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Antibiotic-tolerant *Staphylococcus aureus*

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The phenomenon of antibiotic-tolerance to 7 cell-wall-acting antibiotics and 5 aminoglycosides was studied in 35 strains of *Staphylococcus aureus* isolated from blood cultures. The minimal inhibitory concentration for antibiotic-tolerant *S. aureus* was in the susceptible range but the minimal bactericidal concentration was higher and often in the resistant range. Antibiotic-tolerance was common in strains of *S. aureus* and it involved not only antibiotics which inhibit cell wall synthesis but also the aminoglycosides. There was no uniform cross-tolerance among related antibiotics. Population analyses showed that the susceptibility of the cell population of antibiotic-tolerant *S. aureus* was heterogeneous and that the majority of cells were susceptible to the antibiotic tested. There was no difference in the magnitude or rate of killing of antibiotic-susceptible versus antibiotic-tolerant *S. aureus*.

Introduction

Antibiotics which inhibit bacterial cell wall synthesis such as the penicillins, cephalosporins and vancomycin are usually referred to as bactericidal antibiotics. The aminoglycoside antibiotics which cause misreading of the m RNA and inhibit ribosomal synthesis of susceptible bacteria are also considered bactericidal antibiotics. The minimal bactericidal concentrations (MBC) of bactericidal antibiotics are expected to be the same or only one or two dilutions higher than the minimal inhibitory concentrations (MIC).

Recently there have been reports of *Staphylococcus aureus* with MIC values of oxacillin, nafcillin, cephalothin, vancomycin or the aminoglycosides in the susceptible ranges but with significantly higher MBC values often in the resistant ranges (Gopal, Bisno & Silverblatt, 1976; Mayhall, Medoff & Marr, 1976; Sabath, Wheeler, Laverdiere, Blazevic & Wilkinson, 1977; Wilson & Sanders, 1976). The term tolerance has been used to describe this phenomenon (Sabath *et al.*, 1977). In the present study the phenomenon of antibiotic-tolerance of a large number of *S. aureus* to 7 cell-wall-acting antibiotics and 5 aminoglycosides was investigated.

Materials and methods

Staphylococcus aureus

Thirty-five strains of *S. aureus* isolated from blood cultures of patients with septicaemia and endocarditis were used. They were all coagulase-positive, mannitol-positive and

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belonged to different phage groups. Both β-lactamase producers and β-lactamase-negative strains were included.

Antibiotic susceptibility tests

The MICs and MBCs of methicillin, oxacillin, nafcillin, cephalothin, cephapirin, cefazolin, vancomycin, gentamicin, tobramycin, amikacin, sissomicin and netilmicin were determined by the ICS broth dilution method (Ericsson & Sherris, 1971). Mueller-Hinton broth was the medium and the inoculum was 1 ml of a 10^{-3} dilution of an overnight culture (approximately 10^5 to 10^6 organisms). The final concentration in the first tube was 32 μg/ml and the last tube 0·125 μg/ml. After incubation for 18 to 24 h, 0·01 ml from all the clear tubes were taken out by using a standard loop and plated on Mueller-Hinton agar. The agar plates were incubated at 37°C for 18 to 24 h, the MBC was defined as the minimal concentration of antibiotic in the tube from which $\leqslant 5$ colonies grew on the subculture.

Killing of S. aureus

The time-kill curve method was used to study the dynamics of killing of *S. aureus* with different MBCs. All 35 strains were studied. *S. aureus* were grown overnight and diluted with Mueller-Hinton broth to give between 10^6 and 10^7 organisms per ml and incubated with antibiotics in a water-bath at 37°C. The final concentration of antibiotics were oxacillin, 10 μg/ml, nafcillin, 10 μg/ml and sissomicin, 1 μg/ml. A culture with no antibiotics was set up as a control. At 6, 24 and 48 h of incubation, viable organisms were enumerated by making serial 10-fold dilutions and plating on Mueller-Hinton agar.

Population analyses

Two representative strains with different MIC and MBC values were studied for the number of resistant organisms in a given population of cells at a particular antibiotic concentration. Methicillin, oxacillin, nafcillin, cephalothin, cefazolin and vancomycin were studied. Mannitol-salt agar was incorporated with each of these antibiotics in the final concentration of 32 μg/ml and in a serial twofold decreasing concentrations. Series of agar plates with twofold differences in antibiotic concentrations were inoculated with 0·1 ml of an undiluted and 10^{-1} to 10^{-8} dilutions of an overnight culture. Series of agar plates without antibiotics were inoculated in the same manner and served as controls. Plates were incubated at 37°C and colonies were counted after 48 h of incubation.

Results

Antibiotic susceptibility

Table I lists the MIC and MBC values of the 12 antibiotics for the 35 strains of *S. aureus* respectively. All MIC values were in the susceptible ranges of a given antibiotic. However, many MBC values of all antibiotics tested except tobramycin for varying numbers of strains were in the resistant ranges. The ratio of MBC/MIC of all antibiotics for the 35 strains of *S. aureus* is listed in Table II. The ratio of 1 means that the values of MIC and MBC for that particular strain were the same. Among the 35 strains, MBC/MIC ratios of 1, 2, 4, 8 and $\geqslant 16$ were recorded for almost all the antibiotics tested. For a

Table I. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of 12 antibiotics for 35 strains of *Staphylococcus aureus*

		No. of strains with MIC (μg/ml) upper row or MBC (μg/ml) lower row									
		⩽0·125	0·25	0·5	1	2	4	8	16	32	>32
Methicillin	MIC			3	7	22	3				
	MBC				1	10	8		1		15
Oxacillin	MIC	11	18	6							
	MBC	2	2	10	3	2	5	5		2	4
Nafcillin	MIC	4	22	8	1						
	MBC	2	2	4	2	2	3	6	8		6
Cephalothin	MIC	17	13	5							
	MBC	2	1	5	1	5	2	1	6	7	5
Cephapirin	MIC	19	14	2							
	MBC	2	4	2	1	2	8	3	3	8	2
Cefazolin	MIC	3	20	9	3						
	MBC	2	2	1	1	1	2	1	6	7	12
Vancomycin	MIC		1	16	18						
	MBC						3	7	13	10	2
Gentamicin	MIC	1	8	9	16	1					
	MBC				6	7	6	9	5		2
Tobramycin	MIC	2	13	11	8	1					
	MBC		1	3	11	16	4				
Amikacin	MIC				2	7	18	7	1		
	MBC					2	3	21	7	2	
Sissomicin	MIC	8	16	11							
	MBC	1	4	10	11	5	3		1		
Netilmicin	MIC	3	3	12	14	3					
	MBC		1	1		12	6	3	8	4	

Table II. The differences between minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of 12 antibiotics for 35 strains of *Staphylococcus aureus*

Antibiotics	No. of strains with an MBC/MIC ratio of				
	1	2	4	8	⩾16
Methicillin	3	14	2		16
Oxacillin	1	12	2	3	17
Nafcillin	4	5	1	2	23
Cephalothin	4	2	2	4	23
Cephapirin	3	3	2	2	25
Cefazolin	3	2	2	1	27
Vancomycin			1	7	27
Gentamicin	2	7	3	9	14
Tobramycin	3	11	15	4	2
Amikacin	8	19	4	3	1
Sissomicin	7	9	8	7	4
Netilmicin	2	5	6	9	13

particular strain, there was no correlation among the MBC/MIC ratios of related or unrelated antibiotics. Tables III and IV were examples. For the 16 strains of *S. aureus* with an MBC/MIC ratio of ⩾16 for methicillin, the MBC/MIC ratios for other antibiotics were 1, 2, 4, 8 or ⩾16 (Table III). The same is true for other antibiotics. Table IV shows the data on 14 strains of *S. aureus* with an MBC/MIC ratio of ⩾16 for gentamicin.

Killing of S. aureus

In order to determine whether there is any difference in the magnitude of killing at different time-intervals between the strains with low and high MBC values, data were analysed according to the MBC of the given antibiotic for a particular strain. Table V

Table III. The differences between minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the other 11 antibiotics for 16 strains of *Staphylococcus aureus* with an MBC/MIC ratio of ⩾16 for methicillin

Antibiotics	No. of strains with an MBC/MIC ratio of				
	1	2	4	8	⩾16
Oxacillin	1	7	1	2	5
Nafcillin	3	3		2	8
Cephalothin	1	1		1	13
Cephapirin			1		15
Cefazolin				1	15
Vancomycin				1	15
Gentamicin		3	1	5	7
Tobramycin	2	4	8	2	
Amikacin	4	7	2	3	
Sissomicin	4	2	5	3	2
Netilmicin	1	2	5	3	5

Table IV. The differences between minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the other 11 antibiotics for 14 strains of *Staphylococcus aureus* with an MBC/MIC ratio of ⩾16 for gentamicin

Antibiotics	No. of strains with an MBC/MIC ratio of				
	1	2	4	8	⩾16
Methicillin	1	5	1		7
Oxacillin	1	5	1		7
Nafcillin	1	2		1	10
Cephalothin	1	1	1	1	10
Cephapirin	1	2	1	1	9
Cefazolin	2		1	1	10
Vancomycin			1	1	12
Tobramycin	2	3	5	3	1
Amikacin	3	7	2	2	
Sissomicin	3	2	3	5	1
Netilmicin	2	1	3	3	5

Table V. The effects of antibiotics in the killing of *Staphylococcus auerus* in relation to the MBC

MBC	No. of strains		Viable colonies/ml at 0 h	6 h	24 h	48 h
(a) Nafcillin 10 μg/ml						
⩽8 μg/ml	21	Range	$1{\cdot}0\times10^{6}$–$1{\cdot}2\times10^{7}$	$6{\cdot}9\times10^{6}$–$1{\cdot}6\times10^{7}$	$7{\cdot}6\times10^{3}$–$3{\cdot}3\times10^{6}$	$2{\cdot}7\times10^{3}$–$1{\cdot}3\times10^{5}$
		Mean	$6{\cdot}4\times10^{6}$	$6{\cdot}9\times10^{6}$	$7{\cdot}8\times10^{5}$	$2{\cdot}0\times10^{4}$
⩾16 μg/ml	14	Range	$2{\cdot}0\times10^{6}$–$1{\cdot}1\times10^{7}$	$2{\cdot}2\times10^{6}$–$1{\cdot}0\times10^{7}$	$3{\cdot}0\times10^{3}$–$9{\cdot}2\times10^{5}$	$4{\cdot}0\times10^{3}$–$2{\cdot}9\times10^{4}$
		Mean	$5{\cdot}9\times10^{6}$	$5{\cdot}6\times10^{6}$	$5{\cdot}1\times10^{5}$	$7{\cdot}7\times10^{3}$
(b) Oxacillin 10 μg/ml						
⩽8 μg/ml	29	Range	$1{\cdot}0\times10^{6}$–$1{\cdot}2\times10^{7}$	$1{\cdot}4\times10^{6}$–$1{\cdot}5\times10^{7}$	$1{\cdot}9\times10^{3}$–$1{\cdot}7\times10^{6}$	$2{\cdot}4\times10^{3}$–$1{\cdot}4\times10^{5}$
		Mean	$6{\cdot}4\times10^{6}$	$6{\cdot}8\times10^{6}$	$9{\cdot}3\times10^{5}$	$1{\cdot}1\times10^{4}$
⩾32 μg/ml	6	Range	$2{\cdot}1\times10^{6}$–$7{\cdot}2\times10^{6}$	$3{\cdot}1\times10^{6}$–$1{\cdot}4\times10^{7}$	$3{\cdot}7\times10^{4}$–$5{\cdot}6\times10^{5}$	$1{\cdot}4\times10^{3}$–$3{\cdot}8\times10^{4}$
		Mean	$5{\cdot}2\times10^{6}$	$7{\cdot}1\times10^{6}$	$2{\cdot}6\times10^{5}$	$9{\cdot}5\times10^{3}$
(c) Sissomicin 1 μg/ml						
⩽8 μg/ml	26	Range	$1{\cdot}0\times10^{6}$–$1{\cdot}2\times10^{7}$	$1{\cdot}5\times10^{3}$–$1{\cdot}3\times10^{5}$	$1{\cdot}0\times10^{3}$–$2{\cdot}0\times10^{5}$	$2{\cdot}0\times10^{1}$–$1{\cdot}4\times10^{5}$
		Mean	$6{\cdot}0\times10^{6}$	$1{\cdot}8\times10^{4}$	$1{\cdot}2\times10^{4}$	$1{\cdot}5\times10^{4}$
⩾2 μg/ml	9	Range	$2{\cdot}1\times10^{6}$–$1{\cdot}0\times10^{7}$	$2{\cdot}1\times10^{3}$–$4{\cdot}5\times10^{4}$	$3{\cdot}5\times10^{3}$–$2{\cdot}2\times10^{4}$	$1{\cdot}0\times10^{1}$–$9{\cdot}8\times10^{3}$
		Mean	$5{\cdot}6\times10^{6}$	$1{\cdot}3\times10^{4}$	$7{\cdot}5\times10^{3}$	$2{\cdot}8\times10^{3}$

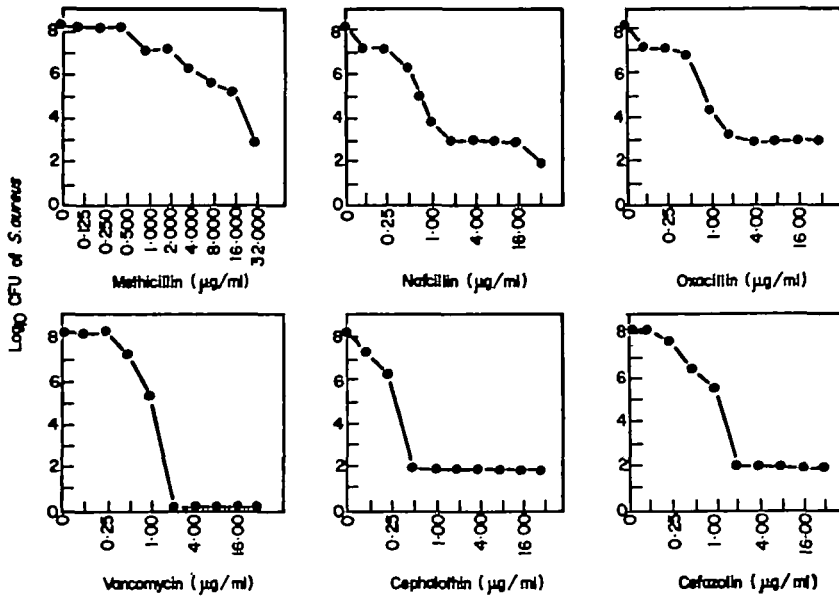

Figure 1. Population analysis of a strain of *Staphylococcus aureus* showing the numbers of organisms resistant to the indicated antibiotic concentrations. MIC/MBC of methicillin was 4/>32, oxacillin 0·5/>32, vancomycin 1/16, cephalothin 0·5/>32, cefazolin 0·5/16 μg/ml for this strain.

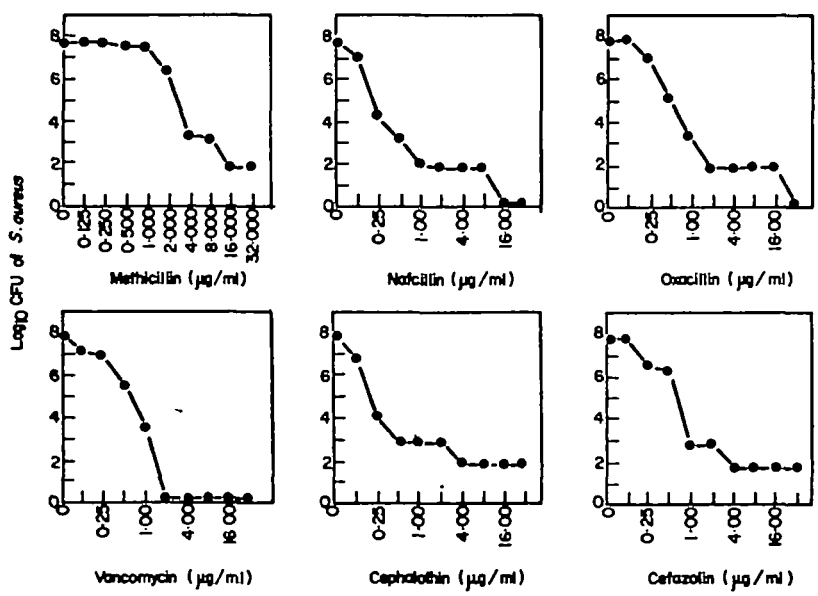

Figure 2. Population analysis of a strain of *Staphylococcus aureus* showing the number of organisms resistant to the indicated antibiotic concentrations. MIC/MBC of methicillin was 2/2, nafcillin 0·25/1, oxacillin 0·25/0·5, vancomycin 0·5/4, cephalothin 0·125/1, cefazolin 0·25/16 for this strain.

lists data on the effect of nafcillin 10 μg/ml in the killing of *S. aureus*. There was no difference in the magnitude of killing at all time-intervals between the 21 strains with MBCs of nafcillin $\leqslant$8 μg/ml and the 14 strains with MBCs of $\geqslant$16 μg/ml. Similar findings are shown for oxacillin 10 μg/ml; there was no difference between results from

the 29 strains with MBCs of oxacillin ⩽8 μg/ml and the 6 strains with MBCs of ⩾32 μg/ml. The results from experiments with sissomicin 1 μg/ml are also listed; again, the effects of killing were the same whether the MBC of sissomicin was ⩽1 μg/ml or ⩾2 μg/ml.

Population analyses

Figures 1 and 2 show the results of population analyses for 2 representative strains of *S. aureus* with different MIC and MBC values for different antibiotics. Within a given population of organisms there is a mixture of cells with different degrees of susceptibility or resistance to a particular antibiotic. By and large, the majority of cells were susceptible to low concentrations of antibiotics, hence the low MIC values. However, when there are significant numbers of resistant organisms, the MBC values increase.

Discussion

The results of this investigation confirm that the phenomenon of antibiotic-tolerance is quite common among strains of *S. aureus* isolated from blood cultures (Mayhall, Medoff & Marr, 1976; Sabath *et al.*, 1977). The 35 strains of *S. aureus* used in this study were taken randomly from the collection of blood culture isolates in our laboratory. There was no uniform cross-tolerance among related antibiotics. The phenomenon of antibiotic-tolerance involved not only the antibiotics which inhibit cell wall synthesis but also the aminoglycosides.

Population analyses showed that the susceptibility of the cell population of antibiotic-tolerant *S. aureus* was heterogeneous and that the majority of cells were susceptible to the antibiotic tested while the minority of cells were resistant in varying degrees. Since the majority of cells in the antibiotic-tolerant *S. aureus* were quite susceptible, there was no difference in the magnitude or rate of killing of antibiotic-susceptible versus antibiotic-tolerant *S. aureus*.

It has been shown that penicillin-tolerant *S. aureus* strains are deficient in autolytic enzyme activity which appears to be necessary for bacteriolysis and the lethal action of penicillin (Sabath *et al.*, 1977). The mechanism of aminoglycoside tolerance of *S. aureus* is unknown at present.

It is not clear how important the phenomenon of antibiotic-tolerance of *S. aureus* is in clinical practice, since this occurs quite commonly. Although slow response or failure to respond to proper antibiotic therapy was documented in infections caused by antibiotic-tolerant *S. aureus* (Gopal, Bisno & Silverblatt, 1976; Mayhall, Medoff & Marr, 1976; Sabath *et al.*, 1977), no unusual response to therapy was noted in patients infected with the strains tested in this study. This finding supports the opinion of Lacey that 'there is no certainty that tolerance is responsible for the therapeutic failure with these drugs' (Lacey, 1977). Should the slow response of serious staphylococcal infection to β-lactam antibiotics (Watanakunakorn, Tan & Phair, 1973) be due to antibiotic tolerance, the enhancement of activity of an antibiotic which inhibits cell wall synthesis in combination with an aminoglycoside (Watanakunakorn & Glotzbecker, 1974; Watanakunakorn & Glotzbecker, 1977) may be beneficial. Such a combination has been shown to be useful in isolated case reports (Gopal, Bisno & Silverblarr, 1976; Mayhall, Medoff & Marr, 1976). However, a recent analysis of a small uncontrolled series of *S. aureus* endocarditis treated with such a combination showed the mortality to be the same as

those treated with a penicillin alone (Watanakunakorn & Baird, 1977). Whether the combination therapy is more effective than single antibiotic therapy must await the results of a well-designed randomized prospective large-scale study.

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