 3.

The group A β-lactams showed dose-dependent and high inducer activities for β-lactamase formation, and the maximum inducer activities of these agents were 0.83 to 1.52 U/mg of protein at initial doses of 25 to 100 μg/ml.

The β-lactams in groups B and C also showed dose-dependent β-lactamase-inducing activity, whereas group B β-lactams had high inducing activities and group C β-lactams had low inducing activities.

The group D β-lactams had moderate to high β-lactamase-inducing activity, with maximum levels of 0.54 to 1.94 U/mg of protein. However, β-lactamase induction was conversely suppressed at high concentrations.

Effect of clavulanic acid on the paradoxical effect. Table 4 shows the effect of clavulanic acid on the paradoxical antibacterial activity of cefmenoxime against *P. vulgaris* 11. Clavulanic acid abrogated the paradoxical effect of cefmenoxime at concentrations of 1.56 μg/ml or above. This abrogating effect of clavulanic acid was also observed in the other combinations with the group B cephalosporin antibiotics.

Affinity for and stability against β-lactamase. Table 5 shows the stability and affinity of each β-lactam agent against and for the partially purified *P. vulgaris* 11 β-lactamase.

The group A cephalosporins showed the lowest stabilities against *P. vulgaris* 11 β-lactamase, with relative V_{max} values of 100 to 594 when the V_{max} of cephaloridine was taken as 100.

The group B cephalosporins also showed insufficient stabilities, showing relative V_{max} values of 50 to 125. On the other hand, the group C agents showed moderate stabilities and the group D agents showed high stabilities, with relative V_{max} values of 4 to 15 and <0.01 to 0.8, respectively.

However, the group A and B cephalosporins showed lower affinities for the β-lactamase. Thus, the actual stabilities of group A and B agents (K_m/V_{max}) were almost equal to those of group C agents. The group D β-lactams had the highest actual stabilities among the four groups.

TABLE 4. Growth inhibitory effect of cefmenoxime against *P. vulgaris* 11 in the presence of clavulanic acid

Clavulanic acid concn (μg/ml)	Growth inhibitory effect at the following concn (μg/ml) of cefmenoxime[a]:															
	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	0.05	0.025	0.013	0.006	0
0	−	++	++	++	+	+	−	−	−	+	++	++	++	++	++	++
0.2	−	−	−	−	+	+	−	−	−	+	+	+	+	++	++	++
0.39	−	−	−	−	+	+	−	−	−	+	+	+	+	++	++	++
0.078	−	−	−	−	−	−	−	−	−	+	+	−	+	++	++	++
1.56	−	−	−	−	−	−	−	−	−	−	−	−	−	++	++	++
3.13	−	−	−	−	−	−	−	−	−	−	−	−	−	++	++	++
6.25	−	−	−	−	−	−	−	−	−	−	−	−	−	++	++	++
12.5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	++
25	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	++
50	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	++
100	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	++

[a] Symbols: ++, good growth; +, growth; −, no growth.

TABLE 5. Stability and affinity of β-lactam antibiotics for the β-lactamase of *P. vulgaris* 11

Group	Antibiotic	Relative V_{max} (%)[a]	K_m or K_i (μmol)[b]	K_m or K_i/relative V_{max}
A	Cephaloridine	100	137	1.37
	Cephalothin	207	56	0.27
	Cefotiam	226	267	1.18
	Cefuroxime	594	455	0.77
B	Cefmenoxime	125	400	3.20
	Ceftriaxone	57	71	1.26
	Cefuzonam	50	50	1.00
	Cefotaxime	53	238	4.49
C	Cefoperazone	4	8	2.00
	Piperacillin	15	15	1.00
D	Ceftizoxime	0.8	62	77.5
	Ceftazidime	0.1	59	590
	Cefbuperazone	<0.01	40[c]	>4,000
	Cefmetazole	<0.01	25[c]	>2,500
	Moxalactam	<0.01	180[c]	>18,000

[a] The relative V_{max} values of antibiotics are expressed in percent, with the value for cephaloridine taken as 100. The specific activity for cephaloridine was 524 μmol/min per mg of protein.

[b] For the determination of K_i, cephalothin was used as the substrate.

[c] K_i values.

DISCUSSION

We have previously shown the paradoxical effect of cefmenoxime in *P. vulgaris*. This paradoxical effect was distinct from the Eagle effect (2, 7) in terms of its mechanism. The Eagle effect could not be explained by the action of β-lactamase, while the paradoxical effect in *P. vulgaris* described here may be accounted for by the relation between the β-lactamase-inducing activity and the stability against β-lactamase. In this study, we found a peculiar cephalosporin group that showed the paradoxical effect frequently in various strains of *P. vulgaris*. These paradoxical antibacterial activities were always confirmed by the paper disk method described previously (4). These drugs produced clear double inhibition zones against *P. vulgaris* 11 at an inoculum size of 1×10^6 to 1.6×10^7 cells per ml. Moreover, the residual activities of these cephalosporins in the culture broth showed almost the same pattern as cefmenoxime did (4). When large amounts of these drugs were added into the culture broths, the β-lactamase activities were increased remarkably and the residual activities decreased rapidly. In contrast, at lower concentrations these high levels of β-lactamase induction and drug inactivation were not observed (data not shown). However, these paradoxical antibacterial effects in *P. vulgaris* were not always observed but were dependent on experimental conditions. These paradoxical phenomena in *P. vulgaris* 11 were generally confirmed at a relatively high inoculum size that was equal or more than 1.3×10^5 cells per ml, whereas at a higher inoculum size, 1.6×10^7 cells per ml, none of these cephalosporins showed the growth-inhibitory activity at concentrations up to 100 μg/ml (data not shown).

On the other hand, none of the group B cephalosporins showed paradoxical antibacterial activities against *P. vulgaris* 11-S, a β-lactamase-noninducing mutant of *P. vulgaris* 11. This suggests that the induction of large amounts of β-lactamase is at least an essential factor for the paradoxical effect of cephalosporins in *P. vulgaris*. We have obtained an interesting finding, in that the combinations of the group B β-lactams with a small amount (0.78 or 1.56 μg/ml) of clavulanic acid, a potent β-lactamase inhibitor (11), prevent the group B β-lactams from showing paradoxical antibacterial activities. The reason is that the β-lactamase induced in the culture broth is inactivated by the supplement of clavulanic acid.

The group B cephalosporins have in common an aminothiazolyl-oxyimino moiety in the 7-acyl side chains. These have generally sufficient stabilities against various types of β-lactamases but have insufficient stabilities against Richmond and Sykes class Ic β-lactamases, such as that of *P. vulgaris* (10). In our study, these aminothiazolyl-oxyimino cephalosporins, except for ceftizoxime and ceftazidime, showed significantly high V_{max} values. Moreover, these antibiotics had lower affinities for this β-lactamase. This may explain the higher rate of hydrolysis of the group B β-lactams at high concentrations. These are the reasons for the dose-dependent inactivation of antibiotics and the appearance of paradoxical antibacterial activities.

The group A cephalosporins also had high inducing activities and insufficient stabilities against the β-lactamase in *P. vulgaris* 11 and were naturally hydrolyzed at high concentrations. Consequently, these drugs did not inhibit the growth of *P. vulgaris* at high concentrations, but the group B cephalosporins did. On the other hand, these cephalosporins showed poor ultimate antibacterial activities and did not even inhibit the growth of *P. vulgaris* 11-S at low concentrations. This may be due to a low affinity for the penicillin-binding proteins or poor permeability through the outer membrane. Thus, the group A cephalosporins did not show antibacterial activities at any of the concentrations tested.

The group C β-lactams did not induce marked levels of β-lactamase at any of the concentrations up to 100 μg/ml. This may be one of the reasons for the proportional antibacterial activities.

The group D cephems also had high β-lactamase-inducing activities and, at the same time, had very strong β-lactamase stabilities. Thus, the antibacterial activities of these agents were not influenced by the presence of β-lactamase.

Okonogi et al. (9) have shown the kinetics of β-lactamase induction by penicillin G in *P. vulgaris*. In our study, the β-lactams of groups A, B, and C induced almost dose-dependent β-lactamase formation. Therefore, the induction kinetics described above may be applied for most of the β-lactams of these groups but not for the β-lactams of group D, because the group D cephems did not show such proportional β-lactamase induction activities. These agents showed peak β-lactamase induction levels at comparatively low concentrations. Thus, above such peak levels the β-lactamase activities were rather suppressed.

Among the β-lactam agents tested, the ideal compounds were those with low β-lactamase inductivities and high β-lactamase stabilities. The group C agents may meet these conditions, even if they are not perfect. We hope now to have β-lactam agents that are incapable of inducing β-lactamase and that have sufficient stabilities against their β-lactamases. It remains to be explored whether the paradoxical antibacterial activity is observable in vivo as well.

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