

Importance of Bacterial Growth Phase in Determining Minimal Bactericidal Concentrations of Penicillin and Methicillin

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The minimal inhibitory concentrations of penicillin against 96 strains of group B streptococci and of methicillin against 10 strains of *Staphylococcus aureus* were unrelated to the growth phase of test bacteria. However, the minimal bactericidal concentrations were significantly higher in the stationary phase than the logarithmic phase for both organisms ($P < 0.001$ and < 0.05 , respectively).

The in vitro determination of bacterial susceptibility to antimicrobial agents is commonly done by disk diffusion. More quantitative determination of growth-inhibiting activity (minimal inhibitory concentration; MIC) is accomplished by agar or broth dilution, and determination of bacterial-killing activity (minimal bactericidal concentration; MBC) is accomplished by a modification of the latter. Although there have been attempts at standardization of these procedures, various methods have been recommended for the preparation of the bacterial inoculum. These include logarithmic, early-stationary, and overnight cultures to be appropriately diluted as the inoculum for the dilution tests (2, 5, 7). In studies of antibiotic tolerance of group B streptococci, we observed that normal, nontolerant organisms were killed more rapidly when exposed to penicillin in the logarithmic phase of growth than when tested after overnight incubation (K. S. Kim and B. F. Anthony, *Pediatr. Res.* 14:560, abstr. no. 808, 1980). The present study extends these observations to examine the effect of the bacterial growth phase on the MICs and MBCs of penicillin against group B streptococci and of methicillin against *Staphylococcus aureus*.

Ninety-six strains of group B streptococci were shown to be nontolerant for penicillin. Briefly, the criteria for tolerance were (i) delayed killing by penicillin concentrations of 16 times the MIC during the logarithmic phase of growth, (ii) additive rather than synergistic response to penicillin-gentamicin combinations, and (iii) relatively deficient autolytic enzyme activity (Kim and Anthony, *Pediatr. Res.* 14:560, abstr. no. 808, 1980). The organisms were isolated from either blood or cerebrospinal fluid of septic infants at the Harbor-UCLA Medical Center, the University of Minnesota Hospitals, the Univer-

sity of Alabama Medical Center, and the Charity Hospital of Louisiana. They were grouped and typed by the methods of Lancefield (4) and Wilkinson et al. (8) with sera provided by the Centers for Disease Control, Atlanta, Ga. Ten strains of penicillin-resistant *S. aureus* were isolated from blood cultures at Harbor-UCLA Medical Center and identified on the basis of colonial morphology, Gram stain, and coagulase reaction. The presence or absence of tolerance was not determined.

Potassium penicillin G in a potency of 1,595 U/mg and sodium methicillin in a potency of 890 $\mu\text{g}/\text{ml}$ (Bristol Laboratories, Syracuse, N.Y.) were dissolved at a concentration of 1 and 10 mg/ml, respectively, in sterile distilled water, passed through a 0.45- μm pore filter (Gelman Instrument Co., Ann Arbor, Mich.), and stored in aliquots at -70°C for use within 4 weeks of freezing.

Logarithmic-phase cultures in Mueller-Hinton (MH) broth (Difco Laboratories, Detroit, Mich.) of each streptococcal strain were stored at -70°C , and staphylococci were stored on agar slants at 4°C . Sheep blood agar plates were inoculated and incubated overnight at 37°C . Four colonies were transferred to 5 ml of MH broth and incubated in a 37°C water bath for 4 h. This late-logarithmic-phase culture was diluted in fresh MH broth to a concentration of approximately 10^5 colony-forming units (CFU) per ml, as determined by light scattering in a Coleman model 9 nephcolorimeter (Coleman Instruments, Oak Brook, Ill.) or by absorbance at 620 nm with a Spectronic 70 spectrophotometer (Bausch and Lomb, Inc., Rochester, N.Y.). Colony counts performed with each experiment showed that this represented a mean streptococcal concentration of 2.4×10^5 CFU/ml and a

mean staphylococcal count of 4.2×10^5 CFU/ml. Stationary-phase cultures were started as above but incubated overnight and diluted 1:1,000 in fresh MH broth. The stationary-phase inocula were determined by colony counts to be a mean of 1.4×10^5 CFU/ml for streptococci and 6.7×10^5 CFU/ml for staphylococci.

Antibiotic solutions were thawed and diluted to 20 μ g of penicillin and 1,000 μ g of methicillin per ml in MH broth, from which twofold dilutions were made in MH broth.

The bacterial inocula in 0.5-ml volumes were added to a series of tubes, each containing 0.5 ml of antibiotic dilution. Control tubes of MH broth without antibiotic were included with each series of dilutions. After 24 h of incubation at 37°C, the MICs were determined as the lowest concentration of antibiotics at which there was no turbidity on visual inspection. Each tube was mixed, and 50- μ l aliquots were pipetted onto a blood agar plate with a semiautomatic pipette (Oxford Laboratories, Inc., Foster City, Calif.) for incubation at 37°C for 48 h to determine the MBC. This was defined as the lowest concentration of antibiotics that resulted in $\geq 99.9\%$ killing or ≥ 3 log reduction of the inoculum count.

Table 1 summarizes the effect of the growth phase of the bacterial inoculum on the penicillin and methicillin MICs and MBCs for 96 strains of group B streptococci and 10 strains of *S. aureus*. The means and ranges of MICs were identical for the logarithmic and stationary phases of growth. However, the effect of growth phase on MBC values was significant, with the mean penicillin MBC for stationary-phase cultures being approximately three times that for the logarithmic-phase cultures of group B streptococci ($P < 0.001$). In the case of the staphylococcus, the mean methicillin MBC for stationary-phase cultures was increased 10-fold over logarithmic-phase inocula ($P < 0.05$). Testing in the logarithmic phase resulted in MBC values within clinically attainable serum levels of meth-

icillin ($\leq 3.1 \mu$ g/ml), whereas testing in stationary phase often gave values clearly beyond this range, (e.g., 34 μ g/ml).

At present, the broth dilution test is usually used for the determination of in vitro bactericidal activity of an antimicrobial agent. However, the methodology has not been thoroughly standardized. Previously, we have shown that the type of medium significantly affects the amount of penicillin required to kill group B streptococci in vitro (3). Variability in antibiotic sensitivity results have also been demonstrated for broth dilution testing with differences in inoculum size, pH, incubation time, and quantitative endpoint for MBC (2, 6, 7). The preparation of a bacterial inoculum containing approximately 10^5 CFU/ml is usually made from either an overnight broth culture (stationary phase) or a broth culture incubated for a shorter time (logarithmic phase) to match a turbidity standard (2, 5, 7). However, to our knowledge, the influence of the different growth phases on MIC and MBC values has not been reported.

It has been appreciated for many years that penicillin exerts its greatest bactericidal activity against sensitive organisms while they are multiplying exponentially in vitro and possibly in vivo (1, 9). Therefore, the significant effect of growth phase upon MBC levels is not surprising. The experiments reported here suggest that the variable of bacterial growth phase should be further evaluated and standardized for quantitative testing of antibiotic susceptibility in vitro. Additional information is needed concerning the importance of the growth phase in killing by agents other than the penicillins and on the correlations between the responses of bacteria to antimicrobial agents in vitro and in vivo.

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TABLE 1. Effect of the bacterial growth phase on the quantitative susceptibility of group B streptococci (96 strains) to penicillin and *S. aureus* (10 strains) to methicillin

Bacteria	Growth phase	MIC (μ g/ml)		MBC (μ g/ml)	
		Mean	Range	Mean	Range
Group B streptococci	Logarithmic	0.04	0.04-0.16	0.08 ^a	0.04-0.31
	Stationary	0.04	0.04-0.16	0.26 ^a	0.04-1.25
<i>S. aureus</i>	Logarithmic	2.1	1.9-3.9	3.1 ^b	1.9-3.9
	Stationary	2.1	1.9-3.9	34.1 ^b	1.9-125

^a $P < 0.001$ (by Student's *t* test) when the penicillin MBCs of stationary-phase cultures of group B streptococci were compared with those of logarithmic-phase cultures.

^b $P < 0.05$ (by Student's *t* test) when the methicillin MBCs of stationary-phase cultures of staphylococcus aureus were compared with those of logarithmic-phase cultures.

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