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Imipenem-Induced Resistance to Antipseudomonal β-Lactams in *Pseudomonas aeruginosa*

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Using clinical isolates of *Pseudomonas aeruginosa*, we studied the ability of imipenem to antagonize the activity of nine other antipseudomonal β-lactam antimicrobial agents. Imipenem caused truncation of the zones of inhibition in a disk diffusion test for 91 to 100% of the strains, depending on the β-lactam tested. Addition of subinhibitory concentrations of imipenem caused a fourfold or greater increase in MICs for 72 of 74 isolates and in 20 to 87% of the tests, again depending on the antibiotic tested. β-Lactamase assays with both whole-cell suspensions and cell sonicates showed that exposure to subinhibitory concentrations of imipenem resulted in a significant increase in enzyme production. Studies with mutant strains with uninducible and constitutive β-lactamase production supported the hypothesis that induction of β-lactamase was responsible for antagonism. In hydrolysis studies with a β-lactamase extract, most of the antagonized drugs were either not hydrolyzed or only poorly hydrolyzed. We conclude that imipenem induces significantly elevated levels of β-lactamase in *P. aeruginosa*. This increase in β-lactamase is associated with increased resistance of the organism to many other β-lactam agents.

Pseudomonas aeruginosa possesses an inducible β-lactamase enzyme which is chromosomally mediated. In addition, *Pseudomonas* isolates can harbor a wide variety of plasmids which may code for other types of β-lactamase (15, 20). Both chromosome and plasmid-mediated β-lactamases have been implicated as factors important in the development of resistance to β-lactam antibiotics. Consequently, many of the newer β-lactams are designed to be β-lactamase resistant to increase their efficacy. However, it has been shown that for many of the *Enterobacteriaceae* species, the β-lactamase-resistant compound cefoxitin can antagonize other β-lactams in vitro (17, 18) and in vivo (3). There is additional evidence that other newer β-lactamase-resistant cephalosporins may also be responsible for induction of β-lactamase, which in turn can result in antagonism against other β-lactam agents (13, 16).

Imipenem (*N*-formimidoyl thienamycin, imipemide, MK 0787) is a new carbapenem which is both highly active in vitro against *P. aeruginosa* and relatively stable against β-lactamase-mediated hydrolysis (8). The compound has also been shown to be a potent inducer of chromosomally mediated β-lactamase in *Enterobacter cloacae* (2, 7).

We investigated the ability of imipenem to induce β-lactamase production in *P. aeruginosa*. We also looked at the effect of such induction on the in vitro activity of other antipseudomonal β-lactams. We found that in 100% of the *Pseudomonas* isolates β-lactam antibiotics were subject to imipenem-mediated antagonism in a disk diffusion test. Virtually all strains, excepting two highly resistant isolates, showed at least a fourfold increase in the MICs of one or more antipseudomonal β-lactam agents when exposed to subinhibitory concentrations of imipenem (0.0625 μg/ml). The amount of β-lactamase produced in strains of *P. aeruginosa* exposed to imipenem increased and appeared to be related to the observed antagonism. Hydrolysis does not appear to account for the increase in resistance for every antagonized drug. We conclude that the inducible β-lactamase is associated with significantly increased resistance to anti-pseudomonal β-lactam agents.

Part of this work has been presented previously (Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, A38, p. 7; Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, C292, p. 285).

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MATERIALS AND METHODS

Bacterial strains. A collection of 97 clinical blood isolates of *P. aeruginosa* was used, consisting of 47 gentamicin-susceptible and 50 gentamicin-resistant strains (MICs of ≤2 and ≥8 μg/ml, respectively). We also tested two mutants and their parent strain (*P. aeruginosa* 50 SAI+), which were kindly provided by N. A. C. Curtis and D. M. Livermore (4). *P. aeruginosa* 50 SAI− is a strain with minimal β-lactamase activity (indicated by a very weak positive reaction with a chromogenic cephalosporin), but it is uninducible. *P. aeruginosa* 50 SAI con2 is a constitutive producer of high levels of β-lactamase. *P. aeruginosa* ATCC 27853 was included as a control when appropriate.

Antimicrobial agents. Commercially prepared disks were purchased from BBL Microbiology Systems, Cockeysville, Md. These were carbenicillin (10 μg), ticarcillin (100 μg), piperacillin (100 μg), mezlocillin (30 μg), cefoxitin (30 μg), cefotaxime (30 μg), cefoperazone (75 μg), and moxalactam (30 μg). Cefsulodin disks (30 μg) were obtained from Difco Laboratories, Detroit, Mich.

Drug solutions for MIC determinations and hydrolysis studies were prepared from standard laboratory powders. The following were kindly provided: imipenem and cefoxitin by Merck, Sharp & Dohme, Rahway, N.J.; cephaloridine and moxalactam by Eli Lilly and Co., Indianapolis, Ind.; cefotaxime by Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N.J.; piperacillin by Lederle Laboratories, Pearl River, N.Y.; ticarcillin by Beecham Laboratories, Bristol, Tenn.; mezlocillin by Miles Pharmaceuticals, West Haven, Conn.; cefsulodin by Abbott Laboratories, North Chicago, Ill.; carbenicillin and cefoperazone by Pfizer, New York,

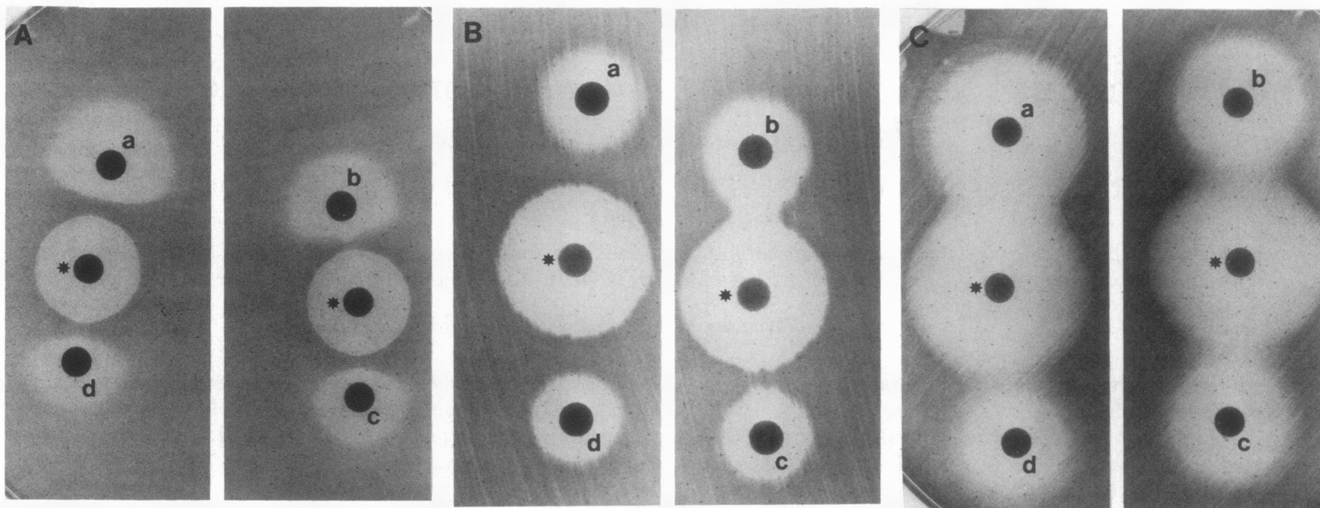

FIG. 1. Disk diffusion test for β-lactam antagonism. Truncation of β-lactam inhibition zones by imipenem in *P. aeruginosa*. (A) *P. aeruginosa* 50 SAI+ (parent strain). (B) *P. aeruginosa* 50 SAI con2 (constitutive β-lactamase production). (C) *P. aeruginosa* 50 SAI− (uninducible for β-lactamase). Disks: imipenem (*); piperacillin (a); cefsulodin (b); moxalactam (c); ceftazidime (d).

N.Y.; and ceftriaxone by Roche Laboratories, Nutley, N.J. Ceftriaxone disks (30 μg) were prepared from standard powder as recommended by the manufacturer. Cephaloridine and imipenem were freshly prepared when needed because of instability.

Disk diffusion assays. All strains were tested for antibiotic antagonism by using a disk diffusion method modified from a method described by C. C. and W. E. Sanders (17). Bacteria were grown to the density of a no. 0.5 McFarland standard in Trypticase soy broth (BBL Microbiology Systems) and inoculated onto Mueller-Hinton agar (MHA) by using a cotton swab. Sterile blank disks, impregnated with 10 μg of imipenem, were placed 5 to 10 mm outside the expected inhibitory zone of disks containing the antimicrobial agents to be tested. Antagonism was visible as a distinct flattening (truncation) of the inhibitory zone around the test antimicrobial agent in the area adjacent to the imipenem disk (Fig. 1A). Truncation was considered positive when the radius of the zone was reduced by 2 to 4 mm depending on the normal size of the inhibitory zone. The strains were called resistant when a zone was absent or so small that truncation was undiscernable. An inhibitory zone was called ambiguous when the degree of truncation was very slight or if it was visible as a "halo" of light growth within a circle of normal growth.

MICs. MICs for imipenem were determined for all *Pseudomonas* isolates according to previously described methods (14). Based on the values obtained we chose an imipenem concentration that was one-half of the lowest MIC with which to investigate the effect of subinhibitory amounts of imipenem on the MICs of other β-lactams. These were ticarcillin, piperacillin, mezlocillin, moxalactam, cefotaxime, and cefoperazone. We performed two parallel sets of MIC determinations, one with and one without imipenem. Of the β-lactams to be tested, twofold serial dilutions were prepared: one set in regular cation-supplemented Mueller-Hinton broth (CSMHB) and one set in CSMHB containing 0.125 μg of imipenem per ml. For the inoculum bacteria were grown in either CSMHB or CSMHB with the same imipenem concentration as above. Upon reaching the density of a no. 0.5 McFarland standard, the cultures were diluted 1:200 in regular CSMHB. An inoculum of bacteria (5×10^5 CFU/ml) grown in the presence of imipenem was added to the serial β-lactam dilutions with imipenem, the effective imipenem concentration becoming 0.0625 μg/ml. Those cultures grown without imipenem were used to inoculate the parallel set of microtiter wells for MIC determinations. A control for growth in 0.0625 μg of imipenem per ml was included. In the case of the mutants, we lowered the imipenem concentration to 0.02 μg/ml because these strains were extremely susceptible to imipenem. The microtiter plates were incubated at 35°C and read after 18 h. MICs were defined as the lowest drug concentration that inhibited growth that was visible macroscopically.

β-Lactamase assays. The β-lactamase activity of intact cells was assayed for 21 strains of *P. aeruginosa* randomly selected from the collection. Using these strains, we compared β-lactamase activity in cultures grown in the presence and absence of imipenem as β-lactamase inducer. We used the following technique, modified from that of C. C. and W. E. Sanders (17), with the spectrophotometric method for measuring β-lactams based on O'Callaghan (12). Strains were grown overnight on regular MHA (uninduced) and on freshly prepared MHA containing 0.25 μg of imipenem per ml (induced). Bacteria were suspended in 0.1 M phosphate-buffered saline (pH 7.0) to the density of a no. 1 McFarland standard. A sample was removed for a colony count, and freshly prepared cephaloridine was added to give a final concentration of 100 μM. The suspensions were incubated for 2 h at 35°C, the bacteria were removed by filtration through a 0.45-μm membrane filter (Millipore Corp.; Bedford, Mass.), and the absorbance of the filtrate was read spectrophotometrically at 255 nm. This procedure was performed five separate times for both samples of each organism. The results were analyzed statistically by using analysis of variance.

The β-lactamase activity of crude β-lactamase extracts was determined in four randomly selected strains of *P. aeruginosa* (B3, B5, B6, and B7) and the ATCC control strain. Bacteria were grown to mid-exponential phase in CSMHB and in CSMHB with imipenem (inducer was added 2 h after inoculation; final concentration, 0.25 μg/ml). The

cells were harvested by centrifugation at 5,000 × *g* at 4°C, resuspended in 1/20 volume of 0.1 M phosphate-buffered saline (pH 7.0), and disrupted by sonication with a 20 kHz Branscom sonifyer. Cell debris was removed by centrifugation, and the supernatant was filtered through a 0.45-μm membrane filter. β-Lactamase activity was determined in 1-ml samples containing 100 μM cephaloridine in 0.1 M phosphate-buffered saline (pH 7.0). Initial rates of hydrolysis were determined at 37°C by measuring the decrease in the A_{255} in a Gilford 240 recording spectrophotometer. Total hydrolysis was achieved by the addition of NaOH to a concentration of 1 N. Enzyme activity was defined as the amount of substrate hydrolyzed under these conditions per milligram of protein. One unit of enzyme is the amount required to hydrolyze 1 μmol of cephaloridine per min. Protein concentration was determined according to the method of Lowry (6) with bovine serum albumin as standard.

Isoelectric focusing. The β-lactamases of three wild-type strains (B5, B6, and B8) and ATCC 27853 were identified by means of isoelectric focusing. Bacteria were induced, and crude extracts were prepared as described above. Focusing was carried out in 1-mm-thick 5% polyacrylamide gels with pH ranges of 3 to 10 and 5.5 to 8.5 (LKB Chemicals, Durham, N.C.) with an LKB 2117 Multiphor. Samples (15 μl) were applied to paper wicks and focused at 0°C for 2 to 2.5 h at constant power (7 W) with voltage ranging from 90 to 1,000 V. Protein isoelectric-point standards were included, as well as a positive control for β-lactamase lactamase type I from *E. cloacae* (Sigma Chemical Co., St. Louis, Mo.). After focusing, the gel was cut, and the reference proteins were stained with Coomassie brilliant blue. The β-lactamases were visualized by overlaying the gel with a 1 mM solution of the chromogenic cephalosporin PADAC[7-(thienyl-2-acetamido)-3-(2-(4-*N*,*N*-dimethylaminophenylazo)pyridinium methyl)-3-ephem-4-carboxylic acid] (Calbiochem-Behring, San Diego, Calif.), solubilized according to the manufacturer's instructions.

β-Lactamase stability of selected β-lactam antibiotics. By using the sonication technique described above, a β-lactamase extract was prepared as described above from a wild-type strain of *P. aeruginosa* (B8) which contained no plasmids and produced only one type of β-lactamase (as detected by isoelectric focusing). UV absorption scans were performed to determine the wavelength at which the difference between the unhydrolyzed substrate and the hydrolyzed product was maximal. Solutions (100 μM) of the following drugs were incubated with the enzyme extract: carbenicillin, ticarcillin, piperacillin, mezlocillin, cefoxitin, cefoperazone, cefotaxime, ceftriaxone, cefsulodin, moxalactam, and imipenem. β-Lactamase activity was determined as described above. The reactions were followed for up to 15 min. The hydrolysis rate is expressed as a percentage of cephaloridine hydrolysis.

TABLE 1. Truncation of inhibition zones by imipenem

Drug	Total no. of isolates tested	No. of isolates		
		With zone present	With ambiguous zone	With truncated zone (%)
Moxalactam	89	88	1	87 (98.9)
Cefotaxime	89	72	2	70 (97.2)
Ceftriaxone	89	55	0	55 (100)
Cefoperazone	89	89	0	89 (100)
Cefsulodin	92	89	3	86 (96.6)
Carbenicillin	97	87	8	79 (90.8)
Ticarcillin	92	88	3	85 (96.6)
Mezlocillin	97	92	0	92 (100)
Piperacillin	97	95	0	95 (100)

TABLE 2. Effect of 0.0625 μg of imipenem per ml on MICs of selected β-lactams

Drug	Total no. of isolates tested	No. of isolates with ≥fourfold increase in MICs (%)[a]
Ticarcillin	71	14 (19.7)
Piperacillin	69	56 (81.2)
Mezlocillin	74	41 (55.4)
Moxalactam	74	40 (54.1)
Cefotaxime	74	64 (86.5)
Cefoperazone	74	47 (63.5)

[a] Of a total of 74 isolates, 72 (97.3%) showed an imipenem-mediated ≥fourfold MIC increase for at least one β-lactam.

RESULTS

Disk diffusion assays. In all isolates (91 to 100% of the tests depending on the antibiotic chosen) β-lactam inhibition zones were truncated by imipenem. The results are summarized in Table 1. On rare occasions a zone was difficult to interpret because of very small zone size (especially with carbenicillin and ticarcillin), indistinctly demarcated zones, or poor growth on MHA. However, only in one strain did we find a dubious result for more than one antibiotic, and this was thought to be due to poor growth on MHA. We were unable to demonstrate any truncation of imipenem inhibition zones by imipenem.

MIC determinations. MICs for imipenem ranged from 0.125 μg/ml (one isolate) to ≥16 μg/ml. Based on these results, we chose 0.0625 μg of imipenem per ml as the subinhibitory concentration with which to investigate the effect of imipenem on MICs of the other β-lactam agents. Table 2 lists the results for all the clinical strains. A fourfold or greater increase in the MICs was seen with 72 of 74 isolates (20 to 87% depending on the antibiotic tested).

β-Lactamase assays. In 21 randomly selected strains the β-lactamase activity of whole-cell suspensions was much higher for bacteria grown in the presence of imipenem than in its absence. Incubation of the former with a 100 μM cephaloridine solution (the A_{255} was 1.4) caused a decrease in the mean absorbance to 0.97. Bacteria grown without imipenem did not alter the absorbance of cephaloridine during the course of the experiment. The mean colony counts were slightly lower for the imipenem-grown strains than for the other strains (3.1×10^8 and 3.9×10^8, respectively). The difference in absorbance was highly significant ($P < 0.0001$) when pairs of cultures of individual strains were

TABLE 3. β-Lactamase activity of cell sonicates[a]

Strain	Sp act (mU/mg of protein)	
	Uninduced	Imipenem induced
ATCC 27853	0.8	307.0
B3	8.0	173.8
B5	2.7	256.6
B6	2.1	503.3
B7	7.9	273.8

[a] One unit of enzyme hydrolyzes 1 μmol of cephaloridine per min at 37°C.

TABLE 4. Hydrolysis of selected β-lactams by chromosomally mediated β-lactamase[a]

Drug	Sp act (mU/mg of protein)	Hydrolysis rate[b]
Cephaloridine	41.9	100
Moxalactam	0	0[c]
Cefotaxime	13.4	3.2
Ceftriaxone	0	0[c]
Cefsulodin	0	0[c]
Cefoperazone	29.3	7.0
Cefoxitin	1.2	0.3
Imipenem	2.0	0.5
Carbenicillin	0	0[c]
Ticarcillin	0	0[c]
Piperacillin	14.3	3.4
Mezlocillin	0	0[c,d]

[a] One unit is defined as the amount of enzyme which hydrolyzes 1 μmol of substrate per min. The enzyme was prepared from strain B8.

[b] Expressed as the percentage of cephaloridine hydrolysis.

[c] Hydrolysis rates listed as zero were below the lower limit of detection of the assay, which ranged from 0.2 mU (ticarcillin) to 0.7 mU (moxalactam).

[d] Mezlocillin appeared to be hydrolyzed very slightly (specific activity measured as 0.7 mU), but this result has to be viewed with caution because it is so close to the lower limit of detection.

compared. Corrections for colony count differences did not affect these results.

Table 3 shows the cephaloridine-hydrolyzing activity of crude β-lactamase extracts of cells grown with and without imipenem. The latter elaborated low levels of β-lactamase (0.8 to 8 mU/mg). Addition of imipenem caused a 21- to 376-fold increase in β-lactamase activity.

Mutant studies. The results obtained with mutants uninducible and constitutive for β-lactamase production and their parent strain 50 SAI+ were as follows. Neither mutant showed imipenem-mediated truncation of the β-lactam inhibitory zone, nor were we able to detect increased β-lactamase production on exposure to imipenem. *P. aeruginosa* 50 SAI− and 50 SAI con2 were very susceptible to imipenem, with MICs of 0.125 μg/ml and 0.25 μg/ml, respectively. (The imipenem MIC for the parent strain, *P. aeruginosa* 50 SAI+, was 2.0 μg/ml.) We therefore chose a lower imipenem concentration than that for the wild-type strains to test for imipenem-mediated MIC increases to other β-lactams. However, in the mutants addition of subinhibitory concentrations of imipenem had an additive effect on the antimicrobial activity of the six other β-lactams. The parent strain showed wild-type behavior for truncation and β-lactamase induction, and MIC increases of at least fourfold were seen in five of six β-lactams, moxalactam being unaffected by 0.0625 μg of imipenem per ml. Figure 1B and C illustrates disk diffusion tests for antagonism with the constitutive and uninducible mutants.

Isoelectric focusing. The crude enzyme extracts all contained a single β-lactamase band which focused between pH 8.5 and 9.1. The β-lactamase used to study hydrolysis of various different β-lactam agents had an isoelectric point of 8.75.

β-Lactam hydrolysis. Table 4 shows the susceptibility of selected β-lactams to hydrolysis by the induced chromosomal enzyme preparation. We were unable to detect any absorbance change during incubation with β-lactamase for moxalactam, ceftriaxone, cefsulodin, carbenicillin, and ticarcillin. The hydrolyses rate for mezlocillin was around the lower limit of detection. Cefoxitin and imipenem were hydrolyzed very slightly cefotaxime and piperacillin at ~ 3% and cefoperazone at 7% the rate of cephaloridine.

DISCUSSION

Our data indicate that exposure of *P. aeruginosa* to subinhibitory levels of imipenem results in an increased resistance in vitro to a wide variety of antipseudomonal β-lactam antibiotics. This was demonstrated by imipenem-mediated truncation of the inhibitory zone of a wide selection of antipseudomonal β-lactams. Increased resistance was not limited to strains with a high overall susceptibility to β-lactams. The collection included many strains resistant to one or more β-lactams; for every isolate, however, there were β-lactams where truncation of the inhibition zone could be observed.

The extent of the increase in resistance was evaluated by determining MICs in the presence of subinhibitory concentrations of imipenem for selected antipseudomonal penicillins and cephalosporins. Under these conditions virtually all (72 of 74) *Pseudomonas* isolates demonstrated a fourfold or greater increase in MICs to these antibiotics, although the number varied depending on the β-lactam tested. MIC increases were not equal for every β-lactam tested; we attribute this to strain variability. Although all strains grew at 0.0625 μg of imipenem per ml as confirmed by the positive growth controls, we cannot rule out the possibility that some isolates may have been partially inhibited by this concentration of imipenem. Finally, similar increases in MICs of antipseudomonal β-lactams for *P. aeruginosa* associated with β-lactamase inducers have been reported recently by other investigators (1, 23).

To determine whether this inducible resistance might be related to β-lactamase induction, we measured the β-lactamase activity of *P. aeruginosa* grown with and without imipenem in cell suspensions and in cultures sonicated to produce cell-free extracts. We found the β-lactamase activity markedly increased over that of base level after growth in imipenem. The β-lactamases induced had isoelectric points compatible with chromosomal enzymes (20). In contrast with the results obtained with wild-type strains and *P. aeruginosa* 50 SAI+, neither the constitutive nor the uninducible mutant showed truncation or increased β-lactamase production on exposure to imipenem. Nor were the MIC values of these mutants to other β-lactams raised by subinhibitory concentrations of imipenem. These findings support the hypothesis that truncation of inhibition zones and increased MIC values are phenomena related to β-lactamase induction.

Imipenem itself did not appear to be affected by induction of β-lactamase, as evidenced by lack of truncation in the disk diffusion test. Moreover, the constitutive β-lactamase producer was more resistant to all β-lactams tested, with the exception of imipenem. In other words, chromosomal β-lactamase did not appear to antagonize the activity of imipenem. However, all other drugs tested were affected.

Finally, we examined the extent to which an extract of the inducible β-lactamase was able to hydrolyze a selection of β-lactam antibiotics. We were not surprised to find that the fourfold increase in MICs was seen most frequently with those β-lactams which were to some extent hydrolyzed by the induced β-lactamase. However, 54% of isolates tested showed a fourfold increase in the MICs of moxalactam, which did not appear to be hydrolyzed easily.

Exactly how induced chromosomal β-lactamase antagonizes β-lactams is not clear at this time. For those drugs which the enzyme readily hydrolyzes, antagonism could be due to hydrolysis of the β-lactam. Many of the new cephalosporins and penicillins do not appear to be hydrolyzed appreciably by inducible chromosomal β-lactamases (5, 16,

19, 21). Yet many of these drugs are antagonized by β-lactamase inducers. One model put forward to explain this phenomenon is the β-lactamase barrier (21). On induction, β-lactamase accumulates in the periplasmic space. The enzyme could bind and trap nonhydrolyzable substrates, blocking the access of the antimicrobial agent to the penicillin-binding proteins on the cytoplasmic membrane. However, for trapping to be an effective resistance mechanism, the entry of drug molecules into the periplasmic space would have to be limited. This is in fact the case: penetration of small water-soluble molecules (including β-lactams) through the gram-negative outer membrane takes place by diffusion through the water-filled channels of porin proteins (9, 10). These porins can effectively control the penetration rate and restrict the entry of β-lactams into the periplasmic space (11, 24). We see the β-lactamase barrier in effect as a "porin blockade" in which accumulated β-lactamase acts in concert with the penetration barrier constituted by the outer membrane. What appear to be nonhydrolyzable β-lactams may be minimally hydrolyzable in vivo under conditions of low drug and high enzyme concentrations. Our data are consistent with an intracellular trapping mechanism of either irreversible binding between β-lactam and β-lactamase or slow hydrolysis.

The extent of the clinical significance of this phenomenon is unclear at this time. However, antagonism of β-lactams by cefoxitin has been demonstrated in vivo (3). The possibility that other β-lactamase-stable β-lactams agents, particularly the newer cephalosporins, may be potential inducers of β-lactamase (2, 7) argues for a cautious approach to the combination of antipseudomonal penicillins and cephalosporins for the treatment of serious *Pseudomonas* infections. Use of such combination therapy might result in induced resistance and treatment failure. Isolated cases in which this may have occurred have already been reported (22).

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