0031-3998/86/2011-1174$02.00/0
PEDIATRIC RESEARCH

Vol. 20, No. 11, 1986
Printed in U.S.A.

Decreased Baseline β-Lactamase Production and Inducibility associated with Increased Piperacillin Susceptibility of *Pseudomonas cepacia* Isolated from Children with Cystic Fibrosis[1]

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ABSTRACT. The incidence of pulmonary infections in children with cystic fibrosis caused by *Pseudomonas cepacia*, an organism which may possess an inducible β-lactamase, has increased since 1978. Seven of 13 sputum isolates of *P. cepacia* from children with cystic fibrosis were classified as inducible by quantitative enzyme production following preincubation with 100, 200, or 400 μg/ml of cefoxitin. The recovery of inducible strains tended to be associated with recent ceftazidime therapy. Susceptibility to aztreonam, ceftazidime, and piperacillin alone or combined with the β-lactamase inhibitors. YTR 830 or sulbactam, and isoelectric focusing for β-lactamase were performed. Inducible isolates produced significantly more β-lactamase than noninducible strains with or wihtout the addition of cefoxitin. Noninducible isolates were more susceptible than inducible isolates to 8 μg/ml of piperacillin, a difference that was eliminated with the addition of either β-lactamase inhibitor. Twelve of 13 strains produced a β-lactamase band in the pH range of 7.9–8.1; no differences in satellite patterns were noted between the two groups of organisms. Increased production of β-lactamase in the absence of an inducer may account for piperacillin resistance in *P. cepacia* in children with cystic fibrosis. (*Pediatr Res* 20: 1174–1177, 1986)

Abbreviations

MIC, minimum inhibitory concentrations
pI, isoelectric point

Pulmonary infections caused by *Pseudomonas cepacia* in children with cystic fibrosis are frequently associated with progressive deterioration in pulmonary function (1). In one series of 38 children with *P. cepacia* isolated from their sputa, the fatality rate was 45% with approximately one-half of the deaths occuring within 3 months of colonization (2). The incidence of infection with this organism in the cystic population has gradually increased since 1978 and is now recovered from 20% of patients in some centers (3, 4).

Sputum isolates of *P. cepacia* from children with cystic fibrosis are resistant to most β-lactam agents *in vitro*. In an *in vitro* study comparing the susceptibilities of 62 consecutive sputum isolates, concentrations of 64 μg/ml of aztreonam and piperacillin were required to inhibit 79 and 87.1% of the bacterial population, respectively; 90% of the isolates were inhibited by 8 μg/ml of ceftazidime (5). *In vitro*, ceftazidime has proven more active against *P. cepacia* than piperacillin, aztreonam, HR-810, or BMY-28142 (5, 6).

Comparative clinical trials of ceftazidime and other drug regimens in the treatment of *P. cepacia* pulmonary infections in children with cystic fibrosis have not been performed. In a noncomparative study, 14 patients colonized with *P. cepacia* received ceftazidime; only six demonstrated clinical improvement (7). Ceftazidime treatment did not significantly reduce sputum concentrations of the organism. Kercsmar *et al.* (8) noted that six of six patients treated with ceftazidime improved. The effect of treatment on the sputum concentration of organisms was not reported in the latter study.

Although the mechanism(s) of resistance of *P. cepacia* to β-lactam agents is unclear, at least three, inducible, isoelectrically distinct β-lactamases have been identified (9–11). Hirai *et al.* (10) noted that the enzyme produced by *Pseudomonas cepacia* GN11164 hydrolyzed cefuroxime, cefotaxime, and cefamandole. The purpose of this study was to ascertain the role played by inducible enzyme production in resistance to piperacillin, aztreonam, and ceftazidime by sputum isolates of *P. cepacia* from patients with cystic fibrosis.

MATERIALS AND METHODS

Test strains. Thirteen isolates of *P. cepacia* recovered from the sputum of 12 children admitted to Rainbow Babies and Children's Hospital with pulmonary exacerbations of cystic fibrosis were studied. With one exception, all of the test strains were recovered between April and December 1984. Seven of the 13 test strains were recovered during or immediately after ceftazidime therapy; two isolates were recovered following ciprofloxacin therapy, one isolate after aztreonam therapy, and three isolates were obtained prior to parenteral antibiotic therapy. *Pseudomonas aeruginosa* ATCC 27853 was used to control the susceptibility and synergy experiments; *Escherichia coli* ATCC 35218, which contains a TEM-1 (pI 5.4) and a chromosomal β-lactamase, was used as a marker for the isoelectric focusing experiments.

Cell-free enzyme preparations. The test strains were subcultured from frozen stock onto Mueller-Hinton agar, incubated

Received March 3, 1986; accepted June 26, 1986.
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Supported by a grant from the Board of Trustees, Rainbow Babies and Children's Hospital.
[1] Presented in part at the 25th Interscience Conference on Antimicrobial Agents and Chemotherapy, Minneapolis, MN, October 1985.
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overnight, and harvested into 10 ml of Mueller-Hinton broth. For the determination of β-lactamase inducibility, aliquots of the suspensions were diluted 10-fold with fresh broth alone and in broth containing 100, 200, or 400 μg of cefoxitin/ml final solution. For isoelectric focusing, suspensions were diluted with fresh broth alone and in broth containing 200 μg of cefoxitin/ml of final solution. After a 2-h incubation in a shaking water bath at 35° C, the organisms were harvested by centrifugation and washed twice with 0.05 M phosphate buffer (pH 7.0). The cell pellets were resuspended in 5 ml of buffer, crushed in a French press at 6000–7000 psi, and centrifuged at 17,000 × *g* for 30 min at 4° C. The supernatant was stored at −20° C.

Determination of enzyme inducibility. The β-lactamase activity of cell-free enzyme preparations preincubated in broth alone or 100, 200, or 400 μg of cefoxitin/ml of final solution was assayed by measuring the rate of nitrocephin hydrolysis at 482 nm over the first min (12, 13). The protein content of each sample was determined by the method of Bradford (14). Specific enzyme activity was expressed as μmol of product formed per min at 30° C/mg of protein (U/mg protein). Strains capable of increasing specific enzyme activity by 25% over baseline following preincubation with any concentration of cefoxitin were classified as inducible.

Sensitivity and synergy testing. The MIC of piperacillin (Lederle Laboratories, Wayne, NJ), aztreonam (E. R. Squibb and Sons, Princeton, NJ), and ceftazidime (Glaxo, Inc., Research Triangle, NC) against the 13 test organisms were determined by agar dilution (15). Inocula for susceptibility and synergy determination were prepared from overnight broth cultures diluted with fresh broth by nephelometry to yield a final inocula of 10^5 CFU/ml. Resistance of the test organisms to the β-lactamase inhibitors sulbactam (Pfizer Central Research, Groton, CT) and YTR 830 (Taiho Pharmaceuticals, Ltd., Tokyo, Japan) was demonstrated by growth on Mueller-Hinton agar containing 32 μg/ml of each compound. Two-fold serial dilutions of piperacillin, aztreonam, and ceftazidime were combined with 5 μg/ml of each of the β-lactamase inhibitors and incorporated into agar; a 4-fold increase or decrease in the MIC of the drug combination compared to the MIC of piperacillin, aztreonam, or ceftazidime alone were respectively defined as antagonism and synergism (16).

Isoelectric focusing of β-lactamase. Isoelectric focusing of the crude enzyme preparations was accomplished by the procedure described by Matthew *et al.* (17) using commercially prepared polyacrylamide gels (pH range of 3.0 to 10.0) and a thermoelectrically cooled horizontal focusing apparatus. The β-lactamase bands were developed by overlaying the gel with filter paper soaked in 0.075% nitrocephin (BBL, Cockeysville, MD) and were recorded photographically on color slide film. The pH gradient for each gel was determined by the migration of known pI standard proteins (Isogel pI Markers, FMC Corporation, Rockland, ME) run in parallel on each gel and stained with Coomassie Blue.

Statistical analysis. The differences in antimicrobial therapy, susceptibility, and synergy were calculated by the Fisher exact test. Comparisons of mean specific enzyme activity between each group of organisms with and without cefoxitin preincubation was determined by the unpaired Student's *t* test. Mean specific enzyme activities within each group of organisms following preincubation with 0, 100, 200, or 400 μg of cefoxitin/ml of reaction mixture were compared using a one-way analysis of variance with repeated measures and Duncan's test (18).

RESULTS

β-Lactamase inducibility. Seven of the 13 test strains satisfied the criteria for inducibility; five of the inducible strains were recovered from patients receiving or recently treated with ceftazidime. The difference in isolation of inducible strains between ceftazidime recipients and nonrecipients approached but did not reach significance ($p = 0.073$). One inducible and one noninducible strain were isolated from the same patient.

The mean specific enzyme activities of the inducible and noninducible strains after preincubation with 0, 100, 200, and 400 μg of cefoxitin/ml of final reaction mixture are shown in Table 1. The inducible isolates produced significantly more β-lactamase without cefoxitin induction than the noninducible isolates. Furthermore, induction with 100, 200, or 400 μg of cefoxitin/ml resulted in significantly higher specific β-lactamase activities for the inducible strains than the noninducible isolates. Following preincubation with any of the concentrations of cefoxitin tested, the noninducible strains did not significantly increase β-lactamase production compared to uninduced preparations as determined by analysis of variance (F ratio = 2.07, p = NS). Conversely, a significant increase in enzyme activity following cefoxitin preincubation was observed for the inducible isolates (F ratio = 6.86, $p < 0.01$). By Duncan's test, preincubation with 200 μg of cefoxitin/ml produced significantly higher specific enzyme activities than with broth alone ($p < 0.01$) or with 100 or 400 μg of cefoxitin/ml ($p < 0.05$).

Quantitative β-lactamase production by the inducible strains following preincubation with broth alone or with broth containing 100, 200, or 400 μg of cefoxitin/ml of final solution is shown in Table 2. Without induction, baseline enzyme concentrations ranged from 0.022 to 0.106 U/mg of protein. Preincubation with 200 μg/ml of cefoxitin produced specific enzyme activities which ranged from 0.049 to 0.314 U/mg of protein and induction indices which ranged from 1.25 to 3.82.

Susceptibility and synergy. The effect of β-lactamase inducibility on the susceptibility of *P. cepacia* to piperacillin, aztreonam, and ceftazidime alone or combined with 5 μg/ml of either β-lactamase inhibitor is shown in Table 3. All of the test strains were resistant to 32 μg/ml of sulbactam or YTR 830

Table 1. *Quantitative β-lactamase production by inducible and noninducible isolates of P. cepacia*

Cefoxitin concentration (μg/ml)	Specific enzyme activity*				
	Inducible ($n = 7$)		Noninducible ($n = 6$)		
	Mean	SD	Mean	SD	*p*-value†
0	0.065	0.03	0.032	0.005	<0.025
100	0.098	0.08	0.024	0.007	<0.025
200	0.158	0.08	0.025	0.004	<0.005
400	0.086	0.07	0.030	0.03	<0.05

* Expressed as μmol of nitrocephin hydrolyzed per min per mgm of protein.

† Unpaired Student's *t* test.

Table 2. *Quantitative β-lactamase production in inducible strains of P. cepacia*

Strain	Concentration of cefoxitin (μg/ml)							
	0		100		200		400	
	SPE*	IInd†	SPE	IInd	SPE	IInd	SPE	IInd
72–70	0.106		0.254	2.41	0.314	2.97	0.260	2.46
81–41	0.048		0.125	2.64	0.182	3.82	0.069	1.45
73–40	0.057		0.045	0.79	0.188	3.29	0.043	0.75
619i	0.086		0.021	0.24	0.136	1.58	0.048	0.56
82–35	0.022		0.041	1.90	0.061	2.81	0.048	2.23
82–14	0.098		0.167	1.71	0.178	1.82	0.11	1.11
82–15	0.039		0.034	0.87	0.049	1.25	0.022	0.56

* Expressed as μmol of nitrocephin hydrolyzed/min/mg protein at 30° C.

† Defined as SPE with inducer/SPE without inducer.

Table 3. *Inhibition of β-lactamase inducible and noninducible P. cepacia by piperacillin, aztreonam, and ceftazidime alone or combined with 5 μg/ml of β-lactamase inhibitors YTR 830 or sulbactam*

Drug	Organisms	Synergy (no. of strains)	Cumulative MIC*								
			1	2	4	8	16	32	64	128	256
Piperacillin	Inducible		0/7	0/7	1/7	1/7	4/7	5/7	7/7		
	Noninducible		0/6	0/6	2/6	5/6	5/6	6/6			
Piperacillin	Inducible	4	2/7	3/7	3/7	4/7	5/7	7/7			
+ YTR 830	Noninducible	4	3/6	4/6	4/6	4/6	5/6	6/6			
Piperacillin	Inducible	5	3/7	4/7	4/7	5/7	5/7	5/7	7/7		
+ sulbactam	Noninducible	4	2/6	4/6	5/6	5/6	5/6	6/6			
Aztreonam	Inducible		0/7	0/7	0/7	0/7	0/7	1/7	2/7	3/7	7/6
	Noninducible		0/6	0/6	0/6	0/6	0/6	0/6	2/6	2/6	6/6
Aztreonam	Inducible	2	0/7	0/7	0/7	0/7	1/7	2/7	3/7	3/7	7/7
+ YTR 830	Noninducible	1	0/6	1/6	1/6	1/6	1/6	2/6	3/6	5/6	6/6
Aztreonam	Inducible	1	0/7	0/7	0/7	0/7	0/7	2/7	3/7	3/7	7/7
+ sulbactam	Noninducible	2	0/6	0/6	0/6	0/6	1/6	2/6	4/6	6/6	
Ceftazidime	Inducible		0/7	0/7	0/7	1/7	1/7	2/7	4/7	5/7	7/7
	Noninducible		0/6	0/6	0/6	0/6	0/6	2/6	5/6	6/6	
Ceftazidime	Inducible	1	1/7	1/7	2/7	2/7	2/7	2/7	2/7	4/7	7/7
+ YTR 830	Noninducible	1	1/6	1/6	1/6	1/6	1/6	1/6	3/6	5/6	6/6
Ceftazidime	Inducible	1	0/7	0/7	1/7	2/7	2/7	2/7	3/7	5/7	7/7
+ sulbactam	Noninducible	3	0/6	3/6	3/6	3/6	3/6	3/6	5/6	5/6	6/6

* Expressed as number of strains inhibited and as μg/ml of piperacillin, aztreonam, or ceftazidime.

alone. Significantly more noninducible strains were inhibited by 8 μg/ml of piperacillin than inducible strains ($p = 0.048$). No differences in susceptibility to ceftazidime or aztreonam alone were observed between the two groups of organisms.

Piperacillin-β-lactamase inhibitor combinations were effective equally against both the inducible and noninducible isolates. In particular, the difference in susceptibility between the two groups of organisms to piperacillin alone was eliminated in the presence of β-lactamase inhibitors. No differences in susceptibility to aztreonam or ceftazidime alone or combined with sulbactam or YTR 830 were observed. Compared to the inhibitory concentration of ceftazidime alone, the combination of ceftazidime-YTR 830 was antagonistic against two inducible and three noninducible isolates while ceftazidime-sulbactam was antagonistic against one noninducible strain.

Isoelectric focusing. Isoelectric focusing of uninduced and cefoxitin-induced cell free enzyme preparations of both groups of organisms were similar. All of the inducible strains produced at least one β-lactamase band which focused in the pH range of 7.9-8.1. Bands that focused in this range were noted in five of the six noninducible strains. There was no statistical difference in the number of satellite bands produced by either group of organisms with or without cefoxitin induction.

DISCUSSION

The production of inducible β-lactamases has been described for most enterobactericeae and pseudomonads (11, 17). Three enzymes from isolates of *P. cepacia* have been identified by isoelectric focusing. Matthew and Harris (11) identified an enzyme from *P. cepacia* 1599E with a pI of 8.0. Bidwell and Reeves (9) described an enzyme from strain 1872E with a pI of 8.5. The β-lactamase partially purified from GN11164 had a pI of 9.0 (10). Of the 13 strains studied in this report, 12 produced bands which focused in the pH range of 7.8 to 8.1. In a previous study, isoelectric focusing of 23 CF isolates of *P. cepacia* from three centers in North America and 17 isolates from patients without cystic fibrosis and from the environment demonstrated β-lactamase bands in the 7.9 to 8.1 pH range for most strains (19). The production of a β-lactamase band in this pH range appears to be common in *P. cepacia* isolated from all sources, including children with cystic fibrosis, and is independent of the inducibility of the organism.

Mutated strains of *P. aeruginosa* unable to produce significant amounts of chromosomally mediated β-lactamase are more susceptible to piperacillin and other β-lactamase-susceptible β-lactam agents than their respective parent strains with normal enzyme production (20, 21). Conversely, mutated strains of *P. aeruginosa* capable of increased β-lactamase production without induction (baseline concentrations) are more resistant to piperacillin than nonhyperproducing strains (22). Noninducible strains of *P. cepacia* have a diminished capacity to produce β-lactamase with or without induction compared to inducible strains. Not surprisingly, these noninducible isolates were more susceptible to piperacillin than the inducible strains, a difference that could be nullified with the addition of low concentrations of a β-lactamase inhibitor. Resistance of *P. cepacia* to aztreonam and ceftazidime was not associated with either β-lactamase inducibility or baseline β-lactamase activity as determined by this study. However, a mutated strain of *P. cepacia* which produces approximately 30 times as much β-lactamase as its inducible parent is significantly more resistant to ceftazidime and piperacillin than the parent strain (Aronoff S, unpublished observations). Resistance to piperacillin appears to require the baseline production of "moderate" concentrations of enzyme while resistance to ceftazidime requires even higher concentrations of β-lactamase. Changes in outer membrane permeability or in penicillin-binding proteins may also play a significant role in ceftazidime and aztreonam resistance. How all of these factors interact and contribute to β-lactamase resistance by *P. cepacia* is unknown.

Induction of chromosomal β-lactamase is a reversible phenomenon, with specific enzyme activities returning to baseline concentrations following withdrawal of the inducer (23). For *P. cepacia*, inducible strains are capable of significantly increasing β-lactamase production following preincubation with 200 μg/ml of cefoxitin and have increased baseline concentrations of enzyme compared to noninducible isolates. As a result, the ability of some strains of *P. cepacia* to produce increased amounts of β-lactamase following exposure to an inducer may be associated with increased baseline enzyme production which, in turn, leads to increased resistance to piperacillin. The frequency of recovery

of inducible strains tends to occur more often in patients treated with ceftazidime and may account for the development of drug resistance during therapy.

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