Penicillin Tolerance in Nutritionally Variant Streptococci

YVETTE HOLLOWAY* AND JACOB DANKERT†

Department of Hospital Epidemiology and Laboratory for Medical Microbiology, University Hospital, Groningen, The Netherlands

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Eleven strains of nutritionally variant streptococci were tested for their susceptibility to penicillin by a broth dilution method. All minimal inhibitory concentrations were low. It was found that plates containing only vitamin B6 (pyridoxal HCl) and cysteine did not reveal true minimal bactericidal concentrations. When penicillinase was added to the subculture medium and a staphylococcal streak was made across the plates, all 11 strains were found to have high minimal bactericidal concentrations and were shown to be tolerant to penicillin.

Satellitoning or nutritionally variant streptococci (NVS) have been isolated from 5 to 6% of patients with viridans streptococcal endocarditis (14). Where the penicillin susceptibility of these strains has been reported and where only the minimum inhibitory concentration (MIC) is given in reports concerning 21 strains, 17 (81%) of them were found to be susceptible to penicillin (3, 6, 12–14), and 4 (19%) strains were found to be resistant to penicillin (12, 14). We have been able to find only two reports (1, 5) where the minimum bactericidal concentration (MBC) was also determined. In the first report (1), both strains tested were resistant to penicillin on both MIC and MBC testing. In the other report (5), one strain was shown to be susceptible to penicillin on both MIC and MBC testing, whereas the other strain had a low MIC but a high MBC (MBC/MIC ratio of 266) and was therefore tolerant to penicillin by the definition given by Sabath et al. (16).

Penicillin carried over from broth dilution tubes can inhibit the growth of microorganisms on subculture medium. The addition of penicillinase can prevent this effect (15), and Horne and Tomasz (10) considered this to be important in a study on penicillin-tolerant viridans streptococci. In a comparative study, using subculture plates containing penicillinase and plates without penicillinase, we demonstrated that more tolerant viridans streptococci were detected when the carry-over effect was inactivated (J. Dankert, Y. Holloway, W. Joldersma, and J. Hess, submitted for publication).

Presuming that this might apply to NVS as well, we collected 11 strains for testing. In addition, the effect of various supplements included in the subculture medium and that of a staphylococcal streak (8) across the MBC plates on the penicillin susceptibility of NVS were determined.

Strains 1, 2, and 7 were isolated by us from children in this hospital. Strains 3, 4, and 5 were sent to us on request by J. Vandepitte, University Hospital, Leuven, Belgium; strain 6 was sent to us by P. C. Appelbaum of the Milton S. Hershey Medical Center, Hershey, Pa., and strains 8 to 11 were sent to us by R. B. Roberts, the New York Hospital-Cornell Medical Center, New York, N.Y. All strains were isolated from blood cultures of patients with bacterial endocarditis with the exception of strain 2, which was isolated from the gingival sulcus of a child with a congenital heart disease. Except for strain 7, which was isolated at a later date, all strains tested in this study are now deposited in the NVS repository of R. B. Roberts.

NVS need supplemental cysteine (3, 7) or vitamin B6 (2, 8) in their culture media. The preliminary screening of our 11 strains showed that they all grew well in either fluid medium or solid medium supplemented with 0.03% L-cysteine-HCl (British Drug Houses, Poole, United Kingdom) together with 0.01% vitamin B6 (pyridoxal HCl; E. Merck AG, Darmstadt, Federal Republic of Germany), and these two supplements were therefore added to all media used in this study. The identification of the 11 strains was performed as previously described for viridans streptococci (9), but with Hiss serum water sugars (4) rather than Minitek (BBL Microbiology Systems, Cockeysville, Md.) disks, and with all media supplemented. Luxurious growth was obtained, and all strains were identified as non-dextran-producing Streptococcus mitior by this method.

The penicillin susceptibility of the NVS was tested with supplemented Todd-Hewitt (TH) broth (Oxoid Ltd., London, England), since the

† Person to whom reprint requests should be sent.
strains grew faster in this medium than in supplemented Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.). The inoculum used for MIC determination was approximately $10^{6}$ colony-forming units (CFU) per ml (range, $9.9 \times 10^{4}$ to $1.5 \times 10^{7}$/ml) as determined by colony count on supplemented blood agar medium. For MBC determination, 0.1 ml from each broth dilution tube was spread out on the subculture media. The MBC was read as the lowest concentration of penicillin providing 99.99% kill (less than 10 CFU).

To avoid the possible pitfall of pH-dependent penicillin tolerance (11), pH measurements were made of all control tubes in the broth dilution rows before and after 24 h of incubation at 37°C in CO₂. The initial pH was about 8.0, and the average pH of the medium after incubation was 7.2 (range of 6.9 to 7.35). As a control for the possible influence of the supplements on the MIC or MBC, a known penicillin-susceptible non-NVS S. mitior (non-dextran producing) was included with all tests.

The MIC and MBC of this strain were 0.01 U of penicillin per ml. After the MICs in supplemented TH broth had been read, subcultures from all tubes were made on blood agar plates containing the following six different supplements for MBC determination: (i) vitamin B6 and cysteine, (ii) vitamin B6, cysteine, and penicillinase (sufficient to counteract 50 U of penicillin per ml), (iii) vitamin B6, cysteine, and a staphylococcal streak (S. aureus, penicillinase producing), (iv) a staphylococcal streak only; (v) penicillinase and a staphylococcal streak, and (vi) vitamin B6, cysteine, penicillinase, and a staphylococcal streak. All plates were incubated in a CO₂ incubator for 48 h, and the colonies were counted.

The MICs of the strains ranged from 0.05 to 0.4 U of penicillin per ml, demonstrating that all 11 strains were susceptible to the MIC of penicillin. It was found that plates containing vitamin B6 and cysteine only gave very low MBCs, with all strains apparently susceptible to penicillin (Table 1). Plates containing the same supplement, together with penicillinase, yielded CFU from high concentrations of penicillin in the case of four (36%) strains, demonstrating the phenomenon of tolerance. Surprisingly, a plain blood agar plate containing only a staphylococcal streak yielded seven (64%) tolerant strains, and plates containing vitamin B6 plus cysteine plus a staphylococcal streak yielded nine (82%) tolerant strains. Plates containing only penicillinase plus a staphylococcal streak yielded 10 (90%) tolerant strains. When all supplements—vitamin B6, cysteine, penicillinase, and a staphylococcal streak—were included, all 11 (100%) strains were shown to be tolerant to penicillin. It can be seen from Table 1 that 9 of the 11 strains of the NVS, in fact, survived incubation in high penicillin concentrations for 24 h in the broth culture tubes. Yet none of these was detected on plates containing only vitamin B6 and cysteine, and not all of them were detected when penicillinase was added.

The control S. mitior strain retained its MBC of 0.01 U/ml on all subculture media, and penicillin tolerance in NVS is therefore not a function of the supplements used. It would seem that the addition of penicillinase to the subculture medium counteracts the carry-over effect and that it enables the detection of penicillin tolerance in NVS, as it does for viridans streptococci (Dankert et al., submitted for publication). But it would seem also that a staphylococcal streak is essential for obtaining the true MBC in the case of NVS. Although the addition of vitamin B6 and cysteine provides adequate growth of NVS on blood agar plates, it appears that another (as yet unknown) factor is supplied by a staphylo-

**TABLE 1. Penicillin MICs of NVS with MBCs as determined on blood agar plates with various supplements**

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>MIC (U/ml)</th>
<th>B + C</th>
<th>B + C + P</th>
<th>B + C + S</th>
<th>S</th>
<th>S + P</th>
<th>B + C + S + P</th>
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<tr>
<td>1</td>
<td>0.05</td>
<td>0.05</td>
<td>&gt;400 (T)</td>
<td>50 (T)</td>
<td>12.5 (T)</td>
<td>&gt;400 (T)</td>
<td>&gt;400 (T)</td>
</tr>
<tr>
<td>2</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>400 (T)</td>
<td>200 (T)</td>
<td>&gt;400 (T)</td>
<td>&gt;400 (T)</td>
</tr>
<tr>
<td>3</td>
<td>0.03</td>
<td>0.05</td>
<td>&gt;400 (T)</td>
<td>0.4</td>
<td>12.5 (T)</td>
<td>&gt;400 (T)</td>
<td>&gt;400 (T)</td>
</tr>
<tr>
<td>4</td>
<td>0.03</td>
<td>0.05</td>
<td>0.05</td>
<td>12.5 (T)</td>
<td>6.3 (T)</td>
<td>&gt;400 (T)</td>
<td>&gt;400 (T)</td>
</tr>
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<td>5</td>
<td>0.05</td>
<td>0.1</td>
<td>0.1</td>
<td>50 (T)</td>
<td>100 (T)</td>
<td>&gt;400 (T)</td>
<td>&gt;400 (T)</td>
</tr>
<tr>
<td>6</td>
<td>0.05</td>
<td>0.1</td>
<td>&gt;400 (T)</td>
<td>12.5 (T)</td>
<td>6.3 (T)</td>
<td>&gt;400 (T)</td>
<td>&gt;400 (T)</td>
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<tr>
<td>7</td>
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<td>0.4</td>
<td>12.5 (T)</td>
<td>400 (T)</td>
<td>6.3 (T)</td>
<td>&gt;400 (T)</td>
<td>&gt;400 (T)</td>
</tr>
<tr>
<td>8</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>6.3 (T)</td>
<td>6.3 (T)</td>
<td>&gt;400 (T)</td>
<td>&gt;400 (T)</td>
</tr>
<tr>
<td>9</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.4</td>
<td>0.8</td>
<td>&gt;400 (T)</td>
<td>&gt;400 (T)</td>
</tr>
<tr>
<td>10</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.4 (T)</td>
<td>0.01</td>
<td>0.03</td>
<td>0.8 (T)</td>
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<td>11</td>
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<td>0.01</td>
<td>0.01</td>
<td>3.1 (T)</td>
<td>0.05</td>
<td>1.6 (T)</td>
<td>6.3 (T)</td>
</tr>
</tbody>
</table>

*Abbreviations: B, vitamin B6 (pyridoxal HCl); C, l-cysteine–HCl; P, penicillinase (50 U/ml); S, staphylococcal (Staphylococcus aureus) streak; T, tolerance.*
cococcus when this is streaked across the plate used for the MBC determination of penicillin. Only when all of these supplements are present can it be demonstrated that the NVS are still alive and have not been killed by penicillin. The NVS tested and subcultured from supplemented TH broth without penicillin grew as normal colonies on plates containing vitamin B6 and cysteine, but reverted to partial satellitism around the staphylococcus at penicillin concentrations below their MIC values; total satellitism was achieved at higher concentrations. This effect was seen clearly in all strains. Plates inoculated from the control tubes yielded colonies covering the entire plate. At very low concentrations of penicillin, this still applied, but for each strain there was a point, ranging from 0.01 U/ml for some strains to 0.4 U/ml for others, where satellites appeared among the normal colonies along the staphylococcal streak. The proportion of satellites to normal colonies increased in parallel with the rise in penicillin concentration, until only satellites were present along the staphylococcal streak. It appears that penicillin, even at very low concentrations, induces streptococcal forms which are unable to multiply on plates containing only vitamin B6 and cysteine, and this may explain why the MBCs were low on plates containing only these supplements, where survivors could not grow, due to the lack of a factor supplied by the staphylococcus. Four strains did yield high MBCs on plates not containing a staphylococcal streak but had very abnormal colony forms, which were tiny compared with the colonies subcultured from control tubes. These colony forms reverted to normal in the presence of a staphylococcal streak as well.

We conclude that NVS need to be tested on media containing penicillinase, cysteine, vitamin B6, and a staphylococcal streak for penicillin susceptibility, as the growth requirements of NVS apparently change in the presence of subminimal concentrations of penicillin.

LITERATURE CITED