

# Inhibition of *Aerobacter* Cephalosporin $\beta$ -Lactamase by Penicillins

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Cephalosporinase ( $\beta$ -lactamase) was obtained from cell washings of *Aerobacter* (*Enterobacter*) *cloacae* as a highly active preparation. An alkalimetric method was used to determine the enzyme activity and to estimate its inhibition by 6-aminopenicillanic acid derivatives. Their order of decreasing inhibitory effect was as follows: cloxacillin, oxacillin, methicillin, ampicillin, and penicillin G. We found that 2 to 3 ng of cloxacillin per ml was sufficient to decrease the enzyme activity by 50% in the presence of 400  $\mu$ g of cephalosporin C per ml. Cloxacillin exerted a potentiating effect on the inhibition of the *E. cloacae* organisms by cephalosporin C.

We have previously described a cephalosporinase ( $\beta$ -lactamase) produced by a strain of *Aerobacter* (*Enterobacter*) *cloacae* (Hospital for Sick Children 18410) and the distribution of cephalosporin-inactivating activity within the *Enterobacteriaceae* (3, 4). Recently, Hennessey (5) has reported on the  $\beta$ -lactamase activity in several strains of *E. cloacae*. In work with *A. (Enterobacter) aerogenes* and *Proteus morganii*, O'Callaghan and co-workers (8) published their results on the inhibition of  $\beta$ -lactamase activity of cephaloridine and cephalothin by derivatives of 7-amino-cephalosporanic acid in which the most potent were four analogues with the 7-acyl group derived from benzoic acid. O'Callaghan and Muggleton (7) also reported the ability of cloxacillin and methicillin to protect cephalothin from the action of  $\beta$ -lactamases from the same organisms. Similar investigations were made by Bach and co-workers (1) on the protective effect against  $\beta$ -lactamases from gram-negative organisms on ampicillin.

In our studies, we had observed an extreme degree of inhibition of the  $\beta$ -lactamase activity from *E. cloacae* on cephalosporin C by penicillinase-resistant penicillins. We now wish to record these findings.

## MATERIALS AND METHODS

**Enzyme preparation.** Cotton-plugged Roux bottles containing 135 ml of Trypticase Soy Agar (BBL) were each inoculated with 5 ml of an overnight culture of *E. cloacae* (HSC 18410) in Trypticase Soy Broth (BBL) and incubated at 30 C for 24 hr. The resulting surface growth, together with the water of condensation, was decanted and centrifuged in the cold at

20,000  $\times$  g for 30 min. The supernatant fluid was then discarded. The resuspended sediment from each Roux bottle was washed with 10 ml of distilled water three times for 10 min at 20,000  $\times$  g in the cold. The three washings were filtered through a 0.22- $\mu$ m membrane (Millipore Corp., Bedford, Mass.) and individually stored at -20 C or lyophilized. The latter process was attended by a variable loss of activity.

**Method of assay and measurement of enzyme inhibition.** A recording pH stat (model TTT/1; Radiometer, Copenhagen) with a constant temperature reaction chamber was used to observe the rate of acid formation resulting from the enzymic hydrolysis of the unbuffered  $\beta$ -lactam substrates at neutral pH by the use of 0.02 N NaOH as titrant.

Enzyme preparations in microliter volumes was added to 5 ml of 0.2% gelatin solution containing appropriate quantities of derivatives of 6-aminopenicillanic acid (inhibitors) and incubated at pH 7 for 10 min at 37 C. The reaction was initiated by the addition of 100  $\mu$ liters of a neutral aqueous solution containing the substrate. In some experiments, the substrate and inhibitor were added to 5 ml of gelatin solution, and the reaction was initiated by the addition of enzyme.

**Combined effect of cephalosporin C and cloxacillin.** After incubation for 24 hr with various inocula of the test organism, minimal inhibitory concentrations of cephalosporin C and cloxacillin were determined by the serial broth dilution method. The least amount of antibiotic in each test resulting in complete inhibition of growth was recorded as the minimal inhibitory concentration. Then the combined effect of the two agents was evaluated by measuring the inhibitory concentration of cephalosporin C in the presence of 50  $\mu$ g of cloxacillin per ml. The combined effect of these two agents was also evaluated by measuring the inhibitory concentration of cephalosporin C in the presence of 100  $\mu$ g of cloxacillin per ml by use of the

plate dilution method. In this test, the antibiotics were incorporated in poured plates containing Penassay agar, and the surface spot was inoculated with 0.02 ml of serial decimal dilutions of a broth culture of the test organism. The number of colonies in each spot was recorded after 24 hr at 37 C.

## RESULTS

When the *E. cloacae* was subjected to multiple washings in distilled water, the major cephalosporin  $\beta$ -lactamase activity was found in the first three washings.

In one example, the activity, in units per milliliter, was 42, 34, and 21 in washings 1, 2, and 3, respectively, but only 5 units/ml in washing 4. One unit is the amount of enzyme which hydrolyzes 1  $\mu$ mole of cephalosporin C in 1 min at pH 7 and 37 C.

**Inhibition of cephalosporin  $\beta$ -lactamase activity.** The inhibitory effect of several penicillin derivatives on the cephalosporin  $\beta$ -lactamase activity was determined; that for cloxacillin is shown in Fig. 1.

The graph depicts the decrease of the initial rate of enzyme activity after preincubation for 10

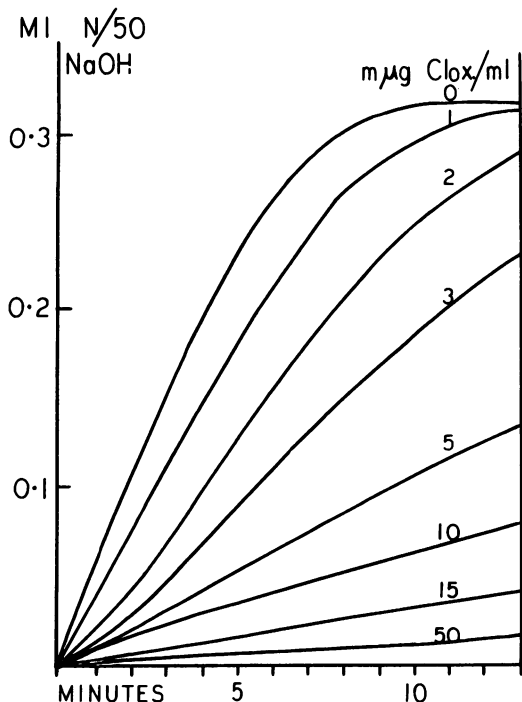


FIG. 1. Inhibitory effect of cloxacillin on the activity of *Enterobacter cephalosporin*  $\beta$ -lactamase on cephalosporin C. Enzyme is 0.63 unit; substrate is 400  $\mu$ g of cephalosporin C per ml; alkalimetric method.

min with increasing concentrations of cloxacillin. We found that 2 to 3 ng of cloxacillin per ml was sufficient to decrease the activity by 50%.

The approximate concentrations of penicillin derivatives required to produce 50% inhibition of the initial rate of cephalosporin  $\beta$ -lactamase activity is shown in Table 1.

The inhibitory effect of benzylpenicillin is best seen when there is no preincubation. The resulting curve is sigmoidal in contrast to the usual hyperbolic form given by the other penicillins tested, including ampicillin (Fig. 2).

**Combined effect of cephalosporin C and cloxacillin on cephalosporin  $\beta$ -lactamase-producing organisms.** The potentiation of the inhibitory effect of cephalosporin C by cloxacillin on the *E. cloacae* strain is shown in relation to inoculum size both by the tube dilution method (Table 2) and by the agar dilution technique (Table 3). This potentiation was also observed with some strains of *Proteus vulgaris* and *Pseudomonas aeruginosa*.

## DISCUSSION

The very powerful inhibition of *Enterobacter cephalosporin*  $\beta$ -lactamase by cloxacillin and other penicillin derivatives is noted here and is of a magnitude similar to that reported for *Pseudomonas cephalosporin*  $\beta$ -lactamase by Jago and co-workers (6). Under the conditions described in our report and when present in an approximate molar ratio of 1:150,000, cloxacillin produced 50% inhibition of the rate of destruction of cephalosporin C by *Enterobacter cephalosporin*  $\beta$ -lactamase (Fig. 1, Table 1). Sabath and Abraham (9) and Sabath et al. (10) suggested that  $\beta$ -lactamase production contributed significantly to the high resistance to the cephalosporins in *P. aeruginosa*. O'Callaghan and her co-workers

TABLE 1. Inhibition of cephalosporin  $\beta$ -lactamase by penicillins

Levels of inhibitor yielding approximately 50% inhibition <sup>a</sup>	
Inhibitor	Level of inhibitor <sup>b</sup>
Cloxacillin.....	2
Oxacillin.....	4
Methicillin.....	20
Ampicillin.....	200
Penicillin G.....	4,000
6-Aminopenicillanic acid.....	40,000

<sup>a</sup> Conditions under which 50% inhibition was achieved were an enzyme concentration of 1.26 units and a concentration of 400  $\mu$ g of cephalosporin C per ml.

<sup>b</sup> Expressed in nanograms per milliliter.

(7, 8) have suggested the use of cephalosporins combined with a semisynthetic penicillin or cephalosporin in inhibiting the  $\beta$ -lactamase of gram-negative organisms.

Since we have previously demonstrated a minor but definite "penicillinase" activity for the *Enterobacter* enzyme (3), it is reasonable to assume that the sigmoidal shape of the curve in Fig. 2, showing the inhibition of hydrolysis by penicillin G, is due to the concurrent hydrolysis of the penicillin G by the enzyme preparation.

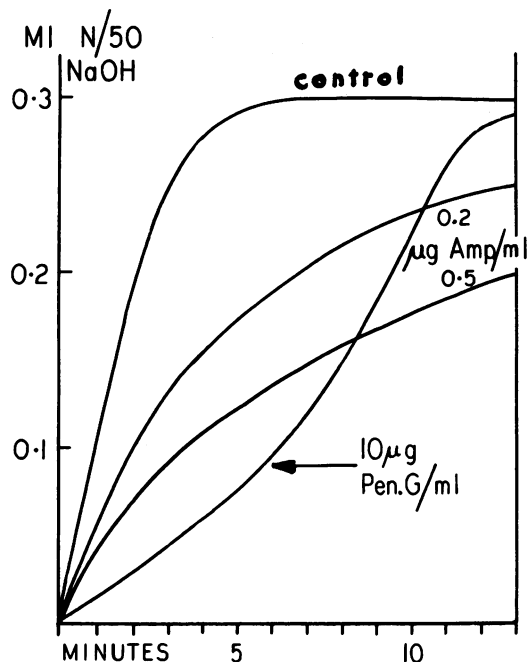


FIG. 2. Inhibitory effect of ampicillin and penicillin G on the activity of *Enterobacter cephalosporin*  $\beta$ -lactamase on cephalosporin C. Enzyme is 1.26 units; substrate is 400  $\mu$ g of cephalosporin C/ml; alkalimetric method.

The lack of this effect in the case of ampicillin indicates the relative stability to this enzyme conferred by the  $\alpha$ -amino substitution (4).

The inhibition of the *Enterobacter* enzyme by cloxacillin, by use of the alkalimetric method, in concentrations which are much below an antibacterial level and are small fractions of that usually attained in serum, would suggest that a useful degree of clinical synergism between cloxacillin and cephalosporins might be obtained (Fig. 1, Table 1). However, in Tables 2 and 3, it can be seen that with the intact *Enterobacter* cells potentiation between cephalosporin C and cloxacillin, although marked, does not result in complete inhibition until much higher levels are attained than seem clinically practical. Moreover, there is the probability that only the free, rather than the bound, cloxacillin is effective as an inhibitor. The results, however, indicate that the complete effect could become manifest in the urine. The in vivo part of the O'Callaghan studies (7, 8) revealed that experimentally infected mice could be protected when treated with synergistic combinations.

The concentration of cephalosporin C required to inhibit the *Enterobacter* organism in minimal inoculum, in the presence of what might be

TABLE 2. Combined effect of cephalosporin C and cloxacillin in broth on *E. cloacae*

Cells inoculated	Minimal inhibitory concentration <sup>a</sup>		
	Cloxacillin only	Cephalosporin C only	Cephalosporin C with cloxacillin (50 $\mu$ g/ml)
$2 \times 10^8$	6,000	25,000	1,250
$2 \times 10^4$	2,500	2,500	625
$2 \times 10^1$	1,000	1,000	312

<sup>a</sup> Values are expressed in micrograms per milliliter.

TABLE 3. Combined effect of cephalosporin C and cloxacillin in agar on *E. cloacae*

Antibiotics in plate <sup>a</sup>	Colony counts with inoculum of approximate size (cells) <sup>b</sup>					
	$2 \times 10^8$	$2 \times 10^6$	$2 \times 10^5$	$2 \times 10^4$	$2 \times 10^3$	$2 \times 10^2$
Control (no antibiotic)	++	++	++	++	26	3
Cephalosporin C (625)	++	++	++	++	+	8
Cloxacillin (200)	++	++	++	++	16	2
Cloxacillin (100)	++	++	++	++	17	2
Cloxacillin (100) + cephalosporin C (325)	+	10	0	0	0	0
Cloxacillin (100) + cephalosporin C (625)	0	0	0	0	0	0

<sup>a</sup> Concentrations of antibiotics are expressed parenthetically in micrograms per milliliter.

<sup>b</sup> Confluent growth is represented by ++; colonies discrete but uncountable are represented by +.

considered adequate excess of cloxacillin, remains at a high figure, 312  $\mu\text{g}/\text{ml}$  (Table 2). It may be that this reflects remaining cephalosporin  $\beta$ -lactamase activity which is not suppressed due to the existence of relative permeability barriers to cloxacillin. This point might be settled by obtaining a cephalosporinase-negative mutant derived from the *Enterobacter* strain. The morphologically and biochemically similar *Klebsiella* group has a lower minimal inhibitory concentration of the cephalosporins (2, 3) as do all the species which we have found to be consistently cephalosporin  $\beta$ -lactamase negative. This is in contradistinction to the uniformly high degree of resistance of the known cephalosporin  $\beta$ -lactamase-producing organisms even when, as in *P. aeruginosa* (10), only small quantities of enzyme are apparently produced. A more direct approach to the problem of cephalosporin resistance would seem to be the development of a cephalosporin  $\beta$ -lactamase-resistant cephalosporin with a suitable degree of inherent activity for gram-negative cells.

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