ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1974, p. 93-97

Vol. 6, No. 1
Printed in U.S.A.

Lack of Activity of Sulfamethoxazole and Trimethoprim Against Anaerobic Bacteria

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Received for publication 15 April 1974

The activity of sulfamethoxazole (SMX), trimethoprim (TMP), and the combination of the two was determined against a variety of anaerobic bacteria. Brucella agar was somewhat inhibitory for SMX and TMP but activity was good and equivalent in Diagnostic Sensitivity Test Agar (Oxoid) and Mueller-Hinton agar and the latter was selected for use in these studies. Agar dilution susceptibility tests showed that 95 of 98 anaerobic isolates were resistant to ≥ 100 μg of SMX per ml and 85 were resistant to ≥ 6.25 μg of TMP per ml. "Checkerboard" agar dilution studies of combined activity showed that 66 of 72 isolates were resistant to $\geq$ (100 μg of SMX per ml + 6.25 μg of TMP per ml) and only six isolates were susceptible to the synergistic activity of the combination. The majority of 32 isolates tested by the disk diffusion method were also resistant to SMX and TMP individually and to the combination 25-μg disk. Correlation between agar dilution minimal inhibitory concentration and disk zone size results was in general good for individual agents. Four *Bacteroides fragilis* isolates were inhibited by the combination 25-μg disk but were resistant to SMX + TMP by agar dilution "checkerboard." This discrepancy may have been due to different incubation periods since disk results also showed resistance when read after 48 h (as is done with agar dilution) rather than the standard 24 h for disk tests. These studies suggest that SMX and TMP, either individually or in combination, are not active against the great majority of anaerobic bacteria.

Co-trimoxazole is an antimicrobial agent recently introduced into the United States for the treatment of chronic urinary tract infections due to susceptible organisms, primarily gram-negative bacilli. This agent has also been used for some time in England and Europe for the treatment of pulmonary infections (4), typhoid fever (2), and gonorrhea (9). Co-trimoxazole is a fixed ratio combination of sulfamethoxazole (SMX) and trimethoprim (TMP). Antimicrobial activity depends upon synergism between SMX and TMP which both interfere with bacterial folic acid activity in the synthesis of nucleic acids, though at different sites (7).

We decided to investigate the potential for use of co-trimoxazole in the treatment of anaerobic infections by studying the activity of SMX and TMP individually and in combination against a variety of anaerobic bacteria. Since the in vitro activity of SMX and TMP is known to be media dependent (12) and since growth of anerobes may vary on different media we initially had to determine the most appropriate medium for these susceptibility studies. Subsequently, we used agar dilution and disk diffusion methods to determine the activity of SMX and TMP against anaerobes.

MATERIALS AND METHODS

Antimicrobials and bacteria. TMP lactate was provided as standard laboratory powder by Burroughs Wellcome and Co., Inc. SMX standard powder was obtained from the Hoffmann-La Roche Co., Inc. Stock solutions of each antimicrobial were prepared in concentrations 10 to 20 times the desired final concentration by using sterile distilled water as the diluent. Addition of a few drops of 1 N NaOH was necessary to dissolve the SMX. Portions of each antimicrobial were dispensed and frozen. Individual TMP (1.25 μg) and SMX (23.75 μg) disks were supplied by Burroughs Wellcome and Co. Combined SMX + TMP (25 μg) disks were manufactured by Difco.

The 98 anaerobe strains tested were stock cultures of the Wadsworth Anaerobe Laboratory. Seventy-three were clinical isolates and 25 were from the normal human fecal flora.

Media and procedures. Thioglycolate broth (without indicator), brucella agar (BA), and brucella broth were BBL products and Mueller-Hinton agar (MHA)

and broth were Difco products. Diagnostic sensitivity test agar (DST) and broth were manufactured by Oxoid Ltd. Fresh defibrinated horse blood was lysed by alternate freezing and thawing. Stock solutions of vitamin K_1, (Nutritional Biochemicals Corp.) 100,000 μg/ml, were prepared in ethanol and refrigerated. MHA and DST were supplemented with 5% lysed horse blood and vitamin K_1 (10 μg/ml), whereas BA was supplemented with 5% sheep blood and vitamin K_1 (10 μg/ml).

Agar dilution (Steer's replicator [10]) and disk diffusion susceptibility tests were performed by using methods described by Sutter et al. (11). Studies of combined SMX + TMP activity were done by the "checkerboard" method by using fixed and varying concentrations of each antimicrobial as described by Sabath (8). SMX in concentrations of 200, 100, 50, and 25 μg/ml was combined with TMP in concentrations of 12.5, 6.25, 3.12, and 1.56 μg/ml. For example, combination plates contained TMP (12.5 μg/ml) and SMX (200 μg/ml), TMP (12.5 μg/ml) and SMX (100 μg/ml), and TMP (12.5 μg/ml) and SMX (50 μg/ml), etc. Disk zone of inhibition diameters were measured by using vernier calipers and agar dilution minimum inhibitory concentrations (MIC) were read as the lowest concentration showing complete inhibition of growth. In a few instances the MIC was read as that concentration allowing growth of three or four colonies when this represented a drastic inhibition of growth compared to the next lower concentration. Anaerobic incubation was carried out by using the GasPak system (BBL). Agar dilution plates were read after 48 h of incubation, and disk plates were read at 24 h (data reported in Results) and again at 48 h.

RESULTS

Anaerobes studied. Table 1 shows the number and kinds of anaerobic isolates studied. The susceptibility of 98 different isolates to SMX and TMP individually was determined by the agar dilution method. Seventy-two of these were included in "checkerboard" studies of combined SMX + TMP activity. A total of 32 isolates was studied by the disk diffusion method and 10 of these were *Bacteroides fragilis*.

Determination of appropriate medium. Initially, the activity of SMX and TMP in BA and MHA was studied by comparing the susceptibility of all 98 isolates in both media by using the agar dilution method. A difference of at least two dilutions in the MIC was considered evidence of a significant difference in antimicrobial activity in the two media. Table 2 shows that SMX was less active in BA than in MHA against 6 of 98 isolates and that TMP was less active in BA against 10 isolates. On the other hand, neither SMX nor TMP were less active in MHA than BA against any of the anaerobes. On the basis of these data suggesting decreased SMX and TMP activity in BA, this medium was eliminated from the study.

Similar agar dilution studies were carried out to compare the activity of SMX and TMP in MHA and DST against 20 anaerobic isolates (Table 3). SMX and TMP were less active in DST than in MHA against one and two isolates, respectively. On the other hand, in no instance was either agent less active in MHA than in DST. On the basis of these data, we determined that there was no significant difference in antimicrobial activity between these two media, and selected MHA as our standard medium for

TABLE 1. *Anaerobes studied for susceptibility to SMX and TMP*

Anaerobes	Agar dilution	Disk diffusion
Bacteroides fragilis	38[a] (26)[b]	10[c]
Bacteroides melaninogenicus	4 (4)	4
Bacteroides oralis	4 (3)	1
Fusobacterium mortiferum	4 (3)	2
Fusobacterium necrophorum	4 (3)	1
Fusobacterium nucleatum	2 (1)	1
Fusobacterium varium	4 (3)	1
Clostridium perfringens	8 (6)	3
Clostridium ramosum	4 (3)	2
Eubacterium limosum	4 (3)	1
Peptococci	7 (6)	1
Peptostreptococci	12 (8)	5
Propionibacterium acnes	3 (3)	0
Total	98 (72)	32

[a] Number of isolates studied for susceptibility to SMX and TMP individually.

[b] Number of isolates studied by the "checkerboard" method for susceptibility to combined activity of SMX + TMP.

[c] Number of isolates studied for susceptibility to individual and combined activity of SMX and TMP by disk diffusion method.

TABLE 2. *Differences in activity of SMX and TMP in different media*[a]

Antimicrobial act in:	SMX	TMP
BA[b] < MHA[c]	6[d]	10
MHA < BA	0[e]	0

[a] Activity studied by agar dilution determination of MIC against 98 anaerobic isolates.

[b] Brucella agar.

[c] Mueller-Hinton agar.

[d] Number of isolates against which SMX (or TMP) was less active in BA than in MHA.

[e] Number of isolates against which SMX (or TMP) was less active in MHA than in BA.

testing susceptibility of anaerobes to SMX and TMP. All subsequently described studies were carried out by using MHA.

Susceptibility of anaerobes to SMX and TMP. Table 4 shows the susceptibility of 98 anaerobic isolates to SMX and TMP, tested by the agar dilution method. Only three isolates had an MIC of SMX $\leq$ 25 μg/ml and would be considered susceptible. The other 95 isolates were resistant to $\geq$ 100 μg/ml. Likewise, only 13 isolates were susceptible to $\leq$ 3.12 μg of TMP per ml (susceptible), whereas 85 were resistant to $\geq$ 6.25 μg/ml. These data indicate that the great majority of anaerobes studied were resistant to SMX and TMP as individual agents.

The susceptibility of 72 anaerobic isolates to the combined activity of SMX + TMP as tested by the agar dilution "checkerboard" method is shown in Table 5. There was no demonstrable synergistic activity between the two agents against 66 isolates. Only six isolates (two *Clostridium ramosum*, two *Peptostreptococcus intermedius*, and two *Bacteroides melaninogenicus*) were susceptible to SMX + TMP synergy by Sabath's criteria (8). These were inhibited by concentrations of SMX and TMP (in combination) which would be considered therapeutically achievable and which approximate the 20:1 concentration ratio thought to be optimum for synergism (25 μg of SMX per ml and 1.56 μg of TMP per ml). The 66 resistant isolates were inhibited only by concentrations of SMX and TMP $\geq$ (100 μg/ml and 6.25 μg/ml), respectively.

TABLE 3. *Differences in activity of SMX and TMP in different media*[a]

Antimicrobial act in:	SMX	TMP
DST[b] < MHA[c]	1[d]	2
MHA < DST	0[e]	0

[a] Activity studied by agar dilution determination of MIC against 20 anaerobic isolates.
[b] Oxoid diagnostic sensitivity test agar.
[c] Mueller-Hinton agar.
[d] Number of isolates against which SMX (or TMP) was less active in DST than in MHA.
[e] Number of isolates against which SMX (or TMP) was less active in MHA than in DST.

TABLE 4. *Susceptibility of 98 anaerobic isolates to SMX and TMP*[a]

Determination	SMX		TMP	
MIC[b] (μg/ml)	$\leq$25	$\geq$100	$\leq$3.12	$\geq$6.25
No. isolates	3[c]	95[d]	13[e]	85[f]

[a] Agar dilution method using MHA.
[b] Minimum inhibitory concentration.
[c] Number of isolates considered susceptible to SMX.
[d] Number of isolates considered resistant to SMX.
[e] Number of isolates considered susceptible to TMP.
[f] Number of isolates considered resistant to TMP.

TABLE 5. *Susceptibility of 72 anaerobic isolates to the combined activity of SMX + TMP*[a]

Determination	Synergism	No synergism
MIC[b] (μg/ml)	$\leq$ SMX 25, TMP 1.56	$\geq$ SMX 100, TMP 6.25
No. isolates	6[c]	66[d]

[a] Agar dilution "checkerboard" method using MHA.
[b] Minimum inhibitory concentration.
[c] Number of isolates against which SMX + TMP showed synergistic activity.
[d] Number of isolates against which SMX + TMP did not show synergistic activity.

The results of disk diffusion susceptibility tests with 32 isolates are shown in Table 6. There are no zone size standards for interpretation of disk tests with SMX, TMP, and the combination. However, 24, 28, and 16 isolates had no inhibition zones when tested with SMX (23.75 μg), TMP (1.25 μg), and combination (25 μg) disks, respectively. These isolates would certainly be considered resistant. Eight, four, and five isolates exhibited inhibition zones $\geq$ 13 mm, with SMX, TMP, and combination disks, respectively. These isolates might be considered susceptible or "moderately resistant" in some instances. There were 11 other isolates with zones around the combination disk but these could be attributed to inhibitory activity of one of the individual component agents rather than synergism of the combination. It should be noted that although the term "synergism" is used to denote enhanced activity with the combination disk, in some instances this activity may be due to additive antimicrobial activity rather than true synergism.

Correlation between MIC and zone sizes for individual agents was generally good. The exceptions were three isolates (two peptostreptococci and a *B. melaninogenicus*) with SMX MIC of $\geq$ 100 and SMX disk zones of 32.3 to 47.0 mm. There is no ready explanation for this discrepancy. Perhaps it was related to poor growth of these isolates on the disk susceptibility plates (although there were no zones around TMP disks in the two isolates with high TMP

MIC) or variability in the interpretation of SMX inhibition of growth on the agar dilution or disk plates or both. Table 7 shows the correlation between MIC and disk zones in those five isolates showing susceptibility to a synergistic effect of SMX + TMP; i.e., the combined disk had an inhibition zone, whereas the individual disks did not. All four *B. fragilis* isolates failed to show susceptibility to SMX + TMP synergism by the agar dilution method. Only against the single *B. melaninogenicus* isolate did the SMX + TMP combination show synergism by both methods. There were six other *B. fragilis* isolates which were totally resistant by the disk method and all *B. fragilis* isolates were totally resistant by agar dilution. An explanation for the discrepancy with disk testing of *B. fragilis* may be found in the time of reading of results. Disk susceptiblity test results were read (and reported here) after 24 h of incubation and in most cases this correlated well with a subsequent 48-h reading. However, with the four *B. fragilis* isolates listed in Table 7, the zone around the combined SMX + TMP disk was markedly reduced at 48 h. In fact, in three of the four isolates there was no zone at 48 h. Therefore, the 48-h disk test reading appears to correlate best with results obtained by agar dilution (read at 48 h), which indicate a lack of any synergistic activity of SMX + TMP against *B. fragilis*.

In spite of these possible discrepancies in results, the great majority of anaerobes, when tested by either the agar dilution or disk diffusion methods, were resistant to SMX and TMP individually and to the combination. No specific anaerobe was consistently susceptible.

TABLE 6. *Susceptibility of 32 anaerobic isolates to SMX, TMP, and the combination by the disk diffusion method*

Zone of inhibition diameter (mm)	SMX (23.75 μg[a])	TMP (1.25 μg)	SMX + TMP (25 μg)
6[b]	24[c]	28	16
≥13	8	4	5[d]

[a] Disk content of each antimicrobial.
[b] No zone of inhibition present since disk diameter is 6 mm.
[c] Number of isolates in each zone size category.
[d] Eleven other isolates had zone sizes in this category which were attributable to the individual activity of SMX or TMP rather than synergism of the combination.

DISCUSSION

This study demonstrates that neither SMX nor TMP, individually or in combination, have

TABLE 7. *Comparative susceptibility of five anaerobic isolates to SMX, TMP, and the combination by the agar dilution[a] and disk diffusion methods*

Anaerobe isolates	MIC[b] (μg/ml)			Disk zone (mm)		
	SMX	TMP	SMX + TMP	SMX 23.75 μg[c]	TMP 1.25 μg	SMX + TMP 25 μg
Bacteroides melaninogenicus	>100	>6.25	25 1.56[d]	6[e]	6	19.5[f]
Bacteroides fragilis #1	>100	>6.25	>100 >6.25	6	6	18.5
Bacteroides fragilis #2	>100	>6.25	>100 >6.25	6	6	15.3
Bacteroides fragilis #3	>100	>6.25	100 3.12	6	6	18.5
Bacteroides fragilis #4	>100	>6.25	>100 >6.25	6	6	13.5

[a] "Checkerboard" method using MHA.
[b] Minimum inhibitory concentration (results read after 48 h of incubation).
[c] Content of antimicrobial in each disk.
[d] Combined MIC of ≤25 μg of SMX per ml and 1.56 μg of TMP per ml indicates synergism and ≥100 μg of SMX per ml and 6.25 μg of TMP per ml indicates no synergism.
[e] No zone of inhibition present since disk diameter is 6 mm.
[f] Zone sizes when read after 24 h of incubation.

significant antimicrobial activity against a variety of anaerobic bacteria. These findings are similar to those of Bushby (1) who could not demonstrate increased susceptibility of three strains of Bacteroides (unspeciated) to the SMX + TMP combination. These strains were reported to be resistant to TMP but susceptible to SMX. Näff's (5) demonstration that feeding of co-trimoxazole to human volunteers eliminated Enterobacteriaceae from their fecal flora but did not affect Bacteroides also suggests lack of activity against these anaerobes. On the other hand, Okubadejo et al. (6) reported inhibition of more than 100 Bacteroides strains by 25-μg co-trimoxazole disks and inhibition of all 60 *B. fragilis* strains tested by an agar dilution method. This report, however, is in the form of a "letter-to-the-editor" and minimal information is provided. The authors do state use of DST and anaerobic incubation in an atmosphere containing 95% H_2 and 5% CO_2. The discrepancies between these results and our own are not easily explained. We demonstrated that results in DST and MHA (used in our studies) should be similar and our anaerobic incubation (GasPak system) should have provided a comparable atmosphere. Supplementation of our media with vitamin K_1, use of a standard heavy inoculum, and incubation of agar dilution plates for 48 h may have produced heavier anaerobic growth than that obtained by Okubadejo et al. This in turn would tend to result in diminished antimicrobial activity. Our methods are similar to those used by Sutter et al. (11) in extensive investigations of the susceptibility of anaerobes to various antimicrobials.

These studies have further demonstrated that BA, a medium recommended for anaerobe susceptibility testing, is not suitable for use with SMX and TMP because of inhibitory activity. Demonstration of adequate anaerobic growth on MHA (containing 10 μg of vitamin K_1 per ml) allowed us to use this medium, which when supplemented with 5% lysed horse blood is not inhibitory for SMX and TMP. Koch and Burchall (3) have recently shown that high concentrations of thymidine in commercially prepared media can reverse the antimicrobial activity of TMP. Although BA was not included in that study, a similar enriched media which allows good anaerobic growth (brain heart infusion) contained as much as 30.9 μg of thymidine per ml, whereas DST and MH broth formulations both contained less than 1.0 μg of thymidine per ml. Excess thymidine content may be one of the factors contributing to BA inhibitory activity against SMX and TMP.

In conclusion, our studies indicate that the majority of anaerobes are not susceptible to SMX, TMP, or the combination, and suggest that co-trimoxazole will not be useful for treatment of anaerobic infections. There is at least one other conflicting report indicating susceptibility of *B. fragilis* to co-trimoxazole. However, this agent should not be used to treat anaerobic infections until further experience with in vitro studies or experimental infections provides information which will help to resolve these questions.

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