Mutagenic Studies of Folic Acid Antagonists¹

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Received for publication 16 February 1977

Compounds that compete with folic acid (folic acid antagonists [FAAs]) become limited in their usefulness in the treatment of leukemia, malaria, and bacterial infections by the rapid development of resistance. Assays of the plasma levels of certain of these FAAs led to the observation, in about 25% of the determinations, that a higher density of growth of *Streptococcus faecium* var. *durans* (ATCC 8043) was obtained at an FAA concentration just below the completely inhibitory level than at one-half this concentration. This and other considerations suggested that FAAs may act not only as selective agents for resistant organisms but also as mutagens. Seven FAAs including amethopterin, pyrimethamine, trimethoprim, chlorguanide triazine, an experimental quinazoline, WR-158,122, and two experimental triazines, WR-99,210 and WR-38,839, were tested for mutagenicity in the *Salmonella* reversion assay developed by Ames et al. (1975). All were found to be negative for strains TA1535, TA1537, TA1538, TA98, and TA100, both with and without microsomal activation. These compounds were then tested as mutagens for three traits in the folic acid-requiring *S. faecium*. FAAs were shown to cause mutations to folic acid independence, rifampin resistance, and FAA resistance. It is postulated that the FAAs induce mutations by causing thymine deprivation in the folic acid-requiring host.

Compounds that compete with folic acid have been used extensively in the treatment of malaria and leukemia and, in combination with sulfonamides, in the treatment of bacterial infections (5, 14, 29). Resistance to these folic acid antagonists (FAAs) is known to develop readily (18, 23). Although data consistent with a mutagenic role for these compounds have been reported, this development of FAA resistance has generally been ascribed to the selection of spontaneously occurring resistant organisms (25). Hemmerly and Demerec (12) and Iyer and Szybalski (19) have reported low-grade mutagenicity of amethopterin and pyrimethamine for the *str*, *pro-1*, and *trp-6* loci of *Escherichia coli*; more recently, Herbold and Buselmaier reported negative results with amethopterin on *Salmonella typhimurium* (13). Prophage induction by aminopterin and amethopterin (11), chromosome abnormalities (16, 22, 24), and teratogenic effects (2, 28) have also been described. The planar polycyclic structure of some of the compounds appears similar to that of known deoxyribonucleic acid (DNA) intercalating agents, and some of these structures may be subject to epoxide formation. It was decided to

investigate further the mutagenic potential of these compounds. The systems used were the *S. typhimurium* histidine auxotroph reversion assay developed by Ames et al. (1) and mutation studies of a folic acid-requiring microorganism, *Streptococcus faecium* var. *durans*.

MATERIALS AND METHODS

***S. typhimurium*.** The mutagen indicator strains were supplied by B. N. Ames, University of California at Berkeley. They consisted of a missense mutant, TA1535, two frameshift mutants, TA1537 and TA1538, and strains TA98 (TA1538/pKM101) and TA100 (TA1535/pKM101); pKM101 is an R factor plasmid that makes these host strains particularly susceptible to the effects of certain mutagens (21). All of the strains are deficient in both cell wall synthesis and DNA repair. Stocks were maintained in dimethyl sulfoxide at -70°C and were used as described by Ames et al. (1).

Livers for microsome preparations were obtained from 150-g male Sprague-Dawley rats that had been pretreated by intraperitoneal injection of 50 mg of Aroclor 1254 per kg for 5 days and then starved for 24 h and killed. Homogenate supernatant S-9 fractions were prepared, maintained, and applied in activation assays essentially according to the methods described by Ames et al. (1). Livers were homogenized in 0.15 M KCl-0.05 M tris(hydroxymethyl)-aminomethane-hydrochloride, pH 7.4, and the homogenate was then centrifuged 20 min at 9,000 × g.

¹ This paper is contribution no. 1454 to the Army Research Program on malaria.

***Streptococcus faecium* var. *durans*.** This is *Streptococcus faecalis* (ATCC 8043), which has been reclassified as *S. faecium* var. *durans* (7). It is one of the lactic acid bacteria that requires folic acid and is widely used for folic acid and FAA assay (15, 27). A fastidious organism, requiring a relatively complex medium (9), it cannot synthesize folic acid from simple precursors. The requirement for folic acid also can be met by providing pterotic acid, a reduced folate, or the products of the folate pathway (20). The organism is a microaerophilic coccus (27) that grows in diplo or short chain form in the liquid test medium. Counts of colony-forming centers are equated with cell count in this study. The strain was maintained by weekly passage on yeast extract agar.

The medium used was the standard folic acid assay medium as described by Flynn et al. (9), with the exception that glucose and folic acid were sterilized separately and added aseptically at the time of the test. This is a semisynthetic medium containing acid-hydrolyzed casein, a vitamin mixture, and purines. For preparation of 10-ml broth cultures, 8-ml quantities of basal medium were sterilized in tubes by autoclaving at 121°C for 10 min. Stock solutions of folic acid were sterilized by membrane filtration and were stored in small quantities at -15°C. Dilutions were prepared in sterile 25% glucose solution and were added in 1-ml quantities to yield final concentrations of 2 ng of folic acid per ml. This quantity of folic acid allows growth to proceed to approximately 7×10^8 cells per ml in medium without drug. Stock solutions of the FAAs or other test drugs, prepared either in self-sterilizing solution or sterilized by membrane filtration, were diluted in water and added to the tubes in 1-ml quantities to give the final volume.

Solid medium was prepared by adding 2% agar (Difco) to the medium described above; since the use of 30 ml gave no advantage, 15 ml in standard 100-mm petri dishes was used routinely. Folic acid, glucose, and test drug were added to the sterile agar medium just before pouring.

Growth conditions. The test inoculum for *S. faecium* was prepared as follows. The strain was subcultured (1/100) twice in standard medium and incubated at 37°C in loosely capped 18-mm test tubes without shaking: the first tube was incubated for 24 h and the second was incubated for 18 to 20 h. The resulting culture was centrifuged, washed twice, and resuspended in sterile saline to form a suspension with turbidity measuring 50 on the Klett-Sumerson photoelectric colorimeter (660-nm filter). Samples, 0.1 ml of this suspension, containing approximately 10^7 cells, either were added to 10-ml culture volumes or were plated. Plating procedures used included standard pour plates, spread plates, or top agar plating in 2 ml of 0.6% molten agar. When spot plates were used, an FAA was added to sterile filter paper placed on the center of the inoculated plate. Plates were incubated in a loosely covered container to retard evaporation. Growth in liquid cultures was read as turbidity in the colorimeter after 22 h of incubation. Colony counts on plates were made at 24 h and at intervals thereafter for up

to 14 days. Colonies were not readily visible until after 48 h of incubation. In experiments in which the organism was first exposed to FAA in liquid culture and then plated, a 10^7 -cell inoculum was cultured overnight in the presence of the drug. These cells were then washed, and a standard inoculum of 10^7 cells was prepared again and subcultured in standard medium for 18 to 20 h to allow expression. Standard inocula were then delivered to selection plates. It was found that approximately 10 times as much FAA was needed in agar plates as in liquid medium for the same degree of inhibition. All plate counts reported are averages of at least duplicate plates.

Mutagens and drugs. Folic acid was obtained from Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y.; chlorguanide triazine (CGT, cycloguanil, 1-*p*-chlorophenyl-1,2-dihydro-2,2-dimethyl-4,6-diamino-*s*-triazine), and chloramphenicol from Parke, Davis and Co., Detroit, Mich.; amethopterin (MTX, methotrexate, 2,4-diamino-*N*¹⁰-methylpteroylglutamic acid) was supplied by James L. Rueggsegger, Lederle Laboratories; pyrimethamine (PM 2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine), trimethoprim [TMP, 2,4-diamino-5-(3',4',5'-trimethoxybenzyl)-pyrimidine], WR-158,122 (2,4-diamino-6-(2'-naphthyl)-sulfonylquinazoline), WR-99,210 [1-(2',4',5'-trichlorophenoxypropoxy)-1,2-dihydro-2,2-dimethyl-4,6-diamino-*s*-triazine], and WR-38,839 (1-[3',4'-dichlorobenzoyloxy]-1,2-dihydro-2,2-dimethyl-4,6-diamino-*s*-triazine) were supplied by the Division of Medicinal Chemistry, Walter Reed Army Institute of Research, Washington, D.C. Structures of the FAAs are depicted in Fig. 1.

FIG. 1. Structures of folic acid antagonists.

Methylmethane sulfonate was obtained from Eastman Chemical Co., Rochester, N.Y.; 2-aminoanthracene and *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine from Aldrich, Milwaukee, Wis.; and 4-nitroquinoline-one-oxide from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England, was provided by Tong Man Ong of the National Institute of Environmental Health Sciences, Research Triangle Park, N.C. Aroclor 1254 was obtained from Supelco, Inc., Bellefonte, Pa.; bromodeoxyuridine and 2-aminopurine from Sigma Chemical Co., St. Louis, Mo.; proflavine from Nutritional Biochemicals, Cleveland, Ohio; quinacrine mustard (NS3424) from the Cancer Chemotherapy National Service Center, and rifampin from Calbiochem, San Diego, Calif.

RESULTS

Studies with *S. typhimurium*. No FAA tested was found to be mutagenic in spot tests with strains TA1535, TA1537, TA1538, TA98, and TA100 without microsomal activation. Since all were somewhat toxic at 100 μ g per plate in spot tests, with narrow zones of inhibi-

tion indicative of poor diffusion by these compounds, experiments were performed with plate assays incorporating graded doses of the FAA. Data for tests of TA98 and TA100 with and without microsomal activation appear in Table 1. Neither TA98 nor TA100 was reverted to a significant extent by PM, MTX, WR-158,122, or WR-99,210 at the doses indicated. WR-99,210 and WR-158,122 proved to be toxic at higher doses; this toxicity was relieved somewhat by the presence of the microsomal system. All FAAs tested in pour plates were toxic for *S. typhimurium* tester strains at 100 μ g per plate. These same FAAs gave similar results with strains TA1535, TA1537 and TA1538; that is, no significant mutagenicity occurred in the presence or absence of activation. Furthermore, varying the concentration of S-9 over a range of 25 to 150 μ l per plate, or employing uninduced, or phenobarbital-, or 3-methylcholanthrene-induced S-9 did not alter the lack of mutagenicity of the FAAs for any *Salmonella* strain (R. S.

TABLE 1. Lack of mutagenicity of folic acid antagonists for *S. typhimurium* in pour plate assays

Mutagen	Amt (μ g/plate)	No. of colonies ^a			
		TA98		TA100	
		-S9	+S9 ^b	-S9	+S9
None		15	27	90	83
2-Aminoanthracene	5	30	580	31	814
Methylmethane sulfonate	97	ND ^c	ND	1,710	ND
4-Nitroquinoline-1-oxide	0.2	180	ND	ND	ND
PM	100	I ^d	I	I	I
	5	2	0	21	48
	1	3	0	20	55
	0.5	0	3	16	45
	0.1	0	1	37	24
MTX	100	I	I	I	I
	10	5	1	15	42
	5	1	3	15	48
	1	7	5	28	36
	0.5	0	6	3	50
	0.1	0	0	24	18
WR-158,122	100	I	I	I	I
	10	I	I	I	I
	5	I	I	I	I
	1	I	I	I	I
	0.5	I	0	I	I
	0.1	3	0	0	23
WR-99,210	100	I	I	I	I
	10	I	I	I	I
	5	I	I	I	I
	1	I	5	10	17
	0.5	5	3	30	55
	0.1	3	3	38	32

^a Based upon quadruplicate platings. Values for spontaneous mutations are averages; other values are averages minus the spontaneous mutations.

^b S9 was a microsome preparation from pooled polychlorinated biphenyl-induced rat liver homogenate and was used at a concentration of 50 μ l/plate.

^c ND, Not done.

^d I, Inhibited growth.

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Studies with *S. faecium*. During the last 3 years, we have been using *S. faecium* var. *durans* in microbiological assays of plasma samples for FAA content. In approximately 20 to 25% of these assays, an unpredicted result was obtained. In these cases more growth occurred at higher concentrations of drug than at one-half these concentrations. For example, in 62 out of 338 assays (18% of tests) with WR-158,122, more growth was obtained at 1 ng/ml than at half the concentration, 0.5 ng/ml (Fig. 2). With WR-99,210 such results were obtained in 35 of 132 trials (26%). This effect had not been recognized in our earlier studies using PM; however, in reviewing our data a few such results were found with that compound.

FIG. 2. Effects of WR-158,122 on growth of *S. faecium*.

Checks of isolates from the tubes having this unexpected growth showed that the organisms had the usual characteristics of *S. faecium* var. *durans* (7) but were approximately five times as resistant to FAA as the stock culture.

Mutation to folic acid independence—spot tests. Typically, when 10^7 cells of a stock culture of *S. faecium* are plated in the absence of added folic acid a few folic acid-independent colonies develop. This suggested that, perhaps, folic acid independence could be mutagenically induced in this strain, so spot tests of various concentrations of FAA or of standard mutagens were conducted on plates lacking folic acid. Rings of folic acid-independent colonies (see Fig. 3) were obtained with bromodeoxyuridine, 2-aminopurine, proflavine, quinacrine mustard, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and with each of the seven FAAs tested: PM, TMP, CGT, MTX, WR-38,839, WR-99,210, and WR-158,122. Rings were obtained with a wide range of concentrations of the FAAs, but not every trial with the known mutagens or with the FAAs yielded positive results (Table 2).

Mutation to FAA resistance—spot tests. Spot tests of the FAAs were also made with plates containing folic acid. Rings of FAA-resistant colonies were formed in the zone of inhibition around the drug spot with the 7 FAAs listed above. Again, positive results occurred with a wide range of concentrations of each drug but were not invariably obtained (Table 2). The position of the ring of mutants and the diameter of the area of inhibition varied with the type and amount of FAA used. However, for

FIG. 3. Spot test of 100 µg of WR-99,210 with *S. faecium* on medium without folic acid. Large squares have an area of 1 cm².

any given FAA, increasing the amount of compound did not increase the diameter of the ring above a certain level. Apparently, FAAs diffuse too slowly to prevent growth beyond a certain distance.

Mutation to rifampin resistance in broth cultures. Since broth growth without folate and resistance to FAA may be related to the folate pathway, an attempt was made to detect unrelated mutagenic effects. Mutation to rifampin resistance was chosen since *S. faecium* is susceptible to this antibiotic at 2.5 µg/ml on agar plates. Spot tests could not be used in this case, since the combined application of FAA and antibiotic eliminated all growth; resistance to this drug combination would require the formation of a double mutant. For this reason a sequential test was employed in which *S. faecium* was first grown in the presence of partially inhibitory concentrations of FAA, subcultured in drug-free medium, and then placed on agar for selection for rifampin resistance or for the properties of folic acid independence or FAA resistance. Typical results (Table 3) show that exposure to FAA increased the number of rifampin-resistant colonies per 10⁷ cells plated from none detected to a range of 39 to 115. In other experiments the number of rifampin-resistant colonies has increased up to 4,500.

Two different levels of rifampin resistance were observed. Of 710 colonies isolated at 5 µg/ml, 61% were able to grow at 100 µg/ml. Others were unable to grow at concentrations above 5 µg/ml.

The possibility was considered that rifampin resistance arose spontaneously in these experiments and that the presence of the FAA in some way favored the multiplication of these rifampin-resistant cells. This was tested in two ways. First, Fig. 4 demonstrates that the parent strain, and two of its derivatives, one a rifampin-resistant (100 µg/ml) strain and one resistant to both rifampin (100 µg/ml) and chloramphenicol (500 µg/ml), all have identical growth rates in the absence of FAA. Second, the double mutant strain was mixed with the parental susceptible strain in the proportions of 1/10⁵ and 1/10⁴. These mixtures were cultured in the presence of varying concentrations of WR-158,122 so as to produce graded degrees of growth inhibition (optical density, 0.5 to 0.1). Resultant cultures were then grown for 22 h in the absence of FAA and plated on rifampin-containing agar. Rifampin-resistant colonies were patch-checked for chloramphenicol resistance. It can be seen in Table 4 that the ratio of chloramphenicol-resistant to rifampin-resistant colonies in the mixed cultures decreased

TABLE 2. Results of spot tests with FAAs on *S. faecium*

Compound	Folic acid independence			FAA resistance		
	Amt spotted (µg)	No. of trials	% Positive ^a	Amt spotted (µg)	No. of trials	% Positive
PM	0.3-100	43	40	0.5-50	28	21
WR-158,122	0.5-20	44	55	0.1-10	28	21
WR-99,210	0.3-20	46	54	0.1-10	29	21
CGT	100-500	12	42	100	8	13
MTX	5-1,000	15	13	5	3	+ ^b
TMP	50-1,000	3	+ ^b	50-1,000	3	+ ^b
WR-38,839	1,000	1	+ ^b	1000	1	+ ^b

^a Positive or negative results were independent of drug concentration used here since both positives and negatives occurred over the entire range reported (see text).

^b All trials were positive.

TABLE 3. Numbers of mutant colonies of *S. faecium* obtained after exposure to FAAs in broth cultures

FAA (ng/ml)	Plate count of colonies resistant to selective agent (ng/ml):					
	Folic acid		Rifampin ^a	WR-158,122 ^a	WR-99,210 ^a	MTX ^a
	(2.0)	(0.0)	(5,000)	(25)	(10)	(100)
None	CF ^b	19	0	23	5	0
WR-158,122 (0.5)	CF	610	75	1,320		
WR-99,210 (0.25)	CF	90	39		1,200	
PM (2.5)	CF	1,300	55			31
MTX (0.25)	CF	205	115			

^a Agar also contained folic acid, 2 ng/ml.

^b CF, Confluent growth.

^c HD, Heavy growth but discrete colonies.

FIG. 4. Growth of *S. faecium* stock, rifampin-resistant and rifampin-resistant, chloramphenicol-resistant strains in test medium.

with increasing exposure to FAA. This would be expected due to the generation of additional rifampin-resistant cells from among the parent, susceptible population. Thus, it does not appear that the original rifampin-resistant, chloramphenicol-resistant cells had a selective advantage in the FAA medium. It appears, rather, that they may not be able to compete successfully with the wild-type organism in mixed culture. Direct plating for the scoring of chloramphenicol resistance was not possible in this experiment due to an apparent diffusion of the chloramphenicol resistance effect, which allowed growth of surrounding cells.

Mutation to folic acid independence and FAA resistance in broth cultures. Cultures exposed to FAA in broth were also checked for numbers of colonies able to grow without folic acid and for the numbers showing FAA resistance. The numbers of folic acid-independent colonies per plate ranged from 90 to 1,300 as compared with 19 on the control (Table 3).

Scoring for FAA resistance is more difficult than scoring for folic acid independence or for rifampin resistance, because the FAAs are only bacteriostatic. Comparison of stock and FAA-exposed cultures made at the same time show reproducible increases in numbers of colonies (Table 3) with resistance to FAA.

It would be expected that, under our condi-

tions of FAA exposure, naturally occurring FAA resistance would be selected. However, highly FAA-resistant cells from the untreated stock culture were undetectable. ($<10^{-10}$).

Independence of mutational events. Cross checks by patch test or replica plating to selective media indicate that the three traits, folic acid independence, FAA resistance, and rifampin resistance arise independently. As presented in Table 5, 6.5% of the colonies that were folic acid independent were resistant to FAA, and a separate 6.5% were rifampin resistant. Of the colonies that were rifampin resistant, 10% were resistant to FAA and a separate 11% were folic acid -independent. Of the colonies resistant to FAA, none was resistant to rifampin and only 30% were folic acid independent.

Relation of growth inhibition by FAAs to mutagenesis. The data for the three types of mutants were examined for possible relationships between the amount of growth inhibition of *S. faecium* and the number of mutants produced. For all three markers, it was necessary that growth be inhibited approximately 50% before the number of mutants exceeded 10 times the mutant level of the untreated control.

For folic acid independence, the marker for which there is the most data, there was a direct relationship between the proportionate increase in number of mutants and the inhibition of growth by the FAAs. Data for PM and WR-158,122 are shown in Fig. 5. The proportionate increase in number of mutants is plotted versus the amount of growth inhibition produced by the FAAs; dotted lines represent the 90% confidence limits of the response. Available data for FAA and rifampin resistance appear to follow the same pattern.

TABLE 4. Percentage of rifampin-resistant, chloramphenicol-resistant colonies when mixtures of susceptible and resistant strains of *S. faecium* were grown in presence of WR-158,122

Optical density after 22 h	No. of rifampin-resistant and % doubly resistant/rifampin-resistant colonies when initial ratio of doubly resistant/susceptible strain was			
	10 ⁻⁵		10 ⁻⁴	
	No.	%	No.	%
Plated immediately	200	100	4,000	100
0.5	140	76	344	60
0.4 ^a	100	78	555	50
0.3	233	50	686	40
0.1	609	44	4,675	4

^a Increasing concentrations of WR-158,122 were employed to retard growth.

TABLE 5. Independence of mutational events

Phenotype selected	No. tested	Colonies showing (%)		
		Folic acid independence	FAA resistance	Rifampin resistance
Folic acid independent	168	100	6.5	6.5
FAA resistant	257	30	100	0
Rifampin resistant	99	11	10	100

FIG. 5. Relationship between -fold increase in number of folic acid-independent mutants and degree of growth inhibition of *S. faecium* by PM or WR-158,122.

DISCUSSION

In most cases, it is generally recognized that the development of drug-resistant populations in laboratory clones is the result of selection of spontaneously occurring resistant mutants (25). It has been shown occasionally, however, that a growth-inhibitory drug may have mutagenic properties (10). The data presented in this paper indicate that drugs that are folic acid antagonists have mutagenic properties for *S. faecium* but are negative in tests with *S. typhimurium*.

When the *S. typhimurium* tester strains of Ames were applied to seven FAAs in our laboratory, the FAAs failed to revert the missense mutation in TA1535 and TA100, as well as the frameshift mutation in TA1537, TA1538, and

TA98 when the FAAs were used in spot tests or at any of several concentrations in pour plate assays. That the FAAs were delivered to the bacteria is evidenced by their toxicity for one or more strains at high doses (100 $\mu\text{g}/\text{plate}$). The toxicity was relieved somewhat by the presence of S-9 liver mix. This effect could be due to a sparing action of the folic acid present in the liver fraction. It is also possible that the liver microsomal fraction was able to bind or to metabolize a portion of the drug to some inactive form. In any case, the S-9 mix did not activate the FAAs to a form capable of reverting the *Salmonella* auxotrophs. This was true both with uninduced microsomal fractions or with a wide range of concentrations of phenobarbital-, 3-methylcholanthrene-, or polychlorinated biphenyl (Aroclor 1254)-induced microsomal fractions (Schoeny et al., manuscript in preparation).

FAAs are far more inhibitory for the folate-requiring *S. faecium* than for the *S. typhimurium* (0.01 to 0.10 $\mu\text{g}/\text{plate}$ for *Streptococcus* as opposed to 100 $\mu\text{g}/\text{plate}$ for *Salmonella*). It may be that the failure to mutate the folic acid-independent *Salmonella* strains is related to their relative nonsusceptibility to these compounds.

Initial evidence for the mutagenicity of FAAs for *S. faecium* was found in tube assays for FAA bacteriostasis, in which more growth occurred at higher concentrations of drug than at one-half those concentrations. When *S. faecium* was used in spot tests for conversion to folic acid independence, positive results were obtained with all seven FAAs tested: CGT, MTX, PM, TMP, WR-38,839, WR-158,122, and WR-99,210. Rings of folic acid-independent colonies were obtained at frequencies of 13 to 54% of trials with various FAAs. Spot tests for FAA resistance were positive at rates of 13 to 21%. That positive results were not achieved consistently is probably due to the delicate balance of interactions between the metabolism of the organisms as they grew on the plate and the bacteriostatic activity of the FAA diffusing from the test spot. We conclude that, for some trials, the bacteriostatic FAA disallowed expression of folate independence or FAA re-

sistance. In sequential tests in which *S. faecium* was first exposed to partially inhibitory concentrations of FAA and then tested for folic acid independence and FAA resistance, the results were more consistent.

Since both markers mentioned above involved the folic acid pathway, it was decided to check the mutagenic effects of FAAs on an unrelated marker. *S. faecium* is a relatively fastidious organism, requiring at least 10 amino acids in addition to other growth factors, so metabolic auxotrophs were not feasible for study. Resistance to rifampin was chosen as a marker since the strain was shown to be quite sensitive to this drug. In assays wherein cells were exposed to FAA, expressed in the absence of FAA, and then plated for selection, the number of rifampin-resistant colonies was increased from 39 to 4,500 times over the number in the unexposed spontaneous control. At least two different levels of rifampin resistance were observed in mutants isolated. These results were obtained with all FAAs tested: CGT, MTX, TMP, PM, WR-158,122, and WR-99,210. Sequential exposure was necessary to avoid the additive growth-inhibitory effects of these compounds. The experiment assumes that previously existing and newly arising rifampin-resistant cells have no selective growth advantage during or after exposure to an FAA. Our data indicate this to be the case for the following reasons.

First, patch-checking and/or replica plating colonies to selective media showed the properties of FAA resistance, rifampin resistance, and folic acid independence to be generally independent events. No colonies selected on FAA plates were rifampin resistant, and only 10% of colonies growing on rifampin were resistant to FAA. If FAA selection were allowing overgrowth of preexisting rifampin-resistant organisms, this percentage would be expected to be higher. Interestingly, even folic acid-independent colonies were generally not FAA resistant, with only 6.5% of such cells having both properties. Levels of folic acid biosynthesis were not examined in these folic acid-independent, FAA-resistant cells. That as many as 30% of the FAA-resistant cells showed folate independence may be due in part to the double exposure these cells received, first in initial exposure for mutagenesis, and second, during isolation as FAA-resistant colonies.

Second, as shown by comparative growth curves, there appears to be no selective growth advantage for rifampin resistance or susceptibility in the absence of FAA. The wild-type organism, a rifampin-resistant strain, and the

strain doubly resistant to rifampin and chloramphenicol all show essentially identical growth rates.

Third, direct seeding experiments were conducted in which rifampin-resistant cells genetically tagged with a chloramphenicol resistance marker were diluted with susceptible cells and carried through the FAA treatment. This assay showed no indication of selective advantage for rifampin-resistant organisms in the presence of FAA. Thus, the increased incidence of rifampin-resistant cells is not due to selective conditions occurring during the FAA treatment procedure.

It has been established that by interfering in the folate pathway, FAAs inhibit the production of thymine, purines, protein, and lipid (2). For *S. faecium*, the requirement for folic acid in the growth medium used here can be replaced by the addition of thymine (17). In the suspension assays for FAA mutagenesis, a certain level of FAA growth inhibition was shown to be necessary. Although the FAA dose required for bacteriostasis and mutagenesis varied somewhat for individual assays, a dose-response relationship could be shown between growth limitation and numbers of folic acid-independent mutants produced.

We suggest that the FAAs, in competing with the folic acid, produce a thymine insufficiency which, although not completely inhibiting growth, does interfere with DNA metabolism so as to cause mutations. Thymine starvation is known to produce mutations in *E. coli* (4, 6, 8), *Bacillus subtilis* (3), and T4 bacteriophage (26). Besides being mutagenic in itself, thymine deprivation could additionally promote mutagenic intercalation of the planar polycyclic FAAs.

That FAAs can effect mutagenesis is of clinical significance. Since the folic acid pathway is common to all cells, the mechanisms operative with *S. faecium* will probably be similar in other types of organisms susceptible to these compounds. When administered repeatedly at low doses, as has been common in the prevention and treatment of malaria, the FAAs are capable of selecting for cells resistant to themselves. On the other hand, high doses delivered for maximum chemotherapeutic effect may be capable of inducing a variety of mutations.

ACKNOWLEDGMENTS

We thank Silvan Witherup for his help in statistical analysis and Steve Glasser for technical assistance.

This work was supported by U. S. Army contract no. DADA 17-73-C-3151, and by Public Health Service grant AI-11516 from the National Institute of Allergy and Infectious Diseases.

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