Mechanisms of Resistance to Beta-lactam Antibiotics in Strains of *Staphylococcus aureus*

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There are three major mechanisms of resistance of *Staphylococcus aureus* to beta-lactam antibiotics: enzyme mediated (penicillinase or beta-lactamase) by which the antibiotic is inactivated; intrinsic, which is not due to drug inactivation, and accounts for methicillin-resistance; and tolerance, in which there is a dissociation of the inhibitory and killing actions of beta-lactam antibiotics. In enzyme-mediated resistance, there are at least three different staphylococcal beta-lactamases, which probably account for differences in the inoculum effect with different cephalosporins. The intrinsic resistance is associated with differences in the affinity of beta-lactams for penicillin-binding proteins, but intrinsic resistance is probably more complex, because the pH of the medium, chelating agents, visible light, and temperature also effect its expression. Tolerance is clearly due to decreased autolytic enzyme activity (reflecting persistance of an enzyme inhibitor) of those tolerant organisms that need 32 (or more) times as much antibiotic for a bactericidal effect as for simple inhibition.

THERE ARE THREE general types of resistance to the penicillins and cephalosporins shown by staphylococci. The best known form of resistance is drug inactivation due to beta-lactam inactivating enzymes properly defined as beta-lactamases. The second form of resistance is intrinsic, due to some mechanism other than the inactivation of the antibiotic. The third form of resistance is tolerance to the killing action of penicillins and cephalosporins. There is considerable information about details of the first form of resistance, and some about the second and third forms. The clinical importance of the beta-lactamase-mediated resistance has been well recognized since the early 1940s. The importance of intrinsic resistance and tolerance has only recently been appreciated. Evidence indicates that each of the three forms of resistance is present in both *Staphylococcus aureus* and coagulase-negative staphylococci. Table 1 shows the features of the three forms. There are instances of a single strain having all three forms of resistance. In this article I will summarize the mechanisms of resistance of *S. aureus* to the beta-lactam antibiotics.

Beta-lactamase Mediated Resistance

The first penicillin-inactivating enzyme was discovered not in *S. aureus*, but in *Escherichia coli* (1). In order to show penicillin-inactivating activity in that organism, it was necessary to disrupt the cells. The discovery of penicillin-inactivating enzymes in *S. aureus* was made somewhat later (2). The epidemiologic increase in the numbers of penicillinase-producing staphylococci progressively limited the usefulness of penicillin G in infections caused by this organism.

The basis for resistance due to drug inactivation is straightforward: An organism produces the beta-lactamase that inactivates the antibiotic before the antibiotic has created irreversible changes in the bacterial cell. The beta-lactamase is most often an inducible enzyme in *S. aureus,* although in some instances it is constituative (3). The dynamics of staphylococcal beta-lactamase interaction with inducible strains have been called a race between the antibiotic's ability to initiate irreversible changes in the organism, and the organism's ability to synthesize and secrete beta-lactamase in sufficient amounts to inactivate the penicillin in its microenvironment.

The consequences of this race between the organism to inactivate the antibiotic, and the antibiotic to cause cell death are easily shown in the inoculum effect. High densities of organisms will have higher minimum inhibitory concentrations, whereas lower densities of organisms will have lower minimum inhibitory concentrations. This difference is due to the fact that only a few organisms in a microenvironment cannot produce enough beta-lactamase to protect themselves, whereas with larger inocula the "factories" for producing beta-lactamase are sufficiently numerous to affect the drug inactivation before all of the cells can be killed. Luria's classic experiment (4) showed that end dilutions of *S. aureus* that produce widely separated colonies on antibiotic-containing plates would have low minimum inhibitory concentrations, although these concentrations would be high if the same strain were tested with heavy inocula.

Although it is widely known that there are many different types of beta-lactamase produced by gram-negative bacilli (5), many physicians and microbiologists are unaware of the fact that there is more than one kind of beta-lactamase produced by *S. aureus.* Work by Richmond and colleagues (6) has shown that there are three different types of beta-lactamases produced by *S. aureus.* Their work is supplemented by that of others, and has shown that these differences are based on four kinds of evidence: immunologic studies (6), differences in inoculum effect (7), differences in substrate profile (6), and differences in inhibitory profiles (6, 8).

The inoculum effect is of use in demonstrating potential clinical differences in the use of different antibiotics, and also reflects the differences in beta-lactamases found in *S. aureus.* The effect of inoculum on activity of six different penicillins against *S. aureus* is shown in Figure 1. The ordinate shows the number of isolates of *S. aureus* from patient material at Boston City Hospital (7). The abscissa shows the factor that was obtained by dividing

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Table 1. A Comparison of Three Types of Penicillin Resistance of *Staphylococcus aureus*

Characteristic	Type of Penicillin Resistance		
	Beta-Lactamase Mediated	Intrinsic	Tolerance
Minimum inhibitory concentration	Very high	High	Normal
Minimum bactericidal concentration	Very high	High	High
Limited to beta-lactam antibiotics	Yes	Yes	No
Approximate phenotypic expression	99.9%	10^{-5}	10^{-2}
Rate of growth of culture	Rapid	Slow	Rapid
Stability of resistance	Stable	Stable	Unstable*
Possible occurrence in hospitals	80% to 90%	1% to 8%†	44%
Clinical importance	Yes	Yes	Yes
Phage types	Many	Few	Many
Protein A	Common	Low	Not tested

* Storage of organisms at 4 °C causes gradual loss of resistance (tolerance), over a period from 4 to 12 months (21). This loss may represent a plasmid locus; compare with the loss of *Staphylococcus aureus* beta-lactamase plasmid with similar storage in some strains (21).

† During nosocomial epidemics, 30% to 40%.

the minimum inhibitory concentration of each organism (determined using an undiluted inoculum) by the minimum inhibitory concentration for that same strain determined with a 10^{-4} dilution of the original inoculum. Thus, the larger the factor the greater the difference between the minimum inhibitory concentration determined with the heavy or light inocula. The results with nafcillin and methicillin are shown to have the lowest factor, the mode factor being 2. This result indicates that even with very heavy inocula there is little reduction in the effect of these antibiotics. The isoxazole penicillins (cloxacillin, dicloxacillin, and oxacillin) gave rather similar results. However, the mode factor with penicillin G is 2048; some strains required 16 000 times as much penicillin G to inhibit the undiluted inocula compared with the lighter inocula, which shows that penicillin G is much less stable to staphylococcal beta-lactamases.

Figure 2 shows the results with the same strains tested with three cephalosporins. With these, cephalothin is clearly the most resistant to the inoculum effect with a unimodal curve with a mode of 4. In contrast, cephaloridine shows a trimodal curve with the portion of its curve on the right being similar to that of penicillin G in Figure 1. This result indicates that for some strains of *S. aureus* the inoculum effect with cephaloridine is similar to that of penicillin G. Most of the strains, however, show a wide curve with factors ranging from 16 to 512 showing intermediary resistance to staphylococcal beta-lactamases, whereas a small number of strains showed little difference in minimum inhibitory concentrations when heavy inocula are used. We feel that this triphasic curve with cephaloridine reflected the three different beta-lactamases present in *S. aureus*. The results of cefazolin show a biphasic curve with about two thirds of the strains showing high resistance to staphylococcal beta-lactamase and similar to results of cephalothin whereas about a third of the strains showed intermediary resistance similar to the bulk of the strains with cephaloridine. Authors (9, 10) have commented on the greater susceptibility of cefazolin to staphylococcal beta-lactamase, and have pointed out that clinical failures of staphylococcal bacteremia may have been due to instability of cefazolin to staphylococcal beta-lactamases.

Intrinsic Resistance

The intrinsic resistance of staphylococci to beta-lactam antibiotics is paralleled in samples of other species that owe their resistance to beta-lactam antibiotics not to drug inactivation, but to a mechanism that as yet remains relatively ill defined. The term intrinsic resistance refers to all forms of increased minimum inhibitory concentration not due to drug inactivation. The examples of clinically important intrinsic resistance to the penicillins are seen in *Neisseria gonorrhoeae,* methicillin-resistant *S. aureus,* methicillin-resistant coagulase-negative strains of staphylococci, carbenicillin-resistant *Pseudomonas aeruginosa,* many gram-negative bacilli, enterococci, *Haemophilus influenzae*, and penicillin-resistant pneumococci.

In this list, it is quite evident that each of the species may have beta-lactamase-mediated resistance as an additional factor in the resistance of the organism mentioned. However, most strains of gonococci and *H. influenzae* do not produce any beta-lactamase at all, and the same may be said for all strains of enterococci. Although studies have been done to determine the basis of intrinsic resistance in each of these species, the possibilities of detailed studies of intrinsic resistance are perhaps best in beta-lactamase-negative segregants of methicillin-resistant strains of *S. aureus.* In this setting, in the absence of drug inactivation, it is possible to study the various biological and biochemical aspects of intrinsic resistance. The general

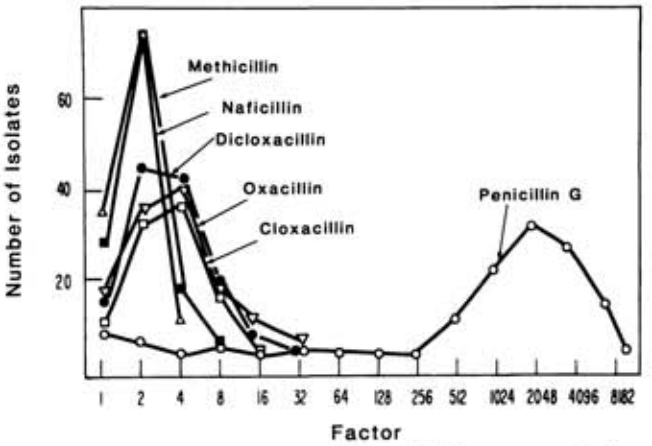

Figure 1. Effect of inoculum on minimum inhibitory concentration of six penicillins for 118 isolates of *Staphylococcus aureus.* The factor for each isolate was obtained by dividing the minimum inhibitory concentration obtained with an undiluted 18-hour culture used as inoculum by the minimum inhibitory concentration obtained with the inoculum diluted by 10^{-4} methicillin (△), nafcillin (▪), dicloxacillin (●), and oxacillin (▽), cloxacillin (▫), or penicilin G (○).

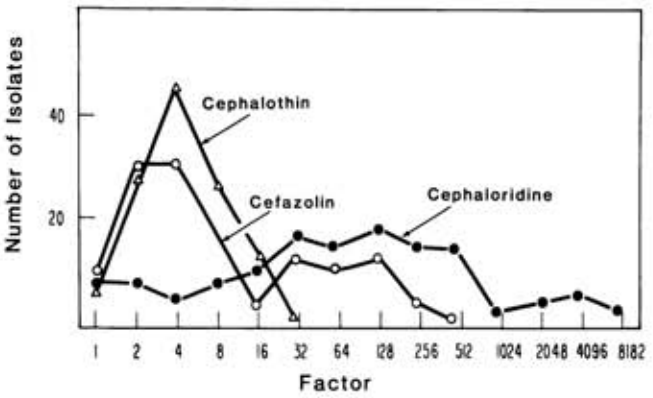

Figure 2. Effect of inoculum on minimum inhibitory concentrations of three cephalosporins for 118 isolates of *Staphylococcus aureus*. The factor for each isolate was obtained by dividing the minimum inhibitory concentration obtained with an undiluted 18-hour culture used as inoculum by the minimum inhibitory concentration obtained when the inoculum had been diluted by 10^{-4} cephalothin (△), cefazolin (○), and cephaloridine (●).

characteristics of methicillin-resistant staphylococci are shown in Table 2.

One major factor that has retarded investigations on the mechanism of resistance of methicillin-resistant staphylococci has been the extreme heterogeneity in the phenotypic expression of the resistance. Numerous observations suggest that virtually all cells from a resistant population harbor the gene or genes necessary for the expression of methicillin resistance. However, as isolated from nature, or the usual laboratory culture, only 1 in 10^5 or 1 in 10^6 of the cells in the usual strain will express the resistance phenotypically at 37 °C. Thus, it would be unlikely that current analytical techniques would have a sensitivity to detect abnormalities occurring in only one part in a million or one part in 100 000.

Some of the workers who have collected information on the basis of resistance have used one of the techniques that influence the phenotypic expression of the resistance trait. Table 3 shows seven chemical or physical factors that may radically change the phenotypic expression.

One surprise is the observation that visible light will influence the phenotypic expression. This influence was especially evident in comparing the effect of visible light on a yellow pigmented strain and a relatively white strain. The experiments (18) showed that the pigment, subsequently shown to be beta-keratinoid, protected the pigmented strain from the effect of visible light, for the variation in percent expression, comparing light and dark room conditions, was only 1.7-fold with pigmented cells. In contrast, the pale strain showed a greater than 6000-fold difference in the proportion of cells expressing methicillin resistance when grown in the dark compared with those grown in the light (18).

Perhaps one of the most important observations concerning factors affecting the expression of resistance was that at pH 5.2 there is no methicillin resistance in *S. aureus,* whereas at pH 7.4 the minimum inhibitory concentrations of resistant strains may be 1600 μg/mL, or higher. This concentration is approximately 1000-fold higher than the minimum inhibitory concentrations of sensitive strains which often have a minimum inhibitory concentration of 1 to 4 μg/mL. It was clear from rather extensive studies that the effect of pH causes suppression of resistance and not an elimination of the resistance trait (16).

Studies involving the binding of radio-labeled penicillin G to penicillinase-negative strains of *S. aureus* showed that the total amount bound was the same when comparing sensitive and resistant strains, but that the affinity for the radiolabeled drug was greater (by a factor of 2) in the sensitive strains compared to the resistant ones. However, when binding studies were compared at pH 7.4 and pH 5.2 both sensitive and resistant strains had approximately a three-fold decrease in their apparent Michaelis constants even though the change in pH has a radical effect on the susceptibility of resistance strains and no effect on the susceptibility of sensitive strains. In addition, there was a greater amount of drug bound at the acid pH but only by a factor of 5% to 10% (unpublished data). Hartman and Tomasz (19) studied binding to specific penicillin binding proteins and found that saturation of the penicillin-binding proteins, numbered 1, 2, and 3, needed higher concentrations of tritiated penicillin for saturation. However, these authors found that by repeating the test at pH 5.2 there was no change in the characteristics of the penicillin binding even though the minimum inhibitory concentration of the resistant strain dropped from 3200 μg/mL at pH 7 to 0.8 μg/mL at pH 5.2. The observations with penicillin binding to whole bacteria (unpublished data) and with binding to specific penicillin binding proteins (19) indicate that differences in penicillin binding do not appear to explain the enormous differences in minimum inhibitory concentrations at the two pHs noted.

An additional set of observations suggests that one of the earliest chemical changes seen after staphylococci are exposed to inhibitory concentrations of penicillins is the leakage of material that probably represents lipo-teichoic acid (20). With methicillin-resistant staphylococci there is little leakage of this material at a concentration of 200 μg/mL whereas at 1600 μg/mL of nafcillin (above the minimum inhibitory concentration of the test strain) there was leakage. These observations, combined with the observations on binding of radiolabeled penicillins, suggest that the earliest defect in methicillin resistance is an abnormality in the control mechanism in the release of an inhibitor of autolytic enzymes (lipoteichoic acid or other

Table 2. Characteristics of Methicillin-Resistant *Staphylococcus aureus*[*]

Minimum inhibitory concentrations less than or equal to 20 μg/mL
Slow growing
Cocci vary in size, somewhat larger than other staphylococci
Heterogeneity of resistance (phenotypic)
Frequent multiple drug resistance
Usually penicillinase positive
Dimorphic morphology (on solid medium)
May cause serious disease and death
Cross-resistance to all beta-lactam antibiotics
Difficult to phage type

[*] From References 11, 12.

Table 3. Chemical and Physical Factors Affecting Expression of Methicillin Resistance in *Staphylococcus aureus**

Heat
Osmolality of medium
Visible light
pH of medium
Chelating agents
Divalent cations
Beta-lactam antibiotics in medium

* From References 11-18.

inhibitors). The experiments of Hartman and Tomasz (19) suggest that this abnormality may be due to low affinities of binding sites, but the fact that this is not changed by low pH means that the specific basis for the failure of penicillins to initiate the inhibitory effect is unknown.

Tolerance to the Killing Action of Beta-lactam Antibiotics

By definition (21), tolerant staphylococci are those with a dissociation between inhibitory and killing action; strains in which the ratio of minimum bactericidal concentration divided by the minimum inhibitory concentration is equal to or greater than 32. In practice most normal strains of staphylococci show a ratio of minimum bactericidal concentration to minimum inhibitory concentration of 1 to 4. Relatively few strains show a ratio of around 32. Most tolerance strains will have ratios considerably in excess of 32. This distinction is relatively easy based on a comparison of values for minimum inhibitory concentration and minimum bactericidal concentration.

The dissociation between inhibitory and bactericidal action of penicillin was first recognized in 1929. In Fleming's original description of penicillin (22) he noted that the antibacterial factor killed bacteria at approximately the same concentration it inhibited them. There have been occasional clinical observations with a discrepancy between the minimum inhibitory concentration and minimum bactericidal concentration of staphylococci but certainly not as clearly noted as in group D streptococci. Tomasz and colleagues (23, 24) showed that factors that interfered with the autolytic system in *Streptococcus pneumoniae* produced tolerance to the killing action of penicillins. This model in the pneumococcus was later shown to have relevance in *S. aureus* when observers noted discrepancies between the minimum inhibitory concentration and minimum bactericidal concentration of *S. aureus* (21, 25-28). Best and associates (25) showed that a strain with a discrepancy between the minimum inhibitory concentration and minimum bactericidal concentration had a decreased amount of autolytic material. We confirmed this finding (21) and showed that the basis for decreased autolytic activity was due to the persistence of an autolysin inhibitory factor. The clinically disturbing fact was that the tolerant organisms appeared to be associated with important staphylococcal infections in humans in which the failure to produce a bactericidal activity at lower concentrations was correlated with treatment failure (21, 29-33).

The interpretation of our group has been that clinical failure in diseases where bactericidal activity is mandatory (as in bacterial endocarditis) is due to the inability of cell-wall-active antibiotics to kill tolerant staphylococci. Because there may be cross-tolerance to either cephalosporins or vancomycin or both (21) we have favored using a second antibiotic that acts on some part of the bacterium other than the cell wall or cell-wall-maintaining mechanisms. In most instances this has been either an aminoglycoside or rifampin.

One additional problem with the study of tolerant staphylococci is the ability to show this trait in the laboratory. The trait appears to be relatively unstable (21). Because of this instability, the origin of tolerant strains appears to be in the patient, or possibly animals, yet on prolonged study in the laboratory the tolerance appears to disappear. Bradley and associates (34) have presented evidence that a bacteriophage may play a role in transferring the tolerance trait from one strain to another. How this happens in nature remains to be shown.

A technical problem in showing tolerance is the possible confusion of the following related phenomena:

1. *Persisters (or "The Persister Phenomenon"):* After overnight testing, some (less than 0.1% of the inoculum) sensitive organisms are not killed. This may be due to the fact that those persisters were dormant and could not be killed by antibiotics, such as penicillins that cannot exert their bactericidal action if the cells are not actively growing. If subcultured, and tested again, less than 0.1% of the inoculum will persist.
2. *Paradoxic Effect ("Eagle Effect"):* With some gram-positive cocci, the rate of killing by penicillins will decrease as the concentration of antibiotic is increased. Thus, a bactericidal effect (greater than 99.9% reduction in viable organisms) noticed at 1 μg/mL, 2 μg/mL, and 4 μg/mL may paradoxically not be obtained at 8 μg/mL, 16 μg/mL, and 32 μg/mL.
3. *Paradoxic Tendency:* Similar to the paradoxic effect is the tendency, where some strains will show more organisms surviving at higher concentrations of a penicillin but not greater than 0.1%, to designate the test as not bactericidal.
4. *Tolerance ($MBC \div MIC \geq 32$):* Whereas most staphylococci are killed at about the same concentration of a penicillin needed to inhibit them, some strains showing tolerance need 32 or more times higher than the minimum inhibitory concentrations to produce the bactericidal effect. Whether this represents an unusually high proportion of persisters or is a very different phenomenon remains unclear.
5. *Technical Inaccuracies of the Test:* The determination of the bactericidal or lethal effect depends on inoculum size, physiologic state of the inoculum, medium, temperature, sample size after incubation, and other factors that may give misleading results. These factors may cause some strains to appear in a category to which they do not belong.

A possible relation between staphylococcal tolerance to the killing action of antibiotics and the "Eagle effect" or paradoxic effect may exist. It is only proper to empha-

size that the precise mechanism for the paradoxic effect has not been shown with data convincing to this author. Although the paradoxic effect is frequently credited to Eagle (36), others appear to have independently made the same observation (37-39). Another important relation that needs elucidation is the possible relation between the persistor phenomenon (40) and the tolerance phenomenon. Our current interpretation is that the persistor phenomenon leads to cells not killed due to metabolic inactivity, whereas the tolerance phenomenon appears to be due to decreased autolytic activity and the persistence of an inhibitor of the bacterial autolysins.

Conclusions

It is clear that there are three very different mechanisms by which *S. aureus* (and other species of coagulase-negative staphylococci) resist the antibacterial action of beta-lactam antibiotics. The differences between the three types of penicillin resistance are shown in Table 1.

Although there is no question about the clinical importance of minimum inhibitory concentrations and minimum bactericidal concentrations, considerable debate continues on the clinical significance of beta-lactam antibiotics. From our knowledge about the basis of these mechanisms, it is quite clear that the basis of the beta-lactamase-mediated resistance is drug inactivation. It is reasonably clear, but on the basis of fewer observations, that the basis for tolerance is due to decreased autolytic activity. Least secure is our knowledge about the basis of intrinsic resistance. Other observations concerning the possible mechanism of resistance of *S. aureus* to methicillin are the observation that methicillin-resistant staphylococci are more resistant to lysostaphin-induced lysis than are other staphylococci (41); and that in spite of this difference in susceptibility to lysostaphin, no difference in amino acid composition or ratios could be shown (42). More recently, it has been seen that there are differences in penicillin sensitivity when comparing cell wall synthesis and cell wall septum synthesis in *S. aureus* (43). The possible relevance of these later observations to our eventual understanding of intrinsic resistance remains to be shown.

ACKNOWLEDGMENTS: The author thanks Jane E. Anderson and Judi Erickson for assistance in the preparation of this manuscript.

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