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Effect of Clavulanic Acid on the Activity of Ticarcillin against *Pseudomonas aeruginosa*

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We studied the ability of clavulanic acid (CA) to induce β-lactamase in *Pseudomonas aeruginosa* isolates and what effect this might have on the susceptibilities to β-lactam agents. We first used a disk approximation method to test 4 laboratory and 16 clinical *P. aeruginosa* isolates against antipseudomonal β-lactam agents for truncation by CA and found this to be very common. All antimicrobial compounds except imipenem demonstrated truncation in the vicinity of CA. We also evaluated the extent to which chromosomal β-lactamase is induced by CA and found this to occur to some degree in most isolates and to be dependent on the concentration of CA. Finally, we performed time kill curves on these isolates to compare bacterial growth in ticarcillin alone with growth in ticarcillin-CA (the CA at 2 or 4 μg/ml). We found that CA at this concentration has neither an antagonistic nor a synergistic antibacterial effect in combination with ticarcillin.

Clavulanic acid (CA) is a naturally occurring β-lactam compound from *Streptomyces clavuligeris*. Although this compound has weak antimicrobial properties, it is a potent inhibitor of β-lactamase types II, III, and IV; it is a less potent inhibitor of β-lactamase type I (16). Because of this inhibitory effect on β-lactamase, CA has been combined with amoxicillin (Augmentin; Beecham Laboratories, Bristol, Tenn.) and more recently with ticarcillin (TIC) (Timentin; Beecham). The rationale of these combinations is the inhibition of β-lactamases to allow the other β-lactam (amoxicillin or TIC) to exert its antimicrobial effect. The most recent combination (TIC-CA) has activity against many pathogens including *Pseudomonas aeruginosa*. In vitro studies (7, 10, 18) and clinical trials with the TIC-CA combination have been promising (6, 19).

Induction of β-lactamase by β-lactamase-resistant agents has been shown to occur frequently in *P. aeruginosa*; this induction has been associated with the development of in vitro resistance to many β-lactam agents (23). CA has been described as a β-lactamase inducer in *Enterobacter cloacae* (14). Therefore, possible effects of CA on *P. aeruginosa* might include induction of β-lactamase (antagonistic effect with other β-lactams) as well as inhibition of β-lactamase (synergistic effect).

This study was designed to answer the following questions. Does CA induce β-lactamase? Might induction of β-lactamase cause increased in vitro resistance to β-lactam agents in *P. aeruginosa*? What is the effect of CA in combination with TIC on the growth of *P. aeruginosa* compared with growth in TIC alone?

(Part of this work has been presented previously [Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, A89, p. 15].)

MATERIALS AND METHODS

Bacterial strains. Laboratory strains used were *P. aeruginosa* ATCC 27853, Ps50SAI− (uninducible for chromosomal β-lactamase), Ps50SAIcon2 (a constitutive β-lactamase producer), and the wild-type parent of these two, Ps50SAI+, all of which have been described previously (13). We also tested 16 blood culture isolates of *P. aeruginosa*.

Antimicrobial agents. Disks containing the following antimicrobial agents were purchased from BBL Microbiology Systems, Cockeysville, Md.: carbenicillin (10 μg), piperacillin (100 μg), TIC (75 μg), mezlocillin (75 μg), moxalactam (30 μg), cefoperazone (75 μg), and cefotaxime (30 μg). Cefsulodin (30 μg per disk) was obtained from Abbott Laboratories, North Chicago, Ill. Aztreonam (30 μg per disk), ceftazidime (30 μg per disk), ceftriaxone (30 μg per disk), imipenem (10 μg per disk), and TIC-CA (75 μg plus 10 μg per disk) were gifts from their respective manufacturers: E. R. Squibb & Sons, Princeton, N.J.; Glaxo Inc., Research Triangle Park, N.C.; Hoffman-La Roche Inc., Nutley, N.J.; Merck, Sharp & Dohme, West Point, Pa.; and Beecham Laboratories.

Disk diffusion tests. Kirby Bauer susceptibility tests were done with disks containing TIC (75 μg) and TIC-CA (75 plus 10 μg, respectively). CA-mediated β-lactam antagonism was tested with a disk approximation test (20). Mueller-Hinton agar plates were seeded with strains ATCC 27853, Ps50SAI+, Ps50SAI−, and Ps50SAIcon2, and PaVU3. The latter is a plasmid-carrying, constitutive producer of β-lactamase.

Disks containing 10, 20, or 50 μg of CA were placed next to disks containing the β-lactam antibiotics carbenicillin, cefsulodin, mezlocillin, piperacillin, ceftazidime, aztreonam, TIC, cefoperazone, ceftriaxone, cefotaxime, moxalactam, imipenem, and TIC-CA. CA-induced β-lactam antagonism also was evaluated in clinical isolates by using moxalactam, TIC, piperacillin, or cefoperazone as the indicator drugs.

Broth dilution tests. MICs for TIC were determined for all strains to determine appropriate concentrations for the initial time kill curve. For five strains (ATCC 27853, PaVU3, and the three Ps50 strains) MICs were determined by both macrodilution (5-ml volumes) and microdilution methods, each according to recommendations of the National Committee for Clinical Laboratory Standards (15). We also checked for differences between results obtained with Mueller-Hinton broth and tryptic soy broth (TSB) and found none. This allowed the use of TSB in some of the time kill curves. For the 15 clinical strains we used the microdilution method with TSB.

Time kill curves. Time kill curves were determined in duplicate or more on separate occasions essentially as de-

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scribed by Krogstad and Moellering (12) and Chalkley and Koornhof (5). This technique was chosen because of the difficulties in interpreting the results of other susceptibility tests (MIC, agar diffusion), which do not monitor bacterial survival over time (22). The initial time kill curve for each isolate contained the same concentration of TIC as the MIC for that organism. Based on the results (rapid killing or rapid regrowth) the range of TIC concentrations for additional time kill curves would be chosen above and below the initial concentration. (For example, PaVU3 [MIC 256 μg/ml] had time kill curves done with 160, 200, 240, 250, 280, 300, 350, and 400 μg of TIC per ml). Time kill curves with TIC were compared with curves drawn up for TIC in the presence of 2 or 4 μg of CA per ml. Time kill curves for strains ATCC 27853, PaVU3, Ps50SAI+, Ps50SAI−, and Ps50SAIcon2 were performed with TIC plus only 4 μg of CA per ml. We, and other investigators (1, 7, 21), chose these two CA concentrations because this range represents the usual achievable tissue and serum levels. Time kill curves for ATCC 27853 were also run with a fixed TIC concentration and various CA concentrations. All broth cultures were done in acid-treated borosilicate bottles which held 30 ml of TSB because this medium has demonstrated the greatest enhancement of β-lactamase production (8). TIC and CA stock solutions were made up according to the recommendations of the manufacturers. The necessary amount of antimicrobial agent, as well as the inoculum, was added to the broth in a small volume (<1 ml) to minimally change the culture volume. The inoculum consisted of logarithmically growing microorganisms with a final density of approximately 10^5 CFU/ml as confirmed by colony counts. The bottles (Johnston Laboratories, Inc., Towson, Md.) were agitated on a shaker during 24 h of incubation. Samples for colony counts were taken at 0, 4, and 8 h, at 12 or 20 h, and at 24 h. In comparing growth with and without CA, increased resistance (antagonism) and increased susceptibility (synergy) were sought in time kill curves by looking for an increase in colony counts (≥2 $\log_{10}$) and a decrease in colony counts (<2 $\log_{10}$), respectively, for the TIC-CA combination as compared with TIC alone (9).

The possibility of TIC carry-over affecting colony counts was investigated with ATCC 27853 and the Ps50 series. We calculated cell densities during culture in the presence and absence of *Bacillus cereus* β-lactamase (Sigma Chemical Co., St. Louis, Mo.). At the TIC concentrations used in the time kill curves drug carry-over was not a problem and did not affect the interpretation of the data.

β-Lactamase induction. As a screen for β-lactamase induction, colonies grown in the vicinity of the inducer disk (CA) and colonies growing far removed from this disk were tested with β-lactamase indicator paper (Oxoid Ltd., Basingstoke, United Kingdom). Isolates were said to have inducible β-lactamase production when the β-lactamase indicator paper gave a positive reaction with bacteria grown in the presence of CA (close to a CA disk) but a negative reaction when bacteria were taken from an area far removed from CA. In other words, the base-line β-lactamase level of *P. aeruginosa* was insufficient to cause a positive color reaction. Isolates were called constitutive when they reacted positively after having been grown under non-β-lactamase-inducing conditions.

The degree of β-lactamase induction by CA was evaluated by using ATCC 27853. TSB (20-ml volumes) was inoculated from a fresh overnight culture. After 1 hour, CA was added to achieve final concentrations of 0, 4, 10, or 40 μg/ml. Incubation was contined for 8 h (because the CA effect on bacterial growth was evaluated at 8 h), then the cells were harvested by centrifugation, and crude β-lactamase extracts were prepared as described previously (23). The β-lactamase activity was measured at 255 nm with 100 μM cephaloridine as the substrate at 37°C by using a Beckman DU 8 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Protein activity was determined by the method of Lowry.

CA stability. The stability of CA as indicated by its UV absorbance was measured at 218 nm after incubation at 37°C for various lengths of time up to 24 h.

TABLE 1. Characteristics of *P. aeruginosa* isolates tested

Organism	Susceptibility[a]		MIC of TIC (μg/ml)	β-Lactam antagonism[b]	β-Lactamase + screen[c]
	TIC	TIC-CA			
ATCC 27853	S	S	32	+	Ind
Ps50SAI+	S	S	16	+	Ind
Ps50SAI−	S	S	0.5	−	Unind
Ps50SAI con2	S	S	16	−	Con
B1	R	R	256	+	Ind
B3	S	S	64	+	Ind
B5	S	S	16	+	Ind
B6	S	S	32	+	Ind
B7	S	S	16	+	Ind
PaVU1	S	S	16	+	Ind
PaVU2	R	R	128	−	Con
PaVU3	R	R	256	−	Con
PaVU4	S	S	16	+	Ind
PaVU6	R	R	256	+	Ind
PaVU7	S	S	16	+	Ind
PaVU10	I	I	32	−	Con
PaVU11	S	I	64	+	Ind
PaVU12	S	S	16	+	Ind
PaVU13	I	S	64	+	Ind
PaVU14	S	S	32	+	Ind

[a] Isolates were labeled susceptible, intermediate, or resistant according to the diameter of the inhibitory zone in routine Kirby-Bauer susceptibility testing. R, Resistant (11 mm); I, intermediate (11 to 15 mm); and S, susceptible (15 mm).

[b] β-Lactam antagonism as indicated by truncation of a β-lactam inhibition zone in the vicinity of a disk containing CA. +, Truncation seen; and −, no truncation evident.

[c] β-Lactamase screen by using β-lactamase paper. Ind, Inducible; unind, uninducible; con, constitutive β-lactamase production.

RESULTS

Susceptibility tests. Table 1 lists the characteristics and susceptibility patterns of the *P. aeruginosa* isolates tested. The macro- and micro-MIC compared well, as did the results with Mueller-Hinton broth and with TSB, results being within 1 tube dilution and showing no trend towards higher or lower resistance. All clinical isolates demonstrated β-lactam zone truncation with at least one of four β-lactam antibiotics (ticarcillin, piperacillin, cefoperazone, and moxalactam), indicating that antagonism is possible in these strains. Strains ATCC 27853, PaVU3, Ps50SAI+, Ps50SAI−, and Ps50SAIcon2 were tested with all 13 β-lactams (Table 2). Strain PaVU3 was resistant to all β-lactams in the concentrations used in the disks, with the exception of imipenem. The imipenem inhibition zone was truncated neither in PaVU3 nor in any of the other strains tested. The constitutive and uninducible β-lactamase mutants did not show truncation with any of the β-lactam agents at any of the CA concentrations.

β-Lactamase screen. The β-lactamase screen correlated well with β-lactam antagonism as indicated by β-lactam inhibitory zone truncation (Table 1).

TABLE 2. CA truncation of β-lactam inhibition zones

β-Lactam	Truncation by CA (μg)[a]	
	ATCC 27853	Ps50SAI+
Carbenicillin	20	20
Piperacillin	20	50
TIC	20	50
TIC-CA	50	20
Mezlocillin	10	50
Aztreonam	20	50
Ceftazidime	20	50
Ceftriaxone	10	20
Moxalactam	50	50
Cefotaxime	10	20
Cefoperazone	10	10
Cefsulodin	20	10
Imipenem	None[b]	None[b]

[a] Micrograms of CA per disk needed to achieve truncation of the β-lactam inhibitory zone.
[b] No truncation of imipenem was seen.

Time kill curves. The results of the time kill curves with and without CA are shown in Table 3. Synergy and antagonism did not occur predictably under any specific conditions but instead occurred randomly. In the majority of cases, the organism appeared to be indifferent to the presence of 2 or 4 μg of CA per ml. Synergy was seen in Ps50SAIcon2 and PaVU3, both constitutive β-lactamase producers, but only six times at 8 h of incubation and three times at 24 h in a total of 37 experiments. We saw no synergy with the other constitutive strains. Antagonism was seen occasionally in 11 of 20 strains.

Figure 1 illustrates the effect of CA in strain PaVU3. At all times the colony counts for the cultures with and without CA were less than 2 $\log_{10}$ apart. Regrowth as shown in Fig. 1 at 24 h was seen quite frequently. In several cases in which bacteria growing at 24 h in high TIC concentrations were subcultured, they were more resistant to TIC than the original isolate (data not shown). Figure 2 shows the growth curve of ATCC 27853 in the presence of 0, 4, 10, and 40 μg of CA per ml with and without TIC. A tendency towards antagonism is evident at 8 h, as is a tendency towards synergy at 24 h, but the actual difference in colony counts is less than 2 $\log_{10}$.

TABLE 3. Effect of CA on the growth rate of *P. aeruginosa* in the presence of TIC[a]

Organism	No. of time kill curves					
	8 h			24 h		
	I	A	S	I	A	S
ATCC 27853	6	2		8		
Ps50SAI+	9			8	1	
Ps50SAI−	7			7		
Ps50SAIcon2	10	2	4	13		3
B1	4	2		6		
B3	5			5		
B5	5			4	1	
B6	5			5		
B7	5			5		
PaVU1	7	1		7	1	
PaVU2	7			7		
PaVU3	19		2	18	3	
PaVU4	10			10		
PaVU6	6	2		8		
PaVU7	6			4	2	
PaVU10	6			6		
PaVU11	6			5	1	
PaVU12	9			9		
PaVU13	5	1		5	1	
PaVU14	9			9		

[a] I, Indifference indicated by ≤2-$\log_{10}$ difference in colony counts between TIC and TIC-CA cultures; A, antagonism (colony count TIC < colony count TIC-CA); S, synergy (colony count TIC > colony count TIC-CA).

TABLE 4. β-Lactamase induction in ATCC 27853 by CA

CA (μg/ml)	β-Lactamase sp act (U[a])
0 (base line)	1.5
4	2.6
10	6.6
40	88

[a] One unit hydrolyzes 1 nmol of substrate per min per mg of protein. Conditions: 100 μM cephaloridine in 0.1 M phosphate buffer, pH 7.0, 37°C.

β-Lactamase induction. The results of the β-lactamase screen with β-lactamase indicator paper are included in Table 1. Table 4 shows the degree of β-lactamase induction by CA.

CA stability. The A_{218} of CA decreased by 20% in 8 h of incubation at 37°C and by 50% after 24 h.

DISCUSSION

There has been concern that the combination TIC-CA might act as both a β-lactam antimicrobial agent plus a β-lactamase inducer when used against *P. aeruginosa*. Our results show that CA can induce β-lactamase in *P. aeruginosa* and that this appears to lead to in vitro antagonism of other antipseudomonal β-lactams (disk approximation data). *Pseudomonas* isolates which have the capacity to significantly raise their production of β-lactamase are most apt to show this type of antagonism. We have previously shown (23) that truncation of β-lactam inhibitory zones by imipenem indicates that antagonism will also be evident as an increase in MICs for β-lactam agents when determined in the presence of subinhibitory concentrations of imipenem.

The time kill curves show in most cases indifference to CA in a low concentration as would be found in tissue and serum. The occasional antagonistic or synergistic effect appears to be random. Comparison of the degree of β-lactamase induction by different concentrations of CA shows that 4 μg/ml raises the β-lactamase activity of ATCC 27853 twofold. Significantly elevated β-lactamase levels (60-fold) can be achieved, however, by higher CA concentrations (e.g., 40 μg/ml). We feel that the absence of an antagonistic effect in our time kill curves is due to the low concentrations of CA. Furthermore, because CA is unstable at 37°C, its concentration decreases significantly during the course of the incubation which would also reduce the tendency towards induction of β-lactamase. Truncation is seen in a disk approximation test because the CA concentration around the disk is proportionally higher than that used in the time kill curves.

Figure 2 shows indeed that when higher concentrations of CA are used there is a tendency towards increased resistance at 8 h. The time kill curves also showed no indication of any beneficial effect of adding CA to TIC for the studied strains which included several constitutive β-lactamase producers and one uninducible strain. Therefore, our findings are in agreement with those of other investigators who used

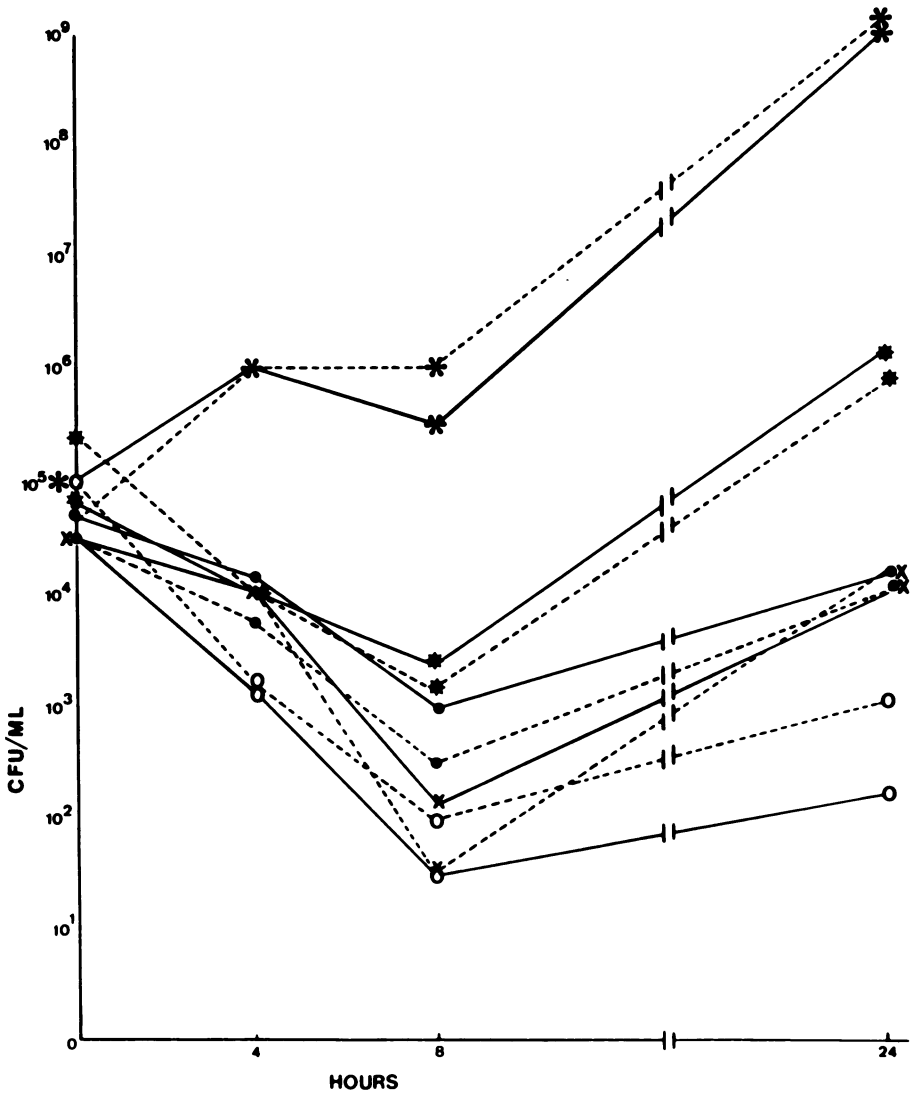

FIG. 1. Time kill curve for PaVU3. Different TIC concentrations with and without 4 μg of CA per ml. —, TIC only; – – –, TIC-CA; *, control (no TIC); ★, 160 μg of TIC per ml; ●, 200 μg of TIC per ml; ×, 240 μg of TIC per ml; ○, 280 μg of TIC per ml.

different techniques and found that CA does not frequently have a synergistic effect in combination with the β-lactams in *P. aeruginosa* (7, 17, 18, 27).

Calderwood et al. have described antagonism between azlocillin and CA in *P. aeruginosa* strains which produced only chromosomal type I β-lactamase (4). They also found synergy between these two β-lactam agents in strains that produced certain plasmid-mediated β-lactamases. The first observation agrees with the assumption that CA can induce chromosomal β-lactamase which in turn can antagonize β-lactam agents. However the second observation suggests that in bacteria whose major β-lactamase production is that of plasmid encoded enzymes, CA is more effective as a β-lactamase inhibitor, and the β-lactam agent is sufficiently protected to be able to cause cell death. Interestingly, Calderwood found that a higher concentration of CA, the synergy observed between azlocillin and CA in plasmid-carrying strains disappeared. This may be explained by increased induction of the chromosomal enzyme which itself is not inhibited by CA. This enzyme produced by *Pseudomonas* isolates may be capable of hydrolyzing agents such as TIC at a low rate. This might be similar to observations by both Vu and Nikaido (24) and White and Curtis (25) who have described hydrolysis of other "β-lactamase stable" β-lactam antibiotics by the inducible chromosomal β-lactamase of *E. cloacae*.

The clinical significance of the in vitro demonstration of synergy or antagonism with TIC-CA is unclear. Exposing *Pseudomonas* isolates to CA can lead to higher production of β-lactamase, which then may provide the cell with a mechanism for greater survival in the presence of other β-lactam agents. Bolivar et al. noted increased resistance in vitro of *P. aeruginosa* to a combination of β-lactams and β-lactamase inhibitors (2). Several investigators have reported emergence of resistant *P. aeruginosa* isolates during therapy with TIC-CA combinations including cases of clinical treatment failure (6, 11, 26). Brittain et al. (3) noted difficulty in eradication of *P. aeruginosa* with these compounds (despite Kirby-Bauer assays which indicated susceptibility). In all these cases derepression of β-lactamase may

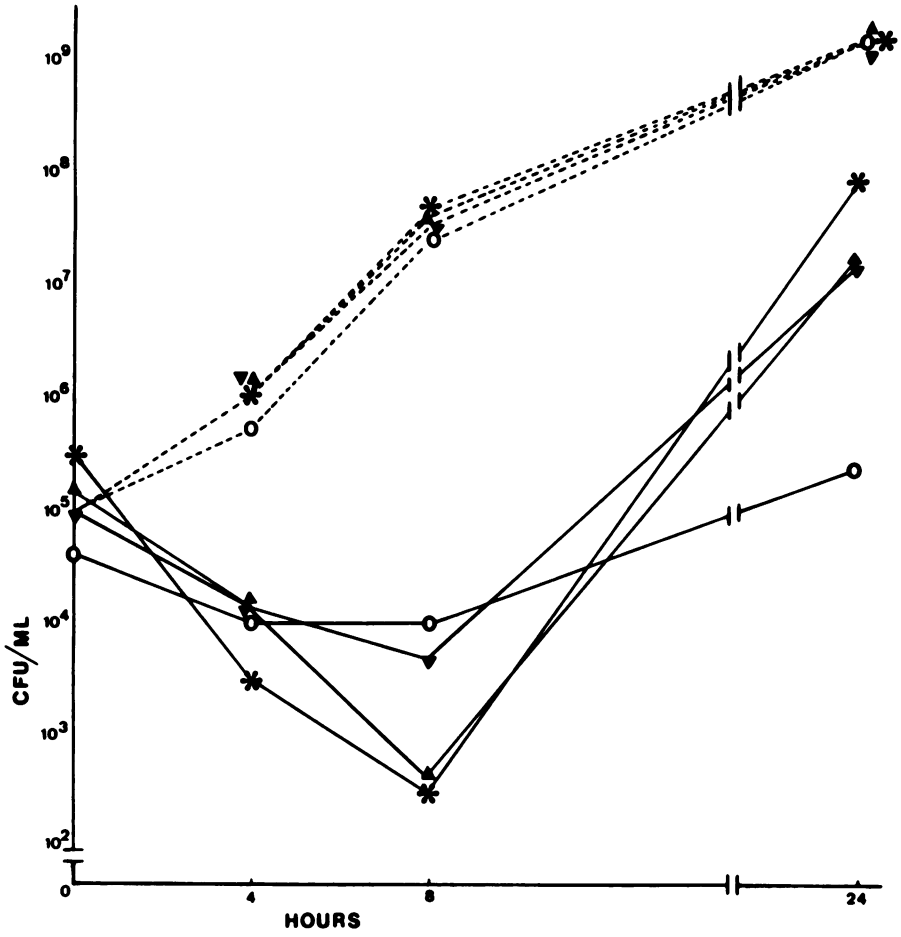

FIG. 2. Time kill curve for ATCC 27853. Constant TIC concentration and various CA concentrations. —, TIC-CA; - - -, CA; *, control (no CA); ▼, 10 μg of CA per ml; ▲, 20 μg of CA per ml; ○, 40 μg of CA per ml.

have delayed initial bacterial killing which would allow resistant mutants to emerge.

In summary, CA can induce the chromosomally mediated β-lactamase enzyme in *P. aeruginosa*. However, clinically achievable concentrations of CA do not cause increased TIC resistance in time kill curves. The combination of TIC-CA may not pose a greater problem than TIC alone with respect to development of inducible resistance in *P. aeruginosa* despite the potential for induction of β-lactamase. More clinical experience with this combination is needed to answer this question. However, because of the potential for antagonism we recommend that TIC-CA be combined with an aminoglycoside in the case of serious infections when *P. aeruginosa* is suspected.

ACKNOWLEDGMENT

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