Penicillin tolerance among oral streptococci

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Summary. Penicillin tolerance was elicited in 18 of 46 strains of viridans streptococci isolated from the mouths of 19 of 20 healthy subjects and in 31 of 54 consecutive blood-culture isolates of streptococci. Enterococci and *Streptococcus sanguis* were the organisms most frequently tolerant but the property was also common among isolates of *S. mutans*, *S. mitior* and Lancefield Group G streptococci. Pneumococci and *S. salivarius* were rarely tolerant. When incubated with penicillin at $5 \times \text{MIC}$ in batch or continuous cultures, both tolerant and sensitive strains of oral streptococci declined in number less rapidly than *S. pyogenes*. However, combinations of penicillin and gentamicin killed tolerant and sensitive oral streptococci.

Introduction

Penicillin tolerance was described in streptococci by Tomasz *et al.* (1970) and Tomasz (1979). A tolerant organism has been defined as one that is inhibited but not killed by antibiotics of the penicillin family, which are normally regarded as bactericidal (Slater and Greenwood, 1983). To be classed as tolerant the minimum bactericidal concentration (MBC) of penicillin must be at least 32 times greater than the minimum inhibitory concentration (MIC) (Dankert *et al.*, 1982).

Although the prevalence of tolerance among several species of streptococci has previously been reported (Holloway *et al.*, 1980; Slater and Greenwood, 1983) there is sparse evidence of the property in different species of oral viridans streptococci that are important causative organisms of infective endocarditis and of endogenous infections in immunocompromised patients.

The principal aims of the present study were to determine the prevalence of penicillin tolerance among the various species of oral streptococci and to investigate its possible nature.

Materials and methods

Isolation and identification of streptococci

Forty-six oral strains were obtained from 20 healthy subjects who had not received penicillin or other

antibiotic in the previous month. A cotton-wool swab moistened in Reduced Transport Fluid (Syed and Loesche, 1972) was gently rubbed across the gingiva and the tip was broken off into 5 ml of Reduced Transport Fluid and agitated in a vortex shaker for a few seconds. Inocula (1 ml each) were spread over the surfaces of three agar plates: blood agar (5% sheep blood in Colombia Agar Base, Oxoid) (BA); sucrose blood agar (BA + sucrose 1%) (SBA); and Gold's medium (Gold *et al.*, 1973) (MSB). Petri dishes were incubated in candle jars at 37°C for 48h. Large, mucoid colonies on SBA were presumptively identified as *Streptococcus salivarius*. MSB was presumed to be selective for *S. mutans* and colonies resembling this species were subcultured for study. From the BA plates, well-isolated colonies were taken at random and re-streaked on BA to obtain pure cultures. It was assumed that this method would yield a representative sample of the streptococcal species present in the mouth of each subject.

The 46 oral isolates were identified by a series of biochemical tests (Hardie and Bowden, 1976). The following six tests were employed: aesculin hydrolysis; ammonia production from arginine detected by Sigma Ammonia Color Reagent, (Sigma); the production of acetoin from glucose (Voges-Proskauer reaction); H_2O_2 production detected by Merck Peroxide test indicator strips (Merck, Darmstadt, DFR); fermentation of sorbitol and mannitol detected by a fall in pH of 1 unit compared with sugar-free controls.

Fifty-four consecutive isolates of streptococci from blood cultures were obtained from the Bacteriology Laboratory of the National University Hospital of Iceland (Landspítalinn). Identification of these strains was by the API-20 Strep system (API La Balme les Grottes, France) and Lancefield groups were determined by the Phadebact test (Pharmacia, Uppsala, Sweden).

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Disk penicillin tolerance test

The method was adapted from Dankert *et al.* (1982). Strains were grown overnight in Todd-Hewitt Broth (Oxoid) (THB), or Brain-Heart Infusion Broth (Oxoid) (BHIB) for pneumococci, then spread uniformly over the surface of a BA plate with a cotton-wool swab soaked in the culture. A disk containing 10 units of penicillin G was placed on the agar surface and the plate was incubated aerobically overnight at 37°C. The zone of inhibition was measured and the disk removed. β -lactamase (Leo Laboratories, Ballerup, Denmark) (25 μ l of a solution containing 2000 U/ml) was placed in the zone of inhibition and the plates were reincubated overnight. In pilot studies we found this method superior to those of Dankert *et al.* (1982) and Slater and Greenwood (1983) who used filter-paper disks containing the enzyme. The β -lactamase diffuses rather slowly and tolerant colonies were observed only very close to the β -lactamase disk. Our method allowed a greater number of tolerant colonies to grow after reincubation. The presence of 10 or more colonies within the inhibition zone was interpreted as evidence of tolerance. Tolerant colonies were subcultured and MIC and MBC values determined. A ratio of MBC:MIC $\geq$ 32:1 indicated tolerance (Slater and Greenwood, 1983).

MIC and MBC determinations

Serial doubling dilutions of penicillin G in THB or BHIB were prepared in 96-well microtitration plates (Nunc, Roskilde, Denmark). The wells were inoculated with overnight broth cultures diluted to a concentration of c. 6000 cfu/well. After overnight incubation at 37°C in a candle jar, the MIC was the lowest concentration of penicillin with which no growth was visible. To determine the MBC, a drop of β -lactamase solution containing 2000 U/ml was added to each well, then a 25- μ l sample from the well was spread on a blood-agar plate. The MBC was the lowest concentration of penicillin at which no growth occurred after overnight incubation.

Effect of penicillin on sensitive and tolerant strains

Two test strains of *S. mutans*, one sensitive (B91) and the other tolerant (TS 26M), were used in a series of experiments to study further the phenomenon of penicillin tolerance. A clinical isolate of penicillin-sensitive *S. pyogenes* was used as a sensitive control.

Studies in batch culture. Test strains were grown in THB for 10 h at 37°C in a candle jar. Pilot studies had indicated that these cultures would be in the logarithmic growth phase and would yield 10^5 – 10^6 cfu/ml. Benzyl penicillin was added to the cultures at a concentration five times the MIC for each strain; 1-ml samples of broth were taken to determine the viable counts. One drop of β -lactamase (2000 U/ml) was added to the sample before serial dilution and culture. Cultures were reincubated and further samples taken at regular intervals over the following 24 h for viable counts.

Studies in continuous culture. To study the effect of penicillin on a steady-state dividing culture, the test strains of *S. mutans* were grown in 75 ml of THB for 8 h in stoppered flasks. Fresh broth was delivered by a peristaltic pump (Gilson, Villiers le Bel, France) once the cultures had become established. Excess broth passed out to a graduated collecting vessel and the culture flasks were continuously stirred on a magnetic stirring plate and all were incubated at 37°C. This simple continuous culture system was allowed to equilibrate for 8 h and the flow-rate was checked. In pilot studies viable counts were determined to check the stability of the continuous culture system. Penicillin was added to the reservoir of broth to give a concentration of $5 \times$ MIC for each test organism and the experiment was run for a further 8 h. Samples were taken from the culture flasks just before the addition of penicillin and at intervals afterwards for determination of viable counts. The experiment was repeated with two lower concentrations of penicillin— $1 \times$ MIC and $0.2 \times$ MIC.

Effect of penicillin and gentamicin in combination. The two test strains of *S. mutans* were inoculated into broths as described above for the batch-culture study. Penicillin $5 \times$ MIC and gentamicin 8 mg/L were added. Viable counts were determined at regular intervals for 8 h after adding the antibiotics. The sample was centrifuged for 15 min and the bacterial pellet resuspended in fresh broth to a volume of 1 ml. This was repeated three times to remove the gentamicin and the sample then used for viable count determination. The effect of these antibiotics was also tested in the continuous culture system.

Inoculum effect. Bactericidal activity of an antibiotic is defined as the killing of 99.9% of the original culture by the antibiotic. The time to achieve this level of killing was determined for the tolerant strain of *S. mutans* (TS26M) with inocula of 10^4 cfu/ml and 10^9 cfu/ml. Penicillin was added to the cultures at a concentration of $5 \times$ MIC and gentamicin at a concentration of 8 mg/L ($8 \times$ MIC). The batch cultures were stirred continuously and samples taken at regular intervals for 20 h for viable counts.

Results

Tolerant bacteria were isolated from the mouths of 19 of 20 subjects and a total of 18 (39%) of the 46 strains isolated were tolerant. *S. sanguis* was the oral species most frequently tolerant (6 of 7 strains tested). *S. salivarius* was rarely tolerant (1 of 14) and about half the strains of *S. mitior* and *S. mutans* were tolerant. None of the strains isolated had a MIC value exceeding 0.4 mg/L but 13 of 18 tolerant strains had MBC values > 100 mg/L; consequently MBC:MIC ratios were $\geq 32:1$, reaching 2048 in one instance (table I).

Of the 54 blood-culture isolates of streptococci examined, 31 (57%) were tolerant. Among the 15 strains belonging to the five species recognised as oral commensals (*S. sanguis*, *S. mutans*, *S. mitior*, *S.*

salivarius and *S. milleri*) 9 were tolerant (60%). Lancefield Group D streptococci were the most common isolates from blood cultures (17 isolates). Eleven of these strains were tolerant and two were resistant to penicillin (MIC > 4 mg/L). Four of five strains of Lancefield Group G streptococci were tolerant. No strain of *S. pneumoniae* was tolerant with MBC values reaching no higher than 0.09 mg/L and MBC:MIC ratios were 1-2:1. The results obtained with the blood-culture isolates are given in table II.

Effect of penicillin on sensitive and tolerant strains

Studies in batch cultures in logarithmic growth phase. Only a slight decline in the viable count of the tolerant organism was seen in 24h (fig. 1) whereas the sensitive strain of *S. mutans* was

eliminated by 24h, although the decline in viable count was slower than for the *S. pyogenes* strain over the first 18h. The decline of the viable count of the sensitive *S. mutans* strain did not represent 99.9% killing of the original cultures until about 20h after penicillin was added.

Studies in continuous culture. The decline in bacterial counts for the sensitive strain of *S. mutans* at three concentrations of penicillin and the control strains of *S. pyogenes* is shown in fig. 2 and in fig. 3 the results are shown for the tolerant strain of *S. mutans*. With concentrations of penicillin at or below the MIC there was only a slight decline in numbers over 8h, although with penicillin at 5 × MIC the culture showed a decline approaching 99.9% killing after 8h for both strains. The viable counts of the sensitive strain of *S. mutans* declined rather more slowly than those of the tolerant test

Table I. Results of tolerance, MIC and MBC tests for the species of streptococci isolated from the mouth

Species	MIC range (mg/L)	MBC range (mg/L)	Range of MBC:MIC ratios	Number of strains tested	Number of MBC:MIC ratios ≥ 32
<i>S. mutans</i>	0.05-0.4	0.05->100	1-512:1	6	3
<i>S. sanguis</i>	0.05-0.4	3.125->100	16-1024:1	7	6
<i>S. mitior</i>	0.05-0.4	0.05-60	1-2048:1	16	7
<i>S. salivarius</i>	0.05-0.4	0.05-25	1-512:1	14	1
<i>S. milleri</i>	0.1	0.1	1:1	1	0
Unidentified	0.1-0.4	0.1->100	1-256:1	2	1
Total	0.05-0.4	0.05->100	1-2048:1	46	18

Table II. Results of tolerance, MIC and MBC tests for the species of streptococci isolated from blood cultures

Species	MIC range (mg/L)	MBC range (mg/L)	Range of MBC:MIC ratios	Number of strains tested	Number of MBC:MIC ratios ≥ 32
<i>S. mutans</i>	0.05-6.3	0.05-6.3	1-2:1	2	0
<i>S. sanguis</i>	0.05-0.78	3.13->100	4-2048:1	7	6
<i>S. mitior</i>	0.2-0.4	0.4-25	2-64:1	2	1
<i>S. salivarius</i>	0.01-0.05	0.01-50	1-256:1	3	2
<i>S. milleri</i>	0.2	0.2	1:1	1	0
<i>S. pneumoniae</i>	0.01-0.05	0.01-0.1	1-2:1	7	0
Lancefield Group A	0.05	0.1	2:1	1	0
Lancefield Group B	0.05-50	0.05-100	1-2:1	2	0
Lancefield Group C	0.05-0.1	0.1->100	1-2048:1	3	2
Lancefield Group D	0.4-6.3	0.4->100	1-128:1	17	13
Lancefield Group G	0.1-3.13	0.1->100	1-64:1	5	4
Unidentified	0.8-1.57	1.57-100	1-128:1	4	3
Total	0.01-50	0.01->100	1-2048:1	54	31

Fig. 1. Effect of adding at time zero penicillin ($5 \times \text{MIC}$) to log-phase batch cultures of tolerant and sensitive strains: ▲, tolerant *S. mutans*; △, sensitive *S. mutans*; ■, sensitive *S. pyogenes*.

Fig. 3. Effect of different concentrations of penicillin added at time zero to a tolerant strain of *S. mutans* in continuous culture: ▲, $0.2 \times \text{MIC}$ penicillin; △, $1 \times \text{MIC}$ penicillin; ■, $5 \times \text{MIC}$ penicillin.

strain. The control strain of *S. pyogenes* declined rapidly with 99.9% killing occurring after 3.5 h at $5 \times \text{MIC}$.

Effect of penicillin and gentamicin in combination. In batch culture (fig. 4) and continuous culture (fig. 5) the test strains declined rapidly with the combination of penicillin and gentamicin; the viable count of the penicillin tolerant strain declined slightly faster in batch culture than that of the sensitive one although both strains have the same MIC of gentamicin (1 mg/L). Killing of 99.9% of the bacteria occurred at 3 h for the tolerant strain and 6.5 h for the sensitive strain in batch culture and for both strains in continuous culture 99.9% killing occurred in 4–5 h.

Inoculum effect. A marked inoculum effect was seen when penicillin and gentamicin were used in

Fig. 4. Effect of adding penicillin ($5 \times \text{MIC}$) and gentamicin ($8 \times \text{MIC}$) at time zero to sensitive and tolerant strains of *S. mutans* in batch culture: ▲, Sensitive *S. mutans*; △, tolerant *S. mutans*.

Fig. 2. Effect of different concentrations of penicillin added at time zero to a sensitive strain of *S. mutans* grown in continuous culture: ▲, $0.2 \times \text{MIC}$ penicillin; △, $1 \times \text{MIC}$ penicillin; ■, $5 \times \text{MIC}$ penicillin; □, *S. pyogenes* $5 \times \text{MIC}$ penicillin (control).

Fig. 5. Effect of adding penicillin ($5 \times \text{MIC}$) and gentamicin ($8 \times \text{MIC}$) at time zero to sensitive and tolerant strains of *S. mutans* in continuous culture: ▲, Sensitive *S. mutans*; △, tolerant *S. mutans*.

batch culture in combination. The culture at a concentration of $9.7 \log_{10}$ cfu/ml did not show 99.9% killing until 10h after the addition of antibiotic whereas the culture starting at $4.8 \log_{10}$ cfu/ml showed this level of killing at 2.75h after the addition of antibiotics.

Discussion

Tolerant streptococci are widely represented in the oral flora of man and in blood-culture isolates. The results obtained in this study of tolerant streptococci from blood cultures are in close agreement with those of Slater and Greenwood (1983) but this study goes further in identifying the viridans group of streptococci to species level. *S. sanguis* was the oral streptococcus most commonly encountered in blood and this species is also the most common cause of infective endocarditis (Bayliss *et al.*, 1983). Our findings confirm the view of Tomasz (1979) that *S. sanguis* is almost always tolerant to penicillin. In the treatment of endocarditis and in the selection of prophylactic measures to prevent it the frequent occurrence of penicillin-tolerant *S. sanguis* and enterococci may well have important implications (Hess *et al.*, 1983; Southall *et al.*, 1983; Holbrook and Benediktsdóttir, 1985). Preliminary screening for tolerance with the disk test and determination of MBC:MIC ratios among strains of other common genera of oral bacteria isolated in this laboratory have not revealed tolerance to penicillin in *Actinobacillus*, *Actinomyces*, *Bacteroides*, *Haemophilus*, *Lactobacillus* and *Neisseria* species (unpublished finding).

Although penicillin tolerance was once thought to be an in-vitro phenomenon of little clinical significance, views have changed in recent years with the appearance of reports of penicillin-tolerant streptococci in endocarditis (Anderson and Cruickshank, 1982; Lewis and Ward, 1983) and cases of prophylactic antibiotic failure (Denning and Hillis, 1984). The failure of amoxycillin in single dose to protect against endocarditis in some animal models (Glauser *et al.*, 1983; Lowy *et al.*, 1983; McGowan *et al.*, 1983) may well be due to the test strains of *S. sanguis* being tolerant to penicillin and amoxycillin.

The mechanism of tolerance is not yet fully understood. Tomasz (1979) has shown that *S. sanguis* lacks murein hydrolase and so cannot be lysed by the action of penicillin but, as shown in this study, some species of oral streptococci, notably *S. mutans*, have within the species strains that are tolerant and strains sensitive to penicillin. In the present study the viable counts of cultures of both sensitive and tolerant strains of oral streptococci,

to which penicillin has been added, declined considerably more slowly than those of *S. pyogenes*.

Figs. 2, 3 and 4 demonstrate clearly that the viable count of the tolerant test strain chosen for this study declined more rapidly than that of the sensitive strain of the same species; both strains had the same MIC. Similar variations in the rates of decline of viable counts have been reported previously (Francioli *et al.*, 1985). The time to achieve 99.9% killing of an inoculum varies greatly even amongst strains of the same species (unpublished data). This degree of bacterial killing is accepted as standard in studies of the action of penicillin and represents bactericidal activity (Eagle and Musselman, 1948; Francioli *et al.*, 1985). The effect of penicillin depends on inoculum size, time and the optimum lethal concentration of the antibiotic (Eagle and Musselman, 1948). This latter factor is strain-dependent making it difficult to control for all variables even when comparing strains within the same species. From figs. 1 and 2 it is possible to conclude that both strains were affected by penicillin but only the tolerant one recovered. When gentamicin was added to the culture neither test strain survived.

The phenomenon of persistence whereby a proportion of any batch culture may be in a non-dividing phase and therefore "persist" in bactericidal concentrations of penicillin has been thought to play some part in tolerance (Greenwood, 1982). In continuous cultures in a steady state all bacteria were dividing but when antibiotic was added to the continuous-culture system in this study the decline in bacterial count was still slow and did not reach 99.9% killing except with $5 \times$ MIC of antibiotic and a time lapse of 8h. This is equivalent to six cell divisions in the culture in the presence of apparently lethal concentrations of penicillin, and the slow decline in viable count was seen with all other strains of oral streptococci subsequently tested. The parameters of continuous culture are obscure once an inhibitory factor such as an antibiotic is added but this experiment served to demonstrate that tolerant organisms are not simply the equivalent of "persisters".

The possibility that tolerance was related to possession of low-affinity penicillin-binding proteins (LAPBP) was studied using some of the isolates from this project but none was found to possess LAPBP (Professor Roberta Fontana, personal communication).

Tolerance is a very stable property not suggestive of a phenotypic characteristic. Attempts to induce tolerance in sensitive strains or to eliminate the property from tolerant strains have met with only

limited success (Holbrook and Benediktsdóttir, 1985). Many of the isolates in this study have been stored frozen in milk at -20°C after initial characterisation and no strain has reverted to being sensitive after previously being characterised as tolerant. It seems unlikely that tolerance in streptococci is merely a phenotypic characteristic of no clinical significance as suggested by Lacey (1984). Greenwood (1985) has challenged this view and stated that penicillin tolerance may have clinical significance.

Although the nature of penicillin tolerance is not yet understood the strong association between the tolerant species, *S. sanguis*, and infective endocarditis and the prevalence of tolerant oral streptococci

in blood cultures makes this an important area for further study. Penicillin tolerance may be found, for example, to be a complicating factor in the use of single-dose amoxycillin prophylaxis for patients at risk of developing endocarditis. This regimen now has wide acceptance among dentists (Holbrook *et al.*, 1987). Further clinical and laboratory studies of the phenomenon of antibiotic tolerance appear to be justified.

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