Effect of pH and Human Serum on the Susceptibility of Group D Streptococci (Enterococci) to Ampicillin In Vitro

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The in vitro susceptibility of group D streptococci (enterococci) to ampicillin was studied comparing the results obtained in Mueller-Hinton broth (MHB) with those obtained in normal human serum (NHS). The rate of enterococcal killing was consistently faster in NHS than in MHB at equivalent ampicillin concentrations. Whereas an increasing media pH appeared to decrease the susceptibility of enterococci to ampicillin by determinations of the minimum bactericidal concentration (MBC) of ampicillin, an opposite increase in susceptibility was observed when the rate of bactericidal activity was studied. This difference may be explainable by the instability of ampicillin at higher pH values. In both MHB and NHS a paradoxical decrease in the rate and extent of enterococcal killing occurred as the ampicillin concentration was increased above the minimally effective concentration. These results demonstrate the inadequacies of the MBC test system and the need for standardizing test media used for determining the susceptibility of enterococci to ampicillin.

Group D streptococci (enterococci) are frequently cultured from patients with pyelonephritis or endocarditis. Since enterococci are relatively resistant to antibiotics, the standard regimen used to treat severe enterococcal infections is a combination of penicillin and aminoglycoside (16). Adverse side effects associated with aminoglycoside antibiotics are frequent enough to make it desirable to find an equally effective, less toxic regimen. Previous reports suggest that in vitro the enterococcus is quite susceptible to ampicillin alone (3, 20, 26, 28, 29, 32). However, single drug therapy of enterococcal endocarditis with ampicillin has given conflicting results (2, 12, 24). The controversy raised by these studies prompted the present evaluation of the in vitro susceptibility of the enterococcus to ampicillin. It was postulated that previous studies might have been in error because of the dissimilarity of standard laboratory culture media and body fluids. For example, the calcium and magnesium concentrations of laboratory culture media are much different than the serum concentrations of these cations. The significant effect of the media calcium and magnesium concentration on the susceptibility of Pseudomonas aeruginosa to gentamicin is well documented (10, 11, 25, 35). For these reasons, susceptibility testing of enterococci to ampicillin was performed in both broth culture media and normal human serum. Since previous reports have described an effect of culture pH on ampicillin activity against the enterococcus, the effect of varying the culture media pH was also studied (33).

MATERIALS AND METHODS

MHB and NHS. A single lot number of Mueller-Hinton broth (MHB) was used throughout these studies (Difco Laboratories). Normal human serum (NHS) was obtained aseptically from three healthy donors and stored in small aliquots at -70 C until used. Serum complement was inactivated by heating at 56 C for 30 min. No variation in results was observed with different sera. For this reason, we arbitrarily used the serum of an individual fasting donor for each experiment.

The osmolality of MHB and NHS was determined with an osmometer (Advanced Instruments, Needham Heights, Mass.). The albumin concentration of MHB and NHS was determined by the HABA dye-binding method (27).

Group D streptococci (enterococci). The 15 strains of enterococci utilized in this study were clinical isolates obtained from the Providence Medical Center Bacteriology Laboratory. In addition to standard criteria for identification of enterococci (1), strains were tested for their ability to reduce litmus milk, survive exposure to 6.5% sodium chloride, and react specifically with group D streptococcal antiserum. Enterococcal strains were speciated according to the criteria of Topley and Wilson (34) as recently modified by P. Toala et al. (32).

MBC. For this study, the minimum bactericidal concentration (MBC) was defined as that ampicillin
concentration which resulted in the survival of 1% or less of an original inoculum of 10^7/ml of enterococci after an 18-h incubation period.

The test strains of enterococci were incubated overnight in MHB at 37 C, diluted 1,000-fold in sterile water, and then diluted 10-fold in either MHB or NHS. The inoculum size was verified in duplicate by standard pour-plate technique. The pH of NHS and MHB was measured and adjusted to 5.0, 7.4, or 9.0 with concentrated acid or base. During initial experiments, it was found that the pH of NHS increased from 7.4 to 8.6 during an 18-h incubation. Subsequently, all experiments involving NHS were done in an atmosphere containing 4% carbon dioxide, which maintained the serum pH within 0.1 pH unit of the original serum pH. The pH of MHB remained constant during incubation in room air. Similarly, after the serum pH was adjusted to 5.0, the pH of NHS remained constant during incubation in 4% carbon dioxide. The pH of NHS adjusted to 9.0 remained constant during incubation in room air.

Serial twofold dilutions of ampicillin trihydrate (Beecham-Massengill, Clifton, N.J.) in either 100% MHB or 100% NHS were performed in sterile disposable tubes. An equal volume of the enterococcal suspension (in 90% MHB or 90% NHS) was then added to each tube, resulting in a final NHS concentration of 95%. An antibiotic-free tube of MHB or NHS was included in each experiment. After 18 h of incubation, a micropipette (Oxford Laboratories, Foster City, Calif.) was used to transfer 0.1 ml from each well to a sheep blood agar plate containing 200 U of penicillinase per ml (Neutrapen, Riker Laboratories, Northridge, Calif.). The sample was spread with a bent glass rod, and the plate was incubated for 18 h. The number of colonies was counted, and the percentage of the original inoculum surviving was determined. Each experiment was repeated a minimum of three times.

Rate and extent of bacterial killing (killing curves). After overnight incubation in MHB, an inoculum of 10^7 enterococci/ml was prepared in either MHB or NHS. Stock ampicillin trihydrate standards of 1,000 μg/ml were diluted in either MHB or NHS to the desired final concentration. The test incubation mixture contained 10^7 enterococci/ml in either 90% MHB or 90% NHS.

In the experiments which depleted NHS of protein, NHS was first passed through an ultrafiltration membrane (Amicon Corp., Lexington, Mass.) which excluded all molecules with a molecular weight greater than 50,000.

Suspensions of antibiotics and bacteria were incubated at 37 C in a shaking water bath, and the number of surviving organisms was determined at time zero and at subsequent intervals by pour-plate technique. Appropriate antibiotic-free controls were performed with both NHS and MHB.

Particle counting. Enterococci and ampicillin were incubated with either MHB or NHS exactly as described for the killing curve studies. Prior to incubation, NHS and MHB were passed through an ultrafiltration membrane which retained constituents with a molecular weight greater than 100,000. Filtration of the culture media was necessary to reduce high background counts.

At intervals during the incubation, the culture was mixed vigorously, and 0.2 ml was withdrawn and diluted 1:100 with isotonic diluting fluid (J. T. Baker Chemical Co., Phillipsburg, N.J.). The number of particles present was determined using an electronic particle counter with a 30-μm aperture size (Coulter Electronics, Inc., Hialeah, Fla.).

Ampicillin stability. Ampicillin concentrations were determined by a disk diffusion microbiological assay using Sarcina lutea ATCC 9341 and antibiotic media no. 1 (Difco Laboratories). The method used was similar to that previously described (14) except 1/4-inch (0.64-cm) diameter paper disks (Schleicher and Schuell, Keene, N.H.) were used in lieu of agar wells or cylinders.

Various concentrations of ampicillin trihydrate were incubated with enterococci in either MHB or NHS adjusted to pH values of 5.0, 7.4, or 9.0. At timed intervals, duplicate samples were obtained for determination of the remaining ampicillin concentrations. Specimens were immediately frozen and stored at −70 C until the ampicillin assay was performed.

RESULTS

Group D streptococci (enterococci). The 15 enterococcal strains employed in this study were speciated as follows: Streptococcus faecalis (three isolates), S. faecalis var. zymogenes (six isolates), and S. faecalis var. liquefaciens (six isolates). Since preliminary tube dilution susceptibility testing indicated similar results with all strains, a single strain of S. faecalis var. zymogenes was used in all killing curve studies.

Osmolality determinations. The initial osmolality of NHS, which contained ampicillin and enterococci, varied between 260 to 280 mosmol. Over the incubation period, the osmolality fell to a low of 215 mosmol after 4 h and then gradually increased to 230 mosmol after 18 h. Under the same conditions, NHS had an initial osmolality between 180 to 190 mosmol. The MHB osmolality remained in this range during the entire incubation period.

MBC results. The MBC of ampicillin against eight strains of enterococci was determined in either MHB or NHS at pH 7.4. For each ampicillin concentration the number of surviving enterococcal colony-forming units was recorded. The mean number of surviving colonies at each ampicillin concentration was calculated, and the results were expressed as percentage of group D streptococci surviving. Figure 1 illustrates the results of a representative experiment. The experiment was repeated four times with similar results.

The maximum susceptibility in MHB of the
eight test strains was 3.12 µg/ml, whereas the maximum susceptibility in NHS was 50 µg/ml (Fig. 1). A paradoxical increase in survival of enterococci occurred in both MHB and NHS, when ampicillin concentrations exceeded the concentration required for maximal bacterial killing. For example, in MHB, over 10 times as many enterococci survived in an 18-h incubation in 800 µg of ampicillin per ml as survived incubation in 3.12 µg of ampicillin per ml.

There was minimal variability between the eight strains of enterococci tested. For this reason, a single *S. faecalis* var. *zymogenes* strain was chosen for killing curve studies. The MBC of ampicillin against this single strain was determined repeatedly in MHB and NHS. The results are shown in Fig. 2 where ampicillin concentration is plotted against the percentage of the *S. faecalis* var. *zymogenes* surviving for 18 h. Each line on the figure represents the mean value of three experiments, with each experiment performed simultaneously in MHB and NHS. As shown, maximal killing in MHB occurred between 0.7 to 3.0 µg/ml, whereas maximal killing in NHS occurred at 50 µg/ml. The ampicillin concentrations necessary for maximal killing were higher in NHS, but the degree of reduction in bacterial survivors was over 99% greater in NHS. As in Fig. 1, a paradoxical increase in bacterial survival occurred in both MHB and NHS at high concentrations of ampicillin.

The effect of the culture media pH on the susceptibility of 15 isolates of enterococci to ampicillin was studied. The MBC of ampicillin appeared to progressively increase as the media pH increased, in both MHB and NHS (Fig. 3). The experiment was repeated a minimum of three times at each culture media pH. The results were highly reproducible, and, hence, the data from a single representative experiment are shown in Fig. 3.

**Rate and extent of enterococcal killing by ampicillin.** The rate and extent of enterococcal killing by ampicillin was compared in MHB and NHS. Each killing curve was repeated several times with the representative single strain of *S. faecalis* var. *zymogenes*. The results at a media pH of 7.4 with ampicillin concentrations of 5 and 20 µg/ml are shown in Fig. 4. Each curve represents the mean results of several experiments. As the curves demonstrate, bacterial death was more rapid in NHS than in MHB at equivalent media ampicillin concentrations.
NHS were very susceptible to low ampicillin concentrations, i.e., 5 µg/ml. During the first 6 h of incubation, the rate of bacterial killing by 5 µg of ampicillin per ml was greater in NHS than MHB (Fig. 4). After 6 h, enterococci frequently multiplied in NHS, but not in MHB, at low ampicillin concentrations, resulting in greater numbers of enterococci counted at 18 h in NHS (data not shown).

Also contrary to the effect of media pH as judged by MBC testing (see Fig. 3), the susceptibility of enterococci to a moderate ampicillin concentration, 20 µg/ml, appeared to increase, not decrease, in an alkaline media by the killing curve technique (Fig. 5). Each curve represents the mean of several experiments, as indicated. The results were similar in MHB and NHS, with the apparent killing rate faster in NHS at any given pH value. The growth behavior of the enterococci after the first 6 to 8 h of incubation may explain the discrepancy with the MBC results. At pH 9.0 early cessation of bacterial killing was observed, especially in NHS (Fig. 5). As described below, the decreased stability of ampicillin at an alkaline pH may contribute to these findings.

In an attempt to explain these results, the effect of the culture media and the media pH on bacterial growth was studied. The growth of the test strain of S. faecalis var. zymogenes in MHB and NHS was determined in media adjusted to pH 5.0, 7.4, or 9.0. No significant differences in bacterial growth were observed under these conditions (Fig. 6). The curves in Fig. 6 are from a single but representative study.

In addition, the effect of binding of ampicillin by media proteins was investigated. NHS and MHB were passed through an ultrafiltration membrane, which excluded proteins with a
molecular weight of 50,000 or greater. Filtration produced a decrease in the serum albumin concentration from 4.4 g per 100-ml prefiltration to 0.3 g per 100 ml. MHB contained no detectable albumin. The rate of enterococcal killing by ampicillin was not affected by the removal of albumin from the NHS (Fig. 7). The killing curve in protein-depleted serum parallels closely the killing curve in NHS. Each curve represents the average values of several experiments with the same representative strain of *S. faecalis* var. *zymogenes*.

**Particle counting.** Bacterial clumping could result in falsely low colony counts by the pour-plate technique. Thus, particle counting studies were performed to ensure that the differences observed in the rate of killing of group D streptococci in NHS, as compared to MHB, and the differences produced by media pH concentrations were not artifactual.
There is a clear relationship between the rate of ampicillin degradation and the alkalinity of either MHB or NHS (Table 1). In MHB the mean \( t_{1/2} \) decreased from 77 to 58 to 21 h at corresponding pH values of 5.0, 7.4, and 9.0. In NHS, the corresponding values were 91, 55, and 7 h at media pH values of 5.0, 7.4, and 9.0. Only at pH 9.0 was there a statistically significant difference in the \( t_{1/2} \) between MHB (21 h) and NHS (7 h, \( P < 0.001 \)). The present results of ampicillin degradation in MHB and NHS at pH 7.4 differ from the much faster degradation rates, reported elsewhere, in citrate phosphate buffer (17).

**DISCUSSION**

**MBC versus killing curves as measure of enterococcal susceptibility to ampicillin.** These studies support the opinion that antibiotic susceptibility testing methods which measure the rate and extent of bacterial killing are superior to determinations of the MBC (6, 18). The discrepancy between these techniques was clearly illustrated by the results of the effect of alkaline pH on the susceptibility of enterococci to ampicillin. The MBC susceptibility studies described above, as well as in previous studies (33), appear to show that enterococci are less susceptible to ampicillin at an alkaline pH. On the other hand, the killing curve data indicates...
Table 1. Effect of media pH on ampicillin degradation at 37°C

<table>
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<tr>
<th>Culture media</th>
<th>Mean t½ ± 2 SE at media pH of</th>
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<tbody>
<tr>
<td></td>
<td>5.0</td>
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<tr>
<td></td>
<td>7.4</td>
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<td></td>
<td>9.0</td>
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<tr>
<td>MHB</td>
<td>77 ± 9 (3)*</td>
</tr>
<tr>
<td>NHS</td>
<td>91 ± 16 (3)*</td>
</tr>
<tr>
<td>Citrate-phosphate buffer</td>
<td>99</td>
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* t½. Time in hours for 50% reduction in ampicillin activity. SE, Standard error.
+ No significant difference between t½ in MHB and NHS, P = 0.1. Number in parentheses indicates number of experiments.
+ Significant difference between t½ in MHB and NHS, P < 0.001.
+ Data from Hou and Poole (17).
+ ND, Not done.

An increased rate of killing at an alkaline pH for the first 6 h of incubation. A major factor in the discrepancy between the MBC and killing curve studies may be the instability of ampicillin at an alkaline pH (Table 1). This is especially true in NHS at pH 9.0, where the half-life of ampicillin is very short (7 ± 2 h). It is under these conditions that regrowth of enterococci was observed at 18 h. This late regrowth of enterococci was not observed in MHB or NHS at pH 5.0 or 7.4, conditions where the t½ of ampicillin is much longer.

Since no data is available on ampicillin stability in MHB and NHS, our data on the stability of ampicillin were compared with published data on the stability of ampicillin in a buffer solution (17). At a media pH of 5.0, the t½ in hours was essentially the same in buffer, MHB, and NHS. However, the t½ in MHB and NHS at pH 7.4 was much longer than the published t½ in buffer. The reported ampicillin t½ in vitro in anephric, nondialyzed patients is 20 h (10), approximately the t½ measured in vitro in a buffer system. The reason for the longer t½ of ampicillin in vitro in MHB and NHS at pH 7.4 is unknown.

A technical point deserves emphasis. The antibacterial activities of several antibiotics against enterococci are reported as being influenced by the pH of the media; i.e., penicillin G, cephalothin, and novobiocin are described as more active at pH 5.0, whereas erythromycin and gentamicin are described as more active at pH 8.5 (33). Because the pH of NHS normally increased upon standing, the pH of NHS must be controlled when NHS is used as a growth medium in antibiotic susceptibility studies. This is easily accomplished by employing a 4% carbon dioxide environment during incubation.

Based on the percentage of organisms surviving 18-h exposure to various ampicillin concentrations, the MBC data suggest that enterococci survive better in NHS than in MHB (Fig. 1 and 2). The killing curve data are contradictory, since the curves indicate the vast majority of enterococci are susceptible to a low concentration of ampicillin, 5 µg/ml in both MHB and NHS. In fact, a smaller percentage of enterococci survive a 6-h exposure to 5 µg of ampicillin per ml in NHS than survive in MHB (Fig. 4). The MBC studies were misleading by obscuring the early rapid ampicillin bactericidal activity against the enterococcus in NHS.

Susceptibility of Group D streptococci to ampicillin in NHS. The data presented suggest an enhanced rate of ampicillin bactericidal activity against the enterococcus in NHS as compared to MHB. Similarly, Bulger and Nelson compared to the rate and extent of killing of one enterococcal isolate by an ampicillin concentration of 20 µg/ml in MHB and NHS (5). Their data shows that after 4 h of incubation there is roughly a 10-fold greater number of bacteria killed in NHS, as compared to the NHB.

Any of several differences between MHB and NHS might explain the increased ampicillin bactericidal activity in NHS. The pour-plate method of bacterial counting might measure fewer viable organisms due to clumping of enterococci in NHS and not in MHB. The particle counting studies failed to support this hypothesis. The osmolality of the culture media is known to affect the activity of ampicillin against Escherichia coli and Proteus mirabilis (13). Hypotonicity of the media enhanced the bactericidal activity of ampicillin against these organisms. Despite the relative hypotonicity of MHB, as compared to NHS, the bactericidal activity of ampicillin against enterococci appears greater in NHS (Fig. 4).

It was considered possible that the greater activity of ampicillin against the enterococcus in NHS was related to the phenomenon in
which there is a paradoxical decrease in penicillin antibacterial activity as the penicillin concentration is increased. Eagle and Musselman first observed that for five of seven strains of *S. faecalis* there was a minimum effective concentration of penicillin G, which produced the maximum rate and greatest absolute amount of enterococcal killing (19). When the concentration of penicillin G was increased beyond this optimal level, the rate and extent to which the organisms died was paradoxically reduced. The optimal killing effect was found within a relatively narrow range of penicillin concentrations. The mechanisms of this phenomenon is unknown.

Approximately 23% of ampicillin in NHS is bound to albumin (2), whereas MHB contains only trace amounts of albumin. If ampicillin is added to NHS and MHB in equal concentration, the unbound (active) ampicillin concentration in NHS would be 23% lower than the unbound ampicillin concentration in MHB. Thus, it seemed conceivable that the enhanced ampicillin bactericidal activity in NHS was due to a lower active antibiotic concentration, which took advantage of the paradoxical phenomenon of Eagle and Musselman. To test this hypothesis, serum was depleted of albumin by membrane ultrafiltration. There was no effect on the rate of enterococcal killing in the albumin-depleted serum (Fig. 7). These observations suggest that other nonidentified factors are responsible for the difference between ampicillin activity in MHB and NHS.

Data has accumulated which suggests that the interaction between the cell walls of susceptible bacteria and penicillin G is an enzymatic reaction (4, 7, 9, 15, 23, 30, 31). At least two bacterial components are known to interact in an enzymatic fashion with penicillin (15, 23). The kinetics of enzymatic reactions are affected by many variable involving the solvent composition, i.e., pH, ionic strength, and other factors (22). The chemical composition of MHB and NHS differs significantly in salt, cation, and protein concentrations. Until the functional importance of these media differences is determined, it would seem judicious to employ NHS, the media which most closely approximates in vitro conditions, as a reference standard for the in vitro susceptibility of enterococci to ampicillin.

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LITERATURE CITED


