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ANTIBIOTIC TOLERANCE AMONG CLINICAL ISOLATES OF BACTERIA

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INTRODUCTION

One of the unique features of β lactam antibiotics and other cell-wall inhibitors like vancomycin and bacitracin is that they can rapidly kill and in many cases lyse susceptible bacteria. Many other types of antibacterial agents (e.g. trimethoprim or chloramphenicol) are primarily bacteriostatic: they inhibit multiplication but do not cause an irreversible inactivation of the cell. In 1970 the characterization of a novel type of pneumococcal mutant was reported in the literature (1). This mutant grows in normal generation times and is as sensitive to growth inhibition by penicillin as the wild-type parent strain. However, while cultures of the parent strain are rapidly lysed and killed during exposure to penicillin, mutant cultures undergo only a very slow loss of viability and do not lyse at all. In other words, in these mutants penicillin and other cell-wall inhibitors act primarily as bacteriostatic agents. The term *antibiotic tolerance* has been coined to describe this novel type of bacterial response to antibiotic treatment.

Since the publication of the report on the pneumococcal mutant, tolerant strains have been isolated in the laboratory from mutagen-treated cultures of a number of other bacterial species (see Table 1). Subsequent reports have established that tolerance is not only a laboratory phenomenon. In 1974, Best and his colleagues tested 60 clinical isolates of *Staphylococcus* and found that one of these (strain Evans) is tolerant to oxacillin (2). Strain Evans has an oxacillin minimum inhibitory concentration (MIC) of 0.8 μg/ml, a value close to that of the majority of oxacillin-sensitive staphylococci. Upon addition of 6 μg/ml of oxacillin to a culture of the Evans strain, though, the bacteria only stop growing; under similar conditions other strains undergo rapid lysis. In

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Table 1 Tolerant mutants isolated in the laboratory

Method of selection	Parent strain	Defective component		References
		Autolysin	Other	
Autolysin defect	*Streptococcus pneumoniae* R36A	N-acetylmuramic acid–L-alanine amidase (amidase)		[redacted]
	Streptococcus faecium ATCC9790	Muramidase		9
	Bacillus subtilis 168	Amidase?		10
	Bacillus licheniformis	Amidase or muramidase		11
	Bacillus subtilis 168	Amidase and glucosaminidase		12
Antibiotic survival	*Escherichia coli* K12	Transglycosylase?		8
	Escherichia coli K1776	Transglycosylase and endopeptidase		7
	Escherichia coli K2452 (temperature sensitive)	Normal level	Trigger pathway?	6
	Streptococcus pneumoniae R36A	Normal level	Trigger pathway?	13
	Staphylococcus aureus Evans	[redacted]	[redacted]	14
	Streptococcus pyogenes T4-56			16
Protease production	*Bacillus subtilis* 168	Normal level	Autolysin inactivation by protease	15

1976, a similar screening by Mayhall et al noted a high prevalence of tolerant cells among clinical isolates of staphylococci (3). Another naturally occurring tolerant strain, *Streptococcus sanguis* Wicky, was described in 1977 (4). Again, this strain is exquisitely sensitive to penicillin inhibition but does not lyse and loses viability only very slowly during treatment with any concentration of penicillin, even up to 10,000 times its MIC value.

A publication of major impact was the report by Sabath and colleagues in 1977 in *Lancet* that described the unsuspected frequency with which tolerant strains of staphylococci can be isolated from clinical specimens originating in deep-seated infections (5). Since then, reports of tolerance among isolates of gram-positive organisms have increased in frequency. A computer-library search (MEDLINE) indicates that five reports about tolerant isolates were published between 1970 and 1977; there were over 35 reports of tolerant clinical isolates between 1977 and 1984, and strains exhibiting the tolerant response now include more than 20 species. Most of these are gram-positive bacteria. The only gram-negative tolerant isolates so far described appear to be laboratory mutants of *Escherichia coli* (6–8).

The better-characterized tolerant laboratory mutants have already been used extensively as experimental tools for the analysis of the mechanism of action of penicillin and other wall inhibitors. The apparently widespread occurrence of tolerant bacteria among natural isolates poses a number of additional questions and moves the phenomenon of antibiotic tolerance into the realm of clinical medicine. Do the naturally occurring tolerant bacteria isolated on the basis of criteria from the clinical diagnostic laboratory have a biochemical basis of tolerance similar to that of the laboratory isolates? Do tolerant bacteria found among clinical isolates arise through antibiotic selection? Do infections with tolerant strains pose any problems in chemotherapy with β lactams or other cell–wall inhibitory antibiotics? The purpose of this review is to attempt a critical evaluation of reports on natural tolerant isolates of bacteria with these three questions in mind.

Antibiotic Tolerance and Antibiotic Resistance

Since tolerance can improve the chances of bacterial survival quite dramatically during antibiotic treatment, the phenomenon is often referred to as a novel form of antibiotic resistance. This terminology can be confusing, since in the mechanistic sense tolerance may actually be contrasted with resistance.

Resistance results in the need for a higher concentration of antibiotic to prevent growth; that is, the MIC is increased. Although higher concentrations are required for growth inhibition of resistant organisms, once exposed to this concentration, the organisms will cease growing and killing (and in some bacteria lysis) will begin. Tolerant organisms, on the other hand, show only small or no change in MIC, but upon exposure to this concentration lysis is blocked and viability is lost only very slowly. Figure 1 illustrates the contrast-

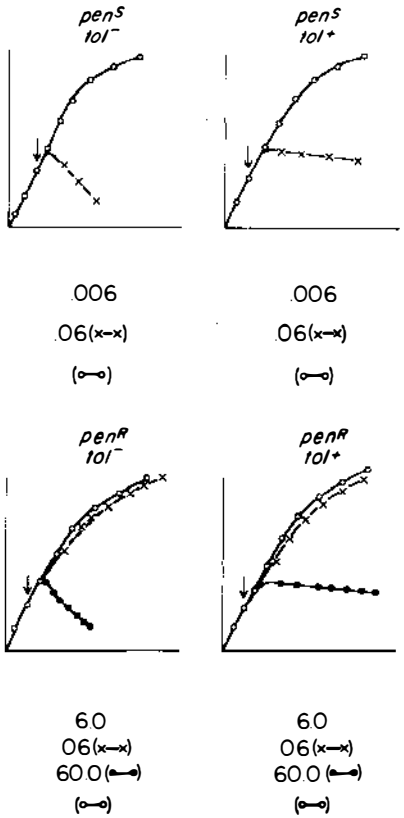

Figure 1 Response of antibiotic-tolerant and antibiotic-resistant strains to penicillin treatment. Viable titer of bacterial cultures (ordinate) is plotted against time (abscissa). Cultures receive penicillin at the times indicated by the arrows. In the upper panel, a penicillin–sensitive nontolerant ($pen^S tol^-$) and a penicillin–sensitive tolerant ($pen^S tol^+$) culture receives 0.06 μg per ml penicillin [corresponding to 10 times the MIC equivalent concentration (x–x–x–x)]. Loss of viability in the $pen^S tol^-$ culture may be contrasted with the primarily bacteriostatic response of the $pen^S tol^+$ bacteria.

In the lower panel, a pair of penicillin-resistant nontolerant ($pen^R tol^-$) and penicillin-resistant tolerant ($pen^R tol^+$) bacteria receive penicillin at two concentration: 0.06 μg (x–x–x–x) and 60.0 μg (←→) per ml. The lower concentration of antibiotic, below the MIC value of these resistant strains (MIC = 6.0 μg/ml), has no effect on growth. The higher concentration of penicillin (concentration to 10 times the MIC value) causes loss of viability in the tol^- strain and bacteriostasis in the tol^+ strain.

ing features and possible combinations of the tolerance and resistance traits. A specific example of the combination of high-level penicillin resistance with tolerance recently has been described in the case of South African pneumococci (17).

Another example of confusing terminology arises when authors refer to

erythromycin or chloramphenicol tolerance. From what has been outlined above, it should be clear that the term *tolerance* refers to bacteria in which a typically bactericidal-bacteriolytic response to antibiotics is changed in the direction of bacteriostasis. There is little use in referring to the response of bacteria to a typically bacteriostatic drug as tolerance.

THE MECHANISM OF THE IRREVERSIBLE EFFECTS OF β LACTAM ANTIBIOTICS

The tolerant phenotype presumably results from some block in the sequence of events between antibiotic exposure and bacterial killing. It is critical, therefore, to understand current models of the mechanism of action of cell–wall active antibiotics, in particular β lactams, in order to comprehend what has gone awry in the tolerant cell. In current models, all the antibacterial effects of β lactam antibiotics, whether inhibition of growth, killing, or lysis of the cells, are initiated by the inhibition of a set of bacterial enzymes that catalyze the terminal stages of cell-wall assembly. These enzymes (penicillin-binding proteins or PBPs) are anchored in the bacterial plasma membrane. Penicillin covalently binds (acylates) the active sites of PBPs, causing inactivation of these enzymes. How then does inhibition of these enzymes cause eventual lysis and death? It appears that antibiotic-induced lysis, and to some extent killing, depends on the activity of a group of ubiquitous bacterial enzymes, called autolysins or murein hydrolases, that can hydrolyze covalent bonds in the cell wall surrounding the bacterial cell (see Figure 2).

The Role of Autolysins in Antibiotic-Induced Bacterial Killing

Mutants defective in autolysins have been isolated in a number of bacterial species. Such mutants have a common phenotype: they do not undergo lysis and lose viability with reduced rates during treatment with β-lactam antibiotics and other cell-wall inhibitors. In other words, these mutants exhibit antibiotic tolerance.

Biochemical and physiological analysis of an antibiotic-tolerant mutant of the pneumococcus was first described in 1970. The tolerant phenotype appeared to be associated with the low specific activity of a cell wall–hydrolyzing enzyme activity, an N-acetylmuramic acid-L-alanine amidase (amidase) (1). This finding has been confirmed by the demonstration that DNA isolated from the mutants can introduce (via genetic transformation) the autolytic defect into lysis-prone recipient cells and the transformants all show a tolerant response to all cell-wall inhibitors. In tolerant mutants subsequently isolated from laboratory mutants of other species, tolerance is also associated with defective autolytic activities.

Tolerant mutants differ in their degrees of tolerance: i.e. in the residual rates

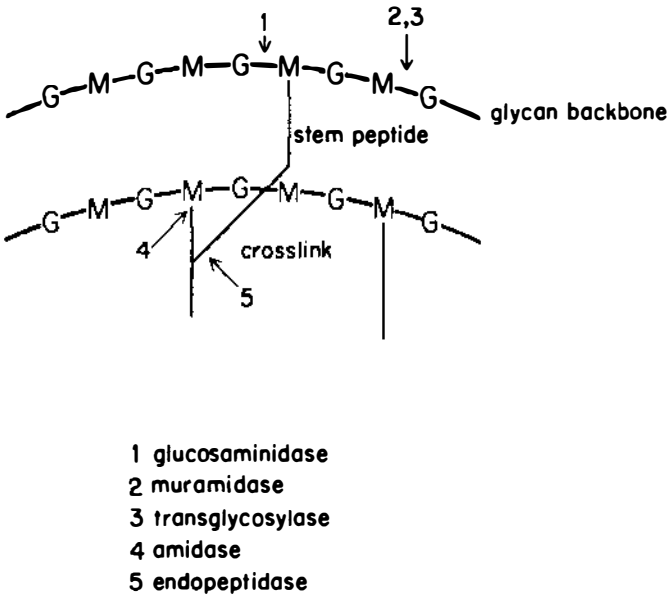

Figure 2 Sites of action of autolytic enzymes; a cartoon of the types of covalent bonds in the cell-wall peptidogylcan: two glycan strands with a pair of cross-linked stem peptides and one uncross-linked stem peptide. Letters M and G represent N-acetylmuramic acid and N-acetylglucosamine residues respectively. Numbers with arrows indicate the sites of action of various autolytic enzymes.

of lysis and viability loss during antibiotic treatment. Whether or not the extent of the defect in autolysin activity determines the degree of resistance to lysis (and killing) is not yet clear. In *E. coli*, which produces several autolytic enzymes, mutants defective in one or two of these enzyme activities have been obtained. The degree of tolerance appears to be related both to the residual enzyme level and to the number of defective autolytic activities (7).

What is the normal role of these hydrolytic enzymes required for antibiotic-induced killing? The type and number of autolytic enzymes differ among bacterial species: e.g. amidase has been described in pneumococci (18); muramidase has been described in lactobacilli; and transglycosylase, peptidase, and amidase activity has been described in *E. coli* (19). None of the mutants shows poor growth or abnormality in functions essential for growth in test-tube cultures, implying that the enzyme activity lost in the mutants does not perform an essential physiological role. The mutants described thus far are not totally devoid of autolysin, however: in each 0.1%–10% of activity remains, and it is possible that this small amount of autolysin activity is sufficient for some essential aspect of cell-wall growth. In several bacteria, autolysins appear to be required for normal cell separation. Autolysin-defective (Lyt^-) mutants show

defective cell separation as manifested by chaining in pneumococci (1) and *Bacillus* species (12) and packet or clump formation in staphylococci (20). Autolysin activity has also been implicated in competence, flagellar extrusion, cell-wall turnover, and sporulation (19).

The major conclusion emerging from the analysis of the autolysin-defective mutants is that interference with cell-wall synthesis in bacteria can rapidly upset the cellular control of autolytic enzymes, triggering their activity on a level that is suicidal for the cell. It is intriguing to consider that the unsurpassed antibacterial power of β-lactam antibiotics may be linked to an enzyme activity that, under in vitro conditions at least, is not essential for the growth of the target bacteria.

The Pleiotropy of Tolerance

Although the great majority of tolerant mutants isolated in the laboratory have defective autolytic systems, one should recall that the majority of these mutants have been selected for an autolytic defect. If selection is for *survival* during antibiotic treatment, as is likely to be the case in the tolerant clinical isolates, then in theory at least any number of complex factors that play a role in bactericidal activity may be altered by mutation and provide antibiotic tolerance. Experience in the laboratory supports this idea (see Table 1 and mechanism 2 below).

It may be worth describing in some detail the types of mutational changes that conceivably could cause the tolerant phenotype. This could also serve to illustrate the variety of factors that can modulate the irreversible effects of β-lactam antibiotics. Figure 3 shows schematically the presumed pathway between β-lactam binding and eventual cell death. Briefly, in this model inhibition of one or more PBPs by β-lactam antibiotics results in inhibition of cell-wall synthesis, generating some regulatory signal in the cell that can trigger the uncontrolled activity of autolytic enzyme(s). These enzymes inflict various degrees of irreparable structural damage on the cell wall, possibly by first cleaving a limited number of covalent bonds in the cell wall, which produces focal nicks too small to be resolved by electron microscopy. Exposure of the underlying plasma membrane would then cause cell death. More extensive damage to the wall would lead to actual wall degradation and anatomical-sized gaps in the wall, followed by rupture of the plasma membrane and escape of the cytoplasmic contents (lysis). [For a more detailed view of the pathways by which cell-wall inhibitors result in bacterial killing, see (19, 21–23).] In Figure 3, sites at which alterations in this pathway might result in tolerance are numbered to correspond to the mechanisms discussed below.

1. It has been shown that β-lactam antibiotics that bind to the penicillin-binding proteins (PBP) 1a and 1b of *E. coli* are those most effective in triggering rapid viability loss and lysis. Preferential binding to other PBPs may

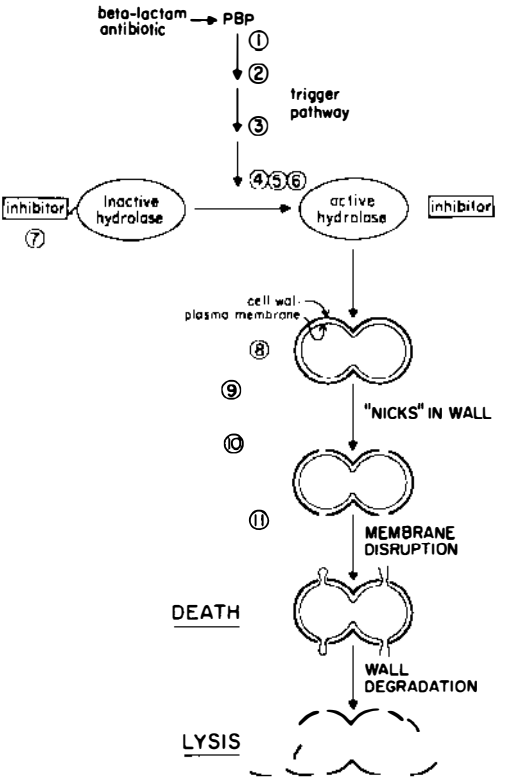

Figure 3 Scheme of the hypothetical pathway leading from antibiotic-inhibited PBPs to cell lysis. See text.

cause only a slow loss of viability, while the rate of killing and lysis by other cell-wall inhibitors would remain unaltered. While such mutants have not yet been reported, drug-specific tolerance (e.g. tolerance to β-lactam antibiotics but not to vancomycin) has been described in both laboratory mutants and clinical isolates (5, 13, 24, 25).

2. A second type of tolerance mutation with normal levels of autolysin activity but without the capacity to induce lytic activity has been described (6, 13). When mutagen-treated pneumococci are exposed to several cycles of treatment with bactericidal doses of ampicillin, among 100 isolates sharing the tolerant phenotype 80 have defective autolysin; the rest have a normally functioning autolytic system. Despite the presence of normal autolytic activity, these mutants are tolerant, i.e. they show slow lysis and death during antibiotic treatment. Williamson & Tomasz have suggested that tolerance may be due to a defect in some signal needed to trigger autolytic activity (13). A similar isolate

has been characterized in *E. coli* (7). Several isolates of resistant South African pneumococci are also highly tolerant but contain only moderately reduced levels of autolysin. These may also contain a defect in the triggering pathway (17).

3. It is well known that non-growing bacteria or bacteria with inhibited protein synthesis are tolerant to cell-wall active antibiotics (26, 27). Normally, the addition of penicillin to growing bacteria results first in interference with cell-wall synthesis and next in inhibition of protein synthesis. In some mutants, the time needed to shut down protein synthesis after penicillin addition may be shortened so that the bacterium in effect assumes the phenotype of a non-growing cell (28).

4. Since the destructive activity of autolysins is provoked by inhibition of cell-wall synthesis, it is conceivable that in some tolerant mutant this suicidal coupling between the halt in wall synthesis and autolysin activity is somehow circumvented.

5. Some autolytic enzymes are sensitive to proteolysis. *Bacillus subtilis* mutants that overproduce extracellular proteases appear resistant to the lytic action of β-lactam antibiotics (15).

6. A lower specific activity of autolytic enzymes seems to be the basis of tolerance in numerous strains of bacteria, as described in the original tolerant laboratory mutants (1, 9, 10, 11, 12). *Streptococcus sanguis* strain Wicky may also represent this type of tolerance (4).

7. If autolysin activity is negatively controlled in vivo, as was first suggested for pneumococci (29, 30), then overproduction of an inhibitor may result in the tolerant phenotype. Lipoteichoic acids appear to inhibit autolysin activity in several bacterial species (29–33). Some tolerant *Staphylococcus aureus* strains produce greater quantities of lipoteichoic acids and secrete greater amounts upon exposure to oxacillin than nontolerant strains (34). Extracts of these cultures added to nontolerant cultures inhibit lytic activity (5). Thus, tolerant staphylococcal strains might represent regulatory mutants that produce increased quantities of autolysin inhibitor.

8. In pneumococci, biosynthetic replacement of choline with ethanolamine results in the formation of cell walls resistant to the effects of autolysin (35). Such bacteria are tolerant to cell-wall antibiotics. An analogous mutational defect resulting in alteration of the autolysin substrate (cell wall) represents still another possible mechanism of tolerance.

9. Persistence, a phenomenon most frequently reported in *S. aureus*, is the survival of a small fraction (less than 0.1%) of cultures despite prolonged antibiotic exposure (36). Subcultures of these survivors yield cultures with the same response, i.e. less than 0.1% survival. This phenomenon may be related to the existence of a stage in the cell-divisional cycle in which autolytic activity is low. Mutants may exist in which the probability of an average cell being in

this low autolytic (tolerant) phase is increased (38). This would raise the percentage of persisters, resulting in apparent tolerance.

Indeed, several authors have suggested that only a fraction of *S. aureus* cultures are actually tolerant in a fashion analogous to the heterogeneity of methicillin-resistant staphylococci (37, 39). While this represents a possible mechanism of tolerance, examination of time-kill studies by these and other investigators indicate that tolerant *S. aureus* show a decreased rate of killing for the culture population as a whole (2, 14, 24, 40). Although survival after 24 hours may differ from strain to strain and reflect a relative degree of tolerance, this situation is not different from that of other tolerant organisms. All tolerant strains, including laboratory autolysin-defective mutants, undergo some loss of viability, albeit slowly, and thus demonstrate variable numbers of survivors after 24 hours (1, 2, 9). This should not be construed to imply that only a portion of the population is tolerant.

10. Bacteria growing at acidic pH values are known to shift to the synthesis of different phospholipids (41) and the plasma membrane of such cells appears to have increased resistance to stress (42). Bacteria growing at low pH values also become tolerant to the killing effect of cell-wall inhibitors (43, 44). Such a membrane alteration conceivably is the basis of tolerance in certain types of bacterial fermentation mutants that overproduce acidic catabolites.

11. Bacterial mutants that produce plasma membranes with altered chemical composition and increased resistance to osmotic lysis may also show the tolerant phenotype.

The main purpose of Figure 3 is to emphasize the complexity of the processes that contribute to the irreversibility of the action of β-lactam antibiotics. Most of the mutational blocks listed are hypothetical. Specific selection processes will have to be designed to test whether or not they actually exist among the numerous tolerant strains described from clinical sources.

THE DETECTION OF TOLERANT BACTERIA: METHODOLOGY

Although tolerance was originally defined as a decreased rate of killing or slow loss of viability upon exposure to bactericidal antibiotics, for clinical purposes tolerance more recently has been considered present when the minimal bactericidal concentration (MBC) is significantly higher (generally 32-fold) than the minimum inhibitory concentration, where the MBC is defined as the concentration of antibiotic yielding 99.9% killing of the inoculum. Notwithstanding the obvious practical values of this assay, one should realize that this method is at best a very insensitive end-point titration and can produce serious artifacts.

A critical review of the literature describing reports of clinical isolates tentatively identified as tolerant makes it apparent that technical problems in

these studies frequently make it difficult to accept their claims. It seems worthwhile to spell out types of problems that contribute to this situation.

Technical Problems with the MBC Determination

Cultures of penicillin-sensitive nontolerant bacteria contaminated with a minority population of either β–lactamase producing or intrinsically resistant cells of the same species may fortuitously produce false tolerance. Subculture and retesting of survivors should eliminate this problem (45). On the other hand, the incidence of tolerance may be falsely decreased due to antibiotic carryover. When a loop full of bacteria from the drug-containing MIC tube is transferred to the surface of drug-free agar, dilution of the drug by the volume of the agar medium does not necessarily occur. Adding penicillinase to cultures prior to agar plating for viability or MBC determinations may be necessary to reliably determine tolerance in some strains (46). However, not all investigators have begun to take this precaution.

Comparison of the rates of viability loss between cultures exhibiting different degrees of chaining can cause obvious problems: longer chains may appear more resisant to killing because of the larger numbers of viable units per chains.

Gwynn and colleagues (47) have observed that bacteria may escape the cidal effect of antibiotics during MIC determination by adhering to test-tube walls above the meniscus of the antibiotic-containing medium. While the factors that can actually contribute to the ability of such bacteria to survive are not completely clear, it is obvious that these cells are reintroduced into the body of the culture fluid during stirring, which many investigators do just prior to testing the viable titers of cells in the MIC tubes. This effect can produce falsely high survival rates and misleadingly indicate tolerance. Taylor et al evaluated the influence of this effect on MBC determinations on 40 strains of *S. aureus,* including several that previously had been described as tolerant. Using the simple technical precaution of mixing the cultures after overnight incubation four hours prior to plating for MBC determination, they found no strains with MBC exceeding the MIC by fourfold or more (48). Presumably organisms adhering to the test tube walls above the meniscus were mixed into the antibiotic-containing broth, eliminating the falsely elevated MBCs (49).

Phenotypic Tolerance

Studies on the mechanism of the cidal and lytic effects of cell-wall inhibitors have revealed that the rate and degree of these irreversible antibacterial effects can be dramatically modulated by factors in the bacterial environment (44, 58). Table 2 is a compilation of some of the factors that can cause phenotypic tolerance in genetically nontolerant bacteria. The biochemical mechanism of these effects is not well understood and discussion of these is outside the scope of this review. Phenotypic tolerance has importance for our discussion in two

Table 2 Environmental factors causing phenotypic tolerance

Factor	Species	Proposed mechanism	References
Low pH	Group B *streptococci, B. subtilis, E. coli, S. aureus, S. pneumoniae*	Suboptimal pH for autolysin and/or membrane stabilization	43, 44
Serum	*S. faecalis*	Diminished autolysin activity or decreased growth rate	50
High Ca or Mg concentration	*N. gonorrhoeae*	Outer membrane stabilization	51, 52
Inhibition of growth (e.g. stationary phase or bacteriostatic antibiotics)	All bacteria	Decreased autolysin activity?	26, 27, 53
High inoculum size	*S. viridans, Listeria monocytogenes*, Group G *streptococci*	?	60, 62, 63, 74
Composition of growth medium	Group B and D *streptococci*, *S. aureus*	May be due to pH effect	24, 57, 61, 87
Protease	*B. subtilis*	Protease sensitive autolysin	15
Forssman antigen	*S. pneumoniae*	Inhibition of autolysin	29, 30
Lipoteichoic acids	*S. aureus, S. fæcalis, Lactobacillus* spp.	Inhibition of autolysin	2, 31, 32
Cardiolipin	*S. faecalis*	Inhibition of autolysin	32

respects. First, conditions may arise during the screening of isolates that result in the appearance of tolerance (see Table 3) and, second, conditions may occur at the sites of infection that promote a decreased rate of killing of (genotypically) nontolerant organisms. Probably the most frequent technical pitfall is the use of stationary-phase cultures in the MBC and viability determinations. It has long been known that β-lactam antibiotics are active against growing cells and that conditions that prevent growth, such as chloramphenicol treatment or amino-acid deprivation, will diminish effective bacterial killing (26, 27, 53). Clearly, then, growing rather than stationary-phase cultures must be used to assess tolerance accurately. The American Society for Microbiology suggests that inocula be prepared by either dilution of overnight cultures or use of growing cultures (54, 55), and many clinical laboratories use overnight cultures to directly inoculate MIC and MBC tubes. Using such stationary cultures will necessarily result in diminished killing rates. Overnight cultures of bacteria that have been in the stationary phase for an unknown length of time cannot be assumed to resume growth instantly upon dilution into fresh medium, particularly not if that medium contains antibiotics. Thus, bacteria reported to be tolerant only after tests using stationary-phase cultures (39, 56) should not be considered truly tolerant unless cultures of growing cells (log phase) demonstrate similar findings.

Additional major variables affecting the rate of killing include media composition, pH, and inoculum size (24, 57–60). Autolysin activity in nontolerant organisms may be decreased with exposure to low pH, causing cultures to appear tolerant (43, 50, 52). Venglarcik et al (61) attempted to correlate the effects of media and growth phase with pH. *S. aureus* cultures showed lowest final inoculum pH after overnight incubation in tryptic soy broth (TSB); highest final pH was obtained after a three-hour incubation in Mueller-Hinton broth (MHB). Those conditions resulting in low-inoculum pH were associated with the highest MBCs, so that 10 of 20 strains appeared tolerant. Under conditions promoting higher final pH, no strains appeared tolerant. Tryptose phosphate and tryptic soy broth seem to allow the lowest pH values and thus the least degree of killing.

The significance of the inoculum effect noted in several species is unclear. Tolerance appears to increase when large inocula are used and to decrease with small inocula (60, 62). Since most authors do not specify whether organisms were diluted to the appropriate concentrations or grown to a given density, it is not apparent whether this is related to growth phase rather than inoculum alone. Best noted no difference in killing rate between staphylococcal cultures with inocula of 10^5 and 10^8 when both cultures were logarithmic phase (14). In group G streptococci, however, inoculum size and growth phase may independently increase the appearance of tolerance. Organisms are killed rapidly when log or stationary-phase cultures are diluted to low inocula (10^4), but log-phase

Table 3 Tolerant clinical isolates

Species/group	Source[a]	Phase of inoculum[b]	Media[c]	MBC≥[d] / MIC	Incidence of tolerance[e]	Comments	References
Streptococcus							
S. viridans	gingivae/ blood	NS	NS	10	15/58	patients receiving pen. prophylaxis	85
S. viridans	blood	NS	NS	10	16/80		107
S. viridans	blood	log	THB	32	0/10		87
S. viridans	mult	stat	THB	32	5/9	penicillinase added	75
S. sanguis	gingivae/ blood	NS	NS	10	4/21	patients receiving pen. prophylaxis	85
S. sanguis	mult	log	THB	32 killing curves	4/5	penicillinase added	46
S. mitior	gingivae/ blood	NS	NS	10	19/94	patients receiving pen. prophylaxis	85
S. mitior (nutritional variants)	blood	log	THB	16	9/11	penicillinase added	74
S. mutans	gingivae/ blood	NS	NS	10	4/15	patients receiving pen. prophylaxis	85
S. milleri	gingivae/ blood	NS	NS	10	1/9	pen. prophylaxis	85
S. salivarius	gingivae/ blood	NS	NS	10	0/17	pen. prophylaxis	85
S. pneumoniae	mult	stat	THB	32	0/11	penicillinase added	75
S. pneumoniae	blood/ CSF	log	C + y	killing curves	5 (only report of tolerant isolates)	also highly resistant	17
Group A	mult	stat	THB	16	11/12		90
Group A	mult	stat	THB	32	1/16	penicillinase added	75
Group B	blood/CSF	log	MHB	32 killing curves	4/100		
Group B	mult	stat	THB	32	13/33		
Group B	mult	stat	THB	32	1/16	penicillinase added	
Group C	mult	log	nutrient broth and serum	32	16/17	serum may increase tolerance	

Group D	mult	stat	THB	32	17/18	penicillinase added	
S. faecalis, S. faecium	blood	log	MHB	32	majority of 34	only mean MBC/MIC data shown	
S. bovis	blood	log	MBH	32	4/4		
Group G	mult	log	THB	32 killing curves	0/9, 0/9 vanco	patients responding poorly to treatment	63
Group G	mult	log	THB	32	0/19, 1/19 vanco		93
Group G	mult	NS	NS	32	1/9, 8/9 vanco		92
Lactobacillus							
Lactobacillus spp.	mult	stat	MHB	achievable serum level	31/40		96
Lactobacillus spp.	mult	stat	MHB	killing curves	16/17		72, 124
Listeria							
L. monocytogenes	mult	NS	dextrose phosphate	killing curves	most of 20	only mean MBC data shown	95
L. monocytogenes	mult	log	TSB	16 killing curves	50/50		62
Clostridium							
C. perfingens	feces	stat	MHB	8	most of 50 tol to vanco	only mean MBC data shown	125
Staphylococcus							
S. aureus	mult	stat	MHB	50 killing curves	33/60 oxa		3
S. aureus	blood	log	MHB	32	28/63 naf	fewer tolerant using 48-hour MBC	5
S. aureus	mult	stat	MHB	100	8/30 cloxa		126
S. aureus	blood	stat	MHB	16 killing curves	16/35 meth, 17/35 oxa, 23/35 ceph		56
S. aureus	blood	stat	MHB	100	34/34 meth		81
S. aureus	blood	log	TSB	10	16/45 oxa		79

S. aureus	bovine mastitis	log	MHB	32	7/24 cloxa	penicillinase added	82
S. aureus	blood	stat	BHI	16	6/13 naf, 5/13 vanco		24
S. aureus	blood	stat	MHB	16	5/13 naf, 7/13 vanco		
S. aureus	mult	NS	MHB	32	2/20 moxalactam		127
S. aureus	mult	NS	BHI	32	9/15 oxa, 4/10 vanco		25
S. aureus	mult	stat	MHB	killing curves	19/30 oxa, 19/30 ceph		40
S. aureus	blood	stat, log	MHB	32	12/15 ceph, 12/15 oxa	penicillinase added	106
S. aureus	blood/endocarditis	stat	TSB	32	32/50 oxa, naf or ceph		80
S. aureus	blood/bacteremia	stat	TSB	32	35/54 oxa, naf or ceph		
S. aureus	mult	stat	MHB	16	25/40 oxa, 20/40 ceph		59
S. aureus	mult	log	MHB	16	0/9 oxa, 0/9 ceph		
S. aureus	mult	log	MHB	8	0/40 oxa	mixed cultures 4 hours prior to plating (see text) penicillinase added	48

[a] Mult = multiple sources, CSF = cerebrospinal fluid
[b] NS = not specified, log = logarithmic phase, stat = stationary phase
[c] MHB = Mueller Hinton broth, THB = Todd Hewitt broth, C + y = casein hydrolysate medium with yeast extract, TSB = tryptic soy broth, BHI = brain heart infusion
[d] Criterion for determination of tolerance; if only numeral given, refers to ratio of MBC to MIC greater than which isolates considered tolerant; killing curves not used unless specified.
[e] Number of tolerant isolates/total number of strains evaluated; refers to penicillin tolerance unless otherwise indicated.
[f] Penicillinase was not added unless specified.

cultures concentrated to high inocula (10^7–10^8) are killed more rapidly than stationary-phase cultures (63).

A more important consequence of phenotypic tolerance concerns its effects on the bactericidal action of antibiotics in vivo. Diminished killing by antibiotics may occur at sites of infection due to changes in the local environment. Low pH, which results in phenotypic tolerance in vitro, may be encountered in phagosomes of polymorphonuclear neutrophils (64), in synovial fluid during septic arthritis (65), in cerebrospinal fluid during meningitis (66), in the endobronchium during pneumonia (67), and in abcesses and empyemas (68). Slow bacterial growth occurs in osteomyelitis (O. Zak, personal communication) and within endocarditic vegetations (69). Additionally, high inocula and stationary-phase growth are associated with phenotypic tolerance, and these conditions may exist in endocarditis, septic arthritis, and other deep-seated infections. Phenotypic tolerance may be superimposed on genotypic tolerance in the laboratory with an additive effect: autolysin-defective pneumococcal mutants show even slower killing by penicillin when growth is inhibited by amino-acid deprivation (S. Handwerger, A. Tomasz, unpublished observations). Such effects may also occur in vivo under conditions disposing to phenotypic tolerance.

Nontechnical Problems with the MBC Determination

Even if standardized, the MBC determination yields only viability at an arbitrary endpoint, with no information about the rate of killing during the preceding 24 hours (see Figure 4). Since tolerance is defined as a slow rate of killing, lysis or viability (time kill) curves that examine multiple time points will necessarily yield more information. In the quantitation of tolerance by the MBC determination, it is assumed that higher concentrations of the drug will cause higher rates of viability loss. This need not be the case. For example, cultures of tolerant *S. sanguis* strains exposed to a wide range of concentrations of penicillin (1–10,000 times the MIC value) lose their viability with the same very slow rate (46). Several investigators have noted that tolerant strains may have an infinite MBC value (37). In nontolerant cells, the rate of cidal action of penicillin increases only up to a certain concentration, above which the rate either stabilizes or actually decreases. In many species of bacteria, it has been shown that the use of increased penicillin concentrations does not increase the rate of killing in either tolerant or nontolerant strains (14, 26, 70, 71). In fact, use of very high concentrations of antibiotic may give precisely the opposite effect. This phenomenon, known as the paradoxical or Eagle effect, was described by Eagle in 1948: in some species of bacteria, the rate of antibiotic killing is diminished at very large multiples of the MIC (70). For this reason, use of a single very high concentration, such as 50–100 times the MIC, for determining tolerance should be avoided.

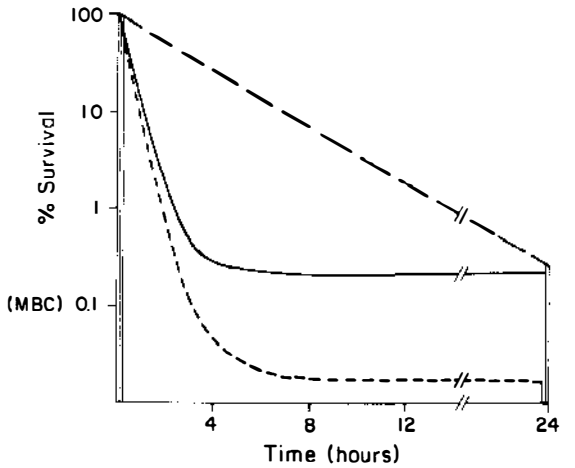

Figure 4 Relationship between killing rates and the MBC value. Curves show three possible types of kinetics for the loss of viability during treatment with penicillin at a concentration of one time the MIC value. The vertical bar at 0 minutes indicates the inoculum; bars at 24 hours represent percent of survival as determined by the MBC value. The lower bar represents less than 0.1% survival reached along the rapidly declining killing curve of the nontolerant bacterium (- - - - -). The higher bars indicate survival of more than 0.1% of the cells, which indicates tolerance by the MBC test. However, rates (and mechanisms) of killing in the two "tolerant" cultures are completely different. The truly tolerant mutant undergoes slow loss of viability (— — —), while the other culture (————) has an initial rapid rate of killing typical of nontolerant cells but a higher survival rate, which may be due to physiologically heterogeneous inoculum (e.g. higher percentage of dormant cells that are phenotypically tolerant). Alternatively, these cells may represent increased persisters or a subpopulation of resistant cells.

In some strains of bacteria, additional problems may hamper the characterization of tolerant isolates. Instability of the tolerant trait (loss during storage of frozen cultures) has been reported in staphylococci (5, 36, 59), and an extreme cell concentration dependence of tolerance has been noted among *S. sanguis* isolates (60). The mechanism of these effects is not understood.

Improved Methods of Detection

Time-kill studies remain the most reliable means of determining tolerance. In many bacterial species, including *E. coli, N. gonorrhoeae, S. pneumoniae,* group A streptococci, lactobacilli, and *S. aureus,* treatment of growing cultures (generation time 30–90 minutes) with 5–10 × the MIC of penicillin results in a 2–3 log decrease in viability within four hours. Treatment with 1–2 times the MIC equivalent causes a similar degree of killing within 6–8 hours (1, 5, 6, 7, 14, 16, 52, 72). In contrast, tolerant mutants of *E. coli,* pneumococci, group A streptococci, lactobacilli, and *S. aureus,* as well as tolerant strains of *Listeria monocytogenes* and *Streptococcus sanguis,* show no decrease in viable titer or, at maximum, a one-log fall (1, 4, 5, 6, 7, 14, 16, 72, 73). [Watanakunakorn

(56) found no difference between rates of killing of staphylococci determined to be tolerant or nontolerant on the basis of high MBC–MIC ratios. However, only stationary-phase cultures were used, and the "nontolerant" strains showed no loss of viability after six hours of incubation with oxacillin.] Thus, within 2–6 hours after the addition of antibiotic, tolerant and nontolerant strains can be differentiated by their relative rates of killing. A reliable and relatively simple method for clinical laboratories might use viability plating just prior to antibiotic addition and, four hours later, at a single antibiotic concentration. This method would not represent a significant increase in workload compared to MBC determinations and would reliably differentiate between rates of antibiotic killing. The same precautions regarding technique outlined for MBC determinations are required for this type of testing.

Recently, more rapid methods have been suggested for the detection of tolerance in the clinical laboratory. A disk-diffusion test substituting a penicillinase disk for a penicillin-susceptibility disk was successful for a small number of *S. viridans* initially screened (74), but use of high inocula caused false-positive results, and further testing with other streptococcal species yielded sensitivities as low as 57% (75). Similar methods have been used for screening a number of other gram-positive isolates (76, 77). A more recently proposed method incorporating penicillin gradient plates and replica plating appears promising but requires further testing (78). Plating technique results have been most frequently compared to MBC–MIC ratios to verify their sensitivity and specificity (75). More detailed analyses comparing test results to viability curves are needed to determine the reliability of these newer techniques.

THE INCIDENCE OF TOLERANCE AMONG CLINICAL ISOLATES

Given the number of possible variables, it is not surprising that estimates of the incidence of tolerance range widely (see Table 2). Estimates of tolerance to β–lactamase resistant penicillins among strains of *S. aureus* generally vary from 30–70% (5, 40, 79, 80), although one report cites 100% of isolates (81) and another no tolerant isolates (48). Tolerant isolates have also been reported in the veterinary literature (82). Staphylococcal isolates generally show cross-tolerance among the β lactams: i.e. strains tolerant to one β lactam show the same response to others. Vancomycin tolerance has also been reported, although the incidence appears lower. Tolerance to vancomycin may accompany β-lactam tolerance or appear independently (5, 83, 84). The mechanism of this phenomenon is unknown. One isolate of *S. epidermidis* has been reported as tolerant to vancomycin, but was only "tolerant" in the stationary phase (39).

Among the streptococcal species, the viridans group shows a high incidence

of penicillin tolerance, with the possible exception of *S. salivarius* (85). A small study of nutritionally variant *S. mitior* found most strains tested to be tolerant (74). A significant fraction of *S. viridans* species may also show tolerance to vancomycin (86). Krogstad et al (87) did not find any tolerance among ten viridans streptococci; however, their methodology differed from that of the former study.

Group D streptococci of both enterococcal *(S. faecalis, S. faecium)* and nonenterococcal *(S. bovis)* groups show a high incidence of tolerance (50, 87, 88, 89). Incidence was reported to be high in Group A strains by one group, but only stationary-phase cultures were used for testing (90). The pediatric literature of the past ten years has noted that in vitro killing of Group B streptococci by penicillin is slow (71); the occurrence of tolerance in clinical isolates (by the MBC–MIC ratio) has been reported as 4% (91), 30% (90), and over 80% (57), depending on the media used for culture. Isolates of Group G streptococci tolerant to penicillin and vancomycin have been reported, but the single report showing a high incidence of tolerance to vancomycin does not specify methodology (92), and other investigators, using time-kill curves and log-phase cultures, have found a low incidence of tolerance (less than 5%) (63, 93). Tolerant isolates also have been reported among group C streptococci (94), but these were tested in medium containing serum, which appears to decrease the activity of autolysin in other streptococcal strains (87).

Listeria monocytogenes strains also show tolerance to penicillin and ampicillin (73, 95). A survey of 50 strains showed that all had MBCs greater than eightfold the MIC, and many were not killed at concentrations one thousandfold greater (62). These findings are confirmed by time-kill studies, which show that viability decreases less than one log after 24 hours over a range of penicillin or ampicillin concentrations. Among 40 strains of *Lactobacillus* spp., 95% were tolerant to ampicillin, 78% to penicillin, and 85% to cephalothin (96).

Will tolerance become an increasingly frequent finding, possibly forcing alterations in standard therapeutic regimens? The effect of environmental pressures on possible selection for tolerance has not yet been investigated. Penicillin-resistant *S. viridans* species arise in the mouth and pharyngeal flora during penicillin therapy. Such strains may appear within the first 48 hours of therapy and persist for several months after treatment is complete (97, 98). They may cause endocarditis in susceptible patients (99, 100). Tolerance similarly might emerge and, in cases where only inhibitory sensitivity testing was done, may have been previously unnoticed. In the laboratory, tolerant mutants may be isolated by repeated passage on penicillin-containing media; such selection might occur in vivo, just as selection of resistant clones has been documented. Holloway et al (85) found a high percentage of tolerant viridans streptococci in patients receiving penicillin prophylaxis. The incidence in

patients receiving no treatment is unknown. In a small study of children with *S. aureus* bacteremia, Hilty and colleagues (79) found a higher incidence of tolerant isolates in patients with prolonged hospital stays, suggesting that tolerant organisms might be a significant source of nosocomial infections. Several of the highly resistant South African pneumococci isolated from hospitalized children also showed a high degree of tolerance (17). Most of the patients had chronic illnesses and had received repeated exposure to antibiotics, and in some children the organisms appeared nosocomially acquired (101). Thus, the antibiotic pressure of the hospital environment might encourage selection of tolerant organisms just as it does highly resistant strains.

A related avenue requiring further investigation is the means of transfer of tolerance among bacteria. In the laboratory, tolerance can be conferred by transformation with relative ease (1, 12). Transduction (102) by bacteriophage may be another means by which tolerance can be transferred. If tolerant organisms can be selected by antibiotic pressure, this trait could then easily spread, increasing the incidence of clinically significant isolates that demonstrate tolerance.

TOLERANT BACTERIA IN VIVO

There is general agreement that the effective cure of bacterial infections at sites of impaired host defense requires the use of bactericidal antibiotics such as β lactams (103, 104). One thus would expect that tolerant bacterial strains with their selective resistance to the irreversible effects of β lactam antibiotics would pose serious problems to chemotherapy in immunocompromised patients or in deep-seated infections such as endocarditis, meningitis, and osteomyelitis. On the other hand, the presence of host factors may make tolerant pathogens more sensitive to the irreversible effects of antibiotics in vivo than in vitro. In tolerant *S. sanguis,* for example, treatment with human lysozyme plus penicillin results in loss of viability, although neither alone has this effect (46). This emphasizes the importance of in vivo investigations of tolerant bacteria.

Animal Studies

The rabbit endocarditis model has been used in several studies attempting to determine the effect of tolerance on the establishment and outcome of infection during antibiotic treatment and prophylaxis. The bacterial pathogens in which tolerance has been most frequently noted, i.e. staphylococci, viridans streptococci, and group D streptococci, are those most frequently causing infective endocarditis. In a Mayo Clinic study of 150 cases of infective endocarditis, 38% were caused by viridans streptococci, 20% by staphylococci, and 18% by group D streptococci (105). Reports on the incidence of tolerant strains among these species indicate high frequencies: up to 50% among *S. sanguis* and other

viridans strains (74, 75, 85), 30–70% among staphylococci (40, 106), and over 90% among group D streptococci (75, 87). Why tolerant strains appear so frequently at this infection site is not clear. Nevertheless, this association makes the use of the endocarditis model particularly suited for testing the in vivo role of tolerance.

While the endocarditis model is presumed to be most useful for the reasons cited above, it is also possible that nontolerant organisms manifest a phenotypically tolerant response while growing on the heart valve due to the high bacterial density, diminished metabolic activity, and slow growth rate that occurs within vegetations (69); thus, differences between tolerant and nontolerant organisms might be less apparent than in a setting where inoculum is lower or growth rate more rapid.

A number of studies with viridans streptococci have noted the relative difficulty of eliminating tolerant over nontolerant isolates from valvular vegetations in experimental endocarditis. A preliminary report comparing a tolerant *S. sanguis I* with a nontolerant *S. sanguis II* in rabbit endocarditis suggested that tolerant organisms are less responsive to killing by penicillin (107). Lowy et al (108) compared the efficacy of treatment regimens on infection with a tolerant *S. sanguis* and a nontolerant *S. mitis*. The high-dose regimen (80,000 U/kg every eight hours) achieved serum concentrations comparable to those in patients treated for endocarditis. Unfortunately, these two viridans strains seem to differ in their virulence properties, since one day after inoculation rabbits receiving *S. mitis* showed greater bacteremia and, left untreated, died sooner than those receiving *S. sanguis*. In animals treated with low-dose penicillin (5,000 U/kg every eight hours), rabbits infected with the *sanguis* strain developed vegetations with significantly higher bacterial counts than those infected with *S. mitis,* despite the apparent greater virulence of the *mitis* strain. However, there was no difference noted between the bacterial counts of *sanguis* and *mitis* vegetations in animals receiving the high-dose penicillin regimen. These authors concluded that, while tolerant pathogens clearly survive better during a lower-dose penicillin regimen, increasing the dosage (i.e. penicillin concentration and length of treatment) successfully eliminates the tolerant bacteria also. This presumably is achieved by the slow rate of killing of *S. sanguis,* which is clearly demonstrable in the test tube during prolonged (several days) exposure to the antibiotic (4).

Brennan & Durack (60) also compared tolerant and nontolerant *S. sanguis* in the rabbit model of endocarditis, using an inoculum and a dose regimen similar to those of the high-dose regimen of Lowy et al. Vegetations excised from untreated animals showed the same number of organisms, suggesting that virulence for the two strains is equal. In rabbits treated with intramuscular (IM) procaine penicillin, the number of organisms surviving five days is significantly greater after inoculation with tolerant than with nontolerant *S. sanguis;* the

addition of streptomycin in a small number of animals results in sterilization of all vegetations by day five. This study suggests that the presence of tolerance may indeed affect the outcome of treatment of viridans endocarditis. The detection of tolerant colonies from vegetations may have been significantly improved in this study with the use of penicillinase in plates for viability counting.

Several studies have used the endocarditis model to test the sensitivity of tolerant versus nontolerant pathogens to prophylaxis with β-lactam antibiotics. While it is generally agreed that treatment of established disease requires bactericidal antibiotics, the issue is less clear regarding prophylaxis. Originally, it was assumed that antibiotic prophylaxis prevents endocarditis by eradicating organisms already lodged on the valve (109). Were this the case, tolerant organisms would evade prophylaxis, as they would grow after the antibiotic is withdrawn. More recently, it has been shown that penicillin also decreases adherence to fibrin-platelet matrices in vitro and to cardiac valves during bacteremia (86, 110, 111). If prevention of adherence is an equally important factor in preventing disease, then tolerance might have less effect on the efficacy of prophylaxis. Experimental tests of these questions have yielded information that suggests that both the cidal effect of the antibiotics and the suppression of adherence play a role in successful prophylaxis and that tolerant bacteria potentially pose problems in chemoprophylaxis.

Hess et al (112) tested four *S. sanguis* strains of relatively similar MIC values (.006–0.1 μg/ml) but vastly different in vitro MBC values (.02 μg/ml–32 mg/ml). The strains appeared to have comparable virulence, i.e. similar abilities to adhere to the traumatized valvular surfaces and give rise to dense populations in untreated rabbits. Rabbits received single intramuscular injection of procaine penicillin 30 minutes prior to inoculation with 10^8 bacteria. Two days later, 44 of 70 (70%) animals infected with a highly tolerant strain had developed endocarditis, while only two of 22 (9%) animals infected with nontolerant strains had detectable bacterial vegetations. The results of this experiment suggest that tolerant bacteria can evade prophylaxis by virtue of their relative resistance to the killing action of penicillin.

However, other studies indicate that penicillin can suppress the ability of both tolerant and nontolerant bacteria to adhere to heart valves during the initial phase of effective contact and colonization, before clearance removes most bacteria from the circulation. Lowy et al (110) briefly exposed tolerant *S. sanguis* to penicillin in vitro. Under the conditions used, bacteria underwent no loss of viability but did secrete over 90% of the acylated lipoteichoic acids, which have been postulated to function as adhesins (113). After removal of penicillin, pretreated bacteria were mixed with an equal number of untreated cells of an isogenic strain distinguishable by genetic markers and the mixed cultures were introduced into rabbits with previously traumatized heart valves.

Animals were sacrificed 15 minutes and two hours after inoculation. Differential counting (using the genetic markers) of bacteria in vegetations at both times revealed ten to one-hundredfold greater numbers of untreated compared to pretreated bacteria. This finding confirms the notion that penicillin treatment interferes with bacterial adherence by a process that does not depend on the cidal activity of the antibiotic. The interplay of cidal activity, which should be relatively ineffective against tolerant cells, and anti-adherence activity, which affects tolerant as well as nontolerant cells, is well demonstrated in the studies of Glauser et al (86, 111). They have shown that antibiotics for which *S. viridans* is tolerant may adequately prevent endocarditis (111) in the rat. Glauser and colleagues (86) further compared amoxicillin prophylaxis of tolerant *S. intermedius* and *S. sanguis* with nontolerant *S. mitior*. Their observations suggest that amoxicillin prevents endocarditis by two mechanisms: (*a*) bactericidal action, a process to which tolerant cells are less sensitive, and (*b*) decreased adherence, which affects tolerant and nontolerant strains equally.

The studies with tolerant viridans streptococci briefly reviewed above outline the types of problems tolerant pathogens may cause in the chemotherapy and chemoprophylaxis of endocarditis. The results with tolerant strains of *Staphylococcus aureus* are less clear. One reason may be the apparent instability of the tolerance trait in staphylococci (5, 59). Using the rabbit endocarditis model, Goldman & Petersdorf (114) found no difference in survival during methicillin treatment after inoculation of 10^4 logarithmic-phase organisms of a tolerant versus nontolerant *S. aureus* strain. (However, the tolerant strain showed a high MBC–MIC ratio at only 24 and not 48 hours.) Survival of the nontolerant strain in vegetations of untreated animals was significantly greater than that of the tolerant, however, suggesting diminished virulence of the tolerant strain. When stationary-phase organisms were used to inoculate the animals, those receiving the tolerant strains survived longer. It is unclear whether these findings are related to tolerance or represent only differences in virulence. In a recent study of bacteremic *S. aureus,* pyelonephritis inoculation with tolerant organisms resulted in a larger renal microbial population after eight weeks than did inoculation with nontolerant organisms. However, animals treated with methicillin showed no difference in clinical outcome between groups (115). These widely disparate results suggest that virulence factors unrelated to antibiotic response may alter the outcome of in vivo studies. Further studies with isogenic strains that show similar virulence are needed before conclusions about the effect of tolerance upon *S. aureus* infections can be drawn.

Clinical Studies

In 1976, Mayhall, Medoff, & Marr (3) described three patients with staphylococcal bacteremia who responded poorly to treatment with oxacillin; the three

strains were subsequently shown to be tolerant. Sabath's report (5) of tolerance in clinical isolates of *S. aureus* described seven patients who had responded poorly to treatment. Since then, there have been multiple case reports of treatment failures and recurrence of infection ascribed to tolerant organisms, including staphylococci and groups A, B, and D streptococci (71, 83, 84, 89, 90, 116–119). The significance of such reports is unclear, since a large proportion of common pathogens, such as staphylococci and streptococci, are tolerant. Multiple other causes, including occult septic foci, exist for treatment failure in invasive bacterial disease, and persistent bacteremia or treatment failure may occur without apparent cause in patients infected with nontolerant organisms (63, 93, 120–122).

In 1979, a retrospective chart review of 20 patients with a variety of *S. aureus* infections first suggested that those with organisms tolerant to the antibiotics received (nafcillin, vancomycin, or cefazolin) have a longer duration of bacteremia and a poorer prognosis, but the two groups were not exactly matched for type of infection or underlying disease (123). A small study of staphylococcal bacteremia in children showed no difference in mortality rate between those with tolerant and those with nontolerant organisms. Duration of fever or positive blood cultures was not reported (79). In 1980, Rajashekaraiah et al (80) evaluated 50 patients with *S. aureus* endocarditis and 54 with *S. aureus* bacteremia. Over 60% of the patients in each group were infected with organisms tolerant by their criterion, an MBC–MIC ratio greater than or equal to 16. Inocula for these determinations were obtained from overnight cultures grown in TSB, conditions likely to overestimate the incidence of tolerance (61). Endocarditis patients infected with tolerant staphylococci had more prolonged fever, more complications, and were more frequently admitted to the intensive care unit than those with nontolerant organisms; mortality was greater in the tolerant group but the difference was not statistically significant. No difference in outcome was detected between groups for patients with bacteremia alone. In a collaborative study of *S. aureus* endocarditis among Veterans Administration hospitals, patients infected with tolerant organisms also had more prolonged fever, although the rate of bacteriologic cure was the same (J. Rahal, personal communication). These studies strongly suggest that infection with tolerant organisms adversely affects the course of treatment of endocarditis. That persistent fever and complications in endocarditis patients do not produce a difference in ultimate mortality is not surprising in light of the laboratory finding that tolerant organisms lose viability more slowly than nontolerant organisms but are ultimately killed by cell-wall antibiotics.

Clearly, more study is needed to further define the effects of infection by tolerant organisms, staphylococci as well as other species, upon the course and outcome of treatment. Investigation is also necessary to determine which antibiotic regimens hasten eradication of infection and whether this prevents increased early morbidity. A randomized study of a large number of patients

with strict definitions of tolerance and prescribed antibiotic therapy is needed. At present, it seems reasonable to suggest that, in patients infected with tolerant organisms, persistent bacteremia or a poor response to antibiotics should prompt a change in therapy to include bactericidal antibiotics.

CONCLUSIONS

We have attempted to evaluate the body of information on tolerance in clinical isolates that has accumulated over the fifteen years since tolerance was first described. The phenomenon of antibiotic tolerance, initially reported as the unique response of lysis-defective pneumococci to penicillin treatment, is clearly not restricted to laboratory mutants. Clinical isolates in which the penicillin-induced loss of viability is substantially slower than in other isolates of the same species have been detected during the last fifteen years among a number of human pathogens, including Listeria, *Staphylococcus aureus, Streptococcus pneumoniae,* and group D and viridans streptococcal species.

It is difficult to compare the mechanistic basis of the tolerance of clinical isolates to those of the autolysin-defective laboratory strains. Most laboratory tolerant strains have been selected for an autolysin defect and clearly demonstrate both diminished lysis and killing upon exposure to cell–wall active antibiotics. Although no mutants show a complete lack of killing, in each autolysin-defective strain the cell population as a whole undergoes loss of viability very slowly. While some natural isolates exist that may demonstrate this type of tolerance, e. g. *S. sanguis,* enterococci, and Listeria, multiple other types of tolerance appear to exist in nature that differ in degree and probably in mechanism. Additionally, the mechanism of killing itself is not clear for all species: although the model of penicillin-induced lysis and death explains the observed phenomena in pneumococci and several other species, cell death may also occur by other routes not accompanied by lysis, as it was first described, e.g. in Group A streptococci. Other possible mechanisms for the production of tolerance have been described in the laboratory and still a further number have been proposed. Many of these are likely to occur in nature and remain to be discovered.

It is also difficult to evaluate the frequency of tolerance among natural isolates since in many cases the sole criterion used for the identification of tolerance has been the elevated MBC–MIC ratio. The insensitivity of this method and the numerous experimental variables that can influence MBC determination, causing unrealistic estimations of tolerance, have been discussed briefly and a more reliable method suggested. Although elevated ratios may serve as a first hint of tolerance in a clinical specimen, such claims should be confirmed by more detailed studies in the microbiology laboratory, using genetically and physiologically homogeneous cultures of the isolate.

The physiological state of the target bacterium and factors in the bacterial environment can profoundly modulate the irreversible effects of β-lactam antibiotics. Phenotypic tolerance due to such factors is likely to be a major variable influencing both the detection of tolerance in vitro and the efficacy of such antibiotics in vivo. Another important question is whether such tolerant bacteria are selected in the natural environment under antibiotic pressure in a fashion similar to antibiotic-resistant mutants.

Tolerant isolates appear to be unusually frequent among bacterial pathogens causing valvular endocarditis in man. The reason for this propensity is not clear. Studies with the rabbit endocarditis model strongly suggest that infections with tolerant isolates of viridans streptococci require more prolonged treatment for cure during penicillin therapy than do infections with nontolerant strains. Sensitivity to the cidal effect of penicillin also appears to be one of the factors influencing the success of prophylaxis in this experimental model. Strain-to-strain differences in virulence complicate the intrepretation of many animal studies. A critical evaluation of the role of tolerance in such models of infection will have to wait until isogenic pairs of tolerant and nontolerant isolates of the same strain of bacteria and of identical virulence properties become available.

Clinical studies thus far suggest that the outcome of treatment of staphylococcal endocarditis with tolerant organisms may not differ but that during the course of treatment prolonged fever and other complications may occur. Based on the present evidence, it seems unwise to change existing successful therapeutic regimens that have shown high therapeutic-to-toxic ratios. However, it seems appropriate to suggest that MBC determinations be routinely done on pathogens isolated from the blood of endocarditis patients. If the presence of tolerant organisms is confirmed (by repeating viability determinations at an earlier time point also, e.g. after four hours incubation of the MIC tubes), then a change to a bactericidal antibiotic regimen should be considered.

Both animals studies and clinical investigations to date have focused on the role of tolerance in endocarditis. The implications of tolerance at other sites of infection remain to be evaluated.

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