ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1976, p. 736-740

Vol. 9, No. 5
Printed in U.S.A.

Activity of Sulfamethoxazole and Trimethoprim Against *Bacteroides fragilis*

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Received for publication 15 December 1975

Minimum inhibitory concentrations (MICs) of sulfamethoxazole (SMX) and trimethoprim (TMP), alone and in three combinations, 20:1, 1:1, and 1:20, were determined on Diagnostic Sensitivity Test (DST) and Mueller-Hinton (MH) agars containing lysed blood for various inocula of 91 strains of *Bacteroides fragilis* from the U.S.A. and U.K. MICs of SMX were high with large inocula and higher on MH than DST, but results for TMP were less affected by these two factors. True SMX resistance was rare: 10 U.S.A. strains previously reported as resistant appeared to be susceptible. Maximum potentiation of MICs was observed when SMX and TMP were combined in ratios close to those of the ratios of their MICs, that is, SMX/TMP 20:1 for large inocula and the reverse for small inocula for determinations on DST and usually 20:1 for all inoculum sizes on MH. These observations explain some of the discrepancies in reports, but defer the problem of potential usefulness of the drugs in the treatment of infection with anaerobes to future study.

There is disagreement on the level of inhibitory activity of sulfamethoxazole (SMX) and trimethoprim (TMP) against *Bacteroides fragilis*. Rosenblatt and Stewart (4) concluded that SMX and TMP, "either individually or in combination are not active against the great majority of anaerobic bacteria." Among the resistant organisms in their study were 38 isolates of *B. fragilis*. In contrast, we concluded that 49 isolates of *B. fragilis* from St. Thomas' Hospital, London, were susceptible to SMX and that there was evidence of potentiation when SMX and TMP were combined in suitable ratios (3). To explain the discrepancies, we have compared some of Rosenblatt and Stewart's strains isolated in the U.S.A. with some, mostly our own, isolated in the U.K.

MATERIALS AND METHODS

Bacteria. The complete collection consisted of 91 isolates of *B. fragilis*. Ten of them that were included in Rosenblatt and Stewart's study were kindly provided by Vera Sutter and were reported to be sulfonamide resistant (U.S.A. strains). British isolates (U.K. strains) included five strains from the National Collection of Type Cultures, NCTC 8560, 9343, 9344, 10534, and 10581 (NCTC strains); two sulfonamide-resistant strains, L284 and L22711, provided by A. T. Willis of the Public Health Laboratory, Luton (L strains); five isolates from blood, BC1-5, included in our previous study; and a further 69 fresh isolates from patients in St. Thomas' Hospital (STH strains).

Antibacterial agents and susceptibility testing. SMX and TMP-lactate were supplied as powders of known purity by the Wellcome Foundation Ltd. Minimum inhibitory concentrations (MICs) of each agent were determined by an agar dilution method. Two media were used: Diagnostic Sensitivity Test (DST) agar (Oxoid CM261) and Mueller-Hinton (MH) agar (Oxoid CM337), both supplemented by the addition of 6% saponin-lysed horse blood. The antimicrobial agents were incorporated in doubling dilutions, SMX from a final concentration of 2,048 μg/ml and TMP from 512 μg/ml. For studies of combinations, the two agents were incorporated in either DST or MH blood agar in suitable doubling dilutions with ratios of SMX/TMP of 20:1, 1:1, and 1:20. Inocula were prepared by suspending growth from blood agar plates, incubated overnight in GasPak jars (BBL), in nutrient broth (Southern Group Laboratories) containing 6% saponin-lysed horse blood. These suspensions were suitably diluted so that a multiple inoculator would deliver the required inoculum, which varied between 10^2 and 10^8 colony-forming units (CFU) in different experiments. In the absence of recognized control strains, we used *Escherichia coli* NCTC 10418 and the Oxford staphylococcus as controls. With an inoculum of about 10^4 CFU these were susceptible to 8 and 32 μg of SMX per ml and 0.25 and 1 μg of TMP per ml, respectively, under anaerobic conditions in GasPak jars.

Plates were examined after incubation for 24 and 48 h in GasPak jars, and MICs were read as the lowest concentration of antimicrobial agent that caused complete inhibition of growth or that permitted growth as a fine haze (5).

These methods were used in three series of experiments.

Series I. Ten U.K. strains (BC1-5 and five NCTC strains) and five U.S.A. strains were used in inocula of 10^2, 10^4, and 10^6 CFU on both DST blood agar and MH blood agar containing SMX and TMP separately, and MICs were read after 24 and 48 h of incubation.

Series II. MICs of SMX were determined for inocula of 10^4 CFU of all 91 strains on DST blood agar. Results were read after overnight incubation of the plates. Twenty-five strains were tested more than once (between two and six times for individual strains).

Series III. MICs of SMX and TMP separately and in the three combinations were determined for inocula of 10^2, 10^4, and 10^6 CFU on DST blood agar and MH blood agar. Twenty-two strains were used in studies on DST blood agar: five NCTC strains, two sulfonamide-resistant L strains, STH BC1-5, and 10 U.S.A. strains. Ten strains were studied on MH blood agar: STH BC1-5 and five U.S.A. strains. Fractional inhibitory concentrations (FICs) were calculated as the MIC of the agent in combination with the other divided by the MIC of the agent acting alone. The sum of the FICs of SMX and TMP was the FIC index (1):

$$\text{FIC index} = \left[\frac{(\text{MIC of SMX in combination})}{(\text{MIC of SMX alone})}\right] + \left[\frac{(\text{MIC of TMP in combination})}{(\text{MIC of TMP alone})}\right]$$

Indexes of less than 1 indicated potentiation, and the lower the figure the greater the potentiation.

RESULTS

Series I. Table 1 shows the results of this series of experiments. MICs of SMX were affected by inoculum size, and when 10^6 CFU was used many strains appeared resistant. With an inoculum of 10^4 CFU all 15 organisms were susceptible in tests on DST blood agar, with MICs between 1 and 8 μg/ml, whereas on MH blood agar the MICs for 10^4 CFU were between 8 and 64 μg/ml. Incubation for 48 h made little difference. The sulfonamide-susceptible control organisms behaved in the same way with regard to both inoculum size and the medium used. MICs of TMP were much less affected by inoculum size in tests in DST blood agar than MH blood agar. In the latter, inocula of 10^6 CFU were particularly resistant, but with 10^4 CFU results were the same in both media. Incubation for 48 h made little difference in the results.

Series II. Table 2 shows SMX MICs for an inoculum of 10^4 CFU of all 91 organisms. MICs were between 1 and 64 μg/ml for all except the two resistant L strains, which were inhibited by 128 and 2,048 μg/ml, respectively. Excluding these two strains, 94% were inhibited by 2 to 32 μg/ml and 72% were inhibited by 4 to 16 μg/ml. All 10 U.S.A. strains were susceptible to SMX, with MICs between 1 and 8 μg/ml. Repeated testing of 25 strains showed that, despite at-

TABLE 1. *Effect of medium, inoculum size, and duration of incubation on MICs of SMX and TMP for 15 strains[a] of B. fragilis*

Antibacterial agent	Medium	Inoculum size (CFU)	Duration of incubation (h)	No. of isolates with MIC (μg/ml of medium) of:										
				≥512	256	128	64	32	16	8	4	2	1	0.5
Sulfamethoxazole	DST	10^6	24	4[b,c]	1				1	1	8			
		10^4								3	3	7	2	
		10^2								1	2	5	4	3
	MH	10^6	24		4	10		1						
		10^4					2	4	6	3				
		10^2						1	1	8	4			
	MH	10^6	48		13	1		1						
		10^4					2	8	2	3				
		10^2						2	2	11				
Trimethoprim	DST	10^6	24					2	8	4	1			
		10^4							1	4	8	2		
		10^2									7	6	1	
	MH	10^6	24		2	8		1	3	1				
		10^4								9	6			
		10^2								4	11			
	MH	10^6	48		2	8	1		4					
		10^4							4	8	3			
		10^2								8	7			

[a] Five NCTC strains, BC1-5, and five U.S.A. strains.

[b] Each figure represents the number of isolates inhibited by the concentrations of drug at the head of the column.

[c] Actual results, >2,048 μg/ml.

TABLE 2. *MICs of sulfamethoxazole for 10 isolates of B. fragilis from the U.S.A. hitherto thought to be resistant, 79 susceptible strains from Great Britain, and 2 known resistant isolates*[a]

Source	No. of isolates	No. of isolates with MIC (μg/ml) of:											
		2,048	1,024	512	256	128	64	32	16	8	4	2	1	0.5
U.S.A.	10									4	3	2	1	
U.K.	79						3	11	20	19	18	7	1	
L[b]	2	1				1								

[a] Inoculum was 10^4 CFU.

[b] Luton Public Health Laboratory sulfonamide-resistant strains.

tempts at standardization of the medium, inoculum, and incubation time, results for individual strains varied over a wide range. Fourfold differences in MICs determined on different occasions for a given isolate were not unusual, and 8- and 16-fold differences were occasionally seen.

Series III. Results for this series of experiments are shown in Table 3. The strains are divided into two groups for each inoculum: those more susceptible to TMP than SMX (group A) and those equally susceptible to both or more susceptible to SMX (group B). Maximum potentiation between SMX and TMP would be predicted, from the ratios of the MICs, for mixtures containing more SMX for group A and for mixtures containing an equal amount or more of TMP for group B.

On DST blood agar (Table 3), because of the considerable inoculum effect with SMX but not TMP, many more organisms fell into group A with an inoculum of 10^6 CFU than with 10^4 or 10^2 CFU. With 10^6 CFU, of 11 organisms more susceptible to TMP than SMX, 10 were observed to have a lowest FIC index for a 20:1 mixture of SMX and TMP. On the other hand, of 11 organisms more susceptible to SMX than TMP, 10 had lowest FIC indexes for 1:1 or 1:20 mixtures of SMX and TMP. Results for 10^2 CFU show the same contrast. Only four strains, including both L strains and two STH strains but none of the U.S.A. strains, were more resistant to SMX than TMP, and three of these had lowest FIC indexes with a 20:1 mixture of SMX to TMP. The remaining 18 strains were as susceptible (3 strains) or more susceptible (15 strains) to SMX, and the best ratios of SMX and TMP in combination were 1:1 or 1:20 for 17 of them. Thus observed results for combinations of the two drugs accord well with predictions from MICs of each agent alone.

Results on MH blood agar (Table 3) were similar to those on DST blood agar for an inoculum of 10^6 CFU, with five strains in each group. However, with an inoculum of 10^4 CFU, in keeping with the observation that MICs of SMX were almost always higher than those of TMP on this medium, a 20:1 mixture of SMX and TMP showed maximum potentiation for all 10 strains, although one would have been predicted to have an optimum of 1:1. With 10^2 CFU, although 6 of the 10 strains were as susceptible to SMX as TMP (none were more susceptible to SMX in contrast with results on DST blood agar), maximum potentiation was observed with a ratio of SMX to TMP of 20:1 for all strains.

In all cases the optimal FIC index was between 0.1 and 0.4, whereas that for *E. coli* was 0.02 to 0.04. This indicates that although there is potentiation between SMX and TMP in appropriate ratios acting on *B. fragilis*, it is of a considerably lower degree than that observed with *E. coli*. In tests on MH blood agar, there was usually some degree of potentiation whatever the ratio of the two drugs, whereas in tests on DST blood agar there was often no potentiation with inappropriate ratios.

Actual concentrations of SMX and TMP in combination required for inhibition were usually of the order of 0.25 to 1 μg/ml of each, in 1:1 mixtures. The two resistant strains were inhibited by 8 to 64 μg of SMX per ml and 0.4 to 3.2 μg of TMP per ml in 20:1 mixtures and 1 to 8 μg of each per ml in 1:1 mixtures, depending on inoculum size.

DISCUSSION

The reported differences in the susceptibility to sulfonamides and TMP of *B. fragilis* isolated in the U.S.A. and Britain can probably be explained by differences in technique, particularly in inoculum size and medium, and in the proportions of SMX and TMP in tests on combinations. The duration of incubation may also have been of minor importance.

The main factor leading to the conclusion by Rosenblatt and Stewart (4), that *B. fragilis* is usually resistant to sulfonamides, appears to be the inoculum size that they used. We calculate that a Steers replicator would deliver 10^5 to 10^6 CFU from a culture of opacity equivalent to a McFarland no. 1 standard and containing therefore 3×10^8 CFU/ml. This is the inoculum

TABLE 3. *Influence of culture medium on the optimal combinations of sulfamethoxazole and trimethoprim required to achieve a standard level of growth inhibition*

Relationship of MICs for SMX and TMP	Evaluation of 22 strains tested on DST blood agar					
	10^{6a}		10^4		10^2	
	20:1[b]	1:1 or 1:20	20:1	1:1 or 1:20	20:1	1:1 or 1:20
SMX > TMP	10	1	4	2	3	1
SMX ≤ TMP	1	10	1	15	1	17
Evaluation of 10 strains tested on MH blood agar						
SMX > TMP	5	0	9	0	4	0
SMX ≤ TMP	0	5	1	0	6	0

[a] Inoculum (colony-forming units).
[b] Optimal ratio of SMX/TMP in combination.

recommended by Sutter and Washington (5) for susceptibility testing of anaerobes and that used by Rosenblatt and Stewart (4). Our results, in keeping with those of others for other organisms (6), show that even sulfonamide-susceptible strains may not be inhibited by 1,000 μg or more of SMX per ml when inocula of 10^6 CFU are used. This is presumably because the organisms in large inocula are able to multiply to produce visible growth during the lag period before sulfonamide begins to inhibit them. TMP MICs and sulfonamide MICs in combinations with TMP are both also affected by inoculum, but to a lesser degree. An inoculum effect was also seen with two sulfonamide-resistant strains, but for inocula of 10^4 CFU these SMX-resistant strains were clearly more resistant than the rest on DST blood agar. It is also possible that the method of preparing the inoculum affects results. Rosenblatt and Stewart grew the organisms in a liquid medium, whereas we scraped organisms from the surface of solid medium and suspended them in a liquid medium. We did not test the two methods in parallel, but our results for other antibiotics are similar to those determined by the method of Sutter and Washington (2, 5).

The second important factor influencing the results of susceptibility testing is the nature of the medium. Rosenblatt and Stewart concluded that MH blood agar and DST blood agar gave similar results for SMX, but did not quote MICs. In contrast, we found SMX MICs four- to eightfold higher on MH blood agar whereas TMP was equally active in both media. It is, however, quite possible that our MH differed from theirs.

The third and least important factor influencing MICs is duration of incubation. We found that a doubling of MIC was not uncommon after 48 h compared with 24 h with MH medium, the only one that we tested in this way.

Rosenblatt and Stewart concluded that there is little evidence of potentiation between SMX and TMP. Theoretically, maximum potentiation is to be expected when the two agents are combined in the ratio of their MICs. Using their strains and MH medium, we found evidence of potentiation for all inoculum sizes and maximal potentiation with a 20:1 mixture of SMX and TMP. This would be predicted from MICs, which were two to four times greater for SMX than TMP on MH blood agar. In disk tests the standard ratio of 20:1 SMX to TMP was used by Rosenblatt and Stewart, and we agree that no potentiation is shown by the use of such disks (3).

On DST blood agar, with SMX MICs considerably lower than on MH but TMP MICs the same, the predicted best ratio was, usually, SMX to TMP 1:2 to 1:4 or more except with very large inocula, when the reverse was found. The observed optima were in accord with predictions. For large inocula, a ratio of SMX to TMP of 20:1 was optimum, whereas for smaller inocula ratios of 1:1 or even 1:20 were better.

We conclude that in the U.K., and possibly in the U.S.A., *B. fragilis* is normally susceptible to sulfonamides. The exact level of activity depends on the inoculum and the medium used, and organisms may thus appear to be either more or less susceptible to sulfonamides than to TMP, whose activity is much less affected by these two factors. Sulfonamide MICs in combinations with TMP are similarly affected by inoculum, making both predictions and actual observations on optimum ratios of the two drugs difficult. In our system, with DST blood agar and an inoculum of 10^4 CFU, predicted and observed optimum mixtures contain more TMP than SMX. It now remains to establish the validity of our conclusions in clinical trials, perhaps with mixtures of SMX and TMP in ratios other than those at present commercially available.

LITERATURE CITED

1. **Elion, G. B., S. Singer, and G. H. Hitchings.** 1954. Antagonists of nucleic acid derivatives. J. Biol. Chem. 208:477-488.
2. **Phillips, I.** 1974. Antibiotic sensitivity of non-sporing anaerobes, p. 37-57. *In* I. Phillips and M. Sussman (ed.), Infection with non-sporing anaerobic bacteria. Churchill Livingstone, Edinburgh and London.
3. **Phillips, I., and C. Warren.** 1974. Susceptibility of *Bacteroides fragilis* to trimethoprim and sulphamethoxazole. Lancet 1:827-829.
4. **Rosenblatt, J. E., and P. R. Stewart.** 1974. Lack of

activity of sulfamethoxazole and trimethoprim against anaerobic bacteria. Antimicrob. Agents Chemother. 6:93–97.

5. **Sutter, V. L., and J. A. Washington II.** 1974. Susceptibility testing of anaerobes, p. 436–438. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington D.C.
6. **Waterworth, P. M.** 1973. Laboratory control, p. 502–503. *In* L. P. Gerrod, H. P. Lambert, and F. O'Grady (ed.), Antibiotic and chemotherapy. Churchill Livingstone, Edinburgh and London.