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Therapeutic Significance of Penicillin Tolerance in Experimental Streptococcal Endocarditis

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Tolerance to penicillin exists among the viridans group of streptococci, but its therapeutic significance is unknown. We studied the effect of penicillin alone and in combination with streptomycin, in vivo and in vitro, on three strains of dextran-producing *Streptococcus sanguis* serotype II which possess widely various degrees of penicillin tolerance. In rabbits with experimental endocarditis, treatment with procaine penicillin (250 mg/kg intramuscularly twice daily for 5 days) decreased the number of viable organisms in valvular vegetations from 8.82 $\log_{10}$ ± 0.98 CFU/g in untreated controls to 5.31 ± 1.19 for a highly tolerant strain, 4.22 ± 1.05 for a less tolerant strain, and 1.79 ± 1.72 for a nontolerant strain ($P \leq 0.01$ for comparison between any of the four groups). None of 36 rabbits infected with tolerant strains were cured by 5 days of treatment with penicillin, but 10 of 23 animals infected with the nontolerant strain were cured ($P = 0.00002$). When streptomycin was given in combination with penicillin, rabbits infected with the nontolerant strain were cured within 3 days, and rabbits infected with the tolerant strain were cured within 5 days. These findings indicate that tolerance can exert a critical influence on the response of *S. sanguis* to penicillin therapy in vivo and that the combination of penicillin plus streptomycin exerts a synergistic effect against tolerant as well as nontolerant organisms.

Tolerance to the lethal action of penicillins among strains of *Staphylococcus aureus* was described by Sabath et al. in 1977 (21). They defined tolerant strains as those that were inhibited by low concentrations of penicillin but were killed completely only at much higher concentrations. A number of both clinical and experimental studies have since been pursued in an effort to determine whether this in vitro phenomenon is of significance in the treatment of infected patients. In clinical studies, Denny et al. (3) and Rajashekaraiah et al. (20) found that infections with tolerant staphylococci seemed to respond less well to treatment than did infections with nontolerant strains. However, for experimental endocarditis, Goldman and Petersdorf (4) found no significant differences between the responses of methicillin-tolerant and nontolerant strains of *S. aureus* to prophylaxis or treatment. Thus, the clinical significance of tolerance in staphylococci for practical therapeutics remains controversial (9).

Tolerance to penicillin is not restricted to *S. aureus*. It has also been demonstrated among enterococci (14) and Lancefield groups B (11), C (18), and G (15), as well as the viridans group of streptococci. Recently, Holloway et al. (6) reported that 19% of viridans streptococci cultured from the gingivae of children, and from their blood after dental extraction, were tolerant to penicillin. In a letter describing preliminary studies, Pulliam et al. (19) reported that for 16 of 80 blood culture isolates of viridans streptococci the minimal bactericidal concentrations (MBCs) of penicillin were at least 10-fold greater than the minimal inhibitory concentrations (MICs). They also found that these tolerant streptococci were killed less rapidly by penicillin than were nontolerant organisms in rabbits with endocarditis.

These observations stimulated us to compare the therapeutic efficacy of penicillin in experimental endocarditis induced by the inoculation of tolerant and nontolerant strains of streptococci.

MATERIALS AND METHODS

Test organisms. Two strains of *S. sanguis* serotype II, originally isolated from the gingivae of children, were supplied by Jaap Dankert from the University Hospital, Groningen, the Netherlands. A third strain was obtained from the blood of a patient with endocarditis. All three produced dextran when grown on sucrose agar (7). Inocula for in vitro and in vivo experiments were prepared by centrifuging an overnight culture of the test organism in tryptic soy broth (Difco Laboratories, Detroit, Mich.), washing it twice,

and suspending the pellet in 0.015 M phosphate buffer in isotonic saline (PBS) at the appropriate concentration.

In vitro susceptibility testing. A serial twofold microdilution technique (personal communication, Clyde Thornsberry, Centers for Disease Control, Atlanta, Ga.) with Mueller-Hinton broth (MHB) supplemented with lysed horse blood was used to determine the MICs and MBCs of the three strains of streptococci. One hundred microliters of MHB (Difco) supplemented with 5% lysed and filtered horse blood was placed in the wells of a microtiter plate (Flow Laboratories, Inc., McLean, Va.). Then, 100 μl of penicillin at a concentration of 4,192 μg/ml was added to the first well of each group, and serial twofold dilutions through 0.002 μg/ml were made. Each well containing penicillin and two inoculum control wells were inoculated with 10 μl of a suspension of streptococci, providing a range of final inoculum sizes from 10^3 to 10^7 CFU/ml. An inoculum of 10^5 CFU of a penicillin-susceptible *S. aureus* strain (ATCC 25923) was placed into a parallel series of wells containing unsupplemented MHB to provide a control for the penicillin assay. One hundred microliters taken from one of the two control wells was serially diluted and cultured on blood agar to quantitate the number of organisms before incubation. The plates were incubated at 37°C in a candle jar for 18 h. The MIC was read as the concentration of penicillin in the first well that showed no visible turbidity. The MBC was determined by plating 50 μl from the last turbid well and all subsequent clear wells onto Columbia agar plates with sheep blood. Concentrated penicillinase (BBL Microbiology Systems, Cockeysville, Md.) was added to the agar at a concentration of 1,000 Kersey units/ml to inactivate any penicillin carried over in the volume plated. The plates were incubated at 37°C in a candle jar for 48 h. The MBC was defined as the lowest concentration that killed 99.9% or more of the total number of organisms originally present.

Production of experimental endocarditis. Male New Zealand White rabbits weighing approximately 2 kg were anesthetized with intramuscular ketamine hydrochloride (75 to 100 mg/kg) plus xylazine (15 to 20 mg/kg). The skin was wetted with alcohol, and the right internal carotid artery was isolated via an incision in the neck. A polyethylene catheter with an internal diameter of 0.76 mm and external diameter of 1.22 mm (Clay Adams, Parsippany, N.J.) was passed in a retrograde fashion down the common carotid artery to reach the aortic valve or left ventricular cavity. Proper position was achieved when pulsation or resistance to further advancement or both were met. The catheter was secured with silk sutures and sealed with a heated spatula; the internal carotid artery was tied off, and the catheter was left in place for the duration of the experiment. The skin was closed with a continuous silk suture.

Eighteen hours after insertion of the catheter, each rabbit was inoculated via an ear vein with 1 ml of a suspension of the appropriate strain. This suspension was derived from an 18-h culture which had been centrifuged, washed in 0.01 M PBS (pH 7.4) and then resuspended in PBS to an optical density of 1.0 ± 0.05 on a spectrophotometer calibrated at 540 nm. The size of the inoculum in terms of CFU was determined by plating 50 μl of serial dilutions of the suspensions in PBS onto blood agar. Colony counts were made after 24 h of incubation at 35°C.

Administration of antibiotics. Antibiotic therapy was begun 24 h after inoculation of streptococci. All treated groups received procaine penicillin G (Wyeth Laboratories, Philadelphia, Pa.) (250 mg/kg) injected into a thigh muscle every 12 h. In addition, some groups received streptomycin sulfate (Eli Lilly & Co., Indianapolis, Ind.) (15 mg/kg) every 12 h, injected into the opposite thigh. Animals treated with penicillin alone were treated for 5 days (10 doses); some treated with penicillin plus streptomycin received the combination for 3 days (6 doses) and others for 5 days (10 doses).

Assay of penicillin concentration in serum. Blood was drawn from the ear veins of four infected animals at 30 min and 1, 3, and 6 h after the first dose of penicillin. The sera were separated and frozen at −20°C for no more than 4 days before assay by the agar well diffusion method of Bennett et al. (1), using *Bacillus subtilis* as the assay organism.

Evaluation of infection. Twelve hours after the final dose of antibiotic, the rabbits were killed by intravenous injection of sodium pentobarbital. The hearts were promptly removed and opened, and the aortic root, aortic valve, and left ventricle were examined for vegetations. The vegetations were dissected away from the endothelium, weighed, suspended in PBS, and homogenized in tissue grinders (American Scientific Products, McGraw Park, Ill.). Serial dilutions of these homogenates were incorporated into blood agar pour plates. Penicillinase was added at a concentration of 1,000 Kersey units per ml. The plates were incubated at 37°C in candle jars, and colonies were counted at 48 h to determine the number of CFU per gram of vegetation. Vegetations were considered sterile if there was no growth on the pour plate prepared from undiluted homogenate.

Eight animals (10%) died early during the course of treatment, seven of these within one day of starting antibiotic therapy, and one on the second day. These animals, distributed among each of the groups, were excluded from the final tabulation of results.

RESULTS

Susceptibility testing. The results of in vitro susceptibility testing are shown in Table 1. All three strains were susceptible to penicillin as defined by a MIC of less than 0.2 μg/ml. At a standard inoculum size of 10^5 CFU/ml, two of the three strains (T1, T2) were tolerant, as defined by an MBC/MIC ratio greater than or equal to 32. The third strain (NT) was susceptible and nontolerant to penicillin at this inoculum. The inhibitory and bactericidal concentrations did not vary by more than one dilution upon repeated determinations.

Although these MICs and MBCs were highly reproducible at the standard intermediate inoculum size of 10^5 CFU/ml, if the concentration of organisms in the microtiter well was varied, the MBCs also varied (Table 1). For example, when the inoculum concentration was raised to 10^7 CFU/ml, even the nontolerant strain had approximately 100 colonies present in 50 μl subcul-

TABLE 1. MICs, MBCs, and MBC/MIC ratios for three strains of *S. sanguis* serotype II demonstrating various degrees of tolerance

Strain	Inoculum size					
	Low (10^3 CFU/ml)[a]		Intermediate (10^5 CFU/ml)[a]		High (10^7 CFU/ml)[a]	
	MBC/MIC (μg/ml)	MBC/MIC (ratio)	MBC/MIC (μg/ml)	MBC/MIC (ratio)	MBC/MIC (μg/ml)	MBC/MIC (ratio)
NT	0.13/0.13	1	0.12/0.13	1	>2,096/0.13	>16,123
T2	0.16/0.16	1	131/0.03	4,367	>2,096/0.03	>69,866
T1	0.008/0.008	1	>2,096/0.008	>262,000	>2,096/0.008	>262,000

[a] Range, 1 $\log_{10}$ CFU/ml.

tured from the wells containing 2,096 μg of penicillin per ml. On the other hand, when the starting inoculum was lowered to 10^3 CFU/ml, the MBCs for both tolerant strains equalled their respective MICs (Table 1). Thus, the definition of tolerance was highly dependent upon inoculum size.

The definition of tolerance for these organisms obtained from our measurement of MICs and MBCs was confirmed by time kill curves which have been previously reported on these strains (J. Hess, J. Dankert, and D. T. Durack, submitted for publication). These curves showed greater than 99.9% killing of the nontolerant strain (NT) by penicillin over 48 h, but less than 99.9% killing for the tolerant strain (T1).

Serum concentrations of penicillin. The concentration of penicillin in serum peaked at a mean value of 12.9 ± 4.0 (standard error) μg/ml 1 h after intramuscular injection and declined to a mean value of 6.0 ± 1.0 μg/ml at 6 h (Fig. 1).

Results of treatment. The inocula in all trials was 3×10^8 to 8×10^8 CFU/ml. All 24 control animals sacrificed 1 to 5 days after inoculation had streptococcal endocarditis. The mean number of organisms in their vegetations was 9.15 ± 0.90 CFU/g or greater. There were no significant differences between the three strains, either in rate of infection or in number of organisms per gram of vegetation in these control rabbits.

Five days of treatment with penicillin reduced the number of organisms for all strains from the mean of 8.82 $\log_{10}$ ± 0.98 CFU/g of vegetation in untreated controls ($P < 0.01$ for each strain). However, the mean number of surviving organisms differed for each strain of streptococci (Table 2). Vegetations from the nontolerant strain (NT) contained a mean count of 1.79 $\log_{10}$ CFU/g, whereas strain T1, which was highly tolerant to penicillin, had a mean count of 5.31 $\log_{10}$ CFU/g ($P < 0.001$). Strain T2, which was tolerant but to a lesser degree than strain T1, had an intermediate mean count of 4.21 CFU $\log_{10}$/g. This was significantly different from both strain NT ($P < 0.001$) and strain T1 ($P = 0.012$).

Of the 23 animals infected with strain NT and treated with penicillin alone, 10 had sterile vegetations after 5 days of therapy. In contrast, there were no sterile vegetations in any of the 36 rabbits infected with the two tolerant strains which were treated similarly. This result was significant for strain NT versus strain T1 ($P = 0.0002$ by Fisher's exact test) and versus strain T2 ($P = 0.015$).

The addition of streptomycin to penicillin therapy resulted in accelerated clearance of streptococci from the vegetations (Table 2). After only 3 days of treatment with streptomycin plus penicillin, rabbits infected with strain T1 had a mean count of 2.25 $\log_{10}$ CFU/g, but all animals infected with strain NT had sterile vegetations ($P = 0.015$). When combination therapy was continued for 5 days, all 5 animals infected with strain T1 had sterile vegetations, contrasting with the mean count of 5.31 $\log_{10}$ CFU/g found in vegetations of rabbits treated with 5 days of penicillin alone ($P < 0.00001$).

DISCUSSION

Approximately 20% of clinical isolates among the viridans group of streptococci are tolerant to

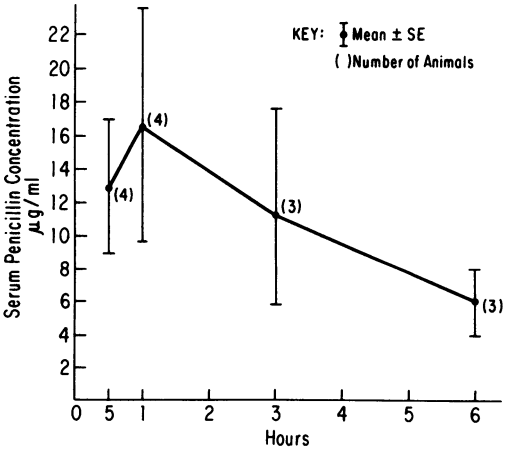

FIG. 1. Serum concentrations of penicillin in rabbits over a 6-h period, produced by intramuscular injection of procaine penicillin (250 mg/kg). SE, standard error.

TABLE 2. Results of treatment with penicillin alone and with penicillin plus streptomycin in rabbits with endocarditis caused by three strains of *S. sanguis* serotype II with various degrees of tolerance

Expt no.	Strain[a]	Treatment regimen		No. of sterile vegetations/total vegetations	Mean (±SD) log CFU/g of culture-positive vegetations
		Drug[b]	Duration (days)		
1	T1	None	−[c]	0/12	9.37 ± 0.79
		Pen	5	0/24	5.31 ± 1.19
	T2	None	−[c]	0/4	9.18 ± 1.10
		Pen	5	0/12	4.21 ± 1.04
	NT	None	−[c]	0/8	9.03 ± 1.03
		Pen	5	10/23	1.79 ± 1.71
2	T1	None	−[d]	0/4	9.27 ± 0.9
		Pen-SM	3	0/7	2.25 ± 0.62
	T1	None	−[d]	0/3	9.36 ± 1.0
		Pen-SM	5	5/5	Sterile
	NT	None	−[d]	0/3	9.65 ± 0.27
		Pen-SM	3	4/4	Sterile

[a] Strains T1 and T2 were tolerant; strain NT was nontolerant.
[b] Pen, Penicillin; Pen-SM, penicillin plus streptomycin.
[c] Controls in experiment 1 were killed on days 1, 3, or 5; mean counts did not vary over this period.
[d] Controls in experiment 2 were killed on the day equivalent to indicated day of treatment.

the bactericidal action of penicillin (6, 19). This observation raises the possibility that penicillin could be less effective for treatment of these organisms than for nontolerant strains. To investigate this question, we utilized three strains of dextran-producing *S. sanguis* serotype II, possessing various degrees of tolerance, in the experimental model for endocarditis in rabbits. Our observations indicate that a striking difference exists between the responses of tolerant and nontolerant streptococci to penicillin in vivo.

The presence or degree of tolerance may vary with the inoculum used (4), the growth phase of the bacteria (10), the number of colonies selected from a plate for testing (2), or the media and pH used for determination of the MIC and MBC (12, 16, 17). Storage of an organism for prolonged periods at −70°C may result in loss of tolerance (13). For the three strains of *S. sanguis* used in this study, we found that the inoculum size did not affect MICs but was a critical factor for MBCs. At low inocula, the MBCs for all three strains were identical to their respective MICs, that is, tolerance was not demonstrated in any strain. At high inocula, MBCs increased to the point that all, even the "nontolerant" strain, appeared to be tolerant to penicillin. Thus, variations in inoculum size alone can determine presence and degree of tolerance for these streptococci. It is noteworthy that the number of streptococci in untreated human vegetations is high, a point demonstrated by histological studies from the preantibiotic era (5).

For these reasons, we carefully standardized the techniques for determination of MICs and MBCs, with particular emphasis on reproducibility of the inoculum. These determinations were repeated periodically to ensure stability of the degree of tolerance shown by each strain of streptococci throughout the duration of the experiment. The supplementation of MHB with lysed horse blood (18) is an important step; this enhances growth of streptococci so that the MICs may be read easily in microtiter wells. Another necessary step is addition of penicillinase to the blood agar plates to inactivate penicillin carried over in the volume of broth or homogenate used for subculture. The necessity for inclusion of penicillinase was well illustrated by the tolerant strain T2, which appeared to be nontolerant when tested in the absence of penicillinase. When penicillinase was included in the blood agar, the MIC was 131 μg/ml, giving an MBC/MIC ratio of 4,367:1.

The results of treatment in vivo demonstrated striking differences between the outcome for animals infected with tolerant and nontolerant strains of streptococci. After 5 days of penicillin therapy, the number of surviving organisms in endocardial vegetations was much higher for the tolerant strains than for the nontolerant strain. Furthermore, treatment with penicillin alone failed to sterilize any of the vegetations infected with the tolerant strains in 5 days, whereas 43% of animals infected with the nontolerant strain were cured. The inverse relationship between MBCs and the efficacy of penicillin therapy is noteworthy. The more tolerant the strain, the more difficult it was to sterilize the vegetation and the higher the number of surviving streptococci, regardless of the fact that all three strains

were highly sensitive to penicillin by MIC.

In vivo synergism between penicillin and streptomycin against the highly tolerant streptococcus (strain T1) was clearly demonstrated. However, even this combination eradicated the tolerant streptococcal strain more slowly than the nontolerant strain ($P = 0.015$).

As far as we are aware, these findings provide the first substantial evidence that tolerance exerts a major influence on the response of streptococcal infection to penicillin treatment in vivo. They confirm and extend the preliminary report of Pulliam et al. (19) as well as recent studies from this laboratory showing that tolerance exerts a major influence on the outcome of attempted prophylaxis of experimental endocarditis (Hess et al., submitted for publication). In view of the well-known vagaries of in vitro tests for tolerance (2, 4, 12, 16, 17), the results of in vivo experiments such as these may provide more meaningful data on the significance of the phenomenon.

The clinical significance of these findings is not yet known. However, the results indicate that accurate determination of both MICs and MBCs of streptococci causing human endocarditis is advisable. Future work may show whether penicillin tolerance is the factor responsible for occasional relapses of streptococcal endocarditis, or for prophylaxis failures. In humans, a short course of penicillin plus streptomycin can cure streptococcal endocarditis with extremely low relapse rates. These experiments suggest that this could hold true for penicillin-tolerant as well as for nontolerant strains of streptococci.

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