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Low Trimethoprim Susceptibility of Anaerobic Bacteria Due to Insensitive Dihydrofolate Reductases

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All the 28 *Bacteroides fragilis* strains investigated were susceptible to sulfamethoxazole (minimal inhibitory concentration < 16 μg/ml) and resistant to trimethoprim (TMP; minimal inhibitory concentration > 4 μg/ml). Synergism between sulfamethoxazole and TMP was present in all strains at a ratio of 1:1. The few clostridia investigated proved more resistant to both compounds. Dihydrofolate reductases from *B. fragilis, C. perfringens,* and some other anaerobic species were isolated. Inhibition profiles with six structurally different inhibitors revealed major differences in all enzymes. For 50% inhibition, the enzyme from *B. fragilis* and all clostridia required concentrations of TMP which were between several hundredfold and 1,000-fold higher than those required for the enzyme of *Escherichia coli,* whereas the enzyme from *Propionibacterium acnes* only needed a threefold higher concentration. In vitro activities of TMP were seen to correspond to the activity at the enzymatic level in *B. fragilis* and *P. acnes,* but corresponded to a much lesser extent to the activity at the enzymatic level in clostridia, where a poor penetration is assumed to be involved. Dihydrofolate reductase inhibitors other than TMP were found to be as active as TMP both at the enzyme and in vitro. In *B. fragilis,* higher concentrations of exogenous thymidine were required for increasing the minimal inhibitory concentration of TMP than in *E. coli* and probably also in *C. perfringens.*

Trimethoprim (TMP) is a highly active compound against a broad range of aerobic bacteria, since it interferes with the synthesis of tetrahydrofolate, derivatives of which are cofactors in several biochemical reactions, the most important being the synthesis of thymidylate (1, 4). Among strictly anaerobic bacteria, however, at least partial resistance to TMP is a widespread feature, the basis of which is not yet understood. Clostridia are resistant in most cases (4, 6–8, 19). Relatively high concentrations of TMP are needed (6, 8, 13, 14, 19) to inhibit *Bacteroides fragilis,* the most frequently isolated species from human anaerobic infections (11).

The choice of media and the inoculum size are known to be crucial factors in determining the sensitivity of anaerobes against TMP and sulfonamides (14, 19). The present study was undertaken to elucidate the basis of the frequent resistance to TMP in anaerobes, mainly *Bacteroides.* Some of the results have been presented recently (17).

MATERIALS AND METHODS

Strains. The strains used included 28 *B. fragilis,* 1 *Bacteroides* species, 3 *Clostridium perfringens,* 1 *C. septicum,* 1 *C. tetanomorphum,* 1 *C. chauvoei,* and 1 *Propionibacterium acnes.* Some of the strains were kindly provided by J. Wüst (University of Zurich). They were maintained on agar plates on diagnostic sensitivity test (DST) agar (Oxoid Ltd., London) containing 7% human blood, in anaerobic jars (Baltimore Biological Laboratory [BBL], Cockeysville, Md.) at room temperature.

Media. The minimal inhibitory concentration (MIC) determinations were performed on DST agar supplemented with hemin chloride (5 μg/ml) and menadione (0.5 μg/ml). The concentration of thymidine in this medium is below the level needed to antagonize the action of antifolate compounds (16). In some cases, other media were used, as indicated below.

MIC determinations. The agar disk method described by Linzenmeier and Neussel (12) for susceptibility testing of aerobic bacteria was modified for anaerobes. Stock solutions of TMP and sulfamethoxazole (SMZ) were appropriately diluted in distilled water. The compounds were incorporated into the medium by adding 13.5 ml of melted DST agar (supplemented) to 1.5 ml of diluted solution of the compound in Folin tubes. The contents of the tubes were poured into 9-cm petri dishes and allowed to solidify.

From the medium prepared in this way, agar disks with a surface of 0.8 cm^2 were punched out and transferred to empty petri dishes, assembling a series of twofold dilution steps of the antibacterial compounds in the same dish.

To prepare the inoculum, an overnight culture in

supplemented sensitivity test broth (Oxoid) was diluted to a concentration of approximately 10^6 organisms per ml. The agar disks were inoculated with 20 μl of this suspension. The plates were placed in GasPak jars (Becton, Dickinson & Co., Rutherford, N.J.) and incubated for 40 h at 37°C. After that period, growth was recorded by interpreting the MIC as the lowest concentration that showed no growth, not more than one discrete colony, or a faint haze.

Escherichia coli B and *Staphylococcus aureus* Schoch were run as controls of TMP-susceptible organisms in some experiments. Synergism was judged by calculating the fractional inhibitory concentrations, as described by Elion et al. (9). Inhibition of growth in liquid medium was determined by monitoring growth at 600 nm with a Beckman DB/GT spectrophotometer.

Propagation of cells. To isolate dihydrofolate reductases (DHFRs; EC 1.5.1.3), cells were grown in increasing amounts of 30 to 500 ml in tryptic soy broth (Difco Laboratories, Detroit, Mich.) in the GasPak system (BBL). This volume was used to heavily inoculate jars, containing 5 to 6 liters each, which had been made airtight after autoclaving. Most strains grew well under these conditions without further precautions. Cells were harvested by centrifugation and stored frozen at −20°C.

P. acnes required 0.05% thioglycolate for better growth. The purity of the cultures was checked by seeding agar plates and incubating them both aerobically and anaerobically. *C. tetanomorphum* was obtained as a frozen cell paste from P. Matzinger (Central Research Units of F. Hoffmann-La Roche). Cells had been grown in a fermentor at pH 7.4 in a semisynthetic medium containing L-glutamic acid, yeast extract, and mineral salts.

Isolation of DHFRs. Cells which had been stored frozen at −20°C were thawed and suspended in 10 mM phosphate buffer (pH 7.0). The suspensions were treated with lysozyme and ethylenediaminetetraacetic acid and disrupted by sonic treatment for 3 to 5 min at intervals. Streptomycin and ammoniumsulfate precipitations were carried out as described (18). The 50 to 90% ammonium sulfate precipitate was either applied to a Sephadex G 100 column (2.5 by 100 cm) or, if larger volumes were to be handled, to a methotrexate-Sepharose affinity column after dialysis. Elution of the activity occurred as described (18). It was essential to remove reduced nicotinamide adenine dinucleotide phosphate oxidase, because nearly all anaerobes tested had very high reduced nicotinamide adenine dinucleotide phosphate oxidase activities, especially the clostridia, which in the crude extracts often completely masked the DHFR activity. It was impossible, therefore, to determine specific activities. In many strains, especially the clostridia, very little DHFR was present, thus allowing only a few determinations. The activity of some reductases, especially those from clostridia, rapidly deteriorated after purification. Addition of 1 to 10 μM dihydrofolate markedly improved the stability of these enzymes.

DHFR assay. The DHFR assay was carried out as described (18) in a volume of 2 ml. Enough enzyme was added to give a change in absorbance of 0.06/min. This corresponds to 9.7 nmol of dihydrofolate reduced per min if a molar absorption coefficient of $\epsilon = 12{,}300$ is assumed for the reaction (10). Determination of the 50% inhibitory concentrations (I_{50}s) occurred by titration with four to six inhibitor concentrations. Double determinations were carried out as far as possible. A statistical comparison of the inhibition profiles obtained for these strains with six structurally different inhibitors was carried out essentially as described (18).

Miscellaneous. Dihydrofolate was prepared from folate as described by Blakley (3). Aminopterin was obtained from Serva (Heidelberg, West Germany), triamterene was from Lederle (Pearl River, N.Y.), reduced nicotinamide adenine dinucleotide phosphate was obtained from Boehringer (Mannheim, West Germany), 2,4-diamino-6,7-diisopropylpteridine was obtained from Calbiochem (Los Angeles, Calif.), and 2,4-diamino-6,7-dimethylpteridine was obtained from Merck-Schuchardt (Munich, West Germany).

RESULTS

In vitro susceptibility to SMZ and TMP. The activity of TMP, alone and combined with SMZ, was investigated in 28 *B. fragilis* strains and 2 *C. perfringens* strains by the agar disk technique with supplemented DST agar as growth medium. All *B. fragilis* strains were susceptible to SMZ, exhibiting MICs of 1 to 16 μg/ml (mostly 2 to 8 μg/ml). The MIC for TMP was in the range of 4 to 16 μg/ml. The two *C. perfringens* strains were highly resistant to both compounds, the MIC being >128 for SMZ and >32 for TMP. Synergism between the two compounds was found to be present in *B. fragilis*, being generally more pronounced with the ratio of 1:1 than with the usual 10:1 or 20:1 for SMZ/TMP. This means that a higher level of TMP is required for optimal potentiation than in many common bacteria, reflecting its lower activity. Fractional inhibitory concentration indexes of 0.19 to 0.63 were observed with the 1:1 ratio.

Sensitivity to TMP of some DHFRs from anaerobes. Inhibition profiles of DHFRs isolated from several strains were recorded with the use of six structurally different inhibitors (Table 1). The figures for *E. coli* B, *S. aureus* Schoch and the rat liver enzyme (as a eucaryotic enzyme) were included for comparison. The statistical analysis of the inhibition data revealed significant differences among all enzymes ($\alpha = 0.01$). All enzymes from anaerobes are very sensitive to the structural analogs aminopterin and amethopterin, as are all DHFRs, with the exception of some R-factor-induced resistant ones (2). However, all these enzymes were found to be considerably less sensitive to the diaminopyrimidine TMP than common pathogens, resulting in ten to several thousandfold higher I_{50}s, in general 100- to 300-fold higher. *C. tetanomor-*

TABLE 1. *Inhibition profiles of several dihydrofolate reductases from anaerobes*[a]

Strain		Amino-pterin	Trime-thoprim	Pyrime-thamine	Triam-terene	2,4-Diamino-6,7-diisopropyl-pteridine	2,4-Diamino-6,7-dimethyl-pteridine
B. fragilis	6	0.0010	0.72	0.9	28.0	1.3	>100
B. fragilis	35	0.0019	2.35	1.3	54.0	2.6	>100
B. fragilis	36	0.0016	1.60	1.2	18.0	1.3	>100
B. fragilis	37	0.0030	10.0	16.0	>100	11.0	>100
B. fragilis	57	0.0022	0.95	0.37	0.42	0.64	130
Bacteriodes species	31	0.0034	0.063	0.73	4.85	0.23	15
C. perfringens	11	—[b]	0.74	—	—	—	—
C. perfringens	40	0.0050	2.55	3.5	32.0	4.1	>100
C. perfringens	41	0.0044	1.30	3.0	14.0	2.3	30
C. septicum	12	0.0010	1.05	0.47	18.0	2.4	>100
C. tetanomorphum		0.0055	20.0	>1,000	>1,000	>2,000	>100
P. acnes	30	0.0100	0.03	0.24	0.63	0.016	1.3
E. coli B		0.0022	0.0087	1.37	4.10	0.071	30.1
S. aureus Schoch		0.0004	0.0125	1.64	4.23	0.065	38.8
Rat liver		0.0065	487	2.03	7.14	2.4	>100

[a] Values indicate I_{50}s (micromolar). Geometric means are given in cases in which more than one determination was carried out. The coefficient of variation was determined to be between 16 and 36%.

[b] —, No determination.

phum seems to be particularly resistant not only to TMP, but also to the other inhibitors tested.

In many of these TMP-insensitive strains, it is striking that pyrimethamine is as active or slightly more active than TMP, the I_{50} being in the range of that needed in the *E. coli* DHFR. The isopropylpteridine is also often as active as TMP in the reductases of anaerobes. *Bacteroides* sp. 31 and *P. acnes* 30 are exceptions, since they responded to TMP concentrations not too dissimilar to those needed in the *E. coli* DHFR.

Despite taking the precaution of adding 10 μM dihydrofolate during the purification procedure, which often stabilized the enzyme, DHFR from *C. perfringens* 11 proved unstable, with the result that not enough enzyme could be obtained to measure more than the TMP value.

In vitro activity of DHFR inhibitors other than TMP. To get some information on the ability of various structures to penetrate the cell of anaerobes, the in vitro activities of the structures used for probing the enzyme were also determined. This was done in 2 ml of Wellcotest broth. Those concentrations leading to I_{50} were determined. With few exceptions, all compounds are considerably less active against clostridia than against *B. fragilis* (Table 2). As with common bacteria, aminopterin is less active than TMP, being especially inactive in *C. perfringens*. The diisopropylpteridine derivative was found to be as active as TMP in vitro, with the exception of *C. perfringens* 11, against which it is much more active. Triamterene is generally less active than TMP, as is the dimethylpteridine, again with the exception of *C. perfringens* 11, which seems to be especially susceptible to 2,4-diaminopteridine structures. This strain was also relatively susceptible to aminopterin, as was *C. septicum*.

At the enzymatic level, pyrimethamine generally exhibited similar activity to that of TMP. This was found to be paralleled by the in vitro findings, not only for the strains listed in Table 2, but also for five additional strains of *B. fragilis* and *C. chauvoei* which were tested.

Influence of the testing medium and thymidine on MICs. Although the results from the preceding sections suggest that the TMP insensitivity of *Bacteroides* and clostridia is due to the insensitive target enzyme, the influence of the testing medium on the MIC of TMP was studied in several anaerobes. DST agar and Wellcotest agar (Wellcome Research Laboratories, Beckenham, Kent, England), which are known to contain little thymidine, were compared to Schaedler medium (Oxoid) which, according to the much higher MIC of TMP obtained in *E. coli* (0.4 μg/ml with considerable trailing, in contrast to 0.04 μg/ml in DST and Wellcotest agar), contains considerable thymidine (0.26 μg/ml in batch 2841570, as microbiologically determined [16]). The usual agar plate technique was used in this comparison, with a low inoculum of only 10^2 to 10^3 cells per ml, to avoid inoculum effects.

In *B. fragilis*, the MIC for TMP was little affected by the medium, if at all. In contrast, the MIC of TMP in *C. perfringens* jumped to very high values if unsupplemented Schaedler medium was used. This effect could be avoided by the addition of 7.5% lysed horse blood. For *P.*

acnes and *C. septicum*, no such comparison could be made, because these strains did not grow in the unsupplemented Schaedler medium.

The different MICs obtained for TMP and the effect of horse blood in *C. perfringens* suggested that the increase in the MIC was due to thymidine. This was proved by the addition of known amounts of thymidine to Wellcotest agar. Heated horse blood was used as the supplement in this case to avoid the destruction of thymidine by thymidine phosphorylase (Table 3). For comparison, *E. coli* B was used as a representative pathogen and showed a tenfold increase in the MIC with 0.1 μg of thymidine per ml. *B. fragilis* was seen to require a higher thymidine concentration to antagonize the activity of TMP, whereas clostridia seem to behave in a similar manner to *E. coli*. With most of our *Clostridium* strains, the influence of thymidine could not be studied adequately because the MIC of TMP was already very high on thymidine-free medium. The results given for the strains in Table 3 were seen to be representative for other strains tested, *B. fragilis* responding to higher concentrations of exogenous thymidine than other common bacteria.

DISCUSSION

The present study was mainly confined to *B. fragilis* and some clostridia as the most important anaerobes (11). As the quite different behavior of *P. acnes* demonstrates, the results obtained cannot be generalized for all anaerobes.

The inhibition profile established for DHFRs with several different inhibitors shows that large differences exist between all enzymes, including those of *E. coli*, *S. aureus*, and rat liver. The differences are statistically significant and often tremendous. Of practical importance is the comparatively low affinity of TMP for many of these enzymes from anaerobes, especially from *B. fragilis* and clostridia, which require several hundredfold more TMP for I_{50} than do common pathogens. Diaminoisopropylpteridine and pyrimethamine are as potent as TMP. The figures for pyrimethamine show relatively little variation in the binding to DHFR from various origins, and it may be assumed that pyrimethamine binds to a site which is highly important for function and hence less subject to alteration during evolution than the TMP binding site.

Whereas *B. fragilis* and clostridia exhibited these relatively TMP-resistant DHFRs, those from other anaerobes such as *P. acnes* were found to be very susceptible to TMP. The variation among various anaerobes with respect to susceptibility to TMP is therefore quite obvious. In most cases, the differential binding of inhibitors to DHFR is reflected by corresponding in vitro potencies. There are exceptions, however,

TABLE 2. *In vitro activity of several dihydrofolate reductase inhibitors in anaerobes*[a]

Strain		Aminopterin	Trimethoprim	Pyrimethamine	Triamterene	2,4-Diamino-6,7-diisopropylpteridine	2,4-Diamino-6,7-dimethylpteridine
B. fragilis	35[b]	30	3.7	3.0	30	3.0	75
	37[b]	44	3.8	6.0	32	2.9	29
	57	24	7.3	0.6	36	5.0	35
C. perfringens	11	9.8	>100	30	24	<0.5	0.7
	40	>100	30	32	57	17	>100
	41	>100	28	38	>100	25	>100
C. septicum	12	8.1	76	75	80	30	>100
P. acnes	30	70	<0.5	0.5	7.1	<0.5	3.2
E. coli B[c]		100	0.1	27	58	2.9	>100

[a] Values indicate I_{50}s (micrograms per milliliter) determined in 2 ml of Wellcotest broth. Inoculum of 2×10^4 cells per ml. Incubation was for 24 h at 37°C in the GasPak system.

[b] Strain was tested in Wellcotest broth supplemented with 0.001% hemin chloride.

[c] *E. coli* B was aerobically run as a control.

TABLE 3. *Influence of thymidine on the MIC (μg/ml) of TMP in Wellcotest agar containing 7.5% heated lysed horse blood*

Strain		MIC with the following amt of thymidine added (μg/ml):					
		0	0.1	0.5	1	5	10
E. coli B		0.04	0.4	7.0	>10	>10	>10
B. fragilis	57	7	7	10	20	100	100
C. septicum	12	100	400	>400	>400	>400	>400

e.g., *C. perfringens,* which exhibits similar I_{50}s for TMP at the enzyme and is much more resistant to it in vitro. Because only low levels of dihydrofolate reductase were detected in the *C. perfringens* strains studied, it seems that TMP, like most of the other compounds, penetrates less well into *C. perfringens* than into *B. fragilis* or other anaerobes.

C. perfringens 11 (which was originally kept as *C. welchii* in our collection) and *P. acnes* proved to be especially susceptible to the diaminopteridine structure in vitro. To some extent, this is also true for *B. fragilis,* against which the dimethylpteridine is of very low activity at the enzyme, but shows some activity in vitro.

Whereas anaerobes such as *P. acnes* may be judged to be fully susceptible to co-trimoxazole, the diaminopyrimidine TMP may not be the optimal structure for reductases of *B. fragilis* and clostridia, which are the most important pathogens in this group (11). More potent structures may well exist and may be worth looking for.

Some problems arose during the isolation of DHFR from anaerobes. All clostridia and many *B. fragilis* strains investigated exhibited very low specific activities of DHFR, whereas a high amount of reduced nicotinamide adenine dinucleotide phosphate oxidase, which interferes with the optical assay of DHFR, was present.

Enzyme with a suitable specific activity could be conveniently obtained after the affinity step with a methotrexate affinity column. A column with a relatively low amount of ligand bound to Sepharose (as judged from the faint yellow color) proved superior to columns with a high amount of bound ligand (the gel being brightly yellow), with respect to elution and recovery. Some enzymes proved unstable during the purification procedure but, with the exception of that of *C. perfringens* 11, dihydrofolate stabilized them sufficiently over the working period.

In vitro, all *B. fragilis* strains were found to be susceptible to SMZ, whereas the two strains of *C. perfringens* tested were found to be resistant. TMP was more active against *B. fragilis* than against the clostridia. Synergism between these two compounds was found in most *B. fragilis* strains, which were almost exclusively studied in this respect.

As with other TMP-insensitive groups of bacteria, e.g., *Neisseria,* a higher amount of TMP is needed to get optimal synergism than the usual 20:1 or 10:1 ratio for SMZ/TMP (15). The optimum found here for *B. fragilis* was a ratio approaching 1:1. These results are consistent with recent findings of other authors (14, 19). Because the TMP levels seem higher in many tissues in which anaerobic infections may be located than it does in blood (5), the SMZ/TMP ratio in tissues would often be in favor of an optimal in vivo activity against these anaerobes. As yet, however, little is known of the clinical efficacy of co-trimoxazole against infections caused by anaerobes.

It was interesting to see that *B. fragilis* seems to be less susceptible to the antagonizing effect of exogenous thymidine than are most other bacteria (except *Neisseriae*). *B. fragilis* seems, in this respect, to differ from clostridia, which exhibit behavior similar to, e.g., *E. coli.* Therefore, it seems worthwhile to look for possible differences in either the thymidine kinase content or its properties in *B. fragilis.* The effect of exogenous thymidine could not, however, be studied very well with most of our clostridia, because the MIC for TMP was already rather high on thymidine-free media.

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