J. MED. MICROBIOL. — VOL. 17 (1984), 37–44

IMPORTANCE OF METHODOLOGY IN DETERMINING BACTERICIDAL AND BACTERIOSTATIC ACTIVITIES OF AZLOCILLIN AND TICARCILLIN AGAINST *PSEUDOMONAS AERUGINOSA*

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SUMMARY. The activities of azlocillin and ticarcillin against *Pseudomonas aeruginosa* were compared by estimating minimum inhibitory and bactericidal concentrations (MIC and MBC) in liquid and solid media, and by constructing killing curves from sequential viable counts. In MIC studies, azlocillin was about three times more active than ticarcillin in solid medium (agar dilution test) and in liquid media (tube and microdilution tests). When the MBC was measured, however, results varied according to the technique used. On agar and in microdilution tests, both azlocillin and ticarcillin were bactericidal, the MBC being 1·3–3 MIC. In the tube test, the MBC for ticarcillin was again about 3 MIC, but azlocillin appeared not to be bactericidal (MBC > 1 mg/ml). However, sequential viable counts of four clinical isolates showed that at 4 MIC both antibiotics reduced viable counts by a factor of 10^4 in 8 h. Our results stress the importance of methodology when assessing the antibacterial activity of an antibiotic.

INTRODUCTION

Pseudomonas aeruginosa most often causes opportunistic infections in compromised patients, and effective treatment must be instituted at once to avoid a high mortality rate. *P. aeruginosa* is not only intrinsically resistant to many antibiotics, but is also able to acquire resistance to others with relative ease. Hence, the search for compounds with antipseudomonal activity has continued. Azlocillin is a ureidopenicillin which has recently been marketed in Great Britain as an alternative to the well-established compound ticarcillin; it is currently in use in this Hospital. Because suggestions have been made that azlocillin has certain shortcomings in respect of its activity against *P. aeruginosa* (Basker, Edmondson and Sutherland, 1979; White, Comber and Sutherland, 1980), we have undertaken a detailed study of a large number of freshly isolated clinical strains.

Different techniques for measuring the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were used, in view of the findings

Received 10 May 1983; *accepted* 6 Jul. 1983.

of Greenwood and Eley (1982) that conventional MIC tests and turbidimetric experiments gave different results when used for assessing the activity of β-lactam antibiotics against *P. aeruginosa*.

MATERIALS AND METHODS

Media. Oxoid Iso-Sensitest Broth (ISB) and Iso-Sensitest Agar (ISA) were used throughout this study.

Organisms tested. Two series of *P. aeruginosa* strains were used. Series A consisted of 110 consecutive isolates obtained recently from clinical material sent to the Diagnostic Microbiology Laboratory at the Royal Free Hospital. Series B consisted of 18 gentamicin-resistant strains collected during a 2-year period from clinical specimens. These strains were epidemiologically distinct; multiple isolates from the same patient were excluded and there were no recorded instances of cross-infection with these strains. These 128 strains were tested for sensitivity by the disk diffusion method (see below). Further experiments were performed with strains selected randomly from the 95 strains in series A that were sensitive to both azlocillin and ticarcillin.

Disk sensitivity tests. Strains were cultured overnight in digest broth, diluted 1 in 10^4 with water, and 0·1-ml amounts were spread on plates of ISA to give an inoculum of *c.* 10^4 cfu/plate. Disks containing azlocillin (75 μg), ticarcillin (75 μg) and gentamicin (10 μg) were placed on the plates, which were then incubated overnight. Strains that gave zones of inhibition of 13 mm or less were regarded as resistant. This is consistent with the criteria laid down by Brown and Blowers (1978) for gentamicin, Phaneuf and Neu (1979) for ticarcillin and Fass (1982) for azlocillin. Disks were controlled with a standard sensitive strain, *P. aeruginosa* NCTC10662.

Determination of MIC and MBC

Agar dilution method. Serial doubling dilutions of azlocillin and ticarcillin in ISA were inoculated with 10^4, 10^5 and 10^6 cfu of 82 strains of *P. aeruginosa*, using a multipoint inoculator (Denly Instruments, Billinghurst, Sussex RH14 9SJ). MICs were read after incubation for 18 h at 37°C; MBCs were determined by replica plating with a velvet pad from the surface of the plates inoculated with 10^5 cfu to fresh ISA, and incubating overnight. Because the efficiency of transfer with the velvet pad is *c.* 1% (Cruickshank, Duguid and Swain, 1969), a 99·8% fall in viable count was represented by the growth of two colonies after this procedure.

Broth dilution method. Serial doubling dilutions of azlocillin and ticarcillin were made in 1-ml amounts of ISB contained in soda lime glass tubes (7·5 cm × 1 cm). Tubes were inoculated with 10^5 cfu of *P. aeruginosa* (9 strains) and the MIC was read after incubation overnight at 37°C. The MBC was determined by sub-culturing 5 μl, with a Nichrome loop of 3·5 mm diameter, on to blood agar from each tube in which no growth was visible. The growth of one colony indicated a 99·8% fall in viable count.

Microdilution method. Serial doubling dilutions of azlocillin and ticarcillin were made in 50-μl amounts of ISB containing triphenyltetrazolium chloride 200 μg/ml in Microtiter "V" plates made of tissue culture grade polystyrene. Each well was inoculated with 150 μl of ISB that contained 4×10^5 cfu of *P. aeruginosa*; 45 strains were tested. Plates were stacked to avoid evaporation and incubated overnight at 37°C. In the wells where growth had occurred the tetrazolium salt was reduced to a red formazan and the appearance of the red colour was used to determine the MIC end-point. The MBC was established by sub-culturing 1 μl from each well of every plate on to ISA, using a multipoint inoculator equipped with a 96-pin head. The growth of four or fewer colonies indicated a 99·8% or greater fall in viable count.

MIC was the lowest concentration of antibiotic that prevented visible growth overnight (agar and tube dilution methods), or prevented reduction of tetrazolium overnight (microdilution method). MBC was the lowest concentration of antibiotic that caused at least a 99·8% fall in viable count during overnight incubation. Although the cut-off points for MBC were slightly different for the three techniques used, there was usually no problem in deciding the MBC

because sub-cultures yielded either no growth, which by all three techniques indicated at least 99·9% fall in viable count, or a heavy growth.

Sequential viable counts. An inoculum of 10^7 cfu of each of four test strains was added to a series of five 250-ml conical flasks each containing 100 ml of ISB to which azlocillin or ticarcillin had been added to achieve concentrations representing 0·5 MIC, 1 MIC, 2 MIC and 4 MIC. An antibiotic-free control was also included. Flasks were swirled at 100 rpm at 37°C and at suitable intervals 0·1-ml amounts were removed from each flask for a viable count on ISA containing penicillinase (Wellcome Laboratories, Beckenham, Kent BR3 3BS) at a dilution of 1 in 250.

Assay for antibiotic destruction. A total of 49 strains of *P. aeruginosa* (44 sensitive and 5 resistant to azlocillin, as judged by the disk test) were cultured in 2·5-ml amounts of broth at 37 C overnight. An equal volume of broth containing azlocillin 1 ng/ml was then added to each bottle; incubation was continued for 6 h and the amount of intact antibiotic in each bottle was estimated by the hydroxylamine assay (Batchelor *et al.*, 1961). Suitable controls were set up to ensure that any breakdown of azlocillin was not due to non-specific causes.

RESULTS

Disk sensitivity testing

In series A (110 consecutive isolates) all the strains were sensitive to gentamicin, and 95 were also sensitive to ticarcillin and azlocillin. Five strains were resistant to both ticarcillin and azlocillin; nine were resistant to ticarcillin but sensitive to azlocillin; one was resistant to azlocillin but sensitive to ticarcillin. Thus, six strains (5·5%) were resistant to azlocillin and 14 (12·7%) to ticarcillin.

In series B (18 gentamicin-resistant strains), 10 strains were sensitive to ticarcillin and azlocillin, six were resistant to ticarcillin but sensitive to azlocillin, and two were resistant to both penicillins. The incidence of resistance to ticarcillin in gentamicin-resistant strains was significantly higher ($P < 0{\cdot}001$) than in gentamicin-sensitive organisms. There was no significant difference in resistance to azlocillin in the two groups.

Cross-resistance between ticarcillin and azlocillin was not total; whereas 7 (32%) of the 22 ticarcillin-resistant strains were sensitive to azlocillin, 7 (88%) of the 8 azlocillin-resistant strains were resistant to ticarcillin.

MIC and MBC

Geometric mean values of MIC and MBC for the two penicillins against *P. aeruginosa* strains are shown in the table. Azlocillin was 3–4 times more active than ticarcillin and there was close agreement between MICs determined by the three methods. However, a major discrepancy appeared when MBCs were considered. When tested by the agar dilution and microdilution methods, the mean MBC was 1·3–3 times greater than MIC for both compounds. The MBC for the great majority of strains was either identical to, or double, the MIC. In the tube dilution test, however, although a similar result was obtained with ticarcillin (MBC = 3 MIC), azlocillin appeared not to be bactericidal (MBC > 1024 μg/ml).

Inoculum size

The effect on MIC determined by the agar dilution technique of increasing the

TABLE

Antibacterial activity of ticarcillin and azlocillin against P. aeruginosa, measured in different ways

Method	Number of strains	Inoculum (cfu)	Antibacterial activity of			
			Ticarcillin		Azlocillin	
			MIC	MBC	MIC	MBC
Agar dilution	82	10^5	40·5	54·4	8·7	15·5
Tube dilution	9	10^5/ml	40	118	6·3	>1024
Microdilution	45	4×10^5/well	38·5	100·5	9·7	26·2

MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; all values are geometric means, expressed as μg/ml.

inoculum from 10^4 to 10^6 cfu was small; mean values of MIC increased only 1·5- and 2-fold, respectively, for ticarcillin and azlocillin.

Sequential viable counts

The MICs of ticarcillin and azlocillin for the four strains used in these experiments ranged from 16 to 64 μg/ml and 2 to 16 μg/ml respectively. The results of sequential viable counts of these strains are shown in the figure. Ticarcillin was somewhat more bactericidal than azlocillin at concentrations of 1 MIC and 2 MIC, but there was no difference in activity at the higher concentration of 4 MIC: viable counts were lowered by a factor of approx. 10^4 in 8 h by either antibiotic.

When cultures were examined 24 h after the start of the experiment, some regrowth had occurred with three of the four strains in the presence of 2 MIC and 4 MIC of azlocillin and with two of the four strains in the presence of 2 MIC and 4 MIC of ticarcillin.

Azlocillin destruction by resistant strains

All five azlocillin-resistant strains of *P. aeruginosa* tested destroyed azlocillin rapidly; in the test conditions, all the antibiotic added was hydrolysed within 6 h. Rapid destruction did not occur with the 44 sensitive strains tested.

DISCUSSION

Azlocillin, to which 94·5% of 110 consecutive clinical isolates of *P. aeruginosa* were sensitive when tested by the disk diffusion method, appeared more active than ticarcillin against this species. Furthermore, certain strains resistant to ticarcillin were sensitive to azlocillin. There was an increased incidence of resistance to ticarcillin, but not to azlocillin, amongst gentamicin-resistant strains. The reason for this is unclear. Resistance to these penicillins in *P. aeruginosa* is usually associated with the presence of plasmid-mediated TEM (class III) or PSE (class V) β-lactamases (Furth, 1979). All the azlocillin-resistant strains tested caused rapid and complete destruction of azlocillin. In our test conditions, sensitive strains did not destroy azlocillin rapidly,

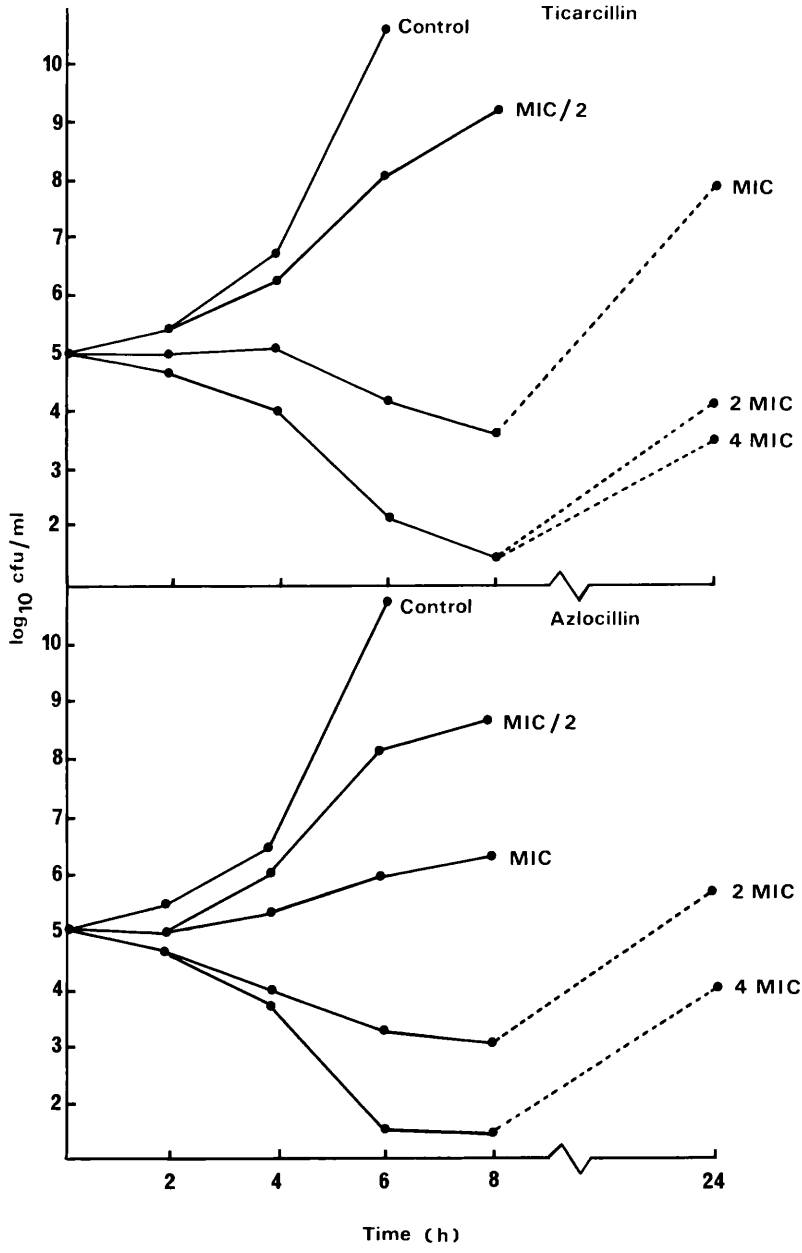

FIG.—Sequential viable counts of *P. aeruginosa* exposed to ticarcillin (upper) or azlocillin (lower) in Iso-sensitest broth. Each point is the mean obtained by testing four different strains.

although other workers (Basker *et al.*, 1979; Gwynn and Rolinson, 1980; White *et al.*, 1980) have shown that the chromosomal β-lactamase (class Id) produced by virtually all *P. aeruginosa* strains hydrolyses azlocillin slowly. Ticarcillin is more stable to the Id enzyme than is azlocillin, but is equally susceptible to the class III and V enzymes (Labia *et al.*, 1982).

The lability of azlocillin to the Id enzyme may explain our findings and those of other workers (Basker *et al.*, 1979; Gwynn and Rolinson, 1980; Greenwood and Eley, 1982) that regrowth sometimes occurs when *P. aeruginosa* strains are cultured in concentrations of ureidopenicillins that are initially bactericidal. However, while recognising the phenomenon, we do not regard it as being necessarily of any relevance clinically, because in this type of experiment only a single dose of the penicillin is used, whereas in therapeutic conditions an 8-hourly dosage schedule is used.

There have been several suggestions that the ureidopenicillins may be deficient in bactericidal activity, especially against *P. aeruginosa* (Kurtz, Holmes and Turck, 1975; Fu and Neu, 1978; Shah *et al.*, 1979). The present study shows that methodology may play a decisive role in whether azlocillin is judged to be bactericidal or not. By three of the four methods used, azlocillin and ticarcillin were approximately equivalent in their bactericidal action; only in liquid medium in static glass tubes did azlocillin appear to be poorly bactericidal. We regard the results of the experiments in which bacteria were shaken in broth in the presence of antibiotic and viable counts made at intervals as being the most reliable indication of bactericidal activity. In these conditions, both penicillins at concentrations of 2 MIC and 4 MIC were progressively bactericidal during a period of 8 h. A concentration of 4 MIC ranged from 8 to 64 μg/ml for azlocillin—readily attainable when 2 gm is given intravenously (Wirth, Schomerus and Hengstmann, 1976)—whereas to achieve equivalent concentrations of ticarcillin (64–256 μg/ml) a dosage of 3–5 g is required.

Some previous comparative studies on the antipseudomonal activity of azlocillin and ticarcillin or carbenicillin (Gwynn and Rolinson, 1980; White *et al.*, 1980; Gwynn, Webb and Rolinson, 1981) have used standard laboratory strains such as *P. aeruginosa* NCTC10662 (synonymous with ATCC27853 and ATCC25668) as test organisms. Such strains do not necessarily represent fresh clinical isolates. We believe that more meaningful information can be obtained by the use of large numbers of unselected fresh clinical isolates.

Alpha-carboxypenicillins and ureidopenicillins both bind strongly to penicillin-binding protein 3 (PBP3) (Curtis *et al.*, 1979). This results in extensive filamentation of the bacteria (Gwynn *et al.*, 1981; Greenwood and Eley, 1982). However, the detailed modes of action of these two groups of penicillins are not identical, because α-carboxypenicillins, but not ureidopenicillins, also bind strongly to PBP1a. Although the production of filaments causes bacteria to die less rapidly than a mode of action which brings about spheroplast formation and rapid lysis, antibiotics that cause filamentation are nevertheless effective *in vitro* (Curtis, 1981) and in experimental animals (Ryan and Monsey, 1981). Further evidence of their efficacy is that azlocillin and ticarcillin have excellent therapeutic records (O'Grady, 1978; Siegenthaler and Weuta, 1980), although in the compromised host—patients with malignancies, or leukopenic subjects—combination therapy with an aminoglycoside is preferred (EORTC International Antimicrobial Therapy Project Group, 1978).

Despite the similarities in overall action between azlocillin and ticarcillin, we did

observe a diminished bactericidal activity of azlocillin in a liquid medium when the test was done in static glass tubes. This phenomenon has also been reported by Kurtz *et al.* (1975) and Fu and Neu (1978). However azlocillin was bactericidal in broth contained in microdilution trays. An obvious difference is that glass is a wettable surface but polystyrene (from which the microdilution trays were made) is not. If filamentous bacteria adsorb on to a glass surface they may be protected in some way from the bactericidal action of azlocillin, but not from that of ticarcillin. In vigorously shaken cultures adsorption would be markedly decreased, which would account for the bactericidal action observed by sequential viable counting. Gwynn *et al.* (1981) have also suggested that adsorption on to glass may protect *P. aeruginosa* from the bactericidal activity of penicillins.

In summary, we have shown, like Greenwood and Eley (1982), that different methods of assessing antimicrobial activity may give different results when *P. aeruginosa* is the test organism. It would be interesting to know if *P. aeruginosa* is unique in this respect, or if susceptibility testing of all organisms should be done by several methods. The question also remains as to which method of testing gives the information that is most clinically relevant.

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