TOLERANCE OF *STAPHYLOCOCCUS AUREUS* TO β-LACTAM ANTIBIOTICS

TOLERANTIE VAN *STAPHYLOCOCCUS AUREUS* VOOR β-LACTAM ANTIBIOTICA

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INTRODUCTION

This study seeks to establish a sound definition of tolerance in Staphylococcus aureus and to investigate its prevalence, mechanism and in vivo significance. Tolerance is a certain form of resistance to antibiotics observed in a number of gram-positive species of bacteria. In general the susceptibility of a bacterial strain to an antibiotic is established by determining the inhibitory action of that antibiotic on the strain. This is expressed as the minimal inhibitory concentration (MIC) of the antibiotic for the strain in question. The lower the value of the MIC, the more susceptible the strain is to a particular antibiotic. The bacterium is said to be resistant if the MIC exceeds a predetermined value. Bacteria which are regularly exposed to an antibiotic easily develop resistance to it. It has been found that bacterial resistance can be produced by different mechanisms. Tolerance is a form of resistance which long remained unnoticed because it is not revealed when the MIC is determined. To effectively combat an infection it is not enough to inhibit the growth of a bacterial strain with the help of an antibiotic; the bacterium must ultimately be killed. As a rule the minimal bactericidal concentration (MBC) and the minimal inhibitory concentration differ little from one another. The MBC is the concentration of the antibiotic which is capable of killing 99.9% of a given bacterial population in 24 hours. Sabath (1) has asserted that tolerant S. aureus strains can be distinguished from strains with a normal susceptibility by the fact that the MBC/MIC ratio is greater than or equal to 32.

Tolerance has been demonstrated in several bacterial species like Streptococcus pneumoniae, Streptococcus mutans, Listeria monocytogenes and Lactobacilli. There are indications that, under certain conditions, infections by tolerant strains of these species react less well to therapy. This is particularly the case in subacute or chronic infections like endocarditis or osteomyelitis localized in tissues that are critical with respect to vascularization.

Chapter I of this study reviews the literature on the in vitro and in

vivo significance of tolerance in several bacterial species. In Chapter II a method is described for a reliable determination of the minimal bactericidal concentration of β-lactam antibiotics for S. aureus. The same method was subsequently used to determine survival of a given inoculum at different concentrations of methicillin. The proportion staphylococci surviving after 24 hours in the presence of high methicillin concentrations is referred to as the tolerance percentage of the strain in question. In Chapter III the tolerance percentages of different collections of staphylococcal strains are investigated. Chapter IV deals with the response of strains with different tolerance percentages to methicillin treatment in an experimental thigh muscle model in mice. In the final chapters the mechanism of tolerance in S. aureus is investigated. Chapter V deals with the course of autolytic activity in cell-bound and extracellular fractions in strains with a low and a high tolerance percentage exposed to different concentrations of methicillin. In Chapter VI the influence of methicillin on the metabolic activity of tolerant and nontolerant strains of S. aureus is studied by measuring the rate of peptidoglycan, RNA and protein synthesis in these strains.

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CHAPTER 1

TOLERANCE OF STAPHYLOCOCCUS AUREUS TO ANTIBIOTICS STUDIED IN VITRO AND IN VIVO

Despite the introduction of new and ever better antibiotics, it is apparent that bacteria have various mechanisms for escaping the killing action of agents of this kind. One of these mechanisms is tolerance to antibiotics. This phenomenon was first observed by Tomasz et al. (69) in a mutant of Streptococcus pneumoniae and thereafter by Best et al. (5) in a clinical isolate of Staphylococcus aureus. Tolerance is a type of resistance in which β-lactam antibiotics and in some cases also vancomycin, inhibit bacterial growth without killing of the micro-organisms. Thus, in these isolates the antibiotic is bacteriostatic rather than bactericidal. In the order in which they are dealt with, the topics discussed in this chapter are the detection of tolerant bacteria, the prevalence of the phenomenon in S. aureus and a number of other species, the possible significance of the phenomenon as regards the effect of antimicrobial therapy or prophylaxis in the animal model and in clinical medicine and, finally, the mechanism underlying the phenomenon.

Detection of tolerant bacteria

Tolerance can be detected in a bacterial strain either by determining the survival rate of the strain at different points of time after exposure to an antibiotic or by determining the number of survivors from a given inoculum at a fixed point of time, usually 24 h after inoculation of the culture. Using either method, there are however several reasons why tolerance can be overlooked. For it appeared from a number of studies that the tolerance phenomenon can be influenced by the following variables: the growth phase of the inoculum (logarithmic or stationary), the composition, the pH and the temperature of the

medium, whether or not the culture tubes are shaken after inoculation or before subculturing, the adherence of the bacteria to the glass of the tubes and any carry-over of the antibiotic from the test tube to the culture plates.

The current method for determining the number of survivors from an inoculum after exposure to an antibiotic for 24 h is as follows. Twofold serial dilutions of the antibiotic are made in a particular growth medium. A known number of viable bacteria are then added to each tube of the series and the series are incubated for 24 h at 37°C. After incubation, a known volume is taken from each test tube and transferred to a solid medium for the purpose of determining the number of surviving bacteria. As a rule the concentration at which the number of viable bacteria is reduced by 99.9% or more of the initial inoculum is defined as the minimal bactericidal concentration of a particular antibiotic for the strain investigated (3). Judging from literature the possible influence of the aforementioned factors on the MBC value may be described as follows.

Contradictory reports exist concerning the influence of the growth phase of the inoculum on the detection of tolerance. Ishida et al. (29) and Venglarcik et al. (74) believe that no tolerant organisms can be detected using logarithmic cultures. In agreement with this is the observation of Taylor et al. (67) that logarithmic cultures are killed more effectively than inocula from stationary cultures. Similar results have also been reported by Kim et al. (33) and Mayhall et al. (43), who found that stationary cultures have higher MBC values than logarithmic cultures. Using inocula obtained from stationary cultures, Bradley et al. (7) was even able to demonstrate tolerant organisms in 100% of the isolates. This does not preclude that Sabath et al. (60), Raynor et al. (56) and Best et al. (5), were able to demonstrate an absence of bactericidal action of β-lactam antibiotics and vancomycin in some strains that were exposed to these antibiotics in the logarithmic phase of the growth curve. Venglarcik et al. (74) pointed out that when stationary cultures are used as inocula, the low pH of these cultures may contribute to the development of high MBC values. The

influence of the composition of media on the detection of tolerance may be due to differences in buffer capacity (41,52) and, hence, indirectly to a pH effect (11,28).

The next two variables relate to the way in which the MBC determination is carried out. Bacteria which adhere to the glass above the meniscus of the growth medium after the inoculum has been added do not come into contact with the antibiotic and are therefore not killed. If the tubes are shaken before subculturing these bacteria can survive because they have been exposed to the antibiotic for only a short time. When transferred to culture plates such samples give rise to MBC values which are too high (20,29,67). To avoid this adherence effect, Taylor et al. (67) proposed to shake the tubes one extra time, four h before subculturing, to ensure sufficient exposure of the bacteria to the antibiotic.

A factor which can give rise to MBC values which are too low is the carry-over effect. By this is meant that when samples are taken, not only viable bacteria, but also antibiotic is transferred on to the culture plates. The influence of the carry-over effect on the MBC values has been studied by Pearson et al. (51). As was to be expected, it was found that the carry-over effect was dependent on the volume of the samples and on the antibiotic concentration. It is therefore recommended that small volumes should be spread on agar containing β-lactamase (24,35,53,73).

An observation which can not be left unmentioned in studying the survival of bacteria which are exposed to an antibiotic is the Eagle effect (13,61). Eagle noted the paradoxical phenomenon that in some strains of streptococci and staphylococci exposed to increasing concentrations of penicillin, the MBC/MIC ratio is much lower and thus more favourable at a low than at a high concentration of the antibiotic. For example, in a particular strain the MBC/MIC ratio might be 4 with a low concentration and 64 with a high concentration. The use of various techniques to eliminate the influence of the above variables has led to different definitions of tolerance. In a frequently cited article Sabath et al. (60) proposed that a staphylococcal strain

should be described as tolerant if it exhibits an MBC/MIC ratio of 32 or more after exposure to nafcillin. This definition is questionable as repeated determinations of survival after 24 h in a strain with a marked Eagle effect can easily give rise to differences in the result of the MBC/MIC ratio. Minor shifting in experimental conditions may cause that such a strain would be classified as tolerant or non-tolerant from one day to another. If the MBC/MIC ratio is used as a borderline it would be advisable to state to what extent the strain in question exhibits the paradoxical phenomenon described by Eagle.

Phenotypic tolerance

It is known that the aforementioned factors such as the growth phase of the inoculum, the pH and the composition of the medium can produce what is referred to as phenotypic tolerance in genotypic nontolerant organisms. Beta-lactam antibiotics have an optimal effect on actively dividing bacteria. It is not difficult to conceive, therefore, that an inoculum obtained from a stationary culture diluted in fresh medium will not immediately resume growth and, hence, that the β-lactam antibiotic will not have a killing action during this period. It is known of low pH values that they have a negative influence on the activity of the autolysins and can lead, therefore, to apparent tolerance (11, 28). The composition of the growth medium is also important in connection with its buffer capacity. Venglarcik et al. (74) has demonstrated that in tryptic soy broth overnight cultures have a low pH and that inocula of such cultures yield higher MBC values. Thus, the choice of growth medium and growth phase is apparently of importance in performing the MBC test.

A phenomenon which may cause confusion with tolerance and which is also detectable by performing MBC tests are the so-called persisters. They were initially described by Bigger (6) in 1944. He observed that in staphylococcal cultures 1 in 10^6 cells survived prolonged exposure to bactericidal concentrations of penicillin. The number of persisters in a culture is influenced by the growth conditions of the inocula. For example addition of inocula to cold broth produces larger numbers

of persisters than addition of cells to broth at 18°C. Gunnison et al. (17) even found that inocula of $2x10^8$ cocci/ml or higher gave a survival of 5-100%. The offspring of such surviving cocci were as susceptible to penicillin as the parent strain. Bigger (6) and Gunnison et al. (17) both concluded that persisters are cocci which survive exposure to penicillin because they are in a dormant non-dividing phase. In view of our remarks on the growth conditions we believe that persistence is identical to the phenotypic form of tolerance.

Prevalence of tolerance to penicillins in various micro-organisms

In view of the role which the foregoing variables can play when the MBC is determined, it is not surprising that different authors give widely differing reports of the prevalence of tolerance. Tolerance has been demonstrated and studied chiefly in streptococcal species (1,24, 32,33,34,36,37,45,50,54,59,62,63,66), S. aureus (7,22,43,44,55,60,67) S. epidermidis (72), Listeria monocytogenes (47,75) and Lactobacillus (4). The prevalence of tolerance in S. aureus found by different authors ranges from 0% to 100%. Taylor et al. (67) did not find tolerance in any of the strains he studied. Other authors (22,44,55, 60) report prevalences of 30-70% in the collections they studied, while Bradley et al. (7) reported that all the strains he studied were tolerant.

Experimental studies

The reaction of tolerant micro-organisms to antimicrobial therapy has been studied in animal models and in patients. Apart from the freedom to choose a particular infection model, an animal study has the advantage that the antimicrobial therapy can be standardized and that strains with the same virulence and growth rate can be compared. Tolerant strains are not easily killed by bactericidal antibiotics, therefore an infection model that normally requires treatment with a bactericidal antibiotic is of particular interest for studying the tolerance phenomenon. Experimental endocarditis is such a model and understandably regularly used for this purpose. Aside from its use for

measuring a therapeutic effect, such a model has also been repeatedly employed to study the prophylactic action of antibiotics. The endocarditis model has the additional advantage that the course of the infection is little affected by cellular defense.

The following studies mostly concern experimental endocarditis caused by streptococcal species in rabbits and rats. In the endocarditis model of Lowy et al. (39) a tolerant Str. sanguis strain was found to have a poorer reaction to penicillin therapy (5,000 U/kg/8h) than a nontolerant Str. mitis strain, despite the fact that the latter was more virulent. When high doses of penicillin (80,000 U/kg/8h) were administered, no difference was found between the numbers of CFUs isolated from vegetations of animals infected with the tolerant or the nontolerant strain. This result is somewhat contradicted by the observations of Wilson et al. (77) who, with a high concentration of penicillin (150,000 U/kg/12h), did find significant differences in reaction in treating infections caused by tolerant and nontolerant viridans streptococci. Brennan et al. (8) reported that the addition of streptomycin to the penicillin therapy led to sterile vegetations on day 5 of the therapy. This applied both to the animals infected with the tolerant strains and those infected with the nontolerant strains. Wilson et al. (77) found a similar synergistic action using streptomycin. Hess et al. (21) investigated whether the infection of cardiac valves by tolerant strains could be prevented by the prophylactic administration of antibiotics. Rabbits were treated prophylactically with penicillin 30 min. before inoculation with tolerant Str. sanguis strains. Section showed that tolerant strains had caused infection in 70% of the rabbits, whereas the corresponding figure for susceptible strains was only 9%. These results appear to be somewhat contradicted by those of Lowy et al. (40) and Glauser et al. (15), who found that the prophylactic administration of amoxycillin influenced the adherence of tolerant and nontolerant strains to cardiac valves equally. All in all the in vivo results obtained with species of Streptococcus seem to point to a positive relationship between tolerance and a reduced response to antimicrobial therapy with β-lac-

tam antibiotics. It has not yet been possible to demonstrate such a relationship in the case of S. aureus. Goldman et al. (16) and Guze et al. (18) were unable to do so with, respectively, an endocarditis and a pyelonephritis model.

Clinical studies

Clinical experience has taught that deep staphylococcal infections respond slowly to treatment with antibiotics. The reason for this is that staphylococcal infections are pyogenic and that the pyogenic nature of these infections makes it difficult for antimicrobial agents to reach the bacteria in the centre of the foci. In fact, antibiotics with a bactericidal action are required to sterilize such foci. In addition to what has been explained in the section on experimental infections it is therefore expected that in particular staphylococcal infections such as endocarditis, osteomyelitis and pneumonia caused by tolerant strains will respond insufficiently or slowly to therapy that is normally considered to be adequate. The number of patients with infections of this type caused by tolerant strains is however so small that it is difficult to determine whether tolerance does have a negative effect on treatment. Reports on this matter are therefore usually of an anecdotal nature. Examples are the studies of Mayhall et al. (43) in 1976 and Sabath et al. (60) in 1977. The latter described 7 patients with deep infections caused by tolerant S. aureus strains. All of the patients appeared to respond less well to antimicrobial therapy. A more comprehensive study was that carried out in 1980 by Rajashekaraiah et al. (55), who compared 50 patients with endocarditis and a group of 54 patients with bacteremia caused by S. aureus strains. As the criteria used for tolerance was an MBC/MIC ratio of 16, which is lower than the ratio indicated by Sabath et al. (60), the proportion of tolerant strains in this series was no less than 60%. The difference in the response to therapy of patients infected with tolerant strains was most marked in the patients with endocarditis as these patients had more complications and longer periods with fever. The difference in mortality in patients with endocarditis and with

bacteremia was however not found to be significant. This is understandable if one realises that tolerant micro-organisms are ultimately killed, but only after a longer period. In 20 patients with deep staphylococcal infections with tolerant strains, Denny et al. (12) found some indications that the blood cultures remained positive longer and caused a higher mortality.

A final judgement whether or not tolerance plays a role in the clinic cannot be given until a study has been carried out in which a recognized method of determining tolerance is used, the patients receive a standardized antibiotic therapy and the groups of patients are defined according to strict criteria.

In the author's own study (46) of 77 patients with positive blood cultures caused by S. aureus it proved to be extremely difficult to say anything definite about the effect of tolerance on the results of antimicrobial therapy. Of the 9 patients with bacteremia caused by a tolerant strain, only 2 or 3 satisfied the above-mentioned conditions. Though they did respond rather slowly to the antimicrobial therapy, so many other factors were involved that it was not possible to make any definite statement about the exact role of tolerance on the outcome of therapy. The conclusion must therefore be that further information on the significance of tolerance for the treatment of staphylococcal infections will have to be gathered from animal models which satisfy the aforementioned conditions.

Mechanism

The bactericidal action of a β-lactam antibiotic, such as penicillin, on susceptible bacteria entails the following important steps. Penicillin is able to inhibit the synthesis of peptidoglycan because it binds covalently to enzymes (PBPs) which are involved in the synthesis of this macromolecule (42,68,78). A continuous inhibition of peptidoglycan synthesis can lead to preponderance of the effect of autolytic enzymes on this compound. The result of this is that the cell wall structure is weakened to such an extent that the cell lyses because of the osmotic pressure differential (71). The last part of the mode of

action was discovered in a study of a pneumococcal mutant which was later defined tolerant (69). In this mutant the antibiotic was able to inhibit but not to kill the strain as the substrate of the autolysins was so modified that they were no longer able to act on it. Though theoretically tolerance could also be based on a change in affinity between certain PBPs and β-lactam antibiotics (19), all of the results thus far considering the cause of tolerance point in the direction of a change in substrate composition (48) or in autolytic enzyme activity (2,14,27,58,65). Mention has already been made of the altered substrate in Tomasz's pneumococcal mutant (69). Staphylococcal strains with diminished autolysin activity after exposure to oxacillin were described as long ago as 1974 by Best et al. (5) and 1977 by Sabath et al. (60). Further, Raynor et al. (56) found that tolerant S. aureus strains exposed to nafcillin produce larger quantities of lipoteichoic acid than normal strains. It is known that lipoteichoic acid can inhibit autolytic enzymes (9,10,25,26,70).

Mutants have also been described which have normal autolysin activity but nonetheless exhibit a tolerant reaction after exposure to a β-lactam antibiotic. According to Williamson and Tomasz (76) this phenomenon is the result of a defect in the signal needed to trigger these autolytic enzymes. In Bacillus subtilis tolerance can be indirectly ascribed to a high turnover of autolytic enzymes following proteolytic breakdown of these enzymes (31). Finally, another possibility, which also leads indirectly to reduced autolysin activity, is the phenomenon that bacteria in which protein synthesis is inhibited are not killed by penicillin (23,30,38,57,64). Mychajlonka et al. (49) demonstrated in a recent study that in tolerant Str. mutans strains penicillin inhibits not only peptidoglycan synthesis but also protein and RNA synthesis. Combined RNA and protein synthesis inhibition can also be brought about by simultaneously adding penicillin and a protein synthesis inhibitor like chloramphenicol to susceptible, i.e. nontolerant, streptococci. As already mentioned this type of tolerance is referred to as phenotypic tolerance. The above-mentioned causes of a tolerant reaction after exposure to a β-lactam antibiotic are only a

few of the many possible causes that can lead to this type of resistance. Further research into the mechanisms of tolerance can contribute to our knowledge, vast as it already may be, of the mode of action of β-lactam antibiotics.

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CHAPTER 2

FACTORS INFLUENCING DETECTION OF TOLERANCE IN *STAPHYLOCOCCUS AUREUS*[*]

W.H.F. Goessens, P. Fontijne, and M.F. Michel

The phenomenon of tolerance to cloxacillin and methicillin was studied in *Staphylococcus aureus*. It was demonstrated that the minimal bactericidal concentrations showed marked differences, depending on the method of detection used. These differences resulted from carry-over of the antibiotic to the subculture plates. When carry-over of the antibiotic was prevented by the addition of β-lactamase to the nutrient medium, the antibiotics were no longer bactericidal. At a certain antibiotic concentration and at higher concentrations, however, each strain showed a certain survival percentage after 24 h. The tolerance percentage was determined for 15 strains. The values found for the individual strains ranged from <0.1 to 11% for cloxacillin and methicillin. Since these percentages were reproducible within narrow limits, they could be regarded as a characteristic of the strains. The tolerance percentage was independent of the growth phase of the initial cultures.

INTRODUCTION

Reports on the phenomenon of tolerance to penicillins, studied mainly in *Staphylococcus aureus* and *Streptococcus* species, have been regularly published in the past few years. The tolerance phenomenon was first described by Tomasz (12) in pneumococci. He observed that, in vitro, tolerant strains were not killed by penicillin in the conventional

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bactericidal concentrations. Subsequently, Best et al. (2) observed the same phenomenon in S. aureus. By using killing curves, they demonstrated that bacteria of certain strains were inhibited, but not killed, by oxacillin. The phenomenon was found to be based on delayed killing of a minority of bacteria in the inoculum. Mayhall et al. (9) and Sabath et al. (11) subsequently attempted to estimate the degree of tolerance. For this purpose, they used the ratio between the minimal bactericidal concentration (MBC) and the minimal inhibitory concentration (MIC). Tolerant strains have a normal MIC but a markedly increased MBC of penicillins. In these studies, the MBC was defined as the concentration at which 99.9% of the original inoculum was killed after 24 h of incubation (1). Sabath accepted a ratio of 32 as borderline between susceptible and tolerant strains.

Several studies have related the tolerance of organisms causing infections to the failure of antimicrobial therapy with penicillins (3,6,11). Differences in the technique of determining the MBC make it difficult to determine whether tolerance is clinically relevant. Data from the literature indicate that the MBC varies with the medium in which it is determined (10) and also with the growth phase of the culture (7,8). Factors such as subculture volume, duration of incubation of the subculture plates, and, possibly, density of the inoculum used may also influence these MBCs.

In the present study, the variables in the test methods were critically analyzed. Next, an attempt was made to evolve, by standardization, a reproducible method of determining the degree of tolerance in S. aureus.

MATERIALS AND METHODS

Bacteria

The staphylococcal strains used in this study were obtained from the bacteriological laboratory of the University Hospital Rotterdam/Dijkzigt, The Netherlands. The organisms were isolated from 15 patients

with a positive blood culture and were identified as S. aureus on the basis of colony form and color, the presence of grapelike clusters in Gram stain, and a positive coagulase test. In addition, all strains were phage typed at the National Institute of Public Health. Of the 15 strains, 13 were susceptible to phages of the S. aureus phage-typing set. All strains were catalase positive. The strains were stored in freeze-dried form.

Antibiotics

The antibiotics used in this study were cloxacillin and methicillin (Beecham Pharmaceuticals, Heppignies, Belgium).

Estimation of MIC and MBC

Estimates of MIC and MBC were made in twofold serial dilutions of the antibiotic in glass tubes containing 2 ml of Mueller-Hinton broth (Difco Laboratories). The serial concentrations were 0.1 to 102.4 μg/ml for cloxacillin and 0.5 to 512 μg/ml for methicillin. For determination, we used logarithmic and stationary-phase cultures of bacteria (see figure legends). These were obtained by suspending 1 loopful from a blood plate (Oxoid Ltd.) in 25 ml of Mueller-Hinton broth and incubating the suspension at 37°C for 4 and 18 h, respectively, on a shaking device. After incubation, the required density of the suspensions was ensured by densitometry at 660 nm in a photometer (Vitatron). The final concentration in the tubes was about 10^5 colony-forming units (CFU) per ml; the correct value was determined by viable counts in duplicate. After addition of the inoculum, the tubes were incubated at 37°C for 24 h without shaking, whereupon the MIC was read. The MIC was defined as the concentration that caused no visible turbidity after 24 h of incubation. The MBC was determined by taking 50 μl from those tubes that showed no growth. Before sampling, the tubes were shaken. The 50-μl volumes were spread on one-third ($\pm$ 20 cm^2) of the surface of nutrient agar (Oxoid) plates which contained 0.15 U of β-lactamase I and 0.015 U of β-lactamase II per ml (Whatman Biochemicals Ltd.). Plates with β-lactamase were used

in all experiments unless otherwise stated. After 48 h of incubation at 37°C, the CFU counts were made and converted to percentages of the initial inoculum. The MBC was defined as the concentration that killed 99.9% of the original inoculum (1). All MIC and MBC estimates were made in duplicate.

RESULTS

In the early phase of this study, rejection values were used to determine the MBC. Given an inoculum of 10^5 CFU/ml and a subculture volume of 200 μl, this value was 20 CFU. At this value, 99.9% of the original inoculum was killed (MBC definition). By using this method, it was found that the MBC/MIC ratio for 15 strains did not exceed 4, and consequently, none of these strains were tolerant according to the definition of Sabath et al. (11). When the subculture volume was reduced from 200 to 50 μl, however, the MBCs for these strains increased. They increased further when the period of incubation of the nutrient plates was increased from 24 to 48 h. With a subculture volume of 50 μl and an incubation period of 48 h, the MBC of methicillin for strains 3 and 11, for example, was 512 μg/ml (Fig. 1A). Since

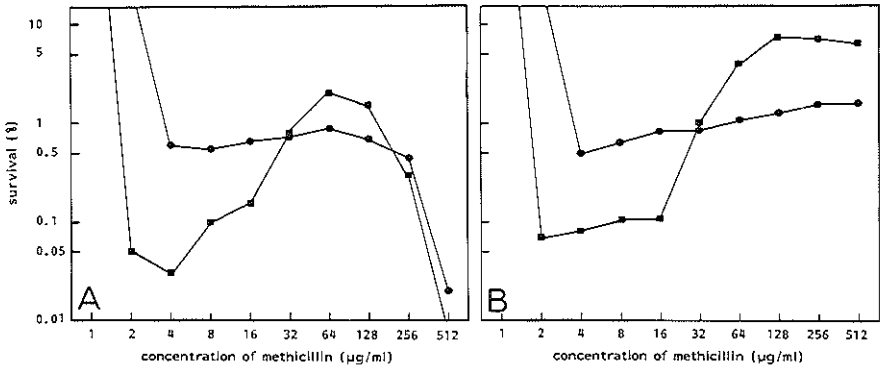

Figure 1: Survival rates of two isolates of S. aureus, strain 3 (■) and strain 11 (●), at various concentrations of methicillin. Stationary-phase cultures were used as inocula. (A) Nutrient agar without β-lactamase. (B) Nutrient agar which contained β-lactamase.

TABLE 1

Estimation of MIC, MBC, and survival percentage for cloxacillin and methicillin in 15 strains of S. aureus with inocula in logarithmic and stationary phases[a]

Strain	Cloxacillin						Methicillin					
	Logarithmic phase			Stationary phase			Logarithmic phase			Stationary phase		
	MIC (μg/ml)	MBC (μg/ml)	Survival (%)	MIC (μg/ml)	MBC (μg/ml)	Survival (%)	MIC (μg/ml)	MBC (μg/ml)	Survival (%)	MIC (μg/ml)	MBC (μg/ml)	Survival (%)
1	0.2	25.6	3.0	0.2	25.6	6.0	1	512	7.0	1	512	8.0
2	0.1	25.6	0.5	0.1	25.6	1.0	2	256	1.0	1	128	2.0
3	0.2	25.6	0.4	0.2	51.2	6.0	2	256	0.9	1	256	6.0
4	0.2	25.6	1.2	0.2	12.8	0.8	2	256	1.7	1	256	2.0
5	0.2	12.8	0.3	0.2	12.8	0.15	2	32	0.5	1	256	0.3
6	0.2	25.6	0.3	0.2	25.6	0.9	2	256	0.3	1	512	1.5
7	0.1	3.2	0.12	0.1	1.6	<0.1	2	64	0.1	2	64	<0.1
8	0.2	25.6	0.4	0.2	25.6	0.3	2	256	0.25	2	128	0.6
9	0.2	0.4	0.12	0.2	25.6	0.4	4	512	0.25	2	256	0.6
10	0.2	12.8	0.12	0.1	6.4	0.15	4	64	0.15	2	128	0.2
11	0.2	12,8	0.5	0.2	25.6	1.0	2	128	0.3	2	512	1.5
12	0.2	51.2	9.0	0.2	51.2	3.0	2	256	11.0	1	512	7.5
13	0.2	0.4	<0.1	0.2	3.2	0.2	1	2	0.1	1	16	0.6
14	0.4	102.4	11.0	0.4	102.4	10.0	2	512	11.0	2	512	10.0
15	0.2	25.6	1.5	0.2	6.4	0.6	1	256	2.8	1	256	2.0

[a] The inocula used for estimation of the MICs were 10^5 CFU/ml. MBCs were obtained by subculturing after 24 h on nutrient agar without β-lactamase. Survival percentages were obtained by subculturing after 24 h on nutrient agar with β-lactamase

the MICs of this antibiotic for these two strains are 1 and 2 μg/ml, respectively, their MBC/MIC ratios are 512 and 256, respectively. Both strains became highly tolerant after the abovementioned modifications were made. Table 1 shows that the MBCs for 15 different strains tested ranged from 0.4 to 102.4 μg of cloxacillin per ml and from 2 to 512 μg of methicillin per ml and that most of the strains tested showed tolerance as a general phenomenon, with the MBC/MIC ratios exceeding 32 according to the modified method. In two strains (9 and 13) the ratio was much lower than 32. Since the results obtained by the modified test method demonstrated that the MBCs were profoundly influenced by carry-over of the antibiotic, β-lactamase was added to the subculture plates in subsequent experiments. Carry-over of the antibiotic was thus prevented. The results of these experiments are presented in Fig. 1B. The antibiotic proved to be no longer bactericidal, and therefore MBCs could not be determined. To indicate the degree of tolerance nevertheless, a different parameter was introduced: the number of viable bacteria expressed as a percentage of the original inoculum after 24 h of incubation. This survival percentage is the plateau value attained when the antibiotic concentration is increased and is called the tolerance percentage of a strain. The tolerance percentage does not change, even in the presence of very high antibiotic concentrations (10 mg/ml). Figure 1B shows tolerance percentages of 1.5 for strain 11 and 6.0 for strain 3.

The tolerance percentage can be regarded as a strain characteristic only if the values found are reproducible within narrow limits. This reproducibility of tolerance percentages was studied by testing two strains four times with methicillin and cloxacillin. Figure 2 shows that the methicillin tolerance percentages of the two strains (14 and 15) are reproducible from a concentration of 128 μg/ml (mean ± standard deviation; 7.7 ± 3.1 and 0.62 ± 0.45 μg/ml, respectively); the cloxacillin tolerance percentages are reproducible from a concentration of 6.4 μg/ml (mean ± standard deviation; 7.6 ± 1.6 and 0.25 ± 0.08 μg/ml, respectively).

Figures 1 and 2 show that there is an optimal antibiotic concentration

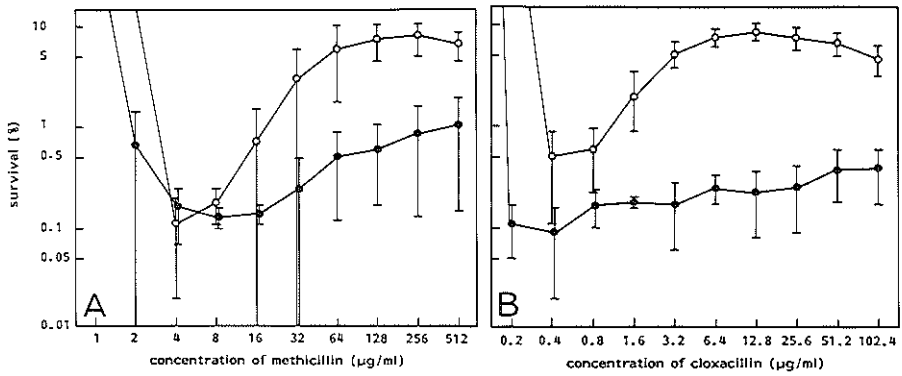

Figure 2: Tolerance percentages for two isolates of S. aureus, strain 14 (O) and strain 15 (●), at various concentrations of methicillin (A) and cloxacillin (B). Stationary-phase cultures were used as inocula. Each point represents the mean of four determinations (± standard deviation).

for the killing effect. This so-called zone phenomenon was observed by Eagle and Musselman (4) as early as 1948. Comparison of Fig. 2A with 2B reveals, moreover, that the killing effect of methicillin exceeds that of cloxacillin.

The results discussed so far were all obtained by proceeding from inoculation of stationary-phase cultures (18 h). In an effort to establish whether the MBCs obtained with a stationary-phase culture differ significantly from those obtained with logarithmic cultures, the cloxacillin and methicillin tolerance percentages of 15 strains were determined with 4-h and 18-h cultures. Table 1 shows that the tolerance percentages of the various strains in 18-h cultures were sometimes higher and sometimes lower than those obtained in 4-h cultures of the same strains.

Our results in terms of tolerance percentages can be plotted, not only against the antibiotic concentration, but also against sampling time (Fig. 3). The curves thus obtained provide more exact information on the degree of bacterial killing at the various concentrations of the antibiotic used. Figure 3 shows, however, that strain 3, with a methicillin tolerance percentage of 6, was killed more rapidly at methicillin concentrations of 4, 8 and 16 µg/ml than strain 2, which has a

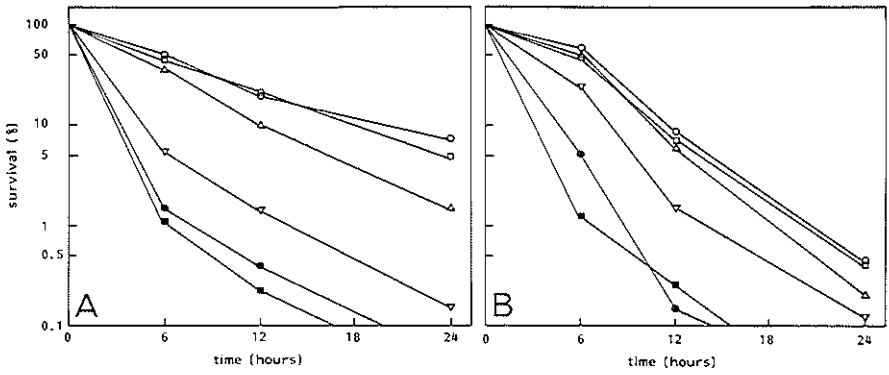

Figure 3: Killing curves of two strains of S. aureus at various concentrations of methicillin with stationary-phase cultures as inocula. (A) Strain 3 has a tolerance percentage of 6.0. (B) Strain 2 has a tolerance percentage of 0.45. Drug concentrations were 4 (■), 8 (●), 16 (▽), 32 (△), 128 (○), and 256 (□) μg/ml.

tolerance percentage of 0.45. These experiments thus demonstrate that a substantial difference in tolerance percentage is not necessarily accompanied by a difference in lysis rate.

DISCUSSION

Previous studies have shown that different techniques are being used to detect tolerance in S. aureus. Best et al. (2) and Sabath et al. (11), for example, studied the decrease in CFU with time spectrophotometrically and were thus able to demonstrate differences between susceptible and tolerant organisms. A variant used by such authors as Mayhall et al. (9) is the killing curve technique to study the decrease in CFU with time. The most widely used technique, however, is the tube dilution method. Several studies have shown that such factors as the medium used and the age of the initial culture influence the MBCs obtained (7,8,10).

We attempted to obtain more detailed information on the causes of these differences in results. It was found that reduction of the subculture volume and prolongation of the period of incubation each

led to an increase in the MBC. A common factor in both modifications of the technique is the reduction of the carry-over of antibiotics to the subculture plates. When carry-over of the antibiotic is prevented by adding β-lactamase to the subculture plates, methicillin and cloxacillin are no longer bactericidal. The curves depicted in Fig. 2 were described by Eagle and Musselman as early as 1948 (4). The course of the latter part of such curves indicates that it is not possible to determine MBCs at high concentrations of these antibiotics. Since in this situation the MBC/MIC ratio can no longer be used as a measure of tolerance, efforts were made to find a different parameter with which the behavior of bacterial strains in response to high concentrations of β-lactam antibiotics could be characterized. This strain characteristic, which we define as tolerance percentage, is the number of bacteria expressed as a percentage of the original inoculum. The tolerance percentage is determined by the lowest concentration at which bacterial survival is stabilized. For each strain, the tolerance percentage is reproducible within narrow limits. Moreover, very high antibiotic concentrations fail to influence this characteristic. The age of the initial culture has no systematic effect on results in terms of tolerance percentage. The observations reported by Mayhall and Apollo (8) and Kim and Anthony (7) could therefore not be confirmed.

The tolerance percentage described in the present study can be explained as the result of the killing of the majority of the bacterial population. The phenomenon is not due to regrowth of part of the population, as described by Gwynn et al. (5). This was concluded from the fact that the number of CFU after 48 h never exceeded the number of CFU after 24 h. Inactivation of the β-lactam antibiotic during the process of subculturing, as first described by Eagle and Musselman (4), allows us to devise a reproducible parameter for estimation of the degree of tolerance. Construction of the entire survival curve is too cumbersome for use as a routine procedure. It is therefore advisable to determine the tolerance percentage at only one particular concentration: 25.6 μg of cloxacillin and 128 μg of methi-

cillin per ml. At these concentrations, the tolerance percentage was found to be stabilized. Survival percentages thus determined may contribute to a better understanding of the clinical relevance of the tolerance phenomenon.

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CHAPTER 3

TOLERANCE PERCENTAGE AS A CRITERION FOR THE DETECTION OF TOLERANT STAPHYLOCOCCUS AUREUS STRAINS*

W.H.F. Goessens, P. Fontijne, M. van Raffe, and M.F. Michel

In this study, the degree of tolerance was determined in several populations of Staphylococcus aureus isolates. The degree of tolerance of a staphylococcal strain can be established in a reproducible way by exposing the strain to increasing concentrations of a β-lactam antibiotic and determining the number of surviving bacteria at each concentration. The number of surviving bacteria was expressed as a fraction of the initial inoculum. By this technique, it appears that for each strain the value of the surviving fraction stabilized above a certain concentration of the antibiotic. This value was called the tolerance percentage of the strain. In 64 S. aureus strains isolated from blood cultures in 1982, the tolerance percentages, after exposure to methicillin, varied from $\leqslant$0.1 to 6; 28% of the strains showed a tolerance percentage of $\leqslant$0.1, and 12.5% showed a tolerance percentage of $\geqslant$ 2. Similar tolerance percentages were found with cloxacillin, nafcillin, cephalothin, and penicillin. Strains with a tolerance percentage of $\geqslant$ 2 showed slow killing and lysis in the presence of a high methicillin concentration. A tolerance percentage of 2 appeared to be the breakpoint between susceptible and tolerant strains. Older collections of S. aureus strains, dating from the years 1951 to 1953 and 1957 to 1958, also included strains with a survival percentage of $\geqslant$ 2, thus indicating that tolerance of S. aureus to β-lactam antibiotics is not a new phenomenon.

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INTRODUCTION

Sabath et al. (11) have described tolerance of Staphylococcus aureus to β-lactam antibiotics as a new type of resistance. This type of resistance has also been observed in other gram-positive bacteria (13). Tolerant strains show a normal MIC and a distinctly increased MBC (MBC:MIC ratio, $\geqslant$ 32) (11), with MBC defined as the concentration at which 99.9% of the inoculum is killed (1).

The efficacy of antimicrobial treatment in infections caused by staphylococci with a high MBC:MIC ratio was studied several times. A negative correlation between tolerance and antimicrobial response was demonstrated in some (3,8,10,11) but not all (6,7) cases. The diverse results obtained in these studies may be based on differences in laboratory conditions used to demonstrate the phenomenon. In a previous study, we have demonstrated that the percentage of surviving bacteria exposed to high concentrations of a β-lactam antibiotic is reproducible constantly within certain limits (5). In the present study, we attempt to indicate the threshold value between susceptible and tolerant strains by determining the tolerance percentage of a number of S. aureus strains. To investigate whether the prevalence of tolerance has increased in the last few decades, two collections of older strains were studied as well.

MATERIALS AND METHODS

Bacteria

The S. aureus strains used in this study are composed of the following collections: 64 strains isolated in 1982 from blood cultures of patients admitted to the Rotterdam University Hospital (group I); 29 strains from the collection of the Statens Seruminstitut, Copenhagen, Denmark, isolated during the period 1957 to 1958 from blood cultures of Danish patients (group II); 29 strains isolated from pus, sputum, or blood of patients admitted to the Leiden University Hospital,

Leiden, The Netherlands, during the period 1951 to 1953 (group III). All strains were identified as S. aureus on the basis of colony form, color, and a positive coagulase test. The strains of groups I and II were phage typed as well. All strains were catalase positive and were freeze-dried for storage.

Antibiotics

The antibiotics used in this study was cloxacillin and methicillin (gifts from Beecham Pharmaceuticals, Amstelveen, The Netherlands), nafcillin and penicillin (Gist-Brocades, Delft, The Netherlands), and cephalothin (Eli Lilly Nederland, Amsterdam, The Netherlands).

Estimation of tolerance percentage

Tolerance percentages were estimated as previously described (5). The method can be summarized as follows: estimations were carried out by incubation of an inoculum of 10^5 CFU/ml in serial twofold dilutions of cloxacillin, nafcillin, cephalothin, methicillin, and penicillin in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) for 24 h at 37°C. After incubation, 50 μl from the concentrations that suppressed visible growth was spread on nutrient agar (Oxoid Ltd., London, England) plates containing 0.15 U β-lactamase I and 0.015 U β-lactamase II per ml (Whatman Biochemicals Ltd.). After 48 h of incubation at 37°C, counts of viable bacteria were made and converted to percentages of the initial inoculum. At higher antibiotic concentrations, survival of the bacteria is no longer dependent on the antibiotic concentration. The plateau of survival level is a characteristic of the strain and is called the tolerance percentage.

Killing curves

For killing curves, 200 μl of a diluted 18-h culture of the strain to be tested was added to a number of tubes containing various concentrations of methicillin in 2 ml of Mueller-Hinton broth. This resulted in an inoculum of 10^5 CFU/ml. Antibiotic concentrations tested ranged from 512 to 0.5 μg/ml. Samples (100 μl) were taken after incubation at

37°C for 0, 3, 6, 12, and 24 h. They were carried through serial 10-fold dilutions in sterile saline, and 50 μl of each dilution was spread on nutrient agar plates containing β-lactamase (as stated above). After incubation for 48 h, CFU counts were made and converted to percentages of the initial inoculum.

Lysis curves

Lysis was measured in an MS-2 research apparatus (Abbott Laboratories, Diagnostic Division, Irving, Tex.). With this apparatus, light transmission is read in a multichamber cuvette, and optical densities are recorded at 5-min intervals on a magnetic tape. Analysis of the data by a microcomputer gives the growth curves on a screen (12).

RESULTS

MBCs of penicillins for S. aureus proved to be influenced by carry-over of the antibiotic (5). By adding β-lactamase to the subculture plates, carry-over of the antibiotic is prevented. The antibiotic proved to be no longer bactericidal, and therefore, MBCs could not be determined. To indicate the degree of tolerance nevertheless, a different parameter was introduced: the number of viable bacteria expressed as a percentage of the original inoculum after 24 h of incubation. This survival percentage is the plateau value attained when the antibiotic concentration is increased and is called the tolerance percentage of a strain. Figure 1 shows a tolerance percentage of 1 to 2.5% for strain 335, determined for five β-lactam antibiotics.

To study the distribution of tolerance percentages in a population of S. aureus strains, we determined these percentages for 64 S. aureus strains isolated in 1982 from positive blood cultures. The values of the tolerance percentages obtained with these strains are shown in Table 1. The majority of these strains (87.5%) had a methicillin tolerance percentage of $\leqslant 1$; in 28%, this percentage was in fact $\leqslant 0.1$

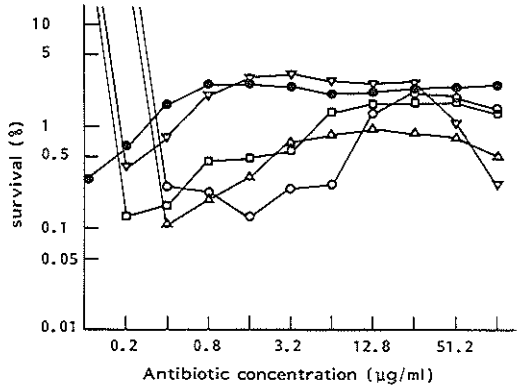

Figure 1: Survival rates of an isolate of S. aureus 335 in the presence of penicillin (●), cloxacillin (□), nafcillin (△), cephalothin (▽), and methicillin (○). Serial concentrations were 0.1 to 102.4 μg/ml for the first four antibiotics. Due to a divergent susceptibility of the strains for methicillin, serial concentrations used in the presence of this antibiotic ranged from 0.5 to 512 μg/ml (data not shown).

TABLE 1: Distribution of tolerance percentages[a] for 64 isolates of S. aureus isolated in 1982 from blood cultures

Antibiotic	No. of strains corresponding to the following ranges of tolerance percentages[b]							
	0-0.5	0.5-1	1-2	2-3	3-4	4-5	5-6	6-7
Methicillin	47(5)	8(1)	1	2(1)	2	1	1	2
Cloxacillin	51(5)	5(1)	1	2(1)	4		1	
Nafcillin	53(6)	5(1)		3	2		1	
Cephalothin	48(5)	9(1)	2	2	2(1)	1		
Penicillin	(5)	(1)		(1)				

[a]Each tolerance percentage represents the mean of duplicate determinations.

[b]Numbers in parentheses denote the number of penicillin-susceptible strains.

(Table 1). The tolerance percentage of the remaining eight strains (12.5%) varied from 2 to 6. A tolerance percentage of $\geqslant 2$ to the other β-lactam antibiotics tested was shown by 10.9, 9.4, 7.8, and 14.3% of the 64 strains.

Tolerance was also looked for in S. aureus strains isolated a few decades ago. For this purpose, we used two collections of 29 strains each dating back to the years 1951 to 1953 (group III) and 1957 to 1958 (group II). Because strains tolerant to methicillin show cross-tolerance to other β-lactam antibiotics, strains of groups II and III were tested only in the presence of methicillin. Both collections show a broad range of tolerance percentages which vary from <0.1 to 12 for group II and from <0.1 to 2.5 for group III. (Table 2).

TABLE 2: Distribution of tolerance percentages[a] for two groups of S. aureus isolates determined for methicillin

Group[b]	No. of strains corresponding to the following ranges of tolerance percentages[c]					
	0-0.5	0.5-1	1-2	2-3	7-8	10-20
II	25	1		1	1	1
III	23(5)	5		(1)		

[a]Each tolerance percentage represents the mean of duplicate determinations.

[b]Group II consists of 29 strains isolated during the period 1957 to 1958; group III consists of 29 strains isolated during the period 1951 to 1953.

[c]Numbers in parentheses denote the number of penicillin-susceptible strains.

Ten strains from the three collections were further studied on the basis of killing and lysis curves. A comparison of the killing curves of the five strains with high ($\geqslant 2$) tolerance percentages to those of five strains with low ($\leqslant 0.1$) tolerance percentages revealed that the tolerance percentage determined after 24 h was highly related to the killing rate (Fig. 2A). However, at relatively low methicillin concentrations (Fig. 2B) there were no longer any demonstrable differences in killing rate between strains with high and low tolerance percentages. That the killing of S. aureus strains is a result of lysis was demonstrated in experiments in which the lysis rate of the same strains in the MS-2 was followed spectrophotometrically during 24 h (Fig. 3). An unmistakable difference in lysis was found between

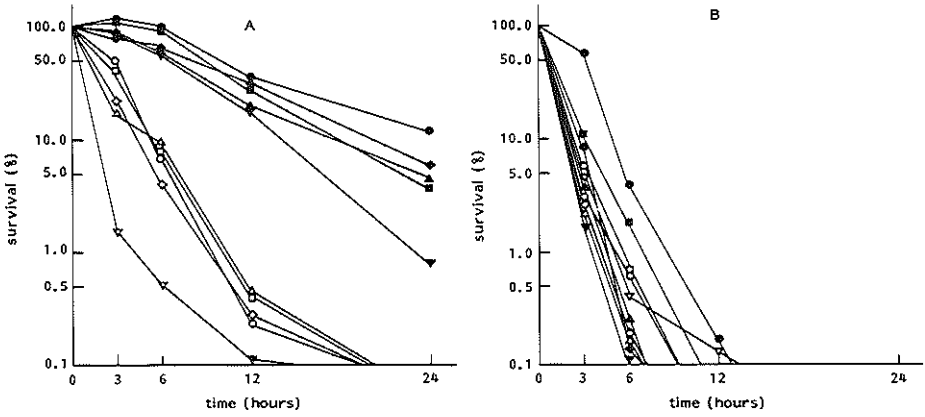

Figure 2: Killing curves of 10 strains of S. aureus at two concentrations of methicillin: 128 μg/ml (A); 4 μg/ml (B). Closed symbols represent five different strains with tolerance percentages of $\geqslant 2$; open symbols represent five different strains with tolerance percentages of $\leqslant 0.1$ for methicillin.

strains with high and low tolerance percentages at a high antibiotic concentration (128 μg of methicillin per ml) (Fig. 3A). The difference in lysis was not demonstrable at a low antibiotic concentration (4 μg of methicillin per ml) (Fig. 3B).

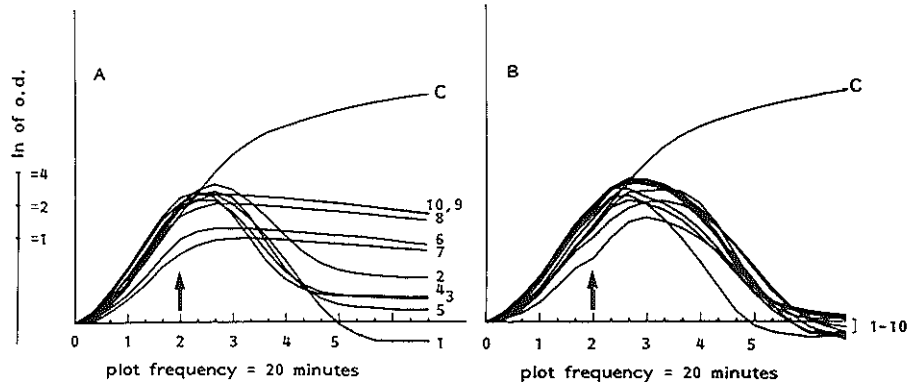

Figure 3: Growth curves of five strains with tolerance percentages of $\geqslant 2$ (numbers 6 to 10) and five strains with tolerance percentages of $\leqslant 0.1$ (numbers 1 to 5) of S.aureus. After 2 h of incubation (arrow), methicillin, 128 μg/ml (A) or 4 μg/ml (B), was added to the log-phase cells.The optical density (o.d.) was measured in an MS-2 apparatus. C, Control.

DISCUSSION

In a previous study, we have demonstrated that the determination of MBCs for S. aureus showed marked differences, depending on the method of detection used. These differences resulted from carry-over of the antibiotic to the subculture plates (5). After minimalization of this carry-over effect, MBCs were no longer found. A certain percentage of the inoculum survived even very high concentrations of the β-lactam antibiotic. This survival percentage proved to be a characteristic of the strain and was called the tolerance percentage of the strain. To gain more insight into the distribution of tolerance percentages in a population of strains, these percentages were measured in 64 S. aureus strains isolated in 1982 from positive blood cultures from hospitalized patients. It is shown that the majority of the strains (87.5%) have methicillin tolerance percentages of $\leqslant 1$. The remaining strains show survival percentages of $\geqslant 2$.

Estimations of the tolerance percentages of a strain to different antibiotics show that these are about the same. This means that cross-tolerance exists, even for penicillin-susceptible strains. On account of the results obtained, we conclude that, apart from a few exceptions, there is a breakpoint between strains with tolerance percentages of < 2 and those with tolerance percentages of > 2. The observations on two other collections of S. aureus strains dated from the periods 1951 to 1953 and 1957 to 1958 seem to corroborate the value accepted as breakpoint. In view of the results of these experiments in vitro, efforts are being made to determine the efficacy of antimicrobial treatment in experimental infections caused by tolerant and susceptible S. aureus strains.

Our S. aureus strains with tolerance percentages of > 2 and $\leqslant 0.1$ showed similar killing and lysis curves as those described by Mayhall et al. (9) and Best et al. (2). This implies that the distribution of tolerance percentages reveals a breakpoint which differentiates between susceptibility and tolerance. It should be borne in mind that the tolerance percentages discussed here were estimated in the

presence of high antibiotic concentrations. Our results show, however, that at low antibiotic concentrations, susceptible and tolerant strains do not differ in lysis or killing rate. This so-called paradoxical zone phenomenon described by Eagle and Musselman (4) has been found to a varying extent for all strains tested so far, including those from groups II and III.

The presence of highly tolerant strains in the old collections indicates that tolerance is probably not a new type of resistance. However, the phenomenon has remained unnoticed because the effect of carry-over of antibiotic in estimating the bactericidal effect of a β-lactam antibiotic was not taken into account.

ACKNOWLEDGEMENTS

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CHAPTER 4

RESPONSES OF TOLERANT AND NONTOLERANT *STAPHYLOCOCCUS AUREUS* STRAINS TO METHICILLIN TREATMENT IN AN EXPERIMENTAL INFECTION IN MICE*

W.H.F. Goessens, P. Fontijne, and M.F. Michel

Staphylococcus aureus strains can be divided into tolerant and nontolerant strains on the basis of their survival in vitro in the presence of high concentrations of methicillin ($\geqslant$ 64 μg/ml). A strain is defined as tolerant if more than 2% of the inoculum survives under these conditions. The response of five susceptible and five tolerant *S. aureus* strains to treatment with methicillin was studied in an experimental thigh infection in mice. Animals were treated with one and two injections of methicillin (2.5 mg per mouse). At the end of treatment, the number of CFUs in the thigh muscles infected with the susceptible strains was found to be significantly lower than that in the thigh muscles infected with the tolerant strains.

INTRODUCTION

The clinical relevance of the phenomenon of tolerance has been studied repeatedly. The results reported by various authors are somewhat contradictory (2,5-9). The question whether infections with tolerant strains are more difficult to treat then infections with susceptible strains can be investigated by means of an experimental infection model in animals. In previous studies (3), a method of distinguishing tolerant from susceptible strains on the basis of their survival in the presence of high concentrations (64 μg/ml) of methicillin has been

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described. A strain is defined as tolerant if more than 2% of the inoculum survives under these conditions. The survival percentage in the presence of high antibiotic concentrations is referred to as the tolerance percentage. In the present study the response of Staphylococcus aureus strains with tolerance percentages of greater or less than 2% to antimicrobial treatment was measured in the model of thigh infection in mice.

MATERIALS AND METHODS

Bacteria

Ten S. aureus strains were studied. Five strains were susceptible and five were tolerant to methicillin. As described previously (4), the tolerance percentages of the five susceptible strains varied from 0.03 to 0.1, and for the five tolerant strains the percentages varied from 2 to 13.

Mice

Female Swiss mice, 11 to 13 weeks old (weight, 18 to 22 g; bred by the Oxfordshire Laboratory Animal Colony, London), were used in all experiments.

Experimental infection

The strain to be studied was incubated for 18 h in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) and were then washed three times with saline. A suspension of ca. 10^8 CFU/ml was obtained. The numbers of viable organisms inoculated in each experiment were determined by plate counts made just before inoculation. One hundred microliters of the suspension was injected into the left thigh muscle of a mouse that was lightly anesthetized with ether, as described by Selbie and Simon (10). In mice sacrificed immediately after the inoculation, 100% of the inoculated bacteria were recovered from the thigh muscle.

Antibiotic treatment

Eighteen mice were inoculated with ca. 10^7 CFU in the left thigh muscle as stated above. Of these 18 mice, 6 were treated subcutaneously in the abdominal region with one dose of methicillin which was given 1 h after the inoculation of the bacteria. Another group of six mice received two doses of methicillin. Doses were administered at 1 and 3 h after inoculation. The remaining six mice did not receive antibiotic treatment. They were sacrificed 1 h after bacterial inoculation and served as the control group for the determination of inoculum size at the time the antibiotic treatment started. Unless stated otherwise, the doses administered were 0.15, 0.3, 0.6, 1.25, and 5 mg per mouse.

Methicillin concentrations in serum

Blood specimens were obtained by orbital puncture of mice under light ether anesthesia at various intervals after the administration of methicillin. Methicillin was assayed by the aid of the agar well diffusion method, with DST agar (Oxoid Ltd., London, England) and Bacillus subtilis as an indicator organism (1).

Therapeutic results

Two hours after the first and second methicillin doses (3 and 5 h after inoculation, respectively), groups of six mice (unless otherwise stated) were sacrificed by cervical dislocation. The infected thigh muscle was removed and immediately homogenized in 20 ml of ice-cold physiological saline, to which 100 μl of β-lactamase (15.2 U of β-lactamase I and 2.3 U of β-lactamase II) (Whatman Biochemicals, ltd.) was added. The tissues were homogenized in a VirTis homogenizer for 1 min. at 7,000 rpm. The homogenates were then diluted 10-fold serially in sterile saline. Each dilution (0.2 ml) was spread on nutrient agar plates, to which 0.15 U of β-lactamase I and 0.015 U of β-lactamase II per ml of agar were added. After 48 h of incubation at 37°C, CFU counts were made and converted to the total number of CFUs per thigh muscle. These numbers were expressed as percentages of the mean value

of the number of CFUs cultivated from muscles of untreated control mice which were sacrificed 1 h after bacterial inoculation as stated above.

Growth rate

The growth rate of the strains in the thigh muscle of untreated mice was chosen as a parameter for virulence. Groups of three mice were infected with 5×10^6 CFUs of each strain. Mice were sacrificed after 1, 3, and 5 h to count the number of CFUs from the thigh muscle.

Statistics

The Wilcoxon rank-sum test was used to determine whether the results of quantitative cultures of thighs infected with susceptible strains differed significantly from those infected with tolerant strains.

RESULTS

The response of five susceptible and five tolerant strains of S. aureus to the administration of methicillin was studied in an experimental infection in mice. In a previous experiment, we demonstrated that the therapeutic response of the five susceptible and the five tolerant S. aureus strains resulted from different tolerance percentages and not from differences in bacterial growth rate in the thigh muscle of untreated mice. Experiments indicated that of the ten strains, 7 show approximately the same increase in CFUs at intervals of 2 and 4 h (Table I). However, strains 372, 914, and 3401 (two tolerant strains and one susceptible strain, respectively) exhibited at an interval of 2 h a slower growth. This difference in growth rate disappeared at the 4-h interval. We concluded that the growth rates of the five tolerant and the five susceptible strains were similar.

On the basis of these results and of preliminary experiments on the therapeutic response of the susceptible strain 5558 and the tolerant

TABLE 1

Growth rate in vivo[a] of five susceptible and five tolerant S. aureus strains

Strain no.	Tolerance (%)[b]	Log CFU (± SD) at[c]: 0 h	1 h	3 h	5 h	Ratio in CFU 3 h/1 h	5 h/1 h
5558	0.09	6.70	6.65(0.18)	7.67(0.02)	8.01(0.14)	10.5	22.9
98	0.07	6.72	6.64(0.02)	7.77(0.04)	8.14(0.07)	13.5	31.6
3401	0.03	6.75	6.54(0.23)	7.21(0.03)	7.93(0.14)	4.7	24.6
355	0.05	6.77	6.66(0.06)	7.65(0.08)	7.96(0.07)	9.8	20.0
477	0.10	6.74	6.88(0.16)	7.81(0.09)	8.33(0.12)	8.5	28.2
194	6.10	6.50	6.50(0.09)	7.57(0.09)	7.70(0.23)	11.8	15.9
5407	5.70	6.57	6.27(0.33)	7.59(0.06)	7.95(0.12)	20.9	47.9
340	2.00	6.57	6.28(0.26)	7.62(0.06)	7.73(0.42)	21.9	28.2
372	6.50	6.55	6.31(0.12)	7.06(0.09)	7.71(0.10)	5.6	25.1
914	13.00	6.72	6.53(0.01)	7.18(0.05)	7.62(0.04)	4.5	12.3

[a]Increase in the number of bacteria in the thigh muscle at 1, 3, and 5 h after bacterial inoculation.

[b]Tolerance percentages are determined in the presence of high concentrations of methicilllin ($\gg$64 μg/ml).

[c]Each value at 1, 3, and 5 h represents the mean log CFU recovered from the thigh muscles of three mice.

strain 194, administration of antibiotic in a dosage schedule consisting of one and two injections at a 2-h interval, starting 1 h after bacterial inoculation, seemed to be the most discriminating for both strains. This dosage schedule was chosen for further studies. The effect of different doses of methicillin (0.15 to 5 mg per mouse) upon the number of bacteria in the thigh muscle is shown in Table 2. After treatment with methicillin doses of 0.6, 1.25, 2.5, or 5 mg per mouse, the number of bacteria cultured from mice infected with the susceptible strain was significantly lower than that of mice infected with the tolerant strain ($P \ll 0.01$). Before studying the therapeutic activity of methicillin in a larger number of susceptible and tolerant strains, serum concentrations after the subcutaneous administration of a single dose of 0.3, 1.25, 2.5, or 5 mg per mouse were determined. Since virtually the same serum levels were obtained after a second antibiotic injection, these values have not been given.

TABLE 2

Survival rates of susceptible S. aureus strain 5558 and tolerant S. aureus strain 194 after treatment with various doses of methicillin

Strain no[a]	Dose of methicillin (mg)	Log CFU (± SD) at[b]: 1 h	3 h	5 h	Inocula recovered (%) at[c]: 3 h	5 h
5558	0.15	6.73(0.09)	7.00(0.19)	6.92(0.15)	186.2	154.9
	0.3		6.14(0.16)	5.51(0.25)	25.7	6.0
	0.6		5.23(0.14)	4.53(0.35)	3.2	0.6
	1.25		5.33(0.07)	4.38(0.28)	4.0	0.5
	2.5		5.17(0.25)	3.71(0.08)	2.8	0.1
	5.0		5.25(0.12)	3.87(0.38)	3.3	0.1
194	0.15	7.00(0.07)	6.84(0.15)	6.71(0.27)	69.2	51.3
	0.3		6.79(0.13)	6.05(0.17)	61.7	11.2
	0.6		6.32(0.08)	5.74(0.02)	20.9	5.5
	1.25		6.56(0.08)	5.64(0.22)	36.3	4.4
	2.5		6.78(0.12)	5.78(0.16)	60.3	6.0
	5.0		6.69(0.08)	5.69(0.10)	49.0	4.9

[a]Log CFU of inocula in the muscle at time zero for strains 5558 and 194 were 6.91 and 6.93, respectively.

[b]Log CFU of inocula at 1 h were determined before the addition of doses of methicillin. Values at 3 and 5 h represent the mean log CFU recovered from the thigh muscles of five mice after one and two doses of methicillin, respectively.

[c]Recovery was calculated as a fraction (%) of the inoculum isolated from the thighs of five untreated control mice which were sacrificed at 1 h after bacterial inoculation.

Table 3 shows that after the administration of a dose of 2.5 mg of methicillin per mouse, methicillin concentrations in excess of the MICs for the S. aureus strains used were present for at least 120 min. This dose was therefore chosen for further studies. The survival of five susceptible and five tolerant strains in the thigh muscle after the administration of one and two doses of 2.5 mg of methicillin are given in Table 4. Bacterial counts for the five tolerant strains were significantly higher, as compared with the five susceptible strains, after treatment with one and two doses ($P \leqslant 0.01$). When the individual susceptible and tolerant strains showing overlap in killing rate are compared, the differences still appear to be significant. The P value was 0.01 in four of the six cases and $\leqslant 0.05$ in the other two.

TABLE 3

Concentration of methicillin in serum at various intervals after subcutaneous administration of various doses[a]

Dose of methicillin (mg per mouse)	Drug concn (μg/ml) in serum ± SD at:						
	5 min	15 min	30 min	45 min	60 min	90 min	120 min
0.30	7.4± 1.5	9.9± 2.9	6.5± 1.0	5.2± 1.8	1.8± 0.3		
1.25	26.1± 5.7	32.4± 3.5	27.3± 9.9	14.9± 2.4	6.6± 2.0	2.2±1.3	
2.50	68.1± 9.3	71.4± 9.6	55.4± 1.4	36.3±10.9	20.3± 4.8	6.2±1.7	4.8±1.6
5.00	66.3±15.1	112.0±13.0	110.0±24.0	68.7± 8.2	24.4±12.4	14.4±5.5	4.6±3.5

[a]Each value represents the mean of five mice plus or minus standard deviation.

TABLE 4

Survival rates of five S. aureus strains with tolerance percentages of $\ll 0.1$ and of five strains with tolerance percentages of $\gg 2$

Strain no.	MIC (μg/ml)	Log CFU (± SD) at[a]:				Inocula recovered (%) at[b]:	
		0 h	1 h	3 h	5 h	3 h	5 h
5558	1	6.37	6.46(0.19)	4.62(0.52)	3.79(0.75)	1.5	0.2
98	0.5	6.91	7.04(0.12)	5.63(0.08)	4.77(0.38)	3.9	0.5
3401	1	6.78	6.73(0.26)	5.26(0.24)	3.86(0.24)	3.4	0.1
355	2	7.23	7.21(0.09)	5.79(0.16)	4.99(0.25)	3.8	0.6
477	1	7.21	7.34(0.13)	6.20(0.22)	5.29(0.21)	7.2	0.9
194	1	6.56	6.30(0.26)	6.08(0.13)	5.24(0.19)	60.3	8.7
5407	2	6.89	6.66(0.20)	6.18(0.21)	5.10(0.27)	33.1	2.8
340	1	7.05	6.55(0.17)	5.75(0.13)	5.24(0.17)	15.9	4.9
372	1	7.08	7.20(0.05)	6.96(0.06)	6.32(0.17)	57.5	13.2
914	1	6.89	6.95(0.08)	6.43(0.10)	5.62(0.18)	30.2	4.7

[a]Each value at 1, 3, and 5 h represents the mean log CFU recovered from the thigh muscles of six mice after one and two doses of methicillin (2.5 mg per dose) at 3 and 5 h, respectively.

[b]Recovery was calculated as a fraction (%) of the inoculum isolated from the thighs of six untreated control mice which were sacrificed at 1 h after bacterial inoculation.

DISCUSSION

In recent years, the relevance of tolerance of S. aureus strains for the therapeutic response was investigated in various studies (5,6,8, 9). Sabath et al. (9) described seven tolerant strains which responded

poorly to penicillin treatment of endocarditis, osteomyelitis and pneumonias caused by staphylococci. Rajashekaraiah et al. (8) compared the results of the treatment of 50 patients with endocarditis and 54 patients with bacteremia caused by tolerant and nontolerant S. aureus strains. Tolerance was found to have an influence on the results of the treatment of endocarditis but not on those of the treatment of bacteremia without endocarditis. Goldman et al. (5) compared the therapeutic response of susceptible and tolerant S. aureus strains to methicillin in an endocarditis model in rabbits. Guze et al. (6) studied the effect of methicillin upon similar strains in a pyelonephritis model in rats. In neither model could significant differences be demonstrated in the number of bacteria or survival of the animals after antibiotic treatment. In the present study, the survival of susceptible and tolerant strains was studied in a thigh muscle infection in mice. In contrast to the results obtained by Goldman et al. (5) and by Guze et al. (6), susceptible and tolerant strains used in the present animal model exhibited significant differences in their survival rates after both one and two doses of methicillin.

Several factors might contribute to the divergent results observed in various studies. For instance, the models used by the authors mentioned are much more complex than a thigh muscle infection in mice. This implies that factors such as virulence of the infecting strains, abscess formation at the site of the infection, and host defense may influence the result of antibiotic treatment. Moreover, the criteria for determining the tolerance of strains used in earlier studies differed from our definition of the phenomenon. Goldman et al. (5) and Guze et al. (6) used strains with an MBC/MIC ratio $\geqslant 32$, whereas our tolerant strains exhibit in vitro survival rates varying from 2 to 13% when exposed to high concentrations of methicillin.

The differences in bacterial survival of tolerant and nontolerant strains in the thighs of mice treated with methicillin support the view that, in some cases, the results of treatment of staphylococcal infections depend not only on the MIC value of the causative agent but also on the killing rate (4).

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CHAPTER 5

CELL-BOUND AND EXTRACELLULAR AUTOLYTIC ACTIVITY OF A TOLERANT AND A NONTOLERANT STAPHYLOCOCCUS AUREUS STRAIN EXPOSED TO METHICILLIN*

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Cell-bound and extracellular autolytic activity was studied in a tolerant and a nontolerant Staphylococcus aureus strain after exposure to low (10 μg/ml) and high (80 μg/ml) concentrations of methicillin. Tolerance was defined as survival after 24 h of over 2% of the inoculum after exposure to high concentrations of methicillin ($\geqslant$ 64 μg/ml).

The nontolerant strain showed an increased cell-bound autolytic activity after exposure to both low and high concentrations of methicillin. The tolerant strain on the other hand selectively showed a reduced cell-bound autolytic activity after exposure to 80 μg/ml of methicillin. No difference in extracellular autolytic activity was found between the nontolerant and tolerant strain after exposure to different concentrations of methicillin. However, in both types of strains extracellular activity was less after exposure to a high concentration of methicillin than after exposure to a low concentration. This phenomenon was caused by the release of an inhibitor of the autolysins under the influence of the high concentration of methicillin. This inhibitor is thermolabile and not dialyzable.

INTRODUCTION

The studies of Rogers (11), Shockman (13) and Weidel et al. (17)

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indicate that the lysis induced by penicillin in some bacteria is not necessarily the result solely of inhibition of the cross-linking process in the peptidoglycan (7,14,19). Tomasz et al. (15) collected evidence of a more complicated mode of action in which autolysins also play a role. They provoked conditions in which mutants of Streptococcus pneumoniae showed a diminished autolytic activity. Under these conditions penicillin had a bacteriostatic action on these mutants, i.e. exposure of the cells to this antibiotic no longer led to lysis. Tomasz called this new form of resistance 'multiple drug tolerance'. Best et al. (1) and Sabath et al. (12) demonstrated this new form of resistance in clinical isolates of S. aureus. These studies showed a diminished specific activity of the autolysins of the strains investigated.

In previous studies we described a method of distinguishing tolerant from nontolerant strains on the basis of their survival in the presence of high concentrations ($\geqslant 64$ μg/ml) of methicillin. A strain was considered to be tolerant if 2% or more of the inoculum survived for 24 h under these conditions and nontolerant if less than 2% survived. In a tolerant S. aureus strain the degree of killing and lysis was found to be dependent on the antibiotic concentration used. Lysis and killing were much more effective with low concentrations (2-4 times the MIC value) in both tolerant and nontolerant strains (3,4). In the present study we, have tried to gain a better understanding of the way in which the tolerance phenomenon works, by determining autolytic activity with lysis-permissive and non-lysis-permissive concentrations of methicillin, in tolerant and nontolerant S. aureus strains.

MATERIALS AND METHODS

Bacteria

Two tolerant (194, 5407) and two nontolerant (5558, 98) S. aureus strains were studied. The minimal inhibitory concentration for methi-

cillin for each of the four strains was $\leqslant 2$ μg/ml. The tolerance percentages (4) were 5.7% and 6.1% for strains 5407 and 194 and 0.09% and 0.07% for strains 5558 and 98.

Growth conditions

Logarithmically growing cultures for autolysin extraction were obtained by inoculating 100 ml of Mueller-Hinton broth (Difco) with cells from an 18 h culture. Cells were incubated at 37°C for 3 h on a shaking device until they reached a density of 0.4 (A_{560}). Ten and 80 μg/ml of methicillin respectively were then added to the cultures (at t = 0 h). Three ml culture samples were taken every 30 min. over a period of $3\frac{1}{2}$ h and centrifuged at 10,000 g for 5 min. The supernatant (culture medium) was frozen at -20°C. The pellet was washed once with physiological saline, following which the cells were suspended in 0.01 M phosphate buffer (pH 7.0) and then frozen at -20°C.
For labeling of the peptidoglycan with ^{3}H-N-acetylglucosamine, 50 μl of an 18 h culture was added to 50 ml of medium containing 0.5% bacto-peptone (Difco), 0.5% yeast extract (Difco), 0.2% glucose, 0.1% K_2HPO_4 and 1 μCi/ml N-acetyl-D-[1-^{3}H] glucosamine (Amersham), (spec. act. 2.84 Ci/mmol).

Autolysin extraction from S. aureus cells

The 'freeze-thaw' method described by Huff et al. (6) and Best et al. (1) was used to extract the autolytic enzymes from the cells. The extract was obtained by thawing the frozen cells (-20°C) in a water bath at 37°C and removing them by centrifugation (5 min. at 10,000 g). This extract and the supernatant of the culture medium were dialyzed against 1000 volumes of 0.01 M phosphate buffer (pH 7.0). The fractions were then frozen at -80°C until testing.
Another procedure used to obtain autolytic enzymes involved extraction of cells with 3 M LiCl (8). Washed cells were suspended in 1 ml of 3 M LiCl at 4°C for 10 min. Cells were removed by centrifugation (5 min. at 10,000 g). The supernatant was dialyzed overnight against

1000 volumes of 0.01 M phosphate buffer (pH 7.0).

Isolation of the radio-active peptidoglycan

Peptidoglycan was isolated according to the method of Park & Hancock (9) as modified by Wilkinson & White (18). After growth for 18 h in the presence of the radio-active precursor the cells were washed three times with distilled water, after which the pellet was suspended in 5% trichloro acetic acid (TCA) for 18 h at 4°C.

The sediment obtained by centrifugation (10 min. at 10,000 g) was then put into 75% ethanol (v/v) for 10 min. at room temperature. After this extraction step the pellet was heated for 6 min. at 95°C in 5% TCA. After being washed once in distilled water, cells were incubated for 2 h at 37°C in Tris buffer (0.02 M, pH 7.9) containing trypsin (1 mg/ml). The peptidoglycan residue was subsequently washed three times with distilled water to remove trypsin and hydrolytic products and then frozen (-20°C) in small aliquots (500 μl) each with an activity of 1-2 x 10^6 cpm/ml. Peptidoglycan prepared according to this method no longer possessed any autolytic activity.

Other substrates used were prepared of cells exposed to various amounts of methicillin. Cells of diluted stationary cultures of strain 194 and 5558 were added to 3 separate flasks (per strain) each containing 40 ml Mueller-Hinton broth and 1 μCi/ml ^{3}H-N-acetylglucosamine (Spec. act. 2.84 Ci/mmol, Amersham). The cells were incubated for 6 h at 37°C on a shaking device until they reached a turbidity (A_{560}) of 0.4. At this moment respectively 0, 10 and 80 μg/ml of methicillin was added. After a 90 min. incubation period in methicillin, cells were harvested and peptidoglycan was isolated as described above.

The autolysin assay

The determination of autolytic activity is based on release of radioactivity from the isolated staphylococcal peptidoglycan. Maximum autolytic activity was measured at 37°C in 0.01 M phosphate buffer (pH 7.0) containing Mg^{2+}ions, which stimulated peptidoglycan hydrolysis in accordance with the results of Huff & Silverman (6). The

incubation mixture contained 20 µl of labelled peptidoglycan (equivalent to 2.5 µg of muramic acid and 10^5 desintegrations per min. (dpm)) 100 µl of $MgCl_2$ (0.56 M), 100 µl of potassium phosphate (0.1 M, pH 7), and autolysin. Distilled water was subsequently added to obtain a final volume of 1 ml.

The assay was started with the addition of the peptidoglycan to the reaction mixtures which had been preheated at 37°C. After 15, 30, 60 and 120 min., 200 µl of the mixture was added to 0.2 ml of ice cold distilled water. This was centrifuged for 5 min. at 10,000 g; 200 µl of the supernatant was put into counting vials and 10 ml of Instagel (Packard) was added. The radio-activity was determined by means of an Isocap scintillation counter. The autolytic activity was expressed in units. One unit of enzyme activity is equivalent to the release of 1 pmol ^{3}H-N-acetylglucosamine per ml of reaction mixture in 1 h.

Muramic acid determination

This was carried out in accordance with the method of Hadzija (5), the peptidoglycan being hydrolyzed in 3M HCl for 4 h at 95°C. The solution was then neutralized with 4M NaOH. This was followed by the colour reaction with copper sulphate and p-hydroxyphenyl reagent in 10 ml of H_2SO_4. This mixture was measured (A_{560}) against a standard curve of muramic acid.

RESULTS

Susceptibility of peptidoglycan prepared from tolerant and nontolerant strains to autolysins from these strains

As penicillin's point of impact is indirect in the peptidoglycan it was investigated whether peptidoglycan from a tolerant strain was less susceptible to autolysins than peptidoglycan from a nontolerant strain. Exposure of peptidoglycan obtained from stationary cultures of two tolerant (5407, 194) and two nontolerant (5558, 98) strains to autolysins from a tolerant (194) and a nontolerant

(5558) strain showed differences in the susceptibilities of the different peptidoglycans to the two autolysins (Table 1). As one strain incorporated more label per μg of peptidoglycan than the other and also released more label when exposed to autolysins, a correction was made for specific activity (quantity of incorporated label per μg of muramic acid). It can be seen in Table 1 that no great differences in released ^{3}H label subsisted after correction.

TABLE 1

Comparison of peptidoglycan susceptibility of tolerant and nontolerant S. aureus strains to autolysins isolated from log-phase cells of a tolerant and a nontolerant strain.

Source of autolysin	Source of peptidoglycan[a]		Release of ^{3}H label (dpm/hour)[b]	Specific activity of peptidoglycan[c]	Corrected autolytic activity (%)[d]
tolerant strain no. 194	tolerant strains	194	21,530	82,890	26.0
		5407	26,470	108,770	24.3
	nontolerant strains	98	36,240	122,700	29.5
		5558	32,050	110,090	29.1
nontolerant strain no. 5558	tolerant strains	194	16,330	82,890	19.7
		5407	18,600	108,770	17.1
	nontolerant strains	98	25,250	122,700	20.6
		5558	24,560	110,090	22.3

[a]Peptidoglycan extracted of stationary cultures of two tolerant (194, 5407) and two nontolerant (98, 5558) S. aureus strains.

[b]Peptidoglycan of the four strains added to the assay in this experiment is equivalent to 5 μg muramic acid and ± 100,000 dpm.

[c]Amount of incorporated ^{3}H label (dpm) per 5 μg muramic acid.

[d]Autolytic activity as the percentage released of the amount of peptidoglycan added to the assay.

Autolysin susceptibility of peptidoglycan isolated after exposure to low and high concentrations of methicillin

It is conceivable that the substrate of autolysin changes in logarithmic cultures exposed to a β-lactam antibiotic and that for instance peptidoglycan from a tolerant strain exposed to a high concentration of methicillin becomes insensitive to its own autolysin. Exposure of peptidoglycan from a tolerant (194) and a nontolerant (5558) strain isolated after exposure to 10 and 80 μg/ml of methicil-

lin to autolysin of strain 194 revealed no differences in susceptibility either before or after correction for specific activity (Table 2).

TABLE 2

Autolysin susceptibility of peptidoglycan extracted from a tolerant (194) and nontolerant (5558) S. aureus strain exposed to methicillin.

Source of autolysin	Source of peptidoglycan[a]		Release of ^{3}H label (dpm/hour)[b]	Specific activity of peptidoglycan[c]	Corrected autolytic activity (%)
tolerant strain no. 194	tolerant 194	0 μg/ml	25,710	48,810	52.7
		10 μg/ml	22,390	50,980	43.9
		80 μg/ml	18,210	41,080	44.3
	nontolerant 5558	0 μg/ml	21,410	50,600	42.3
		10 μg/ml	24,500	44,900	54.6
		80 μg/ml	25,610	49,950	51.3

[a]Peptidoglycan extracted from log-phase cells of strain 194 and 5558 exposed to various amounts of methicillin.

[b]Peptidoglycan added to the assay in this experiment is equivalent to 0.5 μg muramic acid and ± 50,000 dpm.

[c]Amount of incorporated ^{3}H label (dpm) per 5 μg muramic acid.

[d]Autolytic activity as the percentage released of the amount of peptidoglycan added to the assay.

As the aforementioned experiments showed that substrate susceptibility did not vary, the peptidoglycan of strain 5558 was used in all subsequent tests for determining autolytic activity.

Evaluation of enzyme activity

To permit a proper comparison of the autolytic activity of the logarithmic cultures of a tolerant (194) and nontolerant (5558) strain, the enzyme activity of their cell-bound and extracellular fractions was first measured without the addition of antibiotic. It was found that the cell-bound activity remained at a relatively low and constant level, whereas the activity in the culture medium increased sharply as the culture became stationary (Fig. 1).

Decrease of optical density of bacteria exposed to a β-lactam antibiotic is the consequence of combined intracellular and extracellular

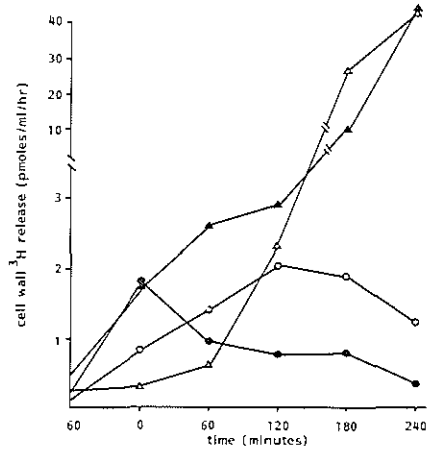

Figure 1: Autolytic activity in various fractions during growth of a tolerant (194, closed symbols) and a nontolerant (5558, open symbols) S. aureus strain. Samples (3 ml) were removed at 60 min. intervals from the cultures.
Symbols indicate extracellular activity (△,▲) as detected in culture medium and cell-bound activity (○,●), expressed as culture yield per ml medium.

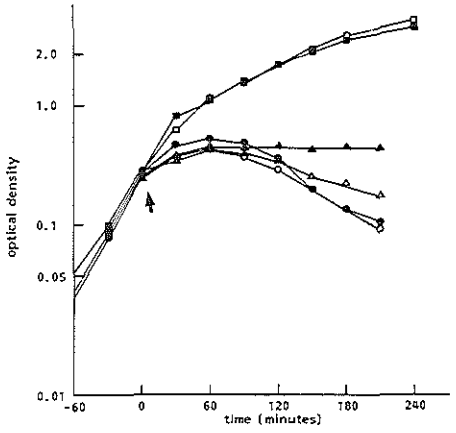

Figure 2: Lysis of a tolerant (194, closed symbols) and a nontolerant (5558, open symbols) S. aureus strain, after exposure to methicillin. Methicillin (10 μg/ml (○,●) and 80 μg/ml (△,▲)) was added to rapidly growing cells in Mueller-Hinton broth, at time indicated by arrow. The culture turbidity was followed spectrophotometrically (Vitatron) at suitable intervals; (□,■) growth control.

autolytic activity. Therefore the course of the optical densities was initially registered in a tolerant and a nontolerant strain exposed to a low and a high concentration of methicillin. All cultures except the tolerant strain exposed to 80 μg/ml of the antibiotic showed a decrease of optical density from 90 min. onwards (Fig. 2).

The addition of 10 μg/ml of methicillin produced an identical rise in cell-bound autolytic activity in the two S. aureus strains. After the addition of 80 μg/ml of methicillin the tolerant strain 194 showed no increase in cell-bound autolytic activity, whereas such an increase was clearly observed in the nontolerant strain 5558 (Fig. 3). Cell-

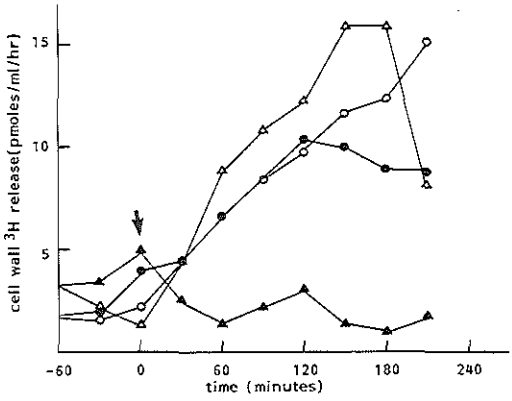

Figure 3: Cell-bound autolytic activity of a tolerant (194, closed symbols) and a non-tolerant (5558, open symbols) S. aureus strain, after exposure to methicillin. Methicillin (10 μg/ml (○, ●) and 80 μg/ml (△, ▲)) was added at time indicated by arrow. Samples (3 ml) were removed at 30 min. intervals and assayed for cell-bound autolytic activity.
Cell-bound activity (freeze-thaw extract) was expressed as activity per optical density unit.

bound autolytic activity corrected for the number of colony forming units gave the same picture. Similar results were obtained when the experiment was repeated or when the autolysins of the cells were extracted with 3 M LiCl.

The course of the extracellular activity of the enzymes clearly differed from the course of the activities observed in the cell-bound

fractions. For both strains autolytic activities ran parallel after addition of the antibiotic with the proviso that from 90 min. onwards 80 μg/ml of methicillin caused lower levels of enzyme activity than 10 μg/ml (Fig. 4).

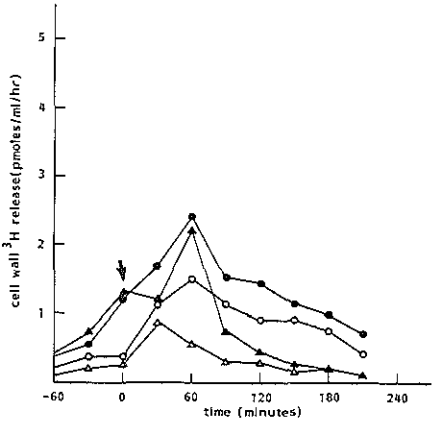

Figure 4: Extracellular autolytic activity of a tolerant (194, closed symbols and a non-tolerant (5558, open symbols) S. aureus strain after exposure to methicillin. Methicillin (10 μg/ml (O,●) and 80 μg/ml (△,▲)) was added at time indicated by arrow. Samples (3 ml) were removed at 30 min. intervals and assayed for extracellular autolytic activity. Extracellular activity was expressed as activity per ml medium.

Inhibition of autolytic activity by fractions from the culture medium of a tolerant and a nontolerant strain

Following the work of Raynor et al. (10) and Sabath et al. (12) an explanation of the low cell-bound autolytic activity in the tolerant strain (194) after the addition of a high antibiotic concentration was sought in a factor having an inhibiting effect on autolytic activity. This was investigated by adding cellular or extracellular samples which might contain an inhibiting factor to a known quantity of autolysin. If the fraction contained an inhibitor, autolytic activity would be suppressed. In the absence of an inhibitor the activity as finally determined would be equal to the expected activity. The application of this procedure by adding cell-bound fractions of the tolerant strain (194), which had been exposed to 80 μg/ml of methicillin for between 0 and 210 min., gave no indication of the presence of

a cell-bound inhibitor. However, autolytic-inhibiting activity was demonstrable from 90 min. onwards in extracellular fractions of cells of both strains that had been exposed to 80 μg/ml of methicillin for between 0 and 210 min. (Fig. 5). The inhibitor was not dialyzable, but sensitive to heating for 30 min. at 100°C (see Fig. 6).

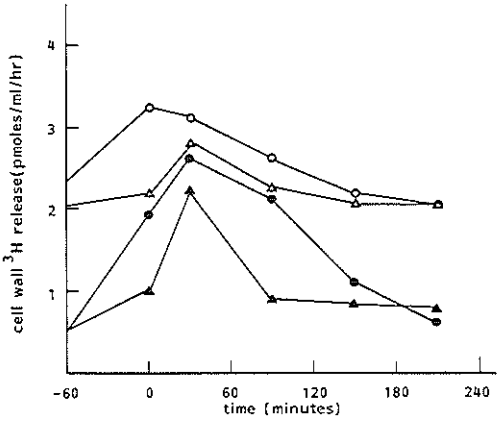

Figure 5: Inhibition of autolytic activity in the culture medium of a tolerant (strain 194, ○ ,●) and a nontolerant (strain 5558, △ ,▲) S. aureus strain after exposure (at time zero) to 80 μg/ml methicillin. Samples of the culture medium of strain 194 and 5558 were mixed with autolysin with an activity of 12,500 dpm per hour (equivalent to 2 units). The mixtures were incubated with ^{3}H labelled peptidoglycan for 1 h and peptidoglycan hydrolysis was determined at regular intervals. The figure shows release of ^{3}H label that might be expected if activities were additive (open symbols) and counts that were actually reached (closed symbols).

DISCUSSION

Eagle & Musselman (2) demonstrated that S. aureus cells usually show a better survival after exposure to a high concentration of penicillin than to a low concentration.

In earlier studies (3,4) we found that the survival rate of a collection of strains of S. aureus exposed for 24 h to high concentrations of methicillin varied from 0.01% to 12% of the inoculum. Strains in which 2% or more of the cells survived under these conditions were

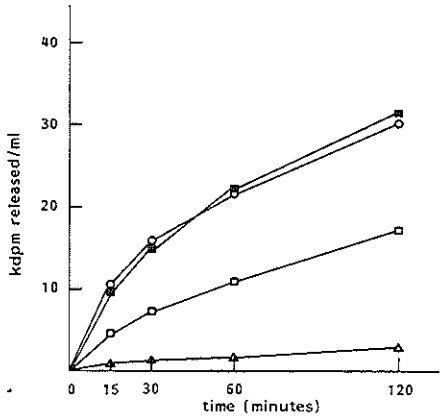

Figure 6: Crude material secreted during methicillin-treatment was used for characterization of the autolysin inhibitor. The reaction mixtures (total volume 1020 μl) contained: 20 μl ^{3}H labelled peptidoglycan substrate (equivalent to 2.5 μg of muramic acid and 10^{5} dpm); 100 μl of buffer (potassium phosphate 0.1 M; pH 7.0) and 100 μl of $MgCl_2$ (0.56 M). To four tubes the following additions were made: To tube 1 100 μl of crude autolysin + 700 μl aqua dest were added (○); To tube 2 100 μl aqua dest + 700 μl of crude secreted material were added (△); To tube 3 100 μl crude autolysin + 700 μl of crude secreted material were added (□); To tube 4 100 μl crude autolysin + 700 μl of crude secreted material heated for 30 min. at 100°C were added (■). The tubes were incubated at 37°C, and the degree of peptidoglycan hydrolysis was determined at the time indicated.

defined by us as tolerant. As our tolerant strains remain, however, extremely susceptible to low concentrations of methicillin they seem to be ideally suited to study autolytic activity at bactericidal and bacteriostatic concentrations.

In the present study we first demonstrated that there was no difference in wall solubilization from tolerant and nontolerant strains, even when these strains had been exposed to low or high concentrations of methicillin. Therefore, the mechanism of the tolerance phenomenon is evidently not based on a difference in susceptibility of the substrates to the autolysins.

Tolerant and nontolerant strains exposed to high concentrations of methicillin clearly differed in autolytic activity of their cell-bound fraction. At this concentration cell-bound autolytic activity increas-

ed in the nontolerant strain whereas such an increase was lacking in the tolerant strain. At low methicillin concentration cell-bound autolytic activity increased in both types of strains.

Best et al. (1) and Sabath et al. (12) found similar results in tolerant S. aureus bacteria exposed to high concentrations of oxacillin and nafcillin, respectively. They did not distinguish cell-bound and extracellular fractions and did not expose their cells to low concentrations of the aforementioned antibiotics.

The constant level of cell-bound autolytic activity in the tolerant strain exposed to a high concentration of methicillin may have several causes. One possibility is that the production of autolysins remains unchanged or is even decreased. The other possibility is that the production of autolysins is increased as usual but that the enzymes cannot be extracted as a consequence of a decreased permeability of the cell wall. As peptidoglycan synthesis of a tolerant and a nontolerant strain exposed to a low or high concentration of methicillin is equally inhibited (unpublished observations W.H.F. Goessens), one may assume that the permeability of the cell walls of both strains for autolysins is identical. Our tentative conclusion at this stage would be that the low level of autolysin observed in a tolerant strain exposed to a high concentration of methicillin has to be ascribed to a low or decreased production of these enzymes.

Extracellular activity appeared to be decreased 90 min. after exposure of both strains to a high concentration of methicillin, as compared to the activity after exposure to a low concentration of the antibiotic. This phenomenon is most easily explained by assuming that with high concentrations an inhibitor is released in the culture medium. A similar explanation has been proposed by Raynor et al. (10). Sabath et al. (12) extended these observations by demonstrating the presence of an inhibitor of autolysins in cell-bound fractions of tolerant S. aureus strains. Although we used the same extraction procedure we were unable to demonstrate a cell-bound inhibitor. If, however, inhibitory activity is looked for in the culture medium such activity was clearly demonstrable in both types of strains exposed to 80 μg/ml

of methicillin. Waks et al. (16) suggested that the release of such inhibitors from the bacterium activated the cell-bound autolysins. According to our observations, however, exposure of the tolerant S. aureus strain to a high methicillin concentration did not give rise to activation of the cell-bound autolysins, despite the presence of an inhibitor of the autolysins in the culture medium. We therefore assume that tolerance in S. aureus is the result of other mechanisms capable of regulating autolytic activity.

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CHAPTER 6

INHIBITION OF RNA SYNTHESIS IN TOLERANT *STAPHYLOCOCCUS AUREUS* STRAINS EXPOSED TO A HIGH CONCENTRATION OF METHICILLIN*

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Exposure of tolerant and nontolerant *Staphylococcus aureus* strains to 10 μg of methicillin resulted in inhibition of peptidoglycan synthesis. The rates of RNA and protein synthesis were found not to be affected by this concentration. In contrast, exposure of tolerant strains to 80 μg of methicillin resulted in inhibition of both peptidoglycan and RNA synthesis. Sixty minutes after exposure to 80 μg of methicillin the rate of RNA synthesis was reduced by 70-90%. At this time the rate of protein synthesis only started to decrease.
In a nontolerant strain the addition of an inhibitor of RNA synthesis (actinomycin D; 0.5 μg/ml) simultaneously with a low concentration of methicillin led to phenotypic tolerance. Thus, for this strain too, methicillin was no longer bactericidal. We assume, therefore, that tolerance in *S. aureus* is caused by inhibition of RNA synthesis when exposed to high concentrations of methicillin.

INTRODUCTION

Penicillin has a bactericidal action on actively dividing bacteria. If protein synthesis of bacteria exposed to penicillin is inhibited, for example by the addition of chloramphenicol or the omission of an essential amino acid from the medium, the bactericidal action of penicillin changes into bacteriostasis (6). Rogers (10) and Shockman (11)

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found with S. aureus and Streptococcus faecalis respectively that inhibition of protein synthesis by chloramphenicol was accompanied by a sharp decline in the autolytic activity of the cells. These results supported their hypothesis that bacteria are lysed under the influence of penicillin because the equilibrium between peptidoglycan synthesis and autolysis shifts in the direction of the latter process as a result of the action of the β-lactam antibiotic. The correctness of the hypothesis of Rogers and Shockman was supported by the work of Tomasz et al. (13), who isolated an autolysin-deficient mutant of Streptococcus pneumoniae which was not killed after exposure to penicillin. Tomasz et al. (13) called the absence of the bactericidal action of penicillin, tolerance for this agent. Since then, tolerant strains belonging to various species of bacteria have been isolated from clinical material. In previous studies (3,4) we showed that S. aureus strains can be divided into tolerant and nontolerant strains on the basis of their survival in vitro in the presence of high concentrations of methicillin ($\geqslant 64$ μg/ml). According to the observations of Rogers (10), the mechanism of the reduced lysis in tolerant S. aureus strains exposed to high concentrations of methicillin could be based on inhibition of protein synthesis. The existence of such a link has already been demonstrated by Mychajlonka et al. (8) for tolerant Streptococcus mutans strains. In the present study we investigated peptidoglycan, RNA and protein synthesis in two tolerant and two nontolerant S. aureus strains after exposure to a low and a high concentration of methicillin.

MATERIALS AND METHODS

Bacteria

Two tolerant (5407, 340) and two nontolerant (5558, 3401) S. aureus strains were studied. The minimal inhibitory concentration of methicillin for each of the four strains was $\leqslant 2$ μg/ml. The survival percentages after exposure for 24 h to high concentrations of methi-

cillin (tolerance percentages) were 5.7% and 2% for strains 5407 and 340 and 0.09% and 0.03% for strains 5558 and 3401 (4).

Growth conditions

S. aureus cells were grown overnight in 30 ml of Mueller-Hinton broth (Difco). After incubation the cells were washed with physiological saline, resuspended in the same volume and diluted 10^{-3}. Logarithmically growing cultures for the determination of the synthesis rate of peptidoglycan, RNA and protein were obtained by adding 100 µl of the washed and diluted cells to 100 ml of preheated semisynthetic medium. The semisynthetic medium (pH 7.3) consisted of 500 mg of yeast extract (Difco) supplemented with 1 mg of cysteine, 5 mg of glycine, 15 mg of thiamin, 5 mg of nicotinic acid, 15 mg of pyridoxine, 1 mg of pantothenic acid, 0.5 mg of riboflavin, 0.5 µg of biotin, 40 µg of folic acid, 5 g of glucose, 3 g of K_2HPO_4, 0.5 g of KH_2PO_4, 0.1 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4$, 5 g of NaCl, 1 mg of $FeSO_4$ and 0.5 mg of $MnCl_2$ per litre. All chemicals were of analytical grade. Cells were incubated for 4½ h on a shaking device (37°C) until they reached a density of 0.4 (A_{560}). Ten and 80 µg of methicillin per ml were then added to the cultures (at t=0 h).

Determination of the synthesis rate of peptidoglycan, RNA and protein

The effect of various concentrations of methicillin on the rates of synthesis of peptidoglycan, RNA and protein in tolerant and non-tolerant S. aureus strains was measured by the pulse-labelling technique. At 20-minute intervals, a series of 0.5 ml samples of cultures taken from 1 h before to 2½ h after the addition of various concentrations of methicillin were mixed with 50 µl of preheated medium containing either 1 µCi of [^{3}H]-N-acetylglucosamine (0.5 µg/0.5 ml, 442 mCi/mmol; for peptidoglycan), 1 µCi of [^{3}H]-uracil (1 µg/0.5 ml, 115 Ci/mmol; for RNA) or 1 µCi of [^{14}C]-leucine (5 µg/0.5 ml, 28.4 Ci/mmol; for protein). After exactly 15 min. at 37°C, 0.5 ml of ice-cold phosphate buffered saline containing 1 mg each of cold N-acetylglucosamine, uracil and leucine per ml was added, and the

tubes were placed in an ice bath. For determinations of RNA and protein 0.5 ml of the chilled samples was added to 0.5 ml of an 18 h culture (carrier) and centrifuged for 5 min. at 10,000 g. The pellet was suspended in 1 ml of preheated phosphate buffered saline, 2 μg of lysostaphin (Schwarzmann) was added and the mixture was incubated for 30 min. at 37°C. After this digestion step RNA samples were added to 10% ice-cold trichloro acetic acid (TCA) and the precipitate was collected on glass fiber filters (Whatman GF/F). Protein samples were mixed with 1 ml of 20% ice-cold TCA and the precipitate was exposed to 95°C for 30 min. and collected on glass fiber filters. Filter precipitates of RNA or protein were washed three times with 5 ml of ice-cold 10% TCA and twice with 5 ml of 75% ethanol and, finally, the filters were dried. Peptidoglycan was isolated according to the method of Park and Hancock (9) as modified by Wilkinson and White (14). Chilled samples were added to 1 ml of an 18 h culture (carrier) and subsequently washed three times with distilled water. The pellet was then suspended in 5% cold TCA. After 18 h at 4°C samples were centrifuged (10 min. at 10,000 g) and pellets were suspended in 75% ethanol ($^{v}/_{v}$) for 10 min. at room temperature. After this extraction step pellets were heated for 6 min. at 95°C in 5% TCA. After being washed once in distilled water, cells were incubated for 2 h at 37°C in Tris buffer (0.02 M, pH 7.9) containing trypsin (1 mg/ml). Pellets were subsequently washed three times with distilled water and finally suspended in 200 μl of water. For the determination of radioactivity 100 μl of the samples was added to counting vials.

Determination of radioactivity

Precipitates dried on glass fiber filters were solubilized by treatment with 1 ml of soluene-350 (Packard) for 2 h at 56°C. Solubilization was followed by the addition of 10 ml instagel (Packard). The radioactivity was determined by means of an Isocap scintillation counter. Corrections were made for quenching and the results were expressed as desintegrations per minute (dpm).

Killing curves

For viability studies samples (0.5 ml) were taken every 20 minutes. They were carried through serial 10-fold dilutions in sterile saline, and 100 μl of each dilution was spread on nutrient agar plates containing β-lactamase (0.15 U of β-lactamase I and 0.015 U of β-lactamase II per ml. Whatman Biochemicals Ltd.). After incubation for 48 h, CFU counts were made.

RESULTS

Tuning of synthesis rate and number of CFU

To determine the synthesis rate of peptidoglycan, RNA and protein, logarithmically growing cells were exposed for 15 min. to radioactive precursors. The radioactivity incorporated in this time interval depends on the number of viable cells and the synthesis rate of the macromolecule in question. If the synthesis rate remains constant the radioactivity measured will be in direct proportion to the number of viable cells. The synthesis rate can be determined by measuring the radioactivity incorporated and the number of viable cells at fixed times. In general the number of viable cells will diminish in the presence of methicillin. The changing number of viable cells, during the pulse label period, was dealt with by taking the samples for the viable counts 10 min. after the pulse label samples. The result of this count represents a mean value for the number of viable cells during the pulse label interval.

Optimal digestion time using lysostaphin

As the cell wall of S. aureus was found to be insensitive to 10% TCA, the cells were treated with lysostaphin, to allow the isolation of labelled RNA and protein. If the digestion time is too long the endopeptidase (5) activity of lysostaphin will lead to the breakdown of labelled protein. Control experiments showed that the amount of labelled RNA and protein decreased 1 h after exposure to lysostaphin.

As it was shown that free label was rapidly (i.e. within 15 min.) released after exposure to lysostaphin the digestion time was set at 30 min.

Rates of synthesis of macromolecules exposed to methicillin

The influence of a low and a high concentration of methicillin on the synthesis rate of the macromolecules was first studied in two susceptible strains. The results obtained with strain 5558 and 3401 are shown in Fig. 1. The addition of 10 and 80 μg of methicillin was found to have the greatest influence on the rate of peptidoglycan synthesis. The radioactivity incorporated decreased sharply over time, the decrease being more rapid with the high concentration of methicillin (Fig. 1C, 1F). Protein and RNA synthesis rates also seemed to be inhibited at the two methicillin concentrations. This apparent inhibition of rates of protein and RNA synthesis was the result of the decline in the number of viable cells in the presence of methicillin. When the amount of radioactivity was corrected for the number of viable cells it was found in these nontolerant strains, that peptidoglycan synthesis was in effect inhibited whereas RNA and protein were not. After correction, strain 5558 even showed an increase in the rate of RNA and protein synthesis when exposed to 80 μg of methicillin. This was ascribed to the fact that the cells of strain 5558 were still able to produce protein and RNA, but were not any longer capable of dividing.

The two tolerant strains, 340 and 5407, exhibited a markedly lower killing rate than the two susceptible strains when exposed to high concentrations of methicillin (Fig. 2A and 2D). Nevertheless, inhibition of peptidoglycan synthesis, in tolerant strains did not differ from the susceptible strains. Also notable in the case of strains 340 and 5407 was that when exposed to 80 μg of methicillin per ml the decline in the rate of RNA synthesis did not parallel the decline in the number of cells. After correction for the number of viable cells the RNA synthesis rate in these strains showed a 70% and 90% inhibition as compared with the controls without addition of

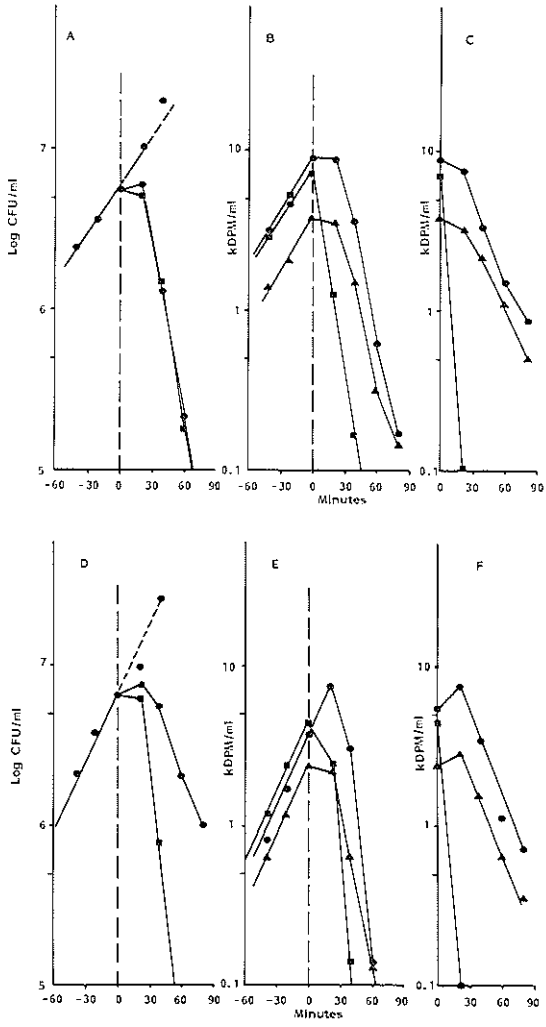

Figure 1: Rates of synthesis of peptidoglycan (■), RNA (●), and protein (▲) as measured by pulse-labelling techniques before and after addition (at time zero) of two different concentrations, 10 μg/ml (Figures B and E) and 80 μg/ml (Figures C and F) of methicillin to logarithmic growing cultures of S. aureus strains no. 5558 (Figures A-C) and no. 3401 (Figures D-F).

Figures A and D represent killing curves of the strains exposed to 10 (■) and 80 μg/ml (●) of methicillin.

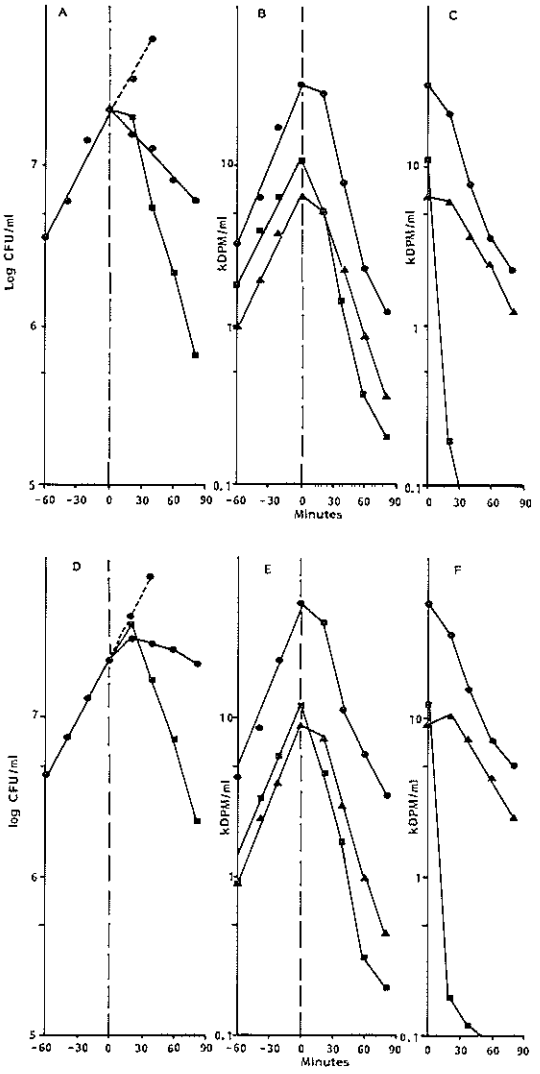

Figure 2: Rates of synthesis of peptidoglycan (■), RNA (●), and protein (▲) as measured by pulse-labelling techniques before and after addition (at time zero) of two different concentrations, 10 μg/ml (Figures B and E) and 80 μg/ml (Figures C and F) of methicillin to logarithmic growing cultures of S. aureus strains no. 340 (Figures A-C) and no. 5407 (Figures D-F).

Figures A and D represent killing curves of the strains exposed to 10 (■) and 80 μg/ml (●) of methicillin.

methicillin. The rate of protein synthesis also declined, but the decline did not begin until 60 min. after exposure to 80 µg/ml of methicillin.

As an example the corrected data for a nontolerant (3401) and a tolerant strain (340) are shown in Fig. 3.

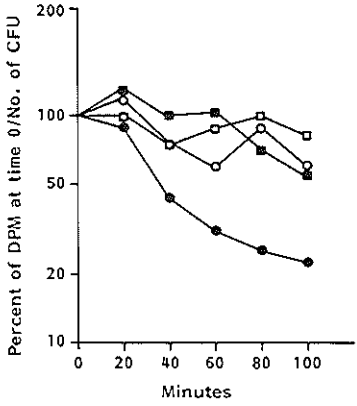

Figure 3: Rates of synthesis (dpm per 0,5 ml per 15 min) of RNA (○, ●) and protein (□, ■) after addition of 80 µg/ml of methicillin (at time zero) to strain 3401 (open symbols) and strain 340 (closed symbols). Synthetic rates at different times after methicillin was added were corrected for the number of viable cells and expressed as the percentage of synthetic rates at time zero.

Induction of phenotypic tolerance through the addition of actinomycin D

The inhibitory action of methicillin on RNA synthesis in tolerant strains prompted further investigation into the role of RNA synthesis in the bactericidal action of methicillin. For this purpose an RNA synthesis inhibitor, actinomycin D (0.5 µg/ml), was added at different times to logarithmic growing cultures of a susceptible (3401) and a tolerant (5407) strain exposed to 6 µg of methicillin per ml. In both strains the addition of actinomycin D at t=0 and t=15 resulted in a sharp reduction in the bactericidal action of methicillin. Addition after 30 and 45 min. still led to reduced bactericidal action

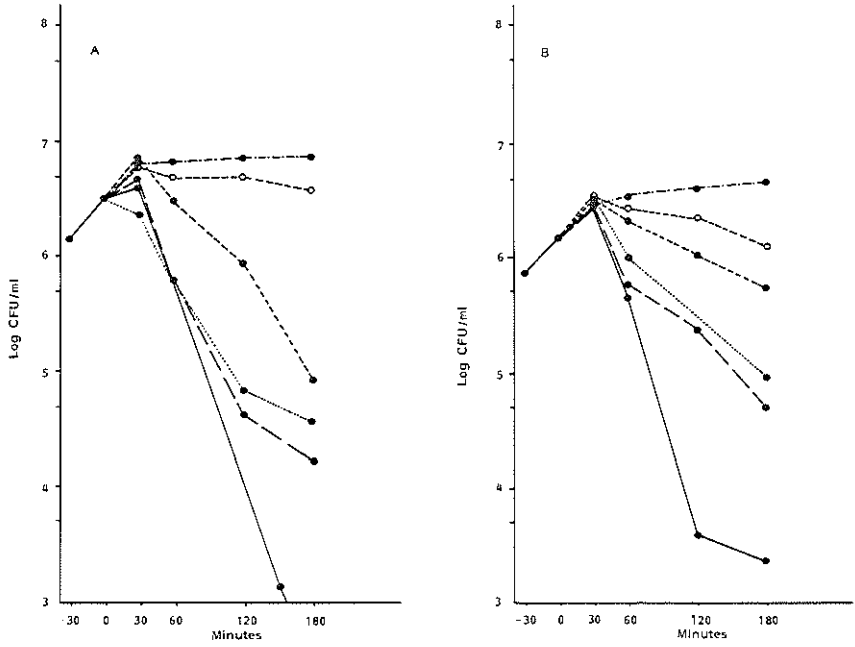

Figure 4: Killing curves of a nontolerant strain (3401, Figure A) and a tolerant strain (5407, Figure B) of S. aureus at 6 μg/ml (●—●) of methicillin with addition of 0.5 μg/ml of actinomycin D at different time intervals (T_0,O----O), (T_{15},●----●), (T_{30},●----●), (T_{45},●—●) and (●---●, actinomycin D without methicillin).

(Fig. 4). When measured 30 min. after exposure to 0.5 μg of actinomycin D per ml, the rates of RNA and protein synthesis were, respectively, 30% and 70% of the control.

DISCUSSION

The most important effect of a β-lactam antibiotic is inhibition of peptidoglycan synthesis (7,12,15). Both the tolerant and the nontolerant strains exhibited an identical concentration-dependent inhibition of peptidoglycan synthesis after exposure to a low and a high concentration of methicillin. Exposure of the two types of

strains to a low concentration of methicillin had no effect on the rates of RNA and protein synthesis. Exposure of the tolerant strains to a high concentration of methicillin resulted in inhibition of RNA synthesis. At this concentration the rate of protein synthesis was also negatively affected but only after a certain delay, suggesting that inhibition of protein synthesis is a secondary effect. The role of RNA synthesis in the mechanism of bactericidal action of methicillin was confirmed in the experiments in which RNA synthesis was selectively inhibited by actinomycin D. In both the nontolerant and the tolerant strains the simultaneous administration of methicillin and actinomycin D resulted in bacteriostasis. In nontolerant S.aureus strains inhibition of RNA synthesis leads to phenotypic tolerance. This lower killing rate is probably not caused by inhibition of protein synthesis by actinomycin D, because protein synthesis is only 30% inhibited.

Mychajlonka et al. (8) reported that inhibition of RNA or protein synthesis, or both, are involved in the tolerant reaction of Str. mutans strains upon exposure to penicillin.

The absence of bactericidal activity of high concentrations of methicillin in tolerant S. aureus strains could be the result of an apparent coupling of cell lysis to RNA synthesis. This is consistent with our finding that at concentrations of methicillin, which do not inhibit RNA synthesis (10 μg/ml), lysis and killing take place.

A possible cause of inhibition of RNA synthesis is that a high concentration of methicillin may lead to magic spot formation (1,2) or binds directly to enzymes which are involved in the regulation of RNA synthesis. Bacteriostasis produced in nontolerant and tolerant strains by simultaneous exposure to low concentrations of methicillin and actinomycin D bears a certain resemblance to the results obtained by Jawetz et al. (6), Rogers (10) and Shockman (11). They eliminated the bactericidal effect of penicillin by directly blocking protein synthesis. Apparently the order in which RNA and protein synthesis are inhibited is not important to bring about phenotypic tolerance; as long as the condition is satisfied that a particular product, presuma-

bly a protein is not formed. An indication that a constant supply of a particular product is necessary for the bactericidal action of the antibiotic is provided by the experiment in which actinomycin D was added 30 and 45 min. after the β-lactam antibiotic. Even at this time point the drug was still capable of slowing down the killing rate. Interrupting the supply of such a product formed during the first 20 min. after exposure to methicillin does not lead to poorer killing.

ACKNOWLEDGEMENTS

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GENERAL DISCUSSION

The object of this study was to determine the prevalence, the in vivo significance and the mechanism of tolerance in Staphylococcus aureus to β-lactam antibiotics, such as methicillin and cloxacillin. The success of the study depended on the development of a reliable method for the detection of tolerance in this species of bacteria.

In Chapter II the factors were studied which can exert a positive or a negative influence on the detection of tolerance. Tolerance in staphylococci was defined arbitrarily by Sabath et al. (9) as an MBC/MIC ratio equal to or greater than 32. Using this definition, we endeavoured to detect tolerant S. aureus strains. A systematic investigation of the factors which could influence the MBC/MIC ratio found, showed that it was influenced most by carry-over of the antibiotic from the culture tube to the culture plate. Whith higher concentrations of antibiotic it was actually necessary to add β-lactamase to the culture plates to break down the residual antibiotic. When this modification was applied it was found that in a large proportion of the 15 strains tested the percentage of surviving bacteria as determined after 24 h was greater than 0.1% of the inoculum. The MBC, however, is defined as the concentration at which 99.9% of the original inoculum is killed (1). In the case, therefore, of strains which have a higher survival rate after exposure to methicillin or cloxacillin, it is no longer possible to give an MBC value for these antibiotics and, hence, no longer possible to use the MBC/MIC ratio as the measure for tolerance. As a result of addition of β-lactamase to the culture plates it was further found that for each strain the value of the surviving fraction stabilized once a certain concentration of the antibiotic had been reached. Repetition of the experiment showed the percentage of surviving cells to be reproducible. We were therefore able to conclude Chapter II with the formulation of a new characteristic of S. aureus bacteria which we call the tolerance percentage. The tolerance percentage of a strain is the number of bacteria which survive after 24 h when the strain is

exposed to a high concentration of a β-lactam antibiotic ($\geqslant$ 64 μg of methicillin per ml or $\geqslant$ 25 μg of cloxacillin per ml). As it had been found in Chapter II that, in addition to strains with a lot of surviving bacteria, there were also strains with few survivors, in Chapter III the distribution of these tolerance percentages was investigated in three collections of strains. In the collection consisting of 64 strains isolated from positive blood cultures in 1982, 87.5% of the strains were found to have tolerance percentages for methicillin of 1 or less. The remaining strains had survival percentages of 2 or more. Similar results were obtained when the strains were tested using cloxacillin, nafcillin, cephalothin and penicillin. In other words, strains with a high tolerance percentage for methicillin behave in the same way with other β-lactam antibiotics. A similar distribution of tolerance percentages was also observed in two collections, each consisting of 30 strains, isolated in the years 1951-1953 and 1957-1958 respectively. In view of the distribution of the tolerance percentages observed for these three collections, the borderline between tolerant and nontolerant strains was put arbitrarily at a tolerance percentage of 2%.

A phenomenon which is exhibited by all strains and must therefore be discussed is the Eagle effect, or, as it is sometimes referred to, the paradoxical zone phenomenon (5). The paradoxical nature of this phenomenon lies in the fact that strains are killed faster in the presence of a low concentration of a β-lactam antibiotic (2-4 times the MIC) than with a high concentration (16-32 times the MIC). The Eagle effect prompted us to investigate the course of lysis and killing in strains with high and low tolerance percentages exposed to low and high concentrations of methicillin. The most notable finding in this study was that strains with high tolerance percentages exhibit delayed lysis and killing after exposure to a high concentration of methicillin. Delayed lysis and killing when exposed to a high concentration of a β-lactam antibiotic is an essential characteristic of the tolerance phenomenon.

In Chapter IV it was investigated by means of an experimental infec-

tion whether, in vivo, tolerance requires adaptation of the dosaging during antimicrobial therapy. For this purpose, 5 strains with high and 5 strains with very low tolerance percentages were compared in a simple thigh muscle model in mice. After 1 and 2 injections respectively with 2.5 mg of methicillin per mouse the number of CFUs isolated from the thigh muscle 2 hours after the antibiotic injections was found to be significantly higher in the case of the tolerant strains than in that of the nontolerant strains. Within the limitations of the model, therefore, a difference was also found to exist in vivo between killing of tolerant and nontolerant strains.

Sabath et al. (9) and Best et al. (2) assumed that the delayed lysis of tolerant strains might be due to a defect in the bacterial cell's autolytic system. In Chapter V, therefore, we measured the cell-bound and extracellular activity of the autolytic enzymes of tolerant and nontolerant strains after exposure to low and high concentrations of methicillin. As regards cell-bound activity, nontolerant and tolerant strains differed from each other in that exposure to high concentrations of methicillin led to an increase in enzyme activity in the former but not in the latter. Under the same conditions, extracellular autolytic activity was found to be reduced in both types of strains. This low extracellular autolytic activity is the result of the presence of an inhibitor of the autolysins. Raynor et al. (7) put forward the presence of this inhibitor as the explanation of the low cell-bound autolytic activity in tolerant strains. However, as we could demonstrate the presence of this inhibitor in the nontolerant strains as well, we believe that tolerance in S. aureus can not be due solely to inhibition of autolysins. This raises the question what, then, is the reason that cell-bound autolytic activity does not increase in tolerant strains after exposure to high concentrations of methicillin. This question was studied in Chapter VI. Our starting-point in attempting to solve the problem were the results of the studies of Jawetz et al., Rogers and Shockman (6,8,10) who observed that in S. aureus and streptococcal species penicillin ceases to be a bactericidal agent and becomes a bacteriostatic agent when protein

synthesis is inhibited by the addition of chloramphenicol or by the omission of an essential amino acid from the medium. This led us to assume that, in addition to inhibiting peptidoglycan synthesis, high concentrations of methicillin lead to the inhibition of protein synthesis in tolerant strains. The inhibition of protein synthesis might in turn be the cause of a decline in cell-bound autolytic activity. To test this hypothesis we measured the rates of protein, RNA and peptidoglycan synthesis in tolerant and nontolerant strains exposed to low and high concentrations of methicillin. It was found that in tolerant strains, aside from inhibiting peptidoglycan synthesis, a high concentration of methicillin also brought about a strong inhibition of RNA synthesis. Apparently the order in which RNA and protein synthesis are inhibited is not important to bring about tolerance, as long as the condition is satisfied that a particular product, presumably a protein is not formed. A possible cause of inhibition of RNA synthesis is that a high concentration of methicillin, may lead to magic spot formation (3,4) or may bind directly to enzymes which are involved in the regulation of RNA synthesis.

The importance of RNA synthesis for the mode of action of β-lactams was confirmed in the experiments in which RNA synthesis was inhibited by the addition of low concentrations of actinomycin D at different points of time after exposure to a low concentration of methicillin. The simultaneous administration of actinomycin D and methicillin prevented normal killing by the latter agent. The administration of actinomycin D, 45 min. after methicillin, still led to delayed killing by methicillin. Our conclusion concerning the mechanism of tolerance to β-lactam antibiotics in S. aureus bacteria is therefore that the phenomenon is ultimately a direct or indirect result of the inhibition of RNA synthesis by high concentrations of the antibiotic.

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SUMMARY

Tolerant strains of Staphylococcus aureus are characterized by a normal MIC value but a markedly increased MBC value for β-lactams or vancomycin. From data described in the literature it appears that the extent of tolerance is clearly dependent on the method used for the detection of the phenomenon. In this study we described a reproducible method for the estimation of the level of tolerance. According to this procedure a certain inoculum of the strain to be studied is exposed during 24 h to high concentrations of a β-lactam antibiotic. Thereupon the number of surviving bacteria is determined and expressed as percentage of the initial inoculum. By this technique, it appears that for each strain the value of the surviving fraction stabilized once a certain concentration of the antibiotic is reached. This percentage is called the tolerance percentage of a strain. In view of the distribution of tolerance percentages observed in a blood culture collection of 64 S. aureus strains, isolated in 1982, a survival rate of 2 % was arbitrarily chosen as the breakpoint between nontolerant and tolerant strains.

In accordance with this finding the response to antimicrobial treatment of S. aureus strains with tolerance percentages of $\leqslant 0.1$ and $\geqslant 2.0$ was compared in an experimental thigh infection model in mice. Two injections of methicillin (2.5 mg per mouse) were given with an interval of 2 h starting 1 h after inoculation of the strain in the thigh. At the end of treatment, the number of CFUs in the thigh muscles infected with the tolerant strains was found to be significantly higher than that in the thigh muscles infected with the nontolerant strains.

In an other series of experiments we investigated the autolytic activity of the cell-bound and extracellular fractions of a tolerant and nontolerant strain exposed to a high (80 μg/ml) or low (10 μg/ml) concentration of methicillin. When a nontolerant strain is exposed to a low or high concentration of methicillin autolytic activity of the cell-bound fractions increases with time. The tolerant strain on the

other hand, electively showed a reduced cell-bound autolytic activity after exposure to a high concentration (80 μg/ml) of methicillin. No difference in extracellular autolytic activity was found between the nontolerant and tolerant strain after exposure to different concentrations of methicillin. However, in both types of strains extracellular activity was less after exposure to a high concentration of methicillin than after exposure to a low concentration. This phenomenon was caused by the release of an inhibitor of the autolysins under the influence of the high concentration of methicillin. This inhibitor appeared to be thermolabile and not dialyzable.

As a decreased autolytic activity in tolerant strains exposed to a high concentration of methicillin might be the consequence of an inhibition of the synthesis of macromolecules, peptidoglycan, RNA and protein synthesis were investigated in a tolerant and nontolerant strain. Using a pulse label technique it was found that RNA synthesis is selectively inhibited in a tolerant strain exposed to a high concentration of methicillin.

Our conclusion is that the mechanism of tolerance of S. aureus bacteria to β-lactams is based, direct or indirect, on inhibition of the RNA synthesis after exposure of the bacteria to high concentrations of the antibiotic.

SAMENVATTING

Tolerante Staphylococcus aureus stammen worden gekenmerkt door het bezit van normale MIC waarden doch sterk verhoogde MBC waarden voor β-lactam antibiotica of vancomycine.
Uit literatuurgegevens bleek dat de mate van tolerantie afhankelijk is van de gebruikte detectiemethode van het fenomeen. In de huidige studie werd een reproduceerbare methode beschreven voor het bepalen van de mate van tolerantie. In deze methode wordt een bepaald inoculum van de te bestuderen stam gedurende 24 uur blootgesteld aan hoge concentraties van een β-lactam antibioticum. Hierna wordt het restant aan levende bacteriën bepaald en omgerekend in procenten van het initieel inoculum. Bij gebruikmaking van deze techniek bleek dat van elke stam een reproduceerbaar aantal bacteriën vanaf een bepaalde antibioticumconcentratie overleeft. Dit percentage overlevende bacteriën werd door ons het tolerantiepercentage van een stam genoemd. De verdeling van de tolerantiepercentages in een collectie stammen, geïsoleerd uit positieve bloedculturen in 1982, deed ons besluiten, op arbitraire gronden een tolerantiepercentage van 2 te hanteren als grens tussen tolerante en nontolerante stammen.
Uitgaande van dit gegeven werd de response van stammen met een tolerantiepercentage $\geqslant 2$ vergeleken met de response van stammen met een tolerantiepercentage $\leqslant 0{,}1$ op antimicrobiële therapie in het dijbeenspiermodel bij de muis. De muizen werden behandeld met 1 of 2 doses methicilline (2,5 mg/muis), toegediend met een interval van 2 uur. De therapie werd 1 uur na inoculatie van de stammen in de dijbeenspier gestart. Na de behandeling, bleek het restant aan CFU in de dijbeenspier geïnfecteerd met de tolerante stammen significant hoger dan dat in de dijbeenspieren geïnfecteerd met de gevoelige stammen.
In andere experimenten werd de extracellulaire en celgebonden autolysine aktiviteit bestudeerd van tolerante en nontolerante stammen blootgesteld aan een hoge (80 µg/ml) en een lage (10 µg/ml) concentratie methicilline. Nontolerante stammen blootgesteld aan lage of hoge concentraties leidt bij beide concentraties tot een toename in

celgebonden autolysine aktiviteit. De tolerante stam daarentegen vertoont een lage celgebonden autolysine aktiviteit na blootstelling aan 80 µg/ml methicilline. Er bleek geen verschil te bestaan tussen extracellulaire autolysine aktiviteit geïsoleerd uit tolerante en nontolerante stammen blootgesteld aan verschillende concentraties methicilline. Echter, bij beide soorten stammen bleek de extracellulaire autolysine aktiviteit lager na blootstelling aan hoge concentraties methicilline dan na blootstelling aan lage concentraties. Dit werd veroorzaakt door het vrijkomen van een remmer van de autolysines onder invloed van hoge concentraties methicilline. Deze remmer bleek thermolabiel en niet dialyseerbaar.

De verlaagde celgebonden autolysine aktiviteit bij tolerante stammen blootgesteld aan hoge concentraties methicilline zou het gevolg kunnen zijn van remming van macromolecuul synthese zoals eiwit of RNA-synthese. Op grond van deze hypothese werd dan ook de peptidoglycaan, RNA- en eiwitsynthese snelheid bestudeerd bij tolerante en nontolerante stammen. Gebruikmakend van de pulse label techniek vonden we een selectieve remming van de RNA-synthese in tolerante stammen blootgesteld aan hoge concentraties methicilline. Onze eindconclusie omtrent het mechanisme van tolerantie voor β-lactam antibiotica bij S. aureus bacteriën luidt dan ook dat het verschijnsel uiteindelijk een direkt of een afgeleid gevolg is van remming van de RNA-synthese bij hoge concentraties van het antibioticum.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd in 1956 te Heerlen geboren. Na het behalen van de diploma's MAVO en HAVO, begon hij in 1974 met de studie M.O. Biologie aan de Rijksuniversiteit te Utrecht.
Deze studie werd afgesloten op 28 april 1980 met het M.O., kandidaats B1 en doctoraalexamen Biologie met als hoofdvakken Endocrinologie en Immunologie en als bijvak Moleculaire Biologie.
Per 1 mei 1980 kwam hij in dienst van de Erasmus Universiteit Rotterdam, alwaar hij het in dit proefschrift beschreven onderzoek heeft uitgevoerd op de afdeling Klinische Microbiologie en Antimicrobiële Therapie.
Sedert 1 januari 1986 is hij werkzaam als wetenschappelijk onderzoekmedewerker bij de vakgroep Biochemie van de Rijksuniversiteit te Leiden.