Reversal of the In Vitro Susceptibility of Enterococci to Trimethoprim-Sulfamethoxazole by Folinic Acid

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The in vitro susceptibilities of 21 clinical isolates of *Streptococcus faecalis* to trimethoprim (TMP) in combination with sulfamethoxazole (SMX) was evaluated in Mueller-Hinton broth (MHB) in the presence and absence of folinic acid as well as in urine. The mean MIC and MBC in MHB, expressed as the TMP concentration, was 0.13 and 0.32 μg of TMP-SMX per ml, respectively. In MHB supplemented with folinic acid, the mean MIC and MBC was 3.3 and 5.5 μg of TMP-SMX per ml, respectively. In urine the mean MIC of TMP-SMX for these isolates was 8.1 μg/ml (range, 1.6 to 50 μg/ml). All isolates were inhibited by less than 0.01 μg of TMP-SMX per ml when methotrexate was added to the urine.

Enterococci are etiologic agents of various human infections, including urinary tract and wound infections, endocarditis, and polymicrobial bacteremia (7, 9, 13). Despite studies showing in vitro susceptibility of enterococci to trimethoprim (TMP)-sulfamethoxazole (SMX) (3, 12), there are reports documenting treatment failures when TMP-SMX is used as therapy for patients with enterococcal infections (6). Earlier studies have demonstrated the ability of enterococci to become more resistant to TMP by incorporating exogenous folates (2). The extent to which this resistance mechanism quantitatively affects the susceptibility of enterococci and accounts for treatment failures with TMP-SMX has not been determined. MICs and MBCs of TMP-SMX in the presence of increasing concentrations of folate have not been previously measured. Susceptibility testing of *Streptococcus faecalis* to TMP-SMX in urine, where it would most frequently be used, also has not been performed. We therefore report findings obtained with clinical isolates of *S. faecalis* isolated from patients at the University of Michigan Hospitals.

All bacteria tested in this study were clinical isolates of *S. faecalis* from specimens submitted to the microbiology laboratory at the University of Michigan Hospital from 1980 to 1984. Strains were stored at −20°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and glycerol (Fisher Scientific Co., Pittsburgh, Pa.). Included were five strains demonstrating high-level (MIC, >2,000 μg/ml) resistance to gentamicin. The media used for susceptibility testing were as follows: Mueller-Hinton broth (MHB) (Difco) supplemented with 60 μg of Ca^{2+} and 30 μg of Mg^{2+} per ml; MHB supplemented with 60 μg of Ca^{2+} per ml; 30 mg of Mg^{2+} per ml, and folinic acid (Lederle Laboratories, Pearl River, N. Y.); two separate samples of freshly voided filter sterilized human urine; MHB supplemented with Ca^{2+} and Mg^{2+}, and urine added in a 1:1 ratio; and urine adjusted to pHs of 7.4 and 9.0. Thymidine phosphorylase was not added. Folic acid concentrations in urine were determined by radioimmunoassay (Bio-Rad Laboratories, Richmond, Calif.).

Susceptibility to TMP-SMX (Hoffman-La Roche Inc., Nutley, N. J.) was tested by a standardized broth dilution microtiter technique as previously described (10). The inoculum used was 5×10^5 CFU/ml. For dilution testing,

TMP was combined in a 1:20 ratio with SMX. TMP and SMX were tested in doubling concentrations of 0.001 to 100 and 0.02 to 2,000 μg/ml, respectively. The MIC was defined as the level of drug resulting in no visible turbidity or deposit at the bottom of the microtiter well. The MBC was obtained by subculturing 0.01 ml from each well onto antibiotic-free brain heart infusion agar and was defined as the lowest concentration of antibiotic that resulted in approximately 99.9% killing after 18 to 24 h of incubation at 37°C. The MIC and MBC results for the combination of TMP with SMX are expressed as the TMP concentration. Methotrexate (Lederle Laboratories) showed no inhibition of the growth of *S. faecalis* when tested at a concentration of 500 μg/ml in MHB supplemented with Ca^{2+} and Mg^{2+}.

The MICs and MBCs of TMP-SMX for the 21 strains of *S. faecalis* are summarized in Table 1. The mean MIC and MBC of TMP-SMX in MHB were 0.13 μg/ml (range, 0.002 to 0.625 μg/ml) and 0.32 μg/ml (range, 0.004 to 0.625 μg/ml), respectively. TMP-SMX inhibited 50% of all organisms at ≤0.16 μg/ml and 90% of all strains at ≤0.3 μg/ml. When MHB was supplemented with 1 μg of folinic acid per ml, the mean MIC for the same organisms increased to 3.3 μg/ml (range, 0.1 to 6.25 μg/ml) and the mean MBC increased to 5.5 μg/ml (range, 0.1 to 12.5 μg/ml). The MIC for 50% of the strains was 3.1 μg/ml, and that for 90% of the strains was 6.25 μg/ml. All but one isolate became more resistant when folinic acid was added. There were no changes in the susceptibilities of any of the strains when the folinic acid concentration in MHB was increased from 1 to 10 μg/ml. A single isolate was tested for susceptibility to TMP-SMX at increasing concentrations of folinic acid (Fig. 1). At 0.0001 μg/ml, the MIC and MBC of TMP-SMX for the organisms were 0.08 and 0.16 μg/ml, values which were unchanged from the susceptibility in the absence of folinic acid. At 0.001 and 0.01 μg of folinic acid per ml, the MIC and MBC of TMP-SMX were 0.125 and 0.2 μg/ml and 0.8 and 0.8 μg/ml, respectively. At 0.1 to 10 μg of folinic acid per ml, the MIC and MBC of TMP-SMX for the organisms were 3.1 and 3.1 μg/ml, respectively. When antimicrobial susceptibility testing of *S. faecalis* to TMP-SMX was performed in urine, the mean MIC was 8.1 μg/ml (range, 1.6 to 50 μg/ml). All organisms were inhibited by <0.01 μg of TMP-SMX per ml when methotrexate was added at a concentration of 100 μg/ml to the urine before susceptibility testing. Seven isolates with a mean MIC in urine of 13.8 μg/ml (range, 3.1 to 50

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TABLE 1. Susceptibility of 21 clinical isolates of *S. faecalis* to TMP-SMX in MHB supplemented with 1 μg of folinic acid per ml and in urine

Medium	Mean (range) MIC of TMP-SMX (μg/ml)[a]	Mean (range) MBC of TMP-SMX (μg/ml)
MHB	0.13 (0.002–0.625)	0.32 (0.004–0.625)
MHB + folinic acid (1 μg/ml)	3.3 (0.1–12.5)	5.5 (0.1–12.5)
Urine	8.1 (1.6–50.0)	ND[b]

[a] MICs are expressed as TMP concentration.
[b] ND, Not done.

μg/ml) were retested with a separate urine specimen which had a measured pH of 5.6, and osmolality of 954 mosmol/kg, and a folic acid concentration of 20 ng/ml. When retested, the mean MIC for these strains was 46.4 μg/ml (range, 25 to 100 μg/ml) and decreased to 35.7 μg/ml (range, 25 to 100 μg/ml), 16.9 μg/ml (range, 6.25 to 50 μg/ml), and 12.5 μg/ml (range, 12.5 to 50 μg/ml), respectively, when this urine sample was either mixed with MHB in a 1:1 ratio or the ph was adjusted to 7.4 or 9.0.

When tested in a medium relatively free of thymidine and thymine, our results supported those of earlier studies (3, 12) showing enterococci to be readily inhibited by low concentrations of TMP-SMX. We found the mean MIC and MBC of TMP-SMX for 21 clinical isolates of *S. faecalis* to be 0.13 and 0.32 μg/ml, respectively, when tested in Difco MHB. Crider and Colby (3), also using Difco MHB supplemented with Ca^{2+} and Mg^{2+}, tested 131 clinical isolates of *S. faecalis* and found the mean MIC and MBC of TMP-SMX to be 0.016 and 0.031 μg/ml respectively.

Two patients described by Goodhart (6) had uncomplicated enterococcal urinary tract infections and were treated with TMP-SMX at doses based on in vitro susceptibility. Both patients developed bacteremia and were cured only after discontinuation of TMP-SMX and the institution of penicillin G or vancomycin. Goodhart suggested that the reason for these treatment failures might be that, unlike other organisms, enterococci are able to utilize exogenous
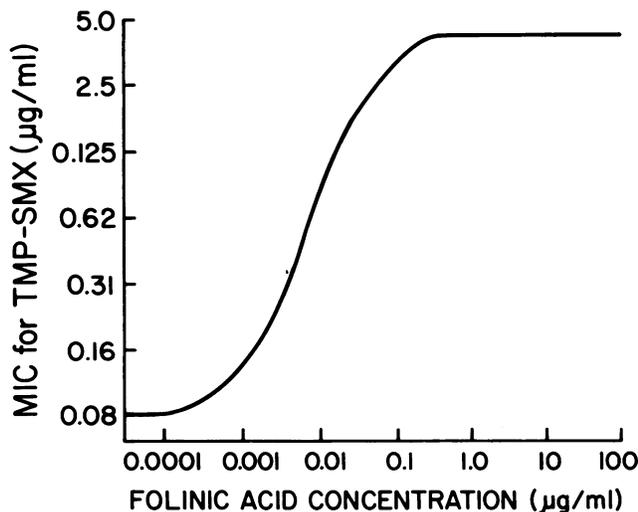

FIG. 1. Effect of folinic acid on the MIC of TMP-SMX for a clinical isolate of *S. faecalis*.

folinic acid and thereby reverse the action of TMP-SMX in vivo (6). The basis for this hypothesis is an earlier study by Bushby and Hitchings (2), who showed that 1 μg of folinic acid per ml in vitro markedly reduced the zone of inhibited growth around a 0.5-μg TMP disk in 16 of 16 enterococci. They also demonstrated that the antibacterial effect of TMP, as expressed by turbidity units of growth of *S. faecalis*, decreased proportionately with the concentration of folinic acid added to the media. This effect occurred at concentrations of folinic acid ranging from 0.1 to 12.5 μg/ml. We extend these findings and show that both the MICs and the MBCs of TMP-SMX increased when MHB was supplemented with folinic acid. Unlike Bushby and Hitchings, however, we found that the response to folinic acid began at as low a concentration as 0.001 μg/ml, and at ≥0.01 μg/ml there was no further response. The response to folinic acid that we observed occurs at concentrations of folinic acid that are achievable in serum or urine, since the normal concentration of folinic acid in serum is 6 to 20 μg/ml and in urine is 2 to 7 μg/ml per 24 h (4, 11). Other evidence that folinic acid inhibits the action of TMP-SMX in vivo is suggested by our finding that the mean MIC of TMP-SMX for the same strains was highest in two separate urine specimens and that this resistance was completely reversed by adding the folic acid analog methotrexate to the urine. We also found that increasing the pH of the urine partially reversed the resistance of enterococci to TMP-SMX, suggesting that variables other than folinic acid concentration may contribute to part of the resistance observed. When urine was added to MHB, the MIC for those strains tested was between that achieved with MHB alone and that achieved with urine alone. This may reflect the combined effect of folinic acid in the urine along with a buffering effect of MHB.

In addition to folinic acid, enterococci are able to utilize exogenous thymidine and thymine. When these substances are added to commercial media, the in vitro activity of TMP-SMX is reduced (1, 8). Thymidine, however, probably did not contribute to the resistance of enterococci to TMP-SMX in urine, since in an earlier study (5) thymidine did not appear to affect the in vivo activity of TMP-SMX. Large doses of thymidine were degraded rapidly when given to hamsters and did not affect the antimicrobial activity (5).

Our findings suggest that the results of in vitro susceptibility testing of enterococci to TMP-SMX, when performed in MHB, may be misleading and not reflect in vivo susceptibility. Since folinic acid in vitro and, potentially, in vivo reverses the antibacterial effect of TMP-SMX, interpretation of susceptibility tests should be made with caution when this fixed combination is used to treat patients with enterococcal infections.

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