IMIPENEM RESISTANCE OF *PSEUDOMONAS AERUGINOSA*

1. RELATIONSHIP OF RESISTANCE BETWEEN IMIPENEM AND OTHER β-LACTAM ANTIBIOTICS

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We determined the susceptibility of 70 clinical isolates of imipenem-resistant *Pseudomonas aeruginosa* to ceftazidime (CAZ), cefsulodin (CFS), cefotaxime (CTX), latamoxef (LMOX), piperacillin (PIPC), and aztreonam (AZT). Twenty strains (29%) produced β-lactamase at low levels, and were susceptible to other antipseudomonal β-lactams (CAZ, CFS, PIPC, AZT). These strains were classified as group A. The production of β-lactamases by the induction of antipseudomonal β-lactams, except imipenem, was low in the group A strains. Resistance of the group A strains to imipenem only was thought to be due to the low permeation of the drug. Fifty strains (71%) showed cross-resistance to other antipseudomonal β-lactams, and these strains were classified as group B. The group B strains produced β-lactamases at high levels by the induction of these antibiotics or dereppressedly. Resistance of the group B strains to imipenem and other antipseudomonal β-lactams was thought to be due to the low permeation of imipenem and the high production of the β-lactamases.

Key words: Resistance, *Pseudomonas aeruginosa*, imipenem, β-lactamase, Inducibility

Introduction

Pseuodomonas aeruginosa strains are generally resistant to most β-lactam antibiotics[1,2], but imipenem is one of the β-lactams which have strong activity against *P. aeruginosa*[3]. In spite of potent activity of the agent, imipenem-resistant clinical isolates have been found in *P. aeruginosa*[4]. Studies on imipenem-resistant mutants have shown that the resistance is due to the decrease of the drug's permeation with diminished production of outer membrane proteins[5,6]. Recently we found a *P. aeruginosa* strain which produced an imipenem-hydrolyzing β-lactamase mediated by a transferable plasmid[7]. To elucidate the epidemiology of imipenem-resistance in *P. aeruginosa*, we determined the antibacterial activity of various β-lactams against imipenem-resistant clinical isolates in *P. aeruginosa*.

Materials and Methods

Bacterial strains. A total of 70 isolates of imipenem-resistant *P. aeruginosa* (MIC; ≥12.5 μg/ml) were examined. These strains were collected from several hospitals throughout Japan from 1988 to 1989.

Antimicrobial agents. Antimicrobial agents were provided as follows: imipenem, Banyu Pharmaceutical Co., Ltd.; ceftazidime and cephaloridine, Nippon Glaxo Co., Ltd.; cefsulodin, Takeda Chemical Industries, Ltd.; piperacillin, Toyama Chemical Co., Ltd.; latamoxef, Shionogi & Co., Ltd.; cefotaxime, Hoechst Japan, Ltd.; aztreonam, Squibb Japan Inc.; benzylpenicillin and ampicillin, Meiji Seika Kaisha, Ltd.

Susceptibility tests. *In vitro* susceptibility of clinical isolates to antibacterial agents was measured by an agar dilution method using sensitivity disk agar (Nissui, Tokyo). A final inoculum of 10^4 CFU per spot was applied to the surface of the drug-containing agar with an inoculating device (Sa-

kuma Seisakusho, Tokyo). MICs were determined after incubation at 37°C for 18 h. The MIC breakpoint for resistance was defined to be ≥12.5 μg/ml for all drugs.

Enzyme assays. β-Lactamase activity was assayed by a spectrophotometric method with a UV-265 spectrophotometer (Shimadzu, Kyoto). Enzyme activity was determined at 30°C in 50 mM phosphate buffer (pH 7.0). Protein concentration was determined by the method of Lowry *et al*[8], using bovine serum albumin as the standard. The relative rate of hydrolysis was expressed as the percentage of hydrolysis of cephaloridine and the concentration of each substrate was 100 μM, except ceftazidime (50 μM). One enzyme unit (1 U) was defined as the amount of enzyme hydrolyzing 1 μmol of cephaloridine per min at 30°C. Detection of the penicillinase production on agar was performed by an iodine reaction; 10 ml of 1% agar containing 1% of soluble starch was mixed with a 1 ml of 160 mM I_2/1.2 M KI, a 1 ml of 20 mg/ml of benzylpenicillin or ampicillin, and the mixture was overlayed on colonies. The halo around a penicillinase-producing colony was determined.

Induction of β-lactamase. Overnight cultures were diluted 1:20 into fresh sensitivity test broth (Nissui) to a final volume of 10 ml. After 2 h of incubation in a shaker at 37°C, each drug was added at a concentration of 1/4 MIC and incubation was continued for another 2 h. Cells were harvested, washed once, and resuspended in 3 ml of 50 mM phosphate buffer (pH 7.0). The cells were disrupted by sonication (1 min at 0°C; output 54 W, TOMY Seiko, Tokyo) and the cellular debris was removed by centrifugation (15,000×g, 15 min, 4°C). β-Lactamase was measured after dialysis against 50 mM phosphate buffer (pH 7.0) at 4°C[9] Cephaloridine was used as the substrate to determine β-lactamase activity.

Results

Susceptibility of clinical isolates. The *in vitro* antibacterial activities of imipenem, ceftazidime, cefsulodin, cefotaxime, latamoxef, piperacillin, and aztreonam against 70 isolates of imipenem-resistant *P. aeruginosa* were determined (Fig. 1). The MICs of imipenem for 50% (MIC_{50}) or 90% (MIC_{90}) of the strains tested was 12.5 μg/ml and 25 μg/ml. The MIC_{50}s of ceftazidime, cefsulodin, cefotaxime, latamoxef, piperacillin, and aztreonam were ≥25 μg/ml. Although all strains were resistant to imipenem, 20, 23, 9, 8, 15, and 24 strains were susceptible (MIC; ≤6.25 μg/ml) to ceftazidime, cefsulodin, cefotaxime, latamoxef, piperacillin and aztreonam, respectively.

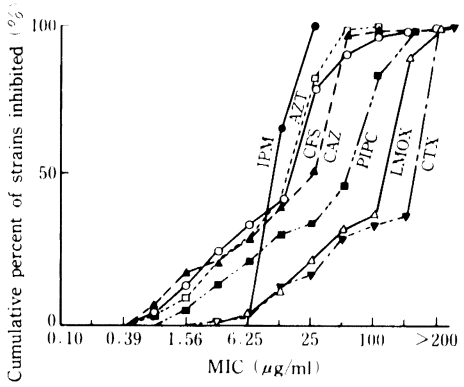

Fig. 1. Susceptibilities of imipenem-resistant *Pseudomonas aeruginosa* isolates to various β-lactams.
Imipenem (IPM), ceftazidime (CAZ), cefsulodin (CFS), cefotaxime (CTX), latamoxef (LMOX), piperacillin (PIPC), aztreonam (AZT).

β-Lactamase-producing levels of clinical isolates. To elucidate the effect of β-lactamase on the cross-resistance between imipenem and other β-lactams, β-lactamase-producing levels of all strains in a non-induced state were determined. In this study, we chose ceftazidime as a representative antipseudomonal β-lactam, and classified imipenem-resistant but ceftazidime-susceptible (IPM^r, CAZ^s) strains as group A, and imipenem- and ceftazidime-resistant (IPM^r, CAZ^r) strains as group B to determine the effect of β-lactamase activity.

The 20 (IPM^r, CAZ^s) group A strains exhibited lower β-lactamase levels of <0.05 U/mg of protein, and the 50 (IPM^r, CAZ^r) group B strains produced β-lactamase at various levels from <0.01 to 0.85 U/mg of protein in a non-induced state. β-Lactamase levels of 17 randomly selected strains

Table 1. MICs and β-lactamase-producing levels of imipenem-resistant strains in *Pseudomonas aeruginosa*

Group	Strain	MIC (μg/ml)					β-Lacatamase sp. act. (U/mg of protein)[b]					
							Noninduced	Induced with				
		IPM[a]	CAZ	CFS	PIPC	AZT		IPM	CAZ	CFS	PIPC	AZT
A	GN17219	12.5	1.56	1.56	3.13	1.56	<0.010	0.76[c]	<0.010	<0.010	0.013	<0.010
	GN17226	12.5	0.78	0.78	1.56	0.78	<0.010	0.16	<0.010	<0.010	<0.010	<0.010
	GN17228	12.5	0.78	0.78	1.56	0.78	<0.010	0.21	<0.010	<0.010	<0.010	<0.010
	GN17235	12.5	0.78	1.56	1.56	1.56	<0.010	0.52	<0.010	<0.010	0.022	<0.010
	GN17263	12.5	1.56	1.56	3.13	3.13	<0.010	0.083	<0.010	<0.010	<0.010	<0.010
B	GN17256	25	12.5	100	50	50	<0.010	0.19	0.020	0.016	0.032	<0.010
	GN17262	12.5	25	200	200	100	<0.010	0.83	0.032	0.036	0.054	0.044
	GN17233	25	50	50	200	25	0.093	1.6	0.66	0.26	0.23	0.30
	GN17259	12.5	50	50	200	25	0.043	1.1	0.72	0.37	0.80	0.44
	GN17269	25	50	25	50	25	0.024	0.73	0.45	0.051	0.088	0.13
	GN17279	12.5	50	25	100	25	0.042	1.4	1.0	0.52	0.97	0.89
	GN17281	25	50	25	50	25	0.023	1.2	0.31	0.087	1.8	2.1
	GN17221	12.5	50	25	200	25	0.44	2.8	1.7	0.88	1.2	1.1
	GN17237	25	25	25	100	25	0.46	1.8	1.1	1.5	1.5	1.9
	GN17253	25	100	50	400	50	0.66	2.2	1.4	1.7	1.7	2.4
	GN17257	12.5	50	50	100	25	0.85	2.1	1.9	1.9	1.6	2.0
	GN17286	25	50	25	100	50	0.58	1.8	1.5	1.7	1.8	2.2

[a] IPM, imipenem; CAZ, ceftazidime; CFS, cefsulodin; PIPC, piperacillin; AZT, aztreonam.

[b] β-Lactamase was induced by a concentration of 1/4 of the MIC of each drug, and the activity was determined using cephaloridine as the substrate.

[c] Underline indicates that the activity in induced state is more than 10 times larger than that in noninduced state.

induced by imipenem, ceftazidime, cefsulodin, piperacillin, and aztreonam are shown in Table 1.

Of five strains in group A, β-lactamase was significantly induced by imipenem but not by ceftazidime, cefsulodin, piperacillin, or aztreonam. In all the strains levels tested in the group B, high levels of imipinem-induced β-lactamase were observed, being more than 8- to 83-fold higher than those in the non-induced state. These strains produced β-lactamase at higher levels induced by ceftazidime, cefsulodin, piperacillin, and aztreonam than those in the non-induced state. The β-lactamase activities in the induced state were 4- to 24-fold (ceftazidime), 1- to 12-fold (cefsulodin), 2- to 80-fold (piperacillin), and 3- to 91-fold (aztreonam) higher than those observed in the non-induced state, respectively.

Table 2. Relative rates of hydrolysis of β-lactam antibiotics by crude enzyme

Strain	Relative rate of hydrolysis (%)					
	IPM[a]	CAZ	CFS	PIPC	AZT	CER
GN17221	<0.10	<0.10	<0.10	4.0	<0.10	100
GN17237	<0.10	<0.10	<0.10	4.4	<0.10	100
GN17253	<0.10	<0.10	<0.10	4.0	<0.10	100
GN17257	<0.10	<0.10	0.10	3.7	<0.10	100
GN17286	<0.10	<0.10	<0.10	4.0	<0.10	100

[a] IPM, imipenem; CAZ, ceftazidime; CFS, cefsulodin; PIPC, piperacillin; AZT, aztreonam; CER, cephaloridine.

Production of penicillinase was detected by an iodometric agar overlay procedure. Four strains in group B were found to produce penicillinases, and

these penicillinases did not hydrolyze imipenem. One strain, GN 17203, produced an imipenem-hydrolyzing β-lactamase[7]

The relative rate of hydrolysis of imipenem and other β-lactams by β-lactamases of derepressedly producing strains of group B is shown in Table 2. These strains highly produced chromosomal β-lactamases. Imipenem, ceftazidime, cefsulodin and aztreonam were stable (<1.0), and piperacillin was slightly hydrolyzed.

Discussion

It has been reported from studies on mutants[4~6] that the imipenem-resistance of *P. aeruginosa* is due to the decrease in drug permeation with diminished outer membrane proteins. These mutants showed no cross-resistance between imipenem and other β-lactams and were supposed to correspond to the group A strains in this study.

We determined the antibacterial activity of various β-lactams against clinically isolated imipenem-resistant strains of *P. aeruginosa*. Of these imipenem-resistant isolates, 71% showed resistance to other antipseudomonal β-lactams, and the type of isolates a (group B) was major.

In previous study, we investigated a novel imipenem-hydrolyzing β-lactamase which was produced by GN 17203. The enzyme was mediated by a transferable plasmid, and dissemination of resistance to imipenem has been speculated[7] In our collection of imipenem-resistant isolates, five strains in all produced extrachromosomal β-lactamase, and only one strain, GN 17203, produced the imipenem-hydrolyzing enzyme.

To elucidate the observed cross resistance between imipenem and other antipseudomonal β-lactams in the group B strains, we investigated their levels of β-lactamase production. The antipseudomonal β-lactam-resistant strains produced β-lactamase at higher levels; β-lactamases were potently induced by these agents or were derepressedly produced. Thus it is likely that the apparent cross-resistance between imipenem and other antipseudomonal β-lactams in the group B strains was due to highly produced β-lactamases, since β-lactamase-stable β-lactams have been found to be affected by highly produced chromosomal β-lactamase, and a reduced activity against the high producers has been found in gram-negative bacteria[10~12].

In β-lactamase inducibility tests, various levels of induction were observed. For example, piperacillin had low β-lactamase-inducing activity in *Enterobacteriaceae*[13]. In our results, piperacillin was a poor β-lactamase inducer in group A strains, like *Enterobacteriaceae*; however, the drug was a potent inducer in some strains of group B. Similar results were obtained with cefsulodin. It was a poor β-lactamase inducer in group A strains, as reported in a *P. aeruginosa* strain[14,15], but had β-lactamase-inducing activity in some strains of group B. To date, the β-lactamase-inducing potencies of β-lactams have been determined for *Enterobacter cloacae*[16] and *Proteus vulgaris*[17]. Our results demonstrated that the β-lactamase-inducing potencies of β-lactams could not be determined for *P. aeruginosa*, since the drugs' induction potencies were different among strains, But the low β-lactamase-inducing potencies of ceftazidime, cefsulodin, piperacillin, and aztreonam in susceptible strains would be an important reason why these agents were active against the strains.

Furthermore, the peculiarity of certain group B strains should be mentioned (GN 17233, GN 17259, GN 17269, GN 17279, and GN 17281). These strains produced β-lactamase at moderate levels in the non-induced state, and highly produced the enzyme by the induction of various β-lactams. To date, highly β-lactamase-producing strains due to changes in the inducer profile have not been determined for clinical isolates of *P. aeruginosa*, Cullmann *et al.*[18] obtained a piperacillin-resistant mutant that was changed so as to potently produce β-lactamase by induction of various β-lactams. Takahashi *et al.*[19] obtained aztreonam-, ceftazidime-, cefotaxime-, or latamoxef-resistant mutants from a β-lactam-susceptible strain, and these mutants moderately produced β-lactamase in the non-induced state, and highly produced the enzyme when induced by imipenem. Our results showed that such strains could be found in clinical isolates.

Potent β-lactamase induction by imipenem was observed in all strains tested, except derepressedly

producing strains, as reported by Tausk *et al.*[20] and all strains showed high levels of β-lactamase activity in the presence of imipenem. The potent β-lactamase-inducing activity of imipenem was supposed to play an important role in imipenem resistance as a basal factor, especially when drug permeation was limited by a change of outer membrane proteins[21].

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Imipenem 耐性 *Pseudomonas aeruginosa* の研究

1. Imipenem 耐性と他の抗緑膿菌 β-lactam 剤耐性の関係について

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臨床分離 imipenem (IPM) 耐性 *Pseudomonas aeruginosa* 70 株の ceftazidime (CAZ), cefsulodin (CFS), cefotaxime (CTX), lactamoxef (LMOX), piperacillin (PIPC), aztreonam (AZT) に対する感受性を検討した。20 株 (29%) は β-lactamase 産生量は低く, 他の抗緑膿菌 β-lactam 剤 (CAZ, CFS, PIPC, AZT) に感受性であり, これらの株を A 群とした。A 群の株の β-lactamase 誘導産生は IPM を除いて誘導がかかりにくかったことから, これらの菌株が IPM 耐性にのみ耐性を示すのは薬剤透過性の低下によることが推論された。50 株 (71%) は他の抗緑膿菌 β-lactam 剤に交叉耐性を示し, これらの株を B 群とした。B 群の株は誘導によって β-lactamase を高度産生するか, あるいは dereppressed の状態で β-lactamase を高度に産生した。B 群の株の IPM および抗緑膿菌 β-lactam 剤に対する耐性には, IPM の薬剤透過性の低下とともに β-lactamase の高度産生の関与が推論された。

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