Resistance of *Escherichia coli* and *Salmonella typhimurium* to Carbenicillin

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(Accepted for publication 30 June 1969)

SUMMARY

Carbenicillin, a 9- α carboxybenzyl penicillin, is bactericidal only for *Escherichia coli* strains which are resistant to less than 125 $\mu\text{g./ml.}$ of ampicillin. All *E. coli* and *Salmonella typhimurium* strains in which penicillinase is a surface enzyme are resistant to carbenicillin. *E. coli* and *S. typhimurium* strains in which production of beta-lactamase is episomally mediated are resistant to both ampicillin and carbenicillin. A non-hydrolyzable penicillin (methicillin or dicloxacillin) does not allow carbenicillin to exert its antibacterial effect against resistant strains of *E. coli*, *Pseudomonas*, *Klebsiella* or *Enterobacter*. Carbenicillin shows no synergy with penicillinase-resistant penicillins.

INTRODUCTION

Carbenicillin is an alpha carboxybenzyl penicillin synthesised from the 6-amino-penicillanic acid nucleus. It differs from ampicillin in that a carboxyl group is substituted for an amino group at the alpha position. The drug is hydrolyzed by β -lactamases to produce alpha carboxybenzyl penicilloic acid. Since its introduction for the treatment of *Pseudomonas* and *Proteus* infections (Brumfitt, Percival & Leigh, 1967), strains of ampicillin-resistant *Escherichia coli* which are also sensitive to this drug have been reported (Acrod *et al.* 1967, Bodey & Terrell, 1968). Previous work from this laboratory on the location of β -lactamases in *E. coli* (Neu, 1968) suggested that carbenicillin sensitivity was related to the low level of penicillinase production of certain *E. coli* strains.

In the present investigation we have studied carbenicillin resistance in *Escherichia coli* and *Salmonella typhimurium* and related it to ampicillin resistance and surface location of penicillinase, whether that enzyme is episomally mediated or not.

Synergism between combinations of penicillins has been reported for ampicillin and penicillinase resistant penicillins such as methicillin and cloxacillin (Sutherland & Batchelor, 1964). We have therefore sought synergism between carbenicillin and the β -lactamase-insensitive penicillins in a variety of Gram-negative rods.

MATERIALS AND METHODS

Materials. Carbenicillin was obtained from Beecham Inc. Dicloxacillin and methicillin were obtained from Bristol Laboratories.

Organisms. *Escherichia coli*, *Pseudomonas*, *Proteus*, *Klebsiella* and *Enterobacter* strains were obtained from the diagnostic laboratories of the Presbyterian Hospital.

Salmonellae were a generous gift of Dr Winter of the Beth Israel Salmonella Centre. *E. coli* isolates had been selected on the basis of resistance to 10 µg. ampicillin discs.

Tube dilution sensitivity tests. A dilution, containing 10^4 organisms, of an overnight trypticase soy broth (BBL) culture of the test organisms was added to tubes containing dilutions of carbenicillin or ampicillin (final vol. 1.0 ml.) and incubated at 37° for 18 hr. and then observed for growth. Bactericidal levels were obtained by plating clear tubes.

Broth dilution tests for synergy. A checkerboard arrangement of test tubes was used to test the effect of pair antibiotics. Tubes were arranged in five rows of 12 tubes each. Each row contained the same concentration of penicillinase inhibitor (methicillin or dicloxacillin; 0, 100, 500, 1000 or 2000 µg./ml.) and successive tubes in an alternate row contained carbenicillin 0, 2, 4, 8, 16, 32, 64, 125, 250, 500, 1000 and 2000 µg./ml. Antibiotics were prepared fresh for each test; inoculation and incubation were as above. The first clear tube was taken as the minimum inhibitory concentration (MIC) for that organism; clear tubes were plated to obtain the bactericidal level.

Carbenicillin assay. A 12 × 12 in. glass plate was poured with 450 ml. of trypticase soy agar (BBL) seeded with 5 ml. of an overnight culture of the Elsworth pseudomonas strain. Wells of 6 mm. were punched in the agar, and 0.06 ml. of the test solution placed in the wells. The plate was incubated at 35° for 18 hr. Concentration of carbenicillin was determined using 5, 10, 20 and 40 µg. carbenicillin standards and measuring diameters in mm. of the zone of growth inhibition.

Procedure for osmotic shock. Stationary-phase organisms were harvested at 16 hr after a 1% inoculation into Penassay broth (Difco) and washed twice at 21° with 0.03 M-NaCl + 0.01 M Tris-HCl (pH 7.3). They were suspended in 0.5 M-sucrose + M Tris-HCl (pH 7.5) at 21° at 10^{10} cells/ml. EDTA was added to 1 mM and mixed by shaking for 5 min. The organisms were removed by centrifugation at 0°, and resuspended in cold distilled water with mixing for 5 min. The organisms were removed by centrifugation and the supernatant fluid assayed for β-lactamase activity.

Bacterial extracts were prepared with a Branson Sonifer Model 75 using 2 min. sonic disintegration at 0° in 15 sec. bursts. R-factors were detected by the method of Watanabe (1964). Penicillinase activity was determined using a modification of Novick's (1962) iodometric assay with benzylpenicillin G as substrate.

RESULTS

Fourteen strains of *Escherichia coli* in which the β-lactamase was released by osmotic shock, and was thus a surface enzyme (Neu & Chou, 1967), were all resistant to carbenicillin. Of 21 strains of *E. coli* in which the penicillinase was not released by osmotic shock 20 or 95% were sensitive to 25 µg./ml. or less of carbenicillin.

Escherichia coli strains which were sensitive to carbenicillin (Table 1) were resistant to less than 125 µg./ml. of ampicillin.

Table 2 is a comparison of the carbenicillin and ampicillin sensitivity of some *Escherichia coli* and *Salmonella typhimurium* strains in which the β-lactamase is either a surface or internally located enzyme. There do not appear to be significant differences in MIC levels of carbenicillin and ampicillin for either high level or low level penicillinase-producing strains. Strains of either organism in which the penicillinase is surface located showed high level resistance. When the penicillinase was an internal enzyme resistance did not exceed 250 µg./ml.

None of 15 *Escherichia coli* and 15 *Salmonella typhimurium* strains in which production of the β -lactamase is episomally mediated were sensitive to either ampicillin or carbenicillin. Several strains of *E. coli* has surface penicillinases but transfer of resistance to another strain was not possible.

Table 1. Sensitivity of ampicillin resistant *Escherichia coli* to carbenicillin

Ampicillin resistance $\mu\text{g./ml.}$	Carbenicillin resistance		Sensitive (%)
	No. tested	No. sensitive*	
10-50	18	16	90
50-250	21	19	90
> 250	14	0	0

* Sensitive to 25 $\mu\text{g./ml.}$ or less of carbenicillin.

Table 2. Comparison of carbenicillin-ampicillin sensitivity and location of penicillinase

<i>Escherichia coli</i> strain	Penicillinase location	Ampicillin $\mu\text{g./ml.}$	Carbenicillin $\mu\text{g./ml.}$
DB 131	Surface	> 4000	> 4000
DB 130	Internal	3.2	3.2
DB 111	Internal	64	6.4
DB 100	Internal	250	100
DB 112	Internal	8	4
FW/R 126	Surface	> 2000	> 2000
DB 103	Surface	> 2000	> 2000
<i>Salmonella typhimurium</i>			
BH 19	Surface	> 8000	> 8000
BH 16	Internal	16	32
A 100	Surface	4000	4000

Table 3. Correlation of growth rates of two strains of *Escherichia coli* with the destruction of carbenicillin*

Time (hr)	<i>E. coli</i> DB 131 (Surface penicillinase)		<i>E. coli</i> DB 307 (Internal penicillinase)	
	o.d.	Carbenicillin % left	o.d.	Carbenicillin % left
0	0.025	100	0.025	100
1	0.130	75	0.085	85
2	0.408	33	0.200	60
3	0.748	5	0.451	50
4	1.020	0	0.785	6

* Overnight cultures were inoculated to 10^4 cells/ml. After 1 hr at 35° on a rotary shaker, 100 $\mu\text{g.}$ per ml. carbenicillin was added. Growth was followed by turbidimetric change at 600 m μ . Carbenicillin was assayed (see 'Methods') on aliquots removed at noted times. DB 131 was resistant to 2000 $\mu\text{g.}$ carbenicillin/ml. and DB 307 was resistant to 125 $\mu\text{g.}$ carbenicillin/ml.

Variation in the size of the *Escherichia coli* inoculum from 10^3 to 10^7 organisms/ml. in the carbenicillin tube dilution did not lower the level of resistance of the high level resistant strains. Strains of *E. coli* resistant to less than 30 $\mu\text{g./ml.}$ rarely showed an effect of inoculum size. Strains such as DB 108, in which the level of resistance was 63 $\mu\text{g./ml.}$ at an inoculum of 10^3 organisms, rose to 200 $\mu\text{g./ml.}$ with an inoculum of 10^7

organisms. Highly resistant strains of *E. coli*. or *Salmonella typhimurium* in which the penicillinase was surface located probably destroyed the carbenicillin so rapidly that the effect of inoculum size was not seen. Table 3 illustrates that the rapid growth rate of high level carbenicillin-resistant, surface penicillinase strains in the presence of carbenicillin, in contrast to those strains with lower resistance and an internal enzyme, is related to destruction of carbenicillin.

Table 4. *Test for synergy of carbenicillin and methicillin*

Strain	MIC with: Carbenicillin ($\mu\text{g./ml.}$)	Methicillin ($\mu\text{g./ml.}$)	Carbenicillin + Methicillin ($\mu\text{g./ml.}$)		
<i>Pseudomonas a. 2</i>	250	1000	4	+	1000
<i>Escherichia coli</i> 132	6	500	2	+	500
<i>E. coli</i> 18	4	500	0.5	+	500
<i>Enterobacter</i> 10	64	1000	4	+	500
<i>Klebsiella</i> 273	250	1000	32	+	1000
<i>Klebsiella</i> 272	125	500	32	+	100
<i>E. coli</i> 131	4000	> 4000	4000	+	> 4000
<i>S. typhimurium</i> A 100	1000	2000	1000	+	1000
<i>Pseudomonas a.</i> 101	8000	16000	4000	+	8000
<i>Proteus m.</i>	4000	> 4000	4000	+	4000

Since carbenicillin is hydrolyzed by β -lactamases of most Enterobacteriaceae we attempted to lower the level of resistance of various organisms by performing the tube dilution in the presence of a penicillinase-resistant penicillin such as methicillin. Using a checkerboard method of broth dilution it is possible to determine whether combinations of antibiotics are synergistic, antagonistic or additive in effect. Using either methicillin or dicloxacillin, only 1 strain of 32 tested showed synergy. Eleven *Pseudomonas aeruginosa*, 8 *Escherichia coli*, 4 *Enterobacter*, 7 *Klebsiella* and 2 *Proteus* were tested. Table 4 demonstrates that only *klebsiella* 272 showed true synergy. In the few cases where the MIC of carbenicillin was lowered the MIC of methicillin in the combination was equal to the MIC of methicillin alone. Fifty per cent of these strains showed a synergistic effect of ampicillin and methicillin.

DISCUSSION

Carbenicillin, which is hydrolyzed by β -lactamases of both Gram-positive and Gram-negative bacteria, had been thought to be active against some ampicillin-resistant *Escherichia coli*. These investigations show that *E. coli* which possess surface penicillinases are resistant to carbenicillin. Not all of these strains with surface penicillinases could transfer resistance to ampicillin and carbenicillin to another strain of *E. coli*. In all of these episome strains the penicillinase was a surface enzyme. Carbenicillin is effective only against ampicillin-resistant *E. coli* in which resistance is at a low level.

It is not readily apparent why carbenicillin shows less synergist action than ampicillin when used with penicillinase-resistant penicillins such as methicillin. It is possible that the entry into the cell of carbenicillin is more rapid or that its affinity for β -lactamases is greater than that of ampicillin. There is no practical value to combinations of carbenicillin with other penicillins since concentrations from 3000 to 20,000 $\mu\text{g./ml.}$ can be obtained in urine (Neu & Swarz, 1969).

Although carbenicillin is unlike ampicillin in that it is active against pseudomonas strains, its activity against members of the Enterobacteriaceae is similar to ampicillin, particularly in regard to β -lactamase producing strains.

REFERENCES

- ACRED, P., BROWN, D. M., KNUDSEN, E. T., ROBINSON, G. N., & SUTHERLAND, R. (1967). New sem synthetic penicillin active against *Pseudomonas pyocyanea*. *Nature, Lond.* **215**, 25.
- BODEY, G. P. & TERRELL, L. M. (1968). In-vitro activity of carbenicillin against gram-negative bacilli. *J. Bact.* **95**, 1587.
- BRUMFITT, W., PERCIVAL, A., & LEIGH, D. A. (1967). Clinical and laboratory studies with carbenicillin, a new penicillin active against *Pseudomonas pyocyanea*. *Lancet* **i**, 1289.
- NEU, H. C. (1968). The surface localization of penicillinases in *Escherichia coli* and *Salmonella typhimurium*. *Biochem. Biophys. Res. Commun.* **32**, 258.
- NEU, H. C. & CHOU, J. (1967). Release of surface enzymes in *Enterobacteriaceae* by osmotic shock. *J. Bact.* **94**, 1934.
- NEU, H. C. & SWARZ, H. (1969). Carbenicillin: use in serious infections caused by *Pseudomonas*. *Clin. Res.* **17**, 372.
- NOVICK, R. P. (1962). Micro-iodometric assay of penicillinase. *Biochem. J.* **83**, 236.
- SUTHERLAND, R. & BATCHELOR, F. R. (1964). Synergistic activity of penicillins against penicillinase from gram-positive and gram-negative bacteria by substrate analogues. *Nature, Lond.* **201**, 868.
- WATANABE, T. (1964). Selected methods of genetic study of episome mediated drug resistance in bacteria. *Meth med. Res.* **10**, 202.