Cultural and electronmicroscopic studies of the effect of penicillin on tolerant oral streptococci

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Summary. Penicillin-tolerant and -sensitive strains of oral streptococci were treated with penicillin to determine the production of a post-antibiotic effect (PAE). No PAE was seen with any of the S. sanguis strains tested but most strains of the other oral streptococcal species produced a PAE. Cultures on nitrocellulose filters treated with penicillin were examined by scanning electronmicroscopy and showed that tolerant and sensitive strains lost the ability to adhere to the filter after application of antibiotic. When the filters were treated with β-lactamase, before processing for microscopy, the tolerant strains but not the sensitive ones recovered and grew in a confluent lawn similar to the control cultures that had not received antibiotic. Transmission electronmicroscopic examination of similarly treated cultures produced comparable results. Bizarre morphological changes were a feature of the tolerant strains of S. sanguis.

Introduction

The prevalence of penicillin tolerance in species of oral streptococci has recently been studied (Holbrook et al., 1988). Organisms that were most commonly tolerant comprised Streptococcus sanguis, S. mutans, S. mitior and enterococci; these streptococci are most frequently involved in infective endocarditis and infections in immunocompromised patients. The concentration of penicillin required to inhibit bacterial growth is not raised in this form of bacterial resistance but tolerant organisms are not readily killed by the antibiotic. The ratio of MBC: MIC for tolerant strains has been defined as ≥32 (Dankert et al., 1982). Despite a considerable accumulation of data in recent years, the mechanisms underlying tolerance are obscure and several problems of determining tolerance in clinical isolates remain (Handwerger and Tomasz, 1985; Sherris, 1986; Tuomanen et al., 1986). The present investigation of the post-antibiotic effect of penicillin on tolerant and sensitive oral streptococci included an electronmicroscopic study of the morphological changes following addition and removal of antibiotic.

Materials and methods

Test strains

Streptococci from the human mouth and from clinical specimens were isolated and identified as described previously (Holbrook et al., 1988). Five strains of S. sanguis, two strains each of S. mitior, S. mutans and S. bovis, and one strain each of S. salivarius, S. milleri, S. faecalis, S. pneumoniae and S. pyogenes were tested. Staphylococcus aureus (Oxford strain) was used as a control. Tolerance to penicillin was determined as described previously (Holbrook et al., 1988).

Determination of post-antibiotic effect (PAE)

The methodology was similar to that of Craig and Gudmundsson (1986). Test strains were grown in 4 ml of Todd-Hewitt Broth (Oxoid) for 8 h at 37°C in candle jars. A 100-µl sample was then removed for determination of the viable count and 2 ml of the culture was transferred to a fresh sterile test tube. Penicillin at a final concentration of 5 × MIC was added to this second tube (time, t = −2 h) and both cultures were reincubated for 2 h (t = 0 h). β-Lactamase (25 µl of 2000 U/ml solution; Leo Laboratories, Ballerup, Denmark) was added to the culture containing penicillin. A 100-µl sample was taken from both cultures at that time and incubation was continued. Further samples were removed from both cultures at the following time intervals: t = 0.5, 1, 1.5, 2,
4, 6, 8, 12 and 24 h. The samples were diluted for determination of viable counts. Growth curves were plotted for each test strain with and without penicillin and the time taken for the culture treated with penicillin to increase by $1 \times \log_{10}$ cfu/ml after $t=0$ h was compared with the time taken by the control culture; the difference in time was recorded as the PAE (Craig and Gudmundsson, 1986).

**Scanning electronmicroscopy**

Representative tolerant strains of *S. sanguis* and *S. mutans*, and sensitive strains of *S. mitior* were grown in Todd-Hewitt Broth for 4 h at 37°C in a candle jar. Cultures were then filtered on to nitrocellulose filters, pore size 0.45 μm, by means of a syringe and filter assembly (Millipore S.A. Molsheim, France). The filters were then transferred to blood-agar plates for further incubation. This method gave an even lawn of bacteria and the culture produced was considered to resemble the form in which bacteria grow on heart valves in vivo (Lorian, 1986). This culture can be handled with the minimum of colony disruption during antibiotic treatment and subsequent preparation for electronmicroscopy (Lorian, 1986). For each test strain one filter was cultured on plain blood agar as a control. Two further filter-disk cultures were prepared but with penicillin disks (2U) placed in the centre of each of the filters. Incubation of all three cultures was continued for 24 h when the penicillin disk was removed from one nitrocellulose filter and replaced by a drop of β-lactamase (25 μl of a 2000 U/ml solution). Incubation of this culture was continued overnight. After the various periods of incubation, wedge-shaped strips of the filters were fixed in glutaraldehyde 3% in sodium cacodylate buffer (300 mOsm, pH 7-4) for at least 24 h at room temperature. The strips were then washed in the same buffer, dehydrated through increasing concentrations of ethanol and critical point dried in a Polaron E-3000 drier with liquid CO₂. After drying, the strips were fastened with double-sided sellotape and coated with gold for 3 min in a sputter coater (Edwards S150) with a gold-plated cathode in an atmosphere of argon. The bacteria on the surface of the strips were examined in a scanning electronmicroscope (Philips 50 at 15 kV with 45° tilt) for changes in bacterial cell morphology.

**Transmission electronmicroscopy**

The test strains examined by scanning electronmicroscopy were also studied by transmission electronmicroscopy. Strains were grown in Todd-Hewitt Broth for 8 h and cultures were transferred to fresh sterile test tubes containing a button of 0.5 ml of semi-solid agar (Gibco) and centrifuged for 20 min at 2500 rpm (MSE Centaur 1). The supernate was discarded and the bacteria impressed into the agar button by centrifugation were prepared for examination by transmission electronmicroscopy.

**Results**

**Post-antibiotic effect**

The growth curve for a sensitive strain of *S. salivarius* illustrating PAE is shown in fig. 1. PAE was seen in all sensitive strains tested and in some that were tolerant to penicillin. None of the five strains of *S. sanguis*, however, showed PAE. The value for the PAE obtained with the control strain of *Staph. aureus* was in close agreement with that reported by Craig and Gudmundsson (1986) and the results for all the strains tested are shown in the table. Pneumococci and *S. milleri* strains grew slowly and each had a particularly extended PAE.

**Electronmicroscopy**

A dense lawn of bacteria was seen on the filters of the control cultures by scanning electronmicroscopy (fig. 2a, b). When penicillin disks were placed on the filters there was marked disruption of both tolerant and sensitive strains. Most of the organisms appeared to have burst or lost the ability to adhere to the filter during fixing after penicillin treatment.
Table. Post-antibiotic effect (PAE) of test strains with penicillin

<table>
<thead>
<tr>
<th>Species</th>
<th>Sensitive/tolerant (s/t)</th>
<th>PAE (h)</th>
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</thead>
<tbody>
<tr>
<td>S. sanguis</td>
<td>t</td>
<td>0</td>
</tr>
<tr>
<td>S. sanguis</td>
<td>t</td>
<td>0</td>
</tr>
<tr>
<td>S. sanguis</td>
<td>t</td>
<td>0</td>
</tr>
<tr>
<td>S. sanguis</td>
<td>t</td>
<td>0</td>
</tr>
<tr>
<td>S. sanguis</td>
<td>t</td>
<td>0</td>
</tr>
<tr>
<td>S. mutans</td>
<td>t</td>
<td>3.75</td>
</tr>
<tr>
<td>S. mutans</td>
<td>s</td>
<td>2</td>
</tr>
<tr>
<td>S. mitior</td>
<td>t</td>
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</tr>
<tr>
<td>S. mitior</td>
<td>s</td>
<td>3.25</td>
</tr>
<tr>
<td>S. salivarius</td>
<td>s</td>
<td>2</td>
</tr>
<tr>
<td>S. milleri</td>
<td>s</td>
<td>&gt;7</td>
</tr>
<tr>
<td>S. bovis</td>
<td>t</td>
<td>0</td>
</tr>
<tr>
<td>S. bovis</td>
<td>t</td>
<td>2.25</td>
</tr>
<tr>
<td>S. faecalis</td>
<td>t</td>
<td>0</td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>s</td>
<td>7</td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>s</td>
<td>3.3</td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>s</td>
<td>1.5</td>
</tr>
</tbody>
</table>

(fig. 2c, d). The filters that were subsequently treated with β-lactamase, however, showed clear differences between sensitive and tolerant strains. Only a few cells of the sensitive strain of S. mitior remained and appeared to have recovered on the otherwise bare filter (fig. 2e) but the culture of the tolerant strain of S. mutans appeared to be as dense as its control (fig. 2f). Few elongated or deformed bacteria were seen in the cultures except for the tolerant strain of S. sanguis (fig. 3a). S. sanguis also showed elongated forms in the preparation examined by transmission electronmicroscopy (fig. 3b) but there was less evidence of bacterial rupture than in the other strains studied. The transmission electronmicrographs of cultures after treatment with penicillin showed clearly that the sensitive S. mitior strain (fig. 4a) and the tolerant S. mutans strain (fig. 4b) were ruptured in considerable numbers. The clearest examples of bacterial disruption were seen in the tolerant strain of S. mutans (fig. 4b).

Discussion

Penicillin-tolerant streptococci have MIC values within the therapeutic range for the antibiotic, but these organisms can evade the bactericidal action of penicillin; the property of tolerance is common among clinical and commensal isolates of “viridans streptococci” (Holbrook et al., 1988). In this study we have shown that cultures of all sensitive strains in early logarithmic growth phase showed a PAE with penicillin according to the criteria defined by McDonald et al. (1977) and Craig and Gudmundsson (1986). However, in some instances, tolerant bacteria were able to grow immediately on removal of the antibiotic. In particular, tolerant S. sanguis strains never showed a PAE with penicillin, suggesting that strains of this species were exhibiting phenotypic tolerance (Tuomanen, 1986; Tuomanen et al., 1986). Organisms may be both phenotypically and genotypically tolerant to penicillin. Phenotypically tolerant bacteria are particularly difficult to eradicate in animal models of experimental endocarditis (Tuomanen et al., 1986). Lowy et al. (1983) found that penicillin reduced the adherence of tolerant S. sanguis strains to heart valves in vitro. The mechanism was thought to be related to the loss of surface lipoteichoic acid after penicillin treatment. Likewise Glauser et al. (1983) have shown that amoxycillin can act in two ways in experimental endocarditis: (i) by bacterial killing to which tolerant bacteria are relatively resistant and (ii) by decreasing adherence to heart valves to which sensitive and tolerant bacteria are equally susceptible. In the present study, the electronmicrographs showed clearly that penicillin disrupted the lawn of bacteria growing on the filters after which the bacteria lost their attachment to the filters. The scanning electronmicrographs illustrate that the penicillin-tolerant bacteria could recover from the effect of penicillin when the penicillin disk was replaced by β-lactamase. Bacteria then regained attachment to the filter and grew up into a lawn of organisms resembling the control culture. Sensitive bacteria were presumably killed or severely damaged by the antibiotic and were largely unable to re-attach to the filter after the addition of β-lactamase. These non-adherent bacteria were then lost from the filter during fixing and processing. The tolerant test strain of S. mutans was presumably only phenotypically tolerant and so only the dormant organisms could resist the killing action of penicillin and the dividing bacteria were killed (Tuomanen et al., 1986). S. sanguis which is genotypically tolerant to penicillin (Tomasz, 1979; Tuomanen et al., 1986) resisted the action of penicillin resulting in bizarre morphological forms of the bacterial cells but no cell rupture occurred (figs. 3a and 3b). Therefore, oral streptococci such as S. sanguis may evade the killing action of β-lactam antibiotics if they are genotypically tolerant. Phenotypically tolerant oral streptococci will persist in the presence of lethal concentrations of antibiotic and in most cases will show a PAE on removal of inhibitory concentrations of penicillin. Survival of these organisms in the presence of penicillin may
Fig. 2. Scanning electronmicrographs of cultures on nitrocellulose filters (magnification 4000). Control cultures of (a) a sensitive *S. mitior* strain and (b) a tolerant *S. mutans* strain show an even lawn of bacteria. After penicillin treatment, *S. mitior* (c) and *S. mutans* (d) strains show a similar loss of organisms from the filters. After treatment with β-lactamase, *S. mitior* (e) remains damaged and *S. mutans* (f) shows the recovery of the tolerant strain.

Fig. 3. The bizarre morphological appearance of *S. sanguis* after treatment with penicillin and β-lactamase seen by (a) scanning electronmicrography (magnification 4000) and (b) transmission electronmicrography (magnification 16 000).
also be related to the paradoxical effect first reported by Eagle and Musselman (1948) and found to be especially common in tolerant streptococci (Meeson et al., 1988; Powley et al., 1989).

Any resistance to the lethal action of β-lactam antibiotics especially among strains of S. sanguis could be expected to be a serious contraindication to the use of single-dose antibiotic regimens for the prophylaxis of endocarditis (BSAC, 1982; Delaye et al., 1985; Cars et al., 1988). Fortunately the effect of the antibiotic in reducing adherence of bacteria to heart valves (Lowy et al., 1983; Glauser et al., 1983) is probably the significant factor in successful prophylaxis in patients at risk of developing infective endocarditis. Penicillin-tolerant streptococci producing endocarditis present particular therapeutic problems. These may best be solved by giving a combination of penicillin and gentamicin, a regimen that has been shown to extend the PAE for enterococci and render the organisms susceptible to the aminoglycoside (Winstanley and Hastings, 1989).

A previous communication from this laboratory showed tolerance to be prevalent among oral streptococci (Holbrook et al., 1988) and the present study illustrates their potential for recovery when antibiotic is removed. The clinician should be cautious, therefore, when using single-dose prophylactic antibiotic regimens.

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